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Determinants of DNA Damage Responses During B Cell Development

Rachel Leigh Johnston
Washington University in St. Louis

WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences
Molecular Cell Biology

Dissertation Examination Committee:
Jeffrey J. Bednarski, Chair
Luis Batista
Susana Gonzalo
Nima Mosammaparast
Zhongsheng You

Determinants of DNA Damage Responses During B Cell Development

by
Rachel Leigh Johnston

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Rachel Leigh Johnston

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Dedicated to my family.

ABSTRACT OF THE DISSERTATION

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Jeffrey J. Bednarski, Chair

Cellular DNA is regularly threatened by both physiologic (normal cellular mechanisms) and genotoxic (external exposures, such as irradiation and chemotherapy) insults. DNA double-stranded breaks (DSBs) are the greatest risk to the genome as they present opportunities for errant repair as mutations or translocations. To minimize these risks, DSBs activate highly conserved signaling pathways to coordinate cell cycle arrest, cell death and DNA repair. During normal development, B cells intentionally create site-specific chromosomal DSBs to assemble the immunoglobulin receptor (*Ig*) genes necessary for specific responses to pathogens. These programmed DNA breaks are generated by the recombinase activating gene (RAG) complex, a specific endonuclease that is selectively expressed in immune cells at discrete developmental stages. While essential for immune development, these RAG DSBs pose a significant risk as errors in the localization or response to these DSBs are a principal driver of oncogenic mutations and translocations in pediatric pre-B cell acute lymphoblastic leukemia (ALL). The cellular mechanisms that limit the extent of DNA damage and prevent oncogenic errors are unknown.

Previous work has established that RAG-mediated DSBs activate a B-cell specific developmental program, known as the non-canonical DNA damage response (ncDDR). Surprisingly, we find that exposure of B cells to ionizing radiation or chemotherapy, which generate DNA damage randomly throughout the genome, does not activate this ncDDR, but instead, promptly triggers rapid activation of cell death pathways, known as the canonical DNA damage response (cDDR). Thus, developing B cells respond to DSBs through distinct signaling pathways depending on the mechanism of DNA injury, as RAG DSBs induce both cDDR and ncDDR, whereas genotoxic DSBs selectively induce only the cDDR. I hypothesize that the specific response to RAG DSBs is determined by either: 1) the unique landscape of the *Ig* locus (i.e., genomic location of the DSB) or 2) distinct domains of the RAG endonuclease itself.

To examine the mechanisms downstream of DNA injury, I designed a novel CRISPR-Cas9 system using a tetracycline-inducible Cas9 in pre-B cells to generate targeted DSBs at precise locations throughout the genome. This approach was used to examine if all DSBs in pre-B cells trigger identical cellular responses. Pre-B cells were transduced with gRNAs to target Cas9 to regions outside of *Ig* loci. These Cas9-mediated DSBs in non-*Ig* genes activated the cDDR but did not trigger ncDDR. Thus, ncDDR signals are not a conserved component of pre-B cell response to all DSBs. Next, Cas9 DSBs were targeted to *Ig* loci to directly compare to a RAG DSB. Cas9-mediated DSBs at *Ig* also only activate cDDR. Therefore, while both Cas9 and RAG DSBs activate cDDR, the ncDDR program is uniquely activated by RAG DSBs. To investigate the underlying mechanisms of this specific response to RAG DSBs, mutagenesis studies of RAG1, a component of the RAG complex, were conducted. These experiments identified that non-core domains of RAG1 are critical for activation of the ncDDR developmental program. Thus, RAG DSBs induce distinct DDR programs in pre-B cells through novel nuclease-independent activities of RAG1.

Ongoing studies are further characterizing the functions of RAG1 in DDR signaling using RAG1-Cas9 fusion constructs to recruit RAG1 to non-RAG-mediated DSBs. Additionally, immunoprecipitation studies will determine the RAG1-binding partners at DSBs that regulate DDR signals, which have not been previously defined.

Overall, my dissertation characterizes the role of RAG1 in dictating DNA damage responses and demonstrates that B cells initiate distinct cellular responses to DNA breaks based on the mechanism of DNA injury. These studies provide key insights into a novel paradigm that directs cellular responses to DNA injury in pre-B cells. Understanding how RAG DSBs activate cellular programs to promote cellular survival and differentiation while maintaining genomic integrity has important implications for lymphocyte development. Selective activation of ncDDR is likely beneficial to developing pre-B cells as it promotes maturation of cells with recombined *Ig* genes. In contrast, non-RAG-mediated DSBs do not activate this process which may function to restrict abnormal B cell maturation and limit autoreactivity or malignant transformation. Further, defining these DSB-specific mechanisms that dictate cellular response may reveal previously unrecognized opportunities to optimize therapies for pre-B cell leukemia.

Chapter 1

Introduction

by
Rachel Leigh Johnston

1.1 Overview

Double stranded breaks (DSBs) are one of the most dangerous sources of DNA damage that can occur in a cell, as they can inactivate an essential gene through loss, amplification, or translocation of chromosomal material, which can lead to tumorigenesis (Khanna and Jackson, 2001). DNA damage can come from exogenous sources, such as environmental agents, as well as endogenous sources, from normal cellular processes, like immune cell development and replication during DNA synthesis (Ciccia and Elledge, 2010). One of these normal physiologic DSBs occurs during the assembly of the immunoglobulin (*Ig*) genes in developing B and T cells. Functional *Ig* genes must be generated by recombining distant gene segments to create the 2nd exon, which encodes the antigen binding portion of the antibody receptor chain (Fugmann et al., 2000a).

The DNA breaks generated for *Ig* gene assembly activate DNA damage responses (DDR) to coordinate DNA repair but also to induce developmental programs to promote B cell maturation. While extensive previous work has defined the factors that regulate DSB repair in developing B cells, the mechanisms that distinguish physiologic from non-physiologic DNA injury and that trigger activation of the developmental program are poorly understood. Recombinase activating gene (RAG)-mediated DSBs activate the serine/threonine kinase Ataxia-telangiectasia mutated (ATM), which promotes a canonical DNA damage response (cDDR) to coordinate DSB repair (Bredemeyer et al., 2008). In response to RAG DSBs, activation of ATM also induces a pre-B cell-specific developmental program (Bednarski et al., 2016; Bednarski and Sleckman, 2019; Bredemeyer et al., 2008). This non-canonical DNA damage response (ncDDR) is critical for continued survival and maturation of pre-B cells following RAG DSBs. Remarkably, exposure of B cells to ionizing radiation (IR) or chemotherapy, does not activate this ncDDR, but instead

promptly triggers rapid activation of only cDDR. My thesis focuses on what factors control the induction of ncDDR in response to a RAG DSB (**Figure 1.1**). Here, I present current knowledge of DDR in B cells and the role of RAG endonuclease, which creates DSBs in lymphocytes. I also present the non-endonuclease functions of RAG outside of DNA break generation and how RAG mutations appear in disease phenotypes. Altogether, this information provides context for my research on the DNA damage responses in lymphocyte development.

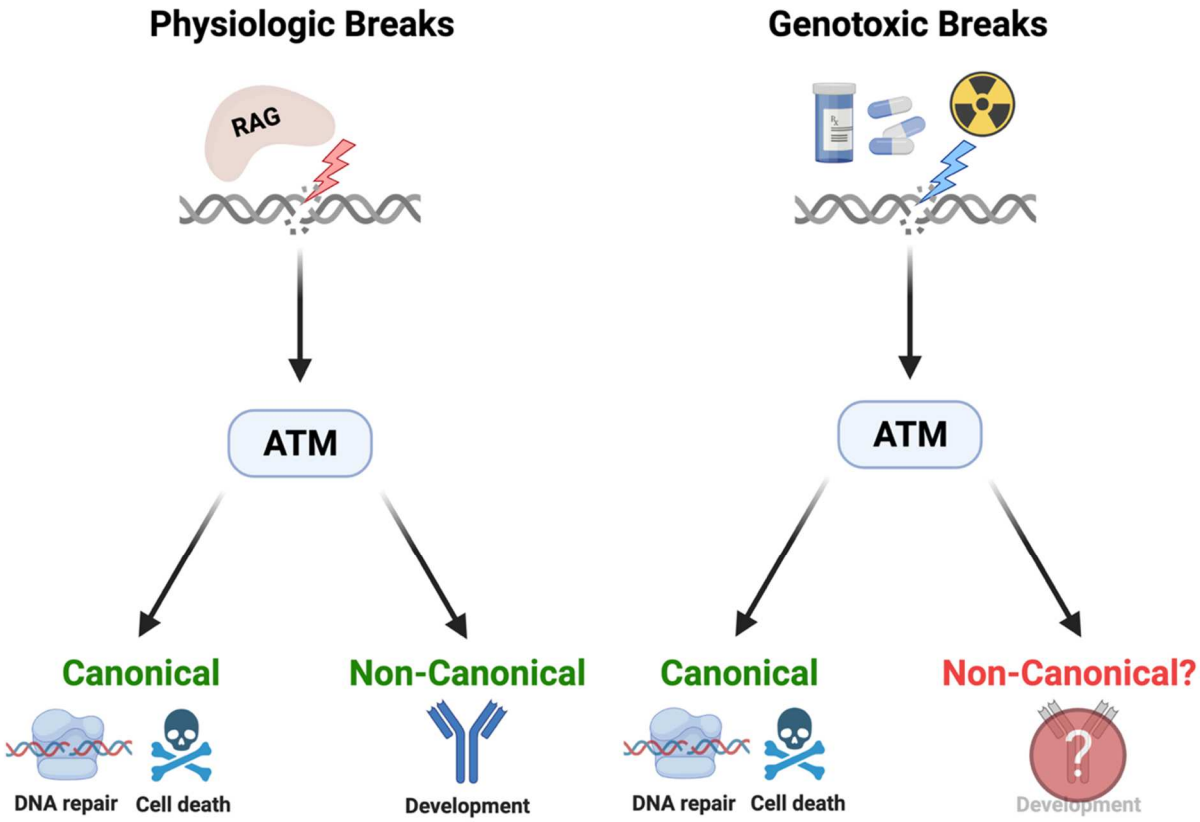

Figure 1.1: Do all DSBs activate the same DNA damage response? Previous work has established that both canonical and non-canonical DNA damage responses are induced following physiologic RAG DSBs. It is unclear if non-canonical DDR can be induced following non-RAG, genotoxic DSBs. Figure created with BioRender.

1.2 B cell Development Requires DNA Breaks

The adaptive immune system requires B cells to build their *Ig* receptors (BCR) (Murphy et al., 2008). Formation of these mature immunocompetent B cells is a highly ordered multistep process that begins in the bone marrow, where hematopoietic stem cells (HSCs) commit to the B cell lineage and move through discrete stages into secondary lymphoid organs as they build their antibody receptor (Melchers, 2015; Nagasawa, 2006). The antibody receptor is essential for antigen recognition and is composed of two heavy and two light chain genes (*Igh* and *Igl*, respectively). In B cells, *Igh* is assembled first in early pro-B cells where it pairs with surrogate light chains to generate the pre-B cell receptor (pre-BCR), which promotes the transition from pro-B to pre-B cells (Herzog et al., 2009). Subsequently, the pre-BCR initiates recombination of the *Igl* chain gene at either the *Igk* or *Igλ* locus. Once expressed, IgL pairs with the IgH chain to generate a mature BCR. Following successful assembly and expression of the BCR, pre-B cells transition to immature B cells and egress from the marrow. Maturation continues in secondary lymphoid organs. (Perez-Vera et al., 2011).

1.2.1 V(D)J Recombination

Immunoglobulin genes are unique in that the second exon of the *Ig* gene must be assembled to build the BCR in a process known as V(D)J recombination. The V(D)J recombination reaction assembles the variable (V), diversity (D), and joining (J) segments of the *Igh* and *Igl* chains to create an exon that encodes the antigen binding portion of the receptor chain (**Figure 1.2**) (Fugmann et al., 2000a). V(D)J recombination is initiated in the G1 phase of the cell cycle of B and T lymphocytes to create functional genes encoding the variable regions of immunoglobulins and T cell receptors (Bassing et al., 2002). V(D)J recombination introduces DSBs between V, D

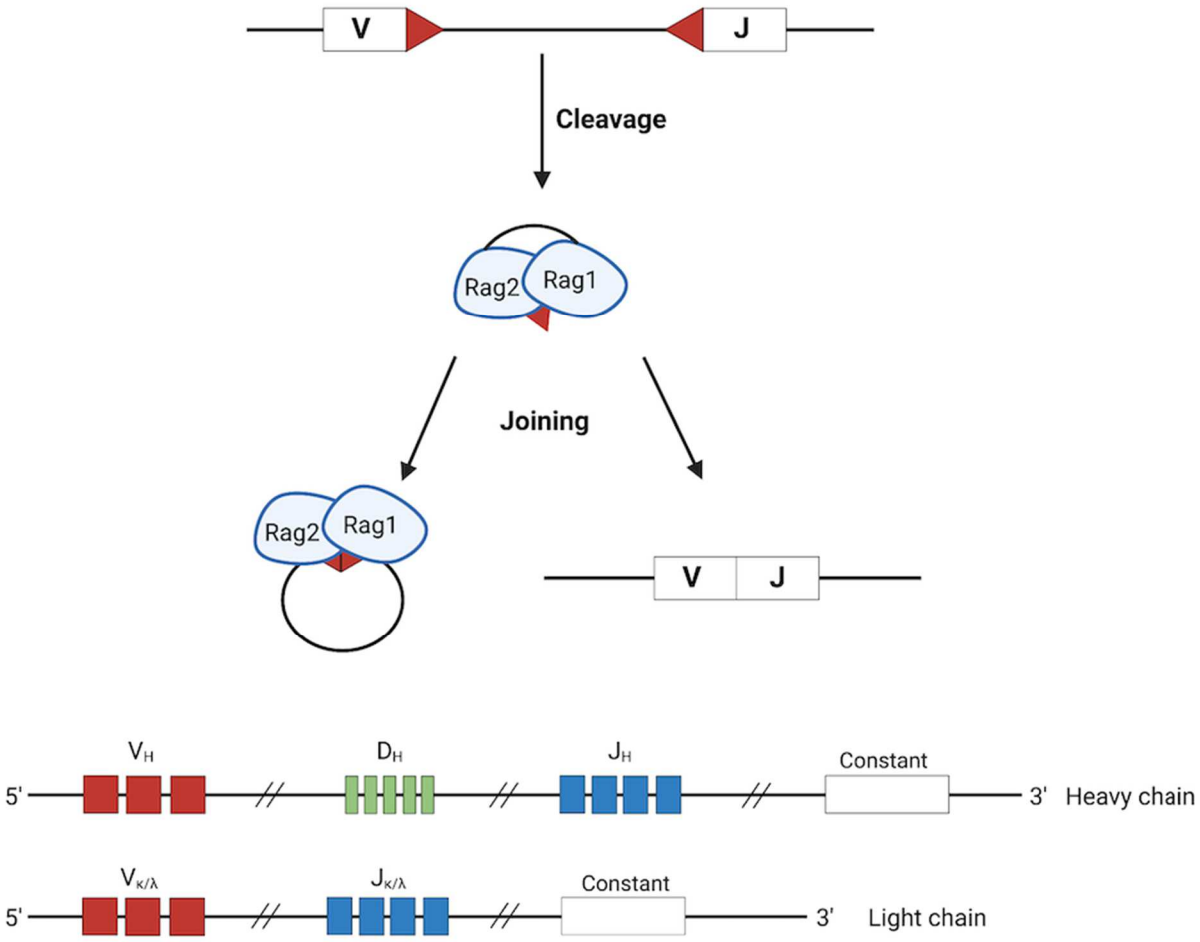

Figure 1.2: The V(D)J Recombination Reaction. Top: V(D)J recombination occurs when the RAG endonuclease, composed of RAG1 and RAG2 proteins binds to recombination signal sequences (RSSs), depicted as red triangles. RAG remains bound to the signal ends while the coding ends are joined. Bottom: V(D)J rearranges the V,D, and J segments of the heavy and light immunoglobulin chain. Figure created with BioRender.

and J coding gene segments (Cerboni et al., 2014). Recombination is directed by the sequence immediately flanking each gene segment known as recombination signal sequences (RSSs). RSSs each contain a well conserved 7-bp sequence (heptamer) and an AT-rich 9-bp sequence (nonamer) that are separated by a poorly conserved spacer whose length is either 12 or 23 base pairs (Fugmann et al., 2000a; Gellert, 2002). RSSs direct the cleavage crucial for the V(D)J reaction, carried out by the proteins encoded by the recombination activating genes *Rag1* and *Rag2* (Bassing

et al., 2002). Following cleavage by the RAG endonuclease complex, a large group of DNA repair enzymes primarily composed of the non-homologous end joining (NHEJ) machinery, including the DNA-dependent protein kinase (DNA-PK), Artemis, and DNA Ligase IV, coordinate to repair the DSB (Schatz and Ji, 2011). Correct repair of cleaved ends is critical to prevent translocations and introduce diversity into the antigen receptor gene. Productive V(D)J recombination leads to expression of IgH and IgL chains as cell surface B cell receptor by newly generated immature B cells.

1.2.2 Class Switch Recombination

The unique ability of B and T cells to somatically alter their genome extends beyond V(D)J recombination. Immature B cells migrate to the spleen and lymph nodes where they can undergo *Ig* gene diversification through class switch recombination (CSR) (Chaudhuri and Alt, 2004). The original BCR expressed on immature B cells contains an IgH expressing the IgM constant region. CSR allows exchange of the IgM constant region with an alternative constant region, thereby changing function of the BCR while maintaining antigen recognition. This allows for isotype switching from IgM to IgG, IgA or IgE, which have different effector functions (Martin and Scharff, 2002). CSR is similar to V(D)J in that an intentional DSB is made. CSR is a deletion-recombination reaction that requires activation-induced cytidine deaminase (AID), rather than RAG (Xu et al., 2012). As with RAG DSBS, breaks made by AID are repaired with NHEJ (Xu et al., 2012).

1.3 DNA Damage Responses in B Cells

DSBs are an immense threat to genomic integrity. Consequently, an elaborate cellular response has evolved to ensure proper repair. DSBs are repaired by either NHEJ or homologous

recombination (HR). NHEJ may introduce errors during re-ligation, while HR precisely restores the sequence of broken DNA ends by utilizing sister chromatids as a template for repair (Ciccia and Elledge, 2010). Beyond DNA repair, DSBs trigger additional cellular programs, including cell cycle arrest, cell death, senescence, and differentiation. These broad cellular programs are coordinated by signaling proteins that are involved in the DNA damage response. (**Figure 1.3**).

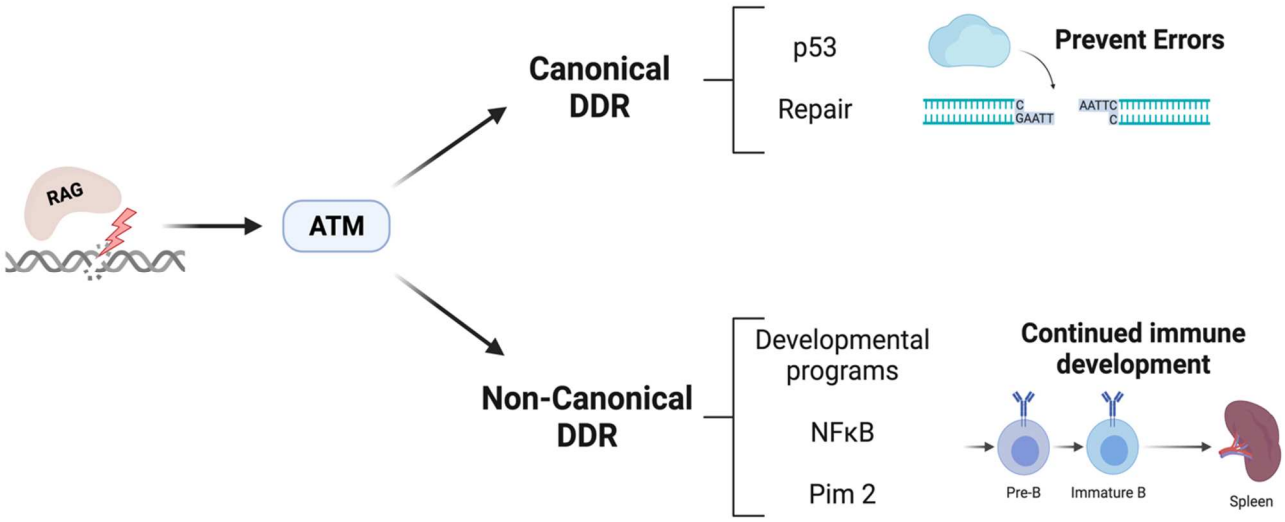

Figure 1.3: RAG DSBs induce canonical and non-canonical DNA damage responses. RAG DSBs signal through ATM which induces both canonical and non-canonical DNA damage responses. Canonical DNA damage responses are critical for preventing errors in the DNA and are characterized by p53-mediated repair or cell death. Non-canonical DNA damage response are characterized by NFκB and Pim2, which are required for continued immune cell development. Figure created with BioRender.

1.3.1 Canonical DNA Damage Responses

Because cells can encounter up to 10^5 lesions per day, an extensive network of maintenance factors must be in place to protect our genetic material (Hoeijmakers, 2009). All DSBs initiate a conserved cDDR in all cells (Ciccia and Elledge, 2010). cDDR during V(D)J recombination coordinates repair of broken DNA ends through NHEJ (Chang et al., 2017). cDDR also activates

checkpoints that prevents cells with DSBs from progressing through the cell cycle, and ultimately kills cells with persistent DSBs that could not be resolved. This prevents aberrant chromosomal rearrangements and cellular transformation (Bednarski and Sleckman, 2019).

In this regard, during *Ig* recombination, the RAG endonuclease produces a blunt signal end and a hairpin-sealed coding (Helmink and Sleckman, 2012). The two hairpin-sealed coding ends are then ligated to form a coding join that completes the second exon of the antigen receptor gene (Rooney et al., 2004). Repair of coding ends is facilitated through NHEJ. The broken DNA ends bind KU70, KU80 and MRE11-RAD50-NBS1 (MRN), which in turn activate DNA-PK and ATM (Bednarski and Sleckman, 2019; Rooney et al., 2004). ATM and another accessory kinase DNA-PK are known as serine/threonine kinases because they phosphorylate a serine or threonine residue when followed by a glutamine (known as "SQ/TQ" motifs) of proteins. ATM phosphorylates numerous proteins to coordinate the DNA damage response (Kastan and Lim, 2000). ATM initiates cDDR by modifying histones leading to recruitment of 53BP1 and phosphorylation of KAP1, which protect DNA ends from degradation (Difilippantonio et al., 2008; Tubbs et al., 2014). Next, the nuclease Artemis forms a complex with DNA-PK and opens the hairpin-sealed coding ends generated by RAG, to be subsequently ligated by DNA Ligase IV and its binding partner XRCC4 (Ma et al., 2002; Rooney et al., 2004).

Another important axis of the cDDR involves the transcription factor p53 (Bednarski and Sleckman, 2019). After a RAG DSB, ATM phosphorylates p53 which is critical for the G1-S cell cycle checkpoint (Helmink and Sleckman, 2012). p53 also promotes expression of proapoptotic genes including Bcl2-associated X protein (*Bax*) to trigger death of cells with persistent unrepaired DSBs (Guidos et al., 1996). Towards this end, mice deficient in p53 and the NHEJ factors required

for RAG DSB repair develop B cell tumors with translocations formed from misrepaired RAG DSBs (Bednarski and Sleckman, 2019; Zhu et al., 2002).

1.3.2 Non-canonical DNA Damage Responses

In addition to canonical repair and cell death pathways, RAG DSBs also upregulate a pre-B cell specific developmental program referred to as the ncDDR (Bednarski et al., 2016; Bednarski and Sleckman, 2019; Bredemeyer et al., 2008). ncDDR includes all processes induced following a DSB that are not related to DNA repair or maintaining genomic integrity. ncDDR includes survival signals that counter p53-mediated cell death pathways to permit time for completion of *Ig* recombination and to promote continued B maturation (Bednarski and Sleckman, 2019). This developmental response is largely regulated by the nuclear factor-κB1 (NFκB1) and NFκB2 transcription factors (Bednarski et al., 2016; Bredemeyer et al., 2008). NFκB1 activation in response to RAG DSBs induces the expression of CD69, L-selectin and switch-associated protein 70 (SWAP70), which are important for cell migration and localization within the bone marrow (Bednarski and Sleckman, 2019; Bredemeyer et al., 2008). NFκB1 also induces expression of the PIM2 kinase which promotes survival to permit multiple *Ig* chain gene rearrangements in a single pre-B cell (Bednarski and Sleckman, 2012).

RAG DSBs also activate the NFκB2 transcription factor (Bednarski and Sleckman, 2019). NFκB2 in turn upregulates SPIC, a transcription factor expressed primarily in macrophages and pre-B cells. (Bednarski et al., 2016; Bednarski and Sleckman, 2019). SPIC binds to promoters of both *Syk* and *Blnk*, which leads to a decrease in PU.1, a transcription factor required for B cell lineage commitment (Schweitzer and DeKoter, 2004; Soodgupta et al., 2019). RAG DSB induction of SPIC leads to repression of *Igl* gene transcription and antagonization of pre-BCR

signals (Bednarski and Sleckman, 2019; Soodgupta et al., 2019). Overall, this genetic program limits proliferation and promotes survival of cells with RAG DSBs to drive continue B cell maturation and prevent generation of additional RAG DSBs and/or translocations.

1.3.3 Response to Irradiation

In addition to DSBs from RAG endonuclease, pre-B cells can encounter DSBs from exogenous sources such IR. IR can be generated from both natural and man-made sources like radon gas, medical devices, and procedures. Failure to repair DSBs from IR can lead to uncontrolled cell death and cancer formation (Canman et al., 1998; Ciccia and Elledge, 2010). DNA breaks from IR also trigger the cDDR to coordinate repair pathways and cell death if unrepaired (Bednarski and Sleckman, 2019; Bredemeyer et al., 2008). Interestingly, IR-induced DNA damage in pre-B cells has been shown to upregulate some of the same transcriptional programs as RAG DSBs, including pre-B cells, including *Cd40*, *Nfκb2* and *Swap70* (Bredemeyer et al., 2008; Innes et al., 2020; Innes et al., 2013). Striking differences were also revealed when comparing irradiation induced breaks to RAG DSBs, such as expression of proto-oncogenes (i.e., *Kras*), increased proliferation and oxidative stress responses (Innes et al., 2020; Innes et al., 2013). This suggests that some of the DDR in pre-B cells is a conserved response to all DSBs but there may also be unique programs activated based on mechanism of DNA injury.

1.4 RAG endonuclease in Lymphocyte Development

The RAG endonuclease is a heterotetramer containing two RAG1 and two RAG2 proteins (Gellert, 2002; Kim et al., 2015). RAG1 contains the DNA binding and endonuclease activity while the RAG2 is critical for binding methylated histones to anchor the complex to the chromatin (Coster et al., 2012; Fugmann et al., 2000a; Gellert, 2002; Matthews et al., 2007). RAG1 and

RAG2 have also been shown to have functions in stabilization of broken DNA ends as well as promoting repair of these ends (Jones and Simkus, 2009). RAG1 and RAG2 contain core domains that are required for recombination activity, i.e. association with chromatin, binding RSS DNA regions, and cleaving DNA. Both proteins also have non-core accessory domains which can have diverse functions (Gellert, 2002; Lescale and Deriano, 2017). Outside of its canonical role in generation of DSBs, RAG has been shown to impact cellular processes and outcomes through genetic and epigenetic changes (Lescale and Deriano, 2017).

1.4.1 RAG Function in DNA Break Generation and Repair

The RAG endonuclease plays an essential role in the generation and repair of DNA breaks during V(D)J recombination. These functions are mediated by the core domains for the RAG1 and RAG2 proteins. The RAG complex binds to the DNA at the RSS and recruits high mobility group 1 protein (HMG1) to facilitate synapsis to capture a partner RSS (Lescale and Deriano, 2017; Swanson, 2002). RAG then generates a DSB, creating 4 broken ends: two blunt 5' phosphorylated signal ends that end in the RSS, and two hairpin-sealed coding ends (Fugmann et al., 2000a). After DSB generation, the RAG proteins remain bound to the DNA ends in a post-cleavage complex to assist in joining (Agrawal and Schatz, 1997; Lescale and Deriano, 2017).

1.4.2 RAG Function in Regulating Protein Stability and Nucleolar Localization

The non-core domains for RAG1 and RAG2 facilitate interaction with other proteins to modulate RAG complex activity. Following DSB generation, RAG1 recruits MDC1, an important DDR protein that amplifies the DNA damage response (Coster et al., 2012). The E3 ligase domain in the N-terminal region (NTR) of RAG1 has been shown to ubiquitinate multiple targets including

KPNA1 and histone H3, which then function to recruit post-cleavage repair proteins (Beilinson et al., 2021; Deng et al., 2015; Simkus et al., 2009). The RAG1 NTR also binds VprBP, a subunit of a RING E3 ligase complex (Beilinson et al., 2021). This interaction is involved in down-regulation of RAG1 protein levels following DSB generation (Kassmeier et al., 2012). This RAG1 NTR also regulates its nuclear localization, more specifically nucleolar import, and subsequent release for efficient V(D)J recombination (Brecht et al., 2020). Similar to RAG1, non-core regions of RAG2 have functions beyond generation of DSBs, including chromatin and phosphoinositide binding and regulation of protein turnover (Elkin et al., 2005; Jones and Simkus, 2009).

1.4.3 RAG Function in DNA Damage Response Signaling

The RAG complex has been implicated in DNA break generation, protein stability and nucleolar localization as detailed above. However, the RAG complex itself has not been shown to play a direct role in regulating the downstream DNA damage response. While it's evident that RAG is essential for creating the DSBs to initiate the signaling, no role has been identified for RAG in the DNA damage response. This topic is the focus of my thesis work.

1.5 RAG endonuclease in Disease

Because RAG endonuclease is essential for V(D)J recombination, disruption of these genes leads to severe immunological phenotypes, including complete loss of B or T lymphocytes, known as severe combined immunodeficiency (SCID) (Notarangelo et al., 2016). Hundreds of RAG1 and RAG2 mutations have been identified in patients, both in core and non-core regions of these proteins (Delmonte et al., 2018). While some variants result in loss of protein, many result in hypomorphic alleles. These hypomorphic RAG proteins result in diverse disease phenotypes.

1.5.1 Immune Deficiency due to Mutations in RAG

One of the main phenotypes in patients with RAG mutations is immune dysregulation. The most severe of these phenotypes is SCID, which results from null RAG mutations that abrogate recombination activity or protein expression/stability. Consequently, B and T cells are not generated resulting in complete absence of normal immune function. SCID patients are prone to life-threatening infections very early on in life (Bosticardo et al., 2021). Omenn syndrome is clinical manifestation of hypomorphic RAG variants, which is characterized by immune dysregulation including absent B cells and activated T cells infiltrating multiple organs (Delmonte et al., 2018). Omenn syndrome most often results from hypomorphic RAG mutations with little recombination activity (Notarangelo et al., 2016). RAG mutations can also result in a delayed onset combined immunodeficiency with granulomas and/or autoimmunity (CID-G/AI). The heterogeneity of RAG mutations that present in humans represent an important diagnostic challenge that often requires complete reconstitution of the immune system. Importantly, many of these mutations occur outside of the core region of RAG1 (Notarangelo et al., 2016). The impact of these non-core mutations on RAG1 function, DNA damaging responses, and lymphocyte development is poorly understood.

1.5.2 RAG in Pre-B Cell Leukemia

Outside of generation of DSBs in V(D)J recombination, RAG has also been shown to generate breaks that lead to chromosomal translocations and deletions associated with lymphoid malignancies (Lieber, 2016; Rommel et al., 2017). One mutation shown to be generated by a RAG DSB is ETV6-RUNX1, which characterizes approximately 25% of pre-B cell acute lymphoblastic leukemia (ALL) (Papaemmanuil et al., 2014). RAG1 and RAG2 mutations have also been implicated in development of follicular lymphoma and mantle cell lymphoma in addition to ALL,

which results from break generation at “cryptic” RSS sites, outside of conserved RSS motifs (Nussenzweig and Nussenzweig, 2010; Rommel et al., 2017). How RAG is regulated to prevent leukemia transformation is not known.

1.5.3 Abnormalities in DNA Damage Responses and Abnormal B cell Development

Defects in downstream DDR signals triggered by RAG DSBs have also been shown to result in clinical manifestations. Loss of ATM leads to ataxia-telangiectasia, a complex disease that involves the nervous, immune and reproductive systems (Concannon and Gatti, 1997). Patients with ATM-deficiency have low B and T cell numbers, poor lymphocyte function and increased risk of lymphoma. Beyond ATM, mutations in other downstream signaling factors like the NFκB essential modulator (NEMO) and PIM2 can also result in clinical phenotypes. NEMO is critical for activation of NFκB1, and mutations in NEMO have been shown to result in X-linked genetic diseases such as Incontinentia pigmenti and anhidrotic ectodermal dysplasia which involve immunodeficiency (Courtois and Gilmore, 2006; Courtois and Smahi, 2006). PIM2 mutations have been identified in hematological malignancies such as B-cell chronic lymphocytic leukemia (B-CLL) (Nawijn et al., 2011). Thus, the downstream DDR signals triggered by RAG DSBs have critical functions in promoting normal B cell development. Given that mutations in RAG outside of core regions can lead to diverse clinical manifestations, it’s conceivable that RAG harbors functions beyond its activity in DSB generation and repair, including a possible role for RAG in modulating DNA damage responses.

1.6 Scope of Thesis

Fundamental work in the field of V(D)J recombination recently has revealed the upregulation of a unique ncDDR that is critical for development of B and T cells in addition to the cDDR signals that upregulate pathways important for repair of DSBs. However, whether this broad cellular response is distinctive to RAG DSBs is poorly understood. Prior work has shown some similarities in transcriptional responses to physiologic RAG DSBs and exogenous, genotoxic DSBs such as irradiation-induced DNA injury in developing B cells. This suggests that pre-B cells are poised to initiate both cDDR and ncDDR to any DSB. Yet, distinctions in gene expression triggered by these modes of DNA injury raises the question of whether specific cellular and molecular mechanisms modulate responses to DSBs to effect differential fates for cells undergoing programmed rearrangement versus unintentional, exogenous DSBs. In the following chapters, I will present my research aimed at delineating the factors that dictate the downstream DNA damage responses to a DNA break in pre-B cells.

In Chapter 2, I report a novel tetracycline-inducible CRISPR-Cas9 system to create targeted breaks anywhere in the genome of a pre-B cell. This system was used to create Cas9 DSBs at *Ig* genes that mirror RAG DSBs. Both RAG- and Cas9-mediated DSBs at *Ig* genes activate cDDR. However, only RAG DSBs, but not Cas9 DSBs, induce the ncDDR-dependent developmental program. This unique response is regulated by non-core regions of RAG1. In Chapter 3, I present experimental tools and approaches I have developed to follow up on the mechanisms behind this unique activation of ncDDR following a RAG DSBs. This Cas9 system was altered to target RAG DSBs to other locations in the genome by fusing RAG1 mutations to the tetracycline-inducible Cas9. Co-immunoprecipitation of RAG1 reveals a unique protein interactome that may have functions in regulating DDR signals. Together, this body of work

provides exciting new knowledge on the role of RAG1 in DNA damage signaling in pre-B cells. It also establishes for the first time that B cells initiate distinct cellular responses to DNA breaks based on the mechanism of DNA injury, which reveals previously unrecognized opportunities to optimize therapies for leukemia and other malignancies.

Chapter 2

The Non-Canonical DNA Damage Response is Uniquely Activated by RAG endonuclease

by
Rachel Johnston, Brendan Mathias, Lynn White,
Abby Green and Jeffrey J. Bednarski

Adapted from submitted manuscript for dissertation:

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2.1 Abstract

Developing B cells generate DSBs to assemble *Ig* genes necessary for expression of a mature B cell receptor. These physiologic DSBs are made by the RAG endonuclease, which is comprised of the RAG1 and RAG2 proteins. In pre-B cells, RAG-mediated DSBs activate the ATM kinase to coordinate canonical and non-canonical DDR that trigger DSB repair and B cell developmental signals, respectively. Whether this broad cellular response is distinctive to RAG DSBs is poorly understood. To delineate the factors that direct DDR signaling in B cells, we expressed a tetracycline-inducible Cas9 nuclease in *Rag1*-deficient pre-B cells. Both RAG- and Cas9-mediated DSBs at *Ig* genes activate cDDR. In contrast, RAG DSBs, but not Cas9 DSBs, induce the ncDDR-dependent developmental program. This unique response to RAG DSBs is regulated by non-core regions of RAG1. Thus, B cells trigger distinct cellular responses to RAG DSBs through unique properties of the RAG endonuclease that promote activation of B cell developmental programs.

2.2 Introduction

Formation of mature immunocompetent B cells is a highly ordered, multistep process that begins in the bone marrow, where HSCs commit to the B cell lineage and move through discrete stages into secondary lymphoid organs as they build their immunoglobulin receptors (Melchers, 2015; Nagasawa, 2006). Each Ig receptor is composed of two IgH and IgL chains (Rajewsky, 1996). The *Igh* gene is assembled first in pro-B cells and pairs with surrogate light chain to generate the pre-BCR, which subsequently activates *Igl* gene assembly at either the immunoglobulin kappa (*Igk*) or lambda (*Igl*) locus. (Herzog et al., 2009; Loffert et al., 1996). *Igh* and *Igl* genes are assembled through the V(D)J recombination reaction, which joins variable (V), diversity (D) and

joining (J) gene segments to generate functional exons encoding the antigen binding domain (Fugmann et al., 2000a). *Ig* gene rearrangement occurs only in G1-arrested lymphocytes and requires generation of DSBs at distant DNA segments followed by excision of the intervening DNA sequence and ligation of the intrachromosomal broken DNA ends (Desiderio et al., 1996). The DSBs needed for *Ig* gene rearrangement are generated by the RAG endonuclease, which is a heterotetramer comprised of two RAG1 and two RAG2 proteins (Fugmann et al., 2000a; Oettinger et al., 1990; Schatz et al., 1989). RAG binds RSSs at the border of V, D, and/or J segments where it subsequently cleaves DNA. Repair of the DNA ends is mediated by NHEJ, which introduces diversity into the antigen receptor gene and is critical for preventing errant repair that could lead to translocations (Bednarski and Sleckman, 2019; Rooney et al., 2004).

RAG-mediated DSBs activate the serine/threonine kinase ATM, which promotes cDDR to coordinate DSB repair (Alt et al., 2013; Bednarski and Sleckman, 2019; Bredemeyer et al., 2008; Helmink and Sleckman, 2012). Through activity of ATM, DDR proteins, including 53BP1, accumulate at sites of DSBs to protect the open DNA ends and recruit repair factors, which join the broken DNA ends (Anderson et al., 2001; Difilippantonio et al., 2008). In response to RAG DSBs, activation of ATM also induces a pre-B cell-specific developmental program (Bednarski et al., 2016; Bednarski and Sleckman, 2019; Bredemeyer et al., 2008). This ncDDR includes activation of PIM2 to promote survival of pre-B cells undergoing *Ig* rearrangement and induction of transcription factors, including NFκB2, which regulate broad gene expression changes (Bednarski et al., 2012; Bednarski et al., 2016; Derudder et al., 2009). NFκB2 induces the transcriptional repressor complex SPIC/BCLAF1, which restricts proliferation of pre-B cells and promotes continued B cell maturation (Bednarski et al., 2016; Soodgupta et al., 2019).

Collectively, the RAG DSB-mediated ncDDR supports B cell development while preserving genome integrity.

In pre-B cells, DNA damage from exogenous sources, such as IR, also triggers the conserved cDDR to coordinate repair pathways and cell death programs if the DNA injury is not resolved (Bednarski and Sleckman, 2019; Bredemeyer et al., 2008; Innes et al., 2006; Innes et al., 2013). However, whether these non-RAG-mediated DNA breaks also initiate the developmental ncDDR is not yet evident. Prior work has delineated some similarities in transcriptional responses to irradiation-induced DNA injury and RAG DSBs in pre-B cells (Bredemeyer et al., 2008; Innes et al., 2020; Innes et al., 2013). These findings suggest that pre-B cells are inherently poised to initiate both cDRR and ncDDR to any DSBs. However, distinctions in gene expression triggered by these two modes of DNA injury raise the question of whether specific cellular and molecular mechanisms may modulate responses to DSBs to effect differential fates for cells undergoing programmed RAG-mediated *Ig* rearrangement versus those with unintended, non-RAG DNA injury.

In addition to its function in V(D)J recombination, the RAG complex interacts with a diverse protein network that regulates protein localization, protein stability, and DSB formation and repair (Brecht et al., 2020; Coster et al., 2012; Elkin et al., 2005; Jones and Simkus, 2009; Kassmeier et al., 2012; Lescale and Deriano, 2017; Matthews et al., 2007). RAG1 harbors the primary DNA-binding and cleavage activity while RAG2 functions as an accessory factor enforcing chromatin association and localization (Alt et al., 2013; Helmink and Sleckman, 2012). The endonuclease activity of RAG1 resides in its core domain (amino acids 384-1008), which is essential for RAG complex localization to RSSs and DSB generation (Sadofsky et al., 1993; Silver et al., 1993). The N-terminal region (NTR; amino acids 1-383) and the C-terminal tail (amino acids

1009-1040) of RAG1, defined as the non-core regions of the protein, have critical functions in regulating its activity through association with interacting proteins (Grundy et al., 2010; Kim et al., 2015). RAG1 NTR interacts with MDC1, SF3A2, VprBP and nucleolar proteins and also contains an E3 ubiquitin ligase domain that regulates accessory factor activities (Brecht et al., 2020; Coster et al., 2012; Deng et al., 2015; Kassmeier et al., 2012; Simkus et al., 2009). The importance of these non-core regions of RAG1 in lymphocyte development are underscored by identification of mutations in these domains in patients with primary immune deficiency (Lee et al., 2014; Notarangelo et al., 2016). Further, mice expressing only core RAG1 exhibit aberrant V(D)J recombination, abnormal lymphocyte development, and increased incidence of malignancies, suggesting a key role of these regions in RAG function and immune development (Beilinson et al., 2021; Deriano et al., 2011; Horowitz and Bassing, 2014; Talukder et al., 2004b).

Here we utilize an inducible Cas9 nuclease to elucidate mechanisms of ncDDR activation in response to DSBs in pre-B cells. We find that both Cas9- and RAG-mediated DSBs initiate cDDR but only RAG DSBs trigger the developmental ncDDR pathways. This distinct response to RAG DSBs is mediated by non-core domains of RAG1 in a manner independent of its E3 ligase activity. Our findings establish that RAG1 has critical functions in regulating DNA damage signaling in pre-B cells undergoing *Ig* recombination

2.3 Results

2.3.1 RAG DSBs at *Ig* alleles activate cDDR and ncDDR in pre-B cells

To elucidate the factors that coordinate ncDDR programs in pre-B cells in response to RAG DSBs, we used *Rag1*$^{-/-}$*:Bcl2* and *Art*$^{-/-}$*:Bcl2* Abelson-kinase transformed pre-B cells (abl pre-B

cells). Expression of the Abl kinase promotes cell proliferation and suppresses *Rag1* and *Rag2* expression. Inhibition of the Abl-kinase with imatinib induces G1 arrest, induction of RAG and recombination of *Igk* (Bredemeyer et al., 2008). Expression of the *Bcl2* transgene in abl pre-B cells supports survival of imatinib-treated, G1-arrested pre-B cells, which permits evaluation of DDR signaling. *Rag1*$^{-/-}$*:Bcl2* abl pre-B cells do not generate RAG DSBs and, thus, do not activate DDR signals (**Figure 2.1 A**). In contrast, since Artemis is required for DSB repair, *Art*$^{-/-}$*:Bcl2* abl pre-B cells generate persistent RAG DSBs at *Igk*, which activate DDR (Bredemeyer et al., 2008; Rooney et al., 2003; Soodgupta et al., 2019). Following imatinib treatment, *Art*$^{-/-}$*:Bcl2* abl pre-B cells generated RAG DSBs in ~50% of *Igk* loci and demonstrated accumulation of the DDR factor 53BP1 in 1-2 foci per cell (**Figure 2.1 A and B**) indicative of RAG DSBs at *Igk* alleles. Additionally, pre-B cells with RAG DSBs had increased phosphorylation of the chromatin modulator KAP1 (**Figure 2.1 C**). Both 53BP1 foci and KAP1 phosphorylation are hallmarks of cDDR signaling (Anderson et al., 2001; Difilippantonio et al., 2008; Tubbs et al., 2014). RAG DSBs at *Igk* also triggered B cell-specific ncDDR as evidenced by activation of the NFκB2 transcription factor (i.e., processing of the p100 precursor to the transcriptionally active p52), induction of *Cd40* and *Pim2* mRNA transcripts, and expression of PIM2 protein in *Art*$^{-/-}$*:Bcl2* abl pre-B cells but not *Rag1*$^{-/-}$*:Bcl2* abl pre-B cells (**Figure 2.1 C and D**). These findings are consistent with previous studies and demonstrate that RAG-mediated DSBs at *Igk* activate both canonical and non-canonical DDR (Bednarski et al., 2012; Bednarski et al., 2016; Bredemeyer et al., 2008).

To determine if RAG DSBs at other *Ig* genes induce similar DDR in pre-B cells, we generated *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2* abl pre-B cells, which do not generate RAG DSBs and are unable to repair DSBs due to absence of DNA Ligase 4 (LIG4), which is required for ligation of broken DNA ends (Helmink and Sleckman, 2012; Purman et al., 2019). *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2* abl pre-B cells

have had no RAG activity and, thus, retain germline *Igh*. In contrast, *Igh* has already been recombined in *Artemis*$^{-/-}$*:Bcl2* abl pre-B cells (**Figure 2.2 A**), which promotes accessibility of *Igk* to RAG and subsequent generation of DSBs at *Igk* (Clark et al., 2014; Johnson et al., 2008). The absence of a recombined *Igh* in *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2* pre-B cells is expected to direct RAG activity to *Igh* rather than to *Igk*. Indeed, retroviral expression of FLAG-tagged RAG1 in *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2* pre-B cells followed by treatment with imatinib resulted in selective induction of DSBs at *Igh* and not at *Igk* (**Figure 2.1 E and Figure 2.2 B**). RAG DSBs were generated at ~50% of *Igh* loci resulting in 1-2 53BP1 foci per cell, similar to the level of DSBs generated at *Igk* in *Artemis*$^{-/-}$*:Bcl2* pre-B cells (**Figure 2.1 E and F compared to Figure 2.1 A and B**). Similar to RAG DSBs at *Igk*, RAG DSBs at *Igh* triggered phosphorylation of KAP1 as well as activation of NFκB2, induction of *Cd40* and *Pim2* transcription, and increased PIM2 protein (**Figure 2.1 G-H**). Thus, RAG-mediated DSBs at either *Igh* or *Igk* similarly induce both cDDR and ncDDR signaling in early B cells.

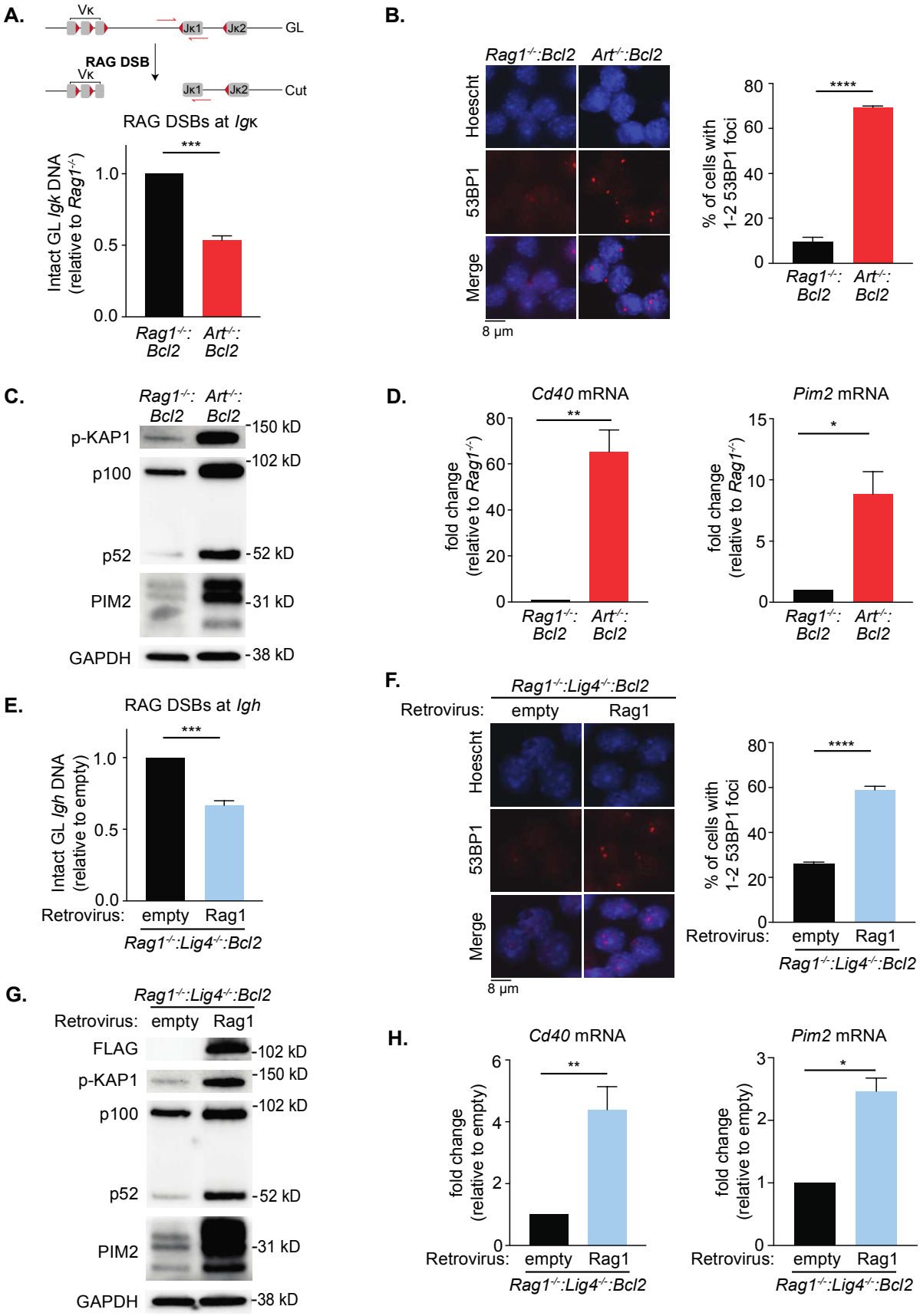
A.
Vκ
Jκ1
Jκ2
GL
RAG DSB
Cut
RAG DSBs at Igκ
Intact GL Igk DNA (relative to Rag1-/-)
***
Rag1-/-: Bcl2
Art-/-: Bcl2
B.
Rag1-/-:Bcl2
Art-/-:Bcl2
Hoescht
53BP1
Merge
8 μm
% of cells with 1-2 53BP1 foci
****
C.
p-KAP1
p100
p52
PIM2
GAPDH
150 kD
102 kD
52 kD
31 kD
38 kD
D.
Cd40 mRNA
Pim2 mRNA
fold change (relative to Rag1-/-)
E.
RAG DSBs at Igh
Intact GL Igh DNA (relative to empty)
Retrovirus:
empty
Rag1
Rag1-/-:Lig4-/-:Bcl2
F.
Rag1-/-:Lig4-/-:Bcl2
G.
FLAG
H.

Figure 2.1 (previous page): RAG DSBs at *Igk* and *Igh* activate cDDR and ncDDR. (A-D) *Rag1*$^{-/-}$*:Bcl2* and *Art*$^{-/-}$*:Bcl2* abl pre-B cells were treated with imatinib for 48 h to induce G1 arrest. **(A)** DSBs quantified by qPCR analysis of *Igk* (*Jk1*) genomic DNA. Schematic shows germline (GL) *Igk* locus, unrepaired Jκ1 coding end (Cut) and primer location. Results are normalized to *Rag1*$^{-/-}$*:Bcl2* abl pre-B cells, which do not generate RAG DSBs and have only germline *Igk* DNA. Loss of *Igk* germline product is representative of DSB generation. **(B)** Representative images of 53BP1 foci. Scale bar denotes 8 mm. Bar graph shows foci quantitation of 300 cells for each condition. **(C)** Western blot analysis of KAP1 phosphorylation (p-KAP1), NFκB2 (p100 and p52) and PIM2. GAPDH is shown as loading control. **(D)** *Cd40* and *Pim2* mRNA expression. **(E-H)** *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2* abl pre-B cells were transduced with an empty vector (control) or vector expressing RAG1 then treated with imatinib for 48 h. **(E)** DSBs quantified by qPCR analysis of *Igh* (J_H1) genomic DNA and analyzed as in A with results normalized to empty vector control, which has only germline *Igh* DNA. **(F)** Representative images of 53BP1 foci. Scale bar denotes 8 mm. Bar graph shows foci quantitation of 300 cells for each condition. **(G)** Western blot analysis of p-KAP1, NFκB2 and PIM2. GAPDH is shown as loading control. **(H)** *Cd40* and *Pim2* mRNA expression. Data in A, B, D, E, F, and H are mean ± SE for three independent experiments. Data in C and G are representative of three independent experiments. Statistical significance was calculated using Student's t-test. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$

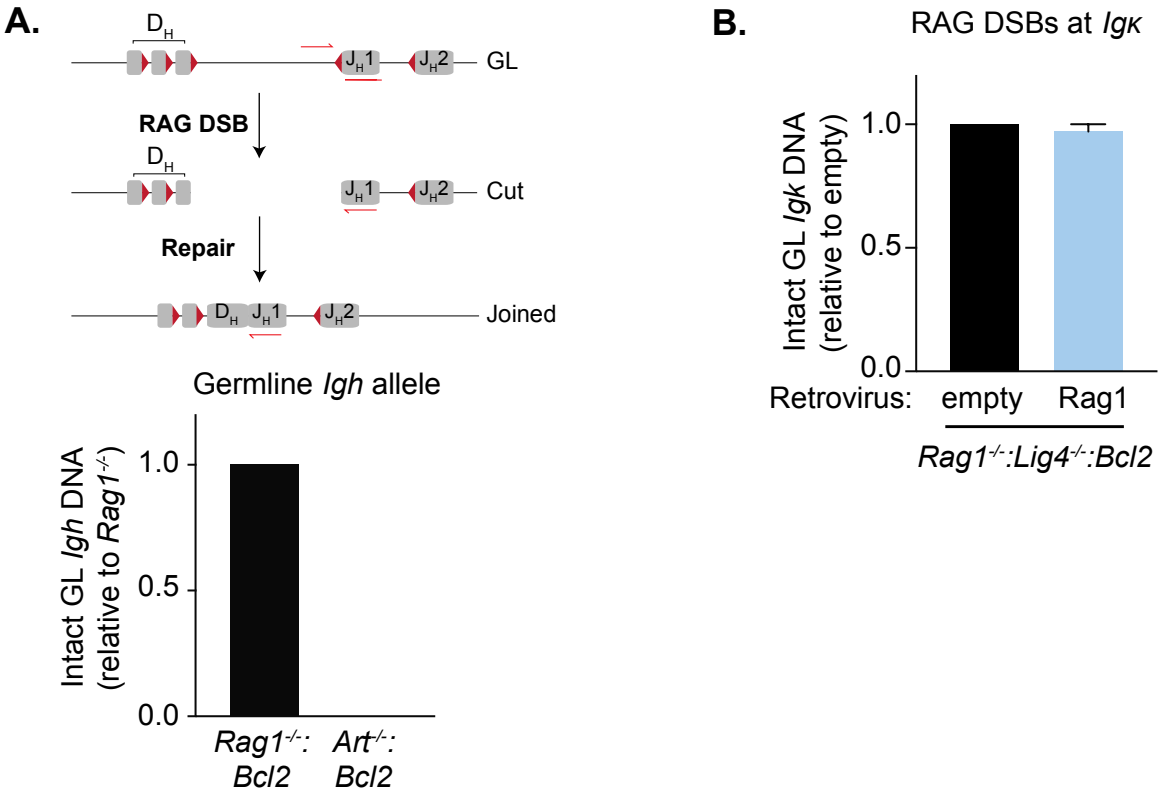

Figure 2.2: RAG DSBs are selectively induced at specific *Ig* loci depending on pre-B cell genotype. (A) Germline *Igh* (*J_H1*) locus quantified by qPCR analysis in *Rag1$^{-/-}$:Bcl2* and *Art$^{-/-}$:Bcl2* abl pre-B cells prior to imatinib treatment. Schematic shows germline (GL) *Igh* locus, unrepaired J_H1 coding end (Cut), representative joined *Igh* locus (Joined), and primer location. Results are normalized to *Rag1$^{-/-}$:Bcl2* abl pre-B cells, which have germline *Igh* DNA. Absence of *Igh* germline product in *Art$^{-/-}$:Bcl2* abl pre-B cells indicates the locus has already rearranged prior to imatinib treatment and induction of RAG. *Art$^{-/-}$:Bcl2* abl pre-B cells had no PCR product for germline *Igh* (absence of column and associated error bars). **(B)** *Rag1$^{-/-}$:Lig4$^{-/-}$:Bcl2* abl pre-B cells were retrovirally transduced with empty vector (control) or vector expressing RAG1 then treated with imatinib for 48 h as in Fig. 1 E. DSBs quantified by qPCR analysis of *Igk* (*Jk1*) genomic DNA as in Fig. 1 A. Data in both panels are mean ± SE for three independent experiments.

2.3.2 ncDDR is not activated by non-RAG, non-*Ig* DSBs in pre-B cells

The similar DDR programs activated by RAG DSBs at *Igh* and *Igk* raised the question whether all DSBs in pre-B cells trigger identical cellular responses. Previous work has shown that IR induced DNA damage results in some of the same cellular responses as RAG DSBs suggesting that pre-B cells may have a generalized response to any DNA insult (Bredemeyer et al., 2008; Innes et al., 2020; Innes et al., 2013). However, there are challenges in comparing IR induced DSBs to RAG DSBs. IR generates DNA breaks, both double-stranded and single-stranded, randomly throughout the genome and concomitantly activates other signals, such as reactive oxygen species. In contrast, RAG DSBs occur at targeted locations and do not induce these other signals. To circumvent these confounding factors, we transduced *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2* pre-B cells with a lentivirus to stably express a tetracycline-inducible FLAG-tagged Cas9 (*Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2:iCas9*) (Purman et al., 2019). Treatment with imatinib and doxycycline induced G1 arrest and Cas9 expression (**Figure 2.3 A**). Subsequent transfection with a guide RNA (gRNA) leads to Cas9-mediated DSBs at a targeted locus; thereby, permitting inducible generation of non-RAG DSBs at any genomic location (Mali et al., 2013; Purman et al., 2019; Sternberg et al., 2014). Of note, Cas9, similar to the RAG complex, remains bound to cut DNA ends (Agrawal and Schatz, 1997; Arnal et al., 2010; Brinkman et al., 2018; Sternberg et al., 2014; Wang et al., 2012). To determine if pre-B cells activate a universal DDR to all DSBs, we independently transfected *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2:iCas9* with gRNAs targeting the T cell receptor α chain gene enhancer (*Eb*) or *Gapdh*. Cas9-mediated DSBs were generated at both targeted loci as demonstrated by loss of germline DNA PCR product (**Figure 2.3 B and C**). DSBs did not occur at putative off-target sites despite persistent expression of Cas9 and gRNAs, which demonstrates the specificity of this approach in generating targeted DSBs (**Figure 2.4 A and B**). To compare DDR signals activated

by Cas9 DSBs and RAG DSBs, cellular responses were measured 48 hours after initiation of DSBs. As expected, Cas9 DSBs activated cDDR as evidenced by accumulation of 53BP1 foci and phosphorylation of KAP1 in cells with targeted gRNA compared to those with no gRNA (**Figure 2.3 D and E**). Of note, 53BP1 was present in 1-2 foci per cell consistent with DSBs occurring only at targeted alleles and not diffusely throughout the genome (**Figure 2.3 D**). Induction of cDDR by Cas9 DSBs was similar to responses triggered by RAG DSBs (**compare Figure 2.3 D and E to Figure 2.1 F and G**). Notably, Cas9-mediated DSBs at *Eb* and *Gapdh* did not trigger activation of NFκB2, increased expression of PIM2, or induction of *Cd40* or *Pim2* mRNA expression compared to cells without gRNA or cells with RAG DSBs (**Figure 2.3 E and F; compare to Figure 2.1 G and H**). Thus, Cas9-mediated DSBs only activated cDDR programs but not ncDDR signaling. These findings reveal that ncDDR signals are not a conserved component of pre-B cell responses to all DSBs.

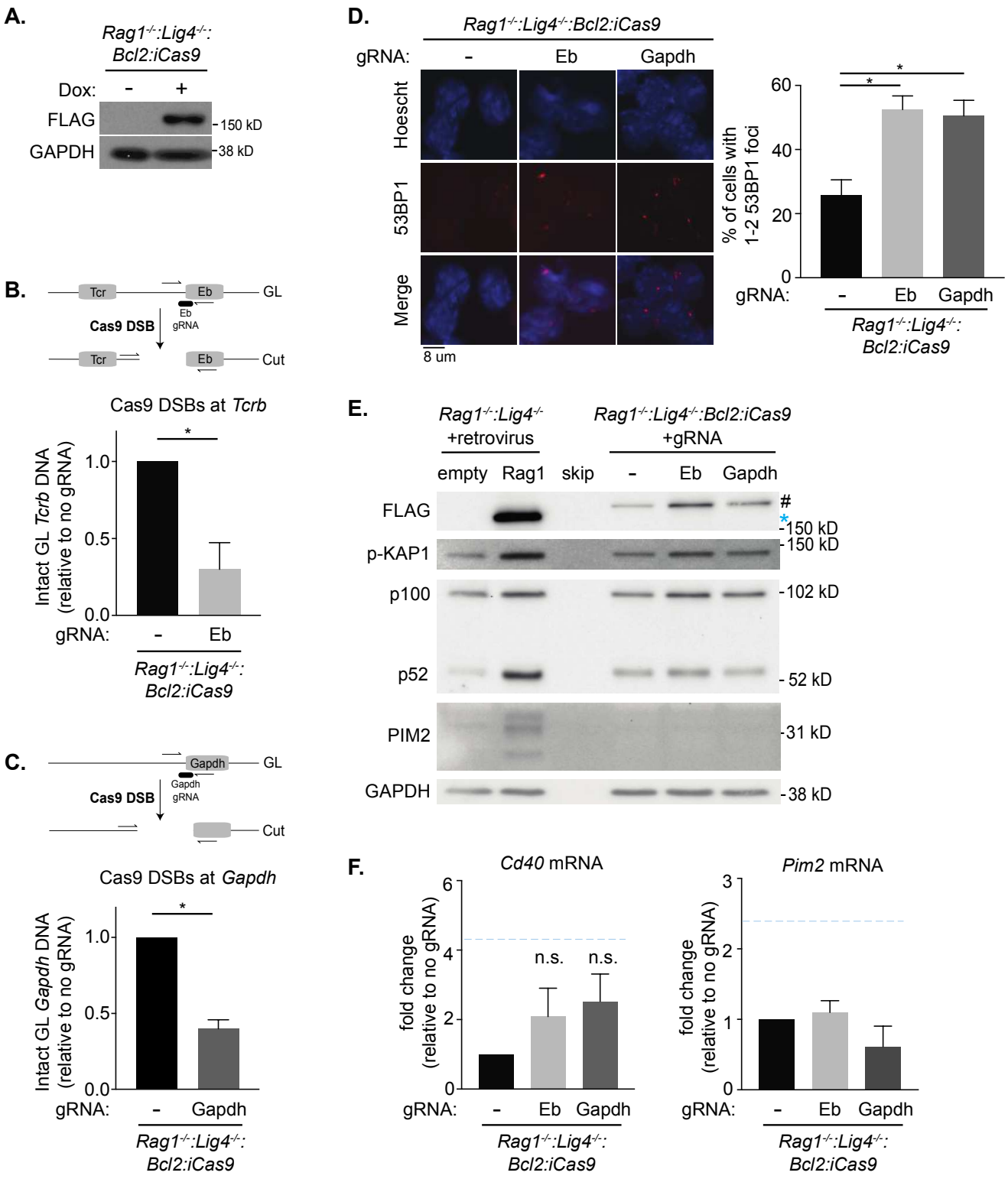
A.
Rag1-/-:Lig4-/-:
Bcl2:iCas9
Dox: - +
FLAG
GAPDH
150 kD
38 kD
B.
Tcr
Eb
GL
Eb
gRNA
Cas9 DSB
Tcr
Eb
Cut
Cas9 DSBs at Tcrb
Intact GL Tcrb DNA
(relative to no gRNA)
1.0
0.5
0.0
*
gRNA: - Eb
Rag1-/-:Lig4-/-:
Bcl2:iCas9
C.
Gapdh
GL
Gapdh
gRNA
Cas9 DSB
Cut
Cas9 DSBs at Gapdh
Intact GL Gapdh DNA
(relative to no gRNA)
1.0
0.5
0.0
*
gRNA: - Gapdh
Rag1-/-:Lig4-/-:
Bcl2:iCas9
D.
Rag1-/-:Lig4-/-:Bcl2:iCas9
gRNA: - Eb Gapdh
Hoescht
53BP1
Merge
8 um
% of cells with
1-2 53BP1 foci
60
40
20
0
*
*
gRNA: - Eb Gapdh
Rag1-/-:Lig4-/-:
Bcl2:iCas9
E.
Rag1-/-:Lig4-/-
+retrovirus
Rag1-/-:Lig4-/-:Bcl2:iCas9
+gRNA
empty Rag1 skip - Eb Gapdh
FLAG
#
*
150 kD
p-KAP1
150 kD
p100
102 kD
p52
52 kD
PIM2
31 kD
GAPDH
38 kD
F.
Cd40 mRNA
fold change
(relative to no gRNA)
6
4
2
0
n.s.
n.s.
gRNA: - Eb Gapdh
Rag1-/-:Lig4-/-:
Bcl2:iCas9
Pim2 mRNA
fold change
(relative to no gRNA)
3
2
1
0
gRNA: - Eb Gapdh
Rag1-/-:Lig4-/-:
Bcl2:iCas9

Figure 2.3 (previous page): Cas9 DSBs at non-*Ig* genes do not activate ncDDR. (A) *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2:iCas9* abl pre-B cells were treated with (+) or without (-) 2 μM doxycycline (Dox) to induce Cas9 as indicated. **(B-F)** *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2:iCas9* abl pre-B cells were treated with doxycycline as in A and imatinib to trigger cell cycle arrest for 24 h. Cells were then transfected with indicated gRNA and maintained in doxycycline and imatinib. All analyses were completed 48 h after gRNA transfection. **(B-C)** DSBs quantified by qPCR analysis of *Eb* (B) and *Gapdh* (C) genomic DNA. Results are normalized to cells without gRNA (-). Schematic shows germline (GL) locus, unrepaired cut end (Cut) and location of gRNA and primers. Loss of germline product is representative of DSB generation. **(D)** Representative images of 53BP1 foci. Scale bar denotes 8 mm. Bar graph shows foci quantitation of 300 cells for each condition. **(E)** Western blot analysis of p-KAP1, NFκB2 and PIM2. GAPDH is shown as loading control. Responses to RAG DSBs in *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2* abl pre-B cells transduced with empty vector or vector expressing RAG1 RAG DSBs from Fig. 1 G are included for direct comparison. # denotes band for FLAG-Cas9. * denotes band for FLAG-RAG1. **(F)** *Cd40* and *Pim2* mRNA expression. Blue dashed line indicates mRNA expression in response to RAG DSBs from Fig. 1 H and is included for comparison. Data in B, C, D, and F are mean ± SE for three independent experiments. Data in A and E are representative of three independent experiments. Statistical significance was calculated using Student's t-test. n.s. = not significant, *$p \leq 0.05$

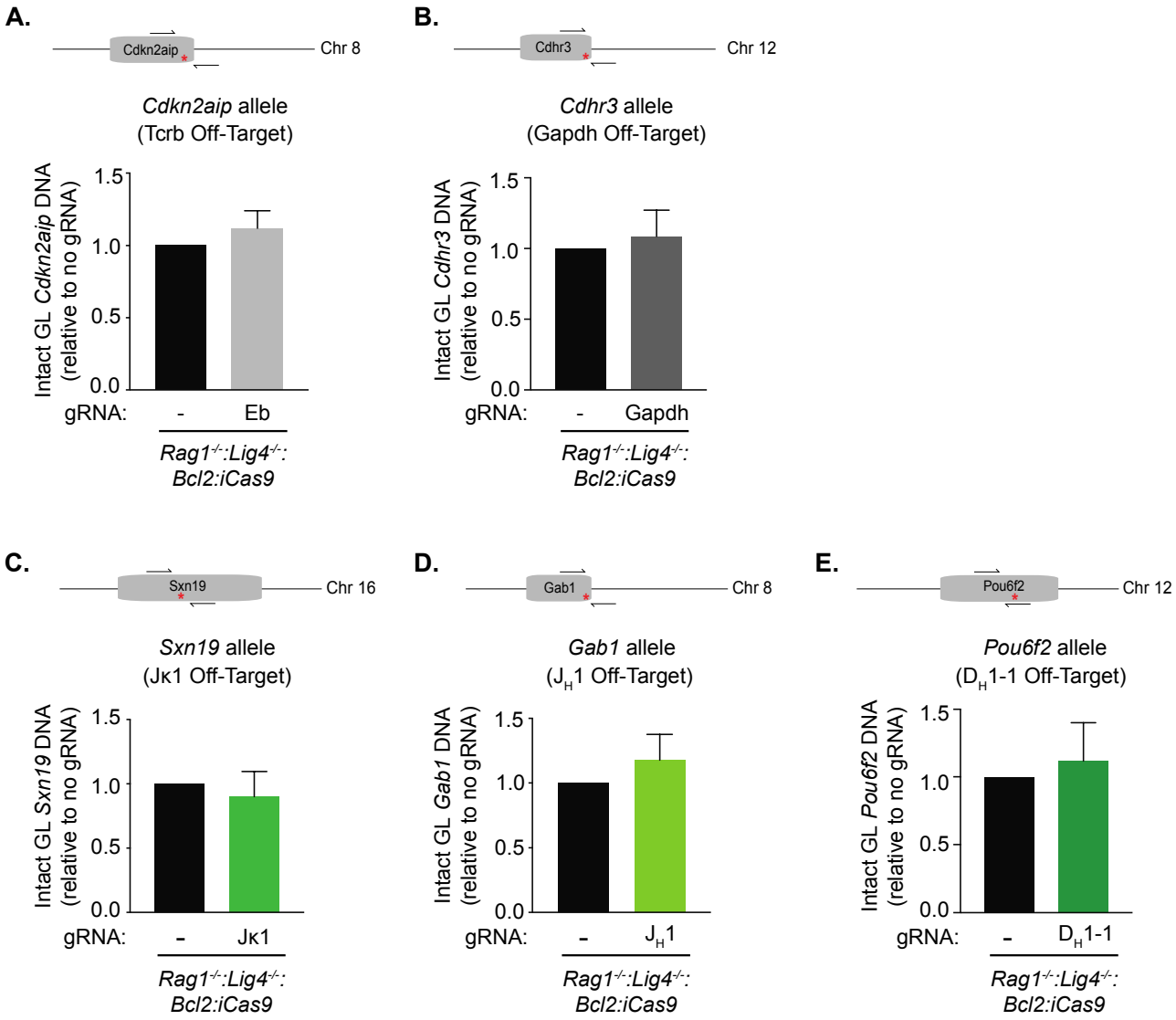

Figure 2.4: Cas9 does not generate breaks at predicted gRNA off-target sites. *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2:iCas9* abl pre-B cells were treated with imatinib and doxycycline then transfected with indicated gRNAs as in Figs. 2 and 3. Following transfection, cells were maintained in doxycycline and imatinib with all analyses completed 48 h after gRNA transfection. **(A-E)** Schematics show putative off-target binding sites of indicated gRNAs (red asterisk), germline (GL) locus of putative off-target sites, and location of PCR primers. Germline locus was quantified by qPCR analysis across *Cdkn2aip* (A), *Cdhr3* (B), *Sxn19* (C), *Gab1* (D), and *Pou6f2* (E). Results are normalized to cells without gRNA (-).

2.3.3 Cas9 DSBs at *Ig* alleles activate cDDR but not ncDDR in pre-B cells

Activation of ncDDR may be regulated by unique features of the RAG endonuclease or specific characteristics of *Ig* loci. To resolve this, *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2:iCas9* were treated with imatinib and doxycycline to induce cell cycle arrest and Cas9 expression then independently transfected with distinct gRNAs targeting the RSS of the *Jκ1*, J_H1, or D_H1-*1* gene segment. All three gRNAs were designed to generate a Cas9 DSB at or very near the location where a RAG DSB would be generated. Cas9 DSBs were generated at each antigen receptor allele to an equivalent extent as observed with expression of RAG (**Figure 2.5 A and B; compare to Figure 2.1 A and E**). As above, Cas9 DSBs did not occur at putative off-target sites (**Figure 2.4 C-E**). Cas9-mediated DSBs at both *Igk* and *Igh* activated cDDR as evidenced by generation of 53BP1 foci and phosphorylation of KAP1 (**Figure 2.5 C and D**). Again, cells generated 1-2 53BP1 foci reflective of DSBs occurring selectively at the targeted alleles. However, Cas9 DSBs at neither *Igk* nor *Igh* triggered ncDDR as they did not induce NFκB2 activation or expression of *CD40* and *Pim2* compared to cells without gRNA (**Figure 2.5 D and E**).

A key difference between RAG- and Cas9-mediated breaks is the number of DSBs generated. RAG generates two simultaneous DSBs. In *Igk*, DSBs are generated in the V and J gene segments (Fugmann et al., 2000a). During *Igh* recombination, RAG first creates DSBs in the D and J gene segments then in the V and DJ gene segment (Fugmann et al., 2000a). In contrast, Cas9 generates a single DSB in the target allele per gRNA. To determine if multiplicity of DSBs impacts DDR signaling, *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2:iCas9* pre-B cells were transfected with two gRNAs to simultaneously generate DSBs at J_H1 and D_H1-*1* to more accurately approximate RAG DSBs (**Figure 2.5 B**). Similar to results with a single Cas9 DSB, generation of paired Cas9 DSBs in *Igh* activated cDDR but not ncDDR (**Figure 2.5 C-E**). Importantly, the absence of ncDDR activation

is not secondary to effects of nucleofection as mock transfection of *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2* pre-B cells expressing FLAG-RAG1 did not alter generation of RAG DSBs or activation of either cDDR or ncDDR (**Figure 2.6**). In sum, these results demonstrate that Cas9 and RAG DSBs at *Ig* alleles activate distinct DDR signals. While both Cas9 and RAG DSBs activate cDDR, the ncDDR program is uniquely initiated by RAG DSBs (**compare Figures 2.1 and 2.5**). Given that Cas9 and RAG DSBs were generated at the same genomic location, these findings suggest that unique features of the RAG endonuclease itself are responsible for coordinating the ncDDR response.

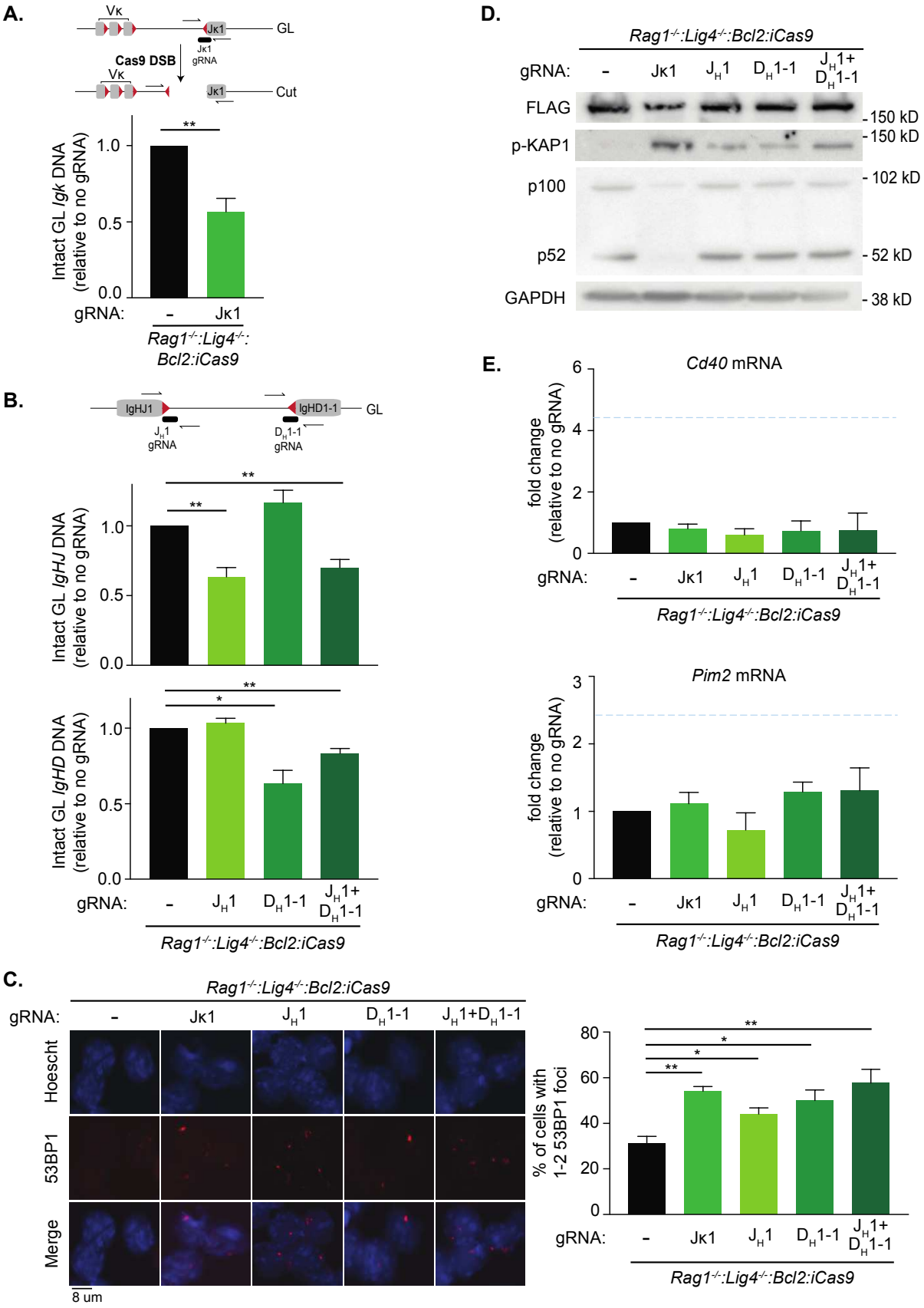
A.
Vκ
Jκ1
GL
Jκ1 gRNA
Cas9 DSB
Vκ
Jκ1
Cut
Intact GL Igk DNA (relative to no gRNA)
1.0
0.5
0.0
**
gRNA:
-
Jκ1
Rag1-/-:Lig4-/-: Bcl2:iCas9
B.
IgHJ1
IgHD1-1
GL
JH1 gRNA
DH1-1 gRNA
Intact GL IgHJ DNA (relative to no gRNA)
Intact GL IgHD DNA (relative to no gRNA)
**
*
gRNA:
-
JH1
DH1-1
JH1+ DH1-1
Rag1-/-:Lig4-/-:Bcl2:iCas9
C.
Rag1-/-:Lig4-/-:Bcl2:iCas9
gRNA:
-
Jκ1
JH1
DH1-1
JH1+DH1-1
Hoescht
53BP1
Merge
8 um
% of cells with 1-2 53BP1 foci
80
60
40
20
0
D.
Rag1-/-:Lig4-/-:Bcl2:iCas9
gRNA:
FLAG
p-KAP1
p100
p52
GAPDH
150 kD
150 kD
102 kD
52 kD
38 kD
E.
Cd40 mRNA
Pim2 mRNA
fold change (relative to no gRNA)
6
4
2
0
3
2
1
0

Figure 2.5 (previous page): Cas9 DSBs at *Ig* genes do not activate ncDDR. *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2:iCas9* abl pre-B cells were treated with imatinib and doxycycline as in Fig. 2 then transfected with *Jk1*, J_H1, J_HD1-*1* or both J_H1 + J_HD1-*1* gRNAs as indicated. Following transfection, cells were maintained in doxycycline and imatinib with all analyses completed 48 h after gRNA transfection. **(A-B)** DSBs quantified by qPCR analysis of *Jk1* (A) and J_H1 and J_HD1-*1* (B) genomic DNA. Schematics show germline (GL) locus and location of respective gRNAs and primers. PCR is normalized to cells without gRNA (-). Loss of germline product is representative of DSB generation. **(C)** Representative images of 53BP1 foci. Scale bar denotes 8 mm. Bar graph shows foci quantitation of 300 cells for each condition. **(D)** Western blot analysis of p-KAP1, NFκB2 and PIM2. GAPDH is shown as loading control. **(E)** *CD40* and *Pim2* mRNA expression. Blue dashed line indicates mRNA expression in response to RAG DSBs from Fig. 1 H and is included for comparison. Data in A, B, C, and E are mean ± SE for three independent experiments. Data in D are representative of three independent experiments. Statistical significance was calculated using Student's t-test. *$p \leq 0.05$, **$p \leq 0.01$.

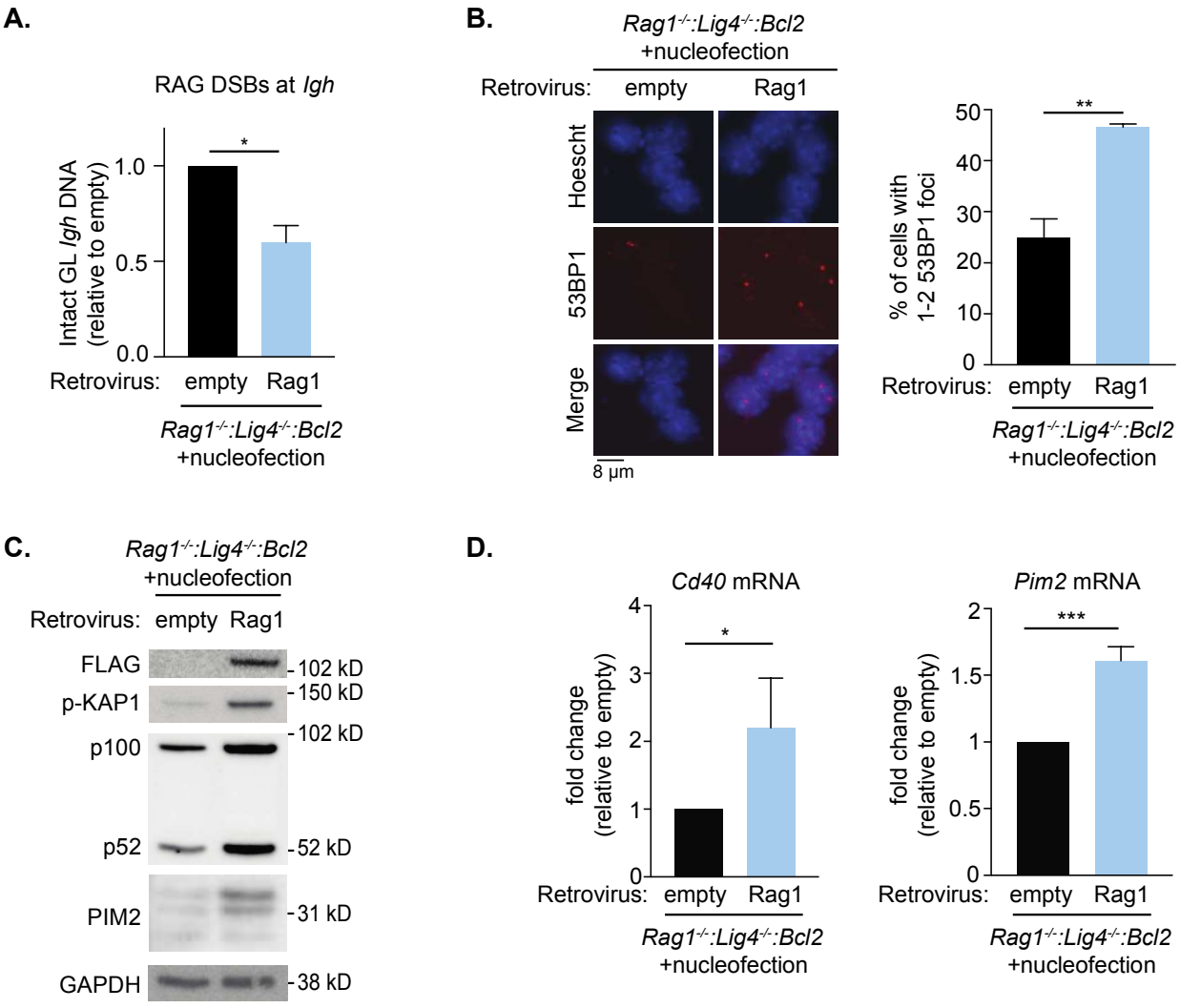

Figure 2.6: Nucleofection does not impair activation of ncDDR by RAG DSBs. (A-D) *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2* abl pre-B cells were transduced with an empty vector (control) or vector expressing RAG1 as in Fig. 1 E. Cells were treated with imatinib for 24 h then nucleofected (no gRNA) and maintained in imatinib as in Figs. 2 and 3. Analyses were done 24 hours after nucleofection (48 hours after imatinib and DSB initiation). **(A)** DSBs quantified by qPCR analysis of *Igh* (J_H1) genomic DNA as in Fig. 1 E. **(B)** Representative images of 53BP1 foci. Scale bar denotes 8 mm. Bar graph shows foci quantitation of 300 cells for each condition. **(C)** Western blot analysis of p-KAP1, NFκB2 and PIM2. GAPDH is shown as loading control. **(D)** *Cd40* and *Pim2* mRNA expression. Data in A, B, and D are mean ± SE for three independent experiments. Data in C are representative of three independent experiments. Statistical significance was calculated using Student's t-test. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$

2.3.4 RAG1 non-core domains are critical for activation of ncDDR

The non-core domains of RAG1, particularly the NTR, have been shown to regulate RAG complex stability, localization and recombination activity (Brecht et al., 2020; Coster et al., 2012; Elkin et al., 2005; Jones and Simkus, 2009; Kassmeier et al., 2012; Lescale and Deriano, 2017; Matthews et al., 2007). To test whether these regions of RAG1 function in DDR signaling we retrovirally transduced *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2* abl pre-B cells with the following RAG1 variants: 1) full-length RAG1, 2) core RAG1 (cRAG1, C), which lacks the entire N-terminal and C-terminal regions but retains the endonuclease domain, 3) RAG1 with inactivating P326G mutation of the E3 ligase catalytic site (RAG1^{P326G}, PG), and 4) endonuclease-inactive RAG1 with D708A mutation within its catalytic domain (RAG1^{D708A}, DA) (**Figure 2.7 A**) (Beilinson et al., 2021; Dudley et al., 2003; Fugmann et al., 2000b; Ji et al., 2010; Kim et al., 1999; Simkus et al., 2007; Yurchenko et al., 2003). Cells were subsequently treated with imatinib to induce G1 arrest and expression of endogenous RAG2. As expected, the catalytically inactive RAG1^{D708A} did not generate DSBs and, accordingly, neither cDDR nor ncDDR programs were activated (**Figure 2.7 B-E**). Expression of cRAG1, RAG1^{P326G} and full-length RAG1 resulted in generation of equivalent levels of DSBs in *Igh* (**Figure 2.7 B**). cRAG1 and RAG1^{P326G} have been shown to have impaired *Igk* recombination (Dudley et al., 2003; Simkus et al., 2007). The similar levels of DSBs between these variants and full-length RAG1 in our studies are resultant of the repair-deficient (*Lig4*$^{-/-}$) background and sufficient time (48 hours) to generate DSBs. Pre-B cells expressing cRAG1 and RAG1^{P326G} had cDDR activation (i.e., 53BP1 foci and p-KAP1) similar to cells expressing full-length RAG1 (**Figure 2.7 B-D**). Despite comparable DSBs and cDDR signaling, expression of cRAG1, in contrast to full-length RAG1 and RAG1^{P326G}, did not activate NFκB2 or trigger increased PIM2 protein (**Figure 2.7 D**). However, cRAG1-mediated DSBs did induce *Cd40* and

Pim2 mRNA transcripts similar to DSBs generated by RAG1 and RAG1^{P326G} (**Figure 2.7 E**). Thus, cRAG1-mediated DSBs activate some but not all components of ncDDR signaling. These findings demonstrate that induction of the ncDDR depends upon generation of a DSB and activities of the non-core regions of RAG1, independent of the E3 ligase domain activity.

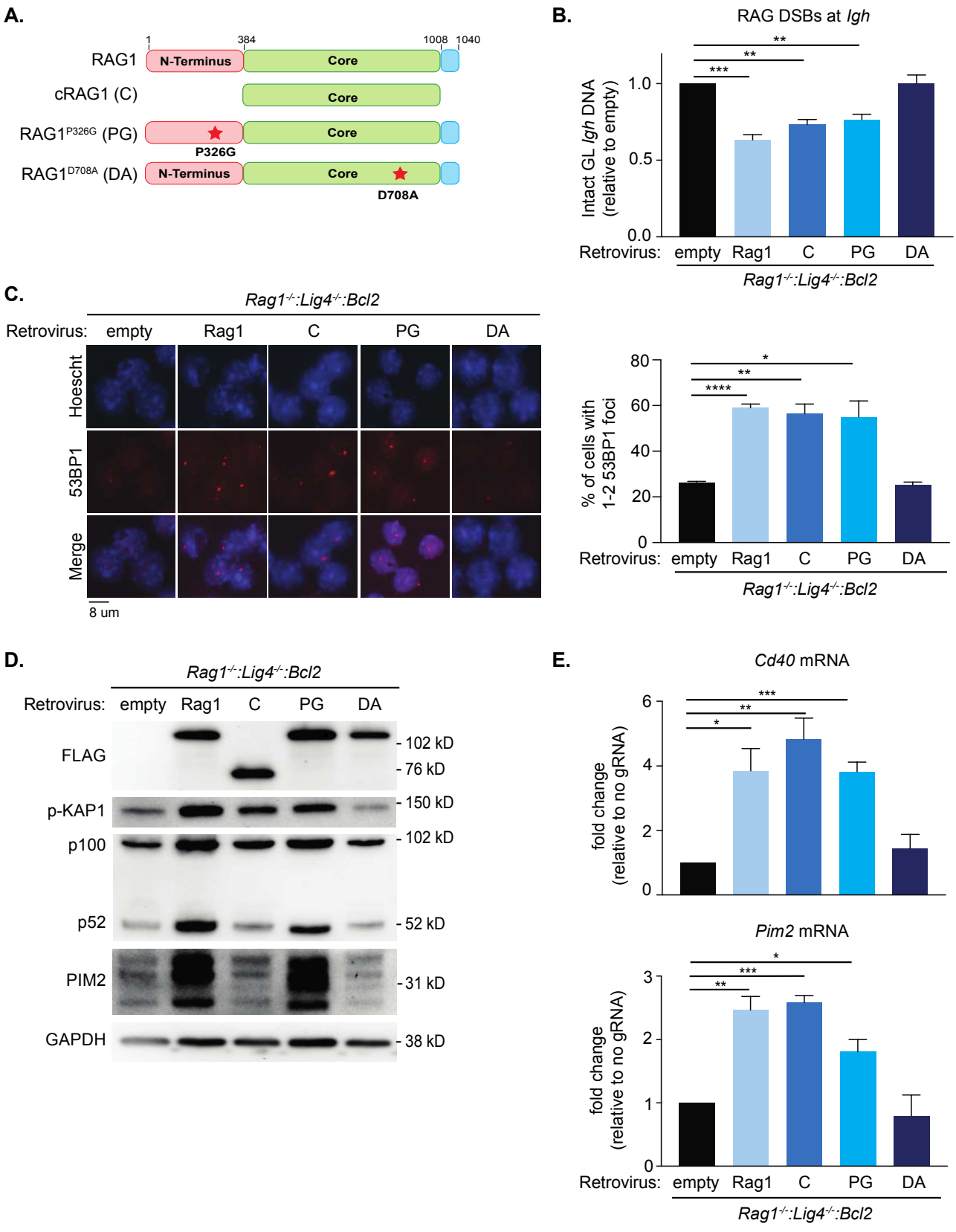
A.
1
384
1008
1040
RAG1
N-Terminus
Core
cRAG1 (C)
Core
RAG1P326G (PG)
P326G
Core
RAG1D708A (DA)
N-Terminus
Core
D708A
B.
RAG DSBs at Igh
Intact GL Igh DNA (relative to empty)
1.0
0.5
0.0
Retrovirus: empty Rag1 C PG DA
Rag1-/-:Lig4-/-:Bcl2
C.
Rag1-/-:Lig4-/-:Bcl2
Retrovirus: empty Rag1 C PG DA
Hoescht
53BP1
Merge
8 um
% of cells with 1-2 53BP1 foci
80
60
40
20
0
Retrovirus: empty Rag1 C PG DA
Rag1-/-:Lig4-/-:Bcl2
D.
Rag1-/-:Lig4-/-:Bcl2
Retrovirus: empty Rag1 C PG DA
FLAG
102 kD
76 kD
p-KAP1
150 kD
p100
102 kD
p52
52 kD
PIM2
31 kD
GAPDH
38 kD
E.
Cd40 mRNA
fold change (relative to no gRNA)
6
4
2
0
Pim2 mRNA
fold change (relative to no gRNA)
3
2
1
0
Retrovirus: empty Rag1 C PG DA
Rag1-/-:Lig4-/-:Bcl2

Figure 2.7 (previous page): Non-core regions of RAG1 are necessary for activation of ncDDR. *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2* abl pre-B cells were retrovirally transduced with empty vector or vector expressing indicated RAG1 variants then treated with imatinib for 48 h. **(A)** Schematic of RAG1 variants. Figure not to scale. **(B)** DSBs quantified by qPCR analysis of *Igh* (J_H1) genomic DNA and analyzed as in Fig. 1 E with results normalized to empty vector control, which has only germline *Igh* DNA. **(C)** Representative images of 53BP1 foci. Scale bar denotes 8 mm. Bar graph shows foci quantitation of 300 cells for each condition. **(D)** Western blot analysis of p-KAP1, NFκB2 and PIM2. GAPDH is shown as loading control. **(E)** *Cd40* and *Pim2* mRNA expression. Data in B, C, and E are mean ± SE for three independent experiments. Data in D are representative of three independent experiments. Statistical significance was calculated using Student's t-test. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$

2.4 Discussion

The differential cellular responses to Cas9- versus RAG-mediated DSBs reveal that pre-B cells trigger distinct DDR signals based on the mechanism of DNA injury. While all DSBs activate canonical pathways to promote DSB repair, RAG DSBs uniquely trigger a developmental program that regulates pre-B cell survival and differentiation. PIM2 supports survival of pre-B cells with RAG DSBs to permit time for completion of *Ig* recombination and for multiple rearrangements necessary for antigen receptor diversification (Bednarski et al., 2012; Derudder et al., 2009). In pre-B cells with RAG DSBs, NFκB2 drives expression of the SPIC/BCLAF1 transcriptional repressor complex, which modulates PU.1 activity to regulate a broad cellular program, including suppression of SYK and pre-BCR signaling (Bednarski et al., 2016; Soodgupta et al., 2019). Selective activation of these programs by RAG DSBs is likely beneficial to developing pre-B cells as it promotes maturation of cells with recombined *Ig* loci. In contrast, this process is not triggered in cells with non-RAG-mediated DSBs where initiation of the developmental DDR program could result in aberrant maturation of B cells that do not express an appropriately rearranged Ig, which poses risks for development of autoreactive clones or leukemic transformation.

We show that the unique ncDDR activated by RAG DSBs is regulated, at least in part, by the RAG protein itself. Specifically, our studies identify a novel, critical activity of non-core regions of RAG1 in coordinating DDR. The non-catalytic domains of RAG1, particularly the NTR, contain highly unstructured or disordered regions that can serve as binding sites for interacting proteins (Jones and Simkus, 2009; Notarangelo et al., 2016). In regards to RAG1 activity in DDR signaling, RAG1 non-core regions may associate with DDR signaling factors to alter their activity or, alternatively, may recruit signaling proteins to sites of DSBs, where they can be modulated by

ATM or other DDR kinases to trigger downstream signaling pathways. Additional studies are needed to delineate the precise regions of RAG1 that function in ncDDR signaling. A function of RAG1 non-core regions in DDR signaling reveals potential mechanisms underlying immune dysregulation in patients with variants in these domains (Lee et al., 2014; Notarangelo et al., 2016). These essential activities of RAG1 ensure that DDR signaling promotes B cell differentiation and diversification while limiting errant B cell development in response to non-programmed DSBs.

2.5 Methods

2.5.1 Cell lines

Rag1$^{-/-}$*:Bcl2* and *Art*$^{-/-}$*:Bcl2* abl pre-B cells were previously described (Bredemeyer *et al.*, 2008; Soodgupta *et al.*, 2019). *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2* abl pre-B cells were generated from *Rag1*$^{-/-}$*:Lig4*$^{flox/flox}$*:Bcl2* abl pre-B cells, which were made from *Rag1*$^{-/-}$ *:Lig4*$^{flox/flox}$*:Bcl2* mice by retroviral transduction of bone marrow cells with MSCV-v-abl plasmid as described previously (Bredemeyer *et al*, 2006). *Rag1*$^{-/-}$*:Lig4*$^{flox/flox}$*:Bcl2* abl pre- B cells were then transiently transfected with MSCV-Cre-IRES-Thy1.1 vector (Addgene, Plasmid #17442) and subsequently subcloned to identify clones with deletion of *Lig4*, which was confirmed by genotyping (PCR) and phenotyping (persistent RAG-mediated DSBs following retroviral expression of RAG1). *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2* abl pre-B cells were transduced with lentiviral vector pFLRU-TRE-Cas9-Ubc-rtTA-IRES-Thy1.2 vector to generate *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2:iCas9* abl pre-B cells with stably integrated inducible Cas9 (Purman *et al.*, 2019). Transduced cells were sorted for Thy1.2 expression and then subcloned. All cell lines were authenticated by genotyping. To induce cell cycle arrest and

induction of RAG DSBs, abl pre-B cells were treated with 3 μM imatinib (10^6 cells/ml) for indicated times prior to harvesting for genomic DNA, protein, RNA and immunofluorescence (Bredemeyer *et al.*, 2008; Johnston *et al*, 2022; Soodgupta *et al.*, 2019). For Cas9 induction, *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2:iCas9* abl pre-B cells were treated with 2 μg/ml doxycycline simultaneously with addition of imatinib.

2.5.2 Mice

Mice were used for generation of abl pre-B cell lines as detailed above. Mice were maintained under specific pathogen-free conditions at the Washington University School of Medicine and were handled in accordance to the guidelines set forth by the Division of Comparative Medicine of Washington University.

2.5.3 cDNA expression

pFLRU-TRE-Cas9-Ubc-rtTA-Thy1.2 lentiviral vector expressing cDNA encoding 5' FLAG tagged *Streptococcus pyogene* Cas9 was a gift from Gene Oltz and Barry Sleckman (Purman *et al.*, 2019). cDNA encoding 5' FLAG tagged RAG1 or cRAG1 was cloned into the pOZ-IRES-hCD25 retroviral vector (Bednarski *et al.*, 2016). RAG1 E3 ligase mutant ($RAG1^{P326G}$) and the catalytically-inactive RAG1 ($RAG1^{D708A}$) were generated using QuikChange II XL (Agilent) according to manufacturer's protocol. Retrovirus and lentivirus were produced in PlatE cells (Cell Biolabs) and 293T cells, respectively, by transfection of the viral plasmids with Lipofectamine 2000 (Life Technologies) (Bednarski *et al.*, 2012; Soodgupta *et al.*, 2019). For lentivirus, pCMV- VSV-G and pCMV-d8.2R were also included in the transfection (Soodgupta *et al.*, 2019; Stewart *et al*, 2003). Viral supernatant was collected and pooled from 24-72 hours after transfection. Abl pre-B cells were transduced with unconcentrated virus in media

with polybrene (5 μg/ml; Sigma-Aldrich) as previously described (Soodgupta *et al.*, 2019). Transduced cells were identified by flow cytometric assessment of hCD25 or Thy1.2 and were sorted using anti-hCD25 or anti-Thy1.2 magnetic beads (Miltenyi Biotec) on MS columns (Miltenyi Biotec) according to the manufacturer's protocol.

2.5.4 Generation of Cas9-mediated DSBs

gRNAs were designed using CHOPCHOP, with the exception of the *Eb* guide, which is previously published (Dorsett *et al*, 2014; Labun *et al*, 2019; Purman *et al.*, 2019). gRNA sequences are listed in **Table 2.1**. gRNAs were generated by *in vitro* transcription using the Precision gRNA Synthesis kit (Invitrogen) per manufacturer's guidelines. *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2:iCas9* cells were treated with doxycycline and imatinib for 24 h. Subsequently, 20 x 10^6 cells and 400 μg of gRNA were resuspended in 100 μl Nucleofector Solution for Human B Cells (Lonza) then nucleofected using an Amaxa Nucleofector II (Lonza), program X-001, according to the manufacturer's instructions. Nucleofected cells were transferred directly to preequilibrated recovery medium containing doxycycline and imatinib at 10 x 10^6 cells/ml and incubated for indicated times prior to harvesting for genomic DNA, protein, RNA and immunofluorescence. Putative off-target gRNA binding sites were identified using Cas- OFFinder (Bae *et al*, 2014).

2.5.5 Flow cytometric analyses

All flow cytometric analyses were performed on a Cytek- modified BD FACScan (BD Biosciences). Antibodies used included phycoerythrin (PE)- conjugated anti-hCD25 (Biolegend) and allophycocyanin (APC)-conjugated anti-Thy1.2 (Biolegend).

2.5.6 Western blot analyses

Western blots were done on whole cell lysates as previously described (Bednarski *et al.*, 2016). Anti-NFκB2 (p100/p52) (clone D9S3M) and anti- GAPDH (clone D16H11) antibodies were from Cell Signaling Technology. Anti-phospho- KAP1 antibody (A300-767A) was from Bethyl Laboratories. Anti-PIM2 (clone 1D12) was from Santa Cruz Biotechnology. Anti-FLAG (clone M2) was from Sigma. Secondary reagents were horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Cell Signaling) or anti-rabbit IgG (Cell Signaling). Westerns were developed with ECL (Pierce) and ECL Prime (Cytiva).

2.5.7 RT-PCR

For genomic DNA isolation, cells were lysed in lysis buffer (100 mM TRIS pH 8.5, 5 mM EDTA, 200 mM NaCl, and 0.2% SDS) and DNA was precipitated by addition of isopropanol, washed with 70% then 100% ethanol, and finally resuspended in water (Johnston *et al.*, 2022; Soodgupta *et al.*, 2019). For PCR over the break assay, genomic DNA was digested with NEBNext dsDNA Fragmentase (NEB) for 10 minutes followed by PCR Cleanup (QIAGEN) as per manufacturer's instructions before RT-PCR. RNA was isolated using RNeasy (QIAGEN) and reverse transcribed using a polyT primer with SuperScriptII (Life Technologies) according to manufacturers' protocol. RT-PCR was performed using Brilliant II SYBR Green (Agilent) and acquired on an MX3000P (Stratagene) or QuantStudio 3 (ThermoFisher). Each reaction was run in triplicate. For PCR over the break analyses, values at targeted sites were normalized to PCR product spanning CD19, a control region of uncut genomic DNA and fold change was determined by the DD cycle threshold method (Johnston *et al.*, 2022). Primer sequences are listed in **Table 2.1**.

Primer Name	Application	Sequence
Jk1_F	qRT PCR for DNA breaks	GCTACCCACTGCTCTGTTCC
Jk1_R	qRT PCR for DNA breaks	CCTTGGAGAGTGCCAGAATC
CD19_F	qRT PCR for DNA breaks	TGTCTCCTTCCTCCTCTTTCT
CD19_R	qRT PCR for DNA breaks	CTCAACTCAGAACCCAGACTTT
IgHJ1_F	qRT PCR for DNA breaks	CCGTTTCAGAATGGAATGTGC
IgHJ1_R	qRT PCR for DNA breaks	TAAGGCAGGATGTGGAGAGA
IgHD1-1_F	qRT PCR for DNA breaks	GGTTGGATTTCTTTGTGGGC
IgHD1-1_R	qRT PCR for DNA breaks	CAACTGAAACTCAACCGTGC
Sxn19_F (Jk1 Off Target)	qRT PCR for DNA breaks	TTAACCCCCATGGGACTGC
Sxn19_R (Jk1 Off Target)	qRT PCR for DNA breaks	TTGGAGAGGCCTCAGGTTCA
Gab1_F (JH1 Off Target)	qRT PCR for DNA breaks	ATCTGGGCTGGGAAAATCAGA
Gab1_R (JH1 Off Target)	qRT PCR for DNA breaks	GGAGAGTTACAAGGTCCCCC
Pou6f2_F (DH1-1 Off Target)	qRT PCR for DNA breaks	GATCAGTGTCCTCAGCCATTGG
Pou6f2_R (DH1-1 Off Target)	qRT PCR for DNA breaks	GAACCACCCCCATGACCAC
Eb_F	qRT PCR for DNA breaks	TCATGTAATGAGTCACAGGAACAGA
Eb_R	qRT PCR for DNA breaks	AGCCCTTACTCACAGAGGATACAC
Gapdh_F	qRT PCR for DNA breaks	AACCCTTAAGAGGGATGCTGC
Gapdh_R	qRT PCR for DNA breaks	TTGCTACGCCATAGGTCAGG
Cdkn2aip_F (Eb Off Target)	qRT PCR for DNA breaks	GCTTACATGGTTAGTTCATGGCA
Cdkn2aip_R (Eb Off Target)	qRT PCR for DNA breaks	CAGTAGAAGGCAAGAAACCAGC
Cdhr3_F (Gapdh Off Target)	qRT PCR for DNA breaks	AAGCAGTATATCCCTCTGTCTGTG
Cdhr3_R (Gapdh Off Target)	qRT PCR for DNA breaks	GATGGCTTCTGGATCCCCCA
Cd40_F	qRT PCR	GGTGGTCAAGAAACCAAAGG
Cd40_R	qRT PCR	ACTGGAGCAGCGGTGTTATG
Pim2_F	qRT PCR	CGGGTGTGATACGCCTTCTTG
Pim2_R	qRT PCR	GCCCCTTCTCTGTGATATAGTCG
B-actin_F	qRT PCR	TCATCACTATTGGCAACGAGCGGTTC
B-actin_R	qRT PCR	TACCACCAGACAGCACTGTGTTGGCA
Jk1	gRNA sequence	GTGGAGTACTACCACTGTGG
IgHJ1	gRNA sequence	GAGTTTTAGTATAGGAACAGAGG
IgHD1-1	gRNA sequence	TGCTGATGGATATAGCACTGTGG
Tcrb	gRNA sequence	GAAAGCCAGCCAATGAATGC
Gapdh	gRNA sequence	CGGTGGCTGTTCTTCAACCG

Table 2.1: Primer Sequences. List of sequences for gRNAs used for Cas9 DSB generation and for primers used for RT-PCR analysis of germline DNA (DSB generation) and quantitation of mRNA.

2.5.8 Immunofluorescence

Immunofluorescence microscopy was done as previously described (Brickner *et al*, 2017). Briefly, cells were applied to coverslips using Cell Tak (Corning) at 37℃ for 20 minutes then

extracted with 0.2% Triton in PBS for 1 minute on ice and fixed with 3.2% paraformaldehyde. Cells were washed with IF Wash Buffer (PBS, 0.5% NP-40 and 0.02% NaN3), then blocked with IF Blocking Buffer (10% FBS in IF Wash Buffer) for 30 minutes at room temperature. Slides were incubated with rabbit anti-53BP1 (Novus; 1:500) in IF Blocking Buffer for 1 hour at room temperature. Slides were washed then stained with goat anti-rabbit IgG conjugated with Alexa Fluor 594 (Invitrogen; 1:1000) and Hoechst 33342 (Sigma-Aldrich) for 30 minutes at room temperature followed by sample mounting with Prolong Gold mounting media (Invitrogen). Microscopy was performed on an Olympus fluorescence microscope (BX-53) using an ApoN 60X/1.49 NA oil immersion lens or an UPlanS-Apo 100X/1.4 oil immersion lens and cellSens Dimension software. Raw images were exported into Adobe Photoshop, and for any adjustment in image contrast or brightness, the levels function was applied. Foci were manually counted in triplicate on at least 100 cells for each biological replicate.

2.5.9 Statistical analysis

Statistical analyses were done by Student's t-test using Prism (GraphPad Software).

Chapter 3

RAG1 recruits signaling factors to the site of a DNA break

by
Rachel Leigh Johnston

3.1 Abstract

RAG DSBs during V(D)J recombination in developing B cells activate both cDDR, which coordinates DSB repair, and ncDDR, which induces B cell developmental programs. My work indicates that ncDDR is uniquely activated by RAG DSBs and this activity is dependent the RAG1 NTR. RAG1 has been previously shown to associate with a broad protein interactome that regulates activity of the RAG complex. Here, I hypothesize that RAG1, through its NTR, recruits signaling proteins to sites of DSBs to activate ncDDR developmental programs in developing B cells. Full-length RAG1 and cRAG1 (lacking the NTR) were immunoprecipitated from pre-B cells with DSBs and interacting proteins were identified by mass spectrometry. Proteins that uniquely associate with full length RAG1 and not with cRAG1 were identified, supporting the idea that the RAG1 NTR recruits distinct factors to sites of DSBs. Several of the RAG1 binding partners have previously identified functions in chromatin remodeling, transcriptional regulation, and nucleolar localization. Function of these RAG1 interacting partners will be tested by knock-down approaches to determine their function in DDR signaling. To further define the distinct function of RAG1 and its interactome in DDR, the RAG1 NTR was fused to Cas9 to generate Cas9-mediated DSBs and tether RAG1 along with its associated proteins to the site of a non-RAG DSB. These studies will define the RAG-specific mechanisms that direct DDR in pre-B cells.

3.2 Introduction

Work outlined in Chapter 2 supports that unique features of RAG1 coordinate the ncDDR. As the RAG endonuclease directs DSBs to the correct location, features of the RAG endonuclease may be responsible for activation of the distinct developmental programming. Further, we find that the RAG1 NTR (amino acids 1-383) has essential functions in regulating the ncDDR. RAG1

has been shown to form a complex with MDC1 to recruit additional DNA damage response proteins (Coster et al., 2012). Additionally, the RAG1 NTR has identified activities in down-regulation of RAG1 protein levels and nucleolar sequestration for RAG1 (Beilinson et al., 2021; Brecht et al., 2020; Kassmeier et al., 2012). Previous work has shown that expression of RAG proteins containing just the core domains required for catalytic activity results in abnormal lymphocyte development (Dudley et al., 2003; Talukder et al., 2004a). These findings suggest that the non-core domains of the RAG proteins may have functions in directing DDR signals that are needed for continued B cell maturation. The functional significance of the RAG1 NTR is further supported by the diverse set of clinical phenotypes human patients with mutations in this region (Notarangelo et al., 2016). However, the role of the non-catalytic regions of RAG1 in DDR signaling has yet to be investigated. Here, I hypothesize that RAG1, through its NTR, recruits proteins to sites of DNA damage that in turn regulate DDR signaling.

In this chapter, I describe an approach and initial results for defining the RAG1 interactome and the role of these RAG1 binding partners in regulating ncDDR signaling. To define the proteins associated with the RAG1 NTR, I immunoprecipitated full-length RAG1 and cRAG1, which lacks the NTR, from pre-B cells with DSBs. Comparison of protein interactomes identified a subset of proteins whose interaction is lost after truncation of the NTR. These N-terminal associated proteins present potential candidates that may regulate ncDDR signaling following a RAG DSB. To examine if recruitment of RAG1-binding proteins to the site of a DSB is critical for activation of ncDDR, catalytically-inactive RAG1 variants were fused to Cas9. These Cas9-RAG1 fusion constructs are expressed in pre-B cells to generate a Cas9-mediated DNA break and recruit nuclease-inactive RAG1 and its binding partners to the site of the DSB. These fusion constructs

provide a powerful new tool to assess the function of distinct regions of RAG1 in ncDDR induction.

3.3 Results

3.3.1 RAG1 interacts with a distinct protein interactome

As previously described, loss of the non-core region of RAG1 results in a defect in DDR signaling (**Figure 2.7**). Here, we hypothesized that the non-core regions of RAG1 bind distinct proteins and recruits them to sites of DSBs where they can modulate DDR. To test this, I used immunoprecipitation and mass spectrometry approaches. FLAG-tagged full length RAG1 or cRAG1 (**Figure 2.7**) were retrovirally expressed in *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2* pre-B cells. Cells were subsequently treated with imatinib to induce G1 arrest, expression of RAG2 and induction of DSBs (as in **Figure 2.1**) (Bredemeyer et al., 2008; Clark et al., 2014). In this system, RAG DSBs are not repaired, and DDR remains persistently activated. The RAG complex remains associated with the broken DNA ends until repair (Helmink and Sleckman, 2012). Thus, this approach allows for identification of RAG1-binding partners at sites of DSBs.

Full-length RAG1 and cRAG1 were independently immunoprecipitated from nuclei of imatinib-treated abl pre-B cells following generation of DSBs using anti-FLAG antibodies (Soodgupta et al., 2019). Associated proteins were identified by tandem mass spectrometry. Protein interactomes for full-length RAG1 and cRAG1 were independently curated using Scaffold and subsequently compared to identify proteins enriched for binding to RAG1. 146 unique binding partners of RAG1 were identified, which supports the hypothesis that RAG1 recruits unique factors to the site of a DSB (**Figure 3.1** and **Table 3.1**). Interestingly, cRAG1 also had 270 unique

binding partners compared to full-length RAG1 suggesting that loss of the NTR permits association of a different protein complex with RAG1 than when NTR is present (**Figure 3.1 and Table 3.2**). As validation of our approach, RAG2 and DDR proteins, including NFκB1, were identified as binding partners common to both RAG1 and cRAG1, (**Table 3.3**). These results demonstrate that RAG1 complexes with a unique interactome through its NTR.

We focused our attention on the unique binding partners of full-length RAG1, i.e., those dependent on NTR, based on our previous work demonstrating this region is necessary for activation of ncDDR. To narrow our investigation, we compared our list of RAG1-NTR associated proteins with known ATM phosphorylation targets, as this is the master DDR regulator following a RAG DSB (Bednarski and Sleckman, 2019; Matsuoka et al., 2007). As shown in **Tables 3.1-3.3**, several of the proteins identified in the immunoprecipitation studies are also known ATM targets. We also compared our list of binding partners to a previous study that performed BioID with RAG1 constructs, which is a method to screen for protein-protein interactions based on proximity (Brecht et al., 2020; Roux et al., 2018). This study focused on identifying RAG1 interacting proteins during V(D)J recombination (Brecht et al., 2020). **Tables 3.1-3.3** again show many proteins from our list of RAG1 interactors that also appeared in this previous BioID study. This acts as validation of our results, as many of the proteins identified as binding partners have been identified previously to be ATM targets or in close proximity to RAG endonuclease during V(D)J recombination.

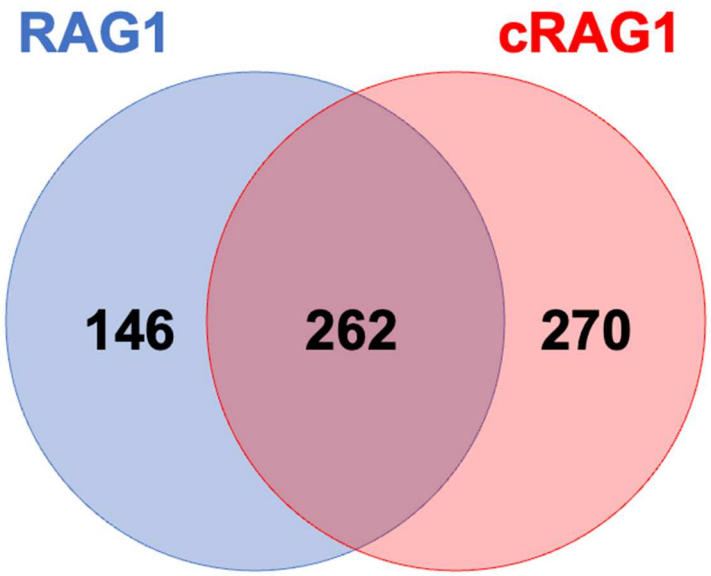

Figure 3.1: RAG1 and cRAG1 unique interacting proteins after MS analysis. Venn diagram of protein interactomes for RAG1 and cRAG1 generated from mass spectrometry.

3.3.2 Recruitment of RAG binding proteins to sites of DSBs

I hypothesize that if RAG1 plays a direct role in regulating the DDR induced by RAG DSBs, then recruitment of RAG1 and its binding partners to the site of a non-RAG DNA break will trigger induction of ncDDR. To examine this hypothesis, Cas9 was fused to a catalytically inactive full length RAG1 (RAG1^{D708A}, **Figure 2.7**) (Cas9-RAG1^{D708A}). In addition, to determine if the RAG1 NTR is sufficient to activate ncDDR, we also generated constructs with 1) the RAG1 NTR fused to Cas9 (Cas9-R1NTR) and 2) the endonuclease-inactive core cRAG1^{D708A} fused to Cas9 (Cas9-cRAG1^{D708A}). These fusion constructs are similar to other Cas9 fusions and are expected to retain Cas9 nuclease activity (Jayavaradhan et al., 2019). This technique allows for RAG1 to be tethered to Cas9-mediated DSBs, resulting in its downstream DDR function independent of its endonuclease activity. These constructs have all been successfully independently expressed in abl pre-B cells (**Figure 3.2 A**). To demonstrate DNA break generation

activity, Cas9-RAG1^{D708A} along with targeted gRNAs were transfected into *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2* pre-B cells following imatinib treatment and associated G1 arrest. Initial experiments show generation of DSBs at *Eb* and *IgHJ1* loci and phosphorylation of KAP1, indicating induction of cDDR (**Figure 3.2 B-C)**. These constructs and experimental approach will be used to determine the nuclease-independent functions of RAG1 in regulating DDR signaling.

3.4 Future Directions

Experiments described above demonstrate significant progress towards assessing the mechanistic role the RAG1 NTR plays in DDR signaling following a RAG DSB. Ongoing and future work described here will be key in assessing this question, as well as examining if the genomic location of a DNA break impacts the downstream DDR.

3.4.1 Do RAG1-NTR interacting proteins regulate ncDDR?

While the results from the initial immunoprecipitation studies are promising and offer targets to follow up on, subsequent studies involve optimization of the mass spectrometry conditions. Initial mass spectrometry results revealed a large number of proteins bound to the empty vector not expressing a RAG construct. To decrease this non-specific binding, extra wash steps have been included following the immunoprecipitation of FLAG-tagged constructs. Non-specific binding was also decreased with an additional pre-clearing step wherein the nuclear lysate was applied to beads that were not conjugated to FLAG-antibody. These optimization steps are critical to ensure that the protein interactome pulled down is an accurate representation of the physiologic RAG1 binding partners.

Once RAG1-interacting proteins have been identified through immunoprecipitation and Cas9-RAG1 fusion experiments, their expression will be suppressed to determine if their loss affects DDR activation in response to RAG DSBs. Target proteins will be inhibited with RNA interference (RNAi). Following confirmation of unique interaction with the RAG1-NTR, each factor will be suppressed by RNAi in *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2* pre-B cells and subsequent induction of RAG DSBs with expression of full length RAG1. After confirming knockdown of protein by Western blot, downstream DDR will be assessed by activation of NFκB2, PIM2 and pKAP1 as previously described. Here, if the binding partner is necessary for activation of ncDDR, then knockdown will impair induction of NFκB2 and PIM2 while retaining 53BP1 foci formation and phosphorylation of KAP1. Once binding partners of RAG1 critical for ncDDR have been identified, additional mutagenesis of the RAG1 NTR can be performed to determine the sequence required to coordinate the binding of these proteins.

3.4.2 Is the N-terminal region of RAG1 sufficient to activate ncDDR?

The studies detailed here provide critical insight into the factors that control induction of the ncDDR. The Cas9-RAG1 fusion proteins described above permit generation of DSBs by Cas9 and recruitment of the RAG1 variants and their associated interacting proteins to the sites of non-RAG mediated DSBs. Subsequent experiments will include characterizing the downstream DDR following DSB generation with Cas9-R1NTR, Cas9-RAG1^{D708A}, and Cas9-cRAG1^{D708A} fusion constructs. Generation of breaks will be quantified by qPCR and 53BP1 foci as previously described. ncDDR will be assessed by activation of NFκB2 and PIM2. If the RAG1 NTR is sufficient to induce the ncDDR, then Cas9-R1NTR will activate NFκB2 and PIM2 to similar levels of RAG1 expression in *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2* pre-B cells. I expect that the Cas9-RAG1^{D708A} will activate ncDDR similar to RAG as well. To confirm that the Cas9-RAG1 fusion proteins complex

with RAG1 binding partners similarly to WT RAG1, Cas9, Cas9-R1NTR, Cas9-RAG1^{D708A}, and Cas9-cRAG1^{D708A} constructs will be immunoprecipitated from nuclei of *Rag1$^{-/-}$:Lig4$^{-/-}$:Bcl2* pre-B cells as detailed above. Binding partners will be sent for mass spectrometry, and I expect that the Cas9-R1NTR and Cas9-RAG1^{D708A} binding partners will associate with similar proteins that are bound to full length RAG1.

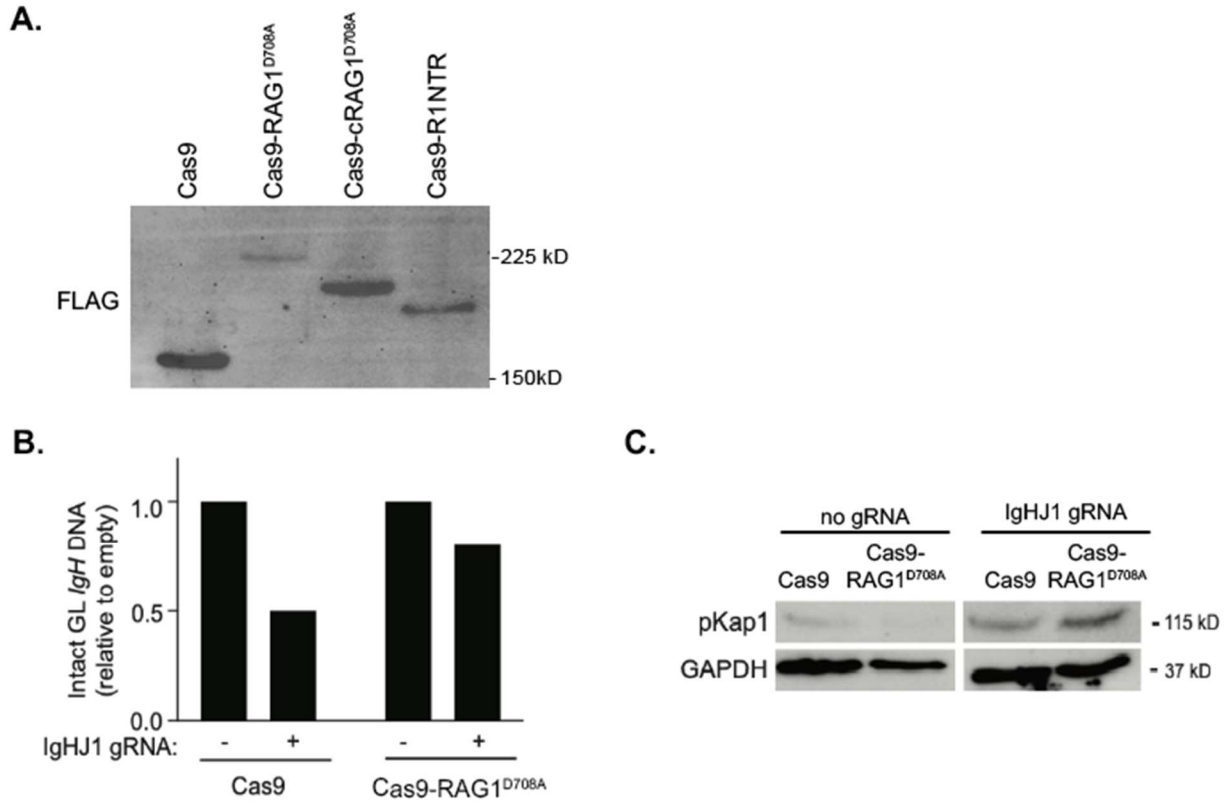

Figure 3.2: Generation of Cas9-RAG1 fusion constructs. (**A**) Western blot shows FLAG-tagged Cas9-RAG1 fusion construct expression in *Rag1$^{-/-}$:Lig4$^{-/-}$:Bcl2* abl pre-B cells transfected with indicated construct, 48h post imatinib treatment. (**B**) qPCR analysis of genomic DNA from *Rag1$^{-/-}$:Lig4$^{-/-}$:Bcl2* abl pre-B cells transfected with Cas9 or Cas9-RAG1^{D708A} fusion protein treated with imatinib for 48 h. (**C**) Western blot shows pKAP1 expression in *Rag1$^{-/-}$:Lig4$^{-/-}$:Bcl2* abl pre-B cells transfected with Cas9 or Cas9-RAG1^{D708A} fusion protein treated with imatinib for 48 h**.**

3.4.3 Does genomic location impact DDR in pre-B cells?

It is plausible that both unique features of the RAG endonuclease and the genomic location of a DSB dictate the downstream DDR and contribute to activation of the ncDDR. If this hypothesis were correct, then DSBs generated in other genomic loci would not activate ncDDR as RAG DSBs do. Therefore, I will test whether RAG-mediated ncDDR only occurs for DSBs at *Ig* loci or if it can be induced by the RAG1 NTR regardless of genomic location of DSB. Here, I will express Cas9-R1NTR in *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2* abl pre-B cells along with gRNAs targeting *Tcrb* and *GAPDH* as in **Figure 2.3**. The Cas9-R1NTR fusion will generate Cas9-mediated DSBs at these loci outside of *Ig* and tether the RAG1 NTR along with its associated binding partners to these DSBs. This approach permits recruitment of the RAG1 protein interactome to any genomic locus in pre-B cells. DSB generation and cDDR induction will be assessed through 53BP1 foci, qPCR over the break and pKAP1 as described previously. ncDDR induction will be measured through upregulation of NFκB2 and PIM2 and compared to ncDDR induction with Cas9-R1NTR and RAG1 at *Ig* loci. If RAG1 and its associated binding partners are the primary determinants of DDR, then Cas9-R1NTR DSBs at all sites are expected to activate the RAG-mediated ncDDR program. If genomic location is a determinant of DDR, then DSBs at *Tcrb* and *GAPDH* will induce responses distinct from those initiated by DSBs at *Ig*. These experiments will delineate how local environment of a DSB influences cellular response to the injury.

3.5 Discussion

These studies identified a unique interactome for the non-core regions of RAG1 and establish an experimental approach for determining the function on these in regulating ncDDR.

Here, I hypothesize that the interaction of distinct binding partners to the RAG1 NTR are critical for inducing the ncDDR in response to a RAG DSB.

One RAG1 interactor of interest is zinc finger protein 91 (Zfp91), an E3 ligase shown to be involved in NFκB2 activation. Activation of NFκB2 is dependent upon NFκB inducing kinase (NIK), which is recruited by TNF receptor associated factor (TRAF) 3 to a complex composed of TRAF2, TRAF3, and cellular inhibitors of apoptosis (cIAP1/2), which in resting cells constitutively ubiquitinates NIK, leading to its degradation (Razani et al., 2011; Zarnegar et al., 2008). Non-canonical NFκB2 precursor protein p100 normally resides in the cytosol, where it remains inactive. Translocation to the nucleus requires processing of p100 to the transcriptionally active p52. p100 processing requires its phosphorylation by the kinase Ikkα, causing proteasomal mediated cleavage to generate p52 (Vallabhapurapu et al., 2008). Prior work shows that RAG DSBs activate NFκB2 through NIK-dependent mechanisms, yet what directs this remains unknown (Bednarski et al., 2016). ZFP91 was recently identified through a microarray analysis of NFκB-regulated genes and is shown to physically associated with NIK to induce conjugation of ubiquitin chains which results in its stabilization and subsequent activation of p100 processing (Jin et al., 2010; Saotome et al., 1995). RNAi experiments in HeLa cells disrupting ZFP91 reveal that it is specific to the non-canonical NFκB2 pathway activation, and not NFκB1, which supports the idea that interaction with the N-terminal region of RAG1 may trigger induction of ncDDR (Jin et al., 2010).

Another Rag1 N-terminal region interactor of particular interest is the transcription factor PU.1. PU.1 is required for B cell lineage commitment and is constitutively expressed during B cell development (Schweitzer and DeKoter, 2004). Previous studies indicate that germline

transcription of *Igk* is dependent upon PU.1 activity (Batista et al., 2017). Importantly, a study from our lab indicates that PU.1 activity is regulated at the pre-B cell stage through RAG DSB-mediated induction of a transcription factor SPIC, which binds chromatin and displaces PU.1 to induce changes in gene expression (Soodgupta et al., 2019). Identification of PU.1 as a unique RAG1 binding partner in immunoprecipitation studies indicates that PU.1 may bind to the N-terminal region of RAG1 to induce SPIC and results in activation of ncDDR.

Identification of unique RAG1 interactors will delineate the factors that associate with the N-terminal region of RAG1 to induce both cDDR and ncDDR. This is a novel function of RAG1 in DDR and these studies will define how RAG1 executes this function in DDR. These studies demonstrate that non-core regions of RAG1 associate with a distinct protein network at sites of DSBs. Future investigations will delineate the function of these RAG1-associated proteins in regulating DDR signaling. Additionally, the Cas9-RAG1 fusions described here are new tools that will provide a novel approach to defining determinants of DDR in B cells but also for tools for customizing DNA damage responses. These studies will reveal new mechanisms of ncDDR signaling in B cells, which provides new insights into B cell development.

3.6 Materials and Methods

3.6.1 Mice

Mice were used for generation of abl pre-B cell lines. Mice were maintained under specific pathogen-free conditions at the Washington University School of Medicine and were handled in accordance to the guidelines set forth by the Division of Comparative Medicine of Washington University.

3.6.2 Cell lines

Rag1$^{-/-}$*Lig4*$^{-/-}$*:Bcl2* abl pre-B cells were generated from *Rag1*$^{-/-}$*:Lig4*$^{flox/flox}$*:Bcl2* abl pre-B cells, which were made from *Rag1*$^{-/-}$*:Lig4*$^{flox/flox}$*:Bcl2* mice by retroviral transduction of bone marrow cells with MSCV-v-abl plasmid as described previously (Bredemeyer et al., 2006). *Rag1*$^{-/-}$*:Lig4*$^{flox/flox}$*:Bcl2* abl pre-B cells were then transiently transfected with MSCV-Cre-IRES-Thy1.1 vector (Addgene, Plasmid #17442) and subsequently subcloned to identify clones with deletion of *Lig4*, which was confirmed by genotyping (PCR). To induce cell cycle arrest and induction of RAG DSBs, abl pre-B cells were treated with 3μM imatinib (10^6 cells/ml) for indicated times prior to harvesting for genomic DNA and protein (Bredemeyer et al., 2008; Johnston et al., 2022; Soodgupta et al., 2019).

3.6.3 cDNA expression

pFLRU-TRE-Cas9-Ubc-rtTA-Thy1.2 lentiviral vector expressing cDNA encoding 5' FLAG tagged *Streptococcus pyogene* Cas9 was a gift from Gene Oltz and Barry Sleckman (Purman *et al.*, 2019). cDNA encoding 5' FLAG tagged RAG1 or cRAG1 was cloned into the pOZ-IRES-hCD25 retroviral vector (Bednarski *et al.*, 2016). RAG1 E3 ligase mutant ($RAG1^{P326G}$) and the catalytically-inactive RAG1 ($RAG1^{D708A}$) were generated using QuikChange II XL (Agilent) according to manufacturer's protocol. Retrovirus and lentivirus were produced in PlatE cells (Cell Biolabs) and 293T cells, respectively, by transfection of the viral plasmids with Lipofectamine 2000 (Life Technologies) (Bednarski *et al.*, 2012; Soodgupta *et al.*, 2019). For lentivirus, pCMV- VSV-G and pCMV-d8.2R were also included in the transfection (Soodgupta *et al.*, 2019; Stewart *et al*, 2003). Viral supernatant was collected and pooled from 24-72 hours after transfection. Abl pre-B cells were transduced with unconcentrated virus in media

with polybrene (5 μg/ml; Sigma-Aldrich) as previously described (Soodgupta *et al.*, 2019). Transduced cells were identified by flow cytometric assessment of hCD25 or Thy1.2 and were sorted using anti-hCD25 or anti-Thy1.2 magnetic beads (Miltenyi Biotec) on MS columns (Miltenyi Biotec) according to the manufacturer's protocol.

3.6.4 Cas9-RAG1 fusion construct cloning

Cas9-RAG1 fusion constructs were generated using the pX458M-53BP1-DN1S plasmid which contains the TGS linker sequence: ACAGGGTCCACAGGATCCACAGGCAGCA CAGGGAGCATGGGA (Addgene #131045). 53BP1 was replaced with $RAG1^{D708A}$, $cRAG1^{D708A}$, or RAG NTR, respectively, using restriction enzyme digest and ligation. For Cas9 alone, 53BP1 was removed through restriction enzyme digest followed by re-ligation of plasmid. RAG1 cDNA was purified from pOZ-IRES-hCD25 constructs as described in Chapter 2.

3.6.5 Generation of Cas9-RAG1 mediated DSBs

gRNAs were designed using CHOPCHOP, with the exception of the *Eb* guide, which is previously published (Dorsett *et al*, 2014; Labun *et al*, 2019; Purman *et al.*, 2019). gRNA sequences are listed in **Table 2.1**. gRNAs were generated by *in vitro* transcription using the Precision gRNA Synthesis kit (Invitrogen) per manufacturer's guidelines. *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2:iCas9* cells were treated with imatinib for 24 h. Subsequently, 20 x 10^6 cells and 5 μg Cas9-RAG1 fusion constructs and 400 μg of gRNA were resuspended in 100 μl Nucleofector Solution for Human B Cells (Lonza) then nucleofected using an Amaxa Nucleofector II (Lonza), program X-001, according to the manufacturer's instructions. Nucleofected cells were allowed to rest at room temperature for 10 minutes then transferred directly to preequilibrated recovery medium

containing imatinib at 10 x 10^6 cells/ml and incubated for indicated times prior to harvesting for genomic DNA and protein.

3.6.6 Tandem Affinity Purification and MS analysis

FLAG-HA-tagged RAG1 and cRAG1 were immunoprecipitated using anti-FLAG antibody as previously described with the following modifications (Mosammaparast et al., 2013; Nakatani and Ogryzko, 2003; Soodgupta et al., 2019). Cells were lysed in low salt MCLB (50mM Tris pH7.5, 0.5% NP-40, and protease and phosphatase inhibitor cocktails (Sigma)) (20 x 10^6 cells in 300ul buffer). The lysate was cleared by centrifugation and incubation with Streptavidin Magnetic Beads (Thermo Fisher). Cleared lysate was bound to Anti-FLAG BioM2 antibody (Sigma) bound to Streptavidin Magnetic Beads (Thermo Fisher) overnight. After extensive washing in the same buffer, magnetic beads were sent to Proteomics Shared Resource at Washington University. Trypsin digestion was performed on the magnetic beads and the detection of complexed proteins was done using timsTOF Pro ion mobility mass spectrometer (Bruker Daltronics) (Turriziani et al., 2014). Interacting proteins were identified by matching protein database with acquired fragmentation pattern by using MaxQuant (Cox and Mann, 2008). Scaffold 5 (Proteome Software, Portland, Oregon, USA) was used to validate protein identification derived from MS/MS sequencing results (Craig and Beavis, 2003). Thresholds were set with MASCOT40+ and 2 peptide minimum for protein identification and analysis (Perkins et al., 1999).

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3.6.7 Western Blot Analyses

Western blots were done on whole cell lysates as previously described (Bednarski et al., 2016). Anti-GAPDH (clone D16H11) antibody was from Cell Signaling Technology. Anti-phospho-KAP1 antibody (A300-767A) was from Bethyl Laboratories. Anti-FLAG (clone M2) was from Sigma. Secondary reagents were horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Cell Signaling) or anti-rabbit IgG (Cell Signaling). Westerns were developed with ECL (Pierce) and ECL Prime (Cytiva).

3.6.8 RT-PCR

For genomic DNA isolation, cells were lysed in lysis buffer (100 mM TRIS pH 8.5, 5 mM EDTA, 200 mM NaCl, and 0.2% SDS) and DNA was precipitated by addition of isopropanol, washed with 70% then 100% ethanol, and finally resuspended in water (Johnston *et al.*, 2022; Soodgupta *et al.*, 2019). For PCR over the break assay, genomic DNA was digested with NEBNext dsDNA Fragmentase (NEB) for 10 minutes followed by PCR Cleanup (QIAGEN) as per manufacturer's instructions before RT-PCR. RT-PCR was performed using Brilliant II SYBR Green (Agilent) and acquired on QuantStudio 3 (ThermoFisher). Each reaction was run in triplicate. For PCR over the break analyses, values at targeted sites were normalized to PCR product spanning CD19, a control region of uncut genomic DNA and fold change was determined by the DD cycle threshold method (Johnston *et al.*, 2022). Primer sequences are listed in **Table 2.1**.

Table 3.1: Unique RAG1 Binding Partners: Proteins identified as bound exclusively to full length RAG1 following immunoprecipitation and mass spectrometry. ATM targets have been previously established (Matsuoka et al., 2007). RAG1 interactors are proteins that appeared in a previously described RAG1 BioID study (Brecht et al., 2020).

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Acsl3	Long-chain-fatty-acid--CoA ligase 3	Ligase		
Alkbh3	Alpha-ketoglutarate-dependent dioxygenase alkB homolog 3	Oxygenase		
Antkmt	Adenine nucleotide translocase lysine N-methyltransferase	Unknown		
Arl14ep	ARL14 effector protein	Unknown		
Aup1	Lipid droplet-regulating VLDL assembly factor	Scaffold		
Brwd3	Bromodomain and WD repeat-containing protein 3	Unknown	x	
Calm1	Calmodulin-1	Calmodulin		
Camk1d	Calcium/calmodulin-dependent protein kinase type 1D	Protein kinase		
Cars1	Cysteine--tRNA ligase, cytoplasmic	tRNA synthetase		
Cbfa2t2	CBFA2/RUNX1 Partner Transcriptional Co-repressor 2	Transcription cofactor		
Ccnb2	G2/mitotic-specific cyclin-B2	Kinase		
Cdk5rap1	Mitochondrial tRNA methylthiotransferase	Unknown		
Cenatac	Centrosomal AT-AC splicing factor	Unknown		
Cnpy2	Canopy FGF signaling receptor 2	Unknown		
Cox10	Protoheme IX farnesyltransferase, mitochondrial	Acyltransferase		
Cpt1a	Carnitine O-palmitoyltransferase 1, liver isoform	Acyltransferase		
Csrp2	Cysteine and glycine-rich protein 2	Actin binding		
Ctdspl2	CTD small phosphatase-like protein 2	Phosphatase		
Dennd4b	DENN domain-containing protein 4B	Nucleotide exchange factor		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Dera	Deoxyribose-phosphate aldolase	Aldolase		
Dnajc10	DnaJ homolog subfamily C member 10	Chaperone		
Dnajc19	Mitochondrial import inner membrane translocase subunit	Unknown		
Dop1b	DOP1 leucine zipper like protein B	Unknown		
Drg1	Developmentally-regulated GTP-binding protein 1	Unknown		
Eif4e3	Eukaryotic translation initiation factor 4E type 3	Translation initiation factor		
Eri3	ERI1 exoribonuclease 3	Exoribonuclease		
Exd2	Exonuclease 3'-5' domain-containing protein 2	Unknown		
Fam172a	Cotranscriptional regulator	Unknown		
Fbxo22	F-box only protein 22	Unknown		
Fra10ac1	FRA10A associated CGG repeat 1	Phosphatase		
Fth1	Ferritin heavy chain	Storage		
Garem1	GRB2-associated and regulator of MAPK protein	Unknown		
Gcfc2	Intron Large complex component	Chromatin binding		
Gemin2	Gem-associated protein 2	RNA splicing factor		
Gemin8	Gem-associated protein 8	Unknown		
Gins1	DNA replication complex GINS protein PSF1	Transcription factor		
Gins3	DNA replication complex GINS protein PSF3	Unknown		
Gmcl1	Germ cell-less protein-like 1	Ubiquitin ligase		
Golph3l	Golgi phosphoprotein 3-like	Unknown		
Gpatch2l	G patch domain-containing protein 2-like	RNA metabolism		
Gpatch4	G patch domain-containing protein 4	RNA metabolism		x
Gstm1	Glutathione S-transferase Mu 1	Transferase		
Gtf2e2	General transcription factor IIE subunit 2	Transcription factor		
Gzf1	GDNF-inducible zinc finger protein 1	Transcription factor		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Heatr5a	HEAT repeat-containing protein 5A	Unknown		
Hook3	Hook microtubule tethering protein 3	Membrane traffic		
Irf5	Interferon regulatory factor 5	Transcription factor		
Itgb2	Integrin beta-2	Integrin		
Itih3	Inter-alpha-trypsin inhibitor heavy chain H3	Protease inhibitor		
Itprid2	ITPR interacting domain containing 2	Unknown		
Kat6a	Histone acetyltransferase KAT6A	Acetyltransferase		
Kdm1b	Lysine-specific histone demethylase 1B	Demethylase		
Kdm2a	Lysine-specific demethylase 2A	Demethylase		
Klhl9	Kelch-like protein 9	Scaffold		
Krt72	Keratin, type II cytoskeletal 72	Unknown		
Krt8	Keratin, type II cytoskeletal 8	Unknown		
Ldlrap1	Low density lipoprotein receptor adapter protein 1	Scaffold	x	
Letmd1	LETM1 domain-containing protein 1	Transporter		
Lrch1	Leucine-rich repeat and calponin homology domain-containing protein 1	Scaffold		
Lrrcc1	Leucine-rich repeat and coiled-coil domain-containing protein 1	Scaffold		
Mecp2	Methyl-CpG-binding protein 2	Chromatin remodeler		
Mib2	E3 ubiquitin-protein ligase	Ubiquitin ligase		
Mis18bp1	Mis18-binding protein 1	Unknown		
Mpnd	MPN domain-containing protein	Translation initiation factor		
Mvd	Diphosphomevalonate decarboxylase	Decarboxylase		
Ncoa3	Nuclear receptor coactivator 3	Chromatin remodeler		
Ndufa11	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 11	Oxioreductase		
Ndufa12	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12	Oxioreductase		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Nedd4l	E3 ubiquitin-protein ligase NEDD4-like	Ubiquitin ligase		
Nomo1	Nodal modulator 1	Unknown		
Nop53	Ribosome biogenesis protein	Unknown		
Nrif2	Neurotrophin receptor-interacting factor 2	Transcription factor		
Nsd3	Histone-lysine N-methyltransferase	Methyltransferase		
Nucb1	Nucleobindin-1	Calmodulin	x	
Nup54	Nuclear pore complex protein	Transporter		
Ociad1	OCIA domain-containing protein 1	Membrane traffic		
Patl1	Protein PAT1 homolog 1	RNA processing factor		
Pdss1	All trans-polyprenyl-diphosphate synthase	Unknown		
Pgm2	Phosphoglucomutase-2	Mutase		
Phf14	PHD finger protein 14	Transcription factor		
Pigu	Phosphatidylinositol glycan anchor biosynthesis class U protein	Unknown		
Pik3ca	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform	Kinase		
Pkn1	Serine/threonine-protein kinase N1	Protein kinase		
Pofut1	GDP-fucose protein O-fucosyltransferase 1	Protein modifying		
Polb	DNA polymerase beta	DNA polymerase		
Pou2f1	POU domain, class 2, transcription factor 1	Transcription factor		
Ppif	Peptidyl-prolyl cis-trans isomerase F, mitochondrial	Chaperone		
Prr12	Proline-rich protein 12	Unknown		
Prr14	Proline-rich protein 14	Unknown		
Ptpa	Serine/threonine-protein phosphatase 2A activator	Phosphatase		
Ptprj	Receptor-type tyrosine-protein phosphatase eta	Phosphatase		
Pwwp3a	PWWP domain-containing DNA repair factor 3A	DNA metabolism		
Rab11b	Ras-related protein Rab-11B	GTPase		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Rab8a	Ras-related protein Rab-8A	Unknown		
Ralgapa2	Ral GTPase-activating protein subunit alpha-2	GTPase activating		
Rasa2	Ras GTPase-activating protein 2	GTPase activating		
Rbak	RB-associated KRAB zinc finger protein	Transcription factor		
Rgn	Regucalcin	Esterase		
Samd4b	Sterile alpha motif domain containing 4B	Unknown		
Sap30	Histone deacetylase complex subunit	Deacetylase		
Slc12a7	Solute carrier family 12 member 7	Transporter		
Slc30a5	Zinc transporter 5	Unknown		
Snx3	Sorting nexin-3	Unknown		
Spi1	Transcription factor PU.1	Transcription factor		
St8sia4	CMP-N-acetylneuraminate-poly-alpha-2,8-sialyltransferase	Transferase		
Stag1	Cohesin subunit SA-1	Chromatin binding		
Stk11	Serine/threonine-protein kinase	Unknown		
Stt3a	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit	Glycosyltransferase		x
Suv39h1	Histone-lysine N-methyltransferase	Methyltransferase		
Taf1	Transcription initiation factor TFIID subunit 1	Transcription factor		x
Tbc1d10a	TBC1 domain family member 10A	GTPase activating		
Tcp11l2	T-complex protein 11-like protein 2	Microtubule binding		
Tgfb1	Transforming growth factor beta-1 proprotein	Growth factor		
Thap1	THAP domain-containing protein 1	Transcription factor		
Timm29	Mitochondrial import inner membrane translocase subunit Tim29	Unknown		
Tm9sf2	Transmembrane 9 superfamily member 2	Transporter		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Tm9sf4	Transmembrane 9 superfamily member 4	Transporter		
Tmem135	Transmembrane protein 135	Unknown		
Tmem209	Transmembrane protein 209	Unknown		
Tmem33	Transmembrane protein 33	Unknown		
Tpp1	Tripeptidyl-peptidase 1	Protease		
Tram1	Translocating chain-associated membrane protein 1	Unknown		
Trappc9	Trafficking protein particle complex subunit 9	Unknown		
Trpc5os	Transient receptor potential cation channel, subfamily C, member 5, opposite strand	Unknown		
Ttc33	Tetratricopeptide repeat protein 33	Unknown		
Tti1	TELO2-interacting protein 1 homolog	Unknown		
Tubb2b	Tubulin beta-2B chain	Tubulin		
Tubb4a	Tubulin beta-4A chain	Tubulin		
Tube1	Tubulin epsilon chain	Tubulin		
Tulp3	Tubby-related protein 3	Scaffold		
Txndc9	Thioredoxin domain-containing protein 9	Transcription cofactor		
Ube2s	Ubiquitin-conjugating enzyme E2	Ubiquitin ligase		
Ufsp2	Ufm1-specific protease 2	Unknown		
Utp23	rRNA-processing protein UTP23 homolog	Unknown		
Wdr89	WD repeat-containing protein 89	Unknown		
Yju2	Splicing factor	RNA splicing factor		
Ykt6	Synaptobrevin homolog	Unknown		
Zbtb7a	Zinc finger and BTB domain-containing protein 7A	Transcription factor		
Zfp91	E3 ubiquitin-protein ligase	Transcription factor		
Zmat2	Zinc finger matrin-type protein 2	RNA processing factor		x
Znf106	Zinc finger protein 106	Unknown		
Znf250	Zinc finger protein 250	Transcription factor		
Znf276	Zinc finger protein 276	Transcription factor		
Znf32	Zinc finger protein 32	Transcription factor		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Znf746	Zinc finger protein 746	Transcription factor		
Zyg11b	Zyg-11 family member b	Unknown		

Table 3.2: Unique cRAG1 Binding Partners: Proteins identified as bound exclusively to cRAG1 following immunoprecipitation and mass spectrometry. ATM targets have been previously established (Matsuoka et al., 2007). RAG1 interactors are proteins that appeared in a previously described RAG1 BioID study (Brecht et al., 2020).

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
A2m	Alpha-2-macroglobulin-P	Protease inhibitor		
Adi1	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	Oxygenase		
Ado	2-aminoethanethiol dioxygenase	Unknown		
Agpat3	1-acyl-sn-glycerol-3-phosphate acyltransferase gamma	Acyltransferase		
Alas1	5-aminolevulinate synthase, nonspecific, mitochondrial	Transaminase		
Aldoc	Fructose-bisphosphate aldolase C	Aldolase		
Ankrd13a	Ankyrin repeat domain-containing protein 13A	Scaffold		
Anxa7	Annexin A7	Calcium binding		
Ap4b1	AP-4 complex subunit beta-1	Membrane traffic		
Ap4e1	AP-4 complex subunit epsilon-1	Membrane traffic		
Arf4	ADP-ribosylation factor 4	G-protein		
Arid4b	AT-rich interactive domain-containing protein 4B	Transcription cofactor		
Asap1	Arf-GAP with SH3 domain, ANK repeat and PH domain-containing protein 1	Unknown		
Asb3	Ankyrin repeat and SOCS box protein 3	Unknown		
Atp11c	Phospholipid-transporting ATPase 11C	Active transporter		
Atp13a3	Probable cation-transporting ATPase 13A3	Primary active transporter		
Atp6v1c1	V-type proton ATPase subunit C 1	ATP Synthase		
Atr	Serine/threonine-protein kinase ATR	Protein kinase		
Aurka	Aurora kinase A	Protein kinase		
Bak1	Bcl-2 homologous antagonist/killer	Unknown		
Becn1	Beclin-1	Protease inhibitor		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Bmp2k	BMP-2-inducible protein kinase	Protein kinase		
Brd3	Bromodomain-containing protein 3	Chromatin remodeler		
Bub1b	Mitotic checkpoint serine/threonine-protein kinase BUB1 beta	Protein kinase		
Cchcr1	Coiled-coil alpha-helical rod protein 1	Unknown		
Cd79a	B-cell antigen receptor complex-associated protein alpha chain	Immunoglobulin receptor		
Cdc37l1	Hsp90 co-chaperone Cdc37-like 1	Chaperone		
Cenpb	Major centromere autoantigen B	Viral		
Cep44	Centrosomal protein of 44 kDa	Centromere DNA binding		
Cep55	Centrosomal protein of 55 kDa	Centromere DNA binding		
Cfap298	Cilia- and flagella-associated protein 298	Unknown		
Chm	Rab proteins geranylgeranyltransferase component A 1	G-protein modulator		
Chmp7	Charged multivesicular body protein 7	Membrane traffic		
Cip2a	Cell proliferation regulating inhibitor of protein phosphatase 2A	Unknown		
Clcn7	H(+)/Cl(-) exchange transporter 7	Ion channel		
Clptm1	Cleft lip and palate transmembrane protein 1 homolog	Unknown		
Cmip	C-Maf-inducing protein	Unknown		
Cnot3	CCR4-NOT transcription complex subunit 3	Transcription factor		
Coa7	Cytochrome c oxidase assembly factor 7	Unknown		
Coasy	Bifunctional coenzyme A synthase	Kinase		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Cog8	Conserved oligomeric Golgi complex subunit 8	Unknown		
Commd10	COMM domain-containing protein 10	Unknown		
Comtd1	Catechol O-methyltransferase domain-containing protein 1	Methyltransferase		
Cops8	COP9 signalosome complex subunit 8	Scaffold		
Cotl1	Coactosin-like protein	Actin binding		
Cox20	Cytochrome c oxidase assembly protein COX20, mitochondrial	Oxidase		
Cr1l	Complement component receptor 1-like protein	Complement component		
Crk	Adapter molecule crk	Scaffold		
Csad	Cysteine sulfinic acid decarboxylase	Decarboxylase		
Cspp1	Centrosome and spindle pole associated protein 1	Centromere DNA binding		
Ctsd	Cathepsin D	Protease		
Cutc	cutC copper transporter	Primary active transporter		
D6Wsu163e	DNA segment, Chr 6, Wayne State University 183, expressed	Unknown		
Dapp1	Dual adapter for phosphotyrosine and 3-phosphotyrosine and 3-phosphoinositide	Unknown		
Dcun1d1	DCN1-like protein 1	Ubiquitin ligase		
Ddx28	Probable ATP-dependent RNA helicase	RNA helicase		
Dhrsx	Dehydrogenase/reductase SDR family member on chromosome X homolog	Dehydrogenase		
Dnajb6	DnaJ homolog subfamily B member 6	Chaperone		
Dop1a	DOP1 leucine zipper like protein A	Unknown		
Dpp8	Dipeptidyl peptidase 8	Protease		
Dus1l	tRNA-dihydrouridine(16/17) synthase [NAD(P)(+)]-like	RNA processing factor		
Dusp11	RNA/RNP complex-1-interacting phosphatase	mRNA capping factor		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Dync2i1	Cytoplasmic dynein 2 intermediate chain 1	Unknown		
Eef1akmt2	EEF1A lysine methyltransferase 2	Methyltransferase		
Eif4enif1	Eukaryotic translation initiation factor 4E transporter	Transporter		
Ell2	RNA polymerase II elongation factor	Transcription factor		
Epn1	Epsin-1	Membrane traffic		
Fancl	E3 ubiquitin-protein ligase	Ubiquitin ligase		
Fig4	Polyphosphoinositide phosphatase	Phosphatase		
Fkbp1a	Peptidyl-prolyl cis-trans isomerase	Chaperone		
Fmc1	Formation of mitochondrial complex V assembly factor 1	Unknown		
G6pc3	Glucose-6-phosphatase 3	Phosphatase		
Galnt7	N-acetylgalactosaminyltransferase 7	Glycosyltransferase		
Gbp2	Guanylate-binding protein 2	G-protein		
Gcc2	GRIP and coiled-coil domain-containing protein 2	Microtubule binding		
Gclc	Glutamate--cysteine ligase catalytic subunit	Ligase		
Ggact	Gamma-glutamylaminecyclotransferase	Unknown		
Gpam	Glycerol-3-phosphate acyltransferase 1, mitochondrial	Acyltransferase		
Gpcpd1	Glycerophosphocholine phosphodiesterase	Phosphodiesterase		
Gphn	Gephyrin	Unknown		
Gramd4	GRAM domain-containing protein 4	Unknown		
Hal	Histidine ammonia-lyase	Lyase		
Haus2	HAUS augmin-like complex subunit 2	Unknown		
Hax1	HCLS1-associated protein X-1	Unknown		
Hbb-y	Hemoglobin subunit epsilon-Y2	Globin		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Hdgfl2	Hepatoma-derived growth factor-related protein 2	Transcription cofactor		
Hirip3	HIRA-interacting protein 3	Unknown		
Hmgn5	High mobility group nucleosome-binding domain-containing protein 5	Unknown		
Hs1bp3	HCLS1-binding protein 3	Unknown		
Hsd17b7	3-keto-steroid reductase/17-beta-hydroxysteroid dehydrogenase 7	Reductase		
Idnk	Probable gluconokinase	Kinase		
Igbp1	Immunoglobulin-binding protein 1	Chaperone		
Igf2r	Cation-independent mannose-6-phosphate receptor	Membrane traffic		
Impact	RWD domain protein	Unknown		
Insr	Insulin receptor	Signal recepor		
Ints11	Integrator complex subunit 11	RNA processing factor		
Iqgap2	Ras GTPase-activating-like protein	GTPase activating		
Irf2bp1	Interferon regulatory factor 2-binding protein 1	Transcription factor		
Irf2bp2	Interferon regulatory factor 2-binding protein 2	Transcription factor		
Jmjd6	Bifunctional arginine demethylase and lysyl-hydroxylase	Demethylase		
Jmy	Junction-mediating and -regulatory protein	Unknown		
Kansl3	KAT8 regulatory NSL complex subunit 3	Unknown		
Kdm2b	Lysine-specific demethylase 2B	Chromatin remodeler		
Kdm6a	Lysine-specific demethylase 6A	Demethylase		
Khdc4	KH homology domain-containing protein 4	Unknown		
Kif18b	Kinesin family member 18B	Microtubule binding		
Kif22	Kinesin family member 22	Microtubule binding	x	
Kif3c	Kinesin family member 3C	Microtubule binding		
Kifap3	Kinesin-associated protein 3	Microtubule binding		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Kmt2c	Histone-lysine N-methyltransferase 2C	Methyltransferase		
Kmt2d	Histone-lysine N-methyltransferase 2D	Methyltransferase		
Lfng	Beta-1,3-N-acetylglucosaminyltransferase lunatic fringe	Glycosyltransferase		
Lpgat1	Acyl-CoA:lysophosphatidylglycerol acyltransferase 1	Acyltransferase		
Lrrc8a	Volume-regulated anion channel subunit	Scaffold		
Lsm6	U6 snRNA-associated Sm-like protein 6	RNA splicing factor		
Lsm8	U6 snRNA-associated Sm-like protein 8	RNA splicing factor		
Lyst	Lysosomal-trafficking regulator	Scaffold		
Maip1	m-AAA protease-interacting protein 1, mitochondrial	Unknown		
Man1a1	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	Enzyme		
Man1b1	Endoplasmic reticulum mannosyl-oligosaccharide 1,2-alpha-mannosidase	Enzyme		
Map3k7	Mitogen-activated protein kinase kinase kinase 7	Protein kinase		
Map4k5	Mitogen-activated protein kinase kinase kinase kinase 5	Protein kinase		
Mboat7	Lysophospholipid acyltransferase 7	Acetyltransferase		
Mbtd1	MBT domain-containing protein 1	Chromatin binding		
Med27	Mediator of RNA polymerase II transcription subunit 27	Transcription cofactor		
Med31	Mediator of RNA polymerase II transcription subunit 31	Transcription factor		
Megf10	Multiple epidermal growth factor-like domains protein 10	Matrix protein		
Melk	Maternal embryonic leucine zipper kinase	Protein kinase		
Metap2	Methionine aminopeptidase 2	Protease		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Mif	Macrophage migration inhibitory factor	Decarboxylase		
Mkln1	Muskelin	Unknown		
Mllt10	Protein AF-10	Transcription factor	x	
Mlst8	Target of rapamycin complex subunit LST8	Unknown		
Mmab	Corrinoid adenosyltransferase	Dehydrogenase		
Mon1b	Vacuolar fusion protein MON1 homolog B	Unknown		
Mrpl14	39S ribosomal protein L14, mitochondrial	Ribosomal		
Mrpl2	39S ribosomal protein L2, mitochondrial	Ribosomal		
Mrpl23	39S ribosomal protein L23, mitochondrial	Ribosomal		
Mtco2	Cytochrome c oxidase subunit 2	Oxioreductase		x
Mthfsd	Methenyltetrahydrofolate synthase domain-containing protein	Ligase		
Mtmr9	Myotubularin-related protein 9	Phosphatase		
Mtnd4	NADH-ubiquinone oxidoreductase chain 4	Oxioreductase		
Mtres1	Mitochondrial transcription rescue factor 1	Unknown		
Mtrf1l	Peptide chain release factor 1-like, mitochondrial	Translation release factor		
Mul1	Mitochondrial ubiquitin ligase activator of NFKB 1	Unknown		
Myb	Transcriptional activator	Transcription factor		
Nck2	Cytoplasmic protein NCK2	Scaffold		
Ndufb4	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 4	Oxioreductase		
Ndufc2	NADH dehydrogenase [ubiquinone] 1 subunit C2	Oxioreductase		
Neu3	Sialidase-3	Hydrolase		
Nfix	Nuclear factor 1 X-type	Transcription factor		
Ngly1	Peptide-N(4)-(N-acetyl-beta-glucosaminyl)asparagine amidase	Hydrolase		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Nr2c2	Nuclear receptor subfamily 2 group C member 2	Nuclear receptor		
Nrdc	Nardilysin	Metalloprotease		
Nrde2	Nuclear exosome regulator	Unknown		
Nt5c3a	Cytosolic 5'-nucleotidase 3A	Esterase		
Nt5dc3	5'-nucleotidase domain-containing protein 3	Phosphatase		
Nuf2	Kinetochore protein	Microtubule binding		
Nutf2	Nuclear transport factor 2	Unknown		
Otulin	Ubiquitin thioesterase otulin	Unknown		
Pag1	Phosphoprotein associated with glycosphingolipid-enriched microdomains 1	Unknown		
Pals1	MAGUK p55 subfamily member 5	Scaffold		
Pals2	MAGUK p55 subfamily member 6	Scaffold		
Papolg	Poly(A) polymerase gamma	mRNA polyadenylation factor		
Pcmt1	Protein-L-isoaspartate(D-aspartate) O-methyltransferase	Methyltransferase		
Pcyox1	Prenylcysteine oxidase	Lyase		
Pdcl3	Phosducin-like protein 3	Viral		
Pex14	Peroxisomal membrane protein	Primary active transporter		
Pex19	Peroxisomal biogenesis factor 19	Membrane traffic		
Pex6	Peroxisome assembly factor 2	Primary active transporter		
Pikfyve	1-phosphatidylinositol 3-phosphate 5-kinase	Protein kinase		
Pitpnb	Phosphatidylinositol transfer protein beta isoform	Transporter		
Pms2	Mismatch repair endonuclease PMS2	DNA metabolism		
Pnpo	Pyridoxine-5'-phosphate oxidase	Oxidase		
Pola2	DNA polymerase alpha subunit B	DNA polymerase		
Pole2	DNA polymerase epsilon subunit 2	DNA polymerase		
Poll	DNA polymerase lambda	DNA polymerase	x	

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Polr3e	DNA-directed RNA polymerase III subunit RPC5	RNA polymerase		
Pon3	Serum paraoxonase/lactonase 3	Unknown		
Postn	Periostin	Cell adhesion		
Pot1	Protection of telomeres protein 1	DNA metabolism		
Ppp3r1	Calcineurin subunit B type 1	Unknown		
Prkag2	5'-AMP-activated protein kinase subunit gamma-2	Kinase modulator	x	
Pten	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase	Phosphatase		
Ptp4a3	Protein tyrosine phosphatase type IVA 3	Phosphatase		
Ptpn23	Tyrosine-protein phosphatase non-receptor type 23	Membrane traffic	x	
Ptpra	Receptor-type tyrosine-protein phosphatase alpha	Phosphatase		
Qsox1	Sulfhydryl oxidase 1	Oxidase		
R3hdm4	R3H domain-containing protein 4	Unknown		
Rab2b	Ras-related protein Rab-2B	GTPase		
Rbbp5	Retinoblastoma-binding protein 5	Unknown	x	
Rbl2	Retinoblastoma-like protein 2	Chromatin binding		
Rbm33	RNA-binding protein 33	RNA binding	x	
Rbm34	RNA-binding protein 34	Translation initiation factor		
Rbpj	Recombining binding protein suppressor of hairless	Transcription factor		
Rc3h1	Roquin-1	Unknown		
Rdh12	Retinol dehydrogenase 12	Dehydrogenase		
Recql5	ATP-dependent DNA helicase Q5	DNA helicase		
Rgs18	Regulator of G-protein signaling 18	GTPase activating		
Rmc1	Regulator of MON1-CCZ1 complex	Unknown		
Rpap3	RNA polymerase II-associated protein 3	Unknown		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Rpp40	Ribonuclease P protein subunit p40	Endoribonuclease		
Rtn4	Reticulon-4	Unknown		
Rttn	Rotatin	Unknown		
Sacs	Sacsin	Unknown		
Scap	Sterol regulatory element-binding protein cleavage-activating protein	Unknown		
Scyl2	SCY1-like protein 2	Protein kinase		
Sdf2	Stromal cell-derived factor 2	Unknown		
Sel1l	Sel-1 suppressor of lin-12-like	Unknown		
Selenot	Thioredoxin reductase-like selenoprotein T	Unknown		
Sesn1	Sestrin-1	Peroxidase	x	
Slc25a51	Mitochondrial nicotinamide adenine dinucleotide transporter	Unknown		
Smap2	Stromal membrane-associated protein 2	Unknown		
Sntb2	Beta-2-syntrophin	Scaffold		
Snx17	Sorting nexin-17	Scaffold		
Soga1	Suppressor of glucose, autophagy associated 1	Unknown		
Sos1	Son of sevenless homolog 1	Nucleotide exchange factor		
Spart	Spartin	Unknown		
Spc24	Kinetochore protein	Unknown		
Spty2d1	Protein SPT2 homolog	Chromatin binding		x
Srp19	Signal recognition particle 19 kDa protein	Primary active transporter		
Srpk2	SRSF protein kinase 2	Protein kinase		
Stam	Signal transducing adapter molecule 1	Scaffold		
Stk11ip	Serine/threonine-protein kinase 11-interacting protein	Scaffold		
Stn1	CST complex subunit	Membrane traffic		
Stx18	Syntaxin-18	SNARE		
Stxbp5	Syntaxin-binding protein 5	Membrane traffic		
Syvn1	E3 ubiquitin-protein ligase synoviolin	Ubiquitin ligase		
Tada1	Transcriptional adapter 1	Scaffold		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Tbc1d1	TBC1 domain family member 1	GTPase activating		
Tbc1d10b	TBC1 domain family member 10B	GTPase activating		
Tbc1d9b	TBC1 domain family member 9B	GTP-ase activating		
Tbck	TBC domain-containing protein kinase-like protein	GTPase activating		
Tdp1	Tyrosyl-DNA phosphodiesterase 1	Phosphodiesterase		
Tdp2	Tyrosyl-DNA phosphodiesterase 2	Phosphodiesterase		
Thumpd1	THUMP domain-containing protein 1	Unknown		
Tkfc	Triokinase/FMN cyclase	Cyclase		
Tle3	Transducin-like enhancer protein 3	Transcription cofactor		
Tnfrsf26	Tumor necrosis factor receptor superfamily member 26	Unknown		
Tnrc6a	Trinucleotide repeat-containing gene 6A protein	RNA metabolism		
Tor1aip2	Torsin-1A-interacting protein 2	Unknown		
Tp53bp1	TP53-binding protein 1	Transcription factor		
Trafd1	TRAF-type zinc finger domain-containing protein 1	Unknown		
Trappc13	Trafficking protein particle complex subunit 13	Unknown		
Trim27	Zinc finger protein RFP	Ubiquitin ligase		
Trmt10a	tRNA methyltransferase 10 homolog A	Methyltransferase		
Tsc1	Hamartin	Unknown	x	
Tyk2	Non-receptor tyrosine-protein kinase	Protein kinase		
Tyw3	tRNA wybutosine-synthesizing protein 3 homolog	Methyltransferase		
Uckl1	Uridine-cytidine kinase-like 1	Nucleotide kinase		
Urgcp	Up-regulator of cell proliferation	Unknown		
Usp38	Ubiquitin carboxyl-terminal hydrolase 38	Protease		
Uvssa	UV-stimulated scaffold protein A	Scaffold		x

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Vangl2	Vang-like protein 2	Unknown		
Vps8	Vacuolar protein sorting-associated protein 8 homolog	Membrane traffic		
Was	Wiskott-Aldrich syndrome protein homolog	Unknown		
Wdfy2	WD repeat and FYVE domain-containing protein 2	Unknown		
Wdr7	WD repeat-containing protein 7	Unknown		
Wnk1	Serine/threonine-protein kinase WNK1	Protein kinase	x	
Wwox	WW domain-containing oxidoreductase	Dehydrogenase		
Zbtb45	Zinc finger and BTB domain-containing protein 45	Transcription factor		
Zbtb5	Zinc finger and BTB domain-containing protein 5	Transcription factor		
Zbtb9	Zinc finger and BTB domain-containing protein 9	Transcription factor		
Zcchc9	Zinc finger CCHC domain-containing protein 9	Transcription factor		
Znf335	Zinc finger protein 335	Transcription factor	x	
Znf574	Zinc finger protein 574	Transcription factor	x	
Znf593	Zinc finger protein 593	Transcription factor		
Znf598	E3 ubiquitin-protein ligase ZNF598	Ubiquitin ligase		
Znf654	Zinc finger protein 654	Transcription factor		
Znf664	Zinc finger protein 664	Transcription factor		

Table 3.3: Shared RAG1 and cRAG1 Binding Partners: Proteins identified as bound to both RAG1 and cRAG1 but not empty vector control following immunoprecipitation and mass spectrometry. ATM targets have been previously established (Matsuoka et al., 2007). RAG1 interactors are proteins that appeared in a previously described RAG1 BioID study (Brecht et al., 2020).

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Abl2	Tyrosine-protein kinase	Protein Kinase	x	
Abraxas1	BRCA1-A complex subunit	Unknown		
Acap2	Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein 2	Unknown		
Acbd5	Acyl-CoA-binding domain-containing protein 5	Carrier		
Acot13	Acyl-coenzyme A thioesterase 13	Esterase		
Aen	Apoptosis-enhancing nuclease	Exoribonuclease		
Ak1	Adenylate kinase isoenzyme 1	Kinase		
Akap1	A-kinase anchor protein 1, mitochondrial	Scaffold		
Ambra1	Activating molecule in BECN1-regulated autophagy protein 1	Unknown		
Ankmy2	Ankyrin repeat and MYND domain-containing protein 2	Unknown		
Ankzf1	Ankyrin repeat and zinc finger domain-containing protein 1	Unknown		
Ap5b1	AP-5 complex subunit beta-1	Unknown		
Apmap	Adipocyte plasma membrane-associated protein	Unknown		
Armh3	Armadillo-like helical domain-containing protein 3	Unknown		
Arrb2	Beta-arrestin-2	Scaffold		
Asf1a	Histone chaperone	Chromatin remodeler		
Atp5pf	ATP synthase-coupling factor 6, mitochondrial	ATP Synthase		
Atp6v1b2	V-type proton ATPase subunit B, brain isoform	ATP Synthase		
Banf1	Barrier-to-autointegration factor	Unknown		
Bbc3	Bcl-2-binding component 3	Unknown		
Bcl2l13	Bcl-2-like protein 13	Unknown		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Bcs1l	Mitochondrial chaperone BCS1	Chaperone		
Blm	Bloom syndrome protein homolog	Helicase		
Cacul1	CDK2-associated and cullin domain-containing protein 1	Unknown		
Ccdc43	Coiled-coil domain-containing protein 43	Unknown		
Ccnh	Cyclin-H	Cyclin kinase		
Ccnl1	Cyclin-L1	Cyclin kinase		
Cdk19	Cyclin-dependent kinase 19	Protein kinase		
Cdkal1	Threonylcarbamoyladenosine tRNA methylthiotransferase	Unknown		
Cers5	Ceramide synthase 5	Transferase		
Chchd6	MICOS complex subunit Mic25	Unknown		
Chd6	Chromodomain-helicase-DNA-binding protein 6	Helicase		
Chd7	Chromodomain-helicase-DNA-binding protein 7	Helicase	x	
Chuk	Inhibitor of nuclear factor kappa-B kinase subunit alpha	Protein kinase		
Ckap2l	Cytoskeleton-associated protein 2-like	Unknown		
Clptm1l	Cleft lip and palate transmembrane protein 1-like protein	Unknown		
Cog4	Conserved oligomeric Golgi complex subunit 4	Membrane traffic		
Colgalt1	Procollagen galactosyltransferase 1	Transferase		
Comp	Cartilage oligomeric matrix protein	Cell adhesion		
Cox15	Cytochrome c oxidase assembly protein COX15 homolog	Chaperone		
Cox4i1	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	Oxidase		
Cox6a1	Cytochrome c oxidase subunit 6A1, mitochondrial	Oxidase		
Cox6b1	Cytochrome c oxidase subunit 6B1	Oxidase		
Crat	Carnitine O-acetyltransferase	Acyltransferase		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Crtc3	CREB-regulated transcription coactivator 3	Transcription cofactor		
Ctdp1	RNA polymerase II subunit A C-terminal domain phosphatase	Phosphatase		
Ctu2	Cytoplasmic tRNA 2-thiolation protein 2	RNA processing		
Cyp20a1	Cytochrome P450 20A1	Oxygenase		
Dbr1	Lariat debranching enzyme	Endoribonuclease		
Ddhd2	Phospholipase DDHD2	Phospholipase		
Dhodh	Dihydroorotate dehydrogenase (quinone), mitochondrial	Dehydrogenase		
Dhrs4	Dehydrogenase/reductase SDR family member 4	Dehydrogenase		
Dlg1	Disks large homolog 1	Scaffold		
Dlg3	Disks large homolog 3	Scaffold		
Dnajc16	DnaJ homolog subfamily C member 16	Chaperone		
Dok1	Docking protein 1	Scaffold		
Dph6	Diphthine--ammonia ligase	Ligase		
Dtx2	Probable E3 ubiquitin-protein ligase	Ubiquitin ligase		
Ecd	Protein ecdysoneless homolog	Unknown		
Eif2ak2	Interferon-induced, double-stranded RNA-activated protein kinase	Protein kinase		
Emc1	ER membrane protein complex subunit 1	Unknown		
Epg5	Ectopic P granules protein 5 homolog	Unknown		
Ercc4	DNA repair endonuclease XPF	Endodeoxyribonuclease		x
Ero1a	ERO1-like protein alpha	Oxioreductase		
Espl1	Separin	Protease		
Fam118a	Protein FAM118A	Unknown		
Fancd2	Fanconi anemia group D2 protein homolog	Unknown	x	
Fgg	Fibrinogen gamma chain	Intercellular signaling		
Fhl3	Four and a half LIM domains protein 3	Transcription cofactor		
Fhod1	FH1/FH2 domain-containing protein 1	Unknown		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Fpgs	Folylpolyglutamate synthase, mitochondrial	Ligase		
Gale	UDP-glucose 4-epimerase	Epimerase		
Gbp5	Guanylate-binding protein 5	G-protein		
Gpat3	Glycerol-3-phosphate acyltransferase 3	Acyltransferase		
Gpn2	GPN-loop GTPase 2	GTPase		
Grn	Progranulin	Unknown		
Gsk3a	Glycogen synthase kinase-3 alpha	Protein kinase		
Gtf2f2	General transcription factor IIF subunit 2	Transcription factor		x
Gyg1	Glycogenin-1	Glycosyltransferase		
H2-L	H-2 class I histocompatibility antigen, L-D alpha chain	MHC		
Heatr6	HEAT repeat-containing protein 6	Unknown		
Homer3	Homer scaffolding protein 3	Unknown		
Inpp5f	Phosphatidylinositide phosphatase SAC2	Phosphatase		
Irak4	Interleukin-1 receptor-associated kinase 4	Protein kinase		
Itch	E3 ubiquitin-protein ligase	Ubiquitin ligase		
Itgb1	Integrin beta-1	Integrin		
Itprip	Inositol 1,4,5-trisphosphate receptor-interacting protein	Nucleotidyltransferase		
Jade3	Jade family PHD finger 3	Transcription factor		
Kdm4a	Lysine-specific demethylase 4A	Chromatin remodeler		
Kif3b	Kinesin family member 3B	Microtubule binding		
Kifbp	KIF-binding protein	Unknown		
Kpna3	Importin subunit alpha-4	Transporter		
Ldah	Lipid droplet-associated hydrolase	Lipase		
Llgl1	Lethal(2) giant larvae protein homolog 1	Membrane traffic		
LRWD1	Leucine-rich repeat and WD repeat-containing protein 1	Extracellular matrix		
Lsm14b	LSM family member 14B	RNA metabolism		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Malsu1	Mitochondrial assembly of ribosomal large subunit protein 1	Unknown		
Man1a2	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IB	Protein modifying		
Man2a1	Alpha-mannosidase 2	Glycosidase		
Man2c1	Alpha-mannosidase 2C1	Unknown		
Map3k1	Mitogen-activated protein kinase kinase kinase 1	Protein kinase		
Map4k2	Mitogen-activated protein kinase kinase kinase kinase 2	Protein kinase		
Mapk8ip3	C-Jun-amino-terminal kinase-interacting protein 3	Scaffold		
Mapkapk2	MAP kinase-activated protein kinase 2	Protein kinase		
Mark3	MAP/microtubule affinity-regulating kinase 3	Protein kinase		
Mcat	Malonyl-CoA-acyl carrier protein transacylase, mitochondrial	Unknown		
Med15	Mediator of RNA polymerase II transcription subunit 15	Unknown		
Med17	Mediator of RNA polymerase II transcription subunit 17	Transcription factor		
Med21	Mediator of RNA polymerase II transcription subunit 21	RNA polymerase		
Med23	Mediator of RNA polymerase II transcription subunit 23	Transcription factor		
Med8	Mediator of RNA polymerase II transcription subunit 8	Transcription factor		
Mettl16	RNA N6-adenosine-methyltransferase	Methyltransferase		
Mga	MAX gene-associated protein	Transcription factor		
Mlh1	DNA mismatch repair protein	DNA metabolism		
Mrpl35	39S ribosomal protein L35, mitochondrial	Ribosomal protein		
Mrps25	28S ribosomal protein S25, mitochondrial	Ribosomal		
Mrto4	mRNA turnover protein 4 homolog	Unknown		
Msmo1	Methylsterol monooxygenase 1	Oxidase		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Mtif3	Translation initiation factor IF-3, mitochondrial	Translation initiation factor		
Mtmr2	Myotubularin-related protein 2	Phosphatase		
Mtss1	MTSS I-BAR domain containing 1	Actin binding		
Mzt2	Mitotic-spindle organizing protein 2	Unknown		
Naa25	N-alpha-acetyltransferase 25, NatB auxiliary subunit	Acetyltransferase		
Napa	Alpha-soluble NSF attachment protein	Membrane traffic		
Nbr1	Next to BRCA1 gene 1 protein	Unknown		
Nceh1	Neutral cholesterol ester hydrolase 1	Deacetylase		
Ncor1	Nuclear receptor corepressor 1	Chromatin remodeler		x
Ndufaf1	Complex I intermediate-associated protein 30, mitochondrial	Unknown		
Ndufaf2	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 2	Chaperone		
Necap2	Adaptin ear-binding coat-associated protein 2	ATP-binding cassette transporter		
Nek1	Serine/threonine-protein kinase	Protein kinase		
Nfkb1	Nuclear factor NF-kappa-B p105 subunit	Transcription factor		
Nle1	Notchless protein homolog 1	Unknown	x	
Nsmce3	Non-structural maintenance of chromosomes element 3 homolog	Scaffold		
Nt5dc1	5'-nucleotidase domain-containing protein 1	Phosphatase		
Nubp1	Cytosolic Fe-S cluster assembly factor	Unknown		
Nubp2	Cytosolic Fe-S cluster assembly factor	Unknown		
Nudt16l1	Tudor-interacting repair regulator protein	Unknown		
Nup62	Nuclear pore glycoprotein p62	Transporter		
Ogfod3	2-oxoglutarate and iron-dependent oxygenase domain-containing protein 3	Chromatin remodeler		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Osbpl2	Oxysterol-binding protein-related protein 2	Carrier		
Osbpl5	Oxysterol-binding protein-related protein 5	Carrier		
Otud6b	Deubiquitinase	Protease		
Pan2	PAN2-PAN3 deadenylation complex catalytic subunit	Exoribonuclease		
Pcmtd1	Protein-L-isoaspartate O-methyltransferase domain-containing protein 1	Methyltransferase		
Pdcd6	Programmed cell death protein 6	Unknown		
Pdxk	Pyridoxal kinase	Kinase		
Phaf1	Phagosome assembly factor 1	Unknown		
Phf10	PHD finger protein 10	Chromatin remodeler		
Phf20	PHD finger protein 20	Unknown		
Pias4	E3 SUMO-protein ligase	Ubiquitin ligase		
Pik3c3	Phosphatidylinositol 3-kinase catalytic subunit type 3	Kinase		
Pip5k1c	Phosphatidylinositol 4-phosphate 5-kinase type-1 gamma	Kinase		
Pisd	Phosphatidylserine decarboxylase proenzyme, mitochondrial	Decarboxylase		
Pmf1	Polyamine-modulated factor 1	Unknown		
Poglut2	Protein O-glucosyltransferase 2	Unknown		
Ppp2r2d	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B delta isoform	Phosphatase		
Ppp4c	Serine/threonine-protein phosphatase 4 catalytic subunit	Phosphatase		
Ppt1	Palmitoyl-protein thioesterase 1	Chromatin remodeler		
Prdm10	PR domain zinc finger protein 10	Transcription factor	x	
Ptk2b	Protein-tyrosine kinase 2-beta	Protein kinase		
Ptpn12	Tyrosine-protein phosphatase non-receptor type 12	Unknown		
Rab2a	Ras-related protein Rab-2A	GTPase		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Rabggtb	Geranylgeranyl transferase type-2 subunit beta	Acyltransferase		
Rag1	V(D)J recombination-activating protein 1	Endodeoxyribonuclease		x
Rag2	V(D)J recombination-activating protein 2	Unknown		x
Rap2c	Ras-related protein 2c	GTPase		
Rassf2	Ras association domain-containing protein 2	Scaffold		
Rb1	Retinoblastoma-associated protein	Chromatin binding		
Rbl1	Retinoblastoma-like protein 1	Chromatin remodeler		
Rbm12	RNA-binding protein 12	RNA splicing		
Rcor2	REST corepressor 2	Chromatin remodeler		
Riok1	Serine/threonine-protein kinase RIO1	Protein kinase		
Riok2	Serine/threonine-protein kinase RIO2	Protein kinase		
Rnf126	E3 ubiquitin-protein ligase	Ubiquitin ligase		
Rnf14	E3 ubiquitin-protein ligase	Ubiquitin ligase		
Rpl24	60S ribosomal protein L24	Ribosomal		
Rpl36a	60S ribosomal protein L36a	Ribosomal		x
Rpp38	Ribonuclease P protein subunit p38	Endoribonuclease		
Rps6ka3	Ribosomal protein S6 kinase alpha-3	Protein kinase		
Rufy1	RUN and FYVE domain-containing protein 1	Unknown	x	
Sav1	Salvador family WW domain containing 1	Unknown		
Scrib	Scribbled planar cell polarity	Scaffold		
Sdcbp	Syntenin-1	Membrane traffic		
Septin11	Septin-11	Cytoskeletal		
Sesn3	Sestrin-3	Peroxidase		
Shprh	E3 ubiquitin-protein ligase	Ubiquitin ligase		
Sirt5	NAD-dependent protein deacylase sirtuin-5, mitochondrial	Chromatin remodeler		
Slc25a16	Solute carrier family 25 member 16	Mitochondrial carrier		
Slc25a4	ADP/ATP translocase 1	Carrier		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Slmap	Sarcolemmal membrane-associated protein	Scaffold		
Smad5	SMAD family member 5	Transcription factor		
Smarca2	Probable global transcription activator SNF2L2	Helicase	x	
Smg5	SMG5 nonsense mediated mRNA decay factor	RNA metabolism		
Snx20	Sorting nexin-20	Scaffold		
Soat1	Sterol O-acyltransferase 1	Acyltransferase		
Sp1	Transcription factor Sp1	Unknown		
Srprb	Signal recognition particle receptor subunit beta	Carrier		
Ssh2	Protein phosphatase Slingshot homolog 2	Phosphatase		
Ssr4	Translocon-associated protein subunit delta	Unknown		x
Ssu72	RNA polymerase II subunit A C-terminal domain phosphatase	Phosphatase		
Stambp	STAM-binding protein	Protease		
Stim1	Stromal interaction molecule 1	Unknown		
Strada	STE20-related kinase adapter protein alpha	Protein kinase		
Sugt1	Suppressor of G2 allele SKP1	Unknown		
Suox	Sulfite oxidase, mitochondrial	Reductase		
Szt2	KICSTOR complex protein	Unknown		
Taf6l	TAF6-like RNA polymerase II p300/CBP-associated factor-associated factor 65 kDa subunit 6L	Transcription factor		
Tapt1	Transmembrane anterior posterior transformation protein 1	Unknown		
Tasor	Transcription activation suppressor	Unknown		
Tbc1d24	TBC1 domain family member 24	GTPase activating		
Tbc1d25	TBC1 domain family member 25	GTPase activating		
Tcf4	Transcription factor 4	Transcription factor		
Tent2	Poly(A) RNA polymerase GLD2	Nucleotidyltransferase		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Thap12	52 kDa repressor of the inhibitor of the protein kinase	Unknown		
Thyn1	Thymocyte nuclear protein 1	Unknown		
Ticrr	Treslin	Unknown		
Tifa	TRAF-interacting protein with FHA domain-containing protein A	Unknown		
Timm23	Mitochondrial import inner membrane translocase subunit Tim23	Amino acid transporter		x
Tmed2	Transmembrane emp24 domain-containing protein 2	Vesicle coat		
Tmx1	Thioredoxin-related transmembrane protein 1	Unknown		
Tnfaip8	Tumor necrosis factor alpha-induced protein 8	Unknown		
Tom1	Target of Myb protein 1	Unknown		
Tox4	TOX high mobility group box family member 4	Unknown		
Tprg1l	Tumor protein p63-regulated gene 1-like protein	Unknown		
Tpx2	Targeting protein for Xklp2	Microtubule binding	x	
Trabd	TraB domain-containing protein	Unknown		
Trappc10	Trafficking protein particle complex subunit 10	Unknown		
Trim34a	Tripartite motif-containing protein 34A	Ubiquitin ligase		
Trmt11	tRNA (guanine(10)-N2)-methyltransferase homolog	Methyltransferase		
Trmt61a	tRNA (adenine(58)-N(1))-methyltransferase catalytic subunit TRMT61A	Methyltransferase		
Tsc2	Tuberin	GTPase activating		
Tsn	Translin	Unknown		
Tuba4a	Tubulin alpha-4A chain	Tubulin		
Twf1	Twinfilin-1	Actin binding		
Twnk	Twinkle protein, mitochondrial	DNA helicase		
Tyw1	S-adenosyl-L-methionine-dependent tRNA 4-demethylwyosine synthase	Lyase		

Gene Symbol	Gene Name	Function	ATM Target	RAG1 Interactor
Ube2j1	Ubiquitin-conjugating enzyme E2 J1	Ubiquitin ligase		
Ube2k	Ubiquitin-conjugating enzyme E2 K	Ubiquitin ligase		
Ube2v2	Ubiquitin-conjugating enzyme E2 variant 2	Ubiquitin ligase		
Ube2z	Ubiquitin-conjugating enzyme E2 Z	Ubiquitin ligase		
Ubr3	E3 ubiquitin-protein ligase UBR3	Ubiquitin ligase		
Uggt1	UDP-glucose:glycoprotein glucosyltransferase 1	Glycosyltransferase		
Ulk1	Serine/threonine-protein kinase ULK1	Protein kinase		
Unc119b	Protein unc-119 homolog B	Membrane traffic		
Unc45a	Protein unc-45 homolog A	Unknown		
Vps33b	Vacuolar protein sorting-associated protein 33B	Membrane traffic		
Vrk3	Inactive serine/threonine-protein kinase	Protein kinase		
Wdr37	WD repeat-containing protein 37	Unknown		
Wdr4	tRNA (guanine-N(7)-)-methyltransferase non-catalytic subunit	RNA processing factor		
Wwp2	NEDD4-like E3 ubiquitin-protein ligase	Ubiquitin ligase		
Zbtb33	Transcriptional regulator Kaiso	Transcription factor		
Zcchc4	rRNA N6-adenosine-methyltransferase ZCCHC4	Methyltransferase		
Zdhhc13	Palmitoyltransferase	Chromatin remodeler		
Zfp646	Zinc finger protein 646	Transcription factor		
Zfp809	Zinc finger protein 809	Transcription factor		
Zmiz1	Zinc finger MIZ domain-containing protein 1	Ubiquitin ligase		
Zmynd11	Zinc finger MYND domain-containing protein 11	Transcription cofactor	x	
Znf445	Zinc finger protein 445	Transcription factor		
Znf787	Zinc finger protein 787	Transcription factor		
Znf800	Zinc finger protein 800	Unknown		
Znhit6	Box C/D snoRNA protein 1	Unknown		

Chapter 4

Conclusions

by
Rachel Leigh Johnston

4.1 Summary

DNA damage is a serious, ongoing threat to the cellular genome. The DDR is a highly complex network that has evolved to combat these dangerous lesions that threaten the integrity of genetic material. This signaling network has evolved to direct cellular responses to various types of injury that DNA is continuously encountering. My thesis focuses on a unique, programmed DNA break in immune cell development – V(D)J recombination during B cell development. The RAG endonuclease creates DSBs in immunoglobulin genes to assemble antigen receptors for specific responses to pathogens. These programmed DSBs activate a specific developmental program that functions to regulate cellular programs required for proper lymphocyte development. Surprisingly, not all DSBs in B cells activate this unique cellular response. Thus, developing B cells respond to different DSBs through distinct signaling pathways. My thesis work centered on determining how cells distinguish between "normal" physiologic RAG DSBs and "toxic", or non-programmed, DSBs to appropriately activate developmental or cell death programs. To do this, I focused on the following questions: 1) Do B cells trigger different DDR programs based on the mechanism of DNA injury? 2) Do unique properties of the RAG endonuclease regulate the DDR signals in B cells?

In this chapter, I summarize the significant findings from my graduate work in answering these questions, discuss the broader significance, and propose future directions. Together, my work advances the DNA damage field by furthering our understanding of how a cell responds to a DNA break to signal appropriately. This work also delineates a direct role for RAG1 in coordinating DNA damage signaling in early B cells. These findings broaden our understanding of both normal B cell development and development of B cell malignancies.

4.1.1 RAG DSBs activate a unique developmental DNA damage response

RAG DSBs are an idiosyncratic DNA injury as they are programmed to occur at precise locations in the genome during specific stages of B and T cell development and occur in the context of other ongoing developmental signals. Thus, the response to RAG-mediated DNA damage must integrate with the developmental processes required for continued B cell development and maturation, which are highly complex in themselves. Intriguingly, RAG DSBs elicit two types of DNA damage responses: 1) cDDR, which coordinates DSB repair, and 2) ncDDR, which activates broad cellular signals that intersect with other developmental programs to promote continued B cell development. Based on these findings, we hypothesized that B cells distinguish non-RAG DSBs, which are detrimental to B cells, from RAG DSBs to restrict activation of ncDDR but still trigger cDDR in order to promote proper DSB repair while preventing errant development. In Chapter 2, I report my findings that RAG-mediated DSBs at *Ig* loci induce both cDDR and ncDDR while Cas9-mediated DSBs either at or outside of *Ig* loci selectively induce only the cDDR. Thus, ncDDR signals are not a conserved response to all DSBs in pre-B cells, but, rather, are uniquely activated by RAG-mediated DSBs generated during *Ig* receptor gene assembly.

4.1.2 The N-terminal region of RAG1 activates the ncDDR

As Cas9-mediated DSBs could not induce ncDDR like RAG-mediated DSBs, this suggests that unique features of the RAG endonuclease itself are responsible for coordinating the ncDDR response. In Chapter 2, I chose to examine whether RAG1 itself has a function in regulating DDR signaling in B cells. Previous work examining the non-core regions of RAG1 demonstrate that these regions regulate RAG complex stability and recombination activity. However, potential functions of these non-endonuclease domains in DNA damage signaling have not previously been investigated. Mutational analysis of RAG1 reveals that induction of non-canonical DDR is

dependent upon generation of DSB and the RAG1 NTR. cRAG1 expression in *Rag1*$^{-/-}$*:Lig4*$^{-/-}$*:Bcl2* pre-B cells was unable to induce ncDDR compared to full length RAG1. These studies reveal for the first time that RAG1 has critical activities in coordinating DNA damage signaling through functions of its non-core domains.

4.1.3 RAG1 recruits signaling proteins to the site of a DSB

In Chapter 3, I examine the role the RAG1 NTR plays in DNA damage signaling. The non-catalytic domains of RAG1 contain highly unstructured regions that can serve as binding sites for interacting proteins. Because of this, I hypothesized that the RAG1 NTR recruits proteins that modulate DDR signaling to the site of the RAG DSB. Using immunoprecipitation and mass spectrometry approaches, the RAG1 NTR was found to associate with a unique protein interactome. These findings support that the RAG1 NTR recruits distinct factors to sites of DSBs. Several of the interacting proteins have functions that could regulate activity of transcription factors, signaling proteins, and gene expression that are uniquely triggered by ncDDR signals induced by RAG DSBs. To determine if the NTR is sufficient to activate ncDDR following a DSB, I generated Cas9-RAG1 fusion proteins to recruit RAG1 and its binding partners to sites of a Cas9 DSB (i.e., a non-RAG-mediated DSBs). Ongoing and future studies will define the function of RAG1-associated proteins in regulating DDR and will determine if the N-terminal region of RAG1 is sufficient to induce ncDDR. The identified RAG1-binding partners and experimental tools created will define novel mechanisms of RAG1 in regulating B cell responses to DNA breaks during *Ig* gene assembly.

4.2 Future Directions

My dissertation research has revealed a novel paradigm for DDR wherein B cells initiate distinct cellular responses to DNA breaks based on the mechanism of DNA injury. Additionally, I have uncovered a critical nuclease-independent function of the RAG complex in DNA damage signaling. These findings reveal previously unrecognized opportunities and problems in the role of RAG DSBs during lymphocyte development and the unique role they play in inducing a cell-type specific response to DNA injury.

This intriguing concept of distinct DNA damage responses based on mechanism of injury merits investigation into other cell-type specific DDR. Mature B cells and T cell development proceeds through break intermediates in V(D)J recombination and CSR as detailed above. These breaks also upregulate both cDDR and ncDDR as in developing pre-B cells (Bednarski and Sleckman, 2019). Similarly, maintenance of genomic stability while also allowing for cell development is critical in hematopoietic stem cells (HSCs). DNA damage accumulates in HSCs, which likely contributes to the functional decline of HSCs over time (Rossi et al., 2007). ATM-mediated pathways also appear to regulate self-renewal and quiescence through inhibition of oxidative stress (Ito et al., 2006; Maryanovich et al., 2012).

Macrophages are another cell type that exhibit cell-type specific DNA damage responses. Macrophages are a key player in inflammatory responses, and once activated become phagocytic to eliminate bacteria and regulate other neighboring cells through the release of cytokines and chemokines (Valledor et al., 2010). An important part of this immune response involves production of reactive oxygen species to eradicate pathogens, but also have the ability to damage host cellular DNA (Nathan and Shiloh, 2000). cDDR is upregulated to respond to these breaks

while ncDDR is induced to control cytokine and chemokine activation, phagocytosis and expression of cell surface receptors (Bednarski and Sleckman, 2019). It is possible that like RAG DSBs in pre-B cells, there could be unique factors recruited to the sites of these programmed DNA breaks to upregulate the macrophage-specific ncDDR. The cGAS/STING pathway is a cytosolic DNA receptor that is a critical mechanism for sensing DNA and inducing an immune response (Decout et al., 2021). cGAS has been shown to move to the nucleus following DNA damage, which in turn activates STING to promote NFκB translocation into the nucleus (Shen et al., 2021). It is conceivable that the cGAS/STING pathway could coordinate unique responses to DSBs in macrophages. To address this question, Cas9 can be expressed in macrophages to generate DSBs in different genomic loci in the context of different inflammatory signals or infectious pathogens and downstream DDR assayed for activation of cDDR and ncDDR.

There is also evidence supporting that natural killer (NK) cells also exhibit cell-type specific DNA damage responses. NK cells are considered the third lineage of lymphocytes, containing germline-encoded antigen receptors but are not thought to require RAG for their development (Kondo et al., 1997; Leavy, 2014). DNA damaging agents or DNA replication inhibitors induce the expression of NKG2D ligands, a stimulatory receptor on the surface of NK cells which induces cytotoxic activity of virally infected cells or tumor cells (Cerboni et al., 2014; Gasser et al., 2005; Gasser and Raulet, 2006). This is an important warning system alerting cytotoxic lymphocytes of stress signals (Cerboni et al., 2014). DNA damage signals in NK cells also downregulate Zap70 and Syk, which allows for termination of NK cell functional activity without the aid of other immune cells (Pugh et al., 2018). Interestingly, while NK cells do not undergo V(D)J recombination, NK cells that have expressed RAG at any point during development subsequently have better fitness for responding to genomic insults later in development (Karo et

al., 2014). This RAG expression may bring along unique binding partners that dictate this phenotype of robust response to viral infection.

Beyond cell-type specific responses, the Cas9-RAG fusion system created here has broad implications for engineering DNA damage signaling following genomic injury. Results of Cas9-RAG1 fusion proteins can be followed up by experiments linking Cas9 to RAG1 binding partners, such as PU.1 or ZFP91 (Cas9-PU.1 or Cas9-ZFP91). Here, the distinct binding partner required for RAG-mediated ncDDR activation can be brought to the site of a DSB to determine if it is sufficient for ncDDR activation in the context of Cas9-mediated DSB. These fusion proteins can be engineered to trigger very refined or customized DDR following a DNA break. These experiments will prove useful for DNA-editing approaches being used broadly for gene therapy in a clinical setting.

4.3 Final Thoughts

The work done throughout my graduate research provide important new insight on DNA damage signaling in developing B cells. I have established that pre-B cells trigger distinct DNA damage responses depending on the mechanism of injury. I also show that RAG1, a component of the RAG endonuclease required for V(D)J recombination, plays a direct role in the downstream DNA damage response. Specifically, the region of the protein that does not contain its catalytic activity is critical for induction of the developmental response following a RAG DSB.

I chose to examine the topics outlined here after a fascinating discovery that irradiation-induced DSBs in pre-B cells do not trigger a developmental response like RAG-mediated DSBs do. Closer examination of this phenomenon resulted in exciting new results that the RAG

endonuclease itself directs this unique developmental response, rather than the location of the break itself. This work allows us to better understand the mechanistic basis of how developing lymphocytes distinguish between programmed and spontaneous methods of damage and subsequently activate a specific cellular program. Defining these DSB-specific mechanisms that dictate cellular response will reveal previously unrecognized opportunities to optimize therapies for leukemia and other malignancies by redirecting cell fate from a cell death pathway to a cell differentiation pathway. This work has also generated new tools for investigating specific responses to DNA injury in other cell types as well, which will have broad impacts on immune development and DNA damage responses.

Chapter 5

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