

**Consequence of a hypomorphic *Artemis* mutation on V(D)J
recombination and proper immune function**

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List of Abbreviations

12RSS	Recombination signal sequence, 12 nucleotide spacer
23RSS	Recombination signal sequence, 23 nucleotide spacer
A-T	Ataxia-telangiectasia
ATM	Ataxia telangiectasia mutated
ATMi	ATM inhibitor KU-55933
ATR	Ataxia telangiectasia and Rad3 related
β -CASP	Metallo- β -lactamase associated CPSF Artemis SNM1/PSO2 domain
BAFF	B-cell activating factor
BCR	B-cell receptor
CAT	Chloramphenicol acetyltransferase gene
CDT	Cytolethal distending toxin
CE	Coding end
CHO	Chinese hamster ovary cell line
CJ	Coding join
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	Double stranded break
dsRED2	<i>Discosoma</i> species red 2 fluorescent protein
DSS	Dextran sulfate sodium
EBV	Epstein-Barr virus
FL	Full length
GFP	Green fluorescent protein
H & E	Hematoxylin and eosin stain
HMGB1	High mobility group box 1
HMGB2	High mobility group box 2
HSC	Hematopoietic stem cells
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IRES	Internal ribosomal entry site
KAP1	KRAB-associated protein 1
MEF	Mouse embryonic fibroblast
MHC	Major histocompatibility complex protein
MRN	MRE11-RAD50-NBS1 complex
M β L/ β CASP	Metallo- β -lactamase/ β -CASP domain
NHEJ	Nonhomologous end joining
PCC	Post-cleavage complex

PIKK	Phosphatidylinositol 3-kinase related kinase
RAG1	Recombination activating gene 1
RAG2	Recombination activating gene 2
RSS	Recombination signal sequence
RS-SCID	Radiosensitive severe combined immunodeficiency
SE	Signal end
SJ	Signal join
SQ	Serine glutamine
TCR	T-cell receptor
TdT	Terminal deoxynucleotidyl-transferase
TQ	Threonine glutamine
v-abl	Viral Abelson kinase
WCL	Whole cell lysate

Abstract

The generation of antigen receptors requires a gene rearrangement process known as V(D)J recombination. Double stranded breaks produced during recombination must be repaired by the nonhomologous end joining pathway. One critical factor important for this recombination event is the Artemis nuclease, which processes the DNA ends before ligation. Patients with mutations affecting the catalytic N-terminal domain of Artemis are radiosensitive and do not produce B and T cells. Hypomorphic Artemis mutations involving the nonconserved C-terminus have also been described. These patients have a partial B and T cell deficiency but exhibit a predisposition to lymphoid malignancies.

Here, I study the regulation of the Artemis nuclease during V(D)J recombination and the genetic interactions between the Artemis C-terminal domain and DNA damage inducible protein kinase, ATM. I define a region in the C-terminus that is required for endonucleolytic activity and preventing aberrant V(D)J recombination. The findings presented here also suggest that there may be previously undescribed regulatory features located in the Artemis N-terminus that are separate from catalytic activity. To analyze the *in vivo* consequences resulting from the loss of the Artemis C-terminus, I utilized a hypomorphic Artemis mouse model harboring an allele that results in C-terminal truncation, Artemis-P70. Cellular and *in vivo* assays suggest that the Artemis C-terminus and the ATM kinase cooperate to facilitate V(D)J recombination and loss of both factors can result in a lymphocyte deficiency that is more severe than in mice deficient for one factor. Although ATM deficient mice succumb to thymic lymphoma, mice harboring homozygous mutations in both Artemis-P70 and ATM are surprisingly tumor-free. These mice, however, had inflamed colons with T cell and neutrophil infiltration.

These findings together emphasize the importance of the regulatory domains of Artemis. A dysregulated nuclease can facilitate aberrant recombination and that can disrupt proper lymphocyte development. A lymphocyte defect may operate in concert

with other factors such as DNA repair deficiency and cytokine imbalance which together could promote chronic inflammation. Finally, results presented here suggest that there may be other means of Artemis regulation that are dependent on features located in the conserved N-terminal domain.

Chapter 1

Introduction

Introduction to V(D)J recombination

The adaptive immune system, present in all jawed vertebrates, can recognize a myriad of invading pathogens [1]. This recognition depends on the B and T lymphocytes, cells which express antigen receptors capable of recognizing antigens from the external environment. When antigen receptor proteins were first sequenced, it was found that these proteins contained regions that were common to other antigen receptor proteins but they also contained other regions of high variability. The antigen receptor protein seemed to originate from one gene in some regions and thousands of genes in other regions. For many years, it was unclear how a protein of this type could be created by the cell (Figure 1.1).

Eventually, it became known that antigen receptors were encoded by a novel process in which gene segments were rearranged to produce the variable regions of the receptors [2]. These gene segments that make up the variable regions are grouped into three categories: variable (V), diversity (D), and joining (J) and accordingly, the process is referred to as V(D)J recombination (Figure 1.2). The rearrangement of these segments is highly regulated and ordered so that only specific types of gene segments are joined and only during specific stages of lymphocyte development.

The development of a T-cell requires that it rearranges its antigen receptor, the T-cell receptor (TCR). TCRs can be generally classified into two categories based on the type of receptor on their surface: those with receptors comprised of an α -chain and a β -chain ($\alpha\beta$ T cells) and those with receptors that are comprised of a γ -chain and a δ -chain ($\gamma\delta$ T cells). B cells on the other hand have B cell receptors (BCRs or

immunoglobulins) which are comprised of two heavy chains (IgH) and two light chains (IgL).

V(D)J rearrangements to generate the TCRs and BCRs begin with the introduction of DNA double stranded breaks (DSBs) by the Rag endonuclease. The Rag endonuclease consists of two lymphocyte-specific proteins, recombination activating gene 1 and 2 (RAG1 and RAG2) [3]. Rag creates a double stranded break at a recognition site referred to as the recombination signal sequence (RSS), a nucleotide sequence adjacent to the gene segments. The RSS contains a conserved 7 nucleotide (heptamer) and 9 nucleotide (nonamer) sequence and a poorly conserved spacer sequence of either 12 or 23 nucleotides. For this reason, the recognition sites are categorized as either 12RSS or 23RSS depending on the nonconserved spacer sequence. The RAG proteins show a preference for the recombination between 12RSSs and 23RSSs [4, 5]. This preference of the RAG machinery adds a level of regulation to ensure the cleavage and joining of the proper gene segments in the correct orientation.

The Rag endonuclease is thought to assemble on a single RSS and this is then followed by the capture of the RSS partner. Some studies have suggested that Rag has a preference for binding and creating a single-stranded nick the 12RSS first and then capturing the 23RSS [6, 7]. However, another study using chromatin immunoprecipitation in lymphocytes found no increased preference for Rag binding to 12RSS over 23RSS [8]. This difference in findings may indicate that while the Rag endonuclease may bind to either 12RSS and 23RSS first, the nicking of DNA may be occurring at a faster rate at 12RSS [9]. (Figure 1.3)

RAG1 and RAG2 are sufficient to bind RSSs and promote cleavage. However, *in vivo*, this process likely occurs with two other proteins important for DNA bending: high mobility group box 1 (HMGB1) and high mobility group box 2 (HMGB2). The presence of these two proteins support Rag binding at 23RSS and cleavage of synapsed RSSs [10]. While the nicking of DNA can occur after Rag binding, the cleavage and generation of hairpin ends requires the synapsis of two paired RSSs [11]. The single-stranded DNA nick creates a 3' hydroxyl group which can act as a nucleophile. The Rag endonuclease uses the hydroxyl group to catalyze a nucleophilic attack of the second

strand cleaving the DNA to produce two types of ends: a covalently sealed hairpin coding end (CE) adjacent to the gene segment and a 5' phosphorylated blunt signal end (SE) that contains the recombination signal sequence. In order to generate the rearranged receptor gene, the hairpin coding ends must be opened and joined to create coding joins (CJs). The signal ends are also joined to create signal joins (SJs) but because they are blunt, they do not require processing. [12]

DNA ends that have been created by the Rag endonuclease are thought to be held in close proximity to facilitate subsequent ligation steps. The Rag endonuclease itself is thought to play an important role in the synapsis of DNA ends. After DNA cleavage, it has been shown that all four DNA ends, two signal and two coding ends, are retained in a protein-DNA complex referred to as the post-cleavage complex (PCC) [13, 14]. Another factor important to DNA end synapsis is the nonhomologous end joining protein (NHEJ) DNA-protein kinase catalytic subunit (DNA-PKcs) [15, 16]. Using electron microscopy, it was shown that DNA-PKcs along with the DNA-end sensor proteins KU70 and KU80 (in complex, referred to as DNA-PK) can bind to the DNA ends of a linearized plasmid and promote a circularized DNA formation.

While these factors may be important for promoting the retention of Rag generated ends, other factors may be important for bringing together gene segments that may be separated by several megabases. Using two-color fluorescence *in-situ* hybridization, one group found that the IgH locus was compacted during the pro-B cell stage, the stage in which B cells rearrange this locus [17]. The compaction of the IgH locus also was not apparent in developing T cells suggesting that this was lineage specific. Using a similar approach, TCR loci compaction has also been observed in developing T cells during rearrangement stages but not in developing B cells [18]. This compaction was not found to be dependent on the Rag endonuclease [17] but instead on the transcription factors Pax5, CCTF, and the protein complex cohesin [19].

Taccioli et al. determined that the Rag generated ends are joined by the NHEJ pathway [20]. This group used several Chinese hamster ovary (CHO) cell lines deficient in various DNA repair pathways including nucleotide excision repair, single stranded break repair, and double stranded break repair and tested the ability of these cells to recombine a plasmid substrate. They found that cell lines deficient in DSB repair were

unable to efficiently join Rag generated breaks. Those DSB-deficient CHO cell lines were later discovered to be deficient in KU80 [21], XRCC4 [22], and DNA-PKcs [23], all proteins that participate in the NHEJ pathway. Interestingly, DNA-PKcs deficient cell lines exhibited impaired coding joining but not signal joining illustrating the differences in the way the two types of DNA ends are handled. This uncoupling of the two joining events was later found to be a product of the requirement for DNA-PKcs-assisted coding end processing.

In addition to cleaving the DNA, the Rag endonuclease is thought to be important for shepherding DNA ends specifically to nonhomologous end joining. Cui and Meek found that DSB-breaks on a plasmid substrate induced by the endonuclease I-Sce1 were repaired efficiently in NHEJ-deficient CHO cell lines [24]. However, when the cleavage of I-Sce1 sites were coupled to the Rag complex by fusing the RAG2 protein with the I-Sce1 endonuclease, efficient repair of the breaks became dependent on NHEJ factors.

Nonhomologous end joining

There are seven key NHEJ factors: KU70, KU80, DNA-PKcs, XRCC4, XLF, LIG4, and Artemis (Figure 1.4). KU70 and KU80 both together form the Ku heterodimer. Ku is the first factor to recognize and bind the double stranded break. DNA-PKcs is then recruited to the breaks and together, DNA-PKcs and KU70/80 form the DNA-PK protein complex. DNA-PKcs is a serine/threonine protein kinase and a member of the phosphatidylinositol 3-kinase-related kinase (PIKK) family which includes the DNA repair kinases ATM and ataxia-telangiectasia and Rad3 related protein (ATR). DNA-PKcs has been shown to phosphorylate the other NHEJ factors but it has not been established that any of these phosphorylation events are functionally important. However, it has been shown that DNA-PKcs autophosphorylation is important for the activity of the Artemis nuclease [25]. Artemis activity is important for the repair of a subset of DNA breaks and has an essential role during V(D)J recombination opening of hairpin coding ends. Because this project focused on the Artemis nuclease, a more detailed review of this protein will follow in the next section.

The final stage of repair involves the ligation of the DNA ends. LIG4 and XRCC4 as a complex perform this last step. Interaction between LIG4 and XRCC4 is required

for LIG4 stability [26]. The most recently discovered NHEJ factor XLF/Cernunnos also participates in this step facilitating the ligation of DNA breaks and during V(D)J recombination [27, 28]. XRCC4 and XLF have also been shown to form filaments and the interaction between the two proteins may aid in bridging DNA ends during coding joining [29, 30].

The joining of coding ends during V(D)J recombination often results in an imprecise joint, that is, nucleotides can be added or deleted from the ends prior to ligation. There are two types of nucleotide additions: P-nucleotides and N-nucleotides. Palindromic additions, or P-nucleotides, arise as a product of Artemis hairpin opening. Artemis will sometimes cleave the DNA at a site proximal to the hairpin apex creating a single-stranded overhang with an inverted repeat. The single-stranded DNA overhang is then used as a template and this region is filled in by either polymerase μ (Pol μ) or polymerase λ (Pol λ) prior to ligation. Nucleotide additions can also be non-templated. N-nucleotides are the product of the lymphoid-specific polymerase terminal deoxynucleotidyl-transferase (TdT) which can add random nucleotides to coding ends prior to ligation [31]. Coding joints can also have nucleotides deleted although it is unclear what exonuclease is responsible for this end 'nibbling' [32]. The nucleotide additions and deletions add another layer of diversity to the antigen receptor repertoire. However, this process can also result in out-of-frame, non-productive rearrangements which will be discussed later in this chapter.

The Artemis nuclease

ARTEMIS was first discovered in radiosensitive severe combined immunodeficiency (RS-SCID) patients of Athabascan American Indian origin [33]. These patients had an absence of B and T cells and patient fibroblasts were sensitive to radiation. The de Villartay group found that these patients had mutations in a previously undescribed gene. They named this new gene after the Greek goddess Artemis, a protector of children, presumably because RS-SCID patients generally die within the first year of life. As the patient phenotypes would predict, fibroblasts from these patients were unable to join two coding ends from a plasmid substrate and had been previously shown to be sensitive to ionizing radiation [34]. They analyzed the sequence of the *ARTEMIS* gene and found that it comprised of a metallo- β -lactamase domain and

another domain that was conserved among members of the SNM1/PSO2 family of proteins. Moshous et al. named this second conserved domain β -CASP (metallo- β -lactamase associated CPSF Artemis SNM1/PSO2 domain). At the time of this first report, it was not known what factor was responsible for the cleavage of hairpin coding ends during V(D)J recombination. Because of what was known about other proteins that shared conserved domains with Artemis, the authors speculated that Artemis could have enzymatic activity and furthermore, that they may have identified the nuclease responsible for opening Rag generated hairpin ends.

Additional support for the role of Artemis in opening V(D)J hairpin ends came in biochemical experiments performed by the Lieber group [35]. Ma et al. found that Artemis did indeed have enzymatic activity. Artemis was able to cleave a 5' and 3' overhang DNA substrate as well as a hairpin substrate. However, this enzymatic activity of Artemis was dependent on its interaction with DNA-PKcs. They found that while Artemis is phosphorylated by DNA-PKcs, phosphorylation of Artemis alone was not sufficient for activity. Rather, DNA-PKcs had to be present in order for Artemis to be active. This group also showed that Artemis was able to cleave Rag-generated hairpin end in a biochemical assay. Together, these data provided strong evidence that Artemis was indeed the hairpin opening factor during V(D)J recombination.

The generation of an Artemis-deficient mouse by the Alt group provided *in vivo* evidence of the role of Artemis in V(D)J recombination [36]. Like the human patients, Artemis-deficient mouse embryonic fibroblasts were sensitive to ionizing radiation and were unable to support coding joining of a plasmid substrate but loss of Artemis did not affect signal joining. As it would be predicted for a V(D)J recombination deficient mouse, these mice also had a B and T cell deficiency. This group showed using ligation-mediated PCR that Artemis-deficient thymocytes accumulated closed coding ends providing *in vivo* evidence that the Artemis nuclease is responsible for opening hairpin coding ends.

The regulation of the Artemis nuclease

The mammalian SNM1 family of DNA nucleases includes two other members, SNM1A and SNM1B/Apollo. These three nucleases share the conserved metallo- β -lactamase domain and the β -CASP domain (M β L/ β CASP) (Figure 1.5) [37]. The

Artemis protein has in its N-terminus the M β L/ β CASP domain and in its C-terminus, a poorly characterized domain unique to Artemis. In this study, we will define the N-terminal domain as the amino acids between 1 and 385 and the C-terminal domain as the amino acids between 385 and 692. The entirety of the C-terminal domain is coded by exon 14. Several studies have found that the N-terminal domain of Artemis is able to functionally complement V(D)J recombination of artificial plasmid substrates indicating that the N-terminus is the catalytic domain [38, 39].

In an attempt to study how Artemis is regulated, many studies have focused on two aspects of the protein: phosphorylation and its interaction with the kinase DNA-PKcs (Figure 1.8). The Artemis protein was shown to interact with DNA-PKcs in biochemical assays and furthermore, incubating the two proteins together resulted in the phosphorylation of Artemis [35, 40, 41]. This suggested that DNA-PKcs could possibly regulate Artemis through its kinase activity. Poinsignon et al. found that Artemis could be phosphorylated in DNA-PKcs-deficient CHO cell lines suggesting that other kinases may also act on Artemis [42]. This study and others have suggested that the kinase ATM can phosphorylate Artemis and that it seemed to be the predominant kinase during recovery from ionizing radiation [42, 43]. However, DNA-PKcs can also phosphorylate Artemis in response to DNA damage [41] and the overlap between the two kinases may make the dissection of their respective roles difficult.

In order to further characterize this possible regulatory event, several groups identified the phosphorylation sites of Artemis and attempted to determine its functional importance. ATM and DNA-PKcs has been reported to phosphorylate its substrates at serines or threonines followed by a glutamine (SQ/TQ). The Artemis protein has 10 SQ/TQ sites, 8 of them in the C-terminal domain (Figure 1.8). Using C-terminal truncations mutants, it was found that most of the phosphorylation occurs at the C-terminus [41, 44]. Other groups have mutated the SQ sites in the C-terminus which prevented phosphorylation showing that these sites are indeed the actual phosphorylation sites [25, 42].

Although there has been much research on the phosphorylation of Artemis, there have not been any definitive findings that show phosphorylation has any functional purpose. Artemis C-terminal phosphorylation-deficient mutants as well as phosphatase-

treated Artemis all have biochemical endonucleolytic activity. The phosphorylation event that seemed to be critical for Artemis activity was the autophosphorylation of DNA-PKcs [25, 40, 42]. Phosphorylation-deficient mutants also were able to rescue V(D)J recombination defects in Artemis-deficient fibroblasts [38, 40]. They were able to rescue the radiation sensitivity of Artemis-null cells, as well [42]. Artemis phosphorylation at S516 was correlated being chromatin-bound but this Artemis phosphorylation-deficient mutant appeared no different when compared to wildtype in its nuclear distribution [41]. These data together all suggest that the phosphorylation of Artemis is not functionally important either biochemically or *in vivo*.

One possible function for Artemis C-terminus phosphorylation has been described more recently [45]. It has been shown previously that cells deficient in the homologous recombination (HR) repair pathway proteins, BRCA1 or BRCA2 exhibit sensitivity to drug inhibitors for poly ADP ribose polymerase (PARP). PARP proteins are involved in base excision repair (BER), a pathway that removes damaged nucleotides from the genome. In PARP inhibited cells, single-nucleotide damage resulting from reactive oxygen species normally repaired by BER are converted into DSBs during transcription or replication. These DSBs are then repaired by HR using the sister chromatid. However, HR deficient cells are unable to properly repair these breaks and as a result, exhibit sensitivity to PARP inhibitors (PARPi). [46]

Loss of the two DNA repair factors 53BP1 and PTIP have been shown to rescue PARPi-sensitivity of BRCA1-deficient cells suggesting that removal of these two proteins restores HR [47]. Wang et al. showed that PTIP associated with Artemis and this interaction was dependent on Artemis C-terminus phosphorylation [45]. Like PTIP and 53BP1, loss of Artemis rescued PARPi-sensitivity in BRCA1-deficient cells. These cells remained resistant to PARPi when an Artemis C-terminal phosphorylation-deficient mutant was expressed. The authors speculated that recruitment of PTIP and Artemis to sites of DNA damage prevented HR and promoted NHEJ. This recruitment of Artemis depended on Artemis C-terminal phosphorylation.

This latest finding suggests a functional role for the phosphorylation of the Artemis C-terminus that is revealed in BRCA1-deficient cells. However, it is unclear whether this function would be important during V(D)J recombination. Earlier studies did

not find a role for Artemis C-terminal phosphorylation in DNA repair or V(D)J recombination. It is possible that the defect of phosphorylation-deficient mutants in earlier studies were masked because of their overexpression. While the loss of Artemis phosphorylation may not completely abrogate localization or its activity in DNA repair or V(D)J recombination, these results do not exclude the possibility that phosphorylation may have more subtle roles that would be apparent if the mutant proteins were expressed from an endogenous promoter.

The structure-function analyses of Artemis have indicated that the interaction between Artemis and DNA-PKcs has some functional importance. Soubeyrand et al. determined that the amino acids L401 and L402 of Artemis were important for DNA-PKcs interaction (Figure 1.8) [41]. Mutating these two sites abrogated *in vitro* phosphorylation by DNA-PKcs and reduced phosphorylation *in vivo* by 2-fold. However, this group found that their Artemis L401G/L402N mutant facilitated V(D)J recombination of a plasmid substrate in Artemis-deficient fibroblasts. A later study used this same mutant in a similar assay using Artemis-deficient human pre-B cells and found that loss of the DNA-PKcs interaction domain did negatively impact V(D)J recombination frequency about 2-fold [48]. The difference in findings demonstrates that cellular environment may influence *ex vivo* V(D)J recombination assays.

Other groups and our lab have shown that the loss of the Artemis C-terminus negatively impacts biochemical endonuclease activity illustrating that this domain is important for promoting activity [44, 49]. However, the C-terminal domain seems to be important for promoting activity in the presence of DNA-PKcs but also inhibiting activity when DNA-PKcs is absent. Wildtype Artemis nuclease has no activity until it is in the presence of DNA-PKcs. However, loss of the entire Artemis C-terminal domain has been reported to result in a low level of DNA-PKcs-independent activity [44, 49]. Thus, the Artemis C-terminus may normally function to inhibit the catalytic activity and interaction with DNA-PKcs relieves this inhibition. How the Artemis C-terminus may promote activity is unclear. Biochemical studies mentioned above using Artemis phosphorylation-deficient mutants indicate that phosphorylation itself does not affect nucleolytic activity. And so the Artemis C-terminus may have structural importance in promoting DNA cleavage possibly by stabilizing interaction with the DNA substrate.

B lymphocyte development

The development of B and T lymphocytes is a highly regulated process. The timing of Rag generated breaks is coordinated with the different stages of lymphocyte development (Figure 1.6) [50]. Hematopoietic stem cells in the bone marrow differentiate into lymphoid progenitors and these progenitors go on to develop into B cells or migrate to the thymus and develop into T cells. The B cell immunoglobulin consists of four components, two identical heavy chain proteins and two identical light chain proteins. Rearrangements to produce the heavy chain occur first during the pro-B cell stage. At the heavy chain locus, recombination of the D_H and J_H segments occurs and is then followed by the V_H segment joining to the recombined D_HJ_H segment. Transcription of this region followed by RNA splicing and translation generates a heavy chain with a V, D, and J segment and a constant region that is present on all newly formed heavy chains ($C\mu$). This constant region of the heavy chain protein determines the isotype of the antibody. In humans, there are five antibody isotypes, IgG, IgA, IgM, IgD, and IgE. The biological properties and distribution of antibodies are determined by its isotype. For instance, IgM, important for fungal and bacterial infections, is primarily found in serum. [51, 52]

The generation of the heavy chain is followed by light chain rearrangement. Light chains are encoded by one of two multigene families, kappa (κ) and lambda (λ). The κ light chain rearranges first. This event only involves the V_k and J_k segments as light chains do not have D segments. If the rearrangement of the κ light chain is successful, the B cell then expresses immunoglobulin consisting of a heavy chain (IgH) and a κ light chain (IgL κ). In the event that κ rearrangement is unsuccessful, the λ light chain genes are rearranged. [52]

There are numerous V, D, and J segments in the recombining loci. For example, the murine heavy chain locus has 134 V_H segments, 13 D_H segments, and 4 J_H segments. The rearrangement of the various segments represents one level of variability that can be generated during this process. Deletions and additions of

nucleotides at the junction between joined segments provide yet another level of diversity. [52]

Although junctional diversity can increase receptor variability, it can also increase the likelihood of an unsuccessful rearrangement. Depending on what and how many nucleotides are added to the junction, this can either generate a productive join or an early stop codon and a nonproductive join. However, because the genome contains two copies of each loci, rearrangements at the second allele are initiated if rearrangement at the first locus is nonproductive. In addition, because there are two multigene light chain families, λ light chain rearrangements will begin if both κ light chain loci recombine nonproductively. In the event that both heavy chain rearrangements or both κ and λ light chain rearrangements are nonproductive, the cell undergoes programmed cell death. [52]

To prevent the expression of more than one recombined immunoglobulin, recombination of the second allele should only be initiated if the first one was unsuccessful. One model that explains the suppression of the second allele, or allelic exclusion, is the feedback inhibition of recombination by the immunoglobulin protein. Support for the feedback inhibition model has been demonstrated in mice expressing an Ig transgene. These transgenic mice do not rearrange their Ig loci suggesting the expression of Ig protein suppresses recombination. [53]

B-lymphocytes that successfully undergo heavy chain and light chain rearrangement then leave the bone marrow. These cells then migrate to the lymph node and spleen where the variable regions of the heavy chain and light chain locus are further diversified by somatic hypermutation. During this process, point mutations, insertions, and deletions are targeted to the rearranged V, D, and J segments. These mutations can either increase or decrease their affinity toward antigen which in turn, plays a role in the selection process of B cells. [52]

T lymphocyte development

There are many parallels between the development of B cells and T cells. T cells begin their lives as lymphoid progenitors in the bone marrow and migrate to the thymus (Figure 1.6). Like B cells, rearrangements in T cells is highly regulated and ordered. T cells can be categorized as $\alpha\beta$ T cells or $\gamma\delta$ T cells depending on whether they express

the $\alpha\beta$ receptor or the $\gamma\delta$ receptor on their cell surface. Analogous to the heavy chain and light chain found in B cells, the T cell β chain δ chain is comprised of V, D, and J segments while the α and γ chains are comprised of V and J segments do not have D segments. [52]

Rearrangements in T cells begin in the double negative stage, a name which refers to the lack of either co-receptors CD4 and CD8 displayed on the cell surface. D β to J β rearrangements at the β -chain occurs first and is followed by V β to D β J β recombination. Cells that are unable to productively rearrange both alleles of their β -chain undergo apoptosis. After successful rearrangements at the β -chain, the TCR β protein is expressed. This protein then associates with a pre-T α chain protein together forming the pre-T-cell receptor or pre-TCR. The pre-TCR initiates signaling pathways that suppresses further TCR β chain rearrangements and promotes the rearrangement of the TCR α chain. The cell then enters the double positive cell stage (CD4+ CD8+) and initiates V to J α chain rearrangement. After successful recombination of the TCR α chain, the T cell then enters the single positive stage where they are either expressing CD4 or CD8 on the cell surface. Unlike BCRs, TCRs do not undergo somatic hypermutation but do exhibit the same types of junctional diversity seen in BCRs.[52]

$\gamma\delta$ T cells rearrange both the γ and δ chain in the double negative stage.

Rearrangement of the γ , δ , and β chain is thought to occur within a similar timeframe. DSBs have been observed at all three loci in the same cell in wildtype thymocytes suggesting that at least some of the time, rearrangements at the three loci occur simultaneously [54]. It is unclear how the cell determines whether to develop into a $\gamma\delta$ T cell or an $\alpha\beta$ T cell but there is some evidence showing that this decision may be determined even before rearrangements at any loci. One group found that in developmental stages before rearrangement, cells expressing high levels of interleukin-17 receptor (IL-17R) are more likely to become $\gamma\delta$ T cells and cells with low levels of IL-17R more likely to become $\alpha\beta$ T cells [55].

B and T cell selection

The survival of lymphocytes depends on many factors. Rearrangements must be productive, generating a functional protein product. Another requirement for the survival of lymphocytes is that they cannot bind to self-antigens. This process of removing

lymphocytes recognizing self-antigens is referred to as negative selection. The negative selection of B cells occurs in the bone marrow. B lymphocytes with BCRs that bind to self-antigens are programmed for cell death (clonal deletion) or become unresponsive (anergic). However, B cells that bind to self-antigens in the bone marrow can undergo receptor editing. In this process, V(D)J recombination is initiated again at the light chain to produce another immunoglobulin, potentially with no reactivity to self-antigens. B cells that leave the bone marrow and may bind to self-antigens that were not present in the bone marrow and will undergo clonal deletion or become anergic. [56]

Unlike B cells, most T cells cannot recognize antigen without its presentation by a major histocompatibility complex (MHC) protein. As a consequence, developing T cells encounter two types of selection, positive selection and negative selection. During positive selection, thymic stromal cells, macrophages, and dendritic cells in the thymus present MHC protein to developing thymocytes. The ability to bind this protein promotes survival. During negative selection, dendritic cells and macrophages in the thymus present developing T lymphocytes with MHC protein and self-antigen. Thymocytes that bind to MHC alone or MHC with self-antigen are also programmed for cell death. Surviving cells then go on to the single positive stage where they are either expressing CD4 or CD8 on the cell surface.[52]

Artemis C-terminal domain

Most human patients with Artemis related RS-SCID have mutations in the N-terminus of the protein (Figure 1.7) [57]. Mutations in this domain often result in loss of the protein or protein activity and these patients are characterized by an absence of B and T cells. Patients with mutations in the Artemis C-terminus have also been reported. Cell-based V(D)J recombination assays expressing C-terminal truncated Artemis protein have previously suggested that this domain is not important for V(D)J recombination [38]. However, human patient disease alleles have indicated that loss of the C-terminus has detrimental effects on lymphocyte development, immunity, and the prevention of tumorigenesis.

Moshous et al. have reported four patients with Artemis C-terminal truncation mutants [58]. Three of these patients had a 7 nucleotide deletion at aspartic acid 451 leading to a premature stop codon 10 amino acids downstream. On their second allele,

these patients had a genomic deletion of exons 1-3 resulting in no mRNA expressed. One of the four patients was a homozygous mutant with 17 nucleotides deleted at threonine 432 resulting in a premature stop codon 15 amino acids downstream. These four patients had mutations that resulted in the loss of most of the C-terminal domain.

The Artemis C-terminal truncation mutant patients all had B and T lymphocytopenia. Two of the patients died from infection and the two others succumbed to Epstein-Barr virus (EBV)-associated B cell lymphoma. Patient fibroblasts did not facilitate V(D)J recombination of a plasmid substrate and were sensitive to ionizing radiation. The patient phenotypes and the phenotypes of their fibroblasts illustrated that the Artemis C-terminal domain does have functional roles in V(D)J recombination and DNA repair. It is likely that in cell-based V(D)J recombination assays done in the past using plasmid-expressed mutant Artemis may have overexpressed the nuclease. However, the human patients and patient fibroblasts expressing Artemis from an endogenous promoter were able to reveal important functional roles of the C-terminus.

In order to better understand the function of the C-terminus, our lab generated a Artemis mutant mouse modeled after a human patient allele which we refer to as Artemis-P70 [49].(Figure 1.8) This Artemis-P70 mouse has a premature stop codon introduced to aspartic acid 449, the murine site orthologous to the human D451. We have previously reported that our Artemis-P70 mouse, like the human patients, have reduced B and T lymphocyte numbers. Artemis-P70 murine fibroblasts are sensitive to ionizing radiation and bleomycin demonstrating the importance of the C-terminus in DNA repair. Our lab has also aged the Artemis-P70 mice and found that 2 of 14 succumb to thymic lymphoma during the course of the 40 week observation period [59]. Together, these findings illustrate that the Artemis C-terminal domain plays important roles in lymphocyte development, DNA repair, and the prevention of tumorigenesis.

Ataxia-telangiectasia mutated (ATM)

Ataxia-telangiectasia mutated (ATM) is a serine/threonine protein kinase that is mutated in patients with ataxia-telangiectasia (A-T). A-T patients are characterized neural degeneration, ataxia, oculocutaneous telangiectasias, mild immunodeficiency, premature aging, and sensitivity to ionizing radiation [60]. Lymphocytes from most A-T patients harbor chromosomal translocations and about 10% of patients develop

lymphoid malignancies [61]. ATM deficient mice have been generated to study this kinase *in vivo* [60, 62]. Like the patients, these mice exhibit mild immunodeficiency and sensitivity to ionizing radiation. Unlike in human patients, neurological effects of ATM deficiency are very mild [60]. In addition, nearly all the mice succumb to thymic lymphoma within 2 to 4 months.

This kinase plays a central role in initiating cell cycle arrest and DSB repair. Cells deficient in ATM have defective G1/S, intra-S, and G2/M checkpoints [63] and are also radiosensitive [61]. About 10% of DSBs seem to require ATM for proper repair [43]. The MRE11-RAD50-NBS1 (MRN) complex is thought to sense DSB ends which then recruits and activates ATM. Inactive ATM exists as a homodimer but when activated, dissociates into monomers [64]. Upon activation, ATM phosphorylates itself as well as about 700 other substrates [65]. A few substrates of note include the cell cycle factors CHK2 kinase and p53 and the repair factors MRN the histone H2AX.

ATM has been shown to be important for the prevention of aberrant events during V(D)J recombination [66]. Bredemeyer et al. showed that pre-B cells deficient in ATM accumulated aberrant hybrid joins, a V(D)J joining event between a coding end and a signal end. Similar results were found when cells were treated with the ATM kinase inhibitor KU-55933 suggesting that it was the kinase activity of ATM that is important for preventing aberrant events. The same group later reported that MRN-deficient pre-B cells exhibited a similar phenotype with increased aberrant hybrid joining when cells were induced to undergo V(D)J recombination [67]. The authors speculated that ATM and the MRN complex may be important for stabilizing the post-cleavage complex (PCC), a complex of proteins thought to be necessary for the synapsis of DNA ends after Rag cleavage. RAD50 protein has been shown to have a “hook domain” that can interact with the hook domain of another RAD50 protein [68] and MRE11 was found to form homodimers that can bind and align DNA ends, facilitating repair [69]. In addition, ATM has been shown to phosphorylate the three components of the MRN complex [65]. The authors hypothesized that ATM phosphorylates members of the MRN complex which regulates the DNA synapsis functions of this complex. Loss of either factors, the MRN complex or the ATM kinase activity, results in a destabilized PCC and coding ends are released with some of them engaging in aberrant rearrangements.

As mentioned above, the ATM kinase can phosphorylate the Artemis protein and seems to be the primary kinase for this event during recovery from ionizing radiation [42, 43]. However, the function of this phosphorylation event is unclear. There have been some reports that suggest Artemis may function epistatically to ATM in the repair of ionizing radiation-induced DSBs [43]. Loss of either factor in fibroblasts results in similar levels of unrepaired breaks after treatment with ionizing radiation. In addition, inhibition of ATM kinase activity with KU-55933 in Artemis-null fibroblasts did not increase levels of unrepaired breaks after ionizing radiation. This same group later reported that Artemis nuclease activity is important in homologous recombination during G2 and this event is mediated by ATM [70]. The authors suggest that while most breaks are repaired in NHEJ in both G1 and G2, heterochromatin associated DSBs in G2 may require ATM and Artemis to process DNA ends prior to resection and initiation of homologous recombination.

Although there is evidence that ATM and Artemis may function epistatically in the repair of ionizing radiation induced breaks, it remains unclear whether ATM and Artemis may function in the same pathway during V(D)J recombination. It has been shown before that ATM cannot activate Artemis endonucleolytic activity in biochemical assays [25]. However, we have noted phenotypic similarities between our Artemis-P70 mice and ATM^{-/-} mice. Because both mice exhibit a mild lymphocyte deficiency and a predisposition to thymic lymphoma, it is tempting to speculate that ATM and Artemis may function epistatically in the prevention of aberrant rearrangements and loss of either factor may result in impaired lymphocyte development and tumorigenesis.

In this study, I further explore the role and regulation of the Artemis nuclease both *in vitro* and *in vivo*. In Chapter 2, I investigate the factors that are important for the regulation of Artemis during V(D)J recombination and examine the consequences of the loss of regulation. My findings suggest that the Artemis C-terminus may have important roles in the prevention of aberrant rearrangement events and that the DNA-PKcs interaction domain may contribute to PCC stability. Results from Chapter 2 also suggest that there may be features of the Artemis N-terminal domain outside of its already established nuclease activity that may facilitate V(D)J recombination.

In Chapter 3, I examine the relationship between the ATM kinase and the Artemis C-terminus *in vivo*. Analyzing Artemis-P70 ATM^{-/-} mice, I find that the loss of both ATM and the Artemis C-terminus have an additive impact on lymphocyte deficiency but unlike ATM^{-/-} mice or Artemis-P70 p53^{-/-} mice, none of these mice develop tumors. Instead these mice exhibit a susceptibility to colitis. These findings suggest that ATM and the Artemis C-terminus cooperate to facilitate lymphocyte development and the severe but not total loss of lymphocyte populations may promote chronic inflammation in the large intestine.

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Figures

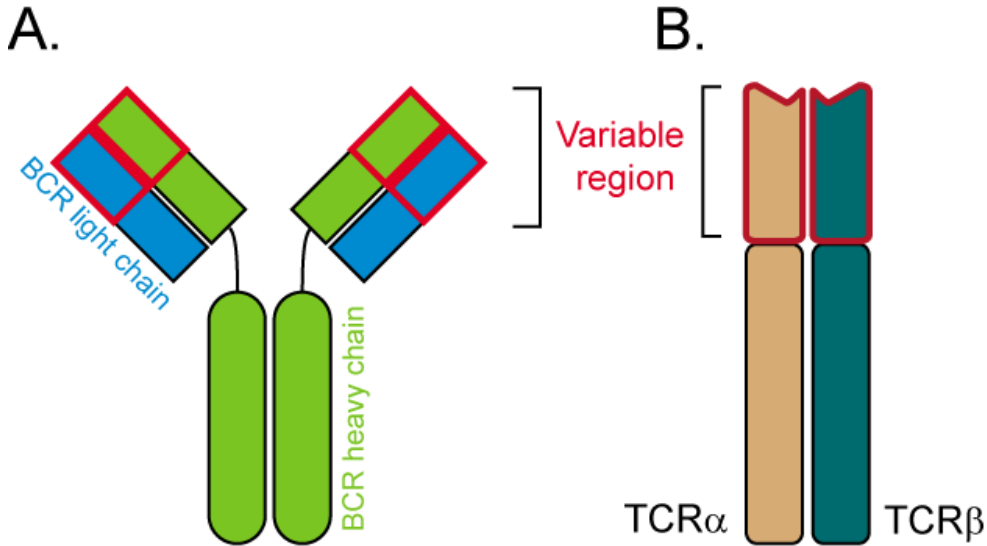


Figure 1. 1 The B-cell receptor (BCR) and the T-cell receptor (TCR). A) The BCR consists of both a light chain (in blue) and a heavy chain (in green). The variable region of the receptor is outlined in red. B) The TCR consists of two chains. Shown is a depiction of the $\alpha\beta$ receptor with the α -chain in beige and the β -chain in dark blue. The variable region is outlined in red.

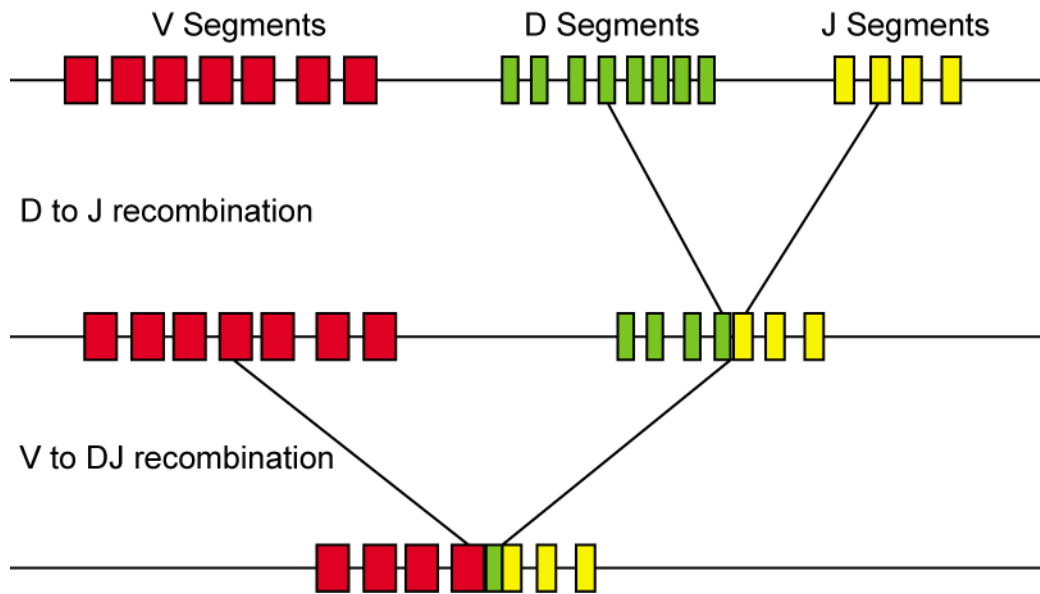


Figure 1. 2 V(D)J recombination. V, D, and J segments are joined to generate the variable region of the antigen receptor. D and J segments are recombined first followed by V to DJ rearrangement. Janeway et al. was used as a source for making this figure [71].

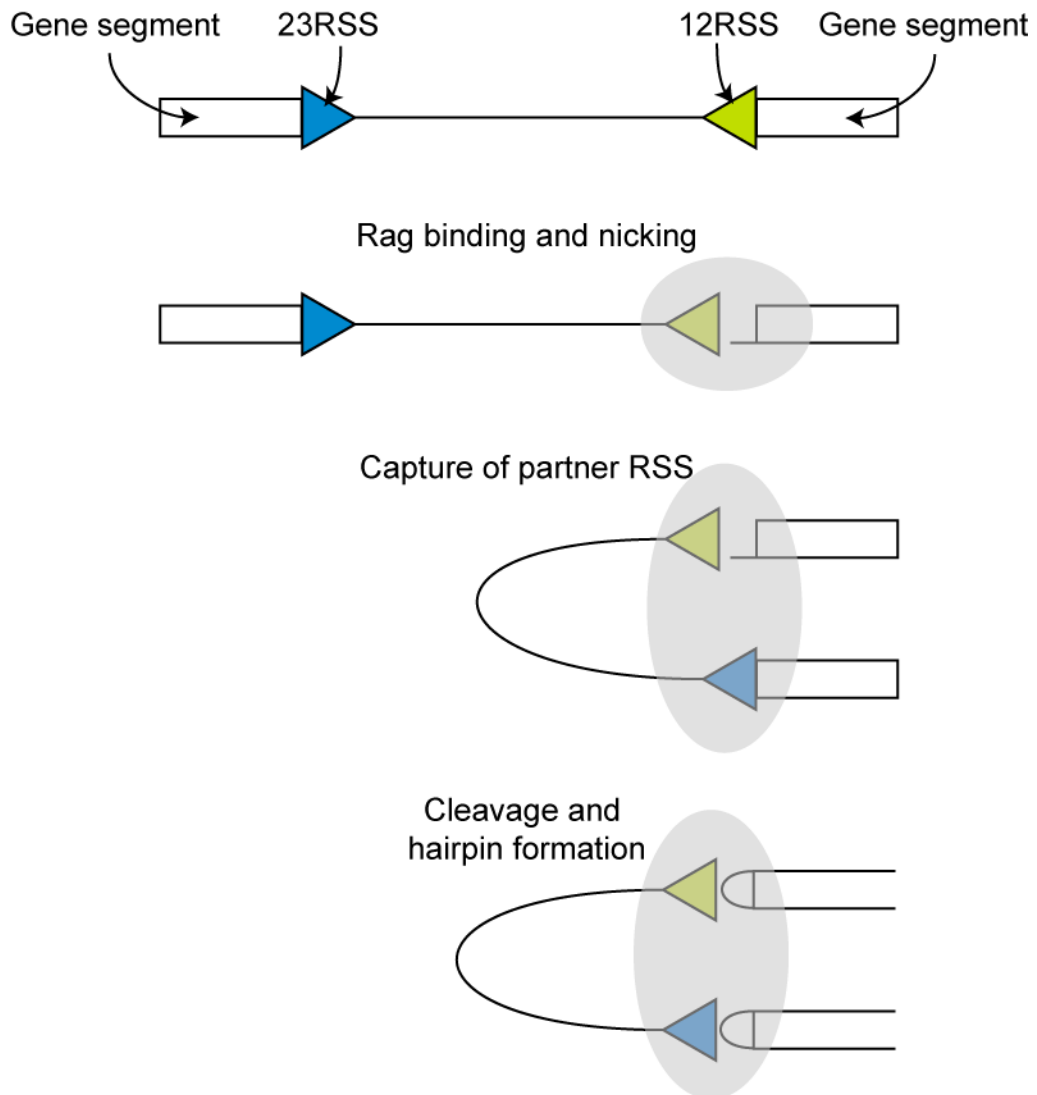


Figure 1. 3 Rag capture of 12RSS and 23RSS. The Rag endonuclease (grey ellipse) binds one RSS (triangle) and may nick the DNA creating a 3' hydroxyl group. The Rag complex then captures the partner RSS and nicks the DNA at the second RSS. A nucleophilic attack on the second strand is catalyzed creating two closed hairpin coding ends.

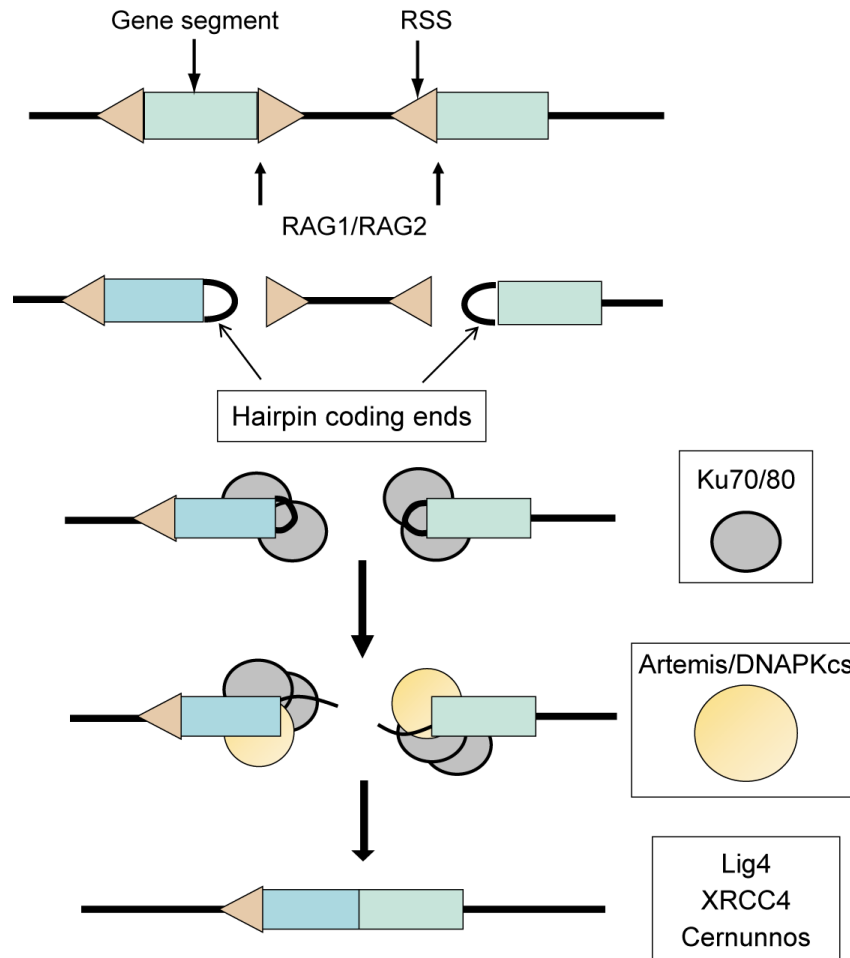


Figure 1. 4 Rag generated ends are joined by the NHEJ pathway. The Rag endonuclease creates a DSB at the junction between the RSS (triangle) and the gene segment (rectangle). This generates a closed hairpin coding end adjacent to the gene segment and a blunt signal end. These ends are then handed off to the NHEJ pathway. KU70 and KU80 (grey ellipse) bind to the DNA ends and recruit DNA-PKcs. DNA-PKcs then recruits Artemis (peach sphere) which opens the hairpin ends. Coding ends are then joined by the LIG4/XRCC4/Cernunnos ligation complex.

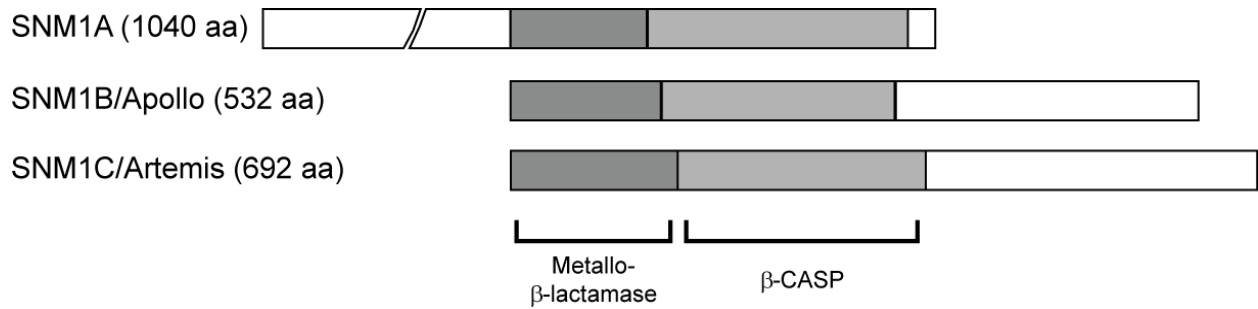


Figure 1. 5 The mammalian SNM1 family of nucleases. Shown here are the three human SNM1 nucleases. The three members of the SNM1 family of proteins share two conserved domains, the metallo- β -lactamase domain and the β -CASP domain. Each member also has regions of the proteins that are unique (white regions). Sengerova et al. was used as a model for this figure [72].

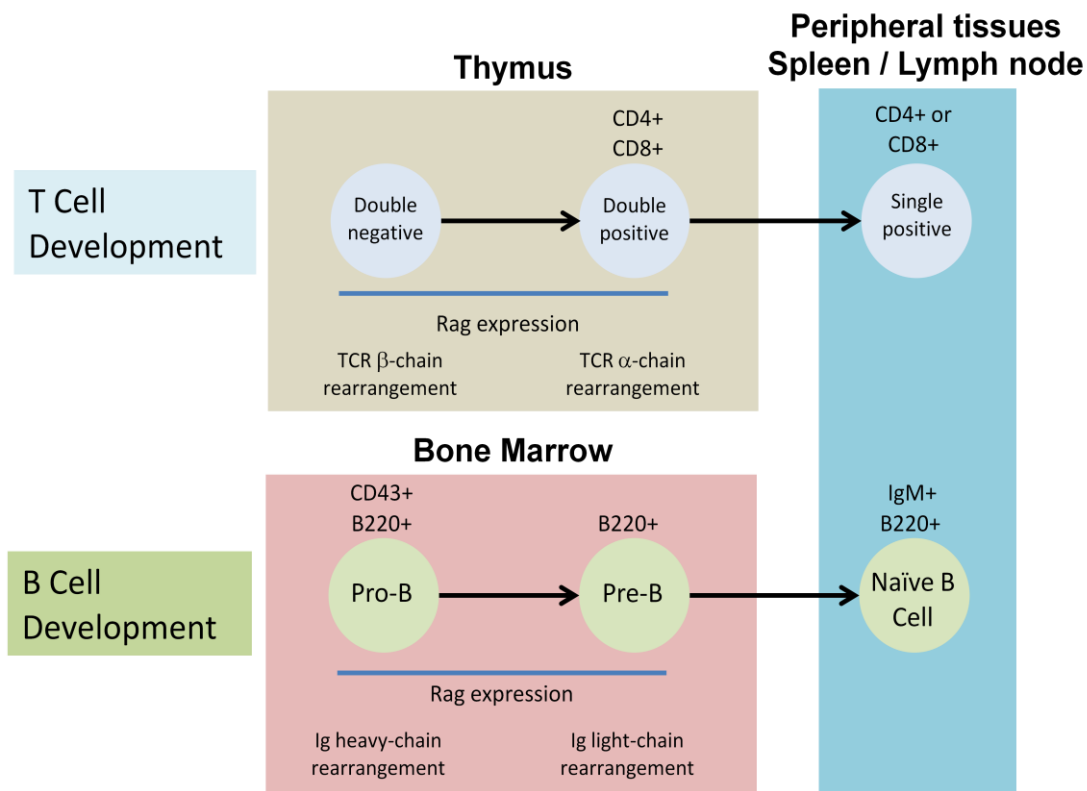


Figure 1. 6 B and T cell development. B and T lymphocytes both arise from hematopoietic stem cells in the bone marrow. T lymphocyte progenitors migrate to the thymus and undergo V(D)J recombination. During the double negative stage (CD4⁻CD8⁻), Rag is expressed and the TCR β chain is rearranged. Double negative cells that successfully rearrange the TCR β chain proceed to the double positive stage (CD4⁺CD8⁺) where rearrangement of the TCR α chain occurs. Developing T cells then undergo positive and negative selection in the thymus. Surviving T cells then migrate to the peripheral tissues as single positive T cells (CD4⁺ or CD8⁺). B cells undergo V(D)J recombination in the bone marrow. Rag expression is initiated at the pro-B cell stage (CD43⁺B220⁺) where the Ig heavy chain is rearranged. Cells that successfully rearrange this locus proceed to the pre-B cell stage (CD43⁻B220⁺) when the Ig light chain is rearranged. These B cells then undergo negative selection in the bone marrow. Cells that survive this process then migrate to the periphery as a naïve B cell (IgM⁺B220⁺).

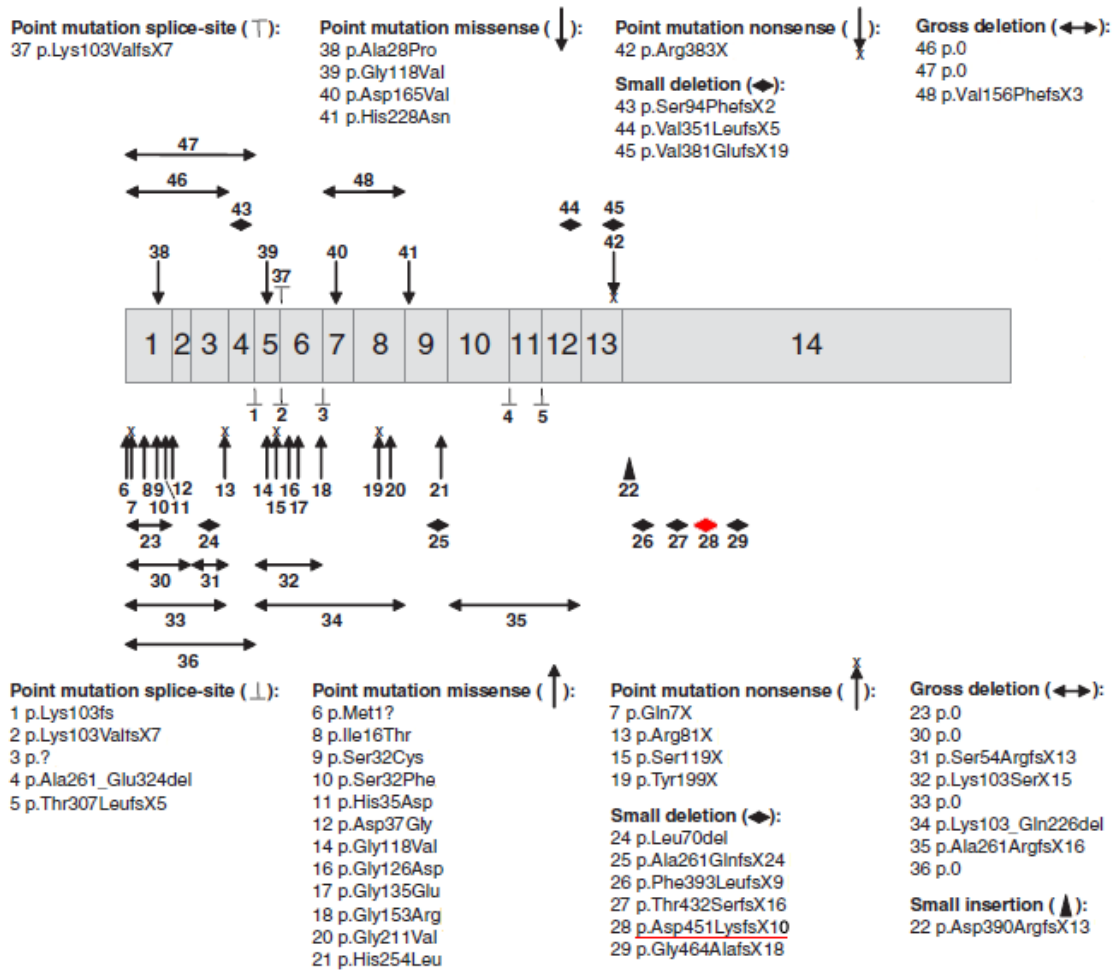


Figure 1. 7 Artemis human patient alleles. The majority of Artemis human patient alleles that have been reported have mutations in the N terminal domain of Artemis. The N-terminal domain, which constitutes the catalytic core of the protein, is coded by exons 1 -13. The last exon, exon 14, codes for the C-terminal domain. Some patient mutations have been reported to occur in this domain. This project focuses on a particular allele which we refer to as Artemis-P70 and is the result of a 7 nt deletion at aspartic acid 451 (mutation number 28 in this figure and highlighted in red). This figure was adopted and modified from Pannicke et al. [57].

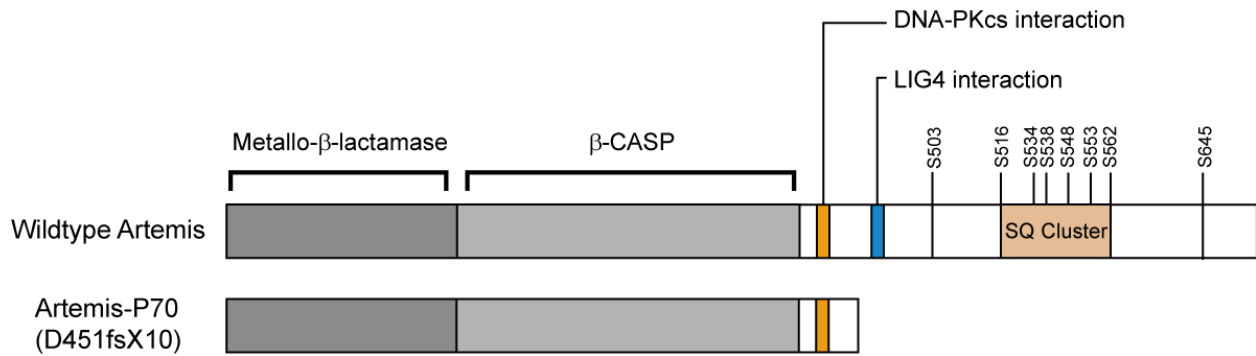


Figure 1. 8 Features of the Artemis protein. Shown here are depictions of human wildtype Artemis and human mutant D451fsX10 Artemis protein. Wildtype Artemis has 8 SQ phosphorylation sites at its C-terminus including 6 that are clustered together. This domain has also been found to interact with DNA-PKcs (L401, R402) and LIG4 (W489). Most human patient mutations in Artemis occur within the metallo-β-lactamase domain or the β-CASP domain. However, mutations at the C-terminal domain have also been reported. The Artemis-P70 human patient allele contains a 7 nucleotide deletion at D451 that results in an early stop codon 10 amino acids downstream. Patients with this allele lose most of the C-terminal domain, the 8 phosphorylation sites, and the LIG4 interaction region.

Chapter 2

The Regulation of Artemis During V(D)J Recombination

Abstract

The Artemis nuclease has important DNA end-processing roles in double-stranded break repair and V(D)J recombination. Disruptions in this gene can result in severe combined immunodeficiency and radiosensitivity (RS-SCID). Artemis hypomorphic mutations involving the C-terminal domain have been described and these patients have been reported to be predisposed to lymphoid malignancies. Here, we investigate the factors involved in the regulation of the Artemis nuclease as well as the consequences of the loss of regulation. First, I examine the role of the Artemis C-terminus in regulating endonucleolytic activity and proper V(D)J recombination. I define a region of the Artemis C-terminal domain that is critical for endonucleolytic activity and also demonstrate that the C-terminus prevents aberrant V(D)J recombination possibly through interaction with the DNA dependent protein kinase catalytic subunit (DNA-PKcs). In addition, I show that the Artemis C-terminus and the ataxia telangiectasia mutated (ATM) kinase work cooperatively to prevent these aberrant products.

In this chapter, I also describe findings that suggest that there may be regulatory features in the conserved N-terminal domain of Artemis that have yet to be defined. I describe two previously unreported regions of the Artemis N-terminus that were important for endonucleolytic activity and also characterize three Artemis mutants based on human patient alleles using cellular and biochemical approaches. Results from this project indicate that phenotypes observed in two human patients may have resulted from protein destabilization. Together, these findings illustrate that loss of Artemis regulation can result in a dysfunctional protein that can catalyze aberrant rearrangements. In addition, these results suggest that there may be other regulatory domains in the Artemis N-terminus that can facilitate V(D)J recombination.

Introduction

B and T cell antigen receptors must be able to recognize and bind to a myriad of substrates including foreign molecules and pathogens. In order to create this level of diversity in antigen receptors, developing B and T cells employ a gene rearrangement strategy referred to as V(D)J recombination. V(D)J recombination occurs during early lymphocyte development and these rearranged gene segments go on to encode the variable regions of antigen receptors. Adjacent to the gene segments are recombination signal sequences (RSSs) recognized by the Rag endonuclease. The Rag endonuclease comprising of the two proteins RAG1 and RAG2, initiates recombination by creating a single stranded nick at the junction between the RSS and the gene segment creating a 3' hydroxyl group. This hydroxyl group acts as a nucleophile and attacks the second strand, creating a double-stranded break (DSB) with one hairpin-sealed coding end and a blunt signal end. In order to join two different gene segments, the hairpin coding ends must be cleaved open. The Artemis nuclease and the serine/threonine protein kinase DNA-PKcs were found to be required for opening these hairpin ends during V(D)J recombination [1].

Artemis belongs to the SNM1 family of proteins which includes the nucleases Snm1A and Snm1B. These three proteins all have the metallo- β -lactamase and β CASP domain which together form the catalytic domain of the protein. The Artemis protein also has a C-terminal domain that is unique among its family members. This C-terminal domain was found to be important for interacting with DNA-PKcs and the nonhomologous end-joining ligase, LIG4 [2, 3].

Human patients with Artemis C-terminal truncations have been reported in the literature. Three human patients reported were found to have a 7 nucleotide deletion that resulted in a frameshift at aspartic acid 451 and an early stop codon 10 amino acids downstream (patient allele referred to as P70). The second allele of these patients, a deletion of exons 1-3, did not produce mRNA. These patients were found to be immunodeficient with low B and T lymphocyte counts. Additionally, human patient fibroblasts were sensitive to ionizing radiation. Because immunodeficiency and ionizing sensitivity was not as severe as human patients with mutations in the catalytic domain,

this mutant was categorized as hypomorphic. Strikingly, two of the three patients succumb to Epstein-Barr virus (EBV)-associated B cell lymphoma. These B cell proliferations were had clonal IgH rearrangements and lymphocytes from these patients harbored chromosomal alterations. Together, these findings suggested the malignancies were at least partially driven by genomic instability. The patient phenotypes indicated that the Artemis C-terminus may have roles in V(D)J recombination, DNA repair in response to ionizing radiation, and preventing lymphoma. [4]

We have previously generated an Artemis-P70 mouse model by introducing a stop codon at aspartic acid 449 of the endogenous murine Artemis gene. Like their human patient counterparts, these mice exhibited reduced numbers of B and T cell lymphocytes with fibroblasts sensitive to ionizing radiation. Additionally, two of fourteen Artemis-P70 mice succumb to lymphoma within 40 weeks. When this mutation is combined with a p53-null background, all mice succumbed to lymphoma. Moreover, Artemis-P70 p53^{-/-} mice die significantly earlier than p53-null single mutant mice. These tumors harbored clonal rearrangements as well as translocations involving the antigen receptor rearranging loci. Together, these data strongly implicate the Artemis C-terminus in V(D)J recombination and preventing tumorigenesis. [5, 6]

Rag-generated DNA ends are held together by a protein-DNA complex referred to as the post-cleavage complex (PCC). The Rag endonuclease itself is thought to be important for preventing both the loss of DNA ends and aberrant rearrangements. Cleavage at RSS has been shown to only require the “core” Rag proteins, the C-terminal domain of RAG1 and the N-terminal domain of RAG2. The non-core N-terminus of RAG1 has been shown in biochemical assays to be dispensable for cleavage of a RSS substrate but was important for the retention of hairpin ends in the PCC [7]. Another group similarly showed biochemically that the RAG2 C-terminus was important for the retention of coding and signal ends in the PCC. Supporting the idea that the RAG2 C-terminus is important for preventing loss of DNA ends, splenocytes from core-RAG2 mice also had increased aberrant rearrangements [8].

The ability of the RAG1 point mutants S723A and S723C in supporting stable PCC have also been studied biochemically. Tsai et al. found that PCCs consisting of

RAG1 point mutants did not bind to DNA ends as well as wildtype protein. [9] Our lab has generated the Rag1 S723C point mutant mouse and found that this mouse exhibits impaired V(D)J recombination *in vivo* and in addition, thymocytes from these mice displayed increased aberrant rearrangements. When these mice were crossed into a p53-null background, double mutant mice succumbed to thymic lymphomas that frequently had clonal translocations making these thymic lymphomas distinct from p53 single mutant lymphomas [10]. Together, these results indicate that the RAG proteins have important roles maintaining PCC stability and that loss of stability can result in aberrant rearrangements and tumorigenesis.

In addition to the Rag endonuclease, other proteins are also thought to be important for preventing aberrant rearrangements by stabilizing the post-cleavage complex. Ataxia-telangiectasia mutated (ATM) is a serine/threonine protein kinase that is mutated in patients with ataxia-telangiectasia. This kinase has important roles in cell cycle regulation as well as DSB repair. Using ATM-null murine pre-B cells, Bredemeyer et al. observed aberrant rearrangement events when these cells were induced to undergo V(D)J recombination [11]. The treatment of these cell lines with the ATM kinase inhibitor KU55933 yielded similar results suggesting it is the kinase activity of ATM that is important for preventing aberrant V(D)J events.

The MRE11-RAD50-NBS1 (MRN) protein complex has also been suggested to be important for PCC stability. This protein complex has roles in DSB repair as well as efficient activation of the ATM kinase. Using cell lines expressing hypomorphic Mre11 and Nbs1, the Helmink et al. found that MRN-hypomorphic cell lines had increased aberrant recombination products similar to results seen in ATM deficiency. [12]

Our characterization of the Artemis-P70 mouse model has led us to suspect that the Artemis C-terminal domain may be important for maintaining post-cleavage complex stability [5, 6]. Lymphocytes from Artemis-P70 mice accumulate coding ends and display increased aberrant rearrangements at antigen receptor loci. We have also found that Artemis-P70/p53 mice succumb to lymphoma with translocations involving rearranging loci. In this study, we further characterize the roles of the Artemis C-terminus during V(D)J recombination and examine what factors are important for proper rearrangement. We identified a part of the Artemis C-terminal domain that is important

for full endonucleolytic activity and that the C-terminus is important for the prevention of aberrant rearrangements. We did not find that loss of the Artemis C-terminus altered ATM activation or MRN stability. However, loss of the DNA-PKcs interacting domain further increased aberrant rearrangements. In this study, we also discovered previously uncharacterized regions of the conserved N-terminal domain that are important for endonucleolytic activity.

Results

The proximal region of the Artemis C-terminus is important for endonucleolytic activity

We and other labs have previously shown that the Artemis C-terminus has important roles in regulating Artemis endonucleolytic activity [5, 13]. The C-terminal domain has been found to be important for promoting endonucleolytic activity in the presence of DNA-PKcs but also seems to inhibit activity in the absence of DNA-PKcs [5, 13]. To further characterize the regulation of activity by the Artemis C-terminus, several Artemis truncation mutants were constructed (Figure 2.1). The M β L/ β CASP construct consists of amino acids 1-385 and is missing the entire C-terminus including the DNA-PKcs and Lig4 interaction domain. The D451X truncation mutant (amino acids 1-451) is modeled after the Artemis-P70 human disease mutation and retains the DNA-PKcs interaction domain (L401 and R402). The Δ 502-692 truncation mutant includes the both the DNA-PKcs and LIG4 interaction domain (W489). The Artemis C-terminus contains a cluster of SQ sites between S516 and S562 which are demonstrated to be phosphorylated by DNA-PKcs and the ATM kinase [2, 14, 15]. The truncation mutant Δ 539-692 and Δ 599-692 include part or all of the SQ cluster domain. Lastly, the Δ 646-692 mutant includes all the features above plus an additional SQ phosphorylation site S645, a site which has been shown *in vivo* to be phosphorylated by ATM [15]. These mutant proteins were expressed in 293T cells and the Artemis proteins were purified from DNA-PKcs. Purification from DNA-PKcs and Artemis protein normalization was confirmed by Western blot analysis (Figure 2.2, A).

I analyzed the ability of these mutants to cleave a 32 P 5' end-labeled 40-bp hairpin substrate. Cleavage at the apex of the hairpin by Artemis yields a 20 nucleotide

product. I found that complete loss of the Artemis C-terminus resulted in low levels of DNA-PKcs-independent activity (Figure 2.2, B., second lane). This result corroborates with previous findings and supports the notion that the C-terminal domain may have an inhibitory function in the absence of DNA-PKcs. Three truncation mutations M β L/ β CASP, D451X, and Δ 502-692 were unable to cleave the hairpin substrate at levels matching the wildtype Artemis protein. However, the three truncation mutants Δ 539-692, Δ 599-692, and Δ 646-692 had wildtype levels of nucleolytic activity indicating that amino acids 599 to 692 are dispensable for full biochemical activity. This result also demonstrates that the C-terminal region between amino acid 385 and 539 contains an important region that is critical for nucleolytic activity. Although these three mutants include either part of or all of the SQ cluster, it is unlikely that full nucleolytic activity can be attributed to the presence of these phosphorylation sites. Past studies using Artemis C-terminal phosphorylation-deficient mutants demonstrated that Artemis phosphorylation did not seem to be important for biochemical activity [14-16]. It is possible that this region may be important for stabilizing interaction with DNA-PKcs or the DNA end.

Increased aberrant rearrangements in Artemis-P70 backgrounds

Our lab has published results showing *in vivo*, B and T cells from Artemis-P70 harbor the products of aberrant V(D)J recombination events [6]. However, B and T cells undergo and selection process *in vivo* and some lymphoid cells that carry out nonproductive rearrangements are removed from the population. By utilizing the transient assay in MEFs, we can take advantage of the lack of selection process and gain a less biased analysis of recombination events.

We previously reported on the proficiency of the Artemis-P70 protein in facilitating V(D)J recombination in the cell-based transient V(D)J assay [5]. We used a deletional coding join plasmid substrate containing two RSSs recognized by the Rag endonuclease. Cleavage by Rag creates two coding ends that are joined by deleting the intervening sequence. We found that while the Artemis-P70 mouse embryonic fibroblasts produced coding joins at low frequencies, the joins formed had normal nucleotide additions and deletions. In contrast, coding joins formed in Artemis-null MEFs typically exhibit larger nucleotide deletions.

In address the role of the Artemis C-terminus in recombination at endogenous loci, our lab analyzed inversional rearrangements at the IgLk light chain locus of splenocytes [6]. We found that Artemis-P70 splenocytes produced aberrant hybrid joins when attempting inversional V(D)J recombination *in vivo*. The hybrid join observed here is an aberrant joining event between a coding end and a signal end. The nature of this event indicates that the inversional, intervening sequence has been lost from the PCC. . These aberrant products were not detected in Artemis-null lymphocytes. In order to test the hypothesis that the Artemis-P70 protein may be more prone to producing aberrant products when attempting inversional recombination, we used the inversional recombination plasmid substrate pJH299 in the cell-based transient V(D)J assay (Figure 2.3). Recombination of this substrate to create a signal joint and a coding joint requires the retention of the intervening sequence. Like at the IgLk locus, the loss of the intervening sequence can result in the formation of an aberrant deletional hybrid joint.

Like our previous experiments using the deletional coding join plasmid substrate, recombination of the inversional substrate occurred at a low frequency in both Artemis-null and Artemis-P70 backgrounds (Figure 2.4). However, aberrant deletional hybrid joins occurred at a higher frequency in Artemis-null and Artemis-P70 backgrounds (wildtype and Artemis-null $p < 0.0001$, wildtype and Artemis-P70, $p < 0.0001$, two-tailed Fisher's exact test) (Figure 2.5). Analyzing junctional sequences revealed differences between the joins formed in Artemis-null MEFs and Artemis-P70 MEFs (Figure 2.6, 2.7, 2.8). The majority of joints formed in Artemis-null mice had larger nucleotide deletions (11 of 13 inversional joins and 14 of 20 deletional hybrid joins had nucleotide deletions larger than 12 nt). Conversely, the majority of joins formed in Artemis-P70 backgrounds did not have large deletions (3 of 10 inversional joins and 4 of 17 deletional hybrid joins had nucleotide deletions larger than 12 nt). Interestingly, one plasmid recovered from Artemis-P70 MEFs was a product of an unexpected aberrant event, joining one signal end to the RAG2 gene (Figure 2.7).

As mentioned above, we previously detected differences in recombination frequency using a deletional coding join plasmid substrate in wildtype and Artemis-P70 MEFs. However, using this inversional recombination plasmid substrate which requires the retention of the intervening sequence, we found that V(D)J recombination facilitated

by the Artemis-P70 protein resulted in increased aberrant deletional joining frequency when compared to wildtype. Recovered recombination substrate from Artemis-P70 and Artemis-null MEFs exhibited differences with regard to nucleotide deletions at coding and hybrid joints. This result indicated that the Artemis-P70 protein facilitated recombination and these joins were distinct from joins facilitated by the yet-undetermined nuclease utilized in Artemis-null MEFs. Together, these results support the hypothesis that loss of the C-terminus destabilizes the PCC. The Artemis-P70 protein retains the ability to open these hairpin ends, but through a yet undefined mechanism, results in the inability of the PCC to retain the intervening sequence.

Loss of the Artemis C-terminus does not disrupt ATM kinase activity or the MRN complex

Our findings that loss of the Artemis C-terminus results in increased aberrant joining *in vivo* as well as *ex vivo* using an inversional recombination plasmid substrate in the cell-based transient V(D)J assay. These findings regarding Artemis-P70 are similar to findings that have been reported for ATM deficiency and MRN deficiency. Loss of ATM, ATM kinase activity, or members of the MRN complex result in increased aberrant rearrangements and therefore, these factors are thought to be important for PCC stability.

We addressed the impact of loss of the Artemis C-terminus on ATM kinase activity and the integrity of the MRN complex. In order to address this issue, Artemis-P70 MEFs were exposed to 10 grays of ionizing radiation and ATM activation was analyzed by Western blotting. Upon exposure to IR, the ATM kinase undergoes autophosphorylation and is activated. ATM then phosphorylates downstream substrates including the histone variant H2AX and the KRAB-associated protein 1 (KAP1). Our lab found that after ionizing radiation, ATM was phosphorylated in both Artemis-null and Artemis-P70 MEFs (Figure 2.4, A). In addition, ATM substrates KAP1 and H2AX were both phosphorylated in mutant MEF lines (Figure 2.4, B). I found that in Artemis-P70 MEFs, NBS1 and RAD50 co-immunoprecipitated with MRE11 indicating that the presence of the Artemis-P70 protein did not disrupt MRN complex stability (Figure 2.4 C). Together, these results indicate that in Artemis-P70 backgrounds, the ATM kinase is active and the MRN complex is intact.

The Artemis C-terminus is important for facilitating chromosomal rearrangement and preventing aberrant products

In order to further characterize the Artemis C-terminal domain in the context of V(D)J recombination, C-terminal truncation mutants were expressed in Artemis-null murine pre-B cell lines using retroviral transduction. This pre-B cell line has been transformed using viral Abelson kinase (*v-abl*) and in addition, has a chromosomally-integrated recombination substrate, pMX-INV. Treating these cells with the Abelson kinase inhibitor STI571 arrests the cells in G1 phase which in turn results in Rag accumulation and the initiation of V(D)J recombination.

pMX-INV contains two recombination signal sequences that flank an anti-sense GFP cDNA. Recombination of this substrate can result in an inversion of the anti-sense GFP and creates both a signal join and a coding join. Cells that have undergone an inversional event will express GFP. Defective recombination can result in the loss of the intervening sequencing and the joining of a signal end and a coding end, a hybrid join. DNA ends released from the recombination protein complex could also engage in transrearrangements with the DNA ends of another rearranging locus. (Figure 2.10)

Wildtype Artemis, empty vector, and Artemis mutant constructs M β L/ β CASP and D451X were stably expressed in the Artemis-null pre-B cells by retroviral transduction. Retroviral construct contained both an Artemis and dsRED2 cDNA separated by an IRES sequence. Transduced cells were then sorted for dsRED2 expression and three clones of each genotype were chosen for further analysis. RT-PCR and Q-PCR demonstrated that Artemis mutant constructs were both being expressed and were expressed at levels that allowed comparison between genotypes (Figure 2.11).

Cell lines were treated with STI571 and harvested at 96 hours post-treatment. Genomic DNA was extracted and rearrangements were analyzed using genomic Southern blot. As expected, Artemis-null pre-B cells transduced with only the retroviral vector did not support V(D)J recombination (Figure 2.12). An accumulation of coding ends indicated that treatment of the cells with STI571 induced recombination but DNA coding ends did not engage in ligation. Expression of wildtype Artemis in these cells rescued the V(D)J deficiency and coding joins were detected by genomic Southern. As reported in the literature, treatment of wildtype Artemis expressing pre-B cells with the

ATM inhibitor KU55933 resulted in increased hybrid joining and the accumulation of coding ends [17].

Surprisingly, expression of the M β L/ β CASP and D451X constructs rescued coding join formation to wildtype levels. These findings are in contrast to recombination frequency using Artemis-P70 MEFs in the transient V(D)J assay was very low. Artemis truncation mutants have low endonucleolytic activity and the M β L/ β CASP construct does not include the DNA-PKcs binding domain. Yet I observed high levels of coding join formation which was likely due to overexpression of the Artemis constructs in these cell lines. In contrast, Artemis-P70 MEFs likely expressed the mutant protein at lower levels from an endogenous promoter. Results using the pre-B cell system indicated that the loss of the Artemis C-terminus resulted in increased hybrid join formation and increased coding end accumulation (Figure 2.12). In order to determine whether these coding ends can go on to engage in aberrant transrearrangements, nested PCR was used to amplify events involving the pMX-INV recombination substrate and the endogenous rearranging IgLk locus (Figure 2.13). Treatment of pre-B cell clones with STI571 alone resulted in transrearrangements in M β L/ β CASP and D451X expressing cells but not in wildtype Artemis expressing cells or Artemis-null cells transduced with empty vector. Treatment of these cells with ATM inhibitor KU55933 and STI571 resulted in higher levels of transrearrangements than treatment with STI571 alone.

Expression of either M β L/ β CASP or D451X Artemis mutants resulted in increased hybrid joining, coding end accumulation, and transrearrangements. We observed more aberrant products in pre-B cell lines expressing M β L/ β CASP Artemis than in pre-B cells expressing the D451X truncation mutant. This difference between the two pre-B cell genotypes suggest a PCC stabilizing role for the DNA-PKcs interaction domain that is present in the D451X truncation mutant but absent in the M β L/ β CASP mutant. We also observed that inhibition of ATM kinase activity increased the levels of transrearrangements observed in both Artemis mutant pre-B cell lines. This result indicates that ATM may be involved in multiple pathways in the stabilization of the PCC but does not rule out the possibility of epistasis between ATM and the Artemis C-terminus specifically in the prevention of aberrant rearrangements.

Uncharacterized regions of the conserved N-terminal domain are important for Artemis activity

We and others have found that expression of the conserved M β L/ β CASP N-terminal domain of Artemis in Artemis-deficient cells fully restores V(D)J recombination defects in the transient assay [5, 13, 18]. Here, I found that M β L/ β CASP expression in the pre-B cell system was able to facilitate wildtype-levels of coding join formation despite producing aberrant products. This was a surprising result because this mutant has very little endonucleolytic activity and does not interact with DNA-PKcs[5, 13]. It is likely that the overexpression of this truncation mutant rescued recombination frequency but also may have revealed an important aspect of Artemis.

In this study, we consider the possibility that the M β L/ β CASP domain has features, outside of its already known catalytic activity, that may aid the protein in facilitating V(D)J recombination. To test the idea that simply overexpression of a nuclease can rescue V(D)J recombination, we overexpressed SNM1B and MRE11 in the transient assay using Artemis-null MEFs. Although both SNM1B and MRE11 have both been shown to be capable of cleaving a DNA hairpin substrate [19] neither overexpressed proteins were able to facilitate recombination (Figure 2.17, A and B)

SNM1B, a member of the SNM1 family of proteins along with Artemis, also has the metallo- β -lactamase and β -CASP domain. Yet, overexpression of this protein did not rescue V(D)J recombination in Artemis-null MEFs. This suggests that the Artemis N-terminal domain has features that allow it to modify Rag-generated breaks and these features are not present in SNM1B. In order to address what these features may be, we searched for regions in the amino acid sequence of the Artemis N-terminus that were unique to Artemis among its family members. To increase the likelihood that these regions are functionally important outside of catalytic activity, we also searched for sequences that were well conserved among Artemis orthologs but were not predicted to be in regions that were involved with metal coordination.

To narrow down candidates, we used previously reported human missense point mutants to help select regions that could be functionally important. We selected three human patient mutants H228N, I16T, and T71P, as a model for making point mutants for study (Table 2.1). The patient with the Artemis-H228N mutation and the patient with

the Artemis-I16T mutation both exhibited absence of B and T cells [20, 21]. The Artemis-H228N patient had a deletion of exon 1-4 on the second allele. The Artemis-I16T patient had a S119X truncation mutation on the second allele, not likely producing a functional protein. Fibroblasts from both patients were sensitive to ionizing radiation. The third patient with the Artemis T71P allele interestingly had some T cells and fibroblasts from this patient did not exhibit sensitivity to ionizing radiation as analyzed by γ -H2AX assay [22]. The second allele in the Artemis-T71P patient contained a deletion of exon 1-3.

A total of five mutations were chosen to be introduced to the M β L/ β CASP truncation construct (Figure 2.14). All amino acids or motifs chosen to mutate were not anticipated to be important for nucleolytic activity based on their positions on a predicted structural model of Artemis generated by the SWISS-MODEL program (Figure 2.15). In addition, amino acid H228 is predicted to be in a shallow groove that others have speculated could be important for DNA binding [23].

To analyze the endonucleolytic activity of the mutant Artemis constructs, Artemis protein was purified from DNA-PKcs and incubated with manganese and either a 32P 5' end labeled DNA overhang substrate or a DNA hairpin substrate. We have previously shown that in the presence of manganese, Artemis does not require DNA-PKcs for activity [5]. M β L/ β CASP-I16A and M β L/ β CASP-H228A could not be consistently expressed at high enough levels to be analyzed for endonucleolytic activity. This suggested that both mutations destabilized the protein and these two regions may have structural roles. We found that mutants M β L/ β CASP-KRR3A and M β L/ β CASP-LHSG4A were not able to cleave either the overhang or hairpin substrate (Figure 2.16). However the T71A point mutation did not have a negative impact on the ability of the M β L/ β CASP protein to cleave either DNA substrates. We also did not observe any impact on endonucleolytic activity when the T71A point mutation was introduced to the full length Artemis protein (FL-T71A).

The two mutants M β L/ β CASP-T71A and FL-T71A were able to facilitate the recombination of the inversional recombination plasmid substrate pJH299. Recombination frequencies observed in T71A expressing MEFs were comparable to wildtype Artemis and M β L/ β CASP expressing MEFs (Figure 2.17, B). Recombined

products were harvested, sequenced, and joining events were categorized as either inversional or deletional (Figure 2.18). We observed increased deletional events in MEFs expressing M β L/ β CASP when compared to wildtype expressing MEFs ($p < 0.0001$, two-tailed Fisher's exact test). However, T71A mutation in either the M β L/ β CASP or full length protein did not impact the ratio of inversional to deletional events (M β L/ β CASP and M β L/ β C-T71A, $p = 1.000$, wildtype and FL-T71A, $p = 0.1461$). We also did not observe any significant differences in nucleotide deletions, additions, or microhomologies when the T71A point mutation was introduced to either the M β L/ β CASP or full length Artemis protein (Figure 2.19, 2.20, 2.21, 2.22).

Here, we report regions of the Artemis N-terminus, KRR (48-50) and LHSG (150-153), that are important for endonucleolytic activity or protein stability despite not being predicted to be important for metal ion coordination. While we cannot rule out that these mutations may alter protein conformation, it is also possible that these two regions may be important for contacting or handling DNA ends. Due to low expression levels, we were not able to analyze the activity of two constructs that were based on human patient alleles, H228A and I16A. It is likely that these two mutations destabilized the protein and this is likely the mechanistic explanation for the patient phenotypes (Table 2.1). Surprisingly, the T71A mutation did not affect biochemical activity or V(D)J recombination despite the report that a patient with an Artemis T71P mutant allele exhibited lymphopenia [22]. However, our results indicate that this amino acid may not be directly involved with activity or recombination but rather the proline substitution in the patient may have altered protein conformation and resulted in a hypomorphic nuclease.

Discussion

We have previously reported that the Artemis C-terminus has important functional roles in V(D)J recombination as well as the repair of general DSBs *in vivo* [5, 6]. In this study, we further demonstrate that the Artemis C-terminus functions in the regulation of the nuclease and the prevention of aberrant events.

It has been previously shown that the Artemis C-terminus is important for full endonucleolytic activity [2, 5, 13]. We show here that the catalytic N-terminal domain

combined with the C-terminal region between amino acid 386 and 538 is sufficient for full endonucleolytic activity in the *in vitro* endonuclease assay. The known features in this region include the DNA-PKcs binding domain, the LIG4 binding domain, and four SQ putative phosphorylation sites (S503, S516, S534, and S538). Because some truncation constructs that contain the DNA-PKcs binding domain and LIG4 binding domain (D451X and Δ 502-692) do not exhibit full activity, it is tempting to conclude that the four SQ phosphorylation sites that are present in the Δ 539-692 construct contribute to its endonucleolytic activity. It has been shown previously that Artemis does not need to be phosphorylated in order to have endonucleolytic activity and the critical phosphorylation event for nuclease recruitment and activity is phosphorylation of DNA-PKcs [14-16, 24]. Thus, this raises the possibility that this region of the C-terminal domain is important for either stabilizing the interaction between Artemis and DNA-PKcs or stabilizing the nuclease interaction with DNA itself.

Artemis-P70 B and T lymphocytes harbor aberrant rearrangements involving the rearranging B and T cell receptor locus and these rearrangements were rarely observed in Artemis-null cells. We also found that Artemis-P70/p53 mice succumb to lymphoma and these tumors were clonal and harbored transrearrangements involving the antigen receptor loci. This led us to hypothesize that the Artemis C-terminus may play a role in PCC stability. We had previously analyzed the ability of Artemis-P70 MEFs to recombine a plasmid substrate. In those experiments we utilized a substrate that recombined by deletion of an intervening sequence to create a coding join. We found that Artemis-P70 MEFs produced much fewer recombinants but recovered plasmids had joint sequences similar to recombinants recovered from wildtype MEFs. In contrast, substrate recovered from Artemis-null MEFs exhibited large nucleotide deletions from both coding ends.

In order to better understand the role of the Artemis C-terminus during inversional recombination, here we performed the transient V(D)J recombination assay using a plasmid substrate that recombines by inverting an intervening sequence generating a signal join and a coding join. We observed very low recombination frequencies in both our Artemis-null and Artemis-P70 MEFs. This was similar to our results using the deletional coding join substrate and likely is due to the low

endonucleolytic activity of the Artemis-P70 protein. Like our *in vivo* results, we found that Artemis-P70 facilitated recombination resulted in increased aberrant hybrid joining when compared to joins produced in wildtype backgrounds. *In vivo*, aberrant hybrid joins were rare events in Artemis-null. Although, we observed similar levels of hybrid joining from our Artemis-null MEFs, the joint sequence from both genotypes were distinct suggesting that two different repair mechanisms were at play. Unlike in Artemis-null MEFs, the majority of joins formed in Artemis-P70 MEFs did not have large nucleotide deletions and the signal ends remained unmodified even in hybrid joins. This result suggests that the Artemis-P70 protein can process these DNA ends but the PCC in these cells is unstable and releases the DNA ends prematurely. Conversely, the PCC in Artemis-null MEFs may be further disrupted by an unknown nuclease resulting in larger nucleotide deletions at both DNA ends.

The Artemis C-terminal domain has several sites that can be phosphorylated by DNA-PKcs and ATM. Because we know that loss of ATM kinase activity can result in aberrant hybrid joining, one simple explanation is that the phosphorylation of the Artemis C-terminus plays a role in stabilizing the PCC. Although others have shown that Artemis phosphorylation-deficient mutants can facilitate V(D)J recombination at wildtype levels, these experiments used plasmids substrates that recombine by deletion to generate a coding join [15]. An inversional recombination substrate may reveal PCC stability defects in Artemis phosphomutant expressing cells.

A second, non-mutually exclusive explanation for increased hybrid joining in Artemis-P70 backgrounds involves the reduced endonucleolytic activity of the protein. A partially active nuclease may cleave only one of two hairpin coding ends in the PCC. Unable to join the two ends, the PCC would eventually collapse and the free ends would be capable of engaging in aberrant joining. Currently, it is not possible to distinguish between the two possibilities or determine if both are at play. One way to address this hypothesis in the future would be to identify N-terminal mutations that partially disrupt nucleolytic activity. This potential separation-of-function mutant could help define the contributions of the Artemis C-terminus and the endonucleolytic activity of Artemis to PCC stability.

Joins formed in Artemis-null MEFs exhibited large nucleotide deletions on both coding ends and signal ends. This result suggests that these ends are processed by a currently unknown alternative nuclease that is capable of disrupting the PCC and the RAG proteins that are normally tightly bound to signal ends. This result also introduces the interesting possibility that the Artemis nuclease may protect signal ends from degradation specifically during inversional recombination. However, it has been previously shown that loss of the Artemis nuclease does not impact signal join formation using a deletional signal join plasmid substrate and Artemis-null ES cells [25]. The majority of signal joins produced in Artemis-null ES cells were precise and did not exhibit end modification. Another report that examined signal joining at the endogenous recombination locus in thymocytes found that the loss of Artemis resulted in *fewer* nucleotide deletions at signal joins when compared to wildtype thymocytes [26]. These two findings from two separate studies illustrate the different ways DNA ends are processed in different environments. The enzymes and nucleases that are differentially expressed in various cell types may contribute to the contradicting observations.

Although the Artemis mutant constructs were overexpressed in the pre-B cell system, we were able to take advantage of this experimental system to further shed light on the Artemis C-terminus in V(D)J recombination. As expected, pre-B cells expressing the Artemis-P70 protein D451X produced aberrant hybrid joins when induced to recombine. We and others have shown that expression of the M β L/ β CASP construct can rescue V(D)J recombination deficiencies of Artemis-null backgrounds. Here, we show for the first time that although M β L/ β CASP can restore recombination, it also facilitates the generation of aberrant hybrid joins and coding end accumulation. The amount of these aberrant products was higher than in D451X expressing clones. This result suggested that the PCC was even more unstable in our lines expressing M β L/ β CASP than in lines expressing D451X. One domain that is present in the D451X construct but absent in the M β L/ β CASP construct is the DNA-PKcs interaction domain. It is possible that there are several factors that contribute to PCC stability and this interaction between Artemis and DNA-PKcs is one of the requirements for stability

The expression of M β L/ β CASP can facilitate V(D)J recombination despite its very low endonucleolytic activity. It is assumed that this is the consequence of mutant

overexpression as all prior experiments testing the protein for V(D)J recombination capabilities have used transient transfection. We hypothesized that the conserved domain of Artemis has features, aside from catalytic activity, that are unique to this nuclease and allow it to facilitate cleavage of Rag-induced hairpin ends. Supporting this idea, overexpression of SNM1B or MRE11 did not support V(D)J recombination in the absence of Artemis. However, in this study, we could not identify regions that disrupted V(D)J recombination but not endonucleolytic activity. We were able to identify two previously undescribed regions, KRR (48-50) and LHSR (150-153), that were important for endonucleolytic activity. Although it we have not ruled out the possibility that these mutations could alter protein conformation, these two regions could potentially be important for contacting DNA during cleavage.

Two amino acids studied H228 and I16, were expressed at very low levels in the transient expression system suggesting these sites are important for protein stability. It is possible that protein instability contributed to or perhaps was even solely responsible for the patient phenotypes observed (Table 2.1, [20], [21]). However, our results do not exclude the possibility that these sites may also have other functions. For instance, in addition to its role in protein stability, H228 has been predicted to be in a groove that may be important for binding to DNA [23]. The presence of this feature may suggest that the Artemis N-terminus may be binding to DNA substrates directly and that Artemis truncation mutants may depend of this feature for activity.

We did not detect an effect of the T71A mutation on either the M β L/ β CASP or full length Artemis protein. T71A mutants readily cleaved the overhang and hairpin substrate and facilitated V(D)J recombination without increasing aberrant events. Yet, the patient with a T71P mutation exhibited lymphocytopenia. The original study sequenced the *RAG1* and *RAG2* genes in addition to *Artemis* [22]. One possibility is that the patient lymphocytopenia was not the caused by any of the three genes analyzed and the T71P mutation was an undisruptive variant. While this possibility cannot currently be ruled out, the evolutionary conservation of T71 and its location on the electropositive surface of the protein suggests it may be functionally important and may even participate in DNA binding [27].

In order to understand the role of this particular T71 site, we used an alanine substitution, a small, inert amino acid that would be less likely to disrupt protein structure. The patient, however, had a point mutation that resulted in a proline substitution which may have altered the secondary structure of the protein more than alanine substitution we used. Because we did not observe any negative impact resulting from an alanine substitution at T71, it is possible that the lymphocytopenia observed in the human patient was due to altered protein conformation.

Our findings provide valuable insight into the importance of the Artemis C-terminal domain. Together, these results indicate that the C-terminus contains regions that are important for DNA end-processing and also for preventing aberrant recombination events likely by stabilizing the post-cleavage complex. Our results also suggest that there may be regions in the N-terminus that are specific for the Artemis nuclease and are important for promoting V(D)J recombination. These findings provide insight into the requirements of end-processing and the prevention of aberrant V(D)J recombination.

Materials and methods

Protein purification

Artemis constructs were cloned into pCDNA6-myc-HIS plasmid. Plasmid constructs were transfected into 293T cells using Lipofectamine 2000 (Invitrogen) transfection reagent. A partial purification of Artemis protein was then performed as described previously [1]. Briefly, cell lysate was then harvested after 48 hours by sonication and centrifugation. Artemis protein was then purified using Ni-NTA beads followed by protein G Sepharose beads (GE Healthcare). Artemis protein bound to protein G beads were resuspended in Buffer G (25 mM HEPES pH 7.9, 10 mM MgCl₂, 10% glycerol, and 2 mM DTT). Artemis protein levels were then normalized by Western blotting. DNA-PKcs was purified from HeLa cells using a previously described protocol [28]

Endonuclease assay

Endonuclease assays were performed as previously described [5]. DNA hairpin substrates and overhang substrates were 5' end labeled using T4 polynucleotide kinase

(NEB) and ^{32}P γ -ATP (Perkin Elmer). End-labeled oligo (36-nt for overhang, 40-nt for hairpin) was then annealed to a complementary oligo (21-nt for overhang, hairpin anneals to self) by heating followed by slow cooling. Annealed substrates were purified using a 17% non-denaturing polyacrylamide gel.

Nuclease reactions with the hairpin substrate were performed using 0.125 pmol of labeled substrate in 25 mM Tris pH 8.0, 10 mM MgCl_2 /10 mM MnCl_2 , 0.05 $\mu\text{g}/\mu\text{l}$ BSA, 0.25 mM ATP, and 10 μl of purified protein in Buffer G in a total volume of 20 μl . Hairpin reactions were incubated for 2 hours at 37 C. Nuclease reactions using overhang substrate were performed in the same buffer conditions except 0.250 pmol of labeled substrate was used instead. Overhang substrate reactions were performed at 37 C for 1 hour. Reactions were stopped with 1 μl of 0.5M EDTA and 10 μl of deionized formamide. Reactions were then visualized by separation in 17% denaturing urea acrylamide gel and autoradiography.

Transient V(D)J recombination assay

SV40 transformed MEFs were transfected using Superfect transfection reagent (Qiagen). The plasmids transfected were the inversional recombination plasmid substrate pJH299, full length murine RAG1 and RAG2 expression constructs, and plasmid constructs expressing a nuclease where indicated. Cells were harvested after 48 hours and plasmids were extracted using alkaline lysis and phenol/chloroform extraction. Plasmids were transformed into MC1061 *E. coli* and plated on ampicillin (250 $\mu\text{g}/\text{ml}$) plates or ampicillin and chloramphenicol (10 $\mu\text{g}/\text{ml}$) double selection plates. Recombination frequency was determined using the ratio of colonies on double selection plates over single selection plates. Colonies were picked from double selection plates and plasmids extracted using Qiagen Mini-prep kit. Plasmids were sequenced to analyze coding and hybrid joints.

Co-immunoprecipitation of the MRN complex

MEFs were grown to confluency, harvested, and lysed by sonication in 25 mM HEPES pH 7.4, 150 mM KCl, 10 mM MgCl_2 , 10% glycerol, 2 mM DTT and protease inhibitors (Roche). 6 mg of protein lysate was incubated with protein G Sepharose beads (GE Healthcare) and α -MRE11 antibody (4.5 μg , Cell Signaling) overnight at 4 C. Beads were then washed in lysis buffer followed by washes in 300 mM KCl lysis buffer.

Immunoprecipitates were analyzed by Western blotting using α -MRE11 (Cell Signaling), α -NBS1 (Novus Biologicals), and α -RAD50 (Bethyl Laboratories).

Retroviral transduction

Artemis cDNA constructs were cloned into an IRES-dsRED2 retroviral vector pMIRA. pCL-Eco was used as the retroviral packaging plasmid [29]. Retrovirus was generated in HEK293T cells using calcium chloride/HEPES transfection. Viral supernatants were collected at 72 and 96 hours post transfection and concentrated using Retro-X Retroviral Concentrator (Clontech). Spinfection of Artemis^{-/-} v-abl pre-B cells (clone c.30) was performed for 90 minutes. Pre-B cell clones expressing dsRED2 were selected by FACS single cell sorting.

Cell culture for pre-B cell system

Artemis^{-/-} v-abl pre-B cells were described previously [11, 17]. These cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, HEPES, non-essential amino acids, glutamine, penicillin/streptomycin, and β -mercaptoethanol. G1 arrest was induced by treating cells with 3 μ M STI571 (Novartis) in DMSO. ATM inhibition was performed using by treating cells with 15 μ M KU-55933 in DMSO 1 hour prior to treatment of STI571.

Quantitative PCR

RNA extraction was performed using RNeasy Mini Kit (Qiagen). RNA was reverse transcribed using M-MLV-reverse transcriptase (Invitrogen). Quantitative PCR was using SYBR Green Reagent (Invitrogen).

Genomic Southern blot

Genomic DNA was extracted from v-abl pre-B cells after indicated treatments. 40 μ g of genomic DNA was digested overnight either with EcoRV-HF (NEB) or double digested with EcoRV-HF and NcoI-HF (NEB). Digestion reaction was concentrated by ethanol precipitation and resuspended in 30 μ l TE. Electrophoresis was performed on digested DNA at 30 V overnight in 0.8% agarose. DNA was then transferred to Zetaprobe membrane (BioRad) and analyzed by Southern blot. C4B probe which was previously described [17] was used to detect digest fragments.

Nested PCR

Transrearrangements between the pMX-INV recombination substrate and IgLk locus were detected using nested PCR and primers flanking Vk6-23 and the IRES sequence of pMX-INV. 100 ng of genomic DNA from v-abl pre-B cells were used as a template with primers JMS528 and JMS579. The first round program was as follows: 25 cycles of 94 C, 60 C, and 72 C at 30 seconds each stage. PCR products were purified using Qiagen PCR Purification Kit. The second round utilized the primers pB and pk6d and the same PCR program as round one. Products were run on a 1.5% agarose gel and transferred to Zetaprobe. pD primer was used as a probe to detect products.

Artemis N-terminus structural modeling

Artemis N-terminus model was constructed using SWISS-MODEL program (swissmodel.expasy.org). This program used the unpublished crystal structure of the SNM1B conserved domain as a template for modeling [30].

Acknowledgements

Full length Artemis, M β L/ β CASP, and D451X pcDNA6 constructs were generated by former members of the Sekiguchi lab. DNA-PKcs purification was performed by a former member of the Sekiguchi lab. ATM autophosphorylation, Kap1 and H2AX phosphorylation Western blots were performed by Cheryl Jacobs-Smith.

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Figures

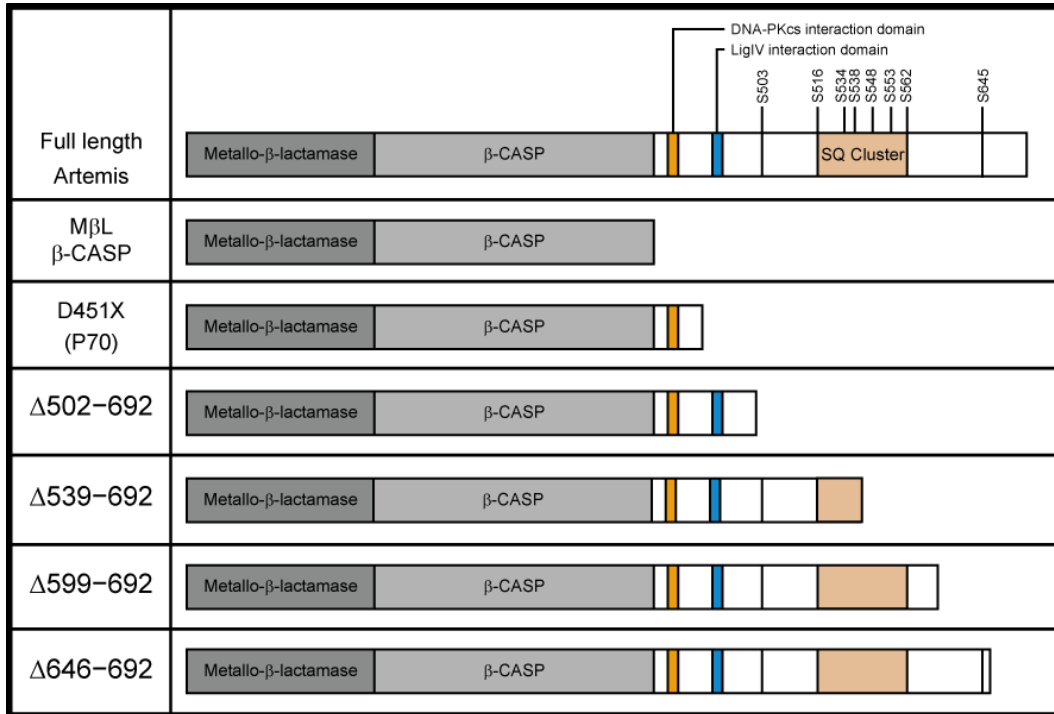


Figure 2. 1 Artemis C-terminal truncation mutants. Six mutants were created and expressed from pCDNA6-myc-HIS vector. The Artemis C-terminus has several SQ sites that can be phosphorylated by either the ATM or DNA-PKcs kinase. The C-terminus also has domains that are known to interact with either DNA-PKcs (orange) or LIG4 (blue).

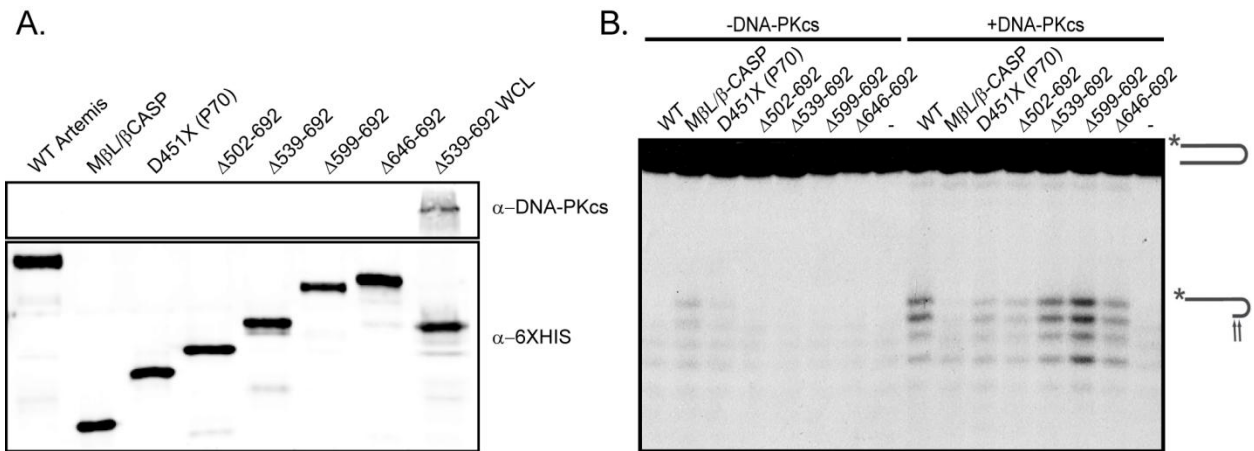


Figure 2. 2 Regulation of endonucleolytic activity. (A) c-myc-6xHIS-tagged Artemis constructs were expressed in 293T cells and purified from DNA-PKcs. Protein normalization and purification from DNA-PKcs was confirmed by Western blot. Whole cell lysate (WCL) was used as a DNA-PKcs positive control. (B) Partially purified Artemis protein was incubated with a 5' ³²P end-labeled DNA hairpin substrate with and without purified DNA-PKcs protein. Reactions were then run on a 17% denaturing polyacrylamide gel and gel was exposed to X-ray film.

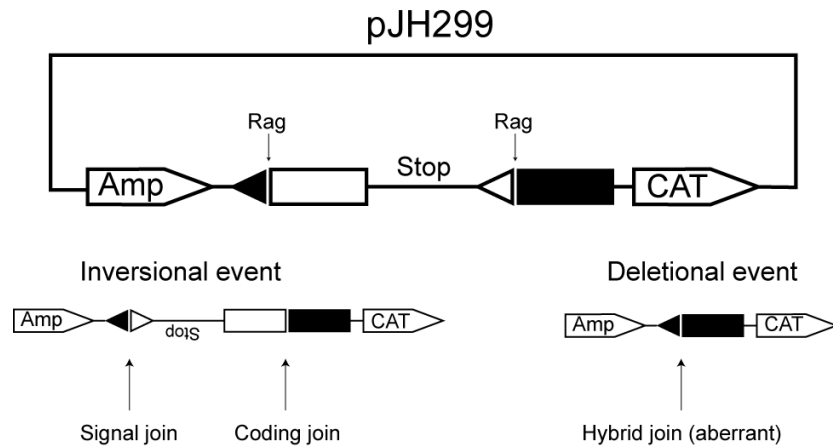


Figure 2. 3 Inversional plasmid recombination substrate pJH299. The pJH299 plasmid substrate has two RSSs which are represented by the triangles. The intervening sequence contains within it a bacterial transcriptional stop sequence. Recombination of the plasmid can generate either an inversional event or a deletional event resulting from the loss of the intervening sequence. Either recombination events will inactivate the stop sequence resulting in bacterial resistance to both ampicillin and chloramphenicol. Amp=ampicillin resistance gene, CAT=chloramphenicol acetyltransferase gene.

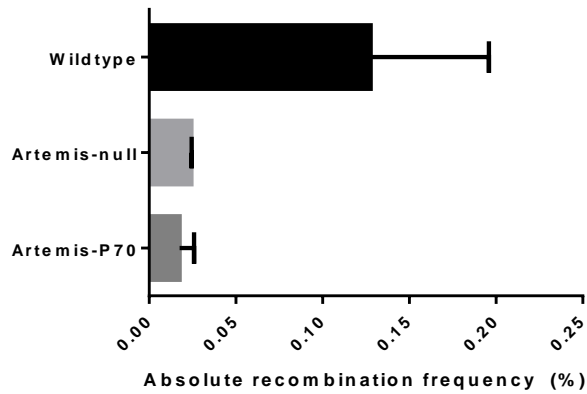


Figure 2. 4 Recombination frequency in Artemis^{-/-} and Artemis-P70 backgrounds. Recombination frequency of inversional plasmid substrate in MEFs derived from Artemis^{-/-} and Artemis-P70 mice were dramatically reduced when compared to recombination frequency in wildtype MEFs. MEFs were transfected with the pJH299 inversional recombination substrate and plasmids expressing the Rag proteins. Plasmid substrate was harvested 48 hours after transfection and analyzed.

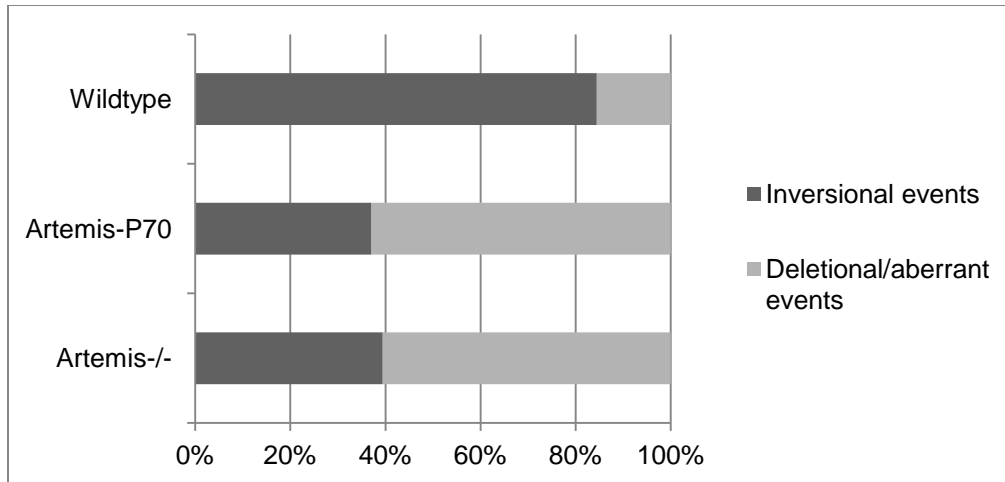


Figure 2. 5 Aberrant events in Artemis-/- and Artemis-P70 backgrounds. Loss of the Artemis or the Artemis C-terminus resulted in increased aberrant joining events. Plasmid – based V(D)J recombination assay was performed using wildtype, Artemis-P70, and Artemis-null MEFs. Recombined plasmid was transformed into bacteria and chloramphenicol and ampicillin resistant bacterial colonies were isolated, plasmids were extracted, and sequenced. Plasmid sequences were analyzed and recombination events were categorized as either inversional or deletional/aberrant. Sample size for WT = 45, Artemis-P70 = 27, Artemis-/- = 33.

RSS23	RSS Joint					RSS12	Coding end					Coding joint					Coding end		MH	Clone
AGTACTACCACTGTG	Δ	P	N	P	Δ	CACAGTGCTACAGAC	TCGATGAGAGGATCC	Δ	P	N	P	Δ	GTCGACCTGCAGCCC							
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGGAT	2				5	CCTGCAGCCC	2	8					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGGA	3				1	TCGACCTGCAGCCC	2	3					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAG	5				3	GACCTGCAGCCC	2	3					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0	G			4	ACCTGCAGCCC	1	3					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGGATC	1				5	CCTGCAGCCC	1	2					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGG	4				8	GCAGCCC	0	2					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGGA	3				3	GACCTGCAGCCC	0	2					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAG	5				2	CGACCTGCAGCCC	0	1					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0	G			7	TGCAGCCC	0	1					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGGAT	2				12	CCC	0	1					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGG	4				14	C	0	1					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGG	4				0	GTCGACCTGCAGCCC	0	1					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGGAT	2				7	TGCAGCCC	0	1					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGGA	3				1	TCGACCTGCAGCCC	2	1					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGGATC	1				10	CAGCCC	1	1					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGG	4				C	0	GTCGACCTGCAGCCC	0	1				
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0	GG			5	CCTGCAGCCC	0	1					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0	GG			2	CGACCTGCAGCCC	0	1					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGGAT	2				6	CTGCAGCCC	1	1					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0				0	GTCGACCTGCAGCCC	0	1					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGG	4				2	CGACCTGCAGCCC	0	1					
AGTACTACCACTGTG	0				0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0				2	CGACCTGCAGCCC	0	1					

38 Total

RSS23	Hybrid joint					Coding end		MH	Clones
AGTACTACCACTGTG	Δ	N	P	Δ	GTCGACCTGCAGCCC				
AGTACTACC	6			8	GCAGCCC	0	1		
AGTACTACCACTGTG	1			4	ACCTGCAGCCC	1	1		
AGTACTACCACTGTG	0			2	CGACCTGCAGCCC	0	1		
AGTACTACCACTGTG	0			12	CCC	1	1		
AGTACTACCACTGTG	0			7	TGCAGCCC	0	1		
AGTACTACCACTGTG	0			3	GACCTGCAGCCC	0	1		
AGTACTACCACTGTG	0			6	CTGCAGCCC	0	1		
						7	Total		

Figure 2. 6 Signal joints, coding joints, and hybrid joints from wildtype MEFs Recombined pJH299 joint sequences showing nucleotide deletions (Δ), N-nucleotide additions (N), and palindromic-nucleotide additions (P). Microhomologies (MH) are labeled in red.

RSS23	Hybrid joint			RSS12	Coding end			Coding joint			Coding end			MH Clones	
AGTACTACCACTGTG	Δ	Insertion	P	Δ	CACAGTGCTACAGAC	TCGATGAGAGGATCC	Δ	P	Insertion	P	Δ	GTCGACCTGCAGCCC			
AGTACTACCACTGTG	0			0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	53				39	GTCGACCTGCAGCCC	G	1	1
AGTACTACCACTGTG	0			0	CACAGTGCTACAGAC	TCGATGAGAGG	4				0	GTCGACCTGCAGCCC		0	1
AGTACTACCACTGTG	0			0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0				5	CCTGCAGCCC		0	1
AGTACTACCACTGTG	0			0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	23			AC	0	GTCGACCTGCAGCCC		0	1
AGTACTACCACTGTG	0			0	CACAGTGCTACAGAC	TCGATGAGAG	5				6	CTGCAGCCC		0	1
AGTACTACCACTGTG	0			0	CACAGTGCTACAGAC	TCGATGAGAGG	4				7	TGCAGCCC		0	1
AGTACTACCACTGTG	0			0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0				0	GTCGACCTGCAGCCC		0	1
AGTACTACCACTGTG	0			1	ACAGTGCTACAGAC	TCGATGAGAGGATCC	0	G			8	GCAGCCC		0	1
AGTACTACCACTGTG	0			1	ACAGTGCTACAGAC	TCGATGAGAGGATCC	23				13	CC		1	1
AGTACTACCACTGTG	0			0	CACAGTGCTACAGAC	TCGATGAGAG	5				3	GACCTGCAGCCC		2	1
														10	Total

RSS23	Hybrid joint			Coding end			MH Clones	
AGTACTACCACTGTG	Δ	Insertion	P	Δ	GTCGACCTGCAGCCC			
AGTACTACCACTGTG	0			3	GACCTGCAGCCC			0 2
AGTACTACCACTGTG	0			10	AGCCC			0 2
AGTACTACCACTGTG	0			5	CCTGCAGCCC			0 2
AGTACTACCACTGTG	0			AC	0	GTCGACCTGCAGCCC		0 1
AGTACTACCACTGTG	0	G		3	GACCTGCAGCCC			0 1
AGTACTACCACTGTG	0			4	ACCTGCAGCCC			1 1
AGTACTACCACTGTG	0			2	CGACCTGCAGCCC			0 1
AGTACTACCA	5			11	GCCC			2 1
	36			23				0 1
AGTACTACCAC	4				RAG2 gene			1 1
AGTACTACC	6	AC	0	0	GTCGACCTGCAGCCC			2 1
	24			16	AG			2 1
AGTACTACCAC	4			80				0 1
	15			15	A			1 1
								17 Total

Figure 2. 7 Signal joints, coding joints, and hybrid joints Artemis-P70 MEFs Recombined pJH299 joint sequences showing nucleotide deletions (Δ), N-nucleotide additions (N), and palindromic-nucleotide additions (P). Microhomologies (MH) are labeled in red.

RSS23	Signal Joint				RSS12	Coding joint					Coding end	MH Clones		
AGTACTACCACTGTG	Δ	N	P	Δ	CACAGTGCTACAGAC	TCGATGAGAGGATCC	Δ	P	N	P	Δ	GTCGACCTGCAGCCC	MH	Clones
AGTACTACCACTGTG	0		0		CACAGTGCTACAGAC		32				23		CGT	3 2
AGTACTACCACTGTG	0		0		CACAGTGCTACAGAC		24				16		A	1 2
AGTACTACCACTGTG	0		0		CACAGTGCTACAGAC		19				6		CTGCAGCCC	2 2
AGTACTACCACTGTG	0		0		CACAGTGCTACAGAC		27				7		TGCAGCCC	1 1
AGTACTACCACTGTG	0		0		CACAGTGCTACAGAC		30				13			0 1
AGTACTACCACTGTG	0		0		CACAGTGCTACAGAC		46				44			0 1
AGTACTACCACTGTG	0		0		CACAGTGCTACAGAC		21				3		CGACCTGCAGCCC	1 1
AGTACTACCACTGTG	0		0		CACAGTGCTACAGAC		176				122		CC	2 1
AGTACTACCACTGTG	0		0		CACAGTGCTACAGAC	TCGATGA	8		T		9		CAGCCC	0 1
AGTACTACCACTGTG	0		0		CACAGTGCTACAGAC	TCGATGAGAGGATCC	0	GG			5		CCTGCAGCCC	0 1
														13 Total

RSS23	Coding joint				Coding end		MH Clones	
AGTACTACCACTGTG	Δ	N	P	Δ	GTCGACCTGCAGCCC	MH	Clones	
	34			31		T	1 2	
	19			19			0 2	
AGTACTACCACTGTG	0		CGAC	0	GTCGACCTGCAGCCC		0 2	
A	14			13		CC	0 1	
AGTACTACCACTGTG	0			14		C	0 1	
AGTACTACCACTGTG	0			5		CCTGCAGCCC	0 1	
	15			10		AGCCC	2 1	
	21			9		CAGCCC	3 1	
	60			29		C	1 1	
AGTACTACCACTGTG	0			10		AGCCC	0 1	
	29			13		CC	1 1	
AGTACCA	8			2		CGACCTGCAGCCC	2 1	
	51			39		G	1 1	
	40			21		GG	2 1	
AGTAC	10		GAC	0	GTCGACCTGCAGCCC		0 1	
	26	T		12		CCC	0 1	
AGTACTACCACTGTG	0	T		9		CAGCCC	0 1	
								20 Total

Figure 2. 8 Signal joints, coding joints, and hybrid joints from Artemis-/- MEFs. Recombined pJH299 joint sequences showing nucleotide deletions (Δ), N-nucleotide additions (N), and palindromic-nucleotide additions (P). Microhomologies (MH) are labeled in red.

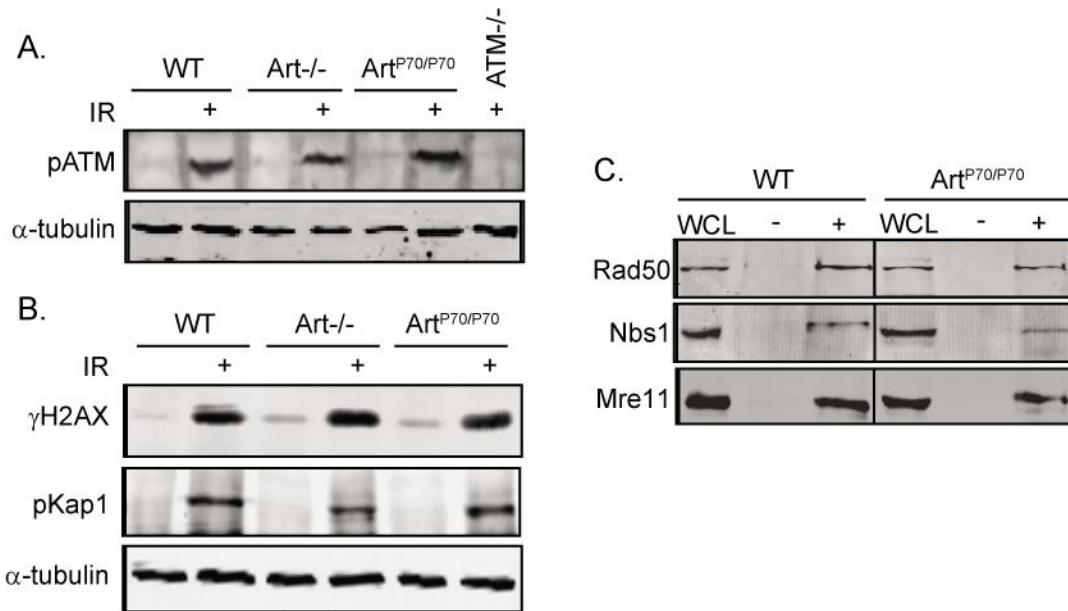


Figure 2. 9 ATM activity and MRN stability in Artemis-P70 backgrounds. (A-B) Wildtype, Artemis-null, Artemis-P70, and ATM-null MEFs were irradiated (10 Gy) and harvested after 1 hour. Phosphorylated ATM was visualized using Western blot and phospho-ATM antibody. Antibodies against phosphorylated ATM substrates gH2AX and Kap1 were used to assess ATM activity on downstream targets. Alpha tubulin was used as a normalization control. (C) Mre11 immunoprecipitation was performed on cell lysates from wildtype and Artemis-P70 MEFs. Co-immunoprecipitation of protein complex partners were detected with antibodies against Nbs1 and Rad50. WCL = whole cell lysate, (-) = no antibody, (+) = with Mre11 antibody.

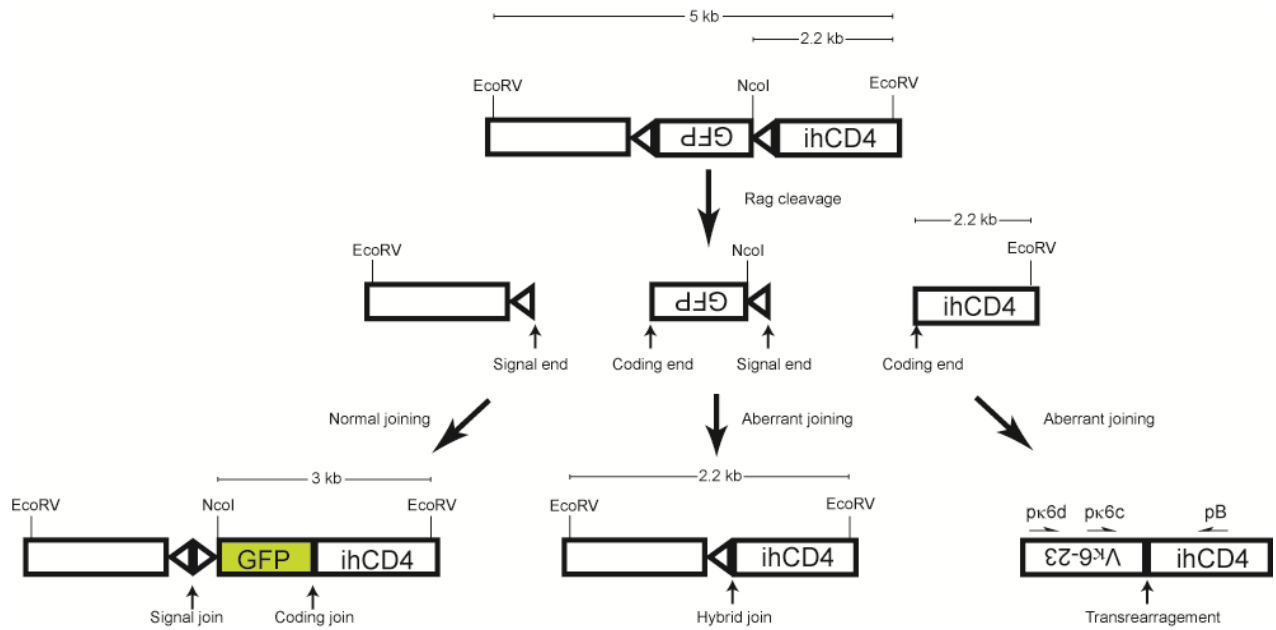


Figure 2. 10 pMX-INV intrachromosomal recombination substrate. pMX-INV was integrated into the chromosome of Artemis-null murine pre-B cells using retroviral transduction. To ensure all cells contain the substrate, cells were sorted for expression of human CD4. Treatment of cell lines with STI571 induces Rag expression and recombination. Recombination events can be analyzed using genomic Southern blot and PCR Southern blot. Coding joins and hybrid joins are analyzed by digesting genomic DNA with EcoRV and NcoI. Hybrid joining and accumulation of coding ends can be determined with EcoRV single digest. Transrearrangements are analyzed using PCR with indicated primers.

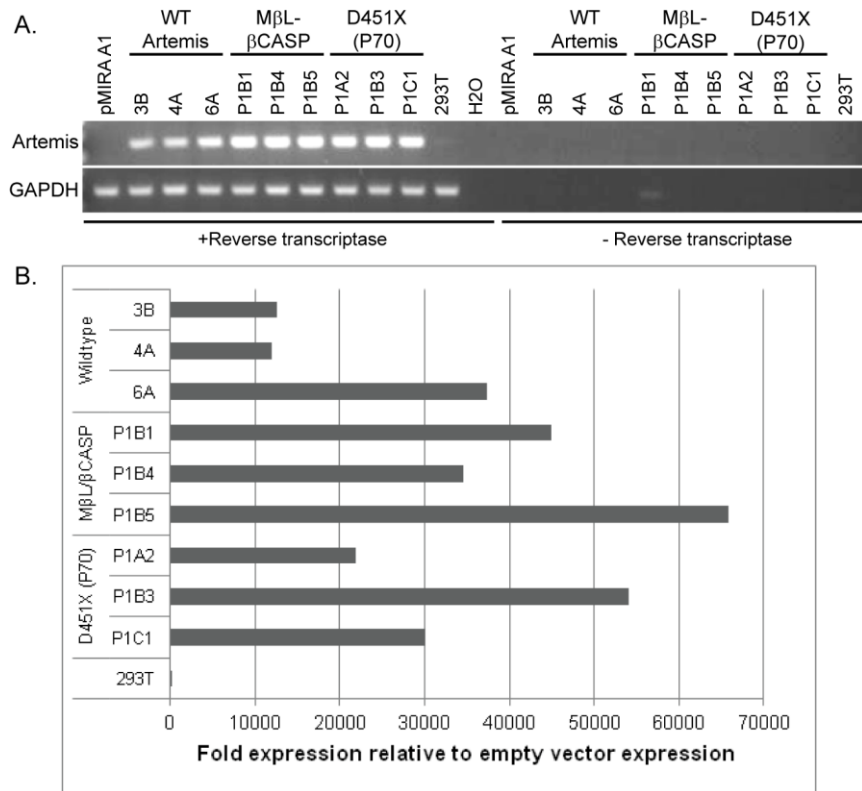


Figure 2. 11 RNA expression level of Artemis constructs in pre-B cell lines. (A) Artemis mRNA analysis of vector-only clone (pMIRAA1), 3 wildtype Artemis expressing clones, 3 MβL/βCASP expressing clones, and 3 D451X expressing clones by RT-PCR. Housekeeping gene GAPDH used as a normalization control. (B) Artemis mRNA analysis of construct-expressing clones using quantitative PCR.

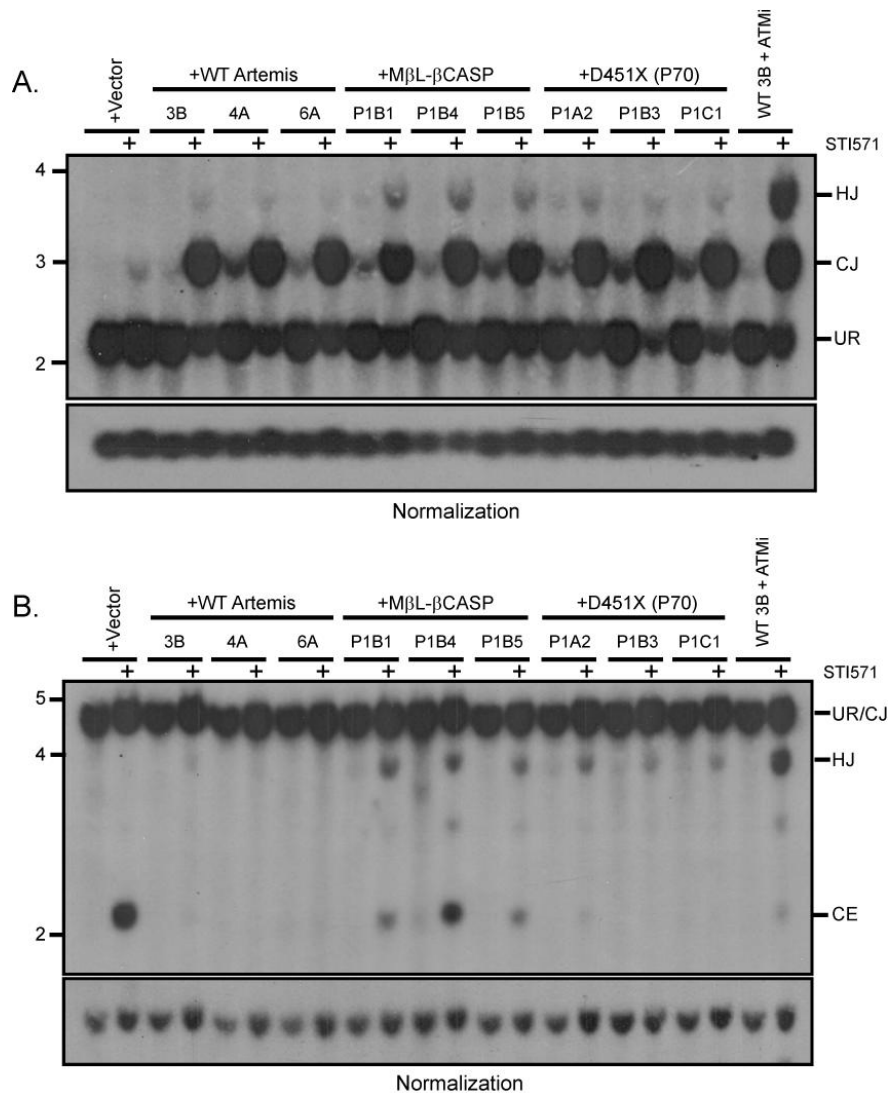


Figure 2. 12 Loss of Artemis C-terminus increases aberrant rearrangement products. Pre B cell lines were treated with STI571 or both STI571 and ATM-inhibitor KU55933 (ATMi). Cells were harvested at 96 hours and genomic DNA isolated. (A) Genomic DNA was digested with either EcoRV or (B) EcoRV and NcoI. Unrecombined substrate (UR), coding joins (CJ), Hybrid joins (HJ), and coding ends (CE) were visualized using genomic Southern blot.

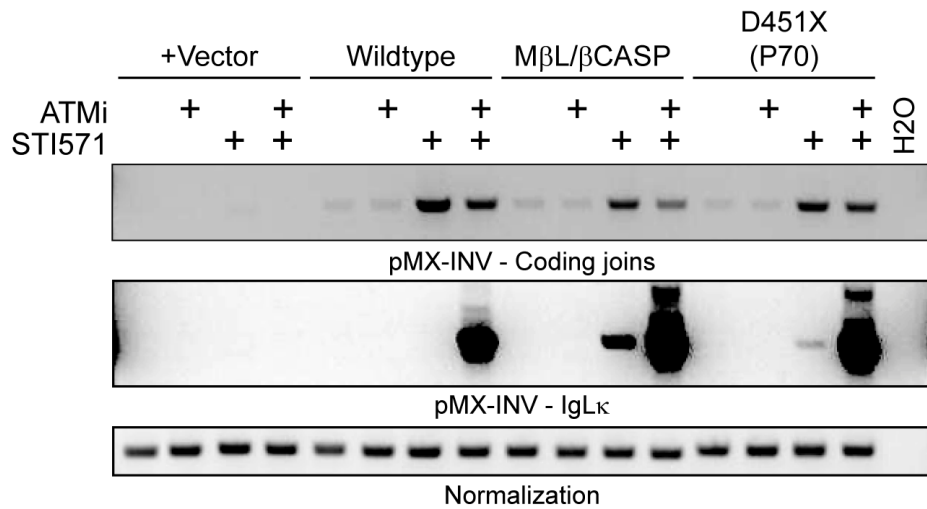


Figure 2. 13 Loss of the Artemis C-terminus results in Increased transrearrangements. Pre-B cell clones were treated with either ATM inhibitor KU55933 (ATMi), STI571, or both drugs. Cells were harvested at 96 hours post STI571 treatment and genomic DNA extracted. Nested PCR was used to amplify transrearrangements (refer to Figure 2.9). Amplification of ATM was used for normalization.

Mutation	Second allele	Lymphocytes	IR sensitivity (fibroblasts)	Study
H228N	Deletion of exon 1-4	Absent	Sensitive	Pannicke et al. 2010
I16T	S119X	Absent	Sensitive	Musio et al. 2005
T71P	Deletion of exon 1-3	Lymphopenia	Normal	Lee et al. 2013

Table 2. 1 Human Artemis N-terminal missense point mutations.

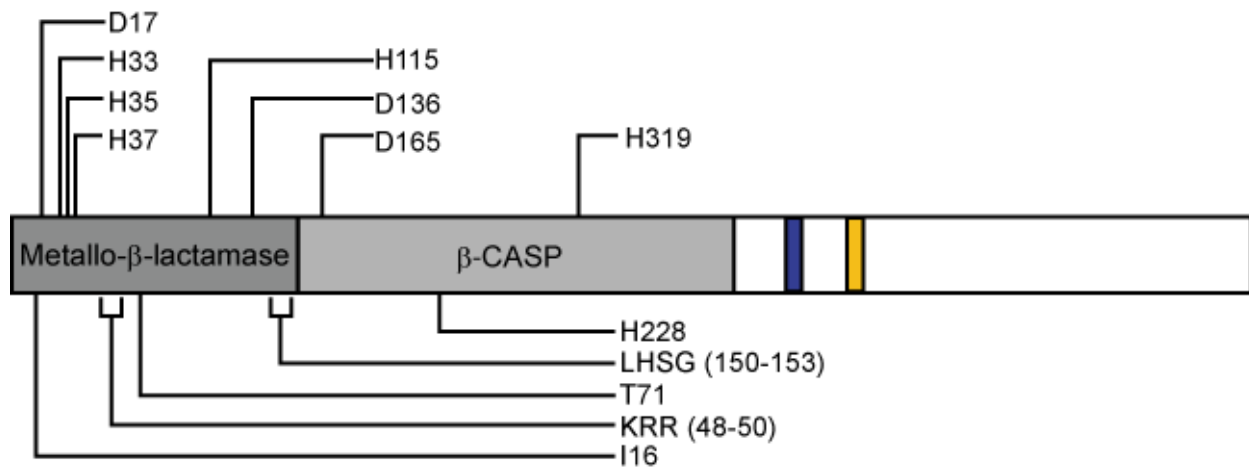


Figure 2. 14 Putative N-terminal regulatory domains. Several sites have been reported in the literature to be important for Artemis activity. These sites are labeled above the Artemis protein diagram. This study focused on sites that may play a role in regulating the nuclease but are unexplored or not well studied (labeled below the protein diagram)

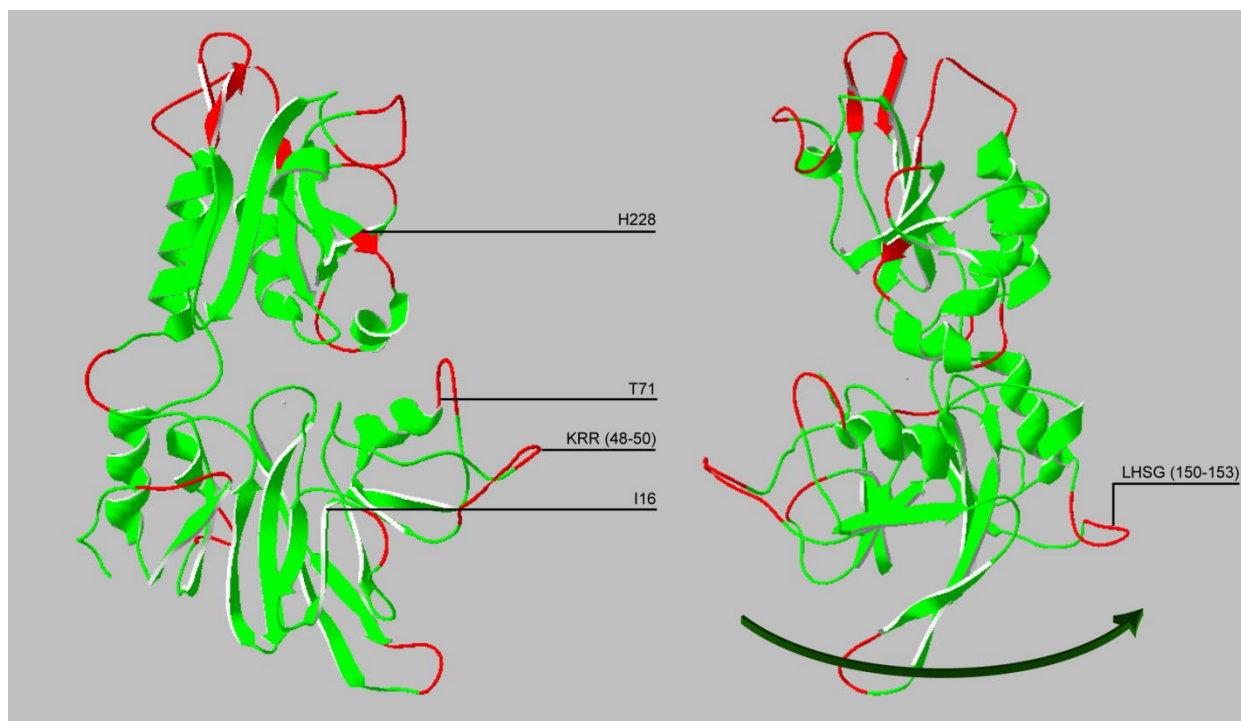


Figure 2. 15 Locations of putative regulatory domains on predicted structure. Artemis N-terminal domain structure prediction was generated by SWISS-MODEL and based on the crystallized structure of the Snm1B conserved domain. The Artemis N-terminus consists of two domains, the metallo- β -lactamase domain and the β -CASP domain. Here, the metallo- β -lactamase domain is depicted below the β -CASP domain with two peptide linkers separating the two. Loops in between the two domains are predicted to be important for coordinating a metal ion important for catalytic function. Red regions represent stretches of Artemis protein sequence that was unique when aligned with Snm1B. Regions that were analyzed by this study are labeled.

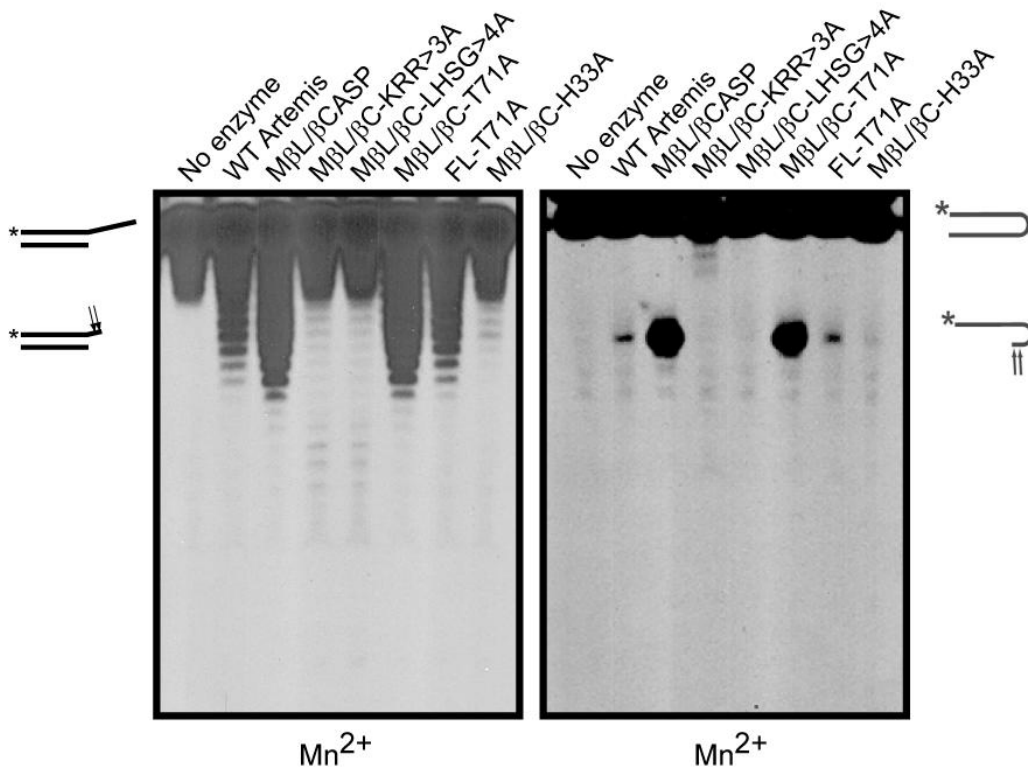


Figure 2. 16 KRR>3A and LHSG>4A mutations disrupt endonucleolytic activity. c-myc-6xHIS-tagged Artemis constructs were expressed in 293T cells and purified from DNA-PKcs. Protein normalization and purification from DNA-PKcs was confirmed by Western blot. Artemis protein was incubated with a 5' ³²P end-labeled DNA overhang (left) and hairpin (right) substrate in the presence of manganese. Reactions were then run on a 17% denaturing polyacrylamide gel and gel was exposed to X-ray film.

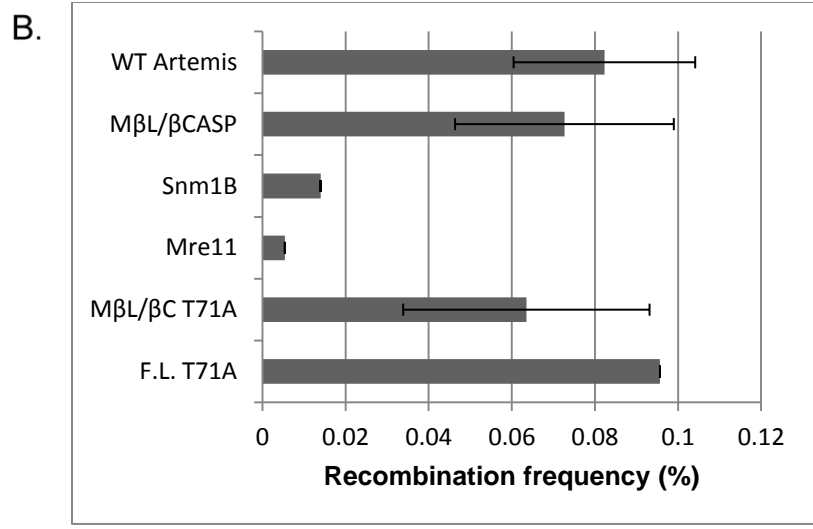
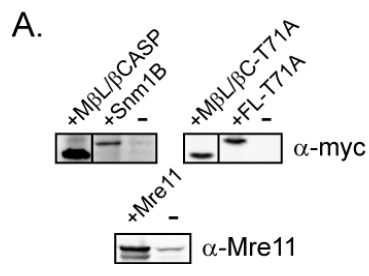


Figure 2. 17 Nuclease overexpression in the transient V(D)J assay (A) Plasmid expression of nucleases in 293T cells. (B) Nucleases were expressed from plasmid in Artemis-null MEFs along with inversional recombination plasmid substrate pJH299 and plasmids expressing the Rag proteins. Artemis truncation mutant and T71A mutant rescues V(D)J recombination defect but overexpression of other nucleases do not facilitate recombination.

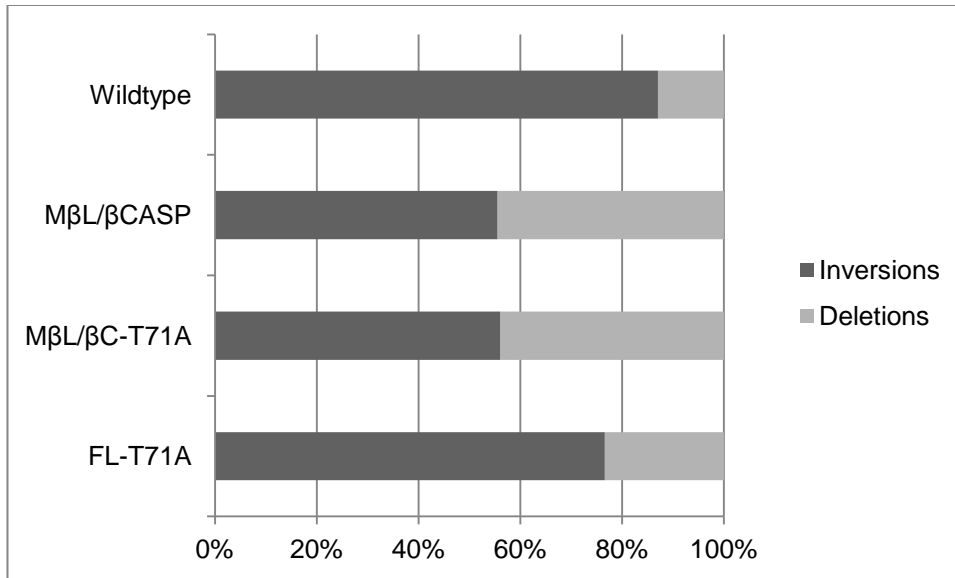


Figure 2. 18

Inversions and deletional joins recovered from transient V(D)J assay. Plasmid –based V(D)J recombination assay was performed using Artemis-null MEFs transiently expressing either wildtype Artemis, MβL/βCASP Artemis, MβL/βCASP-T71A, or full length Artemis-T71A mutant. Chloramphenicol and ampicillin resistant bacteria colonies were isolated, plasmid was extracted and sequenced.

RSS23	Signal joint			RSS12	Coding end				Coding joint	Coding end	MH	Clones
AGTACTACCACTGTG Δ	N	Δ	CACAGTGCTACAGAC	TCGATGAGAGGATCC Δ P	N	P	Δ	GTCGACCTGCAGCCC				
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGGAT	2		5	CCTGCAGCCC		2	10	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGGA	3		C 0	GTCGACCTGCAGCCC		0	8	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAG	5		3	GACCTGCAGCCC		2	7	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0		0	GTCGACCTGCAGCCC		0	7	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGGA	3		1	TCGACCTGCAGCCC		2	6	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAG	5		0	GTCGACCTGCAGCCC		1	4	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0		2	CGACCTGCAGCCC		0	3	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGGATC	1		2	CGACCTGCAGCCC		1	3	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0		1	TCGACCTGCAGCCC		0	2	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGGATC	1		0	GTCGACCTGCAGCCC		0	2	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0		8	GCAGCCC		0	2	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGGAT	2		6	CTGCAGCCC		1	2	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGG	4		6	CTGCAGCCC		0	2	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGGATC	1		9	CAGCCC		1	1	
AGTACTACCACTGTG 0	2	0	CAGTGCTACAGAC	TCGATGAGAGG	4		8	GCAGCCC		0	1	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGGAT	2	T	7	TGCAGCCC		0	1	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAG	5	TT	1	TCGACCTGCAGCCC		0	1	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGA	6		0	GTCGACCTGCAGCCC		0	1	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGGATCC 0 G			2	CGACCTGCAGCCC		0	1	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAG	5		2	CGACCTGCAGCCC		0	1	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGGA	3		7	GCGAGCCC		1	1	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGG	4		10	AGCCC		1	1	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGGA	3		3	GACCTGCAGCCC		0	1	
AGTACTACCACTG 2	0	0	CACAGTGCTACAGAC	TCGATGAGAGGAT	2		6	CTGCAGCCC		1	1	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGG	4		0	GTCGACCTGCAGCCC		0	1	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGGAT	2		8	GCAGCCC		0	1	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGGATCC 0 G			5	CCTGCAGCCC		0	1	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGGATC	1		5	CCTGCAGCCC		1	1	
AGTACTACCACTGTG 0	0	0	CACAGTGCTACAGAC	TCGATGAGAGG	4		5	CCTGCAGCCC		0	1	

74 Total

RSS23	Hybrid joint				Coding end	MH	Clones
AGTACTACCACTGTG Δ	N	P	Δ	GTCGACCTGCAGCCC			
AGTACTACCACTGTG 0			3	GACCTGCAGCCC	0	1	
	17		0	GTCGACCTGCAGCCC	1	1	
AGTACTACCACTGTG 0	G		3	GACCTGCAGCCC	0	1	
AGTACTACCACTGTG 0			2	CGACCTGCAGCCC	0	1	
AGTACTACCACTGTG 0			5	CCTGCAGCCC	0	1	
AGTACTACCACTGT 1	C		29		0	1	
AGTACTACCACTGTG 0		AC	0	GTCGACCTGCAGCCC	0	1	
AGTACTACCACTGTG 0			3	GACCTGCAGCCC	0	1	
	25		29	CA	2	1	
AGTACTACCACTGTG 0			345		0	1	
AGTACTACCACTGT 1			3	GACCTGCAGCCC	1	1	

11 Total

Figure 2. 19 Signal, coding, and hybrid joints from wildtype MEFs. Recombined pJH299 joint sequences showing nucleotide deletions (Δ), N-nucleotide additions (N), and palindromic-nucleotide additions (P). Microhomologies (MH) are labeled in red.

RSS23	Signal joint			RSS12	Coding end	Coding joint				Coding end	MH clones			
AGTACTACCACTGTG	Δ	N	Δ	CACAGTGCTACAGAC	TCGATGAGAGGATCC	Δ	P	N	P	Δ	GTCGACCTGCAGCCC			
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGA	3				1	TCGACCTGCAGCCC	2	6	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATC	1				2	CGACCTGCAGCCC	1	4	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0				0	GTCGACCTGCAGCCC	0	3	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0				2	CGACCTGCAGCCC	0	2	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0				1	TCGACCTGCAGCCC	0	2	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC		40				45		0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC		35				2	CGACCTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAG	5				3	GACCTGCAGCCC	2	1	
AGTACTACCACTGTG	0		177		TCGATGAGAGGAT	2				4	ACCTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAG	5				8	GCAGCCC	1	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC		118				23	C	1	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0				4	ACCTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAG	5				0	GTCGACCTGCAGCCC	1	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0				5	CCTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGAT	2				7	TGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0	GG			5	CCTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0	GG			6	CTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGAT	2		A		4	ACCTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	-	43				68	TC	2	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGA	3				C	0	GTCGACCTGCAGCCC	0	1
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0				C	0	GTCGACCTGCAGCCC	0	1
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGAT	2				5	CCTGCAGCCC	2	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATC	1				5	CCTGCAGCCC	1	1	

35 Total

RSS23	Hybrid joint				Coding end	MH clones	
AGTACTACCACTGTG	Δ	N	P	Δ	GTCGACCTGCAGCCC		
AGTACTACCACTGTG	0		AC	0	GTCGACCTGCAGCCC	0	6
AGTACTACCACTGTG	0			2	CGACCTGCAGCCC	0	5
AGTACTACCACTGTG	0			0	GTCGACCTGCAGCCC	0	4
AGTACTACCACTGTG	0		C	0	GTCGACCTGCAGCCC	0	3
AGTACTACCACTGTG	0			4	ACCTGCAGCCC	1	2
AGTACTACC	6			4	ACCTGCAGCCC	2	1
AGTACTACCACTGTG	0			5	CCTGCAGCCC	0	1
	16			8	GCAGCCC	1	1
AGTACTACCACTGTG	0	G		3	GACCTGCAGCCC	0	1
AGTACTACCACTGT	1			2	CGACCTGCAGCCC	2	1
AGTACTACCACTGTG	0			1	TCGACCTGCAGCCC	1	1
AGTACTACCACTG	2			2	CGACCTGCAGCCC	0	1
AGTACTACCACTGT	1			4	ACCTGCAGCCC	0	1

28 Total

Figure 2. 20 Signal, coding, and hybrid joints from MEFs expressing MβL/βCASP. Recombined pJH299 joint sequences showing nucleotide deletions (Δ), N-nucleotide additions (N), and palindromic-nucleotide additions (P). Microhomologies (MH) are labeled in red.

RSS23	Signal joint			RSS12	Coding joint					Coding end		MH	Clones	
AGTACTACCACTGTG	Δ	N	Δ	CACAGTGCTACAGAC	TCGATGAGAGGATCC	Δ	P	N	P	Δ	GTCGACCTGCAGCCC			
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGA	3				1	TCGACCTGCAGCCC	2	4	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0				2	CGACCTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC		82				2	CGACCTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC		48				2	CGACCTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATC	1				0	GTCGACCTGCAGCCC	0	2	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0				3	GACCTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0				1	TCGACCTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAG	5				3	GACCTGCAGCCC	2	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0	GG			6	CTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGA	3				C	0	GTCGACCTGCAGCCC	0	2
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0				10	AGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGG	4				0	GTCGACCTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGG	4				C	0	GTCGACCTGCAGCCC	0	1
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC		23				1	TCGACCTGCAGCCC	2	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGG	4				8	GCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGA	6				3	GACCTGCAGCCC	1	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0				0	GTCGACCTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGAT	2				8	GCAGCCC	0	1	
23 Total														

RSS23	Coding joint					CJ Right	MH	Clones
AGTACTACCACTGTG	Δ	N	P	Δ	GTCGACCTGCAGCCC			
AGTACTACCACTGTG	0			AC	0	GTCGACCTGCAGCCC	0	3
AGTACTACCACTGT	1				3	GACCTGCAGCCC	1	2
AGTACTACCACTGTG	0				5	CCTGCAGCCC	0	2
AGTACTACCACTG	2			AC	0	GTCGACCTGCAGCCC	0	2
AGTA	11				67	CT	2	1
AGTACTACCACTGTG	0				2	CGACCTGCAGCCC	0	1
	3				24	GT	2	1
	21				9	CAGCCC	0	1
AG	13	A		AC	0	GTCGACCTGCAGCCC	0	1
AGTACTACCACTGTG	0				1	TCGACCTGCAGCCC	1	1
AGTACTACCACTGTG	0				4	ACCTGCAGCCC	1	1
AGTACTACCACTGT	1				2	CGACCTGCAGCCC	2	1
AGTACTACCACTGTG	0				6	CTGCAGCCC	0	1
18 Total								

Figure 2. 21 Signal, coding, and hybrid joints from MEFs expressing MβL/βC-T71A. Recombined pJH299 joint sequences showing nucleotide deletions (Δ), N-nucleotide additions (N), and palindromic-nucleotide additions (P). Microhomologies (MH) are labeled in red.

RSS23	Signal joint			RSS12	Coding joint					Coding end				
AGTACTACCACTGTG	Δ	N	Δ	CACAGTGCTACAGAC	TCGATGAGAGGATCC	Δ	P	N	P	Δ	GTCGACCTGCAGCCC	MH	Clones	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGAT		2			5	CCTGCAGCCC	2	3	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATC		1			2	CGACCTGCAGCCC	1	3	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGA		3			1	TCGACCTGCAGCCC	2	3	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATC		1			0	GTCGACCTGCAGCCC	0	2	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGA		3			7	TGCAGCCC	1	2	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATC		1			5	CCTGCAGCCC	1	2	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC		0			0	GTCGACCTGCAGCCC	0	2	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGAT		2			6	CTGCAGCCC	1	2	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATC		1			4	ACCTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGA		6			6	CTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGA		3		AC	0	GTCGACCTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC		0			2	CGACCTGCAGCCC	0	1	
AGTACTACCACTGTG	2		0	CACAGTGCTACAGAC	TCGATGAGAGGA		3			C	0	GTCGACCTGCAGCCC	0	1
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0	G			0	GTCGACCTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATC	1				8	GCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0	G			4	ACCTGCAGCCC	1	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGAT	2				9	CAGCCC	1	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGG	4				8	GCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGG	4				0	GTCGACCTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0				8	GCAGCCC	0	1	
AGTACTACCACTGT	1		0	CACAGTGCTACAGAC	TCGATGAGAGG	4		T		7	TGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGATCC	0	GG			3	GACCTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGA	8				3	GACCTGCAGCCC	2	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCG	12				6	CTGCAGCCC	0	1	
AGTACTACCACTGTG	0		0	CACAGTGCTACAGAC	TCGATGAGAGGAT	2				7	TGCAGCCC	0	1	
36 Total														

RSS23	Hybrid joint				Coding end	
AGTACTACCACTGTG	Δ	N	P	Δ	GTCGACCTGCAGCCC	MH Clones
AGTACTACCACTGTG	0			2	CGACCTGCAGCCC	0 2
AGTACTACCACTGTG	0			4	ACCTGCAGCCC	1 2
AGTACTACCACTGTG	0		AC	0	GTCGACCTGCAGCCC	0 1
AGTACTACCACTGTG	0			10	AGCCC	0 1
AGTACTACCACTGTG	0			122		3 1
AGTACTACCACTGTG	57			56	AT	2 1
AGTACTACCACTGTG	0			8	GCAGCCC	0 1
AGTACTACCACTGTG	0			5	CCTGCAGCCC	0 1
AGTACTACCACT	3			3	GACCTGCAGCCC	1 1
11 Total						

Figure 2. 22 Signal, coding, and hybrid joints from MEFs expressing full length T71A. Recombined pJH299 joint sequences showing nucleotide deletions (Δ), N-nucleotide additions (N), and palindromic-nucleotide additions (P). Microhomologies (MH) are labeled in red.

Chapter 3

Cooperation between the Artemis C-terminus and the ATM kinase in lymphocyte development and immune regulation

Abstract

Human patients with mutations in the *ARTEMIS* gene exhibit severe combined immunodeficiency and radiosensitivity (RS-SCID) with an absence of B and T lymphocytes. Patients with hypomorphic mutations involving the Artemis C-terminus have been described and these patients exhibit partial loss of B and T lymphocytes but with a predisposition of lymphoid malignancies. Our lab has previously generated a mouse model for human patients with hypomorphic Artemis mutant alleles which we call Artemis-P70. Artemis-P70 mice have an early stop codon that truncates the majority of the Artemis C-terminus. Lymphocytes from these mice produce aberrant V(D)J rearrangement products and in addition, when these mice are crossed into a p53^{-/-} background, all mice succumb to lymphomas that harbor clonal translocations.

To address the genetic interactions between the Artemis C-terminus and the ataxia telangiectasia mutated (ATM) kinase, I crossed our Artemis-P70 mice into mice from an ATM^{-/-} background. Despite the propensity of ATM^{-/-} mice and Artemis-P70 p53^{-/-} mice for tumorigenesis, the double mutant mice do not develop any observable tumors. As a result, these mice survived significantly longer than ATM^{-/-} and Artemis-P70 p53^{-/-} mice. Loss of both the Artemis C-terminus and ATM resulted in a lymphocyte deficiency that was more severe than loss of either factor alone. Hematopoietic stem cell population depletion was not found to be the source of this deficiency. Surprisingly, these mice exhibited thickened and inflamed colons with infiltrating T cells and neutrophils. Together, these results indicate that the ATM kinase and Artemis C-terminus cooperatively facilitate lymphocyte development. The severe, although incomplete, depletion of lymphocytes may give rise to mice that are uniquely susceptible to chronic inflammation.

Introduction

The *ARTEMIS* gene was first discovered in a population of Athabascan speaking American Indians with radiosensitive severe combined immunodeficiency (SCID). These patients had an absence of B and T lymphocytes with a normal population of NK cells. In addition to immunodeficiency, cells from these patients exhibit radiosensitivity [1]. The Artemis protein was soon found to play a critical role in the development of B and T cells and in general double strand break (DSB) repair [2].

The antigen receptors of B and T cells (BCRs and TCRs, respectively) can recognize a large variety of substrates including foreign pathogens and toxins. The process required to generate antigen receptors with this level of diversity is called V(D)J recombination. During this gene rearrangement event, the Rag endonuclease comprised of the RAG1 and RAG2 proteins introduce DSBs at recombination signal sequences (RSS) adjacent to the gene segments that code for the B and T cell receptors. Rag DNA cleavage generates two types of DNA ends, a blunt “signal” end adjacent to the RSS, and a covalently sealed hairpin “coding” end adjacent to the gene segment which codes for the antigen receptor. In order for the gene segments to be joined, the hairpin coding end must be opened. The opening of these hairpin coding ends was found to be dependent on the DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) and the Artemis nuclease. Loss of Artemis results in the accumulation of unopened coding ends and B and T cell development is arrested [1, 2].

Artemis also takes part in the general DSB repair process nonhomologous end joining (NHEJ). There are six other NHEJ factors: KU70, KU80, DNA-PKcs, XRCC4, XLF, and the ligase LIG4. KU70 and KU80 first recognize and bind to the DSB. DNA-PKcs and Artemis is recruited and some of these ends are processed by the Artemis nuclease. It is estimated that about 10% of breaks induced by ionizing radiation requires Artemis to process DNA ends prior to rejoining [3]. The break is then joined by the ligation complex which includes XRCC4, XLF, and LIG4.

The majority of human Artemis mutations occur in the conserved catalytic N-terminal domain resulting in arrested V(D)J recombination and the loss of B and T cells [4]. This N-terminus (a.a. 1-385) consists of a metallo- β -lactamase and β -CASP domain which together forms the active site of the nuclease [1, 5, 6]. Human patient mutations

were soon discovered in the C-terminal domain (a.a. 396-692) [7]. Patients that had nonsense mutations in this domain resulting in early stop codons had a milder B and T cell defect and as a result these mutations are referred to as hypomorphic. Some hypomorphic Artemis mutant patients died of infection and interestingly, some others succumbed to Epstein-Barr virus (EBV) associated lymphoma. These tumors were of clonal origin and had chromosomal alterations indicating genomic instability. The tumor characteristics suggest that the Artemis C-terminus may be important for not only for V(D)J recombination but to prevent tumorigenesis.

The Artemis C-terminus has been shown to be important for interaction with DNA-PKcs at L401 R402 [8] and LIG4 at W489 and these interactions are important for efficient V(D)J recombination [9]. This domain has also been shown to be important for efficient endonucleolytic activity [10, 11] and has multiple SQ phosphorylation sites that can act as a substrate for either DNA-PKcs or the ataxia-telangiectasia mutated (ATM) kinase [8, 12-16]. However, the functional significance of Artemis phosphorylation has yet to be elucidated.

To further investigate the functional role of the Artemis C-terminus *in vivo*, we have generated a mouse model of one of the human hypomorphic Artemis mutant alleles. Human patients with this allele have a 7-nucleotide deletion at aspartic acid 451 which results in a frameshift and an early stop codon (D451fsX10). Our mouse model, which we refer to as P70, has an early stop codon introduced to aspartic acid 449 and like the human patient allele, results in the loss of most of the C-terminal domain. Artemis-P70 mice have reduced B and T cell populations and when crossed into p53-deficient backgrounds, all mice succumb to B and T cell lymphoma. We found that Artemis-P70 thymocytes have increased coding end accumulation and these coding ends can go on to engage in aberrant transrearrangements. [11, 17]

The ATM kinase plays important roles in cell cycle arrest and general DSB repair in response to radiation [18]. Although ATM does not seem to play a direct role in V(D)J recombination, loss of ATM does impact lymphocyte development. ATM^{-/-} mice have a mild B and T lymphocyte deficiency and succumb to thymic lymphoma [19]. ATM-deficient lymphocytes also accumulate coding ends and harbor aberrant rearrangements [20, 21]. These findings have led to the hypothesis that ATM and its

kinase activity may be important for stabilizing the post-cleavage complex (PCC), a complex of proteins thought to be important for the synapsis of Rag-generated DNA ends.

There have been some reports that suggest that the ATM kinase and Artemis may operate in the same pathway during general DSB repair. ATM-deficient and Artemis-deficient fibroblasts were found to display identical levels of sensitivity to ionizing radiation and treatment of Artemis-deficient fibroblasts with the ATM inhibitor KU-55933 did not increase sensitivity to ionizing radiation [3]. Furthermore, one report found the Artemis plays a role in homologous recombination and that its function here was in the same pathway as ATM [22]. As mentioned above, ATM can phosphorylate Artemis at its C-terminus in response to ionizing radiation although the function of this event is unclear [3, 16]. Despite some evidence that ATM and Artemis could function in the same pathway during DSB repair, it is unclear whether this is the case during V(D)J recombination.

In order to address the relationship between the Artemis C-terminus and the ATM kinase *in vivo*, we crossed our Artemis-P70 mouse to an ATM^{-/-} background. Surprisingly, we found that this cross rescued the lymphoma phenotype observed in both single mutants. These mice also lived significantly longer than ATM^{-/-} mice and Artemis-P70 p53^{-/-} mice. Artemis-P70 ATM^{-/-} mice exhibited severely reduced lymphocyte numbers when compared to single mutant mice. Despite the lack of tumors, these mice become moribund earlier than Artemis-P70 littermates and unexpectedly, we find that these mice exhibited thickened and inflamed colons. Together, these results indicate that the loss of both factors, ATM and the Artemis C-terminus, has an additive negative impact on lymphocyte development. However, the severely compromised immunity of these mice may result in a dysregulated response in the colon resulting in chronic inflammation.

Results

Survival of Artemis-P70 ATM^{-/-} double mutant mice

We have previously noted similarities between the phenotypes observed in the Artemis-P70 mice and ATM deficient mice. Both mutant mice have lymphocyte

deficiencies and are prone to lymphoma. ATM deficient mice succumb to thymic lymphoma between 2 and 4 months of age [19, 23]. A proportion of Artemis-P70 mice succumb to T cell lymphoma but when this mutation was crossed into a p53^{-/-} background, all mice died of either thymic lymphoma or pro-B lymphoma [17]. Both genotypes display increased aberrant V(D)J rearrangements [11, 17, 19, 20, 23]. These similarities has led us to speculate that the Artemis C-terminus and the ATM kinase may function epistatically in preventing aberrant V(D)J rearrangements which then result in lymphocyte deficiencies and lymphoma.

In order to determine the impact of the loss of ATM in Artemis-P70 mice on survival and tumorigenesis, Artemis-P70 ATM^{-/-} double mutant mice were generated through mouse breedings. Our lab had in the past generated Artemis-P70 p53^{-/-} mice as well as the Artemis^{-/-} p53^{-/-} mice and found that these mice succumb to lymphoma with a median survival between 11 and 13 weeks [17]. Strikingly, unlike the ATM^{-/-} mice, the Artemis-P70 p53^{-/-} double mutants, and the Artemis^{-/-} p53^{-/-} mice, no tumors were found in the Artemis-P70 ATM^{-/-} double mutant mice at time of death. The lack of tumors extended the lifespan of Artemis-P70 ATM^{-/-} double mutant mice when compared to ATM^{-/-} mice (median survival of 31 weeks for double mutant mice compared to median survival of 21.85 weeks for ATM^{-/-} mice, P=0.02, two-tailed log rank test). Although the double mutant mice were rescued of lymphoma, these mice were small, thin, suffered from diarrhea, and experienced a failure to thrive. (Figure 3.1)

In contrast, Artemis^{-/-} ATM^{-/-} mice have been reported to live up to 36 months without tumors [24]. These mice are reported to appear healthy but smaller. Similarly, my preliminary data (n=6) has shown that these mice do not exhibit lymphoid tumors and seem to survive longer than Artemis-P70 ATM^{-/-} mice with a median survival of 52.15 weeks.

Lymphocyte analysis of Artemis-P70 ATM^{-/-} double mutant mice

Both Artemis-P70 mice and ATM^{-/-} mice have been shown to exhibit mild B and T lymphocyte deficiencies. I utilized the Artemis-P70 ATM^{-/-} double mutant mice to explore the relationship between the Artemis C-terminus and the ATM kinase on lymphocyte development. To this end, mice 6-8 weeks of age were euthanized and lymphoid organs were collected and dissociated. Lymphocytes were stained with

antibodies against B and T cell surface markers and analyzed using flow cytometric analysis (Figure 3.2).

Different cell surface proteins are present during different stages of lymphocyte development. This analysis allows us to determine how the mutant genotypes are affecting specific stages of lymphocyte development. Thymocytes begin rearranging the TCR β -chain genes during the double negative stage, when neither cell surface markers CD4 and CD8 are detectable. These thymocytes then progress to the double positive stage when the TCR α -chain is rearranged. Double positive thymocytes then progress into the single positive stage, either displaying CD4 or CD8 on the cell surface. Single positive cells then migrate out of the thymus and into the peripheral tissues.

Our lab has previously found that Artemis-P70 thymocytes experience a partial block at the double negative stage and this resulted in fewer double positive thymocytes. Artemis-P70 ATM^{-/-} double mutant mice have a more severe defect in double positive thymocytes when compared to either Artemis-P70 or ATM^{-/-} mice (Artemis-P70 = 66%, ATM^{-/-} = 54%, Artemis-P70 ATM^{-/-} = 0.49%). Similarly, there were much fewer single positive peripheral T cells in our double mutant mice lymph nodes than in single mutant mice (CD4⁺, Artemis-P70 = 15%, ATM^{-/-} = 23% Artemis-P70 ATM^{-/-} = 2.3%) (CD8⁺, Artemis-P70 = 12%, ATM^{-/-} = 18%, Artemis-P70 ATM^{-/-} = 2.2%). However, this defect was not as severe as in Artemis^{-/-} mice (CD4⁺ Artemis^{-/-} = 0.46%, CD8⁺ Artemis^{-/-} = 0.03%). (Table 3.1)

We also analyzed the development of B cells in Artemis-P70 ATM^{-/-} mice. B lymphocytes begin development in the bone marrow where the Rag endonuclease initiates recombination in the pro-B cell stage (B220⁺ CD43⁺). During this stage, the immunoglobulin heavy-chain genes are rearranged. After successful rearrangement, the lymphocyte then moves into the pre-B cell stage (B220⁺ CD43⁻) where the light-chain genes are rearranged. Productive rearrangements results in the expression of surface IgM antibody and an exit out of the pre-B cell stage and into the immature B cell stage. These cells then undergo negative selection in the bone marrow to eliminate cells expressing self-reactive antibodies. Surviving cells then leave the bone marrow and migrate to the peripheral tissues

We had previously shown that Artemis-P70 B lymphocytes experience a block between the pro-B and pre-B cell stage resulting in low pre-B cell number and an accumulation of pro-B cells. Our lab had also found that Artemis-P70 mice had fewer IgM expressing lymphocytes than wildtype mice. I analyzed the lymphocytes of the Artemis-P70 ATM^{-/-} double mutant mice and found these mice to have significantly fewer pro-B, pre-B, and IgM⁺ splenocytes than either Artemis-P70 or ATM^{-/-} single mutant mice (Table 3.1). The significant reduction in pro-B cells suggests a more severe defect in V(D)J recombination during the very early stages of heavy-chain rearrangement resulting in the programmed cell death of these cells. Alternatively, this phenotype could suggest that Artemis-P70 ATM^{-/-} mice have a defect in the maintenance of the hematopoietic stem cell (HSC) population.

There are some reports indicating that DNA repair factors are important for the maintenance of HSCs [25, 26]. One report demonstrated that older ATM^{-/-} mice had defective HSC function associated with oxidative stress [27]. To address whether the Artemis-P70 ATM^{-/-} mice had HSC deficiencies, bone marrow from wildtype and double mutant mice between the ages of 5 and 24 weeks was harvested. Bone marrow cells were stained and analyzed using an established protocol identifying HSCs as lineage negative, c-kit positive, Sca-1 positive, CD48 negative, and CD150 positive [28]. We did not detect any significant differences in HSCs in Artemis-P70 ATM^{-/-} mice when compared to wildtype at any age analyzed (Figure 3.3).

Rearrangements in Artemis-P70 ATM^{-/-} backgrounds result in transrearrangements

Our lab had previously found that lymphocytes from Artemis-P70 mice harbored aberrant transrearrangements [17]. We noted the similarities between the Artemis-P70 phenotype and what has been reported in the literature for ATM-deficiency. Thymocytes and splenocytes from ATM-deficient mice have also been shown to harbor aberrant rearrangements and as a result, we asked how the Artemis C-terminus and the ATM kinase could contribute to promoting proper recombination.

I isolated thymocyte DNA from the Artemis-P70 ATM^{-/-} double mutant mice and using nested PCR and Southern blot, rearrangements that occurred within the TCR β locus were analyzed. Nested PCR Southern blot was also utilized to detect aberrant

rearrangements products between the TCR β locus and the TCR γ locus, one locus on chromosomes 6 and the other on chromosome 13, respectively (Figure 3.4 A). As expected, aberrant transrearrangements were detected in both Artemis-P70 and the ATM $^{-/-}$ thymocytes but were rare in wildtype thymocytes. I found that the Artemis-P70 ATM $^{-/-}$ double mutant mice also harbored transrearrangements between the two loci. However, nested PCR amplified fewer aberrant transrearrangements in the double mutant thymocytes when compared to either Artemis-P70 and ATM $^{-/-}$ single mutants. (Figure 3.4 B)

To compare the ratio of aberrant joins to normal rearrangements, the Southern blot was exposed to a phosphorimager screen and band intensity was measured using phosphorimager software (Figure 3.4 C). I found that the ratio of aberrant joins to normal joins in double mutant mice was statistically no different than the ratio found in Artemis-P70 or ATM $^{-/-}$ thymocytes. This result suggests that Artemis C-terminus and the ATM kinase work epistatically in the prevention of aberrant rearrangements in developing thymocytes. However, I have found previously that inhibiting ATM kinase activity in Artemis-P70 protein expressing pre-B cells increased transrearrangements (Chapter 2, Figure 2.13). A possible explanation for this difference is that in Artemis-P70 ATM $^{-/-}$ mice, many thymocytes that harbored aberrant rearrangements may have been eliminated from the population as a result of non-productive rearrangements and therefore, those events were not detected in this experiment.

Artemis-P70 ATM $^{-/-}$ double mutant mice are glucose tolerant

Our lab had previously observed that 2 of our 14 Artemis-P70 mice succumb to thymic lymphoma during a 12 month period [17]. As reported in the literature, nearly all ATM $^{-/-}$ succumb to thymic lymphoma [19] and our ATM $^{-/-}$ mice have a median survival of only 22 weeks. Despite the tumor phenotype of the two single mutant mice, our Artemis-P70 ATM $^{-/-}$ double mutant notably did not have any observable tumors. However, the absence of tumors did not extend the longevity of the double mutant mice to wildtype levels as they had a median survival of only 31 weeks (Figure 3.1). These mice were smaller at birth, did not catch up to their littermates, and at time of death, had fewer fat stores and muscle tissue.

The small size and body wasting observed in the double mutant mice suggested that the double mutant mice may have difficulty absorbing nutrients. One study crossed a *LIG4*^{-/-} mouse into a p53-hypomorphic (R172P) background [29]. *p53*^{R172P} is defective in apoptosis but not cell cycle arrest. The *p53*^{R172P} background rescues the embryonic lethality of *LIG4* deficiency but these mice were shown to have a diabetic phenotype. I asked whether our double mutant mice may also have glucose intolerance. Glucose tolerance is thought to decline with age and so mice of three different ages were tested (6-7 week, 24 week, and 40 week old mice). Age-matched wildtype and *Artemis*-P70 littermates were used as controls. These mice were weighed, fasted for 6 hours, and then administered glucose at 0.7g per kg of mouse body weight. Blood glucose levels were then measured over the course of two hours (Figure 3.5).

While there was variability to the timing of the blood glucose peak and baseline glucose levels, all mice tested recovered from glucose administration within two hours. Additionally, no mouse tested had abnormally high baseline blood glucose. These results indicate that *Artemis*-P70 *ATM*^{-/-} double mutant mice are glucose tolerant and do not have a diabetic phenotype.

***Artemis*-P70 *ATM*^{-/-} double mutant mice have thickened and inflamed colons**

The small size and body wasting phenotype of the double mutant mice suggested that although the mice were rescued from lymphoma, the loss of the *Artemis* C-terminus and the *ATM* kinase have detrimental effects that impact the lifespan of the mouse. To investigate the cause of failure to thrive and early death, I examined the gastrointestinal tract of the mouse at time of death. Surprisingly, 8 of 8 moribund *Artemis*-P70 *ATM*^{-/-} mice had abnormal and thickened colons. No age-matched littermate controls (0 of 4, genotypes *Artemis*-P70 *ATM*^{+/+} and *Artemis*-P70 *ATM*^{+/-}) exhibited this phenotype nor did any wildtype mice (Figure 3.6).

Colon tissues were fixed and sectioned for histology. H&E staining showed that unlike the colons of the wildtype and littermate control mice which have organized crypts that open on the lumen side, crypts from the double mutant colons had unusual branching and were disorganized. Double mutant colons also showed striking hyperproliferation that was not present in any control mice (Figure 3.7). I did not observe any gross colon phenotypes in *Artemis*^{-/-} *ATM*^{-/-} moribund mice (preliminary

data, n=4). However, hyperproliferation was observed in two of four colon sections from moribund mice stained with H&E (ages 48.4 and 56.4 weeks). The lack of gross hyperproliferation suggests that the colon abnormalities in these mice are much milder than in Artemis-P70 ATM^{-/-} mice.

To address the possibility of inflammation in the colon, anti-CD3 immunohistochemistry was performed on sectioned samples to visualize T-cells. Artemis-P70 ATM^{-/-} double mutant colons had increased levels of infiltrating CD3 positive T cells when compared to wildtype and littermate controls. Taken together, these results demonstrate that Artemis-P70 ATM^{-/-} mice colitis, inflammation and hyperproliferation of the colon epithelia. (Figure 3.8)

Pre-moribund mice were also analyzed in order to shed light on the development of colitis in Artemis-P70 ATM^{-/-} mice. I analyzed 10 pre-moribund double mutant mice between the ages of 5.3 and 29.9 weeks, Of these mice, 7 displayed colon hyperproliferation and increased CD3 positive infiltrating T cells (Figure 3.10 A). In contrast, of 8 Artemis-P70 littermates between the ages of 5.3 and 55.7 weeks, none exhibited this phenotype. Additionally, colitis was not observed in any of the 6 ATM^{-/-} mice ages 7 to 26.9 weeks.

Knowing that the Artemis C-terminus and ATM both are important for the repair of DSBs, we hypothesized that the colon inflammation could be brought on by the accumulation of DNA damage in the colon, followed by cell death, and a subsequent immune response to the dying colon epithelial cells. To address this possibility, colon samples with no detectable inflammation were sectioned and stained for phosphorylated histone H2AX (the phosphorylated version of the histone is denoted as γ -H2AX). In response to DNA damage, H2AX is phosphorylated by ATM and DNA-PKcs and is widely used as a marker for DSBs [30]. Both kinases function redundantly in this event and so in our case, the loss of ATM would not abrogate H2AX phosphorylation. We analyzed three Artemis-P70 ATM^{-/-} mice without signs of colitis (8.6, 13, and 29.1 weeks of age) and did not detect increased frequency of γ -H2AX positive cells in the colon tissue when compared to wildtype mice (Figure 3.9).

To better understand the nature of the inflammation we had observed in the Artemis-P70 ATM^{-/-} mice, lymphoid and myeloid cells were harvested from pre-

moribund mice colon lamina propria and these cells were analyzed by flow cytometry. Despite T cell infiltration and hyperplasia observed using immunohistochemistry, B cell infiltration was not observed in the double mutant mice (Figure 3.10 B). Surprisingly, there were fewer B cells in double mutant mice than in wildtype, Artemis-P70, and ATM^{-/-} mice (Artemis-P70 ATM^{-/-} compared to all genotypes $p < 0.05$, Student's unpaired t-test). The B cell frequencies appeared to reflect the mature B cell frequencies observed in the spleens and did not correlate with the inflammatory phenotype (Table 3.1, IgM⁺ splenocytes). Cells harvested from colon tissue were also analyzed by flow cytometry for the presence of the neutrophils. All four Artemis-P70 ATM^{-/-} pre-moribund mice analyzed had elevated levels of infiltrating neutrophils when compared to wildtype, Artemis-P70, and ATM^{-/-} mice (Figure 3.10 B). These findings confirmed that the innate and adaptive immune system both contribute to the inflammatory phenotype.

Several mouse models for colitis have been shown to be dependent on gut bacteria and when these mice are raised in germ free animal facilities, do not develop colitis [31-34]. We asked whether our mice harbored bacteria that have been reported to be associated with colon inflammation. Fecal pellets from our colony were tested for *Helicobacter bilis*, *H. ganmani*, *H. hepaticus*, *H. mastomyrinus*, *H. rodentium*, and *H. typhlonius*. We found that our mice were positive for *H. hepaticus*, and *H. ganmani*, both strains that have been reported to be associated with colitis in mice [35-37]. This finding suggests that the immunodeficiency caused by the loss of the Artemis C-terminus and ATM resulted in a susceptibility to colonization by *Helicobacter*. It has been demonstrated that the presence of some lymphocyte populations are important for the development of severe colitis in response to *H. hepaticus* [35, 36]. Those findings may explain why the double mutant mice, which have low numbers of mature B and T cells (Table 3.1), may be especially susceptible to this type of inflammatory response.

Discussion

In this study, we characterized the *in vivo* consequences of the loss of the ATM kinase in Artemis-P70 mice. Previously, we have shown that the loss of the Artemis C-terminus results in both B and T cell deficiencies [11]. There has been evidence reported that the loss of cell cycle checkpoint regulators ATM or p53 can rescue

embryonic lethality in Lig4^{-/-} and Xrcc4^{-/-} mice [38-40]. NHEJ null mice with lymphocyte deficiencies have been crossed into p53^{-/-} backgrounds resulting in minor increases in progenitor lymphocytes [38, 41, 42]. These findings raise the possibility that loss of ATM could rescue lymphocytes destined for apoptosis and increase the number of lymphocytes seen in Artemis-P70 mice. Another possibility was ATM and the Artemis C-terminus may operate in the same pathway during V(D)J recombination and double mutant mice will have similar levels of lymphocytes as single mutants. Supporting this hypothesis, past studies have shown that ATM can phosphorylate Artemis at its C-terminus and these events were dependent on DNA damage [14-16]. We found that loss of ATM further decreased both B and T lymphocyte counts. One explanation for this finding is that the loss of the Artemis C-terminus and the ATM kinase had a negative impact on HSCs which resulted in fewer lymphocyte progenitors. We found that there were fewer pro-B cells in Artemis-P70 ATM^{-/-} when compared to the single mutants. The pro-B stage is when V(D)J recombination is first initiated but by analyzing cells at this stage, it is unclear whether aberrant recombination is resulting in low cell counts or whether there are simply fewer progenitors entering this stage. To address this issue, we analyzed the HSC population of Artemis-P70 ATM^{-/-} mice but did not observe any deficiencies.

Another explanation for the low lymphocyte counts in double mutant mice is that the loss of both the Artemis C-terminus and ATM resulted in levels of aberrant joining that were that were so excessive that the vast majority of pro-B cells are programmed to undergo apoptosis. We detected increased levels of aberrant transrearrangements in Artemis-P70 ATM^{-/-} thymocytes when compared to wildtype but levels were not higher than Artemis-P70 or ATM^{-/-} single mutant thymocytes. Thymocytes that have undergone nonproductive rearrangements are eliminated from the lymphocyte population by apoptosis. Aberrant rearrangements that are in frame and encode a receptor, such as receptors that are the result of a transrearrangements, would also be selected against if they are unable to recognize the self-MHC molecule. It is possible then, that the double mutant thymocytes have levels of aberrant joins that are far above Artemis-P70 and ATM^{-/-} thymocytes but were not detected in thymocytes dissected directly from mice because they had already been eliminated from the population.

I used the Artemis^{-/-} pre-B cell system described in the previous chapter to address this issue. This system uses pre-B cells that have a chromosomally integrated inversional recombination substrate pMX-INV and can be induced to undergo V(D)J recombination upon treatment with the drug STI571 [20]. These cells do not undergo apoptosis after induction of V(D)J recombination despite harboring aberrant rearrangements [20]. I transduced this line with retrovirus expressing either wildtype Artemis, Artemis-M β L- β CASP, or the Artemis-P70 protein. I found that treatment with both STI571 and ATM kinase inhibitor (KU-55933) further increased transrearrangements in mutant Artemis-expressing lines beyond levels observed when treated with only STI571. This result suggests that loss of ATM in Artemis-P70 lymphocytes increases levels of aberrant events and this may lead to the severely reduced lymphocyte counts observed in double mutant mice. While this does not rule out the possibility that ATM may regulate Artemis through phosphorylation of its C-terminus, it does suggest that ATM may have multiple roles in the prevention of aberrant rearrangements. Others have speculated that ATM may also phosphorylate the members of the Mre11-Rad50-Nbs1 (MRN) complex, a protein complex that acts in DNA DSB repair and has also been shown to be important for preventing aberrant rearrangements during V(D)J recombination [43].

Our lab has previously reported that Artemis-P70 mice bred into a background deficient in the cell cycle regulator p53 all succumb to T or B cell lymphoma [17]. Because ATM has been shown to act upstream and is important for the activation of p53 during DNA damage response [44, 45], we hypothesized that loss of ATM in Artemis-P70 mice would result in tumorigenesis. In addition its role in cell cycle regulation, ATM also functions in DNA repair and the prevention of transrearrangements and so we considered the possibility that mice may succumb to tumors even earlier than Artemis-P70 p53^{-/-} mice [18]. We were surprised to find that we did not observe any lymphoid tumors in any of the Artemis-P70 ATM^{-/-} mice. One possible explanation for the lack of tumors is that lymphocytes of this background exhibit levels of genomic instability so high that they cannot support dysregulated growth rate. This concept is supported by the finding described above in which inhibition of the ATM kinase increased aberrant transrearrangements in Artemis-P70 protein expressing pre-B cells.

Earlier reports have also shown that Lig4^{-/-} ATM^{-/-} mouse embryonic fibroblasts (MEFs) and lymphocytes exhibit increased genomic instability when compared to single mutants and Lig4^{-/-} p53^{-/-} demonstrating the additional role of ATM in providing genomic stability [39]. Together, these results suggest that Artemis-P70 ATM^{-/-} lymphocytes are highly defective in V(D)J recombination and as a consequence, we observed very low levels of developing lymphocytes and no tumors.

Despite being tumor-free, Artemis-P70 ATM^{-/-} mice surprisingly exhibited colon inflammation with hyperproliferation of the colon epithelia, elongated and distorted crypts, and infiltrating T cells and neutrophils. Currently, the mechanism for the development of colitis is unclear. A preliminary test showed that fecal pellets from our colony tested positive for *Helicobacter ganmani* and *H. hepaticus*, two strains of bacteria that have been associated with colitis [37]. *H. hepaticus* and *H. ganmani* have both been shown to induce colitis in IL-10^{-/-} mice, a well studied model for colitis [37]. *H. hepaticus* has also been used to induce colitis in Rag2 deficient mice lacking B and T cells [35]. However, in this model, inflammation seems to require the reconstitution with CD4⁺ T cells from a donor mouse along with *H. hepaticus* infection. This requirement for donor CD4⁺ T cells seems to be strain dependent as the Rag2 mutation in a 129SvEv background do not require lymphocytes for *H. hepaticus*-induced colitis and demonstrate that innate immunity can be sufficient in some cases [46, 47]. Nevertheless, the experiments with CD4⁺ T cell reconstitution in Rag2^{-/-} mice illustrate that in some cases, the presence of T lymphocytes can exacerbate colon inflammation. It is possible that the low levels of B and T cells present in Artemis-P70 ATM^{-/-} mice, unable to respond properly to infection, instead cause chronic inflammation.

A second contributing factor may be the DNA repair defects resulting from Artemis and ATM mutations. One report using a dextran sulfate sodium-treatment (DSS) colitis mouse model found that ATM^{-/-} mice had a more severe immune response to DSS when compared to wildtype mice [48]. The authors speculated that reactive oxygen species created by the inflammatory response were more damaging to the ATM^{-/-} mouse and the increased DNA damage elicited further inflammation. Because our mice have two mutations affecting DNA repair, it is possible that a similar mechanism is contributing to the susceptibility to colitis.

Yet another possible contributing factor may be an imbalance of immune signaling cytokines resulting from loss of ATM. Inhibition of ATM has been shown to increase the production of the cytokine IL-23 from dendritic cells, cells that present antigen to T-cells [49]. Interestingly, IL-23 mediates inflammatory responses and has been shown to be important for the development of colitis in mice [50].

Together these findings demonstrate that the Artemis C-terminus and ATM cooperate to facilitate V(D)J recombination and lymphocyte development. While ATM and the Artemis C-terminus may function in the same pathway in some aspects of DSB break repair, our *in vivo* results illustrate that loss of ATM has a negative impact on lymphocyte development in Artemis-P70 mice. Loss of both factors also resulted in a lack of tumorigenesis but unexpectedly, these mice were susceptible to colitis. This indicated that the Artemis C-terminus and ATM both contribute to the development of a lymphocyte population that may have important roles in the regulation of intestinal homeostasis.

Materials and methods

Mice

Wildtype, Artemis-P70, Artemis-P70 p53^{-/-} Artemis^{-/-}, Artemis^{-/-} p53^{-/-}, ATM^{-/-}, and Artemis-P70 ATM^{-/-} mice were all housed in the same specific pathogen free facility. All mice were on a mixed 129SvEv and C57BL/6 background.

Lymphocyte analysis

Lymphoid organs (bone marrow, thymus, spleen, and lymph nodes) were dissected from mice 6-8 weeks old and single cell suspensions were prepared. T cells were stained with antibodies for CD4, CD8, and TCR β . B cells were stained with antibodies for B220, IgM, and CD43. Stained cells were analyzed using a BD Accuri C6 flow cytometer.

Hematopoietic stem cell analysis

The identification of hematopoietic stem cells has been described previously [28]. Bone marrow was harvested from mice between the ages of 5 and 24 weeks. Single cell suspensions were prepared and the following antibodies were used to exclude lineage positive cells: GR1, CD11c, NK1.1, B220, Ter119, CD19, CD11b, CD8, CD3, and

TCR β . Bone marrow cells were also stained with antibodies to identify hematopoietic stem cells, CD48, CD150, c-Kit, and Sca1. Stained cells were analyzed using a BD FACSCanto flow cytometer.

PCR Southern blot analysis

The PCR analysis of TCR rearrangements used in study have been described previously [17]. Briefly, genomic DNA was extracted from thymocytes from indicated genotypes. Nested PCR was performed using two rounds of the following program: 30 cycles of 95 C for 15 seconds, 55 C for 15 seconds, and 72 C for 30 seconds. PCR products were purified using Qiagen PCR Purification Kit in between the first and second round of PCR. PCR products were run on an agarose gel and transferred to Zetaprobe membrane for Southern blotting analysis. To measure band intensity, Southern blots were exposed to a phosphorimager screen and scanned on GE Healthcare Typhoon Scanner. Typhoon software was used to analyze Southern blot images.

Glucose tolerance test

Mice were first weighed, transferred to new cages, and then fasted for 6 hours with water present. Glucose was administered to these mice by intraperitoneal injection at 0.7g/kg of mouse weight. Tail blood was analyzed for blood glucose levels over the course of 2 hours using a handheld blood glucose monitor.

Tissue histology

Tissues were fixed in 10% formalin overnight and transferred to 70% ethanol. Histology was performed by the University of Michigan Comprehensive Cancer Center Histology and Immunohistochemistry Service.

Hematopoietic-derived cells isolation from colon tissue

Isolation of hematopoietic-derived cells from the lamina propria of colon tissue was performed following a published protocol [51]. Briefly, colons were isolated and washed. Tissues are then minced and digested with collagenase D. Cells released are passed through a 70 μ m strainer. Hematopoietic-derived cells are further isolated using Lymphoprep Gradient Medium (Stem Cell Technologies). Cells are then stained and analyzed with a FACSCanto flow cytometer.

Acknowledgements

Ying Huang analyzed the survival of both the Artemis-P70 p53^{-/-} and Artemis^{-/-} p53^{-/-} mice. Hilary Moale analyzed the survival of ATM^{-/-} mice and assisted with mouse dissections for lymphocyte analysis and colon harvest. Megan Miller assisted with nested PCR to analyze TCR rearrangements. Gabriel Martinez-Santibañez assisted with the glucose tolerance test. Jooho Chung assisted with staining, flow cytometry, and analysis for hematopoietic stem cells and colon lymphocyte/myeloid cell staining and flow cytometry. Tissue embedding and histology was performed by the University of Michigan Comprehensive Cancer Center Histology and Immunohistochemistry Service.

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Figures

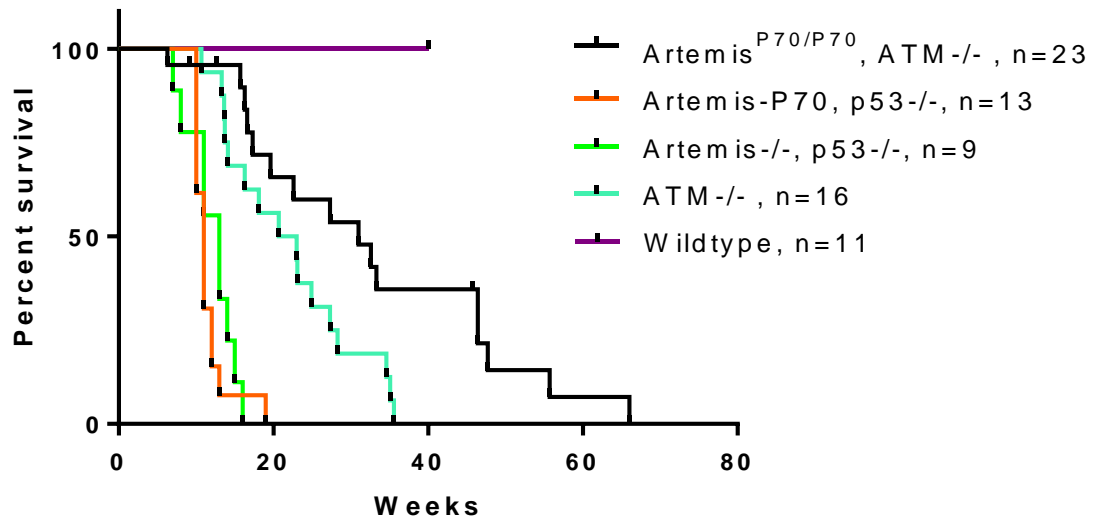


Figure 3. 1 Increased survival of Artemis-P70 ATM^{-/-} double mutant mice. Artemis-P70 ATM^{-/-}, Artemis-P70 p53^{-/-}, Artemis^{-/-} p53^{-/-}, and ATM^{-/-} mice were observed for 66 weeks. Shown is the Kaplan-Meier survival curve representing the life expectancy for mutant mice. Survival curves were compared using two-tailed log-rank test (Artemis-P70 ATM^{-/-} vs. ATM^{-/-}, P=0.02; Artemis-P70 ATM^{-/-} vs. Artemis-P70 p53^{-/-}, P<0.001, Artemis-P70 ATM^{-/-} vs. Artemis^{-/-} p53^{-/-}, P<0.001)

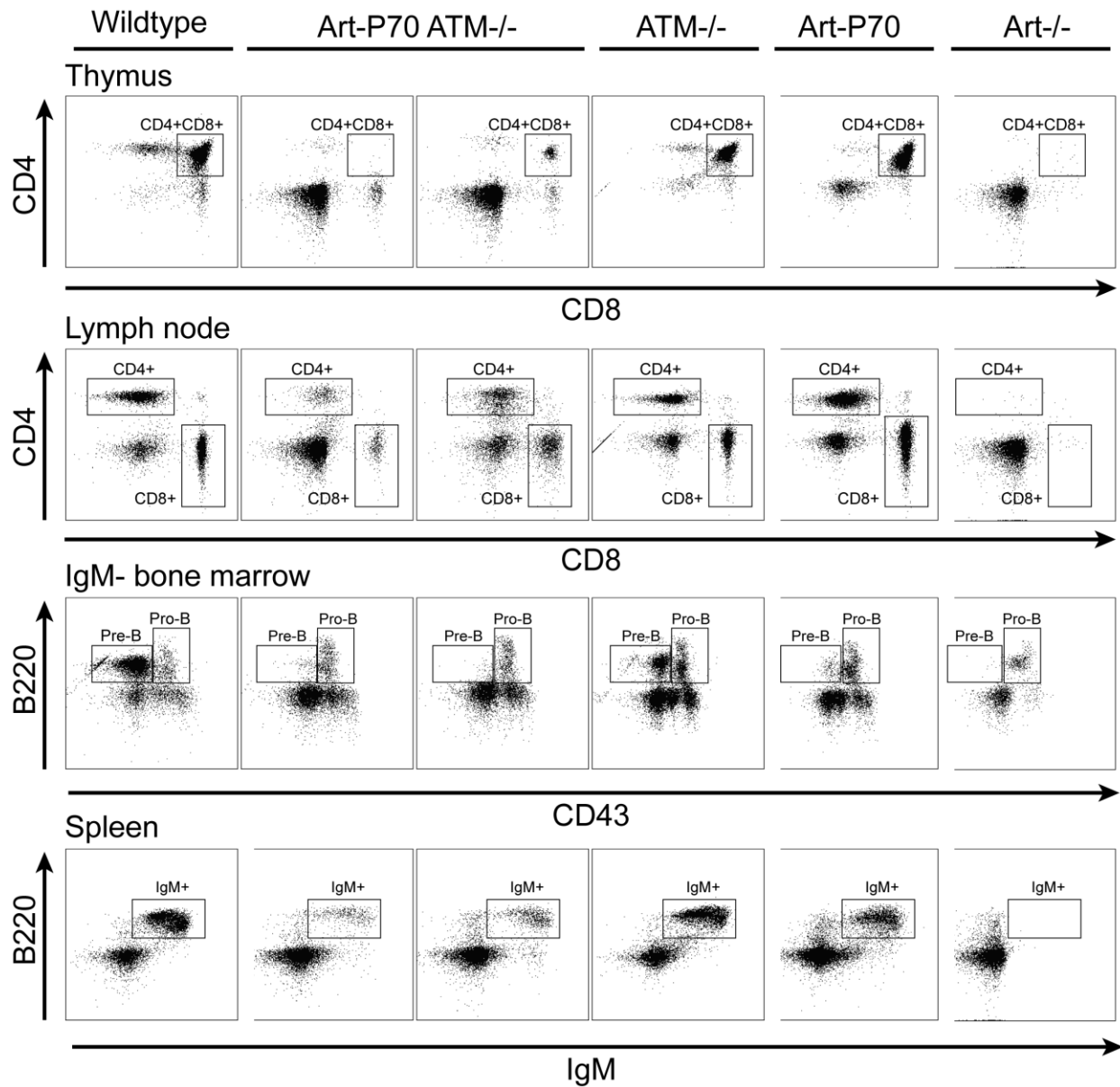


Figure 3. 2 Lymphocyte development in Artemis-P70 ATM^{-/-} mice. Lymphocytes from thymus, lymph nodes, spleen, and bone marrow were removed from wildtype, Artemis-P70, Artemis-P70 ATM^{-/-}, and Artemis^{-/-} mice between the ages of 6 and 8 weeks. Cells were stained antibodies for B and T cell surface markers and then analyzed using flow cytometry.

Genotype	Thymocytes x10 ⁶	CD4+CD8+ Thymocytes %	CD4+LN T cells %	CD8+ LN T cells %	Splenocytes x10 ⁶	Pro-B cells %	Pro-B cells x10 ⁶	Pre-B cells %	Pre-B cells x10 ⁶	IgM+ splenocytes %
Wildtype (n=6)	184 ± 49	70 ± 4.7	35 ± 6.9	17 ± 3.6	43 ± 16	1.6 ± 0.21	0.41 ± 0.18	11 ± 3.2	2.6 ± 1.1	29 ± 4.1
Art-P70 (n=6)	2.6 ± 0.93	66 ± 6.3	15 ± 6.7	12 ± 4.1	5.1 ± 1.4	2.7 ± 0.93	0.38 ± 0.24	1.2 ± 0.43	0.16 ± 0.09	9.8 ± 2.9
Art-P70 ATM ^{-/-} (n=5)	1.9 ± 0.94	0.49 ± 0.59	2.3 ± 0.90	2.2 ± 1.1	5.7 ± 2.3	0.91 ± 0.40	0.10 ± 0.05	0.14 ± 0.090	0.015 ± 0.010	1.3 ± 1.0
ATM ^{-/-} (n=5)	41 ± 23	54 ± 19	23 ± 3.6	18 ± 3.2	19 ± 9.1	2.6 ± 0.38	0.37 ± 0.1	6.4 ± 2.7	0.92 ± 0.47	25 ± 2.9
Art ^{-/-} (n=5)	0.11 ± 0.05	0.04 ± 0.0	0.46 ± 0.49	0.03 ± 0.03	7.1 ± 2.9	1.9 ± 1.5	0.40 ± 0.18	0.22 ± 0.13	0.05 ± 0.02	0 ± 0

Table 3. 1 Lymphocyte counts and percentages of Artemis-P70 ATM^{-/-} mice. Wildtype, Artemis-P70, Artemis-P70 ATM^{-/-}, and Artemis^{-/-} lymphocytes from thymus, lymph nodes, spleen, and bone marrow were harvested from mice between the ages of 6 and 8 weeks. Table represents cell frequencies determined by cell counts and flow cytometric analysis using 5-6 mice per genotype.

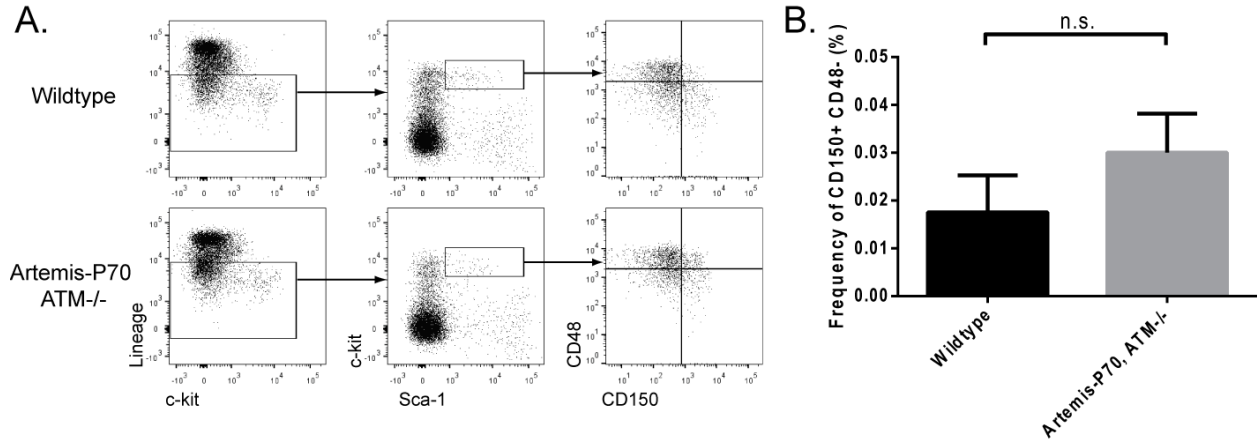


Figure 3. 3 Artemis-P70 ATM^{-/-} mice do not exhibit a HSC deficiency. Bone marrow was collected from wildtype (n = 2) and Artemis-P70 ATM^{-/-} (n = 4) mice between the ages of 5 and 24 weeks. Flow cytometric analysis was performed to measure the frequency of lineage-, c-kit+, Sca1+, CD48-, and CD150+ HSCs. No significant differences in HSC frequency was found in Artemis-P70 ATM^{-/-} mice when compared to wildtype mice.

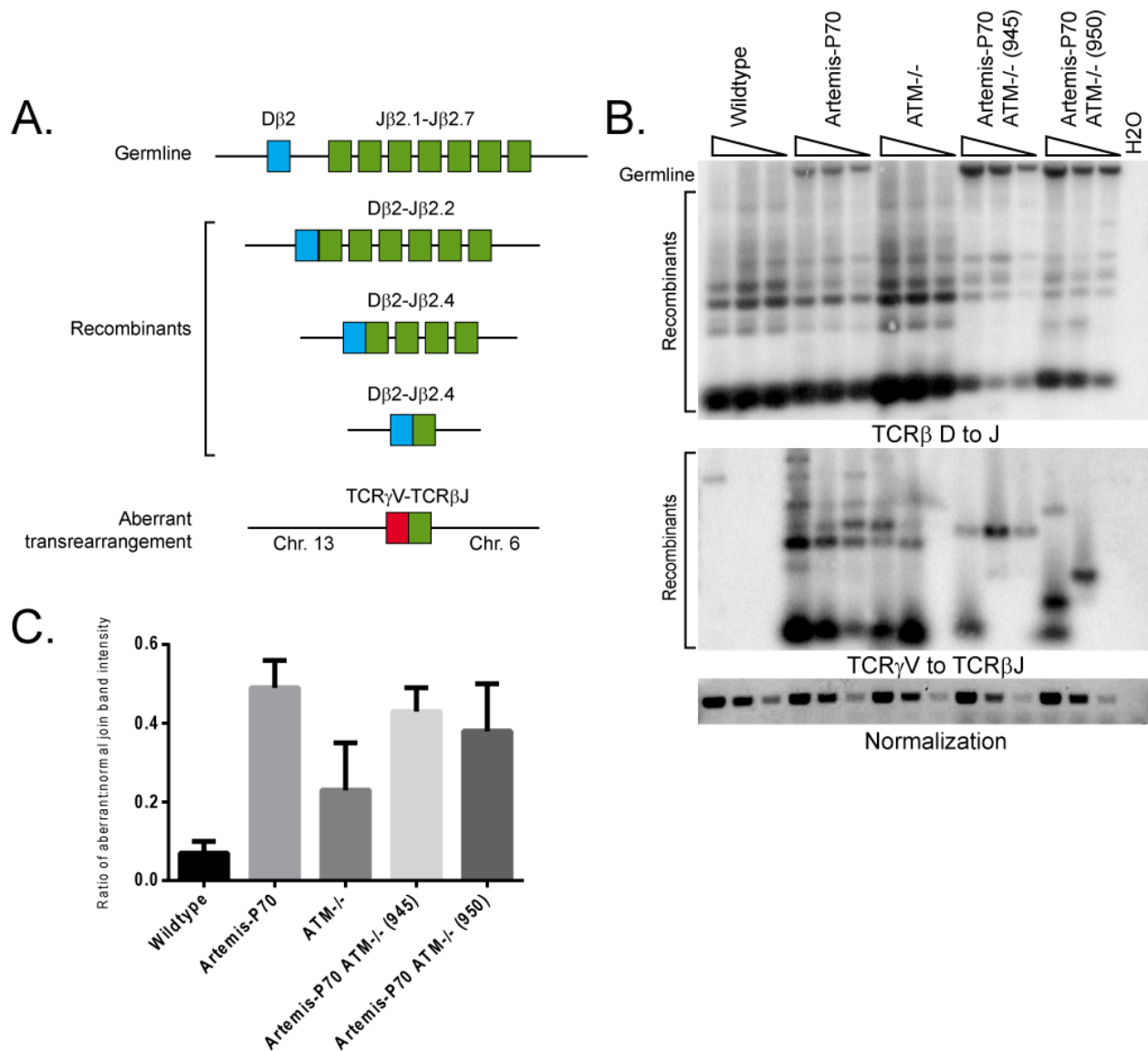


Figure 3. 4 Aberrant transrearrangements in Artemis-P70 ATM $^{-/-}$ thymocytes. A, B) Genomic DNA was isolated from wildtype, Artemis-P70, ATM $^{-/-}$, and Artemis-P70 ATM $^{-/-}$ thymocytes. Intrachromosomal rearrangements within the TCR β locus (D β 2-J β 2, chr. 6) were amplified using nested PCR and visualized using Southern blot. Interchromosomal aberrant transrearrangements between TCR β D2 and TCR γ V (chr. 6 and chr. 13, respectively) were also amplified using nested PCR. Amplification of the Rag2 gene was used as normalization. C) PCR Southern blot from Figure 3.3B was exposed to a phosphorimager screen and band intensity was calculated using phosphorimager software. The graph represents the ratio of aberrant to normal joins based on band intensity.

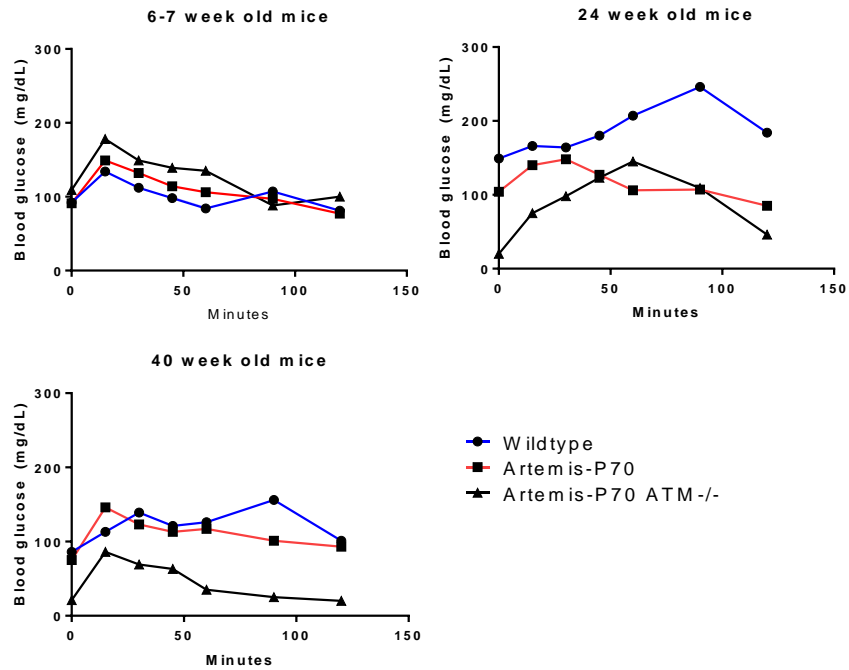


Figure 3. 5 Glucose tolerance in Artemis-P70 ATM^{-/-} mice. Wildtype, Artemis-P70, and Artemis-P70 ATM^{-/-} mice of various ages (6-7 weeks, 24 week, and 40 weeks) were fasted for 6 hours. Mice were then injected with a glucose solution at 0.7g of glucose per kg of mouse body weight. Blood glucose levels during recovery were then measured over the course of 120 minutes.

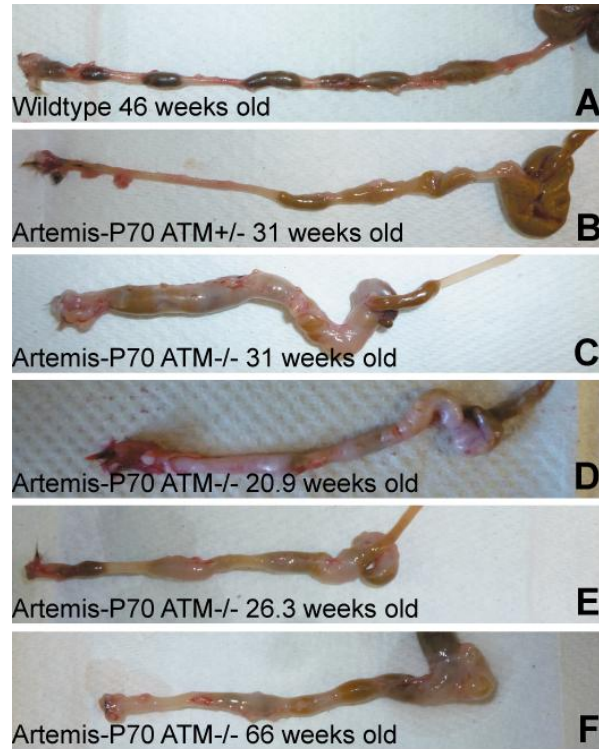


Figure 3. 6 Abnormally thickened colons of Artemis-P70 ATM-/- mice. Gastrointestinal tracts from A) wildtype, B) littermate controls, and C-F) Artemis-P70 ATM-/- double mutant mice were removed from moribund mice. Shown is the general morphology of the colon from these mice.

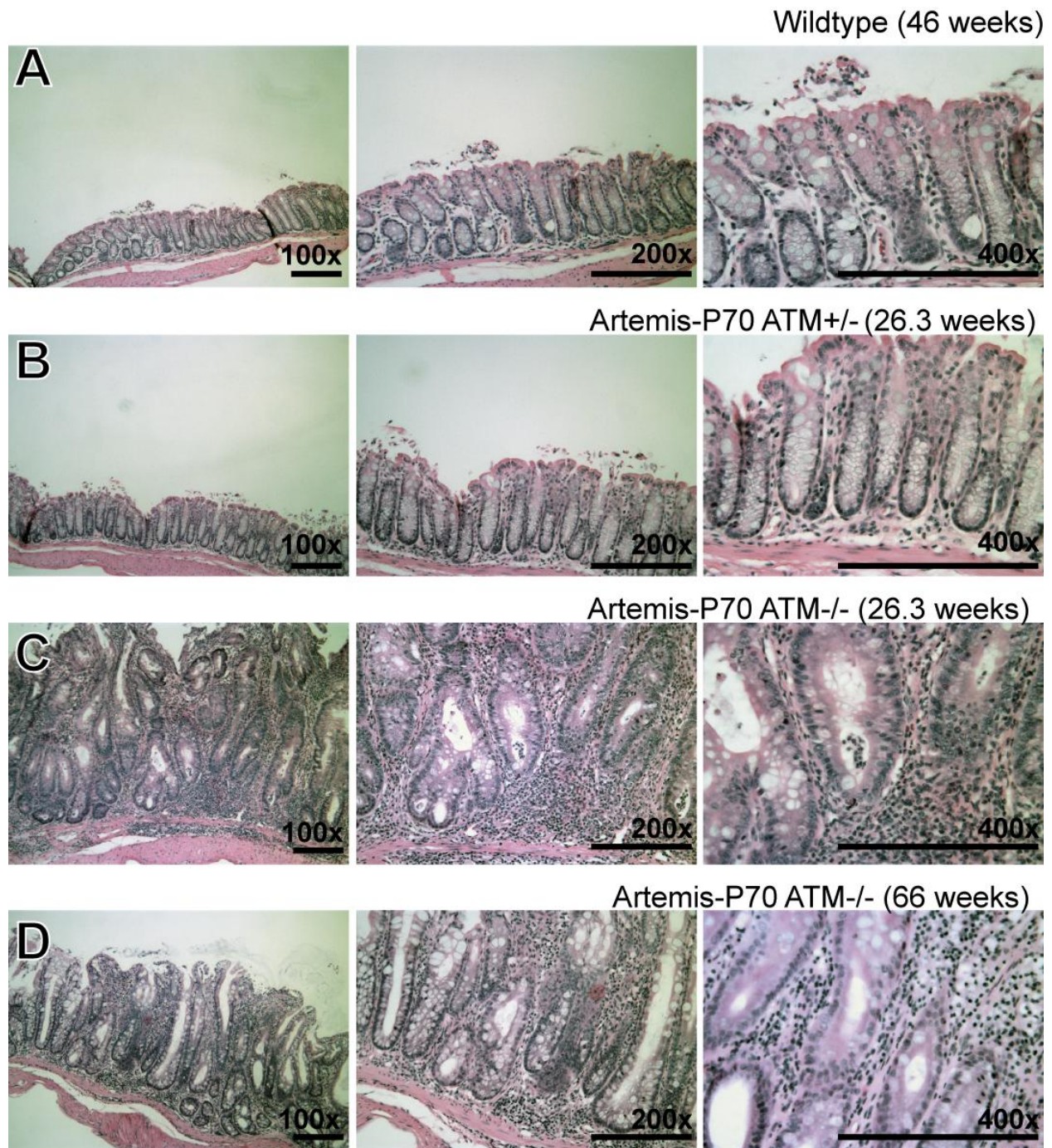


Figure 3.7 H & E colon histology Colons from A) wildtype, B) littermate controls, and C-D) moribund Artemis-P70 ATM^{-/-} double mutant mice were fixed, sectioned, and stained with H&E. Shown are representative images of the distal colon at 100x, 200x, and 400x magnification.

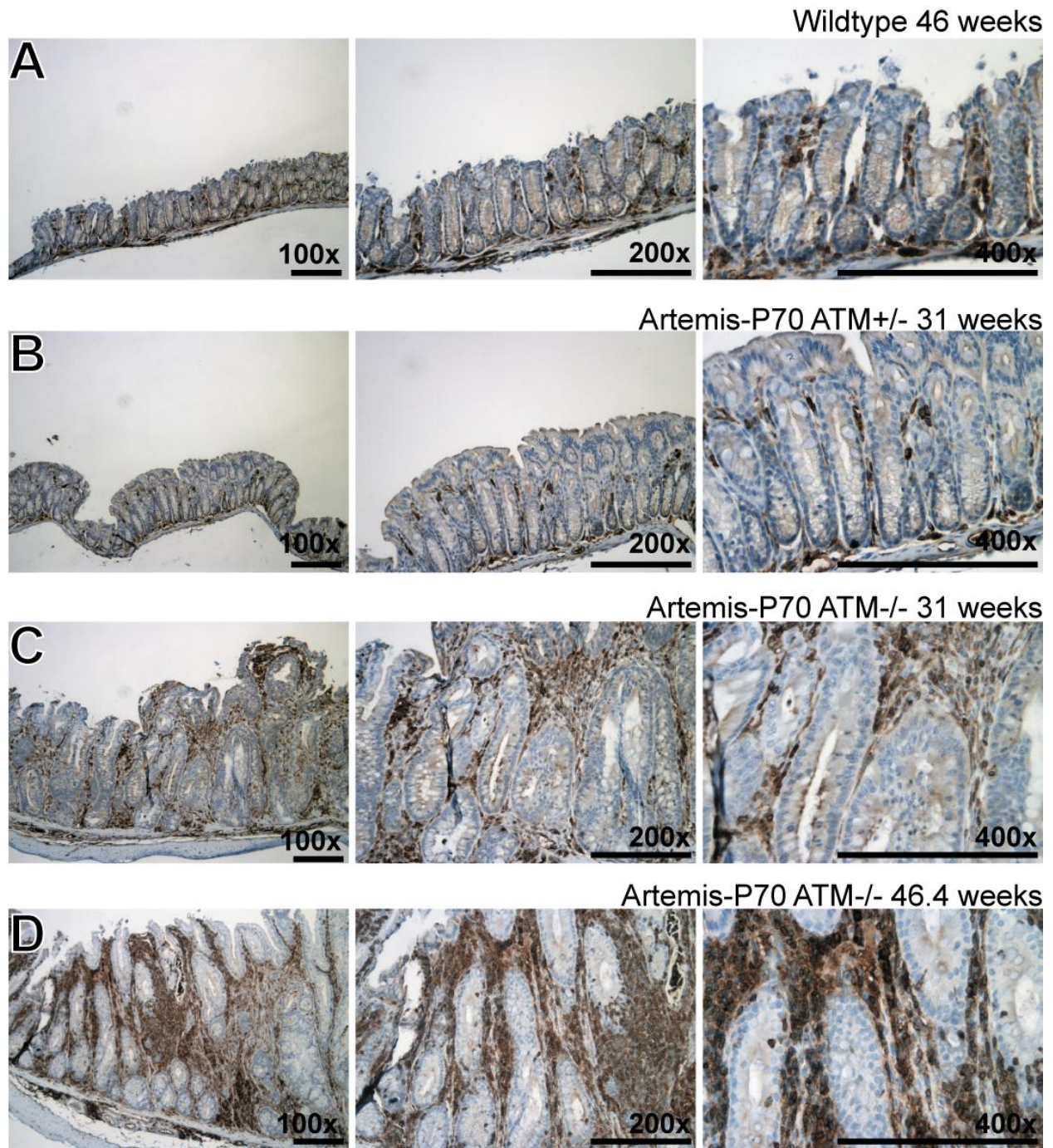


Figure 3.8 CD3 colon histology Colons from A) wildtype, B) littermate controls, and C-D) Artemis-P70 ATM^{-/-} double mutant mice were fixed and sectioned. Immunohistochemistry was performed to visualize CD3 positive T cells in the colon. Shown are representative images from the distal colon at 100x, 200x, and 400x magnification.

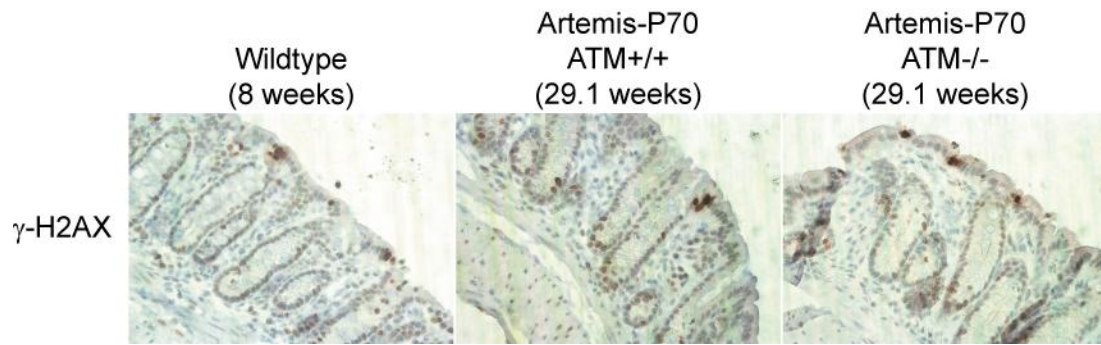


Figure 3. 9 γ -H2AX colon histology Colons from wildtype, Artemis-P70 ATM^{-/-}, and Artemis-P70 ATM^{+/-} or Artemis-P70 ATM^{+/+} littermates were harvested. Immunohistochemistry was performed to visualize phosphorylated histone H2AX in uninflamed colon tissue. All images shown were taken at 400x magnification.

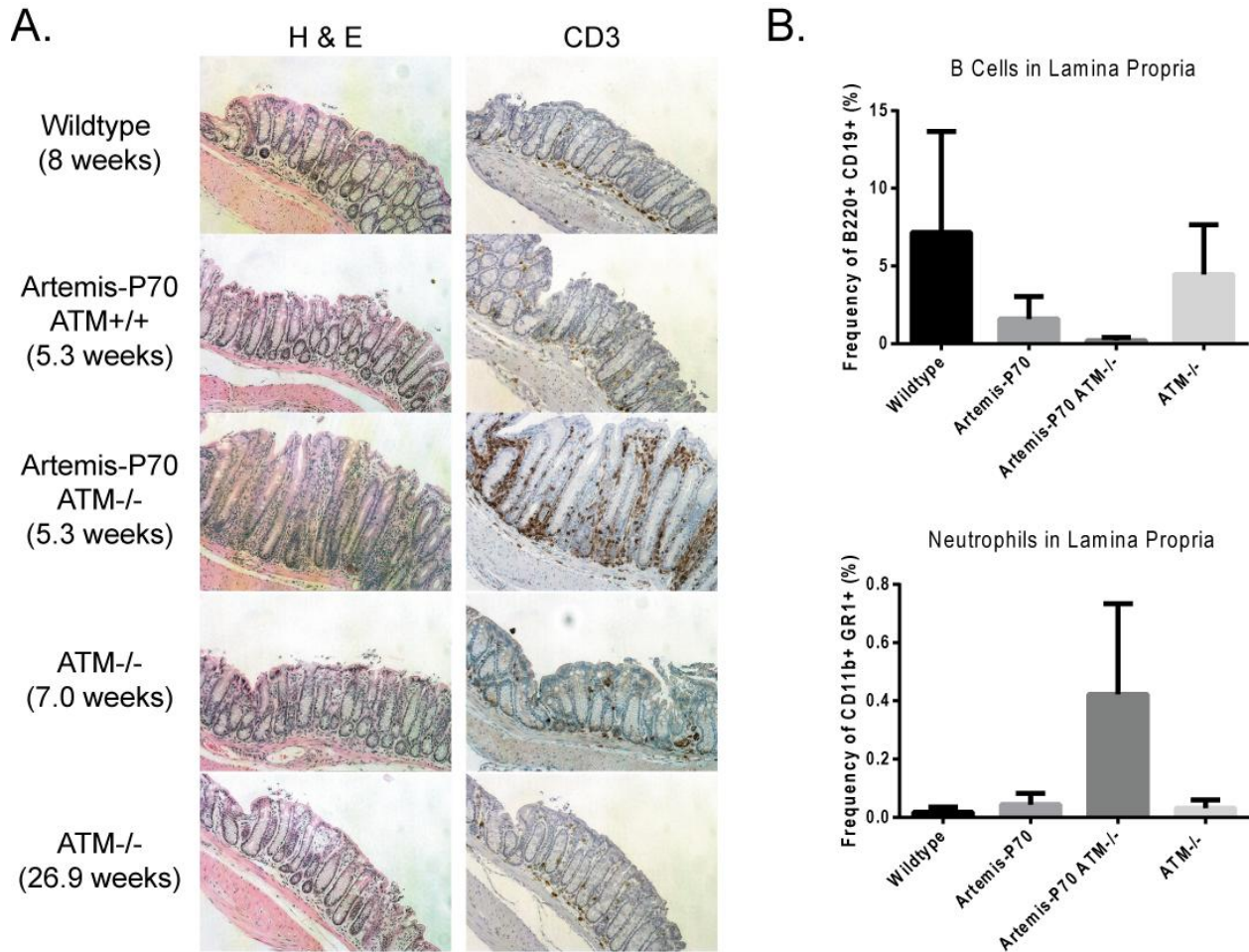


Figure 3. 10 Colon inflammation was observed in mice as early as 5.3 weeks. A) Colons were harvested from non-moribund Artemis-P70 ATM^{-/-} mice and their Artemis-P70 ATM^{+/-} and Artemis-P70 ATM^{+/+} littermates. Colons were also collected from wildtype and ATM^{-/-} mice between 7 and 27 weeks of age. Tissue sections were stained with H & E and immunohistochemistry was performed to visualize CD3⁺ T cells. All images shown were taken at 200x magnification. B) Lymphoid and myeloid cells from the colon lamina propria were harvested and analyzed by flow cytometry. Graphs representing the percent frequency of B cells and neutrophils found in the lamina propria. (B cells: wildtype n=4, Artemis-P70 n= 5, Artemis-P70 ATM^{-/-} n=8, ATM^{-/-} n=4) (Neutrophils: wildtype n=2, Artemis-P70 n=2, Artemis-P70 ATM^{-/-} n=4, ATM^{-/-} n=4)

Chapter 4

Summary of findings and discussion

Mechanisms of post-cleavage complex stability

The Artemis C-terminus phosphorylation and protein interaction

In this body of work, I have demonstrated *in vitro* that the loss of the Artemis C-terminus results in increased aberrant inversional rearrangements and this phenotype was made worse when combined with a deficiency in the cell cycle and DNA repair kinase ataxia-telangiectasia mutated (ATM). The impact of the loss of both factors was additive *in vivo* as well and these mice had a lymphocyte deficiency that was more severe than mice with loss of only one factor. Unexpectedly, this deficiency resulted in a tumor-free mouse with a susceptibility to colitis. I hypothesize the phenotypes observed in the mice can be attributed to the severely defective V(D)J recombination caused by the loss of the Artemis C-terminus and the ATM kinase.

Our results as well as others have suggested that both the Artemis C-terminus and ATM participate in the stabilization of a protein complex important for synapsis of cleaved DNA ends, a complex we and others have referred to as the post-cleavage complex (PCC). The precise mechanism regarding how the Artemis C-terminus and the ATM kinase are facilitating proper recombination through PCC stabilization has not yet been elucidated. In discussing their precise roles, we first should consider the components of the PCC and factors and events that have been shown to be important for the synapsis of DNA ends.

The Rag endonuclease was the first and most established component determined to be important for the synapsis of the DNA ends created by Rag-cleavage. Using *in vitro* assays, it was found that after DNA cleavage, all DNA ends, two signal and two coding ends, were retained in a RAG1/RAG2 synaptic complex [1, 2]. Furthermore, Rag mutations were shown to disrupt this complex despite leaving the Rag cleavage activity unaffected [3, 4]. Using a PCC disrupting point mutant, our lab

generated a RAG1 S723C mutant mouse. Thymocytes from these mice exhibited increased inter-chromosomal transrearrangements and when crossed into a p53^{-/-} background, double-mutant mice succumbed to thymic lymphoma. These tumors harbored clonal rearrangements that were distinct from p53^{-/-} thymic lymphomas [5]. Together, these findings illustrate that after Rag cleavage, coding and signal ends are held together in synaptic complex involving the Rag proteins and disrupting this complex result in the loss of these ends, aberrant rearrangements, and in a p53^{-/-} background, tumorigenesis.

A factor that has been shown to be important for DNA synapsis during double-stranded break (DSB) repair is the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). It is possible that synapsis through DNA-PKcs is one aspect of post-cleavage complex stability. This kinase along with the NHEJ DNA-end sensor proteins KU70 and KU80 (when together, referred to as DNA-PK) were shown to synapse DNA ends and synapsis was shown to increase the kinase activity of DNA-PKcs [6, 7]. The exact function of DNA-PKcs kinase activity and autophosphorylation is complex and has not yet been completely elucidated. Early studies have found that autophosphorylation inactivated the kinase activity of DNA-PKcs and also disrupted its interaction with KU70/80 and DNA [8]. However, the exact phosphorylation sites that are responsible for inactivation and protein-DNA complex dissociation have not been identified.

Phosphorylation of DNA-PKcs seems to have other functions unrelated to complex disruption. DNA-PKcs bound to ends in an *in vivo* phosphorylation-inhibited environment seems to protect the DNA ends from nucleases. However, when autophosphorylation is permitted, DNA-PKcs is thought to undergo a conformational change that allows nucleases to act on DNA ends [7]. Supporting this finding, two clusters of phosphorylation sites referred to as ABCDE and PQR have been characterized and these clusters were found to be important for restricting and permitting nuclease access to DNA ends [9]. More recently, it was found that a DNA-PKcs phosphorylation event was necessary for Artemis recruitment and this event could be carried out by either DNA-PKcs or the ATM kinase. However, this study also showed that there was another phosphorylation event that strictly required DNA-PKcs activity and this was important for ligation [10].

Based on these findings, the phosphorylation events of DNA-PKcs are hypothesized to occur in the following sequence. KU70/80 first recognizes and binds to DNA ends. This is followed by the recruitment of DNA-PKcs which protects the ends from nuclease digestion. DNA ends are then synapsed and phosphorylation is activated which recruits Artemis. The ABCDE cluster is phosphorylated, allowing Artemis and perhaps other nucleases access to DNA ends. The PQR cluster is then phosphorylated, restricting further nuclease activity. DNA-PKcs autophosphorylation occurs at a yet undefined location which promotes ligation and then dissociation.

One possibility is that the Artemis C-terminus plays a structural role in stabilizing the PCC with phosphorylation of this domain by DNA-PKcs and/or ATM facilitating this function. This domain may aid in stabilizing the protein complex at DNA ends, perhaps supporting several weak interactions between different factors. Supporting this hypothesis, I found that loss of the DNA-PKcs interaction domain of the Artemis C-terminus resulted in increased aberrant V(D)J rearrangement products when compared to experiments done with truncation mutants containing this domain.

There are several lines of evidence that suggest phosphorylation of this domain may also play a role. Both DNA-PKcs and ATM have been shown to phosphorylate Artemis at its C-terminus and ATM phosphorylation seems to be the primary kinase during recovery from ionizing radiation [11-16]. We and others have shown that the loss of ATM or inhibition of its kinase activity can result in increased aberrant V(D)J rearrangements and coding end accumulation [17]. This observation was strikingly similar to our results showing increased aberrant rearrangements and coding end accumulation in Artemis-P70 lymphocytes. When I treated pre-B cells expressing Artemis C-terminal truncated protein with an ATM kinase inhibitor drug, I observed an increase in transrearrangements that exceeded the loss of the Artemis C-terminus alone. This finding would suggest that ATM may also phosphorylate other factors important for PCC stability.

Another report found that the DSB repair protein complex MRE11-RAD50-NBS1 (MRN) seemed to function in PCC stability [18]. They found that hypomorphic mutations of Mre11 and Nbs1 resulted in increased aberrant V(D)J rearrangements and coding end accumulation. Because all three members of the MRN complex are phosphorylated

by ATM [19], the authors speculated that ATM may phosphorylate MRN and this regulates a DNA-synapsing activity of the protein complex. Interestingly, when Mre11/Nbs1-hypomorphic cell lines were treated ATM kinase inhibitor, they found that aberrant products were further increased. Although the involvement of other factors cannot be ruled out, one enticing possibility is that ATM may phosphorylate both the MRN complex and the Artemis C-terminus and these phosphorylation events may stabilize the PCC. (Figure 4.1)

Prevention of DNA-PKcs dissociation

One of the early findings regarding DNA-PKcs autophosphorylation was that this event can result in inactivation and dissociation of DNA-PKcs from DNA ends and Ku proteins. [8]. This dissociation has not yet been linked to PCC instability and this possible relationship may be difficult to explore. For instance, it is currently unknown which phosphorylation sites are important for DNA-PKcs dissociation and in addition, it has been shown that DNA-PKcs kinase activity is required for Artemis-mediated DNA hairpin opening [10, 20]. One possible way in which the Artemis C-terminus prevents loss of DNA ends from the PCC is that it may inhibit premature DNA-PKcs dissociation-associated phosphorylation. Perhaps after Artemis cleaves the DNA hairpin, DNA-PKcs phosphorylates the Artemis C-terminus. In this model, this phosphorylation event may not necessarily serve a function. However, without the Artemis C-terminus available as a substrate, DNA-PKcs may phosphorylate itself at sites associated with inactivation and dissociation. The PCC may then become destabilized before DNA-PKcs can facilitate ligation (Figure 4.2). Supporting this hypothesis, it has been shown in the past that incubating DNA-PKcs with a synthetic phosphorylation peptide substrate prevented the self-inactivation of the kinase [8].

Reduced endonucleolytic activity of Artemis-P70 may destabilize the PCC

The work presented in this work as well as previous findings from our lab have suggested the Artemis C-terminus is important for stabilizing the PCC. However, it has been difficult to separate the nuclease function of the Artemis nuclease and its putative PCC-stabilizing function. We have shown in this body of work and in past studies that the Artemis C-terminus is important for endonucleolytic activity [21]. The two truncation mutants M β L/ β CASP and D451X (Artemis-P70) which I and others in our lab have

demonstrated to facilitate aberrant V(D)J rearrangements both have reduced levels of nuclease activity. One possible way in which reduced nuclease activity could result in destabilized PCC is at DNA ends, a partially functional nuclease may only open DNA hairpins a portion of the time. In this scenario, there may be a population of complexes in which only one of the two hairpin coding ends will be opened. Unable to join these two ends, the PCC may eventually collapse resulting in an open hairpin coding that can be joined to a signal end or a Rag-generated DNA end from another locus. (Figure 4.3)

The cause of colitis in Artemis-P70 ATM^{-/-} mice

How the loss of ATM may contribute to colon inflammation

Despite not observing any tumors in our Artemis-P70 ATM^{-/-} mice, surprisingly, all moribund mice exhibited thickened colons with infiltrating T cells and neutrophils. These results suggest that Artemis-P70 ATM^{-/-} mice may be uniquely susceptible to this type of inflammation when compared to their littermates. Here we discuss the potential contributing factors that may be leading to this phenotype in our double mutant mice.

A possible contributing factor to colitis is the impaired DNA repair in the double mutant mice. Although our preliminary data examining γ -H2AX-positive cells did not seem to show increased DNA damage in our double-mutant mice, it is possible that the immunohistochemistry done was not able to detect the accumulation of DSB in non-apoptotic cells. Most positive cells were found at the distal end of the intestinal crypt suggesting that the γ -H2AX-positive cells were apoptotic cells that have undergone DNA fragmentation.

Support for this hypothesis comes from a report using a mouse model for colitis that is induced by oral administration of a sulfated polysaccharide, dextran sodium sulfate (DSS) [22]. Adding DSS to the drinking water of mice induces colitis exhibiting hyperproliferation [23] as well as neutrophils and lymphocyte infiltration [24]. DSS, thought to be toxic to the colon epithelia, damages the barrier between the lumen and mucosa and antigens from the lumen illicit an inflammatory response. Westbrook et al. found that ATM^{-/-} mice treated with DSS exhibited a higher disease activity index, a scoring system they used based on weight loss and stool consistency. Colon tissue

from these mice had higher levels of DNA damage that the authors speculated were a consequence of the inflammatory response.

The authors of that study hypothesized that colon tissue deficient in ATM may not be able to repair the DNA damage induced by the inflammatory response. Cellular stress and DNA damage has been shown to stimulate the immune system by increasing the presence of the cell-surface ligand NKG2D which is in turn recognized by natural killer (NK) cells of the innate immune system [25]. This would result in a positive feedback loop wherein an inflammatory response releases reactive oxygen species, surrounding ATM deficient cells are unable to repair the DNA damage, and this induces further inflammatory response. It is possible that this mechanism is a factor in the development of colitis in our Artemis-P70 ATM^{-/-} mice.

Another possible contributing factor to colitis is that the loss of ATM may increase the production of an immune signaling molecule, the cytokine IL-23 [26]. Wang et al. found that dendritic cells, a type of cell that presents antigens to T cells, increased production of IL-23 when ATM was inhibited by the drug KU-55933. IL-23 is known to promote the survival and expansion of Th17 cells, a subset of T cells that produce an inflammatory cytokine IL-17. Interestingly, IL-17 levels have been shown to be increased in colon tissues from patients suffering from ulcerative colitis and Crohn's disease [27]. Others have found that for one model of murine colitis IL-10^{-/-}, a combined deficiency in IL-23 resulted in disease-free mice suggesting that IL-23 may play a critical role in the development of colon inflammation. [28].

Autoimmunity as a contributing factor

Our lab has in the past generated the Rag1 S723C mouse and these mice exhibited "leaky" severe combined immunodeficiency (SCID) with severely reduced levels of B and T cells [5]. Another group analyzed these mice and found that these mice produced self-reactive autoantibodies [29]. The authors of this paper likened this phenotype to Omenn syndrome, a SCID resulting from hypomorphic Rag endonuclease mutations. Human patients with Omenn syndrome exhibit very low levels of B and T cells, inflammatory skin disease (erythroderma), hair loss, and colitis. Although only 4% of these mice exhibited colitis and hair loss, the authors found that some mutant mice had B and T cell infiltration in the small and large intestine, stomach, kidney, liver, and

lung [29]. In addition, these mice were found to produce autoantibodies. Walter et al. speculated that there were two contributing factors resulting in the development of autoantibodies: impaired receptor editing and B-cell activating factor (BAFF)-mediated survival.

Developing B cells in the bone marrow which have undergone V(D)J recombination may bind to self-antigens. These B cells will then re-express Rag genes and restart V(D)J recombination to rearrange the light chain. The authors found that receptor editing, like V(D)J recombination, was also impaired in Rag1 S723C mice. Walter et al. also found that BAFF, a B-cell survival cytokine was elevated in Rag mutant humans, Artemis mutant humans, and Rag1 S723C mice. This cytokine is thought to be expressed in B-cell lymphopenic environments in order to promote the survival of B-cells. The authors speculate that the high levels of BAFF can rescue self-reactive B cells that were programmed for apoptosis. [29]

We did not analyze our Artemis-P70 ATM^{-/-} mice for self-antibodies and it is unclear whether the mechanisms that played a role in the Rag1 S723C mice also contributed to the colitis observed in our mice. The Walter et al. found that human Artemis patients also had high levels of serum BAFF and because of the very low levels of mature B cells in our mice, it is likely that we would also observe high levels of this cytokine. We also did not examine our Artemis-P70 ATM^{-/-} mice for defective receptor editing but a positive result would be unsurprising given the defects in V(D)J recombination. Therefore, it is plausible that our mice may be producing autoantibodies and this may contribute to the colitis. However, unlike in the Rag1 S723C mice, we did not observe B cell infiltration in the colons nor did we observe lymphocyte infiltration in the kidney and liver. It would also be expected that if autoantibodies were being generated in the double mutant mice in this manner, that we would observe inflammation in other organs. Yet, we observed a very specific and very penetrant phenotype of colon inflammation. And so although the mechanism described by Walter et al, may be contributing, there are likely other factors that are also at play.

Helicobacter-induced colitis

It is been reported that certain immunodeficient mice are susceptible to colitis and these mice do not exhibit this phenotype when raised in germfree conditions [30-

33]. Additionally, the majority of mouse models for inflammatory bowel disease depend on gut bacteria suggesting the bacteria itself plays an important role in the development of colitis [34]. To address whether our mice harbored pathogenic bacteria in the gut, we had histology slides stained and fecal pellets analyzed for the presence of *Helicobacter* species. *Helicobacter* species are commonly found in mouse colonies and have been shown to cause colitis in some immunodeficient mice. One study found that mouse colonies in 6 of 16 U.S. academic institutions tested positive for *H. hepaticus* [35] and another study from Germany found that 35 of 40 mouse strains housed in specific pathogen free animal facilities tested positive for *Helicobacter* bacteria [36]. Although slides stained and analyzed by a pathologist were not found to be *Helicobacter* positive, fecal pellets analyzed by PCR indicated that *Helicobacter ganmani* and *Helicobacter hepaticus* DNA were present, two strains of bacteria that have been associated with colitis [37].

In our animal facility, we house Artemis^{-/-} and Rag2^{-/-} mice along with the Artemis-P70 ATM^{-/-} mice. Although the Artemis^{-/-} and Rag2^{-/-} mice have not yet been tested for *Helicobacter*, it is possible that these strains are also infected. However, we have not observed diarrhea or body wasting in these mice housed in the same room and as mentioned above, we never observed colitis in the Artemis-P70 ATM^{+/-} and Artemis-P70 ATM^{+/+} littermates. These observations suggest that the Artemis-P70 ATM^{-/-} mice may be particularly susceptible to colon inflammation in response to *Helicobacter*. One possible explanation for this sensitivity may lie in the severely reduced, but present B and T cell population in Artemis-P70 ATM^{-/-} mice. Double mutant mice may not have enough lymphocytes to mount a proper immune response to clear the infection but the presence of lymphocytes may be driving a chronic inflammatory response that results in hyperproliferation we observed. Supporting this hypothesis, past studies have found that not all immunodeficient mice exhibit colon inflammation even when administered *H. hepaticus*. Rag2^{-/-} mice, which lack B and T cells, have been used as a model to study *H. hepaticus*-induced colitis [38]. However, using this model required the reconstitution of CD4⁺ T cells along with *Helicobacter* administration, suggesting that in some cases, the presence of lymphocytes is required for inflammation.

Helicobacter species, as well as some other gram negative pathogenic bacteria, employ the bacterial toxin cytolethal distending toxin (CDT) [39]. This toxin has been shown to cause cell cycle arrest and cell death in host cells. CDT consists of three subunits, CdtA, CdtB, and CdtC. The importance of this toxin in virulence was shown using *H. hepaticus* strains in which CDT expression was disrupted [40]. Young et al., using the IL-10 mouse colitis model, infected mice with CDT-mutant *H. hepaticus*, and found that these mice exhibited only mild colitis when compared to mice infected with wildtype *H. hepaticus*. Notably, CdtB was found to have DNase-I-like activity which was abolished with a point mutation to a putative active site [41].

The DNase-I-like activity of CdtB has been shown to activate the ATM phosphorylation cascade in a similar manner as ionizing radiation [42]. Furthermore, ATM-deficient fibroblasts were shown to be more sensitive to *Haemophilis ducreyi* CDT (57% sequence similarity to *H. hepaticus* CdtB [43]). Because our mice are deficient in both ATM and Artemis, it is very possible that if they are *H. hepaticus* positive, that their DNA repair deficiencies may play a role in their development of colitis.

Although we have not fully characterized the mechanism driving colon inflammation in our double mutant mice, I hypothesize that several factors at play make these mice more susceptible. Our preliminary data on the presence of *Helicobacter* DNA in fecal pellets suggest that these pathogenic bacteria may be cause of colitis but further experiments will be necessary to establish that *Helicobacter* is a contributing factor. As a control, our Rag2^{-/-} and Artemis^{-/-} mice should also be tested for infection. Prior research has shown that it is possible these mice could harbor *Helicobacter* species but do not display any signs of body wasting or colitis. Chronic inflammation in the double mutant mice may be partially driven by low levels of B and T cells. Additionally, loss of DNA repair factors may be promoting a positive feedback loop in which reactive oxygen species produced by the inflammatory response to gut bacteria damage the DNA of the colon epithelia, the DNA is inefficiently repaired, and this drives further inflammation. Lastly, loss of ATM may be increasing the levels of proinflammatory cytokines that further promotes colitis.

Future directions

A few experiments can be performed to address the underlying mechanism for the loss of PCC stability in Artemis-P70 lymphocytes. In the first model proposed at the beginning of the chapter (Figure 4.1), the PCC is dependent on the phosphorylation of the Artemis C-terminus as well as its interaction with DNA-PKcs. An Artemis mutant construct containing alanine substitutions at the 8 C-terminus SQ phosphorylation sites as well as mutations at L401 R402, the DNA-PKcs interaction site, can be generated. This mutant can then be expressed in the pre-B cell system with the chromosomally integrated inversional recombination substrate. No study has yet tested a phosphorylation-deficient mutant Artemis construct in inversional recombination and this experiment may reveal the importance of these sites in PCC stability.

In my second model, the Artemis C-terminus serves as a phosphorylation substrate to prevent autophosphorylation leading to inactivation/dissociation of DNA-PKcs (Figure 4.2). One way in which this hypothesis can be tested is to test the kinase activity of DNA-PKcs in the presence of the Artemis C-terminus peptide. DNA-PKcs, in the presence of linear DNA will lose kinase activity over time but the presence of the C-terminus peptide may prevent loss of activity. Dissociation of DNA-PKcs has also been tested *in vitro* with DNA-PKcs-DNA complex formation analyzed by electron microscopy [44]. A similar assay can be performed in the presence of the C-terminus peptide to test its ability to prevent DNA-PKcs dissociation.

I also hypothesized that the reduced endonucleolytic activity may result in a destabilized PCC if only one of two coding ends are opened within one complex (Figure 4.3). To test this hypothesis, a full length Artemis protein with a hypomorphic mutation affecting nuclease activity can be expressed in the pre-B cell system I described chapter 2. Expressing a full-length Artemis protein with reduced nuclease activity could potentially separate the functions of the nuclease domain with the potential functions of the C-terminal domain. In this study, we analyzed an Artemis T71A mutant that was based on a human disease Artemis T71P mutation. Although we found that the Artemis T71A mutation did not alter endonucleolytic activity, it is possible that a proline substitution would be more likely to affect activity. Patients with this mutant allele were lymphopenic with some T cells present [45]. The presence of some lymphocytes in

these patients suggests that this allele is hypomorphic and may be useful in addressing reduced nuclease activity in PCC stability. The full length Artemis-T71P mutant protein, if found to exhibit nuclease activity comparable to Artemis-P70 or M β L/ β CASP, can be expressed in the pre-B cell system described in Chapter 2 and assayed for its propensity for facilitating aberrant V(D)J recombination.

Lastly, it will be important to determine the cause of colitis in the Artemis-P70 ATM^{-/-} mice. Preliminary results suggest that our mice are *Helicobacter* positive. If further experiments indicate that they are indeed positive, this line will have to be rederived to generate a *Helicobacter*-free colony. These mice can then be administered *Helicobacter* bacteria to address its contribution. Other genotypes can also be administered *Helicobacter* to test whether the double mutant mice are uniquely susceptible to colitis.

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Figures

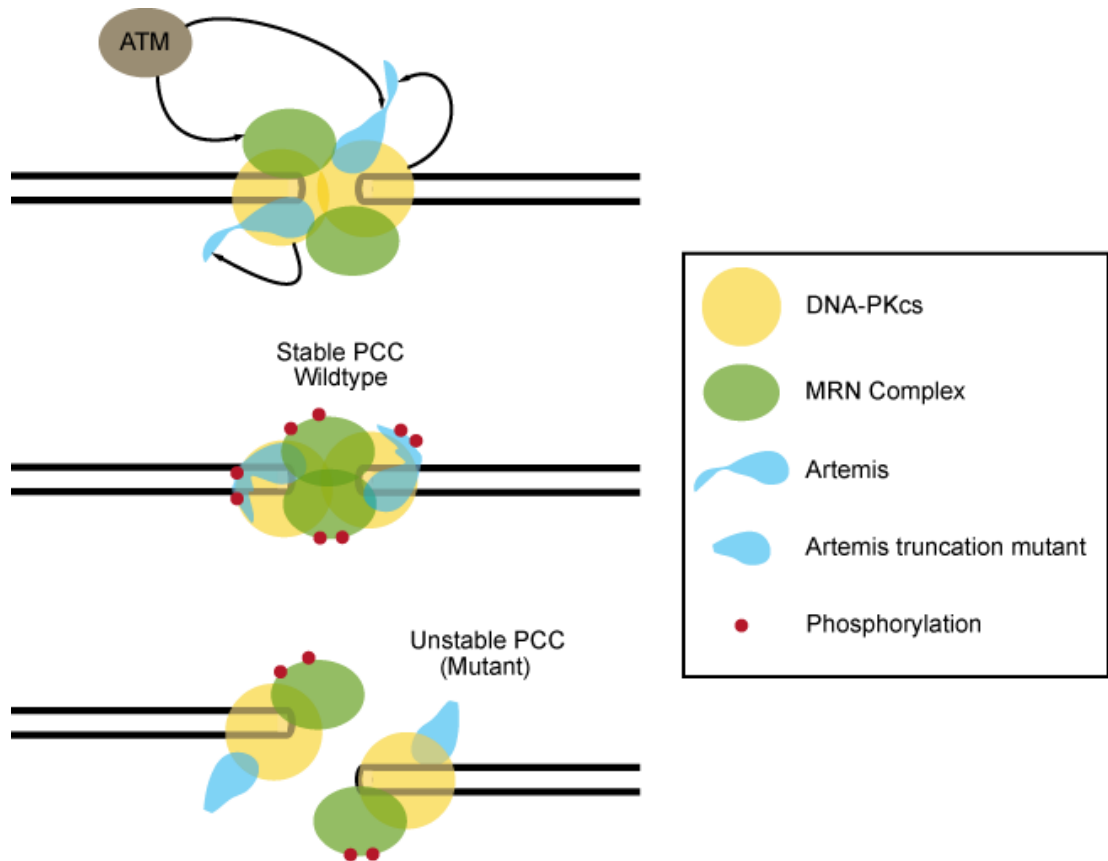


Figure 4. 1 Phosphorylation of the Artemis C-terminus and PCC stability. The stabilization of the PCC by the Artemis C-terminus may depend on its interaction with DNA-PKcs and phosphorylation by DNA-PKcs and ATM. Loss of the C-terminus, like in Artemis-P70 and M β L/ β CASP expressing cells, may result in loss of PCC stability due to loss of phosphorylation sites and in the case of M β L/ β CASP, the additional loss of the DNA-PKcs interaction domain.

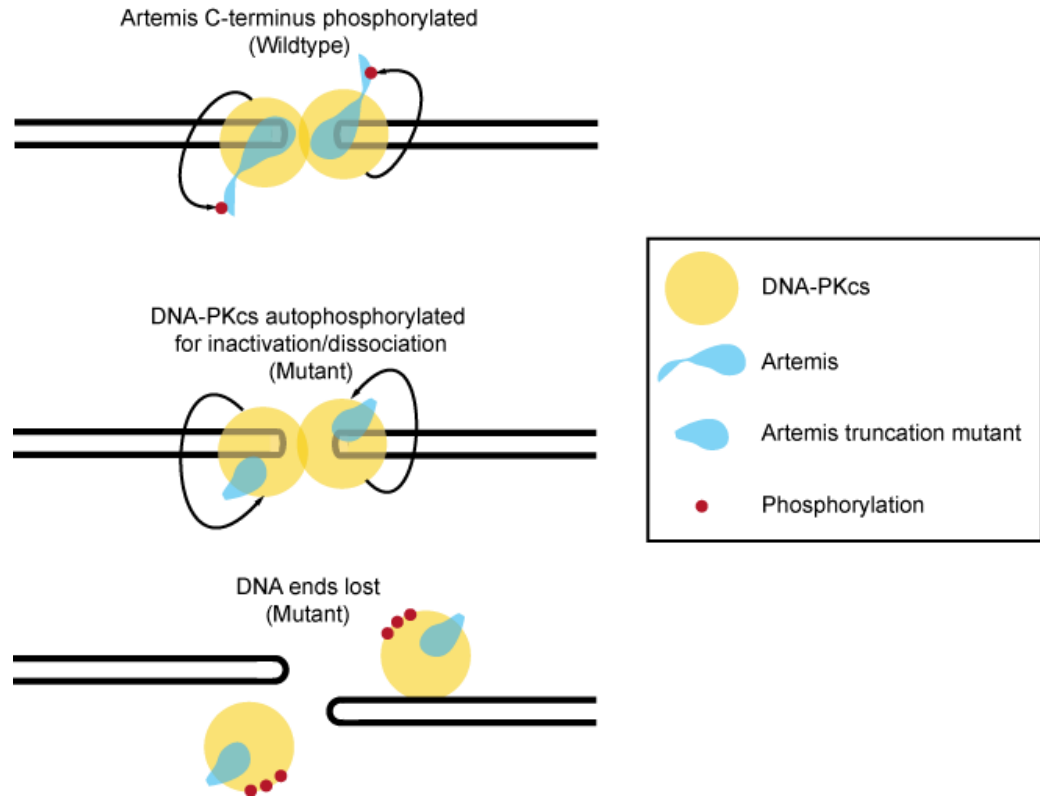


Figure 4. 2 Artemis C-terminus may prevent DNA-PKcs inactivation/dissociation In normal wildtype cells, DNA-PKcs phosphorylates the Artemis C-terminus after binding to DNA ends. When the Artemis C-terminus is not present, DNA-PKcs loses its phosphorylation substrate and instead, phosphorylates itself in a way that results in inactivation and dissociation from the DNA ends.

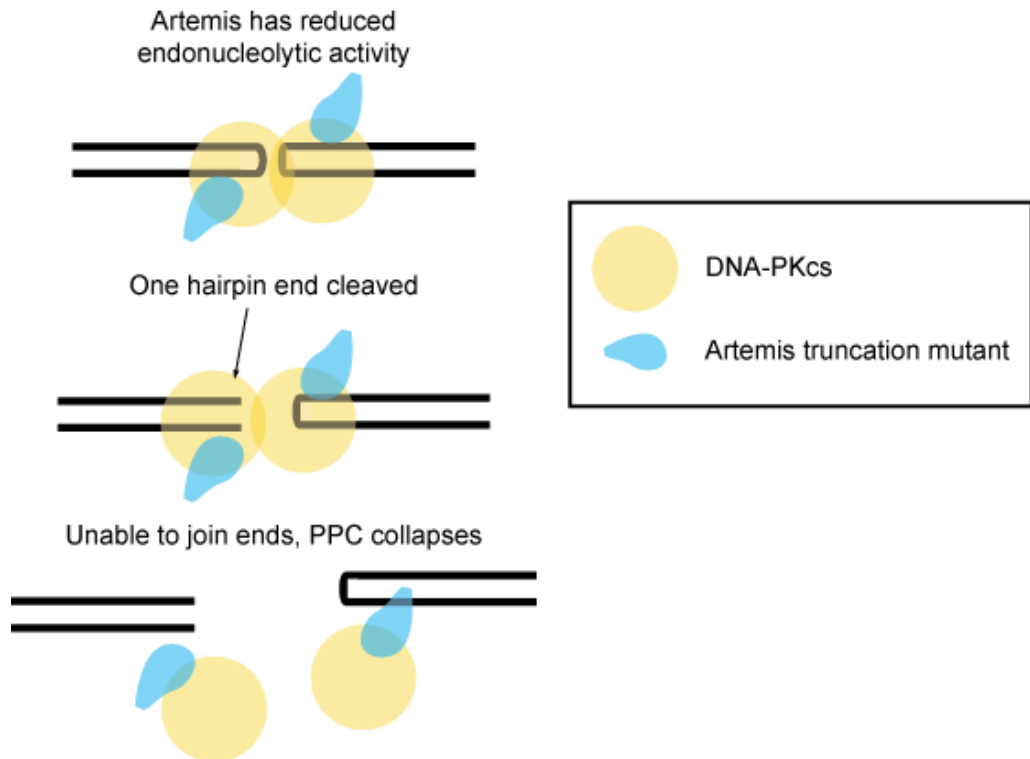


Figure 4. 3 Reduced activity of Artemis mutants may destabilize the PCC Loss of the Artemis C-terminus results in reduced endonucleolytic activity. Only one of two coding ends within the PCC is opened by mutant Artemis because of its reduced endonucleolytic activity. Unable to join the two ends, the PCC then collapses.