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Dna Double Strand Breaks Suppress Expression Of The Rag Recombinase: Mechanisms And Consequences

Megan Rose Fisher
University of Pennsylvania, mefisher@mail.med.upenn.edu

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Abstract
Developing B and T lymphocytes must rearrange the genomic sequence of antigen receptor genes by V(D)J recombination. The lymphocyte-specific endonuclease RAG, composed of Rag1 and Rag2, initiates this process by cleaving specific sites within antigen receptor loci. RAG expression must be carefully regulated to ensure that V(D)J recombination occurs only under appropriate circumstances. The Bassing laboratory has previously demonstrated that Igκ locus cleavage by RAG in pre-B cells initiates a feedback-inhibition signal suppressing RAG expression. Here, we show that DNA double strand breaks (DSBs) induced by a variety of genotoxic agents have a similar effect in suppressing mRNA expression of Rag1 and Rag2. This effect can be observed in pro-B cells, pre-B cells, and DN thymocytes, but is not found in DP thymocytes. Using primary pre-B cells as a model system, we show that DSBs activate ATM and Nemo to rapidly suppress transcription of Rag1 and Rag2. In pre-B cells, loss of Rag1 and Rag2 mRNA expression leads to loss of Rag1 protein, but Rag2 protein is more stable and persists in the absence of Rag2 mRNA. Suppression of Rag1 expression by DSBs is associated with suppressed RAG-mediated cleavage of the Igκ locus or an artificial recombination substrate in Abelson-transformed pre-B cells. However, simply over-expressing Rag1 does not allow cells to complete V(D)J recombination in the presence of DSBs, suggesting that other factors may also play a role in suppressing V(D)J recombination. Parallel studies indicate that that RAG-induced DSBs created during V(D)J recombination activate this suppressive signal to enforce allelic exclusion of IgH, TCRβ, and Igκ antigen receptor proteins. We discuss the importance of Rag1 and Rag2 suppression in the context of allelic exclusion and propose a role in maintaining genomic stability of developing B and T lymphocytes.

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DNA DOUBLE STRAND BREAKS SUPPRESS EXPRESSION OF THE RAG RECOMBINASE: MECHANISMS AND CONSEQUENCES

Megan R. Fisher

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Supervisor of Dissertation

_____

Craig H. Bassing, PhD.

Associate Professor, Pathology and Laboratory Medicine

Graduate Group Chairperson

_____

David Michael Allman, PhD.

Associate Professor of Pathology and Laboratory Medicine

Dissertation Committee

Eline T. Luning Prak, MD, PhD, Associate Professor of Pathology and Laboratory Medicine

Michael L. Atchison, PhD, Professor of Biochemistry

Youhai H. Chen, MD, PhD, Professor of Pathology and Laboratory Medicine

Nancy A. Speck, PhD, Professor of Cell and Developmental Biology

DNA DOUBLE STRAND BREAKS SUPPRESS EXPRESSION OF THE RAG RECOMBINASE: MECHANISMS AND CONSEQUENCES

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ABSTRACT

DNA DOUBLE STRAND BREAKS SUPPRESS EXPRESSION OF THE RAG RECOMBINASE: MECHANISMS AND CONSEQUENCES

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Developing B and T lymphocytes must rearrange the genomic sequence of antigen receptor genes by V(D)J recombination. The lymphocyte-specific endonuclease RAG, composed of Rag1 and Rag2, initiates this process by cleaving specific sites within antigen receptor loci. RAG expression must be carefully regulated to ensure that V(D)J recombination occurs only under appropriate circumstances. The Bassing laboratory has previously demonstrated that Igκ locus cleavage by RAG in pre-B cells initiates a feedback-inhibition signal suppressing RAG expression. Here, we show that DNA double strand breaks (DSBs) induced by a variety of genotoxic agents have a similar effect in suppressing mRNA expression of *Rag1* and *Rag2*. This effect can be observed in pro-B cells, pre-B cells, and DN thymocytes, but is not found in DP thymocytes. Using primary pre-B cells as a model system, we show that DSBs activate ATM and Nemo to rapidly suppress transcription of *Rag1* and *Rag2.* In pre-B cells, loss of *Rag1* and *Rag2* mRNA expression leads to loss of Rag1 protein, but Rag2 protein is more stable and persists in the absence of *Rag2* mRNA. Suppression of Rag1 expression by DSBs is associated with

suppressed RAG-mediated cleavage of the Igκ locus or an artificial recombination substrate in Abelson-transformed pre-B cells. However, simply over-expressing Rag1 does not allow cells to complete V(D)J recombination in the presence of DSBs, suggesting that other factors may also play a role in suppressing V(D)J recombination. Parallel studies indicate that that RAG-induced DSBs created during V(D)J recombination activate this suppressive signal to enforce allelic exclusion of IgH, TCRβ, and Igκ antigen receptor proteins. We discuss the importance of *Rag1* and *Rag2* suppression in the context of allelic exclusion and propose a role in maintaining genomic stability of developing B and T lymphocytes.

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Chapter 1: Introduction

Requirements of the Immune System

All organisms must recognize and react to foreign species that may attempt to colonize them. In many cases the colonizing species may harm or even kill its host, such as the wide variety of pathogens that cause diseases ranging from the common cold to smallpox. In other cases, the relationship between host and colonizer is innocuous or even mutually beneficial, as in the case of the human digestive tract, which relies on a variety of resident bacteria to help break down food into nutrients that human cells can metabolize (Quigley, 2013). An immune system must distinguish between components of the organism (self) and foreign entities (non-self) and neutralize potentially dangerous invaders while minimizing damage to the organism itself and tolerating foreign organisms that benefit their host.

Immune systems employ a wide variety of methods to detect non-self organisms and determine how to respond to them. Components of the immune system are traditionally categorized as "innate" or "adaptive" depending on how specific the response is to a particular stimulus. Innate immune responses are activated when cells recognize characteristic components of a particular class of pathogens, and activate a response targeted to that kind of pathogen. For example, cell-surface receptors that recognize chemicals in the bacterial cell wall would activate an anti-bacterial response if

they detect bacteria where there should be no bacteria. Similarly, intracellular receptors can detect viral DNA or RNA in a host cell's cytoplasm and activate an anti-viral response. Innate immune responses allow rapid responses to pathogens that an organism is pre-programmed to recognize (Brubaker et al., 2015).

However for humans and many other species, innate immunity alone cannot protect the organism from the wide variety of pathogens encountered in daily life (Fischer, 2000). Adaptive immunity allows an organism to recognize unique features of a particular foreign substance, known as antigens. In concert with innate immune components, adaptive immunity allows highly specific, effective responses targeted to particular pathogens (Bonilla and Oettgen, 2010). In humans and other jawed vertebrates, the huge variety of antigen receptors required for adaptive immunity is generated by the process of V(D)J recombination, in which developing B and T lymphocytes rearrange parts of their antigen receptor genes to create unique receptors (Brack et al., 1978; Hedrick et al., 1984a). This process allows each new B or T cell to recognize a different, almost random antigen, so that the full repertoire of B and T cells is capable of recognizing a vast array of unknown antigens with high specificity. Because this process is largely random, many of the receptors produced are autoreactive, meaning they bind to components of the host organism and could theoretically initiate an immune response to the organism itself. Developing lymphocytes must pass through several selection steps that either cause autoreactive lymphocytes to die or otherwise

prevent them from being activated by their cognate antigen. Failure of these selection steps can lead to autoimmune diseases or immunodeficiencies (von Boehmer and Melchers, 2010).

Basic Mechanisms of V(D)J recombination

There are several antigen receptor loci capable of undergoing V(D)J recombination in mammals. In general these loci are composed of many variable (V) segments and several joining (J) segments. Certain loci also have several diversity (D) segments located between the V and J segments. In addition to these segments that encode the variable antigen-recognition sections of an antigen receptor, constant regions (C) encode non-variable regions of the receptor necessary for signaling (Tonegawa, 1983; Hedrick et al., 1984b). Before rearrangement V, D, and J segments span a genomic distance of up to 2.5 megabases. Each of these V, D, and J segments are flanked by recombination signal sequences (RSSs) (Sakano et al., 1979). RSSs are composed of three components. Directly adjacent to the antigen receptor gene segment is a highly conserved seven base-pair heptamer. Next to the heptamer is a poorly-conserved spacer of either 12 or 23 base pairs, followed by a conserved nonamer. An endonuclease complex composed of Rag1 and Rag2 (RAG) binds to and cleaves at RSSs adjacent to two different gene segments, creating a DNA double-strand break (DSB) directly adjacent to each segment (McBlane et al., 1995). Processing of these breaks can

lead to loss and/or addition of several nucleotides at the break site. Finally, non-homologous end joining (NHEJ) factors rearrange and repair the DSBs so that the two selected gene segments are directly adjacent to each other in the repaired DNA sequence (Helmink and Sleckman, 2012). This may be accomplished by removing intervening DNA if the selected DNA segments were in the same orientation in the germline DNA (rearrangement by deletion; Figure 1.1). Alternatively, if the two gene segments were originally in opposite orientations, a section of DNA ending with one of the segments is flipped to bring the two selected segments into proximity (rearrangement by inversion). In either case, the repairs are made such that one repair site includes both gene segments (a coding join) and the other repair site includes both RSSs (a signal join) (Helmink and Sleckman, 2012).

Generally speaking, lymphocytes attempt rearrangement of only one antigen receptor locus at a time (Chan et al., 2013; Chaumeil et al., 2013; Hewitt et al., 2009). However, if an initial rearrangement is non-functional, the cell may subsequently attempt to rearrange the homologous antigen receptor allele, or create additional rearrangements on the first allele. Because nucleotides can be randomly added to or removed from the coding ends of the DSB intermediates, two-thirds of all rearrangements are out-of-frame and therefore non-functional. Inclusion of a stop codon or use of a pseudogene segment in the rearrangement can also produce non-functional antigen receptor genes (Helmink and Sleckman, 2012). Only a functionally

rearranged antigen receptor gene that can produce a protein product expressed on the cell surface can promote lymphocyte survival and development (Miosge and Goodnow, 2005).

At each stage of lymphocyte development, the expression of the V(D)J catalyst (RAG) and the accessibility of the substrate (antigen receptor genes) are each carefully regulated to ensure that the appropriate genes are rearranged at the appropriate time. Antigen receptor genes must be prepared for rearrangement in the appropriate developmental stage by undergoing a process of histone modification, precise nucleosome positioning, and DNA-looping to bring distant gene segments into close proximity (Proudhon et al., 2015). Similarly, RAG expression is strictly limited to B and T cells at appropriate developmental stages (Nagaoka et al., 2000). Different transcription factors and cis-regulatory elements drive *Rag1* and *Rag2* expression in developing B and T cells, but in both cell types rearrangement and subsequent cell surface protein expression of the appropriate antigen receptor gene suppresses further transcription of *Rag1* and *Rag2* (Nagaoka et al., 2000).

An overview of B and T lymphocyte development

V(D)J recombination is absolutely required for B and T cell development, as failure to produce a functional antigen receptor leads to complete developmental arrest of lymphocyte progenitors (Mombaerts et al., 1992a; Shinkai et al., 1992). Lymphocyte

development itself is a complex process in which cells progressively commit to a specific developmental pathway. Transcription factor networks must guide cells down different pathways while integrating environmental cues such as availability of cytokines (Lin et al., 2010). Within this context, V(D)J recombination is regulated to promote the creation of functional antigen receptor genes at the appropriate stages of lymphocyte development (Fig. 1.2).

Both B and T cells develop from a common precursor. Commitment to the B lymphocyte lineage requires a number of transcription factors including Ikaros, E2A, early B-cell factor (EBF1), and Pax-5 (Lin et al., 2010). Among other important functions, many of these factors regulate V(D)J recombination in pro-and pre-B cells (Hsu et al., 2003; Reynaud et al., 2008). These factors are important both for inducing or suppressing expression of the RAG recombinase and for preparing specific antigen receptor loci for recombination.

Pro-B cells first rearrange one Immunoglobulin Heavy Chain (IgH) gene (Alt et al., 1984). An in-frame, functional rearrangement leads to cell surface of the pre-B cell receptor (pre-BCR), composed of the newly produced IgH chain in complex with the invariant surrogate light chains. The pre-BCR sends signals that inhibit further IgH rearrangement and trigger proliferation and progression to the pre-B cell stage (Übelhart et al., 2015). If the first IgH rearrangement is non-functional, the cell will

attempt to rearrange the second IgH allele; if this second attempt creates a productive IgH gene then development proceeds, but if it is unproductive the cell dies. Cells that do proceed to the pre-B cell stage eventually cease proliferating, and begin to rearrange the light chain genes, Igκ and then Igλ (Coffman and Weissman, 1983). Like at the pro-B cell stage, failure to produce a functional light chain gene leads to cell death. Creation of a functional light chain capable of binding to IgH and allowing cell surface expression of a BCR prevents further recombination and causes progression to the immature B cell stage (Melchers et al., 1995). The antigen-recognition portion of the BCR is a heterotetramer containing two IgH chains and two IgL (either Igκ or Igλ) chains (Pleiman et al., 1994). Immature B cells expressing a BCR that recognizes self-antigens may undergo receptor editing, in which they further rearrange their light chain genes in an attempt to produce a non-autoreactive BCR (Gay et al., 1993; Tiegs et al., 1993).

Developing T cells go through a similar process in which CD4 CD8 double negative (DN) thymocytes rearrange the T Cell Receptor β (TCRβ) gene or TCRγ and TCRδ genes. Formation of a complete γδTCR leads to γδT cell development, while functional rearrangement of TCRβ promotes progression to the CD4 CD8 double positive (DP) thymocyte stage (Mombaerts et al., 1992b; Xiong and Raulet, 2007). DP thymocytes continuously rearrange TCRα genes until they produce a functional alpha chain capable of binding the TCRβ chain and allowing cell surface expression and signaling of the $\alpha\beta$TCR by weakly binding MHC presented on thymic epithelial cells

(Mombaerts et al., 1992b; Klein et al., 2014). Failure to create a functional αβTCR leads to "death by neglect", while DP thymocytes expressing a receptor that activates relatively strong signaling may either die or become regulatory T cells that suppress immune activation (Jordan et al., 2000, 2001; Apostolou et al., 2002)

Allelic exclusion in lymphocytes

As with all autosomal genes, cells contain two alleles of each antigen receptor gene. If nothing prevented the expression of multiple receptor chains within the same cell, we would expect these two alleles to rearrange and produce unique antigen receptor chains, leading to expression of multiple antigen receptors on the cell surface. The most extreme case would be a B cell that productively rearranged both IgH, both Igκ, and all Igλ alleles; because an individual receptor contains two IgH and two IgL chains, this cell could express receptors including dozens of combinations of IgH and IgL chains on its cell surface. However, it has long been recognized that this is not the case; instead, most antigen receptor chains are "allelically excluded", meaning an individual cell expresses protein from only one allele. Most B cells express one IgH and IgL chain (Cebra et al., 1966; Pernis et al., 1965). Most T cells express one TCRβ chain, although TCRα is an exception and does not seem to be allelically excluded (Casanova et al., 1991; Padovan et al., 1993). It is important to note that allelic exclusion was originally defined by cell surface expression of a single antigen receptor . Later, it was found that allelic

exclusion is frequently enforced at the level of gene rearrangement, as lymphocytes usually contain only one productive rearrangement for each antigen receptor chain (Alt et al., 1984; Casanova et al., 1991). However, a cell that is "included" at the genetic rearrangement level may still "exclude" at the level of cell surface receptor expression, as several factors may prevent an apparently functional antigen receptor chain from being expressed on the cell surface (ten Boekel et al., 1998; Sant'Angelo et al., 2001; Steinel et al., 2010).

Although allelic exclusion of antigen receptors was discovered over 50 years ago, it is still unclear why it is enforced. An allelically included lymphocyte would express receptors with different specificities on its cell surface. One potential disadvantage of this situation would be inefficient negative selection of allelically included cells expressing both a self-reactive receptor and an innocuous receptor. Studies utilizing transgenic expression of self-reactive antigen receptors show that this is technically possible, but while the "autoreactive cells" can be stimulated by self antigen *ex vivo* (Iliev et al., 1994; Zal et al., 1996), only one study reported autoimmune disease resulting from these cells (Sarukhan et al., 1998). However these studies are limited by the extreme skewing to a single autoreactive receptor, a situation that is not reflective of the diverse antigen receptor repertoire found in healthy individuals. At the same time, it must be recognized that normal individuals do have many allelically included cells, which generally do not harm that individual. Attempting to address this fact,

certain studies have investigated the autoreactive potential of naturally occurring allelically included lymphocytes. Two groups studying different strains of lupus-prone mice came to conflicting conclusions regarding the ability of dual-reactive B cells to promote autoimmunity; it was suggested that differences in tolerance checkpoints between the two models might affect the degree to which allelically included B cells are activated and mediate disease (Fournier et al., 2012; Makdasi and Eilat, 2013). A study examining B cells from healthy mice found that cells expressing two Igκ chains were more likely than single expressers to produce anti-nuclear reactive antibodies, a common screen for self-reactivity (Casellas et al., 2007). More recently, it was found that preventing the normal allelic inclusion of TCRα by deleting one TCRα constant region allele reduced the frequency of T cells responding to an auto-antigen (Ni et al., 2014). While these results suggest that allelically included cells may be overrepresented in the pool of self-reactive lymphocytes, they also highlight the fact that peripheral tolerance mechanisms are usually sufficient to restrain the autoreactive potential of either allelically excluded or included lymphocytes. However, it is possible that normal allelic exclusion represents a "good enough" situation which prevents other tolerance mechanisms from being overwhelmed, and that loss of allelic exclusion might disrupt this balance.

DSBs and allelic exclusion

During Igκ recombination in pre-B cells, RAG-mediated DSBs at one Igκ allele activate the DNA damage response protein Ataxia Telangiectasia Mutated (ATM) to prevent cleavage of the second allele (Steinel et al., 2013). ATM deficiency allows simultaneous rearrangement of Igκ alleles; in this context, the frequency of B cells expressing two unique Igκ chains approximately doubles. These results suggest that monoallelic rearrangement enforced by ATM activation is an important factor in allelic exclusion of Igκ. As described in Chapter 4 of this work, we show that ATM deficient B cells also have increased inclusion of the IgH chain, and ATM deficient T cells have increased inclusion of TCRβ, suggesting that a similar mechanism operates in pro-B cells and DN thymocytes.

Different genetic elements regulate Rag transcription during B and T cell development

Early in the search for Rag1 and Rag2 promoters and enhancers, two general themes emerged. One, it was found that *Rag1* and *Rag2* are generally co-expressed (Yu et al., 1999). This is unsurprising, as the two genes are located in close proximity and could easily be regulated by the same genetic elements. Secondly, it quickly became clear that different sites are important for Rag expression in developing B vs. T cells, and in some cases DN vs. DP thymocytes (Monroe et al., 1999; Yu et al., 1999; Wei et al., 2002). A schematic of the *Rag1/Rag2* gene locus with known genetic regulatory

elements and trans-acting factors is shown in Fig. 1.3. As discussed below, promoter binding sites for different transcription factors are required in B vs. T cells, and only a few factors have been shown to bind in both B and T cells. In all cases, enhancer elements are required for efficient *Rag1* and *Rag2* expression *in vivo*. Developing B cells and DP thymocytes seem to use two entirely distinct sets of enhancers to drive *Rag1* and *Rag2* transcription, while DN thymocytes use enhancers from each set.

The Rag1 promoter was the first element identified. Interestingly, in luciferase assays it can drive limited transcription in mature lymphocyte lines and non-lymphoid cells, in contrast to the restricted pattern of endogenous *Rag1* mRNA expression (Brown et al., 1997; Fuller and Storb, 1997). This may be partially due to the fact that the widely-expressed transcription facto NF-Y seems to be involved in Rag1 promoter activity (Brown et al., 1997; Fuller and Storb, 1997). Therefore, it is believed that endogenous Rag1 transcription must require enhancer elements that are only active in developing lymphocytes. In contrast to the Rag1 promoter, Rag2 promoter activity seems to be intrinsically limited to developing B and T cells (Lauring and Schlissel, 1999; Kishi et al., 2000; Fong et al., 2000). This might be due to a requirement for B and/or T cell restricted transcription factors for promoter activity.

In addition to their promoters, *Rag1* and *Rag2* transcription is affected by a variety of enhancer elements, and all of these elements are preferentially active in

either B or T cells. Four enhancers, all located 5' of the *Rag2* gene, and one silencer element located between the *Rag1* and *Rag2* genes have been identified. Two of these elements, the *Ep* and *Erag* enhancers, demonstrate activity only in developing B cells (Hsu et al., 2003; Wei et al., 2005). The *D3* enhancer (also called *Ed*) is active in pro-B and pre-B cells and in DN thymocytes (Wei et al., 2002). Finally the anti-silencer element (ASE) required to overcome the effect of an active intergenic silencer is absolutely required for *Rag1* and *Rag2* expression in DP thymocytes, is partially responsible for expression in DN thymocytes, but seems to play no role in *Rag1* and *Rag2* expression in developing B cells (Yannoutsos et al., 2004).

The *Erag* enhancer is located approximately 22kb 5' of the first *Rag2* exon (Hsu et al., 2003). Mutation of the endogenous *Erag* enhancer decreases *Rag1* and *Rag2* mRNA expression in pro-B, pre-B, and immature B cells, leading to decreased B cell development; however this mutation has no effect on thymocyte development or *Rag1* and *Rag2* expression in either DN or DP thymocytes (Hsu et al., 2003). *Erag* is also important for *Rag1* and *Rag2* expression at the earlier common lymphocyte progenitor (CLP) stage of development (Borghesi et al., 2005).

The *Ep* enhancer is the most proximal enhancer to the *Rag1* and *Rag2* genes, located approximately 2.6 kb 5' of the first *Rag2* exon. *Ep* was identified as one of three DNase-hypersensitive sites found in lymphocyte cell lines upstream of the Rag2 gene

(Wei et al., 2002). The effect of the Ep enhancer on endogenous *Rag1* and *Rag2* transcription has not been tested, but a transgenic construct containing GFP under the control of the Rag2 promoter and Ep was shown to drive GFP expression in both B220+ IgM- and B220+ IgM+ bone marrow cells, corresponding to pro- and pre-B cells vs. immature and mature B cells (Wei et al., 2005). GFP expression was also found in B220+ splenic B cells, which do not normally express *Rag1* and *Rag2*, suggesting that a third factor normally blocks *Ep*-driven transcription in these cells (Wei et al., 2005).

The *Ed* (or *D3*) enhancer was identified in the same DNase-hypersensitivity screen as the *Ep* enhancer. It is located approximately 8 kb 5' of the *Rag2* gene. Unlike *Ep*, *Ed* promotes *Rag2* promoter activity in both developing B cells and in DN thymocytes, but has no activity in mature lymphocytes (Wei et al., 2002).

The anti-silencer element (ASE) is the only enhancer-like element known to affect *Rag1* and *Rag2* expression in DP thymocytes. It is also the most distant element, located between 71 and 86 kb 5' of the *Rag2* gene (Yannoutsos et al., 2004). It acts by overcoming the effects of the intergenic silencer element located between the *Rag1* and *Rag2* genes (Yannoutsos et al., 2004). In ASE knock-out mice, the silencer element almost completely prevents *Rag1* and *Rag2* expression in DP thymocytes. Absence of the ASE also decreases *Rag1* and *Rag2* expression in DN thymocytes, although less drastically. Experiments using GFP-reporter constructs showed that the silencer does

not prevent Rag expression in developing B cells (Yannoutsos et al., 2004; Yu et al., 1999).

Transcription factors bind that *Rag1* and *Rag2* gene regulatory elements

Just as different genetic elements drive *Rag1* and *Rag2* transcription in developing B and T cells, the transcription factors that bind to these elements are mostly distinct between the two lineages. A comprehensive list of the many factors known to bind the *Rag1/Rag2* gene locus is provided in Fig. 1.3. Unfortunately these factors have been identified in a fairly piecemeal fashion, and so it is difficult to draw a coherent picture of their relative importance in normal developing lymphocytes. Early experiments mostly relied on EMSA to show that factors can bind *Rag1* and *Rag2* regulatory sequences *in vitro*; more recently ChIP has been used to show that some of these factors actually do bind these regulatory sequences *in vivo.* In most cases, luciferase experiments showed that individual factors have the potential to promote (or in a few cases inhibit) *Rag1* and *Rag2* transcription. However knock-out mouse models of these factors often show no effect on *Rag1* and *Rag2* mRNA expression, or may even show the opposite effect of what was predicted by luciferase experiments. The following sections will briefly summarize the findings for several factors that have been implicated in regulating *Rag1* and *Rag2* expression in either developing B or T cells, or in both.

Many of the factors shown to bind the *Rag1/Rag2* gene locus in developing B cells are critical for B cell development in general, including Pax-5, E2A, Ikaros, Bcl11a, and Foxo1 (Bain et al., 1994; Nutt et al., 1997; Dengler et al., 2008; Reynaud et al., 2008; Lee et al., 2013). All have been shown to promote *Rag1* and/or *Rag2* transcription in luciferase assays (Amin and Schlissel, 2008; Fuller and Storb, 1997; Hsu et al., 2003; Lauring and Schlissel, 1999; Lee et al., 2013); however knockouts of these factors produce a variety of results. Pax-5 knockout pro-B cells apparently have normal *Rag1* and *Rag2* expression, although their development is arrested due to other defects (Nutt et al., 1997). E2A knockout fetal liver was shown to lack *Rag1* and *Rag2* expression, although this might be because few cells developed to stages where *Rag* expression is expected to by high (Bain et al., 1994); another paper showed that an E2A knockout pre-B cell line expressed normal levels of *Rag1* and *Rag2* mRNA (Lazorchak et al., 2006). On the other hand, *Rag1* and *Rag2* mRNA expression is impaired in Foxo1, Ikaros, and Bcl11a knockouts, suggesting that these factors are more crucial for *Rag1* and *Rag2* expression (Dengler et al., 2008; Reynaud et al., 2008; Lee et al., 2013). Complicating matters, several of these factors can also regulate the expression of other factors involved in *Rag1* and *Rag2* transcription. For example, both Ikaros and Bcl11a can regulate Foxo1 expression, and so their impact may not entirely be direct (Ferreirós-Vidal et al., 2013; Lee et al., 2013) Ikaros can also suppress expression of the only known

factor that directly inhibits *Rag1* and *Rag2* transcription in B cells, Gfi1b (Schulz et al., 2012; Ferreirós-Vidal et al., 2013).

When studies examining *Rag1* and *Rag2* transcription differentiate between pro- and pre-B cells, they often find little difference between the two stages. In contrast, there are several differences between DN and DP thymocytes. The *Ed* enhancer can drive reporter gene expression in DN but not DP thymocytes, and transcription factor C/EBP can increase *Ed* activity in luciferase assays (Wei et al., 2002). Active NFAT appears to suppress *Rag1* and *Rag2* expression by binding to several sites within the locus in DN cells, but an effect in DPs has not been reported (Patra et al., 2006). In DP thymocytes, the ASE is critical for *Rag1* and *Rag2* expression, but it is less important in DN thymocytes. The ASE is required to overcome the effects of the intergenic silencer, whose effects are believed to be mediated by Runx (Yannoutsos et al., 2004). The protein SATB1, which can be involved in attaching DNA to the nuclear matrix and in forming loops between DNA regions, is required to bring the ASE and the *Rag1* and *Rag2* promoters into close proximity. SATB1-deficiency reduces *Rag1* and *Rag2* expression in DP but not DN thymocytes, although not quite as drastically as ASE deletion (Hao et al., 2015; Yannoutsos et al., 2004).

To date, few factors have been found to bind the *Rag1/Rag2* gene locus in both B and T lineage cells: Sp1 (Miranda et al., 2002), NF-Y (Brown et al., 1997; Fuller and

Storb, 1997), Lef-1 (Okamura et al., 1998; Jin et al., 2002), and c-myb (Fong et al., 2000; Wang et al., 2000; Kishi, 2002; Miranda et al., 2002). However none of these factors have been reported to be essential for *Rag1* and *Rag2* expression in either B or T cells. Sp1 is a ubiquitously expressed transcription factor that may increase the effectiveness of lymphocyte-specific factor in activating *Rag1* and *Rag2* transcription (Miranda et al., 2002). NF-Y is active in many tissues and can drive *Rag1* promoter activity in luciferase assays using developing B or T cell lines (Brown et al., 1997; Fuller and Storb, 1997). However its effect on endogenous *Rag1* expression has not been reported. Lef-1 knockout thymocytes also express *Rag1*, although this may be due to a compensatory effect of TCR-1; *ex vivo* cultured double knock-out thymocytes express only low levels of *Rag1* mRNA. However they are also largely arrested at a developmental stage in which high *Rag1* expression is not expected, making this result difficult to interpret (Okamura et al., 1998). Finally, c-myb overexpression was actually shown to decrease *Rag1* and *Rag2* mRNA expression, although the effect is likely indirect (Timblin and Schlissel, 2013).

As shown above, many factors have been identified that bind to the *Rag1/Rag2* gene locus and seem to regulate its activity. Many of the factors that apparently have the ability to promote *Rag1* and *Rag2* transcription as measured by luciferase assays do not actually seem required to do so in developing lymphocytes. This may be due to

compensation between factors, so that most transcription factors that bind the *Rag1/Rag2* gene locus and promote transcription are not absolutely essential.

Signaling pathways that regulate Rag expression: PI3K-Akt-Foxo1

One of the most critical direct regulators of *Rag1* and *Rag2* transcription in developing B cells seems to be Foxo1. Critical factors for B lineage commitment and development, such as Ikaros, E2A, and EBF1 (Reynaud et al., 2008; Lin et al., 2010), promote Foxo1 expression. Foxo1 binds to the *Erag* enhancer in pre-B cells, and Foxo1 deficient cells have low *Rag1* and *Rag2* mRNA expression. Foxo proteins are rendered transcriptionally inactive and degraded via phosphorylation by Akt (Biggs et al., 1999). Accordingly, the activity of Akt and its upstream activator PI3K suppress *Rag1* and *Rag2* expression in pre-B cells (Amin and Schlissel, 2008), while PTEN, a negative regulator of Akt, promotes *Rag1* and *Rag2* expression (Alkhatib et al., 2012). As both the pre-BCR and BCR activate PI3K, this pathway is believed to be involved in the suppression of *Rag1* and *Rag2* expression upon formation of a productive immunoglobulin heavy chain or light chain (Amin and Schlissel, 2008; Werner et al., 2010).

Signaling pathways that regulate Rag expression: Mapk/Erk

Erk signaling has been implicated in suppressing *Rag1* and *Rag2* expression in both developing T and B cells. In Jurkat T cells and thymocytes, it was shown that tonic TCR signaling activates the Erk and Abl kinases, which cooperate to suppress *Rag1* and

Rag2 mRNA expression (Roose et al., 2003). Similarly, in developing B cells it was found that either active or tonic signaling from the BCR activates Erk in addition to the Akt pathway described above, and that these two pathways cooperate in suppressing *Rag1* and *Rag2* transcription factors. In this context, Erk activity reduced E2A binding to the *Erag* enhancer (Novak et al., 2010).

Signaling pathways that regulate Rag expression: NF-κB

NF-κB signaling plays a fundamental role in lymphocyte development, among other immune system functions. The family of NF-κB transcription factors includes 5 members which can form a variety of transcriptionally active of homo- or heterodimers (Hayden and Ghosh, 2012). Three of these proteins (RelA/p65, RelB, and c-Rel) contain transactivation domains and can promote transcription when bound to a gene locus. The other two (NFKB1/p50 and NFKB2/p52) lack transactivation domains, and can only promote transcription as part of a heterodimer in which the other partner contains a transactivation domain (Hayden and Ghosh, 2012). Although an "NF-κB consensus site" has been defined, different dimers may preferentially bind to somewhat different DNA sequences (Wong et al., 2011).

NF-κB signaling is often generalized as either canonical or non-canonical, depending on the activation pathway and the particular dimer pairs activated by the signal (Hayden and Ghosh, 2012). Both pathways can be activated by a variety of

upstream signaling inputs that converge on the two common pathways. Signals activating the canonical pathway lead to phosphorylation of the Inhibitor of nuclear factor κB kinase subunit β (IKKβ), which in turn phosphorylates the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) (Hayden and Ghosh, 2012). IKKβ normally exists in complex with IKKα and the NF-κB essential modulator (Nemo), and Nemo expression is absolutely required for IKKβ phosphorylation of IκBα and activation of the canonical pathway (Li et al., 1999; Rudolph et al., 2000). IκBα normally binds to NF-κB factor complexes (RelA/p50 most commonly) and prevents their activity; upon phosphorylation it is degraded and RelA/p50 (or other) complexes are free to activate transcription of target genes (Hayden and Ghosh, 2012).

The non-canonical pathway follows a somewhat similar sequence of events requiring different factors. Initial activation depends on stabilization and accumulation of the NF-kB inducing kinase (NIK), and so non-canonical signaling acts relatively slowly as it requires new protein synthesis (Senftleben et al., 2001). NIK phosphorylates IKKα, which in turn phosphorylates p100. Prior to phosphorylation, p100 exists in transcriptionally inactive complex with RelB; upon phosphorylation p100 undergoes partial proteolytic processing to the active p52 form. The p52/RelB dimer can then activate transcription of target genes (Hayden and Ghosh, 2012).

Although both the canonical and non-canonical pathways typically activate gene transcription, NF-κB factors can also inhibit transcription of target genes in some circumstances. For example p50/p50 or p52/p52 homodimers, lacking transactivation domains, can prevent the binding of activating NF-κB complexes (Bohuslav et al., 1998). Cofactors such as Bcl-3 can modify the activity of p50/p50 and p52/p52 homodimers; complexes containing Bcl-3 can either activate or inhibit transcriptional activation, depending on post-translational modifications of the Bcl3 protein (Fujita et al., 1993; Dechend et al., 1999; Grundström et al., 2004).

Although there are several NF-κB binding sites within the Rag gene locus (Verkoczy et al., 2005), the role of NF-κB signaling in regulating Rag expression has been controversial; various studies find that NF-κB activity either promotes, represses, or has no effect on *Rag1* and *Rag2* mRNA expression. Studies showing no effect of NF-κB on *Rag1* and *Rag2* expression focused on basal expression in pre-B cells, showing that neither Nemo-deficiency nor expression of a dominant-negative IκBα construct affected *Rag1* or *Rag2* mRNA expression (Amin and Schlissel, 2008; Derudder et al., 2009). An earlier study implicated NF-κB in promoting *Rag1* and *Rag2* expression during receptor editing in immature B cells (Verkoczy et al., 2005). This study showed that IgM-crosslinking relieved IκBα-mediated NF-κB inhibition and caused several NF-κB factors to bind the *Rag* gene locus. However, the same study showed that p50 knock-out pre-B cells have constitutively high levels of *Rag1* and *Rag2* mRNA, suggesting that this factor

normally suppresses *Rag1* and *Rag2* expression. More recently, IKK inhibition was shown to modestly increase recombination of a reporter substrate in cycling Abelson cells without sti-571 treatment, suggesting that NF-κB activity normally suppresses *Rag1* and *Rag2* expression (Ochodnicka-Mackovicova et al., 2015).

Roles of the DNA damage response in V(D)J recombination

The DSBs produced during V(D)J recombination activate the conserved DNA damage response (DDR), and the signals generated by this pathway are crucial for proper recombination and for normal lymphocyte development (Bredemeyer et al., 2006; Huang et al., 2007; Bredemeyer et al., 2008). The protein kinase ATM plays an important role in this response. ATM is activated by DSBs and coordinates a variety of downstream signals promote DSB repair, arrest the cell cycle to allow time for repair, and promote apoptosis if the DSBs cannot be repaired (Shiloh and Ziv, 2013). Due to these crucial functions, humans and mice deficient in ATM expression are predisposed to develop a genomic instability leading to cancer (Barlow et al., 1996; Taylor et al., 1996; Xu et al., 1996). In pre-B cells, ATM regulates the expression of many genes important for B cell development, implicating the DDR in developmental processes besides V(D)J recombination (Bredemeyer et al., 2008). Although V(D)J recombination can occur without ATM, ATM is normally activated by RAG-mediated DSBs and is required for their efficient and accurate repair (Bredemeyer et al., 2006). Additionally,

the Bassing laboratory has shown that ATM can mediate feedback inhibition of V(D)J recombination, ensuring that only one antigen receptor gene rearranges at a time (Steinel et al., 2013).

Although it is crucial for lymphocyte development, the deliberate formation of DSBs during V(D)J recombination creates a risk of translocation if the process goes awry. RAG-mediated DNA cleavage would be particularly risky in the presence of existing DNA damage, as the presence of two DSBs in a cell increases the risk of translocation by several orders of magnitude (Richardson and Jasin, 2000). Normal cellular metabolism, environmental factors, or off-target activity of RAG itself at cryptic RSSs could all create DSBs with the potential to translocate with cleaved antigen receptor loci. Translocations of antigen receptor loci pose a particular oncogenic risk as they contain enhancers and promoters that are highly active in developing lymphocytes, which if translocated could drive the expression of a proto-oncogene. Indeed, many lymphoid cancers in humans and in mouse models involve clonal AgR translocations that are believed to promote oncogenesis (Leder et al., 1983; Schlissel, 2006). In this context, it is plausible that a developing lymphocyte might avoid creating a DSB at an antigen receptor locus when a DSB already exists at another location in the genome.

Indeed, we find that DSBs suppress RAG expression and V(D)J recombination; this central finding is discussed in Chapter 2. Chapter 3 focuses on the signaling events

necessary for this suppression. Chapters 2 and 3 focus on how exogenous sources of DNA damage suppress RAG expression and V(D)J recombination. However, V(D)J recombination itself requires DNA damage, and in this context suppression of RAG expression can be considered a negative feedback loop. Chapter 4 discusses how defects in this feedback loop can increase allelic inclusion in lymphocytes. Collectively, these results suggest that developing lymphocytes employ a conserved pathway to react to a programmed developmental stimulus (RAG-mediated DSBs) and non-programmed stimulus (exogenous DSBs). While the regulation of RAG expression by developmental cues has been well studied, this work shows that RAG expression can also be regulated by external factors.

Figure 1.1

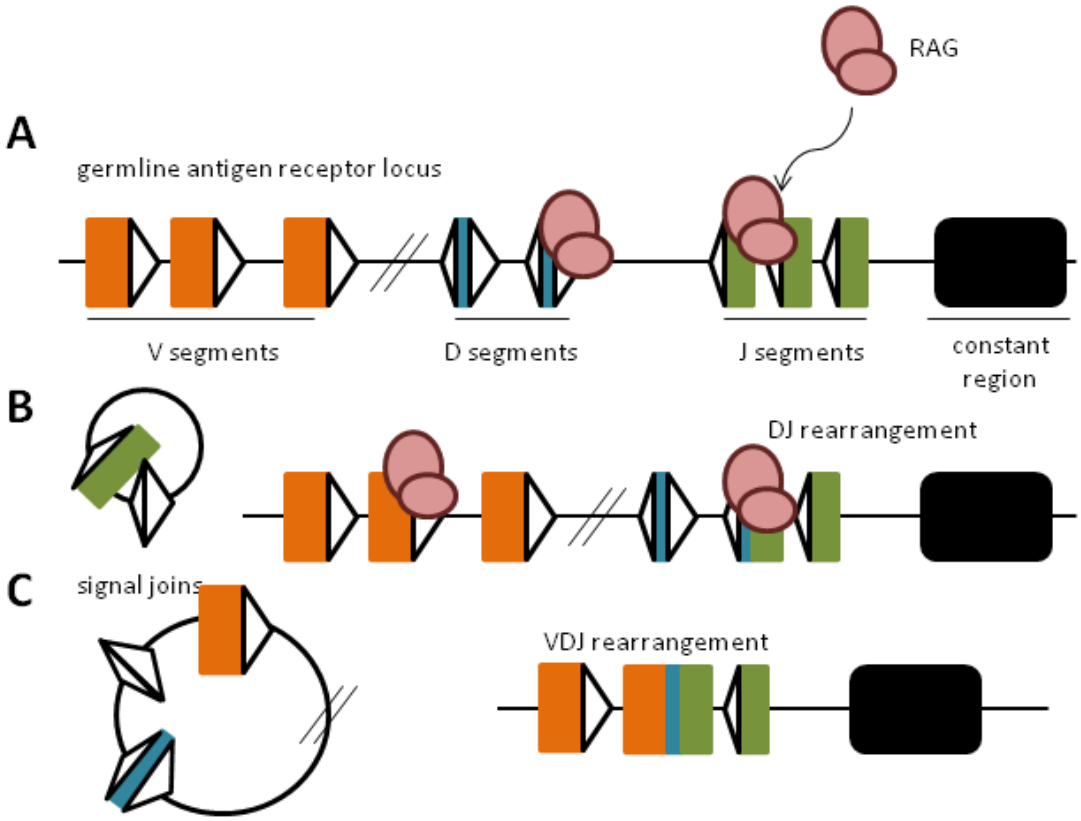


Figure 1.1: Schematic of V(D)J recombination. **(A)** Germline antigen receptor antigen receptor locus. Rag cleaves recombination signal sequences adjacent to D and J segments, creating a DJ join. **(B)** In a second round of RAG activity, a V segment rearranges to the DJ segment to create **(C)** a complete VDJ rearrangement.

Figure 1.2

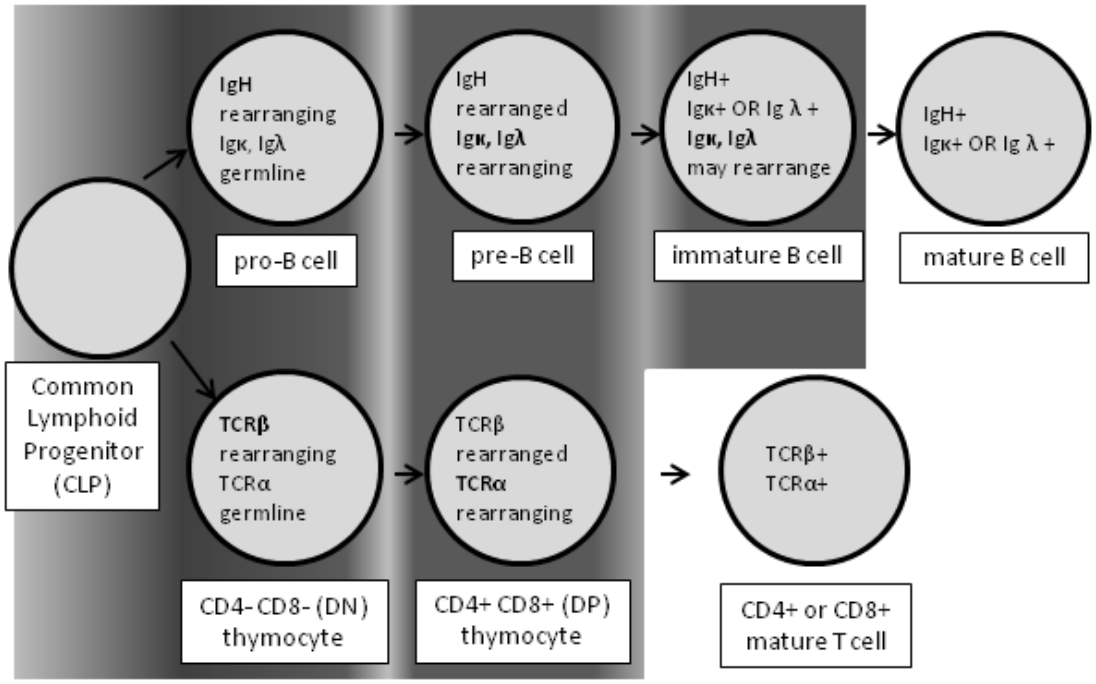
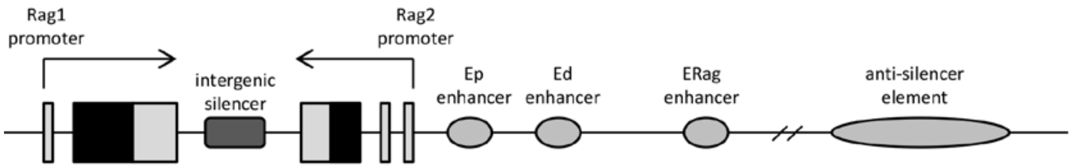


Figure 1.2: Overview of lymphocyte development. B and T lineages diverge from a common progenitor and undergo recombination of specific antigen receptor loci at defined developmental stages. Background shading represents high Rag expression (dark areas) or low Rag expression (light areas) at each developmental stage.

Figure 1.3



B					CLP,B		E2A[1,2,3,4]
B			B	B	B		Ikaros[1,5,6]
					B		Foxo1[7,8,9]
					B		Foxp1[7,8,10]
		B					Pax-5[11,12,13]
B					B		Bcl11a[14]
					B		Gfi1b[15]
B		B		B	B		Nf-κB[16]
		B,T					c-myb[13,17,18,19]
B,T							NF-Y[1,20]
		B,T					Lef-1[21,22]
			B	B, DN T			C/EBP[6,23]
		B,T					Sp1[13]
		B,T					Sp3[13]
		T					Gata-3[12]
	T						Runx[24]
T	T	T				T	SatB1[25]
T							Zfp609[26]
T	T				T		Nfatc1[27]

Figure 1.3: Diagram of *Rag1* and *Rag2* genes and associated regulatory elements. The grid shows proteins known to bind each element. "B" indicates binding in developing B cells, "T" indicates binding in developing T cells, "DN T" indicates binding only in CD4- CD8- thymocytes. "CLP" indicates binding in the common lymphoid progenitor. The factors that regulate *Rag1* and *Rag2* transcription are largely distinct between B and T cells. Sources: [1] (Fuller and Storb, 1997); [2] (Hsu et al., 2003); [3] (Novak et al., 2010); [4] (Borghesi et al., 2005); [5] (Reynaud et al., 2008); [6] (Wei et al., 2005); [7] (Amin and Schlissel, 2008); [8] (Dengler et al., 2008); [9] (Herzog et al., 2008); [10] (Hu et al., 2006); [11] (Lauring and Schlissel, 1999); [12] (Kishi et al., 2000); [13] (Miranda et al., 2002); [14] (Lee et al., 2013); [15] (Schulz et al., 2012); [16] (Verkoczy et al., 2005); [17] (Kishi, 2002); [18] (Fong et al., 2000); [19] (Wang et al., 2000); [20] (Brown et al., 1997); [21] (Jin et al., 2002); [22] (Okamura et al., 1998); [23] (Wei et al., 2002); [24] (Yannoutsos et al., 2004) 2004; [25] (Hao et al., 2015); [26] (Reed et al., 2013); [27] (Patra et al., 2006). Adapted and updated from (Kuo and Schlissel, 2009)

Chapter 2: DSBs suppress Rag1 and Rag2 expression and V(D)J recombination[1]

Abstract

Developing lymphocytes must rearrange the genomic sequence of appropriate antigen receptor genes by V(D)J recombination. The lymphocyte-specific endonuclease RAG, composed of Rag1 and Rag2, initiates this process by cleaving specific sites within antigen receptor loci. RAG expression is regulated to ensure that V(D)J recombination occurs only under appropriate circumstances. The Bassing laboratory previously demonstrated that RAG-mediated DNA cleavage in pre-B cells initiates a feedback-inhibition signal suppressing RAG expression. Here, we show that non-RAG mediated DNA double strand breaks (DSBs) have a similar effect in suppressing mRNA expression of *Rag1* and *Rag2*. This effect can be observed in pro-B cells, pre-B cells, and DN thymocytes, but not in DP thymocytes. In pre-B cells, loss of *Rag1* and *Rag2* mRNA expression leads to loss of Rag1 protein, but Rag2 protein level is unaffected due to high protein stability. Suppression of Rag1 expression by DSBs is associated with suppressed RAG-mediated cleavage of the Igκ locus or an artificial recombination substrate in

[1] Parts of this chapter were published in *The Journal of Immunology*. Copyright © 2017 The American Association of Immunologists, Inc. Fisher MR, Rivera-Reyes A, Bloch NB, Schatz DG, Bassing CH. Immature Lymphocytes Inhibit Rag1 and Rag2 Transcription and V(D)J Recombination in Response to DNA Double-Strand Breaks. J. Immunol. [Epub ahead of print 2017 Feb 17]. Portions of this chapter were prepared jointly with Noah Bloch.

Abelson-transformed pre-B cells. However, simply over-expressing Rag1 does not allow cells to complete V(D)J recombination in the presence of DSBs, suggesting that other factors may also play a role in suppressing V(D)J recombination.

Introduction

Adaptive immunity requires the production of lymphocytes capable of recognizing a broad and often unpredictable array of potential foreign antigens. In jawed vertebrates, B and T lymphocytes achieve the necessary diversity of antigen receptors by V(D)J recombination (Tonegawa, 1983). In this process, the Rag1/Rag2 endonuclease (RAG) makes paired DNA double strand breaks (DSBs) at recombination signal sequences (RSSs) adjacent to variable (V), diversity (D), or joining (J) gene segments within a selected antigen receptor locus; intervening DNA is either deleted or inverted, and the DSBs are repaired by ubiquitous Non-Homologous End Joining (NHEJ) factors (Helmink and Sleckman, 2012). The combination of V, D, and J segments, the semi-random addition and removal of a limited number of nucleotides at the breakpoints, and the use of two independently rearranged antigen receptor chains in each receptor all contribute to the receptor diversity generated by V(D)J recombination (Tonegawa, 1983).

V(D)J recombination is absolutely required for B and T cell development, as failure to rearrange a complete antigen receptor leads to death of progenitor

lymphocytes (Miosge and Goodnow, 2005). At each stage, the expression of the V(D)J catalyst (RAG) and the accessibility of the substrate (antigen receptor genes) are both regulated to ensure that the appropriate genes are rearranged at the appropriate time (Vettermann and Schlissel, 2010). Antigen receptor genes are prepared for rearrangement in the appropriate developmental stage by a process of histone modification, precise nucleosome positioning, and DNA-looping to bring distant gene segments into close proximity (Stanhope-Baker et al., 1996; Mandal et al., 2015; Hu et al., 2015). Furthermore, expression of the RAG endonuclease is restricted to certain stages of B and T cell development. Developing B cells first rearrange the immunoglobulin heavy chain (IgH) chain locus as pro-B cells, and expression of a functional IgH allele suppresses RAG expression and promotes progression to the pre-B cell stage (Übelhart et al., 2015). After a series of cell divisions at the large pre-B1 stage, re-expression of RAG in small resting pre-B2 cells allows light chain rearrangement and expression of a complete B cell receptor (BCR) . Developing αβT cells go through a similar process in which rearrangement of the TCRβ gene in DN thymocytes leads to progression to the DP thymocyte stage and rearrangement of the TCRα gene (Miosge and Goodnow, 2005).

Different transcription factors and genetic regulatory elements drive *Rag1* and *Rag2* expression in developing B and T cells. For example, the *Erag* enhancer promotes *Rag1* and *Rag2* transcription in both pro- and pre-B cells, but does not seem to affect

expression in developing T cells (Hsu et al., 2003). Conversely, interplay between a silencer and anti-silencer element control *Rag1* and *Rag2* expression in DP thymocytes cells, but this system is only partially responsible for *Rag1* and *Rag2* transcription in DN thymocytes, and not at all necessary in developing B cells (Yannoutsos et al., 2004). However in both developing B and T cells, key developmental events either promote or suppress RAG expression; for example, cell surface expression of a functional, non-autoreactive antigen receptor chain suppresses RAG expression in both developing B and T cells (Turka et al., 1991; Li et al., 1993; Verkoczy et al., 2007). Such regulation ensures that V(D)J recombination does not happen at inappropriate developmental stages.

Although crucial for lymphocyte development, the deliberate formation of DSBs during V(D)J recombination creates a risk of translocation if the process goes awry. Richardson et al. show that co-existence of two DSBs at two different loci increases the risk of translocation between those loci by several orders of magnitude (Richardson and Jasin, 2000); therefore RAG-mediated DNA cleavage of an antigen receptor gene pose a risk of translocation if another DSB already exists in the cell. Normal cellular metabolism, genotoxic environmental factors, or off-target activity of RAG itself at sites that mimic RSSs (cryptic RSSs) could all create DSBs with the potential to translocate with antigen receptor loci cleaved by RAG (Schlissel, 2006). Translocations of antigen receptor loci pose a particular oncogenic risk as their enhancers and promoters are

highly active in developing lymphocytes and could drive the expression of a proto-oncogene if translocated. Indeed, many lymphoid cancers in humans and in mouse models involve clonal antigen receptor translocations that are believed to promote oncogenesis (Küppers and Dalla-Favera, 2001).

To combat the risks of DNA-double strand breaks, eukaryotic cells including lymphocytes employ conserved DNA damage response (DDR) mechanisms. DSBs activate the Ataxia Telangiectasia mutated (ATM) protein, which serves as the key regulator of cellular responses to DSBs by phosphorylating a variety of substrates leading to cell cycle arrest and DSB repair (Shiloh and Ziv, 2013). Lack of ATM function allows genomic instability, and both humans and mice deficient for ATM are susceptible to leukemias and lymphomas (Barlow et al., 1996; Taylor et al., 1996). Several studies have demonstrated that RAG-induced DSBs in developing lymphocytes activate ATM and downstream DDR pathways (Bredemeyer et al., 2008). In this context, ATM promotes efficient repair of RAG-induced DSBs (Bredemeyer et al., 2006; Huang et al., 2007). Previous work in the Bassing laboratory has demonstrated that ATM activation by RAG-mediated DSBs can also suppress the induction of RAG-mediated DSBs in pre-B cells. Specifically, RAG-induced DSB on one Igκ allele in pre-B cells suppresses RAG mRNA and protein expression and prevents cleavage of the second Igκ allele in an ATM-dependent manner (Steinel et al., 2013).

As discussed above, the co-existence of multiple DSBs in a cell seriously increases the risk of translocation. Given the need to prevent antigen receptor translocations during V(D)J recombination, we hypothesized that exogenously-induced DSBs would suppress RAG expression and function, just as RAG-mediated DSBs do. We find that etoposide-induced DSBs suppress RAG expression and V(D)J recombination in pre-B cell lines. Exposure to ionizing radiation (IR) to induce DSBs suppresses *Rag1* and *Rag2* mRNA expression in pro- and pre-B cells and in DN thymocytes, but not in DP thymocytes. We discuss the possibility that suppression of RAG expression and activity protects genomic stability of developing lymphocytes by preventing RAG-mediated antigen receptor locus cleavage in the presence of another DSB that could promote translocation.

Results

DSBs in pre-B cells suppress *Rag1* and *Rag2* mRNA expression

The Bassing laboratory has previously shown that RAG-mediated DSBs in pre-B cells activate the DNA-damage response protein ATM to suppress *Rag1* and *Rag2* mRNA expression and inhibit further RAG activity (Steinel et al., 2013). We hypothesized that exogenously-induced DNA damage would share this suppressive effect on RAG expression and V(D)J recombination in pre-B cells. To test this we used the two model

systems previously used to demonstrate the suppressive effect of RAG-mediated breaks: IL-7 withdrawn *ex vivo* pre-B cells, and Abelson-transformed pre-B cell lines.

EμBCL2 primary pre-B cells can be grown for several days *ex vivo* with IL-7 to expand the pre-B cell pool, and then withdrawn from IL-7 to induce RAG expression and V(D)J recombination (Ray et al., 1998). This Bcl2 transgene is expressed in B and T lineage cells and allows these cells to survive in culture and protects cells from apoptosis upon exposure to DNA damage (Strasser et al., 1991a, 1991b). To determine the effect of non-RAG mediated DSBs on pre-B cells, we exposed these cells to 4 Gy ionizing radiation (IR). As a control to show that the cells experienced a DNA damage response, we measured the induction of *p21* mRNA caused by IR; we found that exposure to IR or other DNA damaging agents caused the expected increase in *p21* mRNA expression in all cases (Fig. 2.1a,c,d). From one hour through four hours after IR exposure, *Rag1* and *Rag2* mRNA expression in *ex vivo* pre-B cells decreased by approximately 80%. By 8 hours after IR exposure, *Rag1* and *Rag2* expression in these cells recovered to normal levels (Fig. 2.1a). To rule out any role for RAG-induced DNA breaks in this response, we turned to Abelson-transformed pre-B cell lines pre-treated with sti571 to induce *Rag1* and *Rag2* expression (Muljo and Schlissel, 2003). We compared the effect of IR on wild-type Abelson cells and Abelson cells expressing a cleavage-incompetent Rag1 mutant protein ($Rag1^{D708A}$). We found that both wild-type and $Rag1^{D708A}$ cells suppressed *Rag1* and *Rag2* mRNA expression in response to IR, although the response was slower than in

primary cells (Fig. 2.1b). These results show that IR can suppress *Rag1* and *Rag2* expression without any contribution from RAG-mediated DSBs.

These results suggest that exogenously induced DSBs can suppress *Rag1* and *Rag2* expression. However, IR causes many kinds of cellular damage in addition to DSBs. To more specifically test the effect of DSBs, we tested the effect of two genotoxic drugs on *Rag1* and *Rag2* expression in IL-7 withdrawn pre-B cells. The drug etoposide poisons type II topoisomerase and prevents them from religating the DNA strands (Montecucco et al., 2015). Bleomycin induces DSBs, but may also damage RNA (Hecht, 2000). Exposure to 10μg/mL etoposide leads to a rapid decrease in *Rag1* and *Rag2* mRNA expression by approximately 90% (Fig. 2.1c). Similarly, exposure to 5μM bleomycin leads to an approximately 75% decrease in *Rag1* and *Rag2* mRNA expression (Fig. 2.1d). Collectively, these results show that the DSB response in pre-B cells involves turning off *Rag1* and *Rag2* expression.

DSBs suppress *Rag1* and *Rag2* expression in bone marrow and thymus.

Rag1 and *Rag2* are expressed at defined stages of B and T cell development, and different transcription factors and genetic elements regulate its expression in different cell types (Hsu et al., 2003; Yannoutsos et al., 2004). Because of this heterogeneity, we decided to test whether the effect of DNA damage was specific to pre-B cells, or shared by other RAG-expressing lymphocytes. To survey the effect of DSBs on a variety of

developing lymphocyte populations, we exposed live *EμBCL2* mice to 10Gy IR. The Bcl-2 transgene is expressed in both B and T lineage cells in these mice (Strasser et al., 1991a). We then isolated bone marrow (BM) or thymocytes from irradiated mice 1 or 4 hours after IR, and from unirradiated control mice to measure *Rag1* and *Rag2* mRNA levels by qPCR. As a control to show that the cells experienced a DNA damage response, we measured the induction of *p21* mRNA caused by IR. Upon IR, we found that *Rag1* expression in *EμBCL2* BM decreases by approximately 75%, while *Rag2* expression decreases by approximately 60%. (Fig. 2.2a). To demonstrate that this response is not somehow caused by the *EμBCL2* transgene we performed a similar experiment using wild-type mice. BM from wild-type mice suppressed *Rag1* and *Rag2* expression by approximately 90% in response to IR (Fig. 2.2c). In contrast, IR exposure does not suppress *Rag1* or *Rag2* mRNA expression in *EμBCL2* thymocytes, and may even lead to increased expression (Fig. 2.2b). We found a similar effect using wild-type thymocytes (Fig. 2.2d). In bone marrow of wild-type or *EμBCL2* mice, most RAG-expressing are pre-B or immature B cells, relatively late developmental stages. Similarly, Rag-expressing cells in the thymus consist largely of DP thymocytes, the T cell developmental stage analogous to the pre-B cell stage. Therefore, these results likely reflect the response of pre-B cells and pre-T cells to DSBs, while the effect on pro-B cells ad pro-T cells is masked by their relatively low numbers.

DSBs suppress *Rag1* and *Rag2* expression in early stages of B and T cell development

To determine the effect of IR on *Rag1* and *Rag2* mRNA expression at earlier stages of B and T cell development, we repeated the experiments above using *Rag2*$^{-/-}$ and *Rag1*$^{-/-}$ mice. In these mice, B cell development is arrested at the pro-B cell stage, and T cell development is arrested at the DN thymocyte stage (Hao and Rajewsky, 2001; Mombaerts et al., 1992a). Therefore, in a Rag-deficient mouse, the Rag-expressing cells in the BM consist largely of pro-B cells and earlier precursors, while Rag-expressing cells in the thymus consist of DN thymocytes. Again, increased *p21* expression indicated that these tissues experienced DNA damage (Fig 2.3a-d). *Rag1* expression in both the BM of *Rag2*$^{-/-}$ mice decreases approximately 80% upon exposure to 10Gy IR (Fig. 2.3a); similarly, *Rag2* expression in BM of *Rag1*$^{-/-}$ mice decreases upon exposure to IR (Fig. 2.3b). Interestingly, IR caused an approximately 50% decrease in *Rag1* expression in *Rag2*$^{-/-}$ thymocytes (Fig. 2.3c), and a similar decrease in *Rag2* expression in *Rag1*$^{-/-}$ thymocytes (Fig2.3d). Although less robust than the effect in BM, this suppression is strikingly different from the modest induction of *Rag1* and *Rag2* upon exposure of wild-type thymocytes to IR. Therefore, we conclude that DSBs can suppress *Rag1* and *Rag2* expression at early stages of both B and T cell development; this ability persists in B cells through the pre-B cell stage, but is lost in T cells as they develop to the DP stage. These findings are consistent with the fact that regulation of basal *Rag1* and *Rag2* mRNA

expression in developing B and T cells is relies on different genetic elements and transcription factors, as discussed in Chapter 1.

DSBs suppress Rag1 but not Rag2 protein levels

The previously reported half-lives of both Rag1 and Rag2 half-life are very short, from 10-30 minutes (Grawunder et al., 1996; Lin and Desiderio, 1993; Ochodnicka-Mackovicova et al., 2015). Therefore, we expected the expression of both proteins to decrease rapidly as their mRNA expression was suppressed after IR. However, when we measured protein expression in irradiated IL-7 withdrawn pre-B cells, we unexpectedly found that only Rag1 protein levels decreased within the timecourse of these experiments; Rag2 protein levels remained consistent for several hours after induction of DNA damage by etoposide or IR (Fig. 2.4a&b). The persistence of Rag2 protein expression in the absence of its mRNA suggests that the protein is more stable than previously reported (Lin and Desiderio, 1993). This seemed particularly plausible as the half-life of wild-type Rag2 has only been reported using asynchronously dividing cell lines, and Rag2 is known to be degraded at the G1/S checkpoint (Li et al., 1996).

The half-life of Rag2 is immeasurably long

To measure the half-life of Rag2 protein in G1-arrested cells capable of carrying out V(D)J recombination, we treated IL-7 withdrawn pre-B cells with the ribosome inhibitor cycloheximide (CHX) and quantified Rag2 levels after treatment. We could not

detect a decrease in Rag2 levels earlier than 24 hours after CHX addition, at which point the cells began to die from the treatment (Fig. 2.4c&d). We also measured expression of the short-lived c-myc protein to confirm that the CHX treatment effectively stopped new protein translation. c-myc protein was rapidly depleted by CHX treatment (Fig. 2.4c&d). These results show that the Rag2 protein is remarkably stable, explaining the persistent Rag2 protein expression in the absence of *Rag2* mRNA. In contrast, Rag1 protein levels decrease similarly to *Rag1* mRNA levels. This may be because Rag1 protein is intrinsically less stable than Rag2. Alternatively, IR may induce degradation of existing Rag1 protein in addition to preventing new protein synthesis by suppressing *Rag1* mRNA expression. In either case, transcriptional suppression of both *Rag1* and *Rag2* has a rapid effect on the abundance only of Rag1 protein. While both Rag1 and Rag2 are absolutely required for RAG cleavage activity, Rag1 is normally present at much lower levels than Rag2 in developing lymphocytes (Zhang et al., 2015). Therefore, modulating Rag1 expression might be a more sensitive way to regulate cleavage activity than Rag2 expression.

DSBs inhibit RAG-mediated cleavage of the Igκ allele.

To determine whether suppression of *Rag1* and *Rag2* mRNA expression was associated with inability to initiate V(D)J recombination, we treated an Artemis-deficient Abelson pre-B cell line with the DSB-inducing agent etoposide. Upon addition of the v-

abl inhibitor sti571, these cells arrest in the G1 cell cycle stage, induce Rag expression, and cleave the Igκ locus (Muljo and Schlissel, 2003). Artemis-deficient cells cannot repair RAG-mediated DSBs, so the accumulation of these breaks can be measured by Southern blotting. Etoposide was added to the cells after 24-hours of pretreatment with sti571. Consistent with the results of short-term DSB exposure in primary cells, exposure to etoposide in these experiments suppressed *Rag1* and *Rag2* mRNA expression and induced *p21* mRNA expression (Fig. 2.5a). Furthermore, addition of etoposide halted the accumulation of cleaved Igκ loci, while untreated cells continued to accumulate cleaved loci over the following 24 hours (Fig 2.5b). We found the same suppressive effect on Rag expression and Igκ cleavage by treating the cells with bleomycin instead of etoposide (Fig. 2.5c&d).

Unfortunately, Rag1 and Rag2 protein expression in these cells is too low to measure reliably by western blot. Therefore we could not definitively determine whether Rag1 and Rag2 protein expression in Abelson cells exposed to etoposide or bleomycin for long time periods follows the same pattern found in primary pre-B cells exposed to IR, in which Rag1 protein decreases while Rag2 is maintained.

We repeated this experiment using an Artemis-sufficient Abelson cell line, to rule out the possibility that abnormal repair of Rag-mediated DSBs in the absence of Artemis causes the decrease in RAG activity. In this case, RAG makes breaks at Igκ loci which are

rearranged in a variety of configurations, leading to loss of the germline Igκ band. Using wild-type Abelson cells, we found that addition of etoposide prevents disappearance of the germline Igκ band, indicating that V(D)J recombination is halted (Fig. 2.5f). As in the Artemis-deficient lines, exposure to etoposide suppressed *Rag1* and *Rag2* mRNA expression and induced *p21* expression throughout the course of the experiment (Fig. 2.5e). These results show that exposure to DSBs prevents the RAG-mediated initiation of V(D)J recombination in pre-B cells.

Suppression of V(D)J recombination is not specific to the Igκ locus.

In principle, V(D)J recombination can be regulated globally, for example by manipulating factors like RAG necessary for recombination, or locally, for example regulating accessibility of a specific antigen receptor locus to RAG. To determine whether the inability of etoposide-treated pre-B cells to undergo V(D)J recombination extends beyond the Igκ locus, we measured recombination of an artificial recombination substrate (pMX-INV) in an etoposide-treated Abelson line (Gapud et al., 2011). This substrate is chromosomally integrated, and therefore could be subject to broadly-acting alterations in chromosome that could affect RAG activity. However, we do not expect it to be specifically targeted in the way the Igκ locus could be. This substrate contains an anti-sense GFP gene flanked by RSSs. RAG activity on this substrate inverts the GFP gene, turning on GFP expression (Fig. 2.6a). We first treated these cells with sti-571 for

24 hours. Within this time, *Rag1* and *Rag2* mRNA expression turned on, and approximately 10% of the cells expressed GFP, indicating they had rearranged the substrate (Fig. 2.6b&c). After 24 hours of sti-571 treatment, the culture was divided and treated with either 10μg/mL etoposide or vehicle control. The control cell continued to upregulate *Rag1* mRNA expression, and the proportion of GFP+ cells increased to 40%. In contrast, *Rag1* and *Rag2* expression decreased dramatically in etoposide-treated cells, and the proportion of GFP+ cells remained at 10%, as it was at the time of etoposide addition (Fig. 2.6b&c). We conclude that DSBs suppress V(D)J recombination in general, and this effect does not require factors specific to the Igκ locus.

Enforced *Rag1* expression does not restore the ability to complete V(D)J recombination in the presence of DNA damage

Next, we tested the ability of Rag1-overexpression to overcome inhibition of V(D)J recombination by DSBs. We tested the effects of Rag1 alone, because the experiments shown in Fig. 2.4 show that Rag2 protein has a long halflife, so we do not expect that its expression is affected by DSBs. We transfected pMX-INV+ cells with retrovirus containing *Rag1* cDNA or an empty vector. We then treated these cells as described above, first with sti-571 for 24 hours, then adding etoposide or vehicle control for an additional 48 hours. Empty-vector transduced cells behaved similarly to the untransduced cells, in that etoposide treatment inhibited both *Rag1* and *Rag2*

expression and recombination of the artificial substrate (Fig. 2.6d&e). As expected, the *Rag1*-transduced cells express far higher levels of *Rag1* mRNA than vector-transduced cells at all timepoints (Fig. 2.6d). Like in vector-transduced cells, *Rag2* mRNA expression is high in vehicle-treated cells and low in etoposide treated cells (Fig. 2.6d). *Rag1* overexpression does not allow these cells to undergo pmx-INV recombination in the presence of etoposide; among cells exposed to vehicle control the GFP+ cells increase from 10% to 45% over 48 hours, while cells exposed to etoposide halt rearrangement and remain approximately 10% GFP+ (Fig. 2.6e). We conclude that simply maintaining *Rag1* mRNA expression at a high level in the presence of etoposide does not prevent inhibition of V(D)J recombination by DSBs.

Discussion

These studies show that DSBs caused by a variety of factors can suppress *Rag1* and *Rag2* mRNA expression in pre-B cells, leading to a loss of Rag1 protein. *In vivo* studies suggest that this response is shared by pro- and pre-B cells, and to a lesser extent DN thymocytes. However, we found no evidence that DSBs inhibit *Rag1* or *Rag2* expression in DP thymocytes. We found that exposure to DSBs prevented cleavage of the Igκ locus and rearrangement of an artificial recombination substrate in pre-B cell lines. However, this inhibition was not relieved by constitutive expression of *Rag1* mRNA, suggesting that additional factors may play a role in inhibiting V(D)J recombination in response to DSBs.

The loss of *Rag1* and *Rag2* mRNA leads to selective loss of Rag1 protein, while the relatively stable Rag2 protein persists in the absence of its mRNA. Both *Rag1* and *Rag2* are absolutely required for RAG-mediated cleavage. At basal levels, Rag1 protein is far less abundant than Rag2 protein (Zhang et al., 2015). Therefore Rag1 availability might be a limiting factor and a more responsive target for regulating V(D)J recombination activity.

The failure of constitutive *Rag1* mRNA expression to restore V(D)J recombination activity in the presence of etoposide suggests that additional signals beyond suppression of Rag1 expression are involved. Possibilities include post-translational modifications that inactivate any Rag1 protein that persists, or inactivating modifications of Rag2 protein. Both Rag1 and Rag2 contain S/TQ motifs, the target sequence for the ATM kinase and related PI3K-like kinases including DNA-PKcs (Gapud et al., 2011). Indeed, DNA-PKcs can phosphorylate Rag2 *in vitro* (Hah et al., 2007). Phosphorylation by other factors might also play a role. For example, the metabolic sensor AMPK was shown to increase Rag1 cleavage activity by phosphorylating Rag1 on amino acid S528 (Um et al., 2013); this might be prevented by DSB. Other post translational modifications might also be affected, such as the autoubiquitylation of Rag1 that normally increases its cleavage ability (Singh and Gellert, 2015).

In fact there is already evidence that Rag2 is phosphorylated in the presence of DSBs. Rodgers et al. have shown that a portion of the Rag2 protein pool is exported from the nucleus following IR of Abelson-transformed pre-B cells (Rodgers et al., 2015). The purpose of this export is unclear, particularly as over half of total Rag2 protein remains nuclear. However, export was shown to require the cdk-phosphorylation site T490, suggesting that at least some fraction of available Rag2 is phosphorylated at this site following IR. Phosphorylation at T490 of Rag2 is normally associated with degradation (Lin and Desiderio, 1993); however the persistent Rag2 expression observed in our study and in the Rodgers study suggests that this is not the case in the context of DSBs. Skp2-SCF, the E3 ligase implicated in Rag2 ubiquitylation after T490 phosphorylation, is poorly expressed in G1 phase until just before the G1/S transition (Jiang et al., 2005; Bilodeau et al., 1999); therefore, T490 phosphorylation of Rag2 in G1-arrested cells, like those used in our study, may lead to fates other than degradation. Alternatively, Rag2 protein might be inactivated by post-translational modifications that leave its expression intact.

Another potential method of suppressing RAG activity is to decrease the accessibility of target antigen receptor loci. It was recently found that RAG-mediated DSBs induce expression of the Ets-family member Spi-c, which competes with transcriptionally active Ets family members for binding at the Igκ locus and decreases Igκ accessibility and RAG-mediated cleavage (Bednarski et al., 2016). We also find

induction of *Spi-c* mRNA expression in our recombination assays, although etoposide treatment causes only a slight increase in *Spi-c* expression above the level induced by RAG-mediated DSBs alone (discussed further in Chapter 4). However, the ability of etoposide treatment to inhibit recombination of an artificial recombination substrate suggests that locus-specific factors such as Spi-c are not required to inhibit recombination, even if they do contribute to regulation of endogenous loci.

It is also possible that DSBs normally suppress V(D)J recombination by suppressing Rag1 expression, but that details of our experimental setup account for the failure of constitutive *Rag1* mRNA expression to rescue. For example, the 48 hour DSB exposure used in our experiments is far longer than the time normally required to repair DSBs (Rothkamm et al., 2003). This long DSB exposure might have effects that would not be relevant to a developing lymphocyte experiencing only normal levels of DSB exposure. For example, Rag2 protein expression might decrease during this long time course. We assume that Rag2 protein half-life is long in Abelson cells, as it is in primary pre-B cells. However, we cannot directly measure this because basal expression of Rag2 in Abelson cells is much lower than in primary cells and cannot be reliably detected by western blotting. Indeed, the fact that both Rag1 and Rag2 proteins have low expression in Abelson cells but mRNA expression is comparable with primary cells suggests that 1) translation of these mRNAs is lower in Abelson cells, or 2) the half-life of the proteins is shorter. Therefore, it is possible that in the systems we used to measure V(D)J

recombination, both Rag1 and Rag2 protein expression decreases in the presence of DSBs, and that constitutive expression of both proteins would allow V(D)J recombination activity in the presence of DSBs.

The differential regulation of *Rag1* and *Rag2* mRNA expression observed in different lymphocyte subsets is not entirely surprising, as it has long been known that different transcription factors and different enhancer elements drive basal *Rag1* and *Rag2* expression in different kinds of lymphocytes. The *Erag*, *Ep*, *and Ed* enhancers are all active in pro- and pre-B cells (Hsu et al., 2003; Wei et al., 2002, 2005). *Erag* is also active in the common lymphoid progenitor (Borghesi et al., 2005). The *Ed* enhancer is also active in DN thymocytes, but none of these three elements affect *Rag1* or *Rag2* expression in DP thymocytes. Instead, the ASE overcomes a highly active silencer element to allow *Rag1* and *Rag2* expression in DP thymocytes; the ASE has some activity in DN thymocytes, but none in pro- or pre-B cells (Yannoutsos et al., 2004). The pattern of required enhancers is reminiscent of the pattern of *Rag1* and *Rag2* suppression by DSBs: a strong effect in pro- and pre-B cells, which generally use the same regulatory elements for *Rag1* and *Rag2*; modest suppression in DN thymocytes, which uses one enhancer element in common with developing B cells; and no suppression in DP thymocytes, which uses an entirely different enhancer to regulate *Rag1* and *Rag2* transcription. While not conclusive, this pattern suggests that 1) DSBs suppress *Rag1* and *Rag2* mRNA expression by inhibiting their transcription, and 2) this regulation

involves regulatory elements used by pro- and pre-B cells as well as DN thymocytes. The *Ed* enhancer is the only identified element that fits this description; additional involvement of the B cell-specific *Erag* and *Ep* enhancers might explain the more effective suppression in pro- and pre-B cells. Additional experiments will be necessary to determine whether these elements are the main targets of DSB signaling that lead to the suppression of *Rag1* and *Rag2* expression.

Identification of critical genetic elements regulating *Rag1* and *Rag2* expression in response to DSBs could allow us to mutate these elements and selectively abrogate this response in *in vivo* developing lymphocytes. This would allow us to explore the role of this response in lymphocyte development. As discussed in the introduction to this chapter, we suggest that this signal plays a role in maintaining genomic stability by preventing the creation of RAG-mediated DSBs at an antigen receptor locus while another DSB exists that might lead to a translocation. Future experiments will attempt to address this hypothesis by preventing the normal suppression of *Rag1* and *Rag2* expression during lymphocyte development *in vivo*, and measuring the frequency of antigen receptor translocations and/or development of lymphoid malignancies. We also have reason to believe that RAG-mediated DSBs activate the same signal to help prevent simultaneous rearrangement of two antigen receptor alleles, enforcing allelic exclusion. This effect will be discussed in Chapter 4.

Figure 2.1

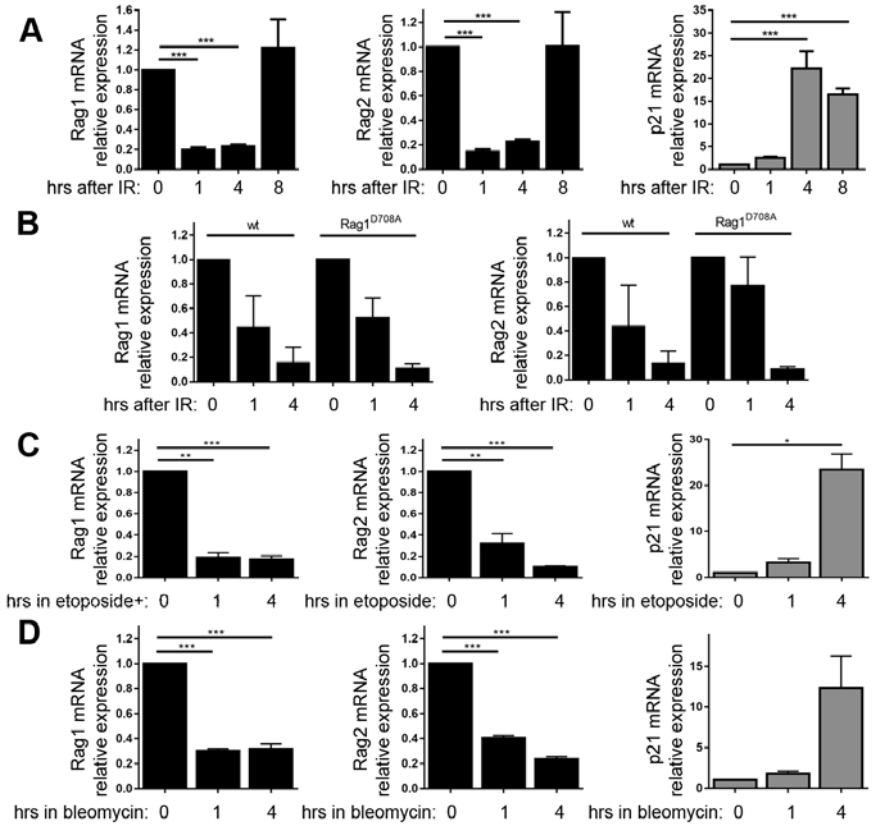


Figure 2.1: DSBs induced by a variety of agents suppress *Rag1* and *Rag2* mRNA expression in pre-B cells. **(A)** qRT-PCR quantification of *Rag1*, *Rag2*, and *p21* mRNA in non-irradiated and irradiated *EμBCL2* pre-B cells at indicated times after exposure to 4 Gy IR. Data are from 11 independent experiments. **(B)** qRT-PCR quantification of *Rag1* and Rag2 *mRNA* expression in a wild-type Abelson cell line and a Rag1-mutant cell line at indicted times after exposure to 4 Gy IR. Data are from 3 independent experiments. **(C)** qRT-PCR quantification of *Rag1*, *Rag2*, and *p21* mRNA in *EμBCL2* pre-B cells at indicated times after addition of 10 μg/mL etoposide to culture media. Data are from 3 independent experiments. **(D)** qRT-PCR quantification of *Rag1*, *Rag2*, and *p21* mRNA in *EμBCL2* pre-B cells at indicated times after addition of 5 μM bleomycin to culture media. Data are from 3 independent experiments. **(A-D)** Data are normalized to 1.0 for untreated cells within each experiment. For treated cells, data averages are shown with error bars indicating SEM. p-values calculated using Dunnett's post-test after ANOVA.

Figure 2.2

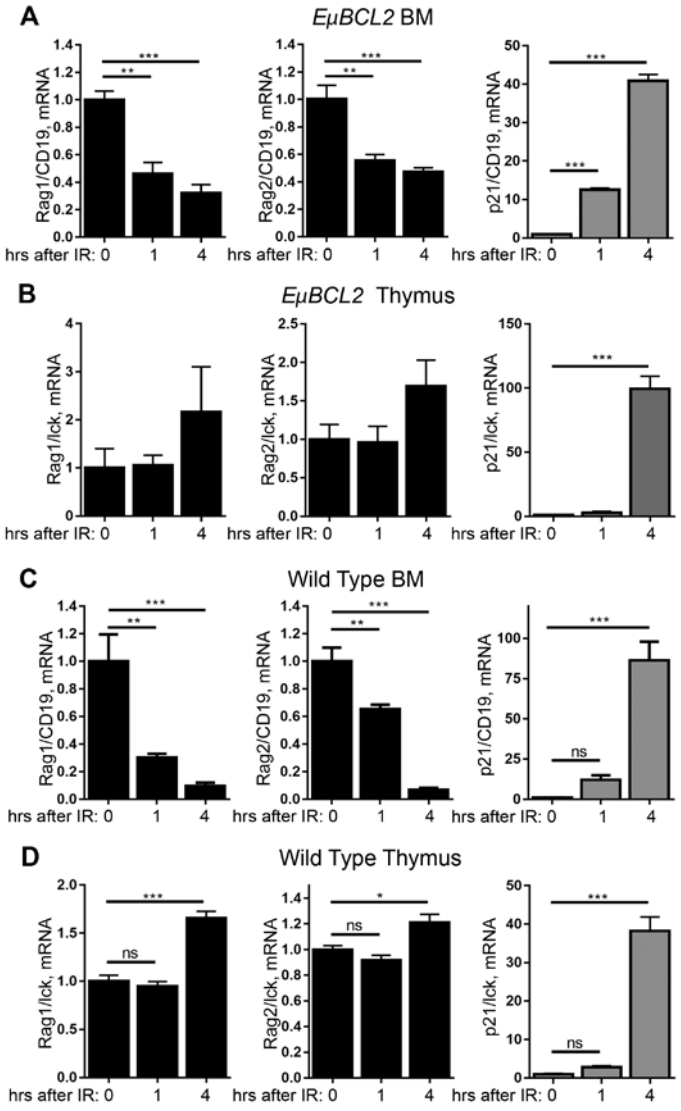


Figure 2.2: DSBs suppress *Rag1* and *Rag2* mRNA expression in BM but not thymus. **(A)** qRT-PCR quantification of *Rag1*, *Rag2*, and *p21* mRNA in BM from non-irradiated *EμBCL2* mice or *EμBCL2* mice at indicated times after exposure to 10 Gy IR. Data are from one experiment with 3-4 mice for each timepoint. **(B)** qRT-PCR quantification of *Rag1*, *Rag2*, and *p21* mRNA in thymuses from non-irradiated *EμBCL2* mice or *EμBCL2* mice at indicated times after exposure to 10 Gy IR. Data are from three independent experiments including a total of 4-6 mice per timepoint. **(C)** qRT-PCR quantification of

Rag1, *Rag2*, and *p21* mRNA in BM from non-irradiated wild-type mice at indicated times after exposure to 10 Gy IR. Data are from two independent experiments including a total of 4-5 mice per timepoint. **(D)** qRT-PCR quantification of *Rag1*, *Rag2*, and *p21* mRNA in thymus from non-irradiated wild-type mice or wild-type mice at indicated times after exposure to 10 Gy IR. Data are from two independent experiments including a total of 4-5 mice per timepoint. **(A-D)** Data averages are shown with error bars indicating SEM. p-values calculated using Dunnett's post-test after ANOVA.

Figure 2.3

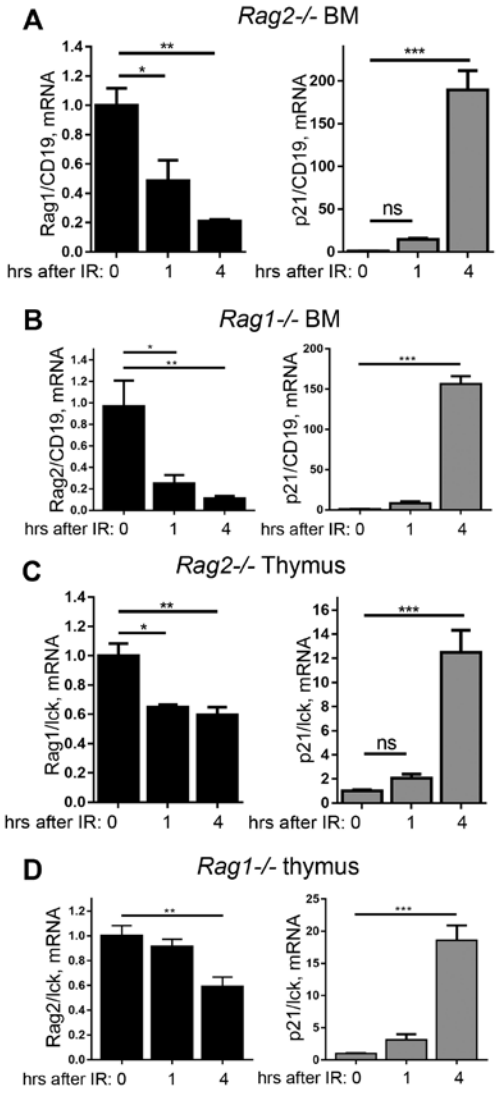


Figure 2.3: DSBs suppress *Rag1* and *Rag2* mRNA expression in both pro-B cells and pro-T cells. **(A)** qRT-PCR quantification of *Rag1* and *p21* mRNA in BM from non-irradiated *Rag2*$^{-/-}$ mice or *Rag2*$^{-/-}$ mice at indicated times after exposure to 10 Gy IR. Data are from one experiment with 3 or 4 mice per timepoint. **(B)** qRT-PCR quantification of *Rag2* and *p21* mRNA in BM from non-irradiated *Rag1*$^{-/-}$ mice or *Rag1*$^{-/-}$ mice at indicated times after exposure to 10 Gy IR. Data are from three independent experiments with 4-6 mice

per timepoint. **(C)** qRT-PCR quantification of Rag1 and p21 mRNA in thymus from non-irradiated *Rag2*$^{-/-}$ mice or *Rag2*$^{-/-}$ mice at indicated times after exposure to 10 Gy IR. Data are from one experiment with 3 or 4 mice per timepoint. **(D)** qRT-PCR quantification of *Rag2* and *p21* mRNA in thymus from non-irradiated *Rag1*$^{-/-}$ mice or *Rag1*$^{-/-}$ mice at indicated times after exposure to 10 Gy IR. Data are from three independent experiments with 4-6 mice per timepoint. **(A-D)** Data averages are shown with error bars indicating SEM. p-values calculated using Dunnett's post-test after ANOVA.

Figure 2.4

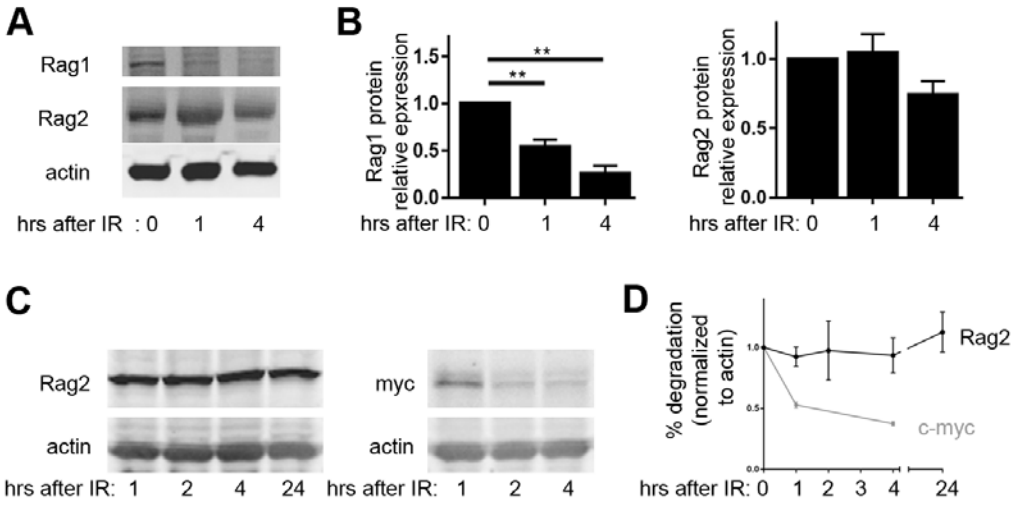


Figure 2.4: Rag1 protein expression decreases after IR, but Rag2 protein is maintained. **(A)** Representative western blot analysis depicting Rag1 and Rag2 protein in non-irradiated or irradiated *E*μ*BCL2* pre-B cells at indicated times after exposure to 4 Gy IR. **(B)** Quantification of (A). Data are from 8 independent experiments. **(C)** Representative western blot analysis depicting expression of Rag2 and c-Myc protein in cycloheximide-treated *E*μ*BCL2* pre-B cells. **(D)** Quantification of (C). Data are from 3 independent experiments. **(A-D)** Data are normalized to 1.0 for untreated cells within each experiment. For treated cells, data averages are shown with error bars indicating SEM. p-values calculated using Dunnett's post-test after ANOVA.

Figure 2.5

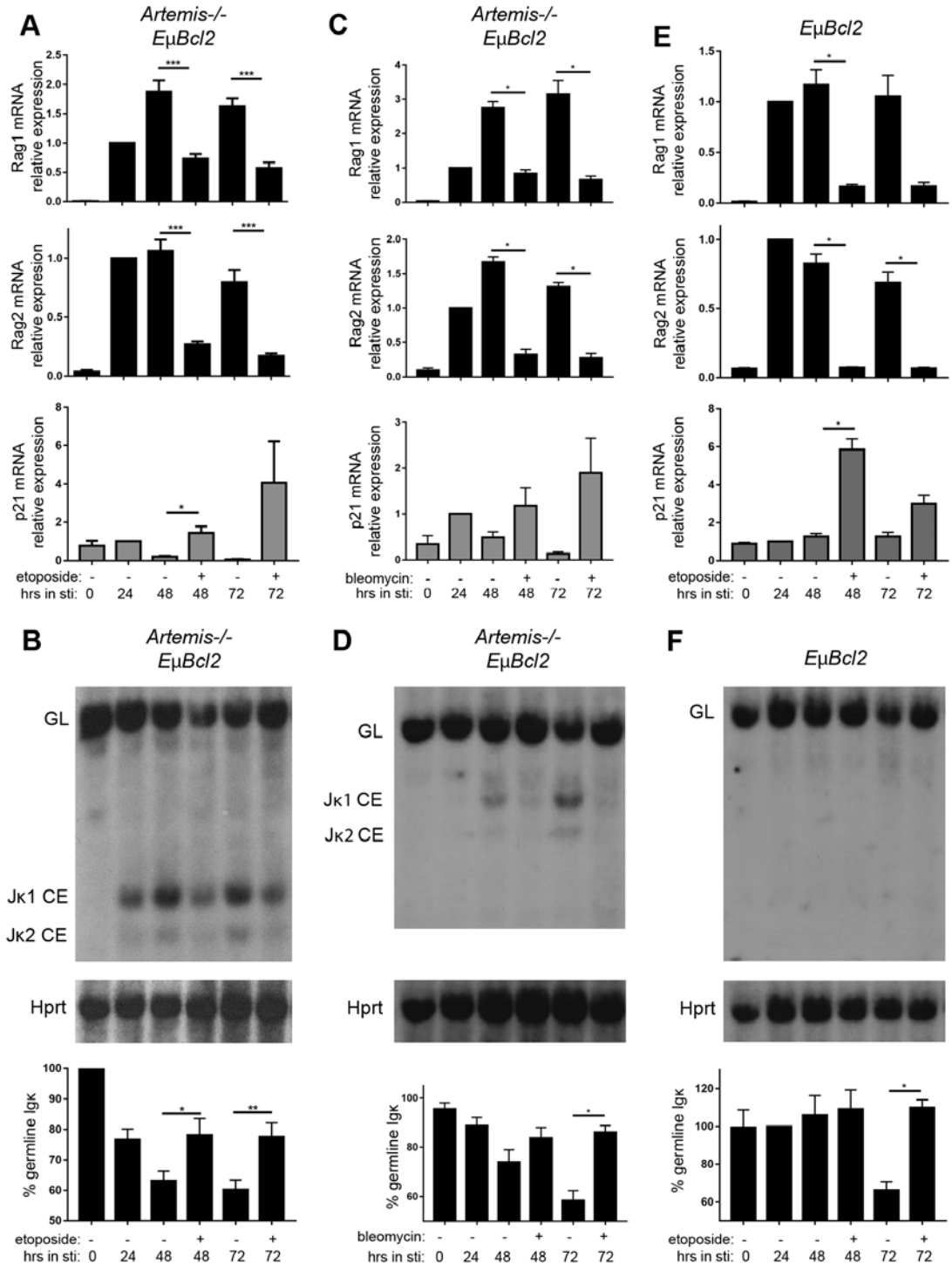

A
Artemis-/- EμBcl2
Rag1 mRNA relative expression
Rag2 mRNA relative expression
p21 mRNA relative expression
etoposide: - - - + - +
hrs in sti: 0 24 48 48 72 72
C
Artemis-/- EμBcl2
Rag1 mRNA relative expression
Rag2 mRNA relative expression
p21 mRNA relative expression
bleomycin: - - - + - +
hrs in sti: 0 24 48 48 72 72
E
EμBcl2
Rag1 mRNA relative expression
Rag2 mRNA relative expression
p21 mRNA relative expression
etoposide: - - - + - +
hrs in sti: 0 24 48 48 72 72
B
Artemis-/- EμBcl2
GL
Jκ1 CE
Jκ2 CE
Hprt
% germline Igκ
etoposide: - - - + - +
hrs in sti: 0 24 48 48 72 72
D
Artemis-/- EμBcl2
GL
Jκ1 CE
Jκ2 CE
Hprt
% germline Igκ
bleomycin: - - - + - +
hrs in sti: 0 24 48 48 72 72
F
EμBcl2
GL
Hprt
% germline Igκ
etoposide: - - - + - +
hrs in sti: 0 24 48 48 72 72

Figure 2.5: DSBs inhibit V(D)J recombination in pre-B cells. **(A)** qRT-PCR quantification of *Rag1*, *Rag2*, and *p21* mRNA in *Artemis*$^{-/-}$ *EμBCL2* Abl cells untreated or treated with STI571 or STI571 and etoposide for the indicated amounts of time. Data are from 6 independent experiments. **(B)** Representative Southern blot analysis and graphical quantification of *Jκ* cleavage in *Artemis*$^{-/-}$ *EμBCL2* Abl cells treated with STI571 or STI571 and etoposide for the indicated amounts of time. Data are from 6 independent experiments. **(C)** qRT-PCR quantification of *Rag1*, *Rag2*, and *p21* mRNA in *Artemis*$^{-/-}$ *EμBCL2* Abl cells untreated or treated with STI571 or STI571 and bleomycin for the indicated amounts of time. Data are from 3 independent experiments. **(D)** Representative Southern blot analysis and graphical quantification of *Jκ* cleavage in *Artemis*$^{-/-}$ *EμBCL2* Abl cells treated with STI571 or STI571 and bleomycin for the indicated amounts of time. Data are from 3 independent experiments. **(E)** qRT-PCR quantification of *Rag1*, *Rag2*, and *p21* mRNA in *EμBCL2* Abl cells untreated or treated with STI571 or STI571 and etoposide for the indicated amounts of time. Data are from 3 independent experiments. **(F)** Representative Southern analysis and graphical quantification of *Igκ* recombination in *EμBCL2* Abl cells untreated or treated with STI571 or STI571 and etoposide for the indicated amounts of time. Data are from 3 independent experiments. **(A-F)** Data averages are shown with error bars the SEM. p-values were determined by Dunnett's post-test after ANOVA.

Figure 2.6

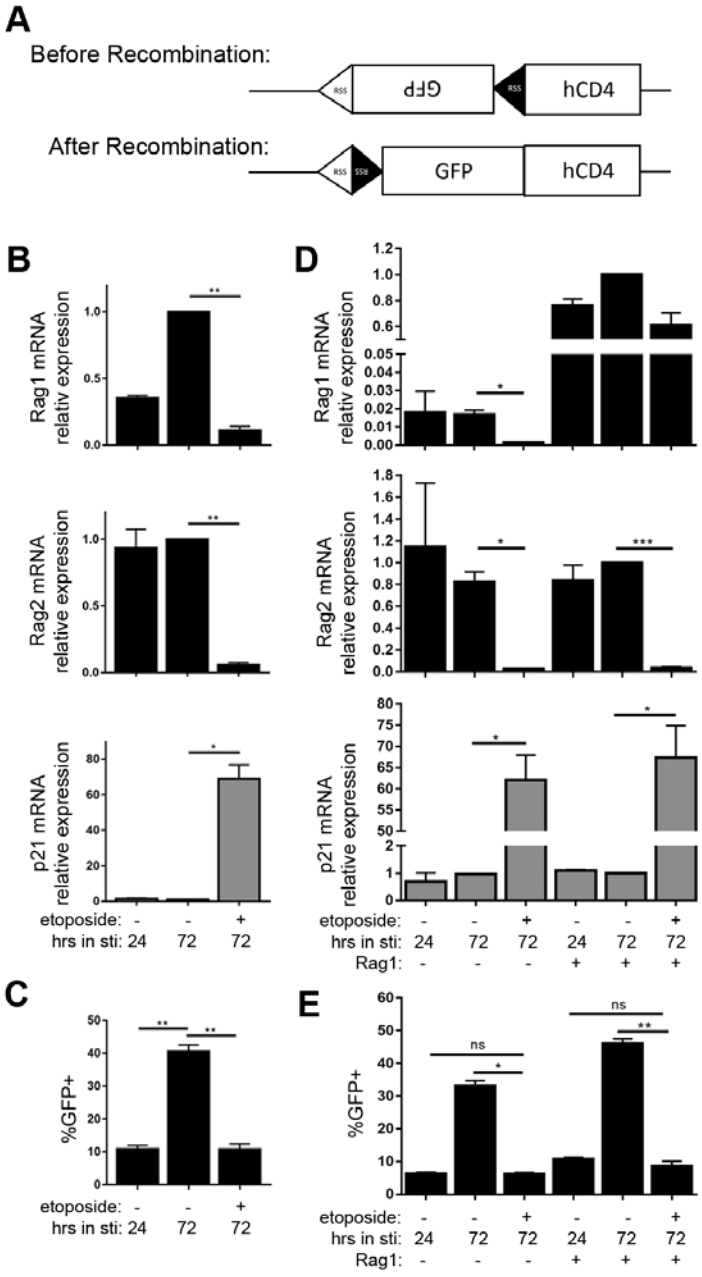


Figure 2.6: DSBs inhibit recombination of an artificial recombination substrate, and constitutive *Rag1* expression does not overcome inhibition of recombination. **(A)**

Schematic of the *pMX-INV* recombination substrate before and after RAG-mediated recombination. (**B**) qRT-PCR quantification of *Rag1*, *Rag2*, and *p21* mRNA in *EμBCL2-pINV* Abl cells treated with STI571 or STI571 and etoposide for the indicated amounts of time. Data are from 3 independent experiments. **(C)** Quantification of GFP+ cells among hCD4+ *EμBCL2-pINV* Abl cells as determined by flow cytometry. Cells were treated with STI571 or STI571 and etoposide for the indicated amounts of time. Data are from 3 independent experiments. **(D)** qRT-PCR quantification of *Rag1*, *Rag2*, and *p21* mRNA in *EμBCL2-pINV* Abl cells transduced with empty vector or retrovirus containing Rag1 and treated with STI571 or STI571 and etoposide for the indicated amounts of time. Data are from 3 independent experiments. **(E)** Quantification of GFP+ cells among hCD4+ *EμBCL2-pINV* Abl cells as determined by flow cytometry. Cells were transduced with empty vector or retrovirus containing Rag1 and treated with STI571 or STI571 and etoposide for the indicated amounts of time. Data are from 3 independent experiments. **(B-E)** Data averages are shown with error bars the SEM. p-values were determined by Dunnett's post-test after ANOVA.

Chapter 3: Signaling mechanisms leading to suppression of Rag1 and Rag2 expression by DSBs[1]

Abstract

The work presented in Chapter 2 shows that DSBs induced by exogenous factors can also suppress *Rag1* and *Rag2* mRNA expression and V(D)J recombination. Here, we discuss the role of specific signaling pathways, particularly ATM and NF-κB, in suppressing *Rag1* and *Rag2* mRNA expression. We find that suppression of *Rag1* and *Rag2* mRNA expression is caused by rapid inhibition of *Rag1* and *Rag2* transcription. This inhibition absolutely requires the activity of the ATM kinase. Inhibition is also partially dependent on Nemo and IKKβ activity. Because suppression of *Rag1* and *Rag2* expression does not require new protein synthesis, we propose that the ATM/Nemo/IKKβ signaling axis leads to post-translational modification of a transcriptional regulator present in the cell before DSB exposure. Classically, the ATM/Nemo/IKKβ pathway acts by activating NF-κB transcription factors; possible roles for NF-κB factors in suppressing *Rag1* and *Rag2* transcription are discussed.

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[1] Parts of this chapter were published in *The Journal of Immunology*. Copyright © 2017 The American Association of Immunologists, Inc. Fisher MR, Rivera-Reyes A, Bloch NB, Schatz DG, Bassing CH. Immature Lymphocytes Inhibit Rag1 and Rag2 Transcription and V(D)J Recombination in Response to DNA Double-Strand Breaks. J. Immunol. [Epub ahead of print 2017 Feb 17].

Introduction

The regulation of basal *Rag1* and *Rag2* expression is complex; over a dozen transcription factors have been implicated in some way in regulating *Rag1* and *Rag2* transcription (see Fig. 1.3). Most studies focus on factors that promote *Rag1* and *Rag2* transcription in the pro- and pre-lymphocytes expected to complete V(D)J recombination, while a few studies examine the ways a functional antigen receptor turns off *Rag1* and *Rag2* expression. One of the clearest findings is that B and T cells use largely distinct mechanisms to turn *Rag1/Rag2* transcription on and off, employing lineage-specific transcription factors that act at different genomic regulatory elements(Yu et al., 1999). This is particularly relevant, as we see that developing B cells suppress *Rag1* and *Rag2* expression robustly in response to DSBs, while DN thymocytes have a modest response and DP thymocytes do not suppress *Rag1* and *Rag2* in response to DSBs.

In developing B cells, the forkhead-box protein Foxo1 is one of the most crucial transcription factors promoting *Rag1/Rag2* transcription (Amin and Schlissel, 2008; Dengler et al., 2008). Several transcription factors necessary for *Rag1* and *Rag2* expression also promote Foxo1 expression, suggesting that their effect is at least partially indirect; Ikaros and Bcl11a fall into this category (Lee et al., 2013; Reynaud et al., 2008). Gfi1b, a negative regulator of *Rag1/Rag2* transcription, also works partially by suppressing Foxo1 expression (Schulz et al., 2012). Foxo1 binds to the *Erag* enhancer

element, which is critical for *Rag1* and *Rag2* transcription in developing B cells (Hsu et al., 2003; Dengler et al., 2008). Foxo1 activity is terminated by tonic signaling from a functional antigen receptor that activates Akt to phosphorylate Foxo1 (Verkoczy et al., 2007; Amin and Schlissel, 2008; Herzog et al., 2008).

Previous work from the Bassing laboratory implicated the DNA damage response protein ATM in regulating *Rag1* and *Rag2* expression. Activation of ATM by DSBs normally promotes repair and cell cycle arrest (Shiloh and Ziv, 2013). It was known that RAG-mediated DSBs activate ATM, and that ATM activity is important for the efficient repair of these breaks and for ensuring developing lymphocytes with cleaved antigen receptor loci do not enter cell cycle (Bredemeyer et al., 2006; Callén et al., 2007; Dujka et al., 2010).The Bassing laboratory showed that in addition to these functions, RAG-induced DSBs at the Igκ locus in pre-B cells suppress *Rag1* and *Rag2* expression. Furthermore, ATM prevents pre-B cells from cleaving a second Igκ allele before the first is repaired; this signal is believed to be important for normal allelic exclusion of Igκ (Steinel et al., 2013).

Rag1 and *Rag2* are by no means the only targets of ATM activation by RAG-mediated cleavage of the Igκ locus; in fact, ATM activates hundreds of genes important for genomic stability and lymphocyte development (Bredemeyer et al., 2008). Many of these targets also require NF-κB activity (Bredemeyer et al., 2008; Bednarski et al.,

2016). ATM can cooperate in activation of canonical NF-κB signaling by phosphorylating the NF-κB essential modifier (Nemo) (Huang et al., 2003). In this context, activated ATM and Nemo are exported from the nucleus and promote IKKβ activation and transcription of NF-κB target genes (Huang et al., 2000; Verma, 2003). Interestingly, ATM can also phosphorylate p65 to suppress transcription of a small subset of p65 target genes, opposing the effect of activated Nemo on these genes (Sabatel et al., 2012).

DNA damage activates NF-κB signaling in many but not all cell and tissue types. Notably, irradiation strongly activates NF-κB activity in bone marrow, but not in thymocytes (Zhou et al., 1999). The mechanistic studies discussed in this chapter focus on pre-B cells, as these cells can readily be cultured *ex vivo.* However, we believe that similar mechanisms operate in pro-B cells and to some extent in DN thymocytes. In pre-B cells, we show that DSBs act through the ATM-Nemo signaling pathway to suppress *Rag1* and *Rag2* transcription.

Results

DSBs inhibit *Rag1* and *Rag2* transcription, while mRNA turnover is mostly unaffected

In most identified cases, regulation of *Rag1* and *Rag2* mRNA occurs through transcriptional regulation. However, steady state mRNA levels can be regulated either by changes in input or outflow, ie either by changes in transcription or degradation. Therefore, the decreased total mRNA levels upon DSBs exposure shown in Chapter 2

could be caused either by decreased *Rag1* and *Rag2* transcription or increased degradation of these mRNAs. To identify the mechanism by which DSBs suppress *Rag1 and Rag2* expression, we measured turnover and transcription of these mRNAs in either irradiated or unirradiated IL-7 withdrawn pre-B cells. For these experiments we used Click-iT® technology, in which cells are cultured in media containing ethyl uridine (EU); EU is incorporated into mRNA transcripts generated in its presence, and can be specifically pulled down so that labeled RNA can be measured by qPCR.

To measure mRNA turnover, EU was added to the media of IL-7 withdrawn *ex vivo* pre-B cells for 16 hours, and then washed out immediately before the cells were either exposed to 4 Gy IR or left unexposed to IR. Loss of labeled transcripts over time was measured by qPCR. Half-life was determined by normalizing the values measured for labeled *Rag1* and *Rag2* mRNAs to labeled *18S* RNA, which has a long half-life and therefore undergoes negligible degradation within the timecourse of our assay. We found that the half-life of both *Rag1* and *Rag2* mRNAs in unirradiated cells was about 40 minutes (Fig. 3.1a), similar to previously published results using nuclear run-on assays (Verkoczy et al., 2005). IR did not change the half-life of *Rag1* mRNA, while the half-life of *Rag2* mRNA decreased modestly, by approximately 25% (Fig. 3.1a). A 25% decrease in *Rag2* mRNA half-life is not sufficient to explain the rapid (<1hour) and substantial (80%) decrease in total *Rag2* mRNA measured in these circumstances (see figure 2.1a).

To measure transcription, EU was added to the media of IL-7 withdrawn *ex vivo* pre-B cells immediately after these cells were either exposed to 4Gy IR or left unirradiated. The accumulation of new labeled transcripts was measured by qPCR and normalized to values measured for EU-labeled Hprt, a transcript we do not expect to be affected by IR. These data are presented as the ratio of new *Rag1* or *Rag2* transcripts in irradiated vs. unirradiated transcripts; a ratio of 1 would indicate that IR has no effect on transcription. As shown in Fig. 3.1b, by 45 minutes after irradiation irradiated cells have produced significantly less new *Rag1* and *Rag2* mRNA than unirradiated cells, and a trend in this direction can be observed as early as 30 minutes after irradiation. This demonstrates a rapid and profound decrease in transcription of both the *Rag1* and *Rag2* genes following IR exposure. Therefore, we conclude that transcription is the major mechanism through which DSBs suppress *Rag1* and *Rag2* mRNA expression, and regulation of mRNA turnover does not play a major role.

Although these results show that *Rag1* and *Rag2* mRNA transcription is profoundly reduced following IR of *ex vivo* cultured primary pre-B cells, it is formally possible that this is not because *Rag1* and *Rag2* transcription is specifically targeted by the DNA damage response (DDR), but rather because the extreme stress caused by exposure to a high dose of IR leads to a general transcriptional repression of genes not required for the DDR. One piece of evidence against this scenario is that the values for newly transcribed *Rag1* and *Rag2* mRNA are normalized to newly transcribed *Hprt*

mRNA; if all of these mRNAs were affected by a general transcriptional repression, then there would be no apparent difference in the normalized values of irradiated and unirradiated samples. Of course Hprt might be an exception, and so we also measured transcription of another gene that is not required for the DDR, *Wasp*. We found that irradiation caused a modest decrease in accumulation of new *Wasp* transcripts, but this decrease was slower and much less profound than the decrease observed for *Rag1* and *Rag2* transcripts (Fig. 3.1b). These data support our hypothesis that *Rag1* and *Rag2* expression is specifically targeted by the DDR in pre-B cells.

Suppression of *Rag1* and *Rag2* mRNA by DSBs does not require new protein synthesis

In broad terms, DSBs could suppress *Rag1* and *Rag2* transcription either by increasing the expression and/or activity of a transcriptional repressor, or by decreasing the expression and/or activity of a transcriptional activator. However, the speed with which DSBs suppress *Rag1* and *Rag2* transcription suggests that the mechanism does not require the synthesis of a new repressor, as this would be likely to act more slowly. To test this directly, we treated IL-7 withdrawn and irradiated pre-B cells with cycloheximide to determine whether new protein synthesis was required for suppression of *Rag1* and *Rag2* transcription by DSBs. Cycloheximide treatment without IR lead to an increase in *Rag1* and *Rag2* mRNA expression (Fig. 3.2a), as previously reported (Verkoczy et al., 2005). However, cycloheximide treatment did not prevent the

suppression of *Rag1* and *Rag2* mRNA expression by IR (Fig. 3.2a). This result indicates that the suppressive signal acts through factors that are present in undamaged cells, and does not require the synthesis of a new transcriptional inhibitor.

Suppression of *Rag1* and *Rag2* mRNA by DSBs does not require protein degradation

We next tested whether protein degradation was required for the suppression of *Rag1* and *Rag2* mRNA expression by DSBs, as we would expect to be the case if the signal depended on loss of a transcriptional activator. We find that suppression of *Rag1* and *Rag2* mRNA is unaffected by the proteosome inhibitor Mg132 (Fig. 3.2b). This results shows that protein degradation is not absolutely required for inhibition of *Rag1* and *Rag2* transcription by DSBs. However, it is possible that the activity of a transcriptional activator might be suppressed by post-translational modification rather than by degradation.

ATM activity is required for suppression of *Rag1* and *Rag2* expression by DSBs

Having established general rules that the suppression of *Rag1* and *Rag2* expression by DSBs seems to follow, we investigated the involvement of specific signaling pathways. Because RAG-mediated DSBs act through ATM to suppress *Rag1* and *Rag2* mRNA expression (Steinel et al., 2013), we tested ATM's role in the response to IR-induced DSBs. We first determined the effect of ATM inhibition on DSB-mediated suppression of *Rag1* and *Rag2* mRNA expression in ATM-sufficient IL-7 withdrawn

primary pre-B cells. Either the ATM inhibitor KU55933 or vehicle control was added to these cells at the same time as IL-7 withdrawal. 48 hours later, these cells were either exposed to 4 Gy IR or left unirradiated. ATM inhibition led to increased basal *Rag1* and *Rag2* mRNA expression in unirradiated cells. More importantly, ATM inhibition completely prevented the suppression of *Rag1* and *Rag2* mRNA expression caused IR (Fig. 3.3a). ATM inhibition also prevented the loss of Rag1 protein expression that normally occurs after exposure to IR (Fig. 3.3b). To further confirm the role of ATM in suppression of *Rag1* and *Rag2* mRNA expression, we exposed *Mb1cre*$^{+}$ *Atm*$^{f/f}$ IL-7 withdrawn pre-B cells to 4 Gy IR and compared their *Rag1* and *Rag2* expression to unirradiated controls. We found that IR exposure does not decrease *Rag1* or *Rag2* mRNA expression in ATM-deficient cells, in contrast to the suppression observed in *EμBCL+* ATM-sufficient cells. Collectively, these data show that DSBs act through the DNA-damage response protein ATM to suppress *Rag1* and *Rag2* mRNA expression, leading to decreased Rag1 protein expression.

Suppression of Gadd45α expression is not required to suppress *Rag1* and *Rag2* mRNA expression

Previous work from the Bassing laboratory showed that RAG-mediated DSBs suppress expression of Gadd45α mRNA and protein via ATM activity (Steinel et al., 2013). Because other work showed that Gadd45α can indirectly regulate *Rag1* and *Rag2*

transcription by promoting activation Foxo1 activity (Amin and Schlissel, 2008), we hypothesized that DSBs suppress *Rag1* and *Rag2* expression by decreasing Gadd45α expression. However, we found that IR only modestly suppressed Gadd45α mRNA expression, by about 50% (Sup. Fig. 3.1a). Furthermore, although *Gadd45α* transcription was decreased by IR, this decrease occurred more slowly than the decrease in *Rag1* and *Rag2* transcription (compare Fig. 3.2 and Sup. Fig. 3.1b). Finally, we performed several experiments in which Gadd45α was constitutively expressed in a variety of conditions, including using either an unmodified Gadd45α construct or a tamoxifen-inducible version, and using either primary pre-B cells or Abelson-transformed pre-B cell line. Sup. Fig. 3.1c shows the results of a representative experiment in which *EμBCL2+* Abelson cells were transduced either with empty vector or ER-Gadd45α. These cells were then treated with tamoxifen and 2 hours later exposed to 4Gy IR or unexposed. In this experiment, as in all conditions tested, we found that Gadd45α overexpression had no effect on either basal *Rag1* and *Rag2* mRNA expression or in the suppression caused by IR. Therefore, we believe that the decrease in Gadd45α expression caused by both RAG-mediated and IR-induced DSBs is coincidental, and does not mediate suppression of RAG expression.

Activation of Nemo is required for optimal suppression of *Rag1* and *Rag2* by DSBs

Although ATM has many substrates and orchestrates a complex network of responses to DSBs, two important branches of this signaling network act through p53 and Nemo (Miyamoto, 2011; Shiloh and Ziv, 2013). Therefore, we next investigated the roles of p53 and Nemo in suppressing *Rag1* and *Rag2* expression following the induction of DSBs in primary pre-B cells. To determine whether Nemo or p53 is required for suppression of *Rag1* and *Rag2* expression by DSBs, we exposed cells deficient for each factor to 4 Gy IR and compared their *Rag1* and *Rag2* mRNA expression to unirradiated cells. We observed normal downregulation of Rag1 and Rag2 mRNAs following irradiation of primary pre-B cells cultured from *VavCre*$^{+}$*Tp53*$^{flox/flox}$ mice with hematopoietic lineage-specific deletion of *p53* (Sup. Fig. 3.2). In contrast, *EμBCL+ Mb1cre*$^{+}$ *Nemo*$^{f/f}$ IL-7 withdrawn pre-B cells did not suppress *Rag1* and *Rag2* mRNA expression as efficiently as wild-type cells. Although *Rag1* and *Rag2* mRNA expression is lower in irradiated Nemo-deficient cells than in unirradiated cells at 1 hour after exposure, the decrease is less than that seen in Nemo-sufficient cells (Fig. 3.4a&b). By 4 hours after IR exposure, *Rag1* and *Rag2* mRNA expression in irradiated Nemo-deficient cells recovers to normal levels, while expression in Nemo-sufficient cells is still repressed this timepoint (compare Fig. 3.4a and Fig. 2.1a). The partial suppression observed 1 hour after IR exposure suggests that a Nemo-independent mechanism may act very early to

suppress *Rag1* and *Rag2* expression. However, Nemo is absolutely required for sustained suppression of *Rag1* and *Rag2* mRNA expression by DSBs.

Activation of IKKα/β in pre-B cells exposed to IR

In most documented cases, Nemo regulates gene expression by allowing activation of canonical NF-κB signaling pathway (Miyamoto, 2011). DNA damage has been shown to activate NF-κB signaling in many cell and tissue types, including whole bone marrow and pre-B cell lines (Zhou et al., 1999; Bredemeyer et al., 2008). One of the hallmarks of NF-κB activation is phosphorylation of the key kinases IKKα (which activates the non-canonical pathway) or IKKβ (which activates the canonical pathway). To determine whether IL-7 withdrawn pre-B cells exposed to IR activate this pathway within the timeframe that *Rag1* and *Rag2* expression are repressed, we measured accumulation of phospho-IKK. The antibodies used bind both IKKα and IKKβ. Because these two kinases have a similar molecular weight, we cannot distinguish between them in this experiment. We found that phospho-IKKα/IKKβ accumulates as early as 30 minutes after IR exposure and persists for at least 60 minutes (Sup. Fig. 3.3). Notably, phospho-IKKα/IKKβ is completely lost by 4 hours after IR exposure, although *Rag1* and *Rag2* mRNA expression are still suppressed at this point (compare Sup. Fig. 3.3 and Fig. 2.1a). Therefore if NF-κB signaling is required for suppression of *Rag1* and *Rag2* mRNA

expression, the suppressive effect can persist for some time after active signaling is terminated.

Two IKK inhibitors have different effects on basal *Rag1* and *Rag2* expression and response to DSBs.

As noted above, the most prominent role for Nemo in cell biology is through its involvement in canonical NF-κB signaling. In the absence of Nemo, IKKβ is unable to phosphorylate IκBα and activate NF-κB activity (Rudolph et al., 2000; Schröfelbauer et al., 2012). Therefore, we hypothesized that lack of IKKβ activity would have a similar effect on *Rag1* and *Rag2* mRNA expression as Nemo deficiency. We initially chose the IKK inhibitor BMS-345541 because a recent paper showed that this inhibitor could act synergistically with Akt inhibition to activate *Rag1* mRNA expression in Abelson-transformed cells without sti571 treatment, a circumstance in which *Rag1* expression is normally strongly suppressed (Ochodnicka-Mackovicova et al., 2015). We first pre-treated IL-7 withdrawn *EμBCL2+* primary-pre-B cells with BMS-345541; after 2 hours the culture was split and either exposed to 4 Gy IR or unirradiated. To our surprise, BMS-345541 treatment alone inhibited *Rag2* mRNA expression to approximately the same extent as IR alone; there was no significant additional effect with combined BMS-345541 treatment and IR (Fig. 3.5a). We also found a trend towards decreased *Rag1* mRNA expression in the BMS-345541 treated cells (Fig. 3.5a). However whereas IR had a

negligible effect on *Rag2* expression in BMS-345541 treated cells, *Rag1* expression appeared to follow a pattern similar to that in *Nemo*-deficient cells, in which *Rag1* expression first decreases modestly and then recovers to normal levels (compare Fig. 3.5a and Fig. 3.4a).

Notably, BMS-345541 inhibits IKKα as well as IKKβ, although the IC50 for IKKα inhibition is approximately an order of magnitude greater (Burke et al., 2003). Therefore, we considered that our results using BMS-345541 might be confounded by inhibition of non-canonical NF-κB pathway in addition to the canonical pathway. To address this issue, we repeated these experiments using the inhibitor ML120B, which selectively inhibits IKKβ and has no effect on IKKα even at high doses (Nagashima et al., 2006). In contrast to BMS-345541, treatment with ML120B caused a trend towards increased *Rag1* and *Rag2* mRNA expression (Fig. 3.5b). When ML120B-treated pre-B cells were exposed to IR, they experienced an early decrease in *Rag1* and *Rag2* mRNA expression but began to recover by 4 hours after IR exposure; cells without the inhibitor still showed strong suppression of *Rag1* and *Rag2* mRNA expression at this time point (Fig. 3.5b). This is very similar to the effect of Nemo-deficiency, consistent with the hypothesis that Nemo regulates *Rag1* and *Rag2* expression through its ability to potentiate IKKβ activity.

Nfkb1/p50 is not required for suppression of *Rag1* and *Rag1* mRNA by DSBs

Of the five NF-κB family transcription factors, only p50 has been specifically shown to have any effect on *Rag1* and *Rag2* expression; p50 knockout pre-B cells have approximately 3-fold higher *Rag1* and *Rag2* mRNA levels than wild-type cells (Verkoczy et al., 2005). As this data suggests that p50 normally has a suppressive effect on *Rag1* and *Rag2* expression, we hypothesized that p50-deficient pre-B cells would be unable to suppress *Rag1* and *Rag2* expression in response to DSBs. Because NF-κB signaling normally promotes cell survival, we were concerned that the p50-deficient cells would not survive the week-long culture used in most of our *ex vivo* pre-B cell experiments. Therefore, we isolated total BM from *Nfkb1-/-* mice and pre-incubated them for only 2 hours before splitting the cells from each mouse into two pools, either unirradiated or exposed to 4 Gy IR; RNA was collected from each pool 4 hours after IR exposure. *Rag1* and *Rag2* expression decreased in the p50-/- BM to a similar extent as in wild-type pre-B cell cultures (Fig. 3.5c). We conclude that p50 is not required for normal suppression of *Rag1* and *Rag2* mRNA expression in developing B cells.

LPS treatment suppresses *Rag1* and *Rag2* mRNA expression

The role of Nemo and IKKβ in suppressing *Rag1* and *Rag2* expression in response to DSBs led us to wonder whether stimuli that activate NF-κB signaling independent of ATM activity would also suppress *Rag1* and *Rag2* expression. To test this we exposed IL-

7 withdrawn pre-B cells to LPS, a potent inducer of NF-κB activity in pre-B cells (Sen and Baltimore, 1986). LPS treatment suppressed *Rag1* and *Rag2* mRNA expression approximately 80% within 4 hours of exposure. This is similar to the reduction caused by IR, etoposide, or bleomycin. These results show that DSBs are not the only stimulus that can suppress *Rag1* and *Rag2* expression, and are consistent with a central role for NF-κB signaling in this pathway. However, both DSBs and LPS activate a multitude of intracellular signaling pathways, and so additional work will be necessary to confirm the importance of NF-κB factors in response to each stimulus.

Discussion

These studies show that suppression of *Rag1* and *Rag2* mRNA expression by DSBs depends on rapid inhibition of *Rag1* and *Rag2* transcription. This inhibition requires neither new protein synthesis nor protein degradation, suggesting that the mechanism does not require production of a new transcriptional activator or destruction of an existing transcriptional activator. We found that the DNA damage response protein ATM is absolutely required for suppression of *Rag1* and *Rag2* mRNA expression by DSBs. Nemo and IKKβ, upstream activators of canonical NF-κB signaling, are also required for maximal suppression of *Rag1* and *Rag2* expression by DSBs. However, the limited suppression observed in the absence of these factors suggests that a second, more transient mechanism may also be involved in suppression of *Rag1* and *Rag2* expression. Finally, the ability of LPS stimulation, like DSBs, to suppress *Rag1* and

Rag2 expression suggests that other stimuli can activate NF-κB signaling to suppress *Rag1* and *Rag2* expression.

Several studies have implicated ATM in regulating V(D)J recombination to promote mono-allelic rearrangement of antigen receptor alleles. Previous work from the Bassing laboratory showed that RAG-mediated DSBs activate ATM and suppress *Rag1* and *Rag2* expression (Steinel et al., 2013). The experiments presented in this dissertation show that this signal is not unique to RAG-mediated DSBs, but rather can be activated by DSBs induced by a variety of agents. This signal represents a highly cell-type specific application of a ubiquitous DDR factor conserved from yeast to mammals, highlighting the ability of evolution to employ ancient systems for new functions.

The Bassing laboratory previously showed that RAG-mediated DSBs suppress expression of Gadd45α, a factor which had been shown to promote *Rag1* and *Rag2* expression by increasing Foxo1 activity (Amin and Schlissel, 2008; Steinel et al., 2013). However, the work presented here does not support a role for Gadd45α in suppressing *Rag1* and *Rag2* transcription. Gadd45α overexpression was not able to prevent suppression of *Rag1* and *Rag2* expression; we found similar results when over-expressing Foxo1 (data not shown). Admittedly, these experiments cannot rule out the possibility that post-translational modifications inhibit Gadd45α or Foxo1 activity; indeed, it is well known that Foxo1 activity and stability in is inhibited through

phosphorylation by Akt (Tzivion et al., 2011; Verkoczy et al., 2007; Amin and Schlissel, 2008). A recent study confirmed many of our central findings regarding the ability of DSBs to suppress *Rag1* and *Rag2* expression via ATM activity in pre-B cell lines. Additionally, this group found that DSBs lead to Foxo1 protein degradation and loss of Foxo1 binding to the *Erag* enhancer in B-ALL cell lines (Ochodnicka-Mackovicova et al., 2016). However, we find no decrease in Foxo1 protein or increase in Foxo1 phosphorylation in primary pre-B cells exposed to IR (data not shown). Although we have not assayed Foxo1 binding to the *Rag1/Rag2* gene locus, our data suggest that the regulation of Foxo1 in primary pre-B cells does not completely recapitulate the regulation found in B-ALL cell lines. However, it is possible ATM activates two diverging pathways activating Nemo and inhibiting Foxo1 binding to the *Rag1* and *Rag2* locus, and that both pathways contribute to optimal suppression of *Rag1* and *Rag2* expression. This scenario could explain the limited suppression of *Rag1* and *Rag2* mRNA expression we find in Nemo-deficient mice.

The effect of NF-κB activity on *Rag1* and *Rag2* expression has been controversial, perhaps partially because of the complexity of NF-κB signaling itself. One widely-cited study found IgM crosslinking in immature B cells increases *Rag1* and *Rag2* expression by activating NF-κB factors. However, the same study found that *Nfkb1* knockout cells have increased *Rag1* and *Rag2* expression; the authors suggested that transcriptionally inactive p50/p50 homodimers might compete with transcriptionally active p65 and c-Rel

containing complexes (Verkoczy et al., 2005). Several studies find that DN IκBα, which inhibits canonical NF-κB activation, has no effect on *Rag1* and *Rag2* expression in pre-B cells (Amin and Schlissel, 2008; Cadera et al., 2009). On the other hand, inhibition of IKKβ increased *Rag1* expression in certain pre-B cell lines (Ochodnicka-Mackovicova et al., 2015).

Here, we add to this confusing mess of data points by showing that Nemo and IKKβ participate in suppressing *Rag1* and *Rag2* expression in pre-B cells exposed to DSBs. Furthermore, we found that an IKKα/IKKβ inhibitor seriously impaired *Rag2* expression but had little effect on basal *Rag1* expression, a notable exception to the general rule that expression of *Rag1* and *Rag2* mRNAs are coordinately regulated. In contrast, an IKKβ-specific inhibitor does not suppress, and may even increase, *Rag1* and *Rag2* mRNA expression. Although only specific NF-κB transcription factor tested, Nfkb1/p50, was not required for suppression of *Rag1* and *Rag2*, this does not rule out a role for NF-κB signaling as other NF-κB factors are still active in these cells (Sha et al., 1995).

Together, these results suggest several potential explanations for the confusion in the literature. First, apparently contradictory findings might be due to the different methods used to manipulate NF-κB, which affect different steps of NF-κB activation and may have narrower or broader effects on the various NF-κB homo- and heterodimers

and the IkB factors that regulate them. Indeed, our IKK inhibitor experiments suggest that IKKα and IKKβ activity might have opposing effects on *Rag1* and *Rag2* expression. Secondly, most of this activity seems to be induced by stimulus-induced, and may be less important under normal conditions; this would explain the lack of effect previously seen on basal *Rag1* and *Rag2* expression. Additionally, the expression of many NF-κB factors and their regulatory proteins is known to change throughout B cell development, particularly at the pre-B to immature B transition (Sup. Fig. 3.4 and Kistler et al., 1998). This raises the possibility that NF-κB activity might have different effects on *Rag1* and *Rag2* activity depending on the developmental stage of the cell.

Although the bulk of this our work focuses on the effect of DSBs on *Rag1* and *Rag2* expression, the apparent involvement of NF-κB factors led us to question whether other stimuli that activate NF-κB signaling would also suppress *Rag1* and *Rag2* expression in pre-B cells. Indeed, we find that exposure to the bacterial endotoxin LPS suppresses *Rag1* and *Rag2* mRNA expression. LPS is an inflammatory stimulus, and it has been thoroughly documented that inflammation suppresses B cell development at multiple stages (Cain et al., 2009). It will be important to confirm that NF-κB factors play a role in the suppression of *Rag1* and *Rag2* expression by LPS, and determine whether other inflammatory stimuli have a similar effect. Interestingly, it was recently shown that chronic inflammation can suppress *Rag1* expression in the common lymphoid progenitor, the earliest developmental stage in which it is normally expressed (Baratono

et al., 2015). In itself, the ability of LPS to suppress *Rag1* and *Rag2* mRNA expression simply tells us that DSBs are not unique in their ability to suppress *Rag1* and *Rag2* expression. Of course, these two stimuli are very different in nature. DSBs represent a threat within the developing lymphocyte itself; as discussed in Chapter 2, initiation of V(D)J recombination in the presence of DSBs might lead to oncogenic antigen receptor translocations. LPS and other inflammatory stimuli do not necessarily represent a threat to the health of an individual lymphocyte, but rather to the larger organism. Therefore on a more speculative level, our results suggests that two quite different "danger" stimuli activate a conserved pathway to suppress *Rag1* and *Rag2* expression in situations where V(D)J recombination may be undesirable.

Figure 3.1

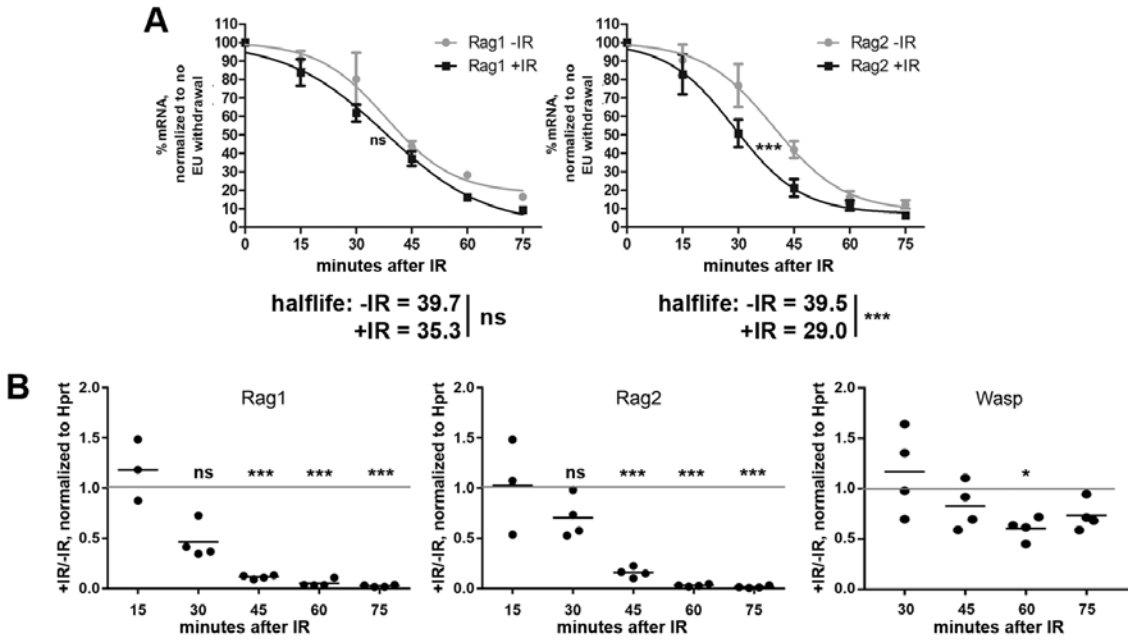


Figure 3.1: DSBs cause a rapid and specific decrease in *Rag1* and *Rag2* transcription. **(A)** qRT-PCR quantification of EU-labeled Rag1 and Rag2 mRNA levels relative to EU-labeled *18S* RNA levels in non-irradiated or irradiated *EμBCL2* pre-B cells at indicated times after EU washout and/or exposure to 4 Gy of IR. Data are from 4 independent experiments. Data averages are shown with error bars the SEM. Prism 5 was used to calculate best-fit curves, half-lives and p-values. ***p<0.001. **(B)** qRT-PCR quantification of EU-labeled *Rag1*, *Rag2*, and *Wasp* mRNA levels relative to EU-labeled *Hprt* mRNA levels in non-irradiated or irradiated *EμBCL2* pre-B cells at indicated times after addition of EU and exposure to 4 Gy of IR. Data are presented as the ratio of relative levels of each mRNA in irradiated cells compared to non-irradiated cells. The gray line represents a value of 1, which would indicate that IR had no effect on the transcription rate of a gene. Data are from 4 independent experiments. p-values for whether the average ratio at each time point is different from 1.0 was determined using one-tailed T test with Bonferroni's correction for multiple testing.

Figure 3.2

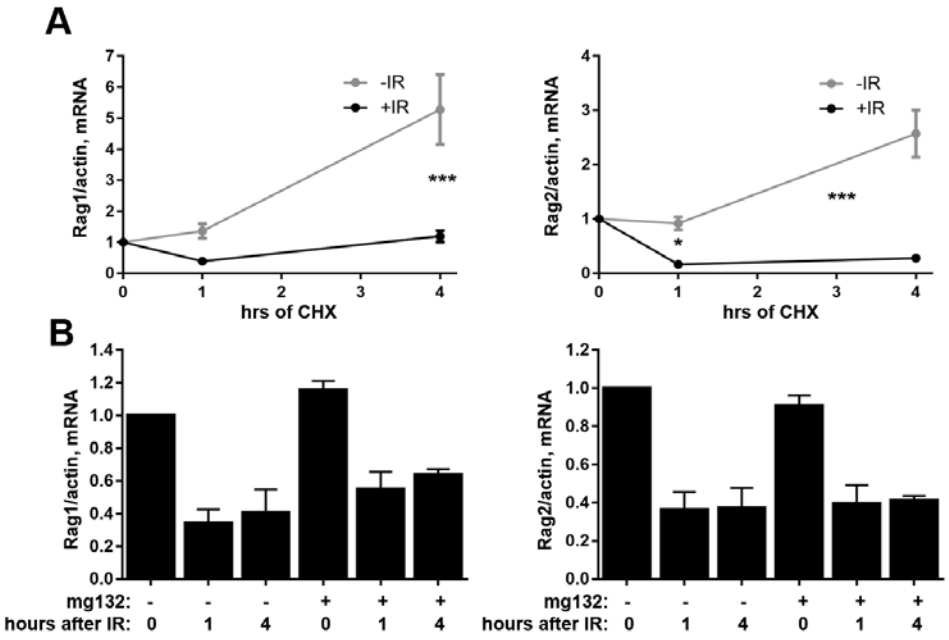


Figure 3.2: DSBs do not require new protein synthesis or protein degradation to suppress *Rag1* and *Rag2* mRNA expression. **(A)** qRT-PCR quantification of *Rag1* and *Rag2* mRNA in *EμBCL2* pre-B cells treated with cycloheximide and then not irradiated exposed to 4 Gy IR. Data are from 3 independent experiments. **(B)** qRT-PCR quantification of *Rag1* and *Rag2* mRNA in *EμBCL2* pre-B cells treated with mg132 or vehicle control and non-irradiated or irradiated with 4 Gy IR at indicated times after exposure IR. Data are from 3 independent experiments. **(A&B)** Data averages are shown with error bars the SEM. p-values were calculated by T test with Bonferroni's correction for multiple testing.

Figure 3.3

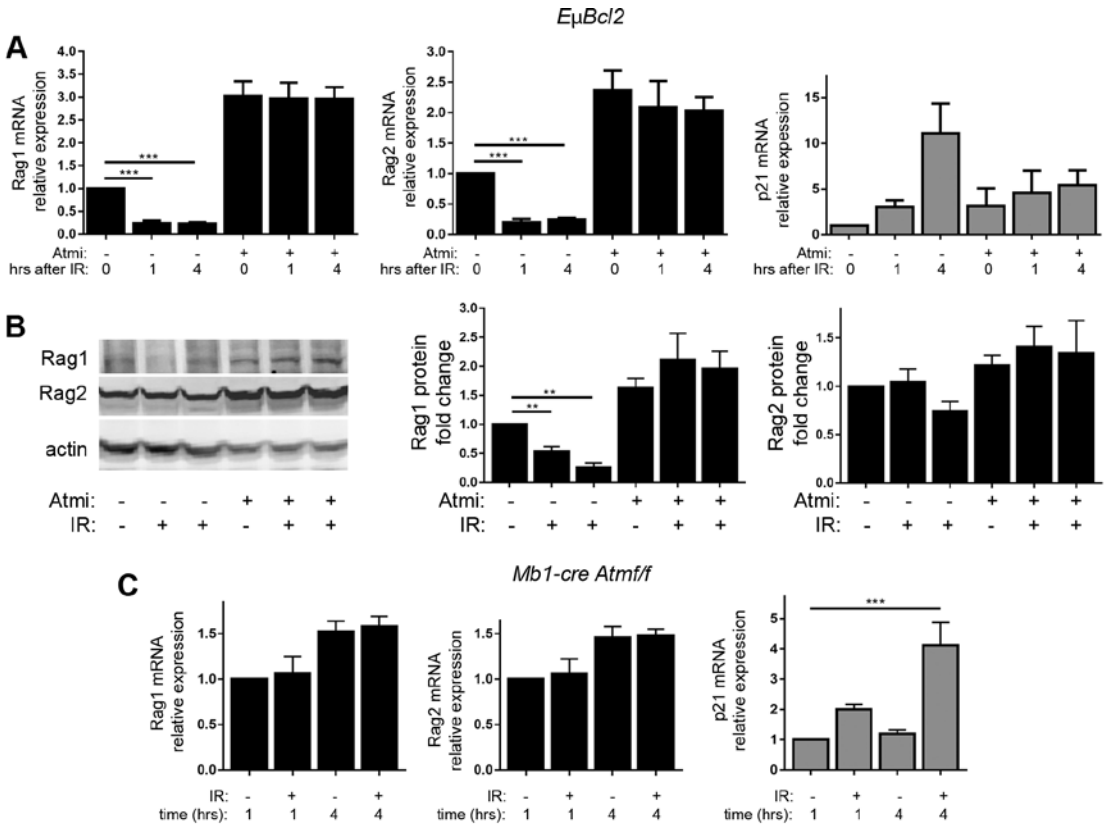


Figure 3.3: ATM is required for DSBs induced in pre-B cells to downregulate *Rag1* and *Rag2* expression. **(A)** qRT-PCR quantification of *Rag1*, *Rag2*, and *p21* mRNA in non-irradiated or irradiated *EμBCL2* pre-B cells at indicated times after exposure to 4 Gy of IR. Cells were treated with 15 μM of the KU55933 ATM kinase inhibitor or vehicle (DMSO) for 48 hours prior to irradiation or harvesting of non-irradiated cells. Data are from 8 independent experiments. **(B)** Representative western blot and quantification from experiment described in (A). **(C)** qRT-PCR quantification of *Rag1*, *Rag2*, and *p21* mRNA in non-irradiated or irradiated *Mb1Cre*$^{+}$*Atm*$^{flox/flox}$ pre-B cells at indicated times after exposure to 4 Gy IR. Data are from 3 independent experiments. **(A-C)** Data are normalized to 1.0 for non-irradiated cells harvested one hour following IR exposure of irradiated samples. For other samples, data averages are shown with error bars indicating SEM. p-values were calculated by Dunnett's post-test after ANOVA.

Figure 3.4

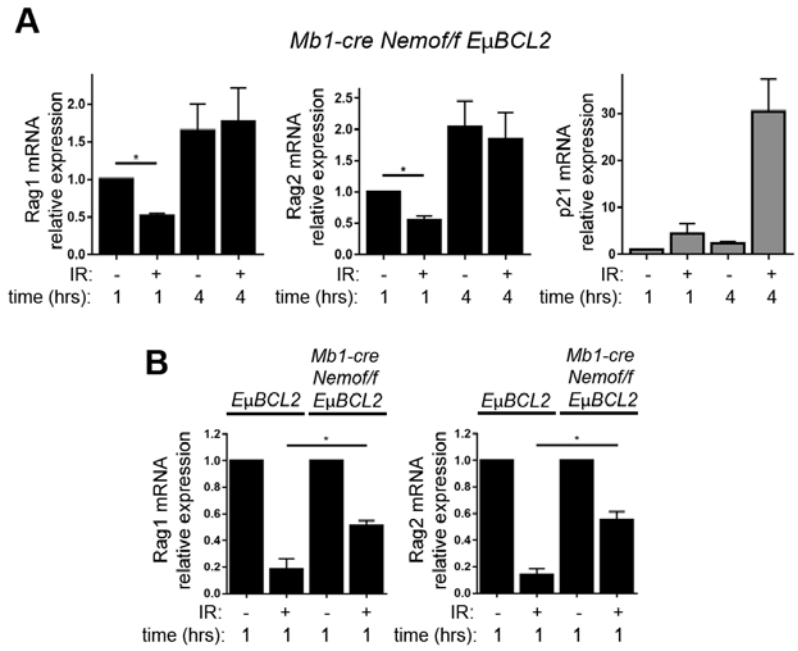


Figure 3.4: Nemo is required for pre-B cells to normally downregulate *Rag1* and *Rag2* expression in response to DSBs. **(A)** qRT-PCR quantification of *Rag1*, *Rag2*, and *p21* mRNA in non-irradiated *Mb1Cre*$^{+}$*Nemo*$^{flox/flox}$*EμBCL2* pre-B cells or irradiated *Mb1Cre*$^{+}$*Nemo*$^{flox/flox}$*EμBCL2* pre-B cells at indicated times after exposure to 4 Gy IR. Data are from 3 independent experiments. p-values were calculated by Dunnett's post-test after ANOVA. **(B)** qRT-PCR quantification of *Rag1* and *Rag2* mRNA in non-irradiated or irradiated pre-B cells from *EμBCL2* or *Mb1Cre*$^{+}$*Nemo*$^{flox/flox}$*EμBCL2* mice at 1 hour after exposure to 4 Gy IR. Data are from 3 independent experiments. p-value was calculated by T test. **(A&B)** Data are normalized to 1.0 for non-irradiated cells harvested one hour following IR exposure of irradiated samples. For other samples, data averages are shown with error bars indicating SEM.

Figure 3.5

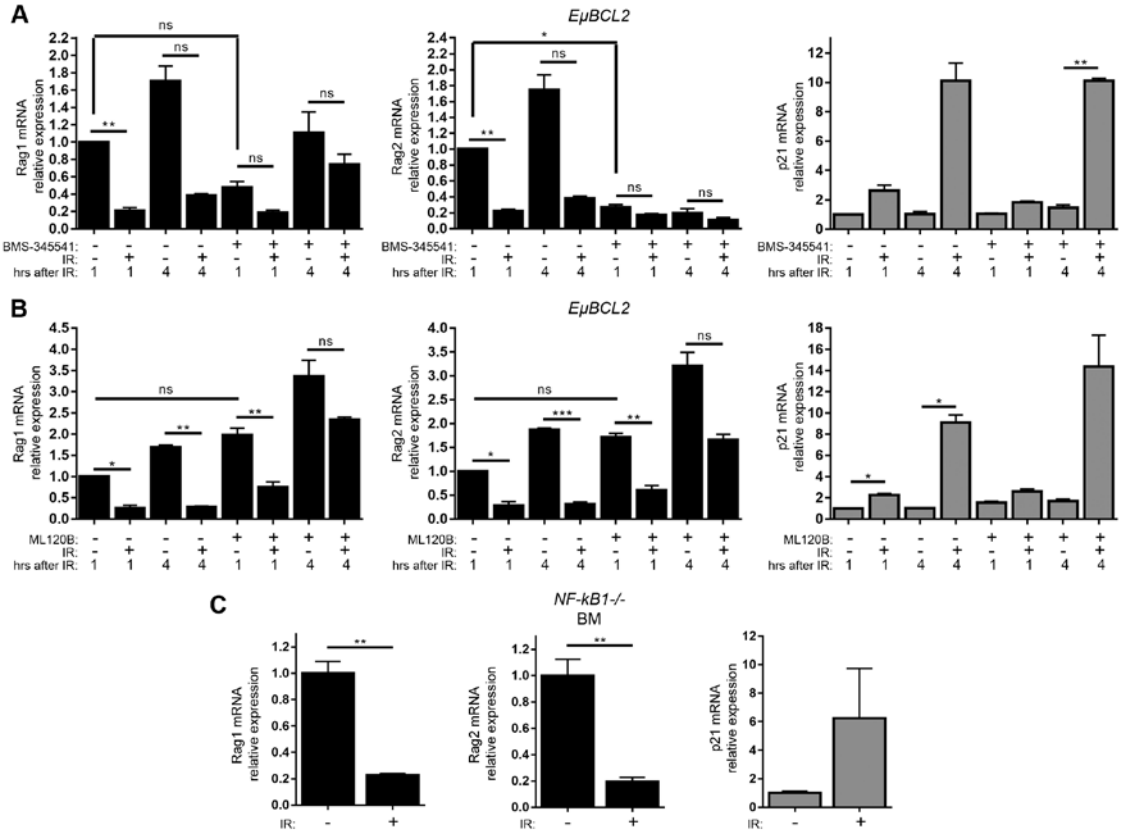


Figure 3.5: Variable effects of IKK inhibitors on basal *Rag1* and *Rag2* expression and on suppression by DSBs. **(A)** qRT-PCR quantification of *Rag1*, *Rag2*, and *p21* mRNA in non-irradiated or irradiated *EμBCL2* pre-B cells at indicated times after exposure to 4 Gy of IR. Cells were treated with 5 μM BMS-345541 IKK kinase inhibitor or vehicle (DMSO) for 2 hours prior to irradiation. Data are from 3 independent experiments. **(B)** qRT-PCR quantification of *Rag1*, *Rag2*, and *p21* mRNA in non-irradiated or irradiated *EμBCL2* pre-B cells at indicated times after exposure to 4 Gy of IR. Cells were treated with 10 μM of the ML120B IKKβ kinase inhibitor or vehicle (DMSO) for 2 hours prior to irradiation. Data are from 3 independent experiments. **(C)** qRT-PCR quantification of *Rag1*, *Rag2*, and *p21* mRNA in BM from *NF-κB1-/-* mice. BM was harvested and pre-incubated for 2 hours at 37°. After pre-incubated, BM from each mouse was divided into two samples and either unirradiated or exposed to 4 Gy IR; RNA was collected 4 hours later. Data are from one experiment with 4 mice.

Figure 3.6

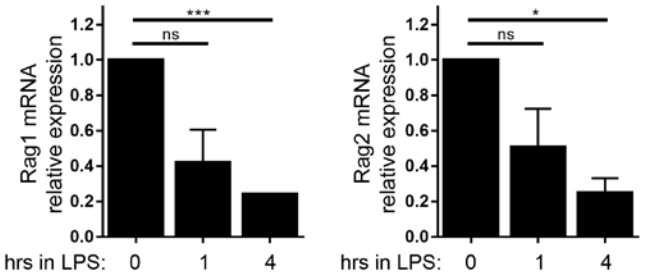
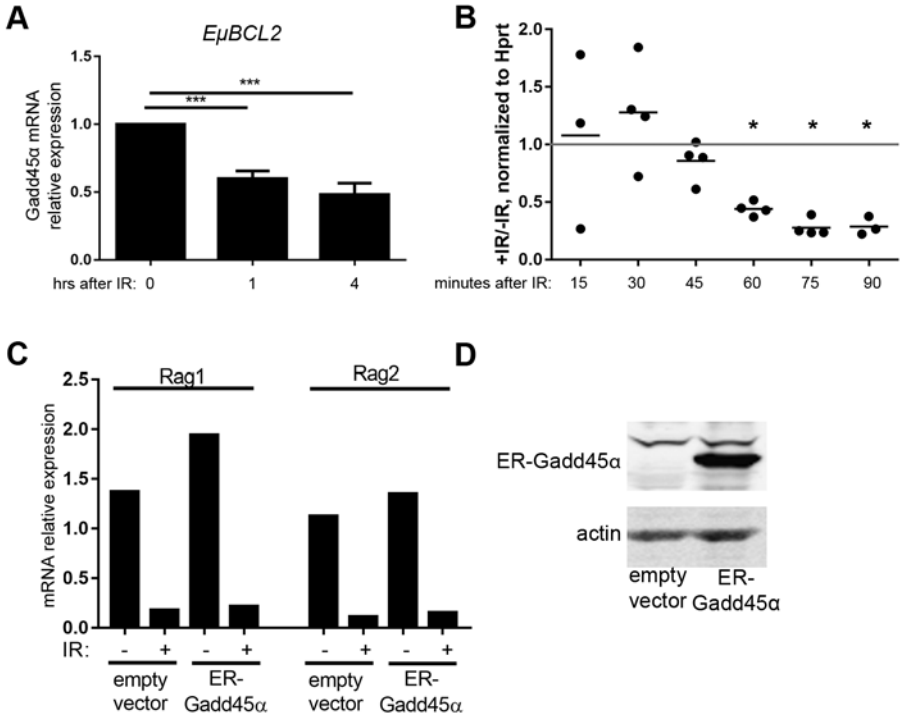


Figure 3.6: LPS treatment suppresses Rag1 and Rag2 mRNA in a manner similar to DSBs. qRT-PCR quantification of *Rag1* and *Rag2* mRNA in *E*μ*BCL2* pre-B cells treated with 15 μg/mL LPS or vehicle (water) for the indicated time periods. Data are from 3 independent experiments.

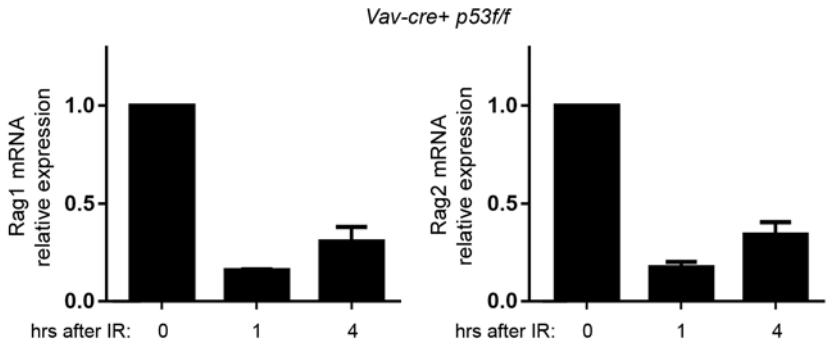
Supplemental Figure 3.1



Sup. Fig. 3.1: Constitutive *Gadd45α* expression does not prevent suppression of Rag1 and Rag2 mRNA expression by DSBs. **(A)** qRT-PCR quantification of *Gadd45α* mRNA in non-irradiated or irradiated *EμBCL2* pre-B cells at indicated times after exposure to 4 Gy IR. Data are from 11 independent experiments. Data are normalized to 1.0 for non-irradiated cells. For irradiated cells, data averages are shown with error bars indicating SEM. p-values calculated using Dunnett's post-test after ANOVA. ***p<0.001. (**B**) qRT-PCR quantification of EU-labeled *Gadd45α* mRNA levels relative to EU-labeled *Hprt* mRNA levels in non-irradiated *EμBCL2*pre-B cells and irradiated *EμBCL2* pre-B cells at indicated times after addition of EU and exposure to 4Gy of IR. Data are presented as the ratio of relative levels of each mRNA in irradiated cells compared to non-irradiated cells. The dotted line represents a value of 1, which would indicate that IR had no effect on the transcription rate of an assayed gene. Data are from 4 independent experiments. p-values were determined using one-tailed T test with Bonferroni's correction for multiple testing. ***p<0.001.(**C**) qRT-PCR quantification of *Rag1* and *Rag2* mRNA in non-irradiated and irradiated *Rag1D708A* Abl pre-B cells transduced with an empty retroviral vector or the same retroviral vector containing an ERGadd45α cDNA. For 2 hours before

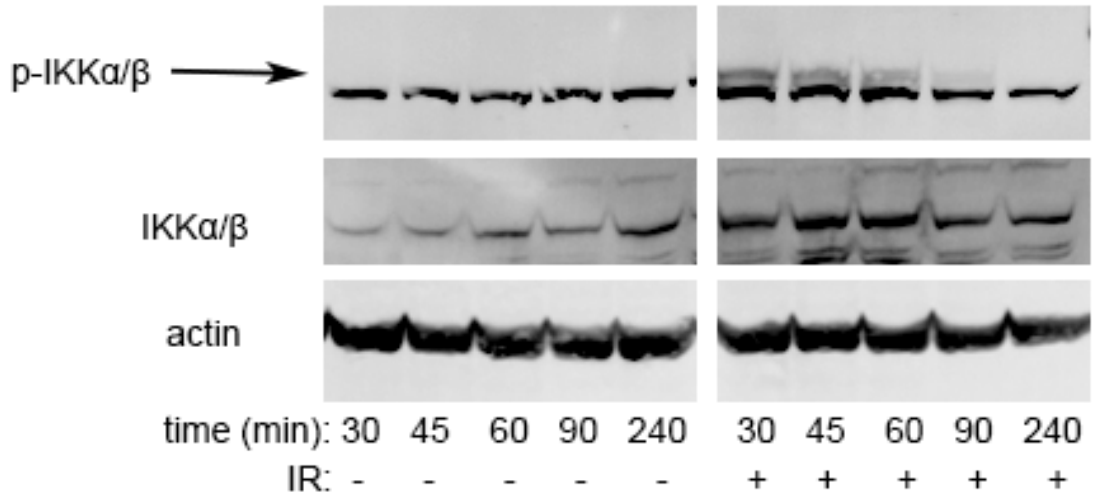
harvesting non-irradiated cells or exposing to IR the irradiated cells, all cells were treated with tamoxifen to induce nuclear translocation of ER-Gadd45α. Data are from one representative experiment. **(D)** Western blot showing ER-Gadd45α and actin expression in *Rag1*^{D708A} Abl pre-B cells transduced with an empty retroviral vector or the same retroviral vector containing an ER-Gadd45α cDNA. Data are from one experiment.

Supplemental Figure 3.2



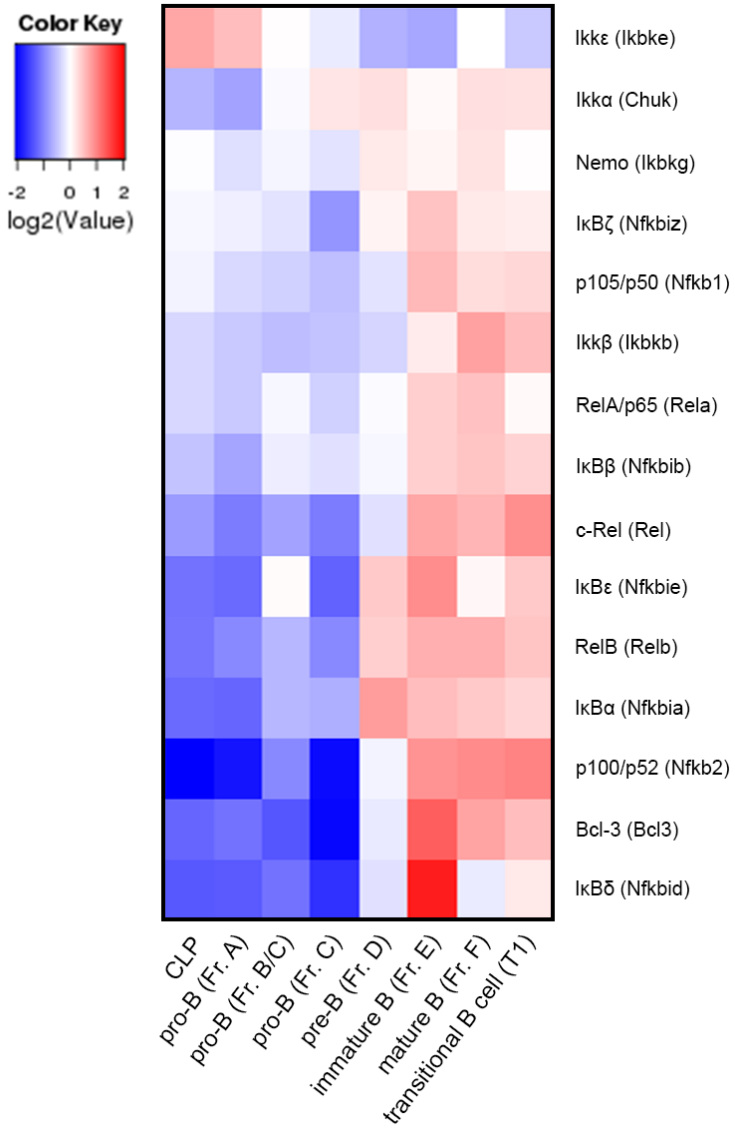
Sup. Fig. 3.2: p53 activity is not required for suppression of *Rag1* and *Rag2* mRNA expression by DSBs. qRT-PCR quantification of *Rag1* and *Rag2* mRNA in non-irradiated V*avCre*$^{+}$*Tp53*$^{flox/flox}$ pre-B cells or irradiated *VavCre*$^{+}$*Tp53*$^{flox/flox}$ pre-B cells at indicated times after exposure to 4 Gy IR. Data are from 2 independent experiments. Data are normalized to 1.0 for non-irradiated cells harvested one hour following IR exposure of irradiated samples. For other samples, data averages are shown with error bars indicating SEM.

Supplemental Figure 3.3



Sup. Fig. 3.3: Phosphorylated IKKα/IKKβ accumulates rapidly and transiently in IL-7 withdrawn pre-B cells in response to DSBs. Western blot showing phosphorylated and total IKKα/IKKβ in unirradiated pre-B cells or pre-B cells exposed to 4 Gy IR. The antibodies used cannot distinguish between IKKα and IKKβ.

Supplemental Figure 3.4



Sup. Fig. 3.4: Expression of factors involved in NF-κB signaling changes throughout B cell development. The heatmap was generated using "my dataset" tool in the Immgen database (www.immgen.org). All BM B cell subsets and an early splenic subset (T1) were used to generate the heatmap. The list of factors involved in NF-kB signaling was based on a comprehensive review of NF-kB published in 2012 (Hayden and Ghosh, 2012).

Chapter 4: Impact of DSB signaling on allelic exclusion[1]

Abstract

Chapters 2 and 3 described the ability of exogenously induced DSBs to activate ATM and Nemo to suppress *Rag1* and *Rag2* expression in pro-B cells, pre-B cells, and DN thymocytes. A similar suppressive signal activated by RAG cleavage in pre-B cells had previously been correlated with increased Igκ allelic inclusion. Here, we show that ATM also regulates allelic exclusion of the IgH chain rearranged in pro-B cells and the TCRβ chain rearranged in DN thymocytes. Increased TCRβ allelic inclusion is established early in T cell development and is correlated with an increased frequency of T cells containing two TCRβ rearrangements, indicating a failure of allelic exclusion at level of TCRβ rearrangement. We also investigated the role of several factors proposed to cooperate with or act downstream of ATM in promoting allelic exclusion. We found that the cell cycle regulator cyclin D3 has only a minor role in Igκ allelic inclusion despite its more prominent role in IgH and TCRβ allelic inclusion. Spi-C, which has been proposed to suppress Igκ locus accessibility in the presence of DSBs, was found to be unnecessary for allelic exclusion. In contrast, we find that Nemo-deficient B cells have increased Igκ

[1] Parts of this chapter were published in *The Journal of Immunology*. Copyright © 2014 The American Association of Immunologists, Inc. Steinel NC, Fisher MR, Yang-Iott KS, Bassing CH. The Ataxia Telangiecetasia Mutated and Cyclin D3 Proteins Cooperate to Help Enforce TCRβ and IgH Allelic Exclusion. J. Immunol. 193:2881-2890. Portions of this chapter were prepared jointly with Natalie Steinel.

allelic inclusion, further supporting a role for Nemo-mediated suppression of *Rag1* and *Rag2* expression in promoting allelic exclusion.

Introduction

Even before the discovery of V(D)J recombination, it was recognized that each B cell expresses only one IgH and one IgL chain on their cell surface, despite the availability of multiple alleles; this phenomenon is known as allelic exclusion (Bernier and Cebra, 1964; Pernis et al., 1965). At a genetic level, it was found that B cells typically contain only one productively rearranged IgH gene and one functional IgL gene, while T cells have only one productively rearranged TCRβ gene (Alt et al., 1980, 1984; Casanova et al., 1991). However, the TCRα chain does not appear to be allelically excluded at either the level of genetic rearrangement or cell surface expression (Casanova et al., 1991; Heath et al., 1995).

If allelic exclusion did not occur at the genetic level, it would be predicted that V(D)J 100% of cells would contain two rearrangements for each antigen receptor chain, and that many cells would contain two productive rearrangements. For IgH and TCRβ loci the upper limit of cells that could contain two productive rearrangements is 20%, as 2/3 of all rearrangements are out-of-frame, and each IgH or TCRβ allele can undergo rearrangement only once. On the other hand, if allelic exclusion operated perfectly at the genetic level, it would be predicted that approximately 60% of B and T cells would

have only one fully rearranged IgH or TCRβ gene (Alt et al., 1984; Malissen et al., 1992). By definition, 0% of those lymphocytes would contain two productive rearrangements for the same antigen receptor gene in this case. In fact, IgH, Igκ, and TCRβ rearrangements show strong evidence for allelic inclusion at the genetic level, in that close to 60% of B and T cells contain only 1 rearranged IgH or TCRβ allele, and very few lymphocytes contain two productive rearrangements for the same antigen receptor chain (Alt et al., 1984; Casanova et al., 1991). However, allelic inclusion at the genetic level is generally somewhat higher than inclusion at the level of cell surface antigen receptor expression. Factors accounting for this gap include the requirement for pairing of IgH and IgL or TCRβ and TCRα chains and post-transcriptional silencing of one allele (Brady et al., 2010)

Several mechanisms cooperate to enforce allelic exclusion of antigen receptor gene rearrangements. Most famous is feedback inhibition by the protein products of functionally rearranged antigen receptor genes. These proteins assemble into cell surface complexes that generate signals to inhibit subsequent gene rearrangement. In pro-B cells that create a productive IgH rearrangement, the new IgH chain pairs with surrogate light chains (VpreB and λ5) and the signaling components of the BCR (Igα and Igβ) to form the pre-BCR; inability of this heavy chain to signal prevents feedback inhibition and allows cells to create multiple productive IgH rearrangements (Kitamura and Rajewsky, 1992; Papavasiliou et al., 1995; Schweighoffer et al., 2003). Similarly,

creation of a functional light chain in pre-B cells allows cell surface expression of the complete BCR, which signals feedback inhibition of further Igκ or Igλ rearrangement (Grawunder et al., 1995). A similar situation is found in DN thymocytes; a newly synthesized TCRβ chain pairs with the pre-Tα chain to form the pre-TCR, and inability of the pre-TCR to signal leads to the generation of cells with two productive TCRβ alleles (Aifantis et al., 1997, 1999). The feedback signal acts both by turning off RAG expression (Grawunder et al., 1995; Hoffman et al., 1996; Li et al., 1993), and by alterations in chromatin structure and 3D DNA organization of the excluded locus to preclude RAG activity (Corcoran, 2005; Roldán et al., 2005; Kondilis-Mangum et al., 2011).

It is important to note that feedback inhibition can only prevent allelic inclusion if rearrangements do not occur simultaneously. Indeed, several studies using FISH and γ-H2AX staining to mark antigen receptor loci with DSBs show that normally, only one cleaved allele exists in a cell (Hewitt et al., 2009; Chaumeil et al., 2013; Chan et al., 2013). The Bergman laboratory has provided evidence that one of the two homologous alleles for IgH, Igκ, and TCRβ is marked to rearrange earlier than the other by epigenetic marks established early in development, so one allele is predisposed to initiate recombination before the other (Mostoslavsky et al., 1998, 2001; Goldmit et al., 2005). Additionally, it is possible that initiation of rearrangement is a relatively rare event, so that the likelihood of two rearrangements occurring simultaneously in the same cell is very low.

The Bassing laboratory showed that in pre-B cells, a third allelic exclusion mechanism acts in the time between initial cleavage of one Igκ allele and initiation of feedback inhibition by cell surface expression of a complete BCR. Cleavage of Igκ activates the DNA damage response protein ATM, which inhibits cleavage of the second allele before the first is repaired (Steinel et al., 2013). Importantly, inhibition of V(D)J recombination must be transient, as a second recombination event will occur if the first is non-productive.

ATM deficient mice lacking this transient inhibition of V(D)J recombination have an increased frequency of B cells expressing two different Igκ chains on the cell surface, a failure of allelic exclusion (Steinel et al., 2013). More recently, we have found that ATM deficient mice also have increased allelic inclusion of IgH and TCRβ chains which rearrange in pro-B cells and DN thymocytes, respectively. We have further explored the genetic and developmental basis of increased TCRβ allelic inclusion in ATM-deficient T cells. We find that increased ATM-deficient T cells have a relatively high proportion of cells with two rearranged TCRβ alleles, consistent with our hypothesis that the defect in allelic exclusion is at the level of rearrangement. Furthermore, we find that this defect is already present in pre-selection DP thymocytes. Finally, we examined the effect of several molecules proposed to either participate in or cooperate with the ATM-mediated inhibition signal on Igκ allelic exclusion. We find that the cell cycle regulatory protein cyclin D3 has a modest role in promoting Igκ allelic exclusion. The Igκ locus

regulatory protein Spi-C was not required for allelic exclusion of the Igκ locus. In contrast, Nemo-deficient B cells show a defect in allelic exclusion of approximately the same magnitude as ATM-deficient cells.

Results

Increased frequency of lymphocytes expressing two IgH or TCRβ chains in *ATM*$^{-/-}$ mice

The importance of ATM-mediated inhibition of V(D)J recombination for Igκ allelic exclusion led us to question whether a similar mechanism operates in pro-B and pro-T cells, which rearrange IgH and TCRβ genes, respectively. To measure IgH allelic inclusion we took advantage of naturally occurring IgM allotypes, IgM^{A} vs. IgM^{B}. We performed cell surface staining for these two markers using *ATM*$^{+/+}$ *Igμ*$^{A/B}$ and *ATM*$^{-/-}$ *Igμ*$^{A/B}$ BM cells. We found that approximately 0.4% of *ATM*$^{-/-}$ *Igμ*$^{A/B}$ BM cells expressed both IgM^{A} and IgM^{b}, compared to approximately 0.25% of *ATM*$^{+/+}$ *Igμ*$^{A/B}$ cells (Fig. 4.1a&b). Although these frequencies are low, it is important to note both asynchronous initiation of rearrangement and feedback inhibition from a complete antigen receptor are not expected to be affected by ATM deficiency, and would both still limit the frequency of allelically included cells. We performed similar flow cytometry experiments to measure allelic inclusion of the TCRβ chain. Lacking allotypic markers for TCRβ, we conducted this analysis using pairs of antibodies that recognize different Vβ segments. Because *ATM*$^{-/-}$ thymocytes exhibit defects in both D-to-J rearrangement and V-to-DJ rearrangement

which might limit the frequency of productive biallelic rearrangement, we used *Jb1*$^{DJ/DJ}$ mice in these analyses to limit the opportunity for aberrant recombination events that would decrease observed allelic inclusion. In these mice, the TCRβ loci contain a Dβ1-to-Jβ1 rearrangement encoded in the germline and deletion of Dβ2 and Jβ2, and so only undergo V-to-DJ rearrangement of TCRβ (Carpenter et al., 2009). For most Vβ pairs tested, we found that *ATM*$^{-/-}$ *Jb1*$^{DJ/DJ}$ thymocytes showed a trend towards increased double expression, and this difference was significant for several pairs (Fig. 4.1c&d). These results support a role for ATM in enforcing allelic exclusion of both IgH and TCRβ.

Increased allelic inclusion of TCRβ is evident in DP thymocytes

As described in Chapter 3, ATM inhibits RAG expression in response to DNA damage. This suggests that ATM regulates allelic exclusion at the level of antigen receptor gene rearrangement. However, it is also possible that ATM affects selection processes that might affect the ability of cells expressing two different IgH or TCRβ chains to survive and progress through development (Balomenos et al., 1995). To address this issue we measured TCRβ inclusion in earlier stages of T cell development in thymocytes from either developing *ATM*$^{+/+}$ *Jb1*$^{DJ/DJ}$ and *ATM*$^{-/-}$ *Jb1*$^{DJ/DJ}$ mice. We performed surface staining for CD4 and CD8 to divide cells into DN, DP, and SP developmental stages. We then performed intracellular staining for pairs of Vβ chains, to allow detection of TCRβ chains not yet expressed on the cell surface. As expected,

relatively few cells in the DN population expressed Vβ chains. Unfortunately, this fact combined with the low frequency of DN thymocytes prevented us from reliably detecting dual Vβ expressing cells among either $ATM^{+/+}$ $Jb1^{DJ/DJ}$ or $ATM^{-/-}$ $Jb1^{DJ/DJ}$ cells (Fig. 4.2a). In contrast, dual Vβ expressing cells were easily detected among DP thymocytes (Fig. 4.2a). For each Vβ pair tested, $ATM^{-/-}$ $Jb1^{DJ/DJ}$ DP thymocytes had an increased frequency of dual expressing cells (Fig. 4.2b). Although we cannot completely rule out a role for selection, this result shows that increased allelic exclusion of TCRβ in $ATM^{-/-}$ cells is established early in thymocyte development.

ATM-deficient T cells exhibit increased biallelic rearrangement of TCRβ genes

In the case of Igκ allelic exclusion, ATM was shown to promote allelic exclusion by inhibiting RAG-mediated cleavage of a second allele before repair of the first cleaved Igκ allele. If a similar mechanism operates in pro-B cells rearranging IgH genes and in DN thymocytes rearranging TCRβ genes, we would predict that an increased frequency of mature lymphocytes would have two completely rearranged IgH or TCRβ genes. We used two methods to test this prediction. We used a fluorescence *in situ* hybridization (FISH) assay to measure the frequency of $ATM^{+/+}$ $Jb1^{DJ/DJ}$ and $ATM^{-/-}$ $Jb1^{DJ/DJ}$ T cells with one vs. two V-to-DJ rearrangements of the TCRβ locus. In this assay, we labeled cells with two probes; one that hybridizes to the region downstream of the J segments, which is present on all intact TCRβ alleles. The second hybridizes between the V

segments and the pre-rearranged DJ segment. This probe only hybridizes if the allele has not undergone rearrangement. Thus, a VDJ rearrangement is identified by labeling with only the downstream probe, while an unrearranged allele is labeled with both probes (Fig. 4.3a). Certain aberrant rearrangement events can also be identified in this system; for example hybridization of the two probes to different chromosomes indicates a translocation event, while hybridization of probe(s) to only one chromosome in a cell indicates a large deletion event on one allele. Examples of these scenarios are shown in Fig. 4.3b. Notably, small deletion events that might nevertheless affect the productivity of a rearrangement are not detected by this method. In the case of perfect allelic exclusion, we would predict that 60% of T cells would contain one complete VDJ rearrangement. Using this technique, we found that approximately 65% of $ATM^{+/+}$ $Jb1^{DJ/DJ}$ T cells contained only one complete VDJ rearrangement, similar to the 60% predicted in the case of perfect allelic exclusion. In contrast, only 53% of $ATM^{-/-}$ $Jb1^{DJ/DJ}$ T cells contained one complete V(D)J rearrangement, a significant decrease compared to the wild type cells (Fig. 4.3c). We also found an increased frequency of aberrant rearrangement events in $ATM^{-/-}$ $Jb1^{DJ/DJ}$ T cells (Fig. 4.3d).

The FISH analysis cannot address the question of whether any of the cells with two complete rearrangements contain two productive rearrangements. To address this question, we generated a panel of T cell hybridomas from $ATM^{-/-}$ $Jb1^{DJ/DJ}$ spleen cells and analyzed the rearrangement status of their TCRβ loci by Southern as previously

described (Khor and Sleckman, 2005). We obtained a total of 171 clones in this analysis; 38 were rejected from the analysis because only a single TCRβ band could be detected by southern. This might be caused by loss of an allele during hybridoma fusion or aberrant rearrangement, both of which might be affected by ATM deficiency. Of the remaining 133 clones, 87 (65.4%) contained only one complete VDJ rearrangement (Fig. 4.3e). We further analyzed the 46 hybridoma lines containing two complete rearrangements to determine if any of these contained two productive rearrangements. We first identified the Vβ segments involved in rearrangement by a PCR screen, and then sequenced the PCR products obtained. Four lines were excluded at this step because we were unable to obtain two sequences. Of the remaining 42 clones, 3 (7.1%) contained two productive rearrangements (Fig. 4.3f). A older study examining wild-type hybridomas detected no examples of T cell hybridomas with two productive rearrangement (Khor and Sleckman, 2005). The frequency of $ATM^{-/-}$ $Jb1^{DJ/DJ}$ containing two VDJ rearrangements (65.4%) is similar to the amount predicted in the case of perfect allelic exclusion, but the presence of 3 lines containing two productive rearrangements indicates a defect in allelic exclusion.

Cyclin D3 deficiency leads to a minor defect in allelic exclusion of Igκ

Having established the importance of ATM for allelic exclusion of IgH, TCRβ, and Igκ loci, we wanted to identify other factors that either participate in the ATM-mediated

regulation of allelic exclusion or complement ATM's activity. Work by Natalie Steinel in the Bassing lab showed that the cell cycle regulatory protein cyclin D3 helps enforce allelic exclusion of the IgH and TCRβ loci, and that double deficiency of ATM and cyclin D3 have an additive effect on allelic inclusion (Steinel et al., 2014). Normally, cyclin D3 promotes the proliferative expansion that occurs upon expression of a pre-BCR or pre-TCR, as developing lymphocytes transition from the pro- to pre-B or T cell stage (Sicinska et al., 2003; Cooper et al., 2006). As this proliferation is believed to play a part in both RAG downregulation and chromatin alterations of IgH and TCRβ loci, it was suggested that cyclin D3 deficiency increases allelic inclusion in *ATM+/+* or *ATM-/-* lymphocytes by increasing the time available for a second recombination event after the first is completed. However, cyclin D3 was also shown to repress germline V_H transcription in pro-B cells, indicating that it may also promote allelic exclusion by directly suppressing accessibility of antigen receptor loci to the RAG recombinase (Powers et al., 2012). As pre-B cells do not undergo a proliferative burst following expression of the BCR, cyclin D3's role in promoting proliferation would not be expected to affect Igκ allelic exclusion in pre-B cells. However, if cyclin D3 has a role in directly regulating Igκ accessibility, it might impact Igκ allelic exclusion in this way. To test the role of cyclin D3 in Igκ allelic exclusion, we used the mice in which one Igκ constant region allele was replaced with the equivalent human gene ($Ig\kappa^h$), while the other allele was wild type ($Ig\kappa^m$) allowing the two Igκ alleles to be stained by two different antibodies (Casellas, 2001). We found

that the frequency of cells expressing Igκ^{m} and Igκ^{h} together was slightly higher in *Ccnd3*$^{-/-}$ *Igκ*$^{h/m}$ BM cells than in *Ccnd3*$^{+/+}$ *Igκ*$^{h/m}$ BM cells, approximately 2.25 % compared to 1.9% (Fig. 4.4a&b). Among splenic B cells, no significant increase in Igκ biallelic expression was found in *Ccnd3*$^{-/-}$ *Igκ*$^{h/m}$ cells (Fig. 4.4 a&b). This very small increase in Igκ allelic inclusion, which does not persist in the periphery, is far less than the approximately 2-fold increase in IgH allelic inclusion found in *Ccnd3*$^{-/-}$ B cells. Therefore, cyclin D3 appears to play at most a minor role in promoting Igκ allelic exclusion.

Spic deficiency does not affect allelic exclusion of Igκ

It has been proposed that DSBs regulate accessibility of the Igκ locus in addition to suppressing *Rag1* and *Rag2* expression. In this context, RAG mediated DSBs have been shown to act through ATM to increase expression of the Ets family protein Spi-C (Bednarski et al., 2016). Spi-C lacks the ability to activate transcription, and has been shown to inhibit the activity of transcriptionally activate Ets family members by competing for binding (Hashimoto et al., 1999; Schweitzer et al., 2006). In the context of RAG-mediated DSBs, Spi-C overexpression was shown to partially inhibit Igκ cleavage in *ATM*$^{-/-}$ pre-B cells that would otherwise experience no inhibition of Igκ cleavage (Bednarski et al., 2016). In light of these findings, we hypothesized that increased Spi-C expression may decrease accessibility of the Igκ locus and cooperate with the suppression of *Rag1* and *Rag2* expression to promote allelic exclusion. Indeed, in the Igκ

cleavage assays discussed in Chapter 2 (Fig. 2.5a), the accumulation of cleaved Igκ alleles that occurs upon treatment of *Artemis*$^{-/-}$ cells with sti571 is accompanied by increased *Spic* mRNA expression; addition of the DNA damaging agent etoposide might cause a slight additional increase (Fig. 4.5a). To determine whether Spi-C expression is necessary to enforce allelic exclusion of the Igκ locus, we performed flow cytometry on *Spic*$^{+/+}$ *Igκ*$^{h/m}$ and *Spic*$^{-/-}$ *Igκ*$^{h/m}$ BM cells and splenocytes. We observed approximately normal frequencies of pre-B cells in *Spic*$^{-/-}$ *Igκ*$^{h/m}$ BM cells, although they express to have slightly increased levels of CD43 expression, and slightly decreased levels of B220 (Fig. 4.5b); this might be due to defects in regulating pre-B cell-specific gene expression. Nevertheless, we found no defect in Igκ allelic exclusion in the BM, and actually found a slight decrease (approximately 20%) in allelic inclusion among *Spic*$^{-/-}$ *Igκ*$^{h/m}$ splenocytes (Fig. 4.5c). These results show that Spi-C expression is not necessary for normal Igκ allelic exclusion.

Nemo deficiency causes an increase in Igκ allelic inclusion

In Chapter 3 we described a role for Nemo in mediating suppression of *Rag1* and *Rag2* expression. As Nemo is a known target of ATM activity, we propose that Nemo acts downstream of ATM to mediated suppression. As discussed above, developing lymphocytes that lack this pathway due to ATM-deficiency exhibit increased allelic inclusion of IgH, TCRβ, and Igκ (Fig. 4.1 and Steinel et al., 2013). To determine if Nemo-

deficiency has a similar effect on allelic inclusion of Igκ, we performed flow cytometry on *Nemo*$^{+/+}$ *Igκ*$^{h/m}$ and *Nemo*$^{-/-}$ *Igκ*$^{h/m}$ BM cells and splenocytes. In accordance with the known survival defect of mature *Nemo*$^{-/-}$ B cells, we found relatively few of the most mature B220^{high} cells in *Nemo*$^{-/-}$ *Igκ*$^{h/m}$ BM (Fig. 4.6a and Sasaki et al., 2006). As we have previously observed that these mature cells tend to exhibit the highest levels of allelic inclusion, we analyzed Igκ^{h} and Igκ^{m} expression both in total B220^{+} CD43^{-} cells (Fig. 4.6a, red box), and in the less mature B220^{intermediate} CD43^{-} population (Fig. 4.6b, blue box). In both cases we found an approximately 50% increase of Igκ^{h}/Igκ^{m} dual expressing cells among *Nemo*$^{-/-}$ *Igκ*$^{h/m}$ BM cells compared to *Nemo*$^{+/+}$ *Igκ*$^{h/m}$ (Fig. 4.6b). We found a similar 50% increase in allelic inclusion in *Nemo*$^{-/-}$ *Igκ*$^{h/m}$ splenocytes. In contrast, *Atm*$^{-/-}$ *Igκ*$^{h/m}$ BM cells and splenocytes exhibit an approximately 2-fold increase in Igκ allelic inclusion (Steinel et al., 2013). These results are consistent with the mechanistic data presented in Chapter 3, where we show that ATM is absolutely required for suppression of *Rag1* and *Rag2* expression in response to DSBs, while Nemo is only partially responsible for this suppression. Together, these data are consistent with a role for Nemo in enforcing allelic exclusion, likely as one downstream effecter of ATM activity.

Discussion

Steinel et al. originally proposed that ATM regulates allelic exclusion through its role in inhibiting *Rag1* and *Rag2* expression in response to a RAG-mediated DSB (Steinel et al., 2013). Here, we demonstrate several parallels between the regulation of allelic

exclusion and the regulation of *Rag1* and *Rag2* expression by DSBs. First, we find that DSBs suppress *Rag1* and *Rag2* expression in pro-B and pre-B cells and in DN thymocytes, the cell subsets that rearrange the allelically excluded antigen receptor chains IgH, Igκ, and TCRβ. Conversely, pre-T cells rearranging the non-allelically excluded TCRα chain do not suppress *Rag1* and *Rag2* expression in response to DSBs. This finding suggests suppression of *Rag1* and *Rag2* expression by DSBs is common mechanism for enforcing allelic exclusion, similar to the common role of inhibition from cell surface antigen receptors and asynchronous initiation of recombination (Mostoslavsky et al., 2004). Furthermore, we find that ATM is important for promoting allelic exclusion of IgH and TCRβ, consistent with a role for DSB-mediated suppression of *Rag1* and *Rag2* expression in promoting allelic expression in pro-B cells and DN thymocytes. Finally, we find that the NF-κB regulatory protein Nemo plays a role in both regulation of *Rag1* and *Rag2* expression by DSBs and in allelic exclusion. Collectively, these results provide strong support for the hypothesis that feedback inhibition by RAG-mediated DSBs plays an important role in promoting allelic exclusion.

Notably, the inability of constitutive *Rag1* expression to restore V(D)J recombination activity in pre-B cells experiencing etoposide-induced DSBs (Fig. 2.6e) suggests that the inhibitory signal may involve more than simply suppression of *Rag1* and *Rag2* transcription. Additional factors might involve post-translational modification of Rag1 and/or Rag2 proteins or decreased accessibility of antigen receptor loci.

Additional experiments will be necessary to determine the relative importance of *Rag1* and *Rag2* mRNA suppression and alternative mechanisms in promoting allelic exclusion.

We did not find a role for Spi-C in enforcing Igκ allelic exclusion, despite the published finding that Spi-C expression is induced by RAG-mediated cleavage of Igκ and can suppress Igκ cleavage in the absence of ATM activity (Bednarski et al., 2016). The published data shows Spi-C induction and activity over the course of days; it is possible that Spi-C induction is irrelevant in the much shorter timeframe of normal V(D)J recombination cleavage and repair. Indeed, we see induction of *Spic* mRNA expression when measuring Igκ cleavage in a pre-B cell line over the course of three days; however we do not see any induction within four hours of exposing primary pre-B cells to IR or etoposide (Fig. 4.5a and data not shown). Furthermore, the effect of Spi-C on Igκ cleavage was only shown in ATM-deficient cells; it is possible that it is relatively unimportant in the presence of normal ATM activity. In this respect, it is possible Spi-C induction complements the function of other factors acting downstream of ATM, such as suppression of *Rag1* and *Rag2* expression. Additional experiments will be necessary explore the possibility that Spi-C induction and suppression of *Rag1* and *Rag2* expression act synergistically to inhibit V(D)J recombination and promote allelic exclusion.

Figure 4.1

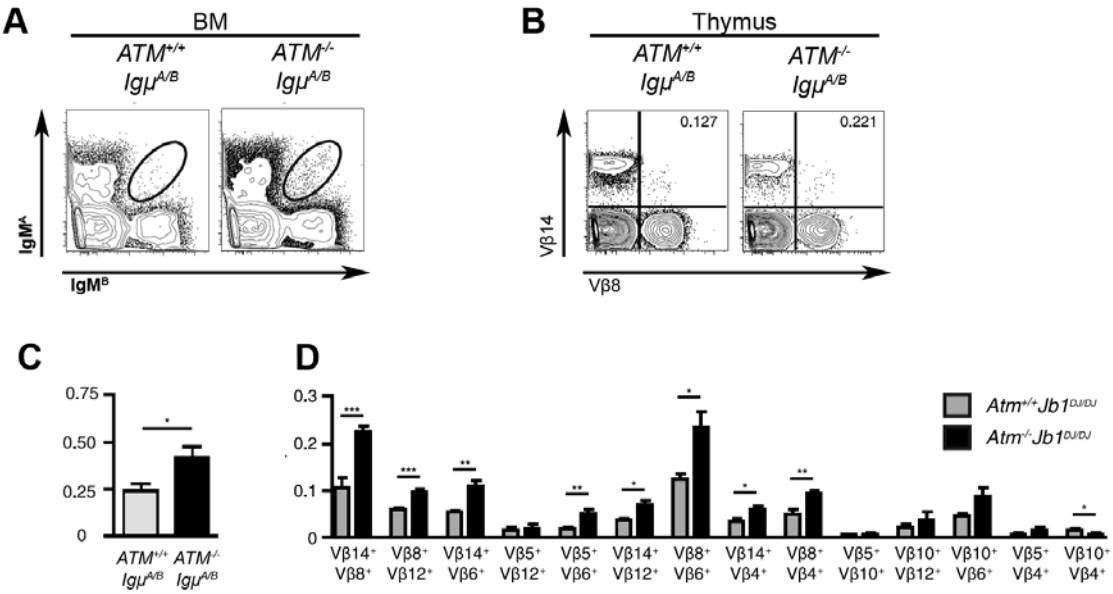


Figure 4.1: Increased allelic inclusion of IgH and TCRβ in ATM-deficient mice. **(A)** Representative flow cytometry analysis showing cell surface expression of allotypically marked IgM proteins in $Atm^{+/+}Jb1^{DJ/DJ}$ or $Atm^{-/-}Jb1^{DJ/DJ}$ mice B220+ BM cells. **(B)** Representative flow cytometry analysis showing cell surface expression of Vβ8 and Vβ14 among $Atm^{+/+}Jb1^{DJ/DJ}$ or $Atm^{-/-}Jb1^{DJ/DJ}$ mice TCRβ+ thymocytes. **(C)** Quantification of dual IgM expressing B cells as shown in (A). **(D)** Quantification of thymocytes with dual expression of the indicated Vβ pairs as shown in (B). p values determined by t-test. Error bars indicate SEM.

Figure 4.2

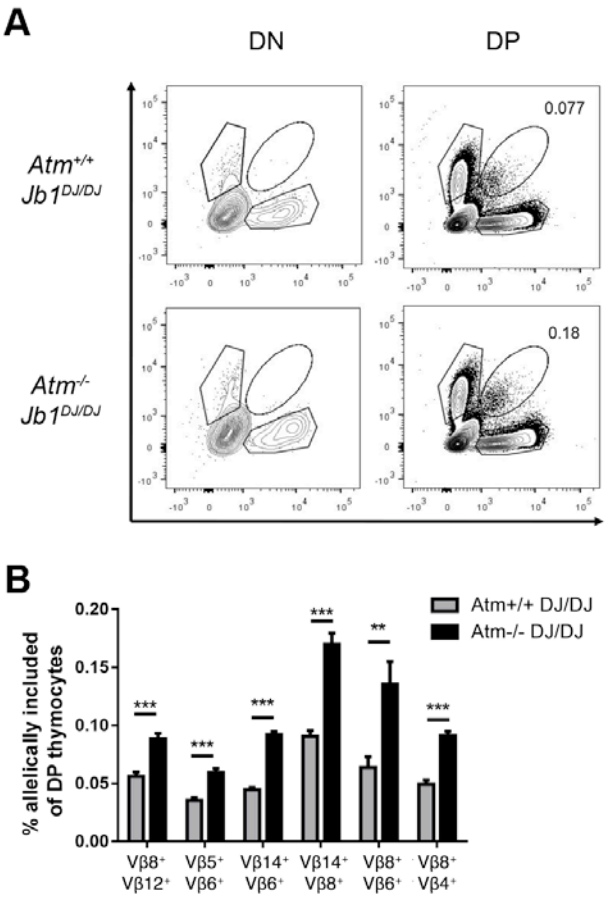
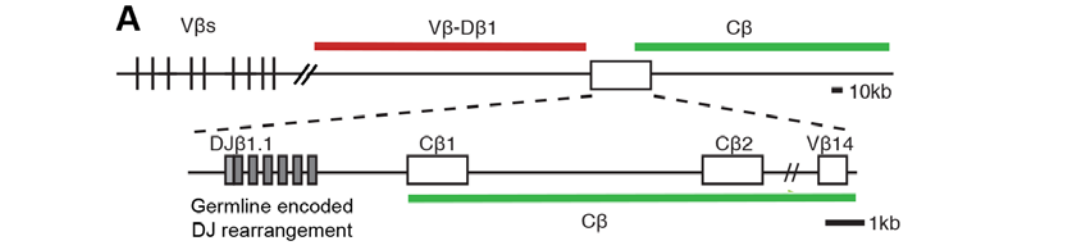
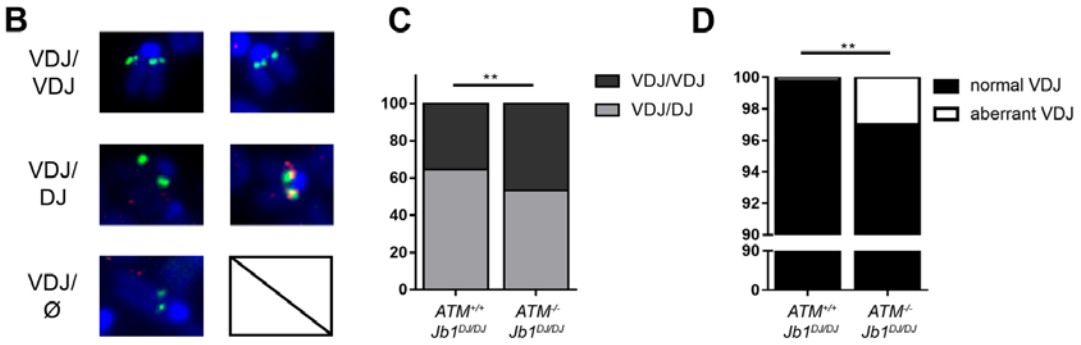


Figure 4.2: Increased allelic inclusion in ATM-deficient thymocytes is established by the DP stage of development. **(A)** Representative flow cytometry analysis of intracellular expression of Vβ14 and Vβ8 expression in DN or DP thymocytes isolated from *Atm*$^{+/+}$*Jb1*$^{DJ/DJ}$ or *Atm*$^{-/-}$*Jb1*$^{DJ/DJ}$ mice. The circle gates indicate where cells expressing equivalent high levels of both Vβ14 and Vβ8 should be visualized. DN thymocytes lacked detectable populations of Vβ14^{+}Vβ8^{+} cells, whereas DP thymocytes contained populations that comprised the indicated percentage of DP cells. **(B)** Quantification of the average frequencies of DP thymocytes that express each indicated combination of Vβ chains in their cytoplasm as depicted in (A). Data are from two independent experiments conducted on a total of seven *Atm*$^{+/+}$*Jb1*$^{DJ/DJ}$ and eight *Atm*$^{-/-}$*Jb1*$^{DJ/DJ}$ littermate mice. Error bars indicate SEM.

Figure 4.3





E

ATM$^{-/-}$ DJ/DJ	#	%
VDJ/DJ	87	65.4
VDJ/VDJ	46	34.6
	133	

F

ATM$^{-/-}$ DJ/DJ	#	%
VDJ+/VDJ+	3	7.1
VDJ+/VDJ-	39	92.9
total	42	

Figure 4.3: ATM-deficient T cells include a high frequency of cells with two TCRβ VDJ rearrangements. **(A)** Schematic of the TCRβ locus including with pre-rearranged *Jb1*$^{DJ/DJ}$ allele. Red and green lines indicate areas of probe hybridization for FISH analysis. **(B)** Examples of cells containing 2 rearranged TCRβ alleles (VDJ/VDJ), 1 rearranged allele and 1 unrearranged allele (VDJ/DJ), or 1 rearranged allele and a deletion of the second allele (VDJ/Ø). **(C)** Frequency of *Atm*$^{+/+}$*Jb1*$^{DJ/DJ}$ or *Atm*$^{-/-}$*Jb1*$^{DJ/DJ}$ spleen cells with one vs. two rearranged TCRβ alleles, as measured by FISH. p value calculated by chi-square test. **(D)** Frequency of aberrant rearrangement events (deletions, translocations, duplications) in *Atm*$^{+/+}$*Jb1*$^{DJ/DJ}$ vs. *Atm*$^{-/-}$*Jb1*$^{DJ/DJ}$ spleen cells containing at least one TCRβ rearrangement, as measured by FISH. p value calculated by chi-square test. **(E)** Frequency of cells with one vs. two TCRβ rearrangements as determined by Southern analysis of *ATM*$^{-/-}$ *Jb1*$^{DJ/DJ}$ T cell hybridoma lines. **(F)** Frequency of cells with one vs. two productive TCRβ rearrangements as determined by PCR and Sanger sequencing of TCRβ rearrangements from *ATM*$^{-/-}$ *Jb1*$^{DJ/DJ}$ T cell hybridoma lines.

Figure 4.4

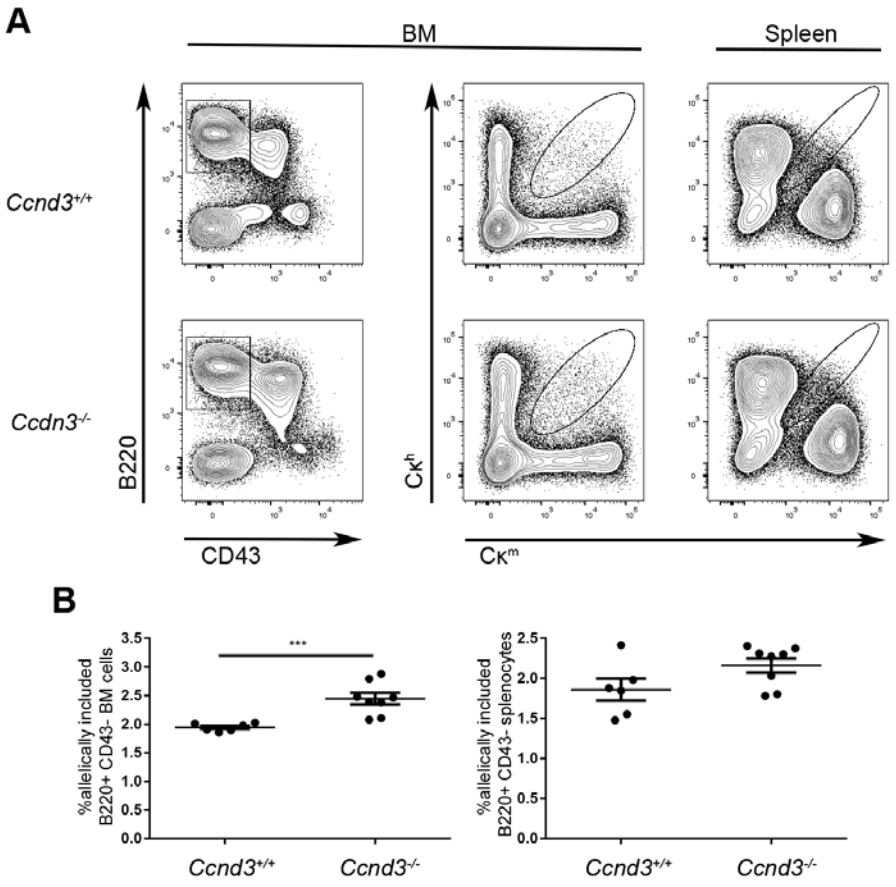


Figure 4.4: *Ccnd3*$^{-/-}$ B cells exhibit a modest increase in Igκ allelic inclusion. **(A)** Representative FACS plots showing Igκ^{h} and Igκ^{m} expression in *Ccnd3*$^{+/+}$ *Igκ*$^{h/m}$ and *Ccnd3*$^{-/-}$ *Igκ*$^{h/m}$ BM and spleen cells. The gate represents cells expressing equivalently high levels of both Igκ^{h} and Igκ^{m}. **(B)** Quantification of B220^{+} CD43^{-} *Ccnd3*$^{+/+}$ *Igκ*$^{h/m}$ and *Ccnd3*$^{-/-}$ *Igκ*$^{h/m}$ BM and spleen cells expressing both Igκ^{h} and Igκ^{m} as depicted in (A). p-values determined by t-test. Error bars represent SEM.

Figure 4.5

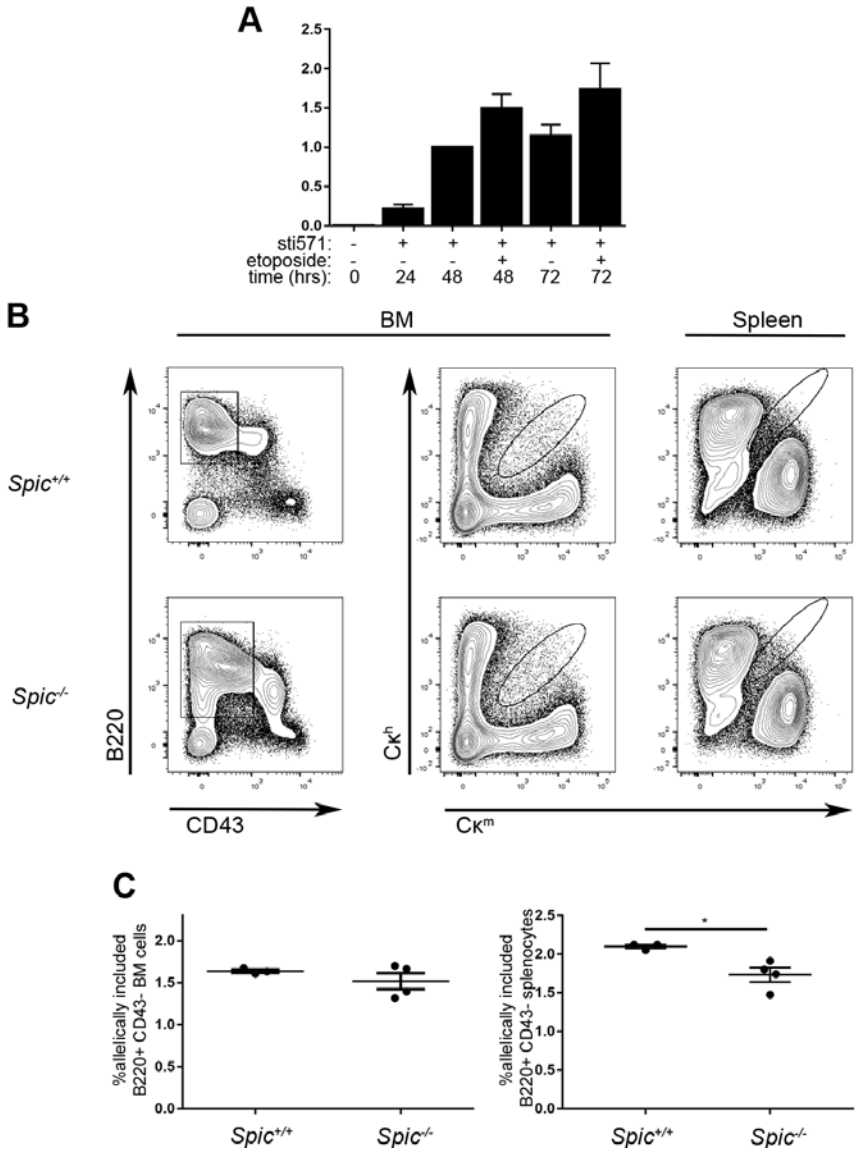


Figure 4.5: *Spic*$^{-/-}$ B cells exhibit normal Igκ allelic inclusion. **(A)** Spic mRNA expression in *Artemis-/-* Abelson pre-B cells exposed to sti571 alone or sti571 and etoposide for the indicated times. **(B)** Representative FACS plots showing Igκ^{h} and Igκ^{m} expression in *Spic*$^{+/+}$ *Igκ*$^{h/m}$ and *Spic*$^{-/-}$ *Igκ*$^{h/m}$ BM and spleen cells. The gate represents cells expressing equivalently high levels of both Igκ^{h} and Igκ^{m}. **(C)** Quantification of B220^{+} CD43^{-} *Spic*$^{+/+}$ *Igκ*$^{h/m}$ and *Spic*$^{-/-}$ *Igκ*$^{h/m}$ BM and spleen cells expressing both Igκ^{h} and Igκ^{m} as depicted in (B). p-values determined by t-test. Error bars represent SEM.

Figure 4.6

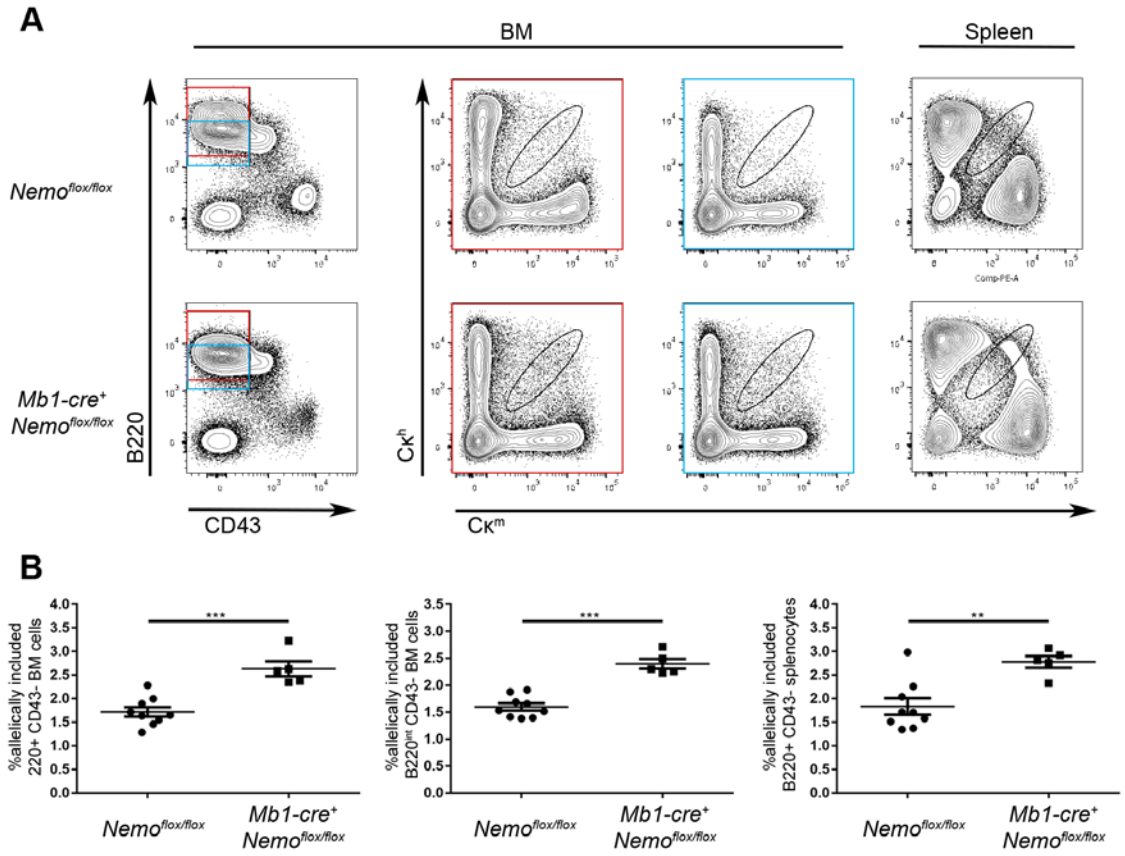


Figure 4.6: *Nemo*$^{-/-}$ B cells exhibit increased in Igκ allelic inclusion. **(A)** Representative FACS plots showing Igκ^{h} and Igκ^{m} expression in *Nemo*$^{+/+}$ *Igκ*$^{h/m}$ and *Nemo*$^{-/-}$ *Igκ*$^{h/m}$ BM and spleen cells. The gate represents cells expressing equivalently high levels of both Igκ^{h} and Igκ^{m}. **(B)** Quantification of B220^{+} CD43^{-} cells or B220^{intermediate} CD3^{-} *Nemo*$^{+/+}$ *Igκ*$^{h/m}$ and *Nemo*$^{-/-}$ *Igκ*$^{h/m}$ BM and spleen cells expressing both Igκ^{h} and Igκ^{m} as depicted in (A). p-values calculated by t-test. Error bars represent SEM.

Chapter 5: Discussion

V(D)J recombination is crucial for lymphocyte development and immune function, but poses risks at multiple levels. Deliberate cleavage and rearrangement of DNA could lead to genomic instability and malignant transformation (Schlissel, 2006). The largely random nature of the final product necessitates stringent selection processes to promote the development of cells with useful receptors and eliminate those with potentially harmful ones (Pelanda and Torres, 2012; Xing and Hogquist, 2012). In light of these hazards, it has long been recognized that lymphocytes carefully regulate V(D)J recombination in many respects. Most obviously, V(D)J recombination is strictly limited to developing lymphocytes, and specific rearrangement events are restricted to certain developmental windows. Within those windows, it is generally found that only one antigen receptor gene rearranges at a time (Hewitt et al., 2009; Chan et al., 2013; Chaumeil et al., 2013). This asynchronous recombination, combined with feedback signals generated by newly synthesized antigen receptors, leads to allelic exclusion of several antigen receptors (Brady et al., 2010). In this work, we describe a signal originating from DNA double strand breaks, which activates a conserved DNA damage response pathway involving ATM and Nemo. We build on previous work from the Bassing laboratory to show that this pathway helps to promote allelic exclusion in developing B and T cells.

Regulation of Rag1 and Rag2 expression

The regulation of *Rag1* and *Rag2* expression has mostly been studied in relationship to developmental signals. Most notably, many factors that turn on *Rag1* and *Rag2* expression at appropriate stages of T and particularly B cell development have been identified. The previously identified suppressive signals originate from cell-surface antigen receptors, which have long been known to turn off *Rag1* and *Rag2* expression (Li et al., 1993; Turka et al., 1991). The work presented in this dissertation shows that a very different kind of stimulus, DNA damage, can also turn off *Rag1* and *Rag2* expression. We also find that LPS stimulation suppresses *Rag1* and *Rag2* expression in pre-B cells. These findings suggest that *Rag1* and *Rag2* expression is subject to more complex regulation than has generally been considered. Additional work will be necessary to determine how these signals affect lymphocyte development *in vivo*.

A model of our findings and open questions regarding the mechanism by which RAG-mediated or exogenous DSBs suppress V(D)J recombination is depicted in Fig. 5.1. To summarize, we have shown that DSBs activate ATM, which is required for transcriptional suppression of *Rag1* and *Rag2*. Loss of *Rag1* and *Rag2* transcripts is accompanied by loss of Rag1 protein. Lack of the complete RAG recombinase prevents cleavage of antigen receptor loci. However, the inability of Rag1 reconstitution alone to

promote Igκ cleavage in the presence of DSBs (Fig. 2.6) suggests that additional signals may act in parallel to suppress V(D)J recombination in the presence of DSBs.

The role of NF-κB factors in regulating *Rag1* and *Rag2* expression will be of particular interest. As discussed in Chapter 3, the literature on this subject and our own work present several apparent contradictions. Ambiguity in precisely which NF-κB factors are manipulated in different studies may account for some of this confusion. Our own work shows that two different IKKβ inhibitors have different effects on *Rag1* and *Rag2* expression. The more specific inhibitor leads to a slight increase in *Rag1* and *Rag2* expression in pre-B cells and partially prevents suppression upon IR exposure. Another inhibitor that affects IKKα as well as IKKβ leads to a slight decrease in *Rag1* expression and a substantial decrease in *Rag2* expression, which makes the effect of IR difficult to interpret. These results suggest that both IKKs have a modest basal effect on *Rag1* and *Rag2* expression, and that these effects oppose each other.

Another possibility is that the effect of NF-κB signaling may change during the course of development. Expression of many NF-κB factors changes as cells approach maturity (Sup. Fig. 3.4 and Kistler et al., 1998); a change in the relative expression in different factors might change the target genes they bind to and their activating or inhibitory effects on transcription of those genes. One study to date that shows an inhibitory effect of NF-κB signaling on *Rag1* and *Rag2* expression dealt with expression

in immature B cells (Verkoczy et al., 2005). It is possible that NF-kB signaling earlier in development could have different effects. It is also possible that different stimuli that activate NF-κB might simultaneously activate other signaling pathways that interact with and modify NF-κB signals.

Potential role for parallel signals in suppressing V(D)J recombination

The failure of constitutive *Rag1* mRNA expression to restore V(D)J recombination activity in the presence of etoposide suggests that additional signals beyond suppression of Rag1 expression are involved. Given the demonstrated involvement of ATM, direct phosphorylation of Rag1 or Rag2 proteins may occur. Inactivation by phosphorylation could have the advantage of acting more rapidly than transcriptional suppression of *Rag1* and *Rag2*, as it takes some time to deplete the Rag1 protein pool by degradation. Both proteins contain motifs that could be targeted by ATM or DN-PKcs, a related kinase that is also activated by RAG-mediated DSBs (Gapud et al., 2011).

Phosphorylation by other kinases might also play a role. One study demonstrated that some Rag2 protein is exported from the nucleus following IR, and that export requires the cdk-phosphorylation site T490 (Rodgers et al., 2015). Regulated degradation of Rag2 at the G1/S checkpoint also depends on phosphorylation of T490 (Jiang et al., 2005). However given that neither we nor the Rodgers group observe

degradation of Rag2 protein upon IR, T490 phosphorylation may play another role in this circumstance.

Another potential method of suppressing RAG activity is to decrease the accessibility of target antigen receptor loci. In this regard, we looked for a role for the Ets-family member Spi-C, which was recently shown to decrease Igκ locus accessibility, presumably by competing with transcriptionally active Ets family (Bednarski et al., 2016). Although we confirmed the finding that Spi-C is induced in cells accumulating RAG-mediated DSBs, we did not find a role for this protein in allelic exclusion. It is possible that it plays a secondary role, perhaps complementing the effect of suppression of *Rag1* and *Rag2* expression. As-yet unidentified factors acting at antigen receptor loci may also play a role.

Allelic exclusion and genomic stability

The results presented in Chapter 4 and previously published studies suggest that ATM suppresses allelic inclusion by promoting monoallelic rearrangement of antigen receptor alleles (Chaumeil et al., 2013; Steinel et al., 2013). Monoallelic rearrangement may also be important for genomic stability of developing lymphocytes, as formation of a productive rearrangement that leads to developmental progression could lead to aberrant rearrangement of a second allele that remains unrepaired. The proliferative burst that occurs after the pro-B cell or DN thymocyte stages may pose a particular

hazard in this regard, which may be one reason that allelic exclusion is enforced more strictly for the IgH and TCRβ chains than for Igκ and TCRα (Brady et al., 2010).

Allelic exclusion and autoimmunity

Although allelic exclusion of antigen receptors was first described over fifty years ago, its importance is still debated. A long-standing hypothesis posits that allelic exclusion is required for proper positive and negative selection of developing lymphocytes (Vettermann and Schlissel, 2010). Central tolerance mechanisms that cull self-reactive B and T cells depend stimulation of those cells through their cell surface receptors. In an allelically included cell, the cell-surface concentration of an autoreactive receptor might be diluted by a

+ second innocuous receptor so that it does not trigger central tolerance mechanisms that would normally lead to removal of the autoreactive receptor from the repertoire. However, if the cell later became activated through the innocuous receptor, the activity of the self-reactive receptor might lead to autoimmune disease (Vettermann and Schlissel, 2010).

Experimental evidence regarding this idea has been mixed. Several groups have shown that cells engineered to express antigen receptor chains likely to mediate self-reactivity have shown that co-expression of an endogenous receptor can indeed allow development of cells carrying self-reactive receptors; however in most cases resulting

autoimmune disease was not reported, perhaps because peripheral tolerance mechanisms provided sufficient protection (Iliev et al., 1994; Zal et al., 1996; Gerdes and Wabl, 2004). In contrast, one study employing transgenic expression of both TCR and the cognate antigen found that co-expression of an endogenous TCRα chain could allow T cells to evade tolerance mechanisms and cause diabetes (Sarukhan et al., 1998). A limitation of all these studies is the extreme overrepresentation of a single self-reactive receptor, a situation that is not representative of a normal immune system. One study instead used transgenic expression of an Igκ chain that was not expected to be particularly self-reactive; this led to a relatively high frequency of Igκ/λ inclusion, but not to noticeably high levels of autoantibody production or autoimmune disease (Sirac et al., 2006). Finally, several groups have examined the self-reactivity of naturally occurring allelically included cells; conflicting results have come from these studies. In studies of lupus prone mice, one group found that cells expressing two Igκ chains were more likely than single-expressers to produce autoantibodies, while another group using a different lupus model found the opposite (Fournier et al., 2012; Makdasi and Eilat, 2013). The use of two different models suggest that strain differences may account for this contradiction. It is possible that lapses in other tolerance mechanisms would be necessary to reveal any increased autoreactive potential possessed by allelically included lymphocytes.

Steps toward a new model of allelic inclusion

A tool that has been lacking in this field is a model of increased allelic inclusion in the absence of other immune system abnormalities. Antigen receptor transgenic models skew the immune repertoire unnaturally. Unfortunately, although deficiency of either ATM or Nemo increases allelic inclusion, these mice are also not ideal models. ATM deficient mice are somewhat lymphopenic, have impaired specific antibody production, and develop lymphoma by four months of age, all of which confound investigations into of the effects of allelic inclusion on the immune system (Xu et al., 1996). Nemo-deficient B cells develop fairly normally but do not die upon reaching maturity (Sasaki et al., 2006). It is our hope that further elucidation of the signaling events leading to the suppression of RAG expression and V(D)J recombination will allow a more targeted disruption of the signal *in vivo*, increasing allelic inclusion without impairing critical immune functions.

A first, relatively blunt approach will be to constitutively express Rag1. Constitutive expression of both Rag1 and Rag2 was previously reported; in this case a decrease in allelic exclusion was reported, but this might be due to the severe impairment of B and T cell development reported in these mice (Barreto et al., 2001). It was surmised that impaired development was due to RAG activity during inappropriate developmental stages; by constitutively expressing only Rag1 we hope to avoid this

problem. Our finding that Rag2 protein persists in the presence of DSBs due to its long half-life suggests that maintaining expression of Rag1 alone will be sufficient to overcome the transient suppressive signal activated by a RAG-mediated DSB and promote allelic inclusion. However, a more thorough understanding how *Rag1* and *Rag2* transcription is suppressed by DSBs might allow us to take a more nuanced approach to disrupting this signal. For example, if we identify a suppressive factor that turns off *Rag1/Rag2* transcription, it may be possible to mutate its binding site to specifically prevent suppression of *Rag1* and *Rag2* expression.

V(D)J recombination and genomic stability

In addition to promoting allelic exclusion, we propose that suppression of Rag expression and V(D)J recombination serves to limit the probability of antigen receptor translocation. When antigen receptor loci are involved in translocations, they can drive the expression of proto-oncogenes and promote malignant transformation (Schlissel, 2006). A translocation necessarily requires DNA breaks at two genomic locations. Accordingly, it has been formally shown that increasing the frequency of DSBs at a locus increases the frequency of translocation (Richardson and Jasin, 2000). Therefore, cleavage of an antigen receptor locus in the presence of a pre-existing DSB is likely to be particularly risky. The models proposed above to study allelic exclusion will also be

helpful in testing the hypothesis that suppression of *Rag1* and *Rag2* expression by DSBs helps to prevent oncogenic antigen receptor translocations.

Concluding remarks

The work presented in this dissertation shows how developing lymphocytes use the conserved DNA damage response to address lymphocyte-specific developmental needs. These data add to our understanding of Rag expression and its role in allelic exclusion. The ability of DSBs to regulate *Rag1* and *Rag2* expression in both developing B and T cells, which otherwise regulate *Rag1/Rag2* using largely distinct mechanisms, suggests that this signal plays an important role in lymphocyte biology.

Figure 5.1

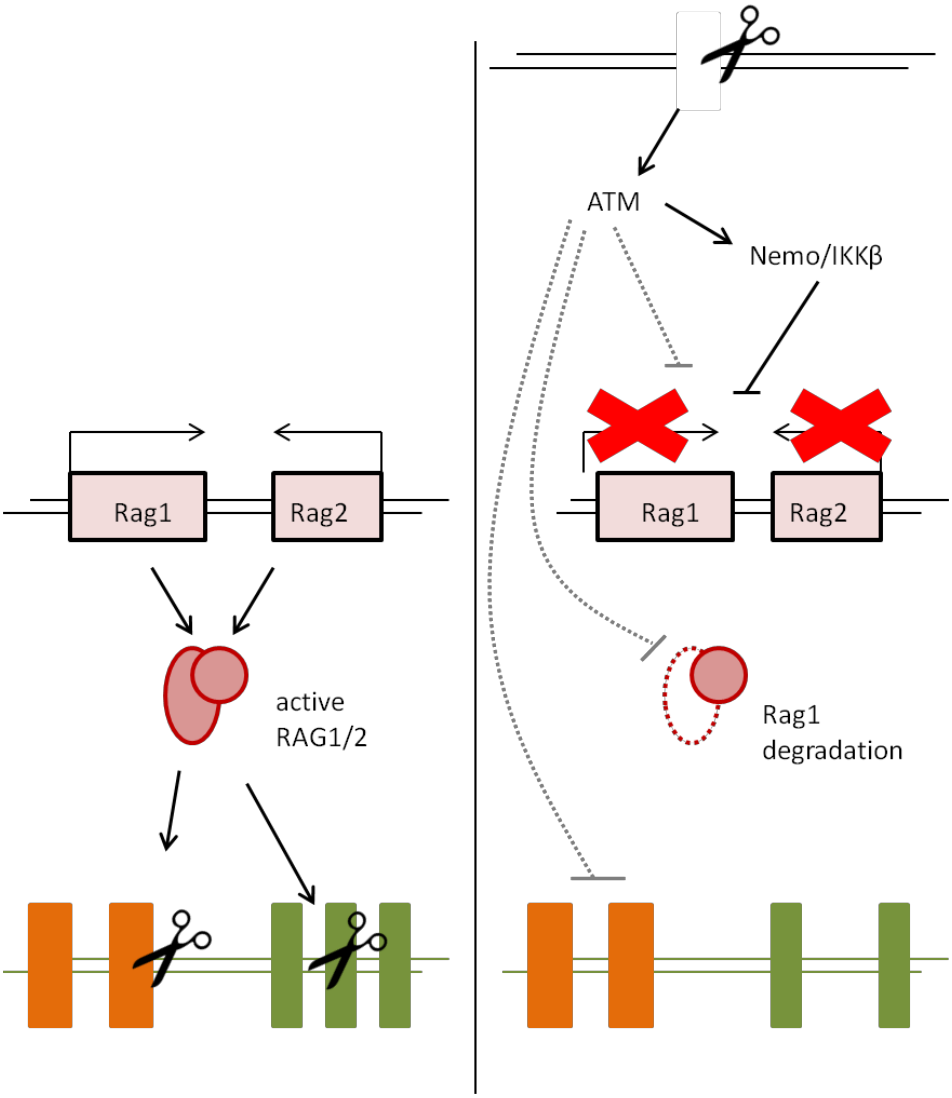


Figure 5.1: Model of inhibition of V(D)J recombination by DSBs. Left panel shows a normal situation in which *Rag1* and *Rag2* are transcribed and produce protein, leading to RAG-mediated cleavage of an antigen receptor locus. Right panel shows DSB signaling to suppress V(D)J recombination. Black, solid lines represent signals described in this dissertation. Gray, dotted lines represent hypothetical signals that might cooperate with loss of *Rag1* and *Rag2* transcription and Rag1 protein in suppressing V(D)J recombination.

Methods

Mice

All mice used within this study were housed, bred, and used under pathogen-free conditions at the Children's Hospital of Philadelphia (CHOP). All experiments were performed using 4-6 week old mice using both male and female mice. Experimental mice were euthanized by exposure to CO_2 followed by cervical dislocation. Animal husbandry and experiments were performed in accordance with national guidelines and regulations and approved by the CHOP Institutional Animal Care and Use Committee. The *EμBCL2* (Strasser et al., 1991b) $Atm^{-/-}$, $Mb1Cre^{+}Atm^{flox/flox}$(Steinel et al., 2013), $VavCre^{+}p53^{flox/flox}$ (DeMicco et al., 2013), $Mb1Cre^{+}Nemo^{flox/flox}$ (Derudder et al., 2009) mice were on a mixed 129S1/SvImJ and C57BL/6 background, while $Rag1^{-/-}$ (Mombaerts et al., 1992a) and $Rag2^{-/-}$ (Hao and Rajewsky, 2001) mice were on the C57BL/6 background.

Irradiation

All mice were subject to irradiation using an XRAD320 X-ray irradiator (Precision X-Ray) and a dose rate of 0.74 Gy/min. Primary cells and cell lines were subject to irradiation using a Gammacell 1000 Cs-137 irradiator (Nordion Inc) and a dose rate of 1.8 Gy/min.

qRT-PCR quantification of Rag1, Rag2, and p21 mRNAs

Non-irradiated and irradiated cells were harvested at indicated time points and immediately lysed in Trizol (Life Technologies) and processed according to the manufacturer's instructions. Isolated RNA was treated with DNase (Promega) according to manufacturer directions, primed with random nonamers (New England Biolabs, NEB), and reverse transcribed with M-MuLV (NEB) to generate cDNA. The cDNAs were then used as template for qRT-PCR reactions were performed with SYBR green mastermix (Applied Biosystems) and run on an Applied Biosystems 7500 Fast machine. Values were normalized to housekeeping genes as indicated in the text and fold change was determined by the ΔΔCT method. The primers used for qRT-PCR reactions are listed in Table 1. When pre-B cells were subject to irradiation, non-irradiated pre-B cells also were transported to the irradiator, but not exposed to ionizing radiation along with irradiated samples. The irradiated and non-irradiated pre-B cells were each placed back into culture until they were harvested. Since pre-B cells were transported to and from the irradiator at room temperature, the basal levels of Rag1, Rag2, and p21 mRNAs were lower at 1 hour after the irradiation time point than at 4 hours after the irradiation time point.

Small molecule inhibitors

To inactivate the ATM kinase, the KU55933 ATM kinase inhibitor was added to media at a concentration of 15 μM. To block new protein synthesis, the ribosome inhibitor cycloheximide was added to media at a concentration of 10 μg/mL. To block protein degradation, the proteosome inhibitor MG132 was added to media at a concentration of 10 μM. To inhibit IKKβ either 5μM BMS-345541 or 20μM ML120B was used. To induce DSBs by genotoxic drugs, etoposide was added to the media at a concentration of 10 μg/mL or bleomycin was added at a concentration of 5 μM.

Primary pre-B cell cultures

Primary bone marrow (BM) cells were harvested by flushing BM from all leg bones of at least four mice of the appropriate genotype for each experiment. These BM cells were cultured for 3-5 days in RPMI supplemented with 10% FBS, 10 mM HEPES, 1 x non-essential amino acids, 1 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL pen-strep, 30 μM β-mercaptoethanol and 50 ng/mL IL-7. Cells were plated at a density of 5 million cells per mL of media. Each day, cells were harvested and put back into culture in fresh media at a density of 5 million cells per mL. To induce G1 arrest and activate transcription of *Rag1* and *Rag2* by IL7 withdrawal, cells were pelleted by centrifugation, re-suspended in the same media lacking IL7 at a density of 5 million cells per mL, and treated as described for each experiment.

Western blots

Pre-B cells were washed with PBS and re-suspended in ice cold lysis buffer (20 mM Tris pH 7.4, 20 mM glycerol phosphate, 10 mM sodium orthovanadatem, 10% glycerol, 0.5 mM EDTA, 0.5 mM $MgCl_2$, 200 mM NaCl, and 0.2% Triton-X) and then sonicated at intervals of 30 seconds on and 30 seconds off for 8 minutes at 4°C. The sonicated cells were incubated on ice for 10 minutes and then centrifuged to remove insoluble material. Laemmli buffer was added and then samples were boiled for 5 minutes. Lysates from equivalent numbers of pre-B cells were loaded and run on NuPage tris-glycine gels (Life Technologies). Electrophoresed proteins were transferred to Immobilon-FL PVDF membrane (EMD Millipore). Membranes were blocked with Odyssey blocking buffer (Li-Cor) according to manufacturer's instructions. Antibodies used are: anti-Rag1 or anti-Rag2 monoclonal antibody (Coster et al., 2012); anti-actin antibody (Sigma AV40173); anti-Gadd45α (Santa Cruz sc-797); anti-phospho IKKα/IKKα (Cell Signaling Technology 2697S); anti-IKKα/IKKα (Santa Cruz sc-7607). Blots were washed and incubated with appropriate IRDye800 secondary antibodies (LiCor) according to manufacturer's instructions. After washing, blots were scanned on an Odyssey infrared scanner (Li-Cor).

Click-It analysis of Rag1 and Rag2 mRNA turnover and Rag1 and Rag2 transcription

These assays were conducting using Click-It® Nascent RNA Capture kits (Life Technologies). For mRNA turnover assays, ethynyl uridine (EU) was added to medium of IL7 withdrawn pre-B cell cultures at a final concentration of 0.2 mM for the final 16 hours of culture time. Pre-B cells were washed, placed into media lacking EU, and then split into pools that were immediately irradiated or left non-irradiated. Cell pools were collected for RNA isolation immediately before EU removal or at indicated times after EU removal or EU removal and irradiation. For transcriptional assays, pre-B cells were grown in media lacking EU. Cultures were split into pools that were either irradiated or left non-irradiated. Immediately after irradiation of some pools, EU was added to the media of all pools at a final concentration of 0.5 mM. After the indicated times, cells were collected for RNA isolation. For both assays, RNA was isolated using Trizol (Life Technologies) according to the manufacturer's instructions. Click chemistry and streptavidin pull down of EU-labeled RNA were performed according to the Click-It Nascent RNA Capture kit's instructions. Pulled-down RNA was reverse transcribed and analyzed by qRT-PCR as described above. The loading controls for mRNA turnover and transcription experiments were 18S RNA and Hprt mRNA, respectively.

Abelson pro-B cell cultures

The *EμBCL2* (A2) and *Artemis*$^{-/-}$*EμBCL2* (Art2.1) Abelson transformed and immortalized pro-B cell lines were previously described (Bredemeyer et al., 2006; Savic et al., 2009). We made the *EμBCL2-pINV* Abelson pro-B cell line using the same procedures that we previously used to generate cell lines with recombination substrates (Gapud et al., 2011). Abelsons pro-B cells were cultured in RPMI media supplemented with 10% FBS, 100 U/mL pen-strep, and 30 μM μ-mercaptoethanol To induce G1 cell cycle arrest, differentiation into pre-B cells, expression of *Rag1* and *Rag2* mRNA, and V(D)J recombination, 5 μM STI571 was added to the culture media.

Southern blot analysis of Jκ cleavage

Southern blotting was performed as described (Savic et al., 2009). Briefly, 15-20 μg of genomic DNA was digested with Sac1-HF (NEB) and EcoR1-HF (NEB). Southern blot membranes were probed with a *3'Jκ* probe and then a *5'Hprt* probe as a loading control. The intensities of bands were quantified using ImageJ software (NIH). For *Artemis*$^{-/-}$ *EμBLC2* cells, the percentage of *Jκ* cleavage at each time point was calculated by dividing the total intensities of *Jκ* coding end bands by the combined intensities of the germline *Jκ* band and *Jκ* coding end bands (Yin et al., 2009). For *EμBLC2* cells, the percentage of *Jκ* cleavage at each time point was calculated by dividing the intensity of the germline *Jκ* band by the intensity of the *5'Hprt* control band.

Flow cytometry for pMX-INV rearrangement

For analysis of GFP expression in *EμBCL2-pINV* cells, cells were washed in FACS buffer (PBS with 3% FCS and 0.25 mM EDTA) and then stained with PE-conjugated anti-human CD4 antibody (BD Pharmigen, clone RPA-T4) to stain cells containing the substrate. Flow cytometry was conducted using an LSR-II flow cytometer (BD Biosciences) and data was analyzed using FlowJo 10.

Flow cytometry for IgM allelic inclusion

Stains were conducted using the following Abs or reagents: FITC-anti-IgMa (DS-1; BD Biosciences), PE-anti-IgMb (AF6-78; BD Biosciences), Biotin-anti-CD23 (B3B4; BD Biosciences), PerCP/Cy5.5 anti-CD21/35 (7E9; BioLegend), and PE/Cy7-SA (BD Biosciences). Flow cytometry was conducted using an LSR-II flow cytometer (BD Biosciences) and data was analyzed using FlowJo 10. Surface IgM expression was assayed on singlet, live, B220+ cells.

Flow cytometry for intracellular TCRβ allelic inclusion

Surface stains were conducted using the following Abs or reagents from BD Biosciences: PE-anti-B220 (RA3-6B2), APC-anti-TCRβ (H57-597), V450-anti-CD4, TxRed-anti-CD8, . After surface staining, cells were permeabilized using BD Fix-Perm solution according to the manufacturer's instructions. After permeabilization cells were stained with

combinations of FITC or biotin conjugated: anti-Vb5 (MR9-4), anti-Vb14 (14-2), anti-Vb8 (F23.1), anti-Vb4 (KT4), anti-Vb6 (RR4-7), anti-Vb12 (MR11-1), and PE/Cy7-streptavidin. Flow cytometry was conducted on an LSR-II flow cytometer (BD Biosciences) and data was analyzed with FlowJo 10. Internal Vβ expression was assayed on singlet, live, cells.

Stimulation of αβ T cells for generation of hybridomas and for two-color fluorescence in situ hybridization assays

Single-cell suspensions were isolated from the spleens of 6-wk-old mice and depleted of RBCs with NH_4Cl lysis buffer prior to stimulation. Each spleen was stimulated for 48 h in 40 U/ml IL-2 and 5 mg/ml Con A at 4 ml/spleen in DMEM containing 15% FBS, 1% penicillin/streptomycin, 1% L-glutamate, and 30 mM β-mercaptoethanol. Additional medium was added to the stimulation after 24 h.

Two-color fluorescence in situ hybridization assay

T cells stimulated for 48 h were arrested in metaphase by incubating with colcemid (KaryoMax) and 0.45 mM BrdU (Sigma-Aldrich) for 2 h. Metaphase-arrested cells were isolated by hypotonic treatment (40 mM KCl, 0.5 mM EDTA, and 20 mM HEPES [pH 7.4]) and fixation in methanol:acetic acid (3:1 volume). The fixed cells were dropped on slides at 4°C and dried at 75°C for 5 min. Metaphase spreads were hybridized overnight with *Tcrb* bacterial artificial chromosome probes Vb-DbJb1, RP23-203H5 and Cβ, 164G11. Cβ probe was labeled using DIG-NICK Translation Mix (Roche). Vb-DbJb1 probe was labeled

using BioPrime DNA Labeling System (Invitrogen). Probes were detected using FITC-anti-digoxin Fab (Roche) and Texas Red-streptavidin (Vector Laboratories). Coverslips were mounted with Vectasheild mounting medium with DAPI (Vector Laboratories). Images were captured and analyzed using Case Data Manager (Applied Spectral Imaging).

Fusion and Analysis of T Cell Hybridomas

αβ T cell hybridomas were produced by fusion of ConA and Il-2 stimulated T cells with BW-1100.129.237 thymomas. Southern analyses of *Tcr*β rearrangements were performed as previously described (Khor and Sleckman, 2005).

PCR Analysis and Sequencing of Vβ Rearrangements in Hybridomas

Hybridoma genomic DNA was isolated and Vβ rearrangements were amplified by PCR using Vβ-specific primers and 3'Jβ1.2 primer (P2) as previously described (Bassing, Alt et al. 2000; Steinel, Brady et al. 2010). PCR products were resolved on a 1% agarose gel to identify which Vβs were utilized. PCR products were purified (Qiagen, QIAquick PCR Purification Kit, 28104) and sequenced using the 3'Jβ1.2 primer (P2).

Statistical analyses

All statistics were generated using GraphPad Prism 5 or Prism 7 software.

Table 1: qPCR primers	
18S F	CGCGGTTCTATTTTGTTGGT
18S R	AGTCGGCATCGTTTATGGTC
β actin F	TCATCACTATTGGCAACGAGCGGTTC
β actin R	TACCACCAGACAGCACTGTGTTGGCA
CD19 F	ACCAGTACGGGAATGTGCTC
CD19 R	TTCATAGGCCTCCCCTTCTT
Gadd45α F	CCGAAAGGATGGACACGGTG
Gadd45α R	TTATCGGGGTCTACGTTGAGC
Hprt F	CTGGTGAAAAGGACCTCTCG
Hprt R	TGAAGTACTCATTGTAGTCAAGGGCA
Lck F	CCTTCGGGATCTTGCTTACA
Lck R	GTTGTCAGGTCTCACCATGC
p21 F	GACATTCAGAGCCACAGGCAC
p21 R	GTCAAAGTTCCACCGTTCTCG
Rag1 F	TGGGAATCGTTTCAAGAGTGAC
Rag1 R	CATCTGCCTTCACGTCGATCC
Rag2 F	ACACCAAACAATGAGCTTTCCG
Rag2 R	CCGTATCTGGGTTCAGGGAC
Spic F	AAACATTTCAAGACGCCATTGAC
Spic R	CTCTGACGTGAGGATAAGGGT
Wasp F	GCTCCCTCCTACTCCAGTGTC
Wasp R	AGGGCACCTACTAGGCCTTC

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