Characterizing the role of the DNA Damage Response in Class switch recombination.

by

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Department of Immunology
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Abstract

B cells undergo secondary antibody diversification to elicit an effective immune response. Secondary antibody diversification generates antibodies with high affinity for the cognate antigen, and antibodies of different classes. Different classes of antibodies mediate different effector functions, enabling the humoral response to be fine-tuned to the infection at hand. The different classes are generated by a process called class switch recombination (CSR), which requires the generation and repair of double-stranded DNA breaks (DSBs) at the antibody heavy chain locus, and relies on the 53BP1-dependent DNA damage response (DDR). However, the molecular mechanism and function of DDR in CSR is not fully understood. To characterize the role of DDR in CSR, we first established an in vitro CH12F3-2 mouse B cell line/ RNA interference system and used this system to demonstrate that the DDR factors RNF8 and RN168, play a role in CSR. Using this system, we then developed and conducted a genome-wide screen to identify novel CSR factors, followed by a secondary screen to identify DDR factors among the CSR candidates. Based on this study, we selected one candidate, the Drosophila enhancer of yellow 2 homolog (Eny2), for further characterization. Eny2 is a structural component of the H2B-deubiquitination module of the SAGA complex. We found that Eny2 likely acts downstream of the DSBs generated for CSR, and that Eny2 might act with the SAGA complex in
mediating H2B-deubiquitination to facilitate CSR. We also provide evidence that, in addition to its role in CSR, Eny2 is important for H2B-deubiquitination upon irradiation-induced DNA damage, and may be required for optimal DNAPK activation.
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DISCUSSION AND FUTURE DIRECTIONS

RNF8 and RNF168 play a role in CSR.

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List of Abbreviations

3'RR  3' Regulatory Region  
53BP1 p53 Binding Protein 1  
dA  Adenine  
AID Activation-Induced cytidine Deaminase  
APOBEC Apolipoprotein B mRNA-editing catalytic  
APRIL A proliferation-inducing ligand, TNFSF 13a  
A-T Ataxia-telangiectasia  
ATM Ataxia-telangiectasia Mutated  
ATR Ataxia-telangiectasia and Rad3 related  
ATRIP ATR interacting protein  
BAFF B cell activation factor of the TNF family, TNFSF 13b  
Bcl2 B cell leukemia/lymphoma 2  
Bcl6 B cell leukemia/lymphoma 6  
BER Base Excision Repair  
BRCA1 breast cancer 1  
C Constant  
Chk1 Checkpoint kinase 1  
Chk2 Checkpoint kinase 2  
c-myc myelocytomatosis oncogene  
CSR Class Switch Recombination  
CtIP CtBP-interacting protein; retinoblastoma binding protein 8  
D Diversity  
dA Deoxyadenosine  
dC Deoxycytosine  
DDR DNA Damage Response  
dG Deoxyguanosine  
DNAPK DNA-dependent protein kinase  
DNAPKcs DNA-dependent protein kinase catalytic subunit  
DSB Double-Strand Break  
dT Deoxythymidine  
dU Deoxyuridine  
Eµ Heavy chain µ intronic enhancer  
Eny2 Enhancer of Yellow 2 homolog (Drosophila)  
Exo1 Exonuclease 1  
Fc Fragment crystallisable  
Fucci Fluorescent Ubiquitination based cell cycle indicator  
GCV Gene Conversion  
HR Homologous Recombination  
Hsp90 Heat shock protein 90
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>I</td>
<td>Intronic promote</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon γ</td>
</tr>
<tr>
<td>Igα</td>
<td>Immunoglobulin α chain</td>
</tr>
<tr>
<td>Igβ</td>
<td>Immunoglobulin β chain</td>
</tr>
<tr>
<td>IL4</td>
<td>Interleukin 4</td>
</tr>
<tr>
<td>IL5</td>
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</tr>
<tr>
<td>IRIF</td>
<td>Irradiation-induced nuclear foci</td>
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<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>J</td>
<td>Joining</td>
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<tr>
<td>K</td>
<td>Lysine</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>mAG1</td>
<td>Azami-Green1</td>
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<td>MDC1</td>
<td>Mediator of DNA damage checkpoint 1</td>
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<td>Transformed mouse 3T3 cell double minute 2</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<td>Mlh1</td>
<td>mutL homolog 1</td>
</tr>
<tr>
<td>MMEJ</td>
<td>Microhomology-mediated end joining</td>
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<tr>
<td>MMR</td>
<td>Mismatch repair</td>
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<td>Msh2</td>
<td>mutS homolog 2</td>
</tr>
<tr>
<td>Msh6</td>
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<tr>
<td>mTOR</td>
<td>mechanistic target of rapamycin (serine/threonine kinase)</td>
</tr>
<tr>
<td>Nbs1</td>
<td>nijmegen breakage syndrome 1 protein</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
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<tr>
<td>p53</td>
<td>tumor protein p53</td>
</tr>
<tr>
<td>PI3-Kinase</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PI3-KK</td>
<td>phosphatidylinositol 3-kinase-like kinase</td>
</tr>
<tr>
<td>pIgR</td>
<td>polymeric Ig receptor</td>
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<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
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<td>Pms2</td>
<td>postmeiotic segregation increased 2 (S. cerevisiae)</td>
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<tr>
<td>PTIP</td>
<td>Pax Transactivation-Domain Interacting Protein</td>
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<tr>
<td>R</td>
<td>Purine base pairs</td>
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<td>Rad50</td>
<td>Rad50 homolog (S. cerevisiae)</td>
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<td>RAG1</td>
<td>Recombination activating gene 1</td>
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<td>RNF8</td>
<td>Ring finger protein 8</td>
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<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>RPA</td>
<td>Replication Protein A</td>
</tr>
<tr>
<td>S</td>
<td>Switch</td>
</tr>
<tr>
<td>SAGA</td>
<td>Spt-Ada-Gcn5 acetyltransferase complex</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
</tr>
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<td>Ser</td>
<td>Serine</td>
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<tr>
<td>SHM</td>
<td>Somatic Hypermutation</td>
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<tr>
<td>SIM</td>
<td>SUMO-Interacting Motif</td>
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<td>SMG1 homolog, phosphatidylinositol 3-kinase-related kinase (C. elegans)</td>
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<td>S/TQ</td>
<td>Serine/Threonine-Glutamine</td>
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<td>SUMO</td>
<td>Small Ubiquitin-like Modifier</td>
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<td>TGFβ</td>
<td>Transforming growth factor, beta</td>
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<td>Threonine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TRRAP</td>
<td>transformation/transcription domain-associated protein</td>
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<td>ubiquitin conjugating enzyme 13</td>
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<td>UNG</td>
<td>uracil-DNA glycosylase</td>
</tr>
<tr>
<td>V</td>
<td>Variable</td>
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<tr>
<td>W</td>
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INTRODUCTION

1.1 B CELLS AND THE HUMORAL IMMUNE RESPONSE

1.1.1 Humoral immune response

Humoral immunity is responsible for protecting an organism by eliminating extracellular pathogens or toxins, and by preventing the spread of intracellular infections. The humoral immune response is carried out by B lymphocytes. There are two types of mature B lymphocytes, called B-1 and B-2 cells. B-1 cells are considered innate-like, mainly express CD5, undergo self-renewal, mainly reside within the peritoneal and pleural cavities and produce natural serum antibodies (Baumgarth 2011). B-2 cells are the conventional B cells which arise in the bone marrow, function within the lymphoid tissue and produce antibodies in response to an infection. Upon activation with its cognate antigen, B cells can either differentiate into memory cells or plasma cells. Memory cells are long-lived quiescent B cells that get activated upon subsequent exposure to the same pathogen that they previously encountered. Upon activation, they typically produce high-affinity antibodies. On the other hand, plasma cells are short-lived cells that secrete large amounts of high affinity antibodies to the pathogen that serve to eliminate the infection.

Antibodies can eliminate an infection by three different mechanisms: neutralization, opsonization, and complement activation. Neutralization is achieved, for example, when antibodies bind to receptors on a pathogen that are used by the pathogen to infect cells, thus disabling the pathogen from infecting cells (Smith et al. 1996; Edelson et al. 2001; Haberstroh et al. 2008). Opsonization occurs when antibodies coat the surface of a pathogen, but in this case, the bound antibody promotes phagocytosis of the antibody-coated pathogen, by phagocytic
immune cells such as macrophages (Joller et al. 2011). Upon phagocytosis, the pathogen is enveloped within the phagosome, which fuses with the lysosome, in turn causing degradation of the pathogen (Joller et al. 2011). Complement deposition is achieved by the activation of the complement cascade by pathogen-bound antibody (Mold et al. 2002). Complement activation can either augment opsonization or create pores in the pathogen membrane, directly inducing lysis of the pathogen (Dunkelberger et al. 2010). All three of these functions of antibodies play a key role in controlling extracellular bacterial infections. Antibodies can also cause antibody-dependent cellular cytotoxicity, in which case the antibodies bind to an infected cell and this is typically recognized by natural killer kills that induce lysis of the infected cell (Bradford et al. 2001; Sulica et al. 2001). In addition to natural killer cells, macrophages, myeloid cells and neutrophils can also mediate antibody-dependent cellular cytotoxicity (Siders et al. 2010; Braster et al. 2013).

1.1.2 B cell activation and signalling

Naïve B cells are activated when they first encounter foreign antigens, leading to antibody secretion and the generation of a germinal center response. B cell activation involves cross-linking of the B cell receptor expressed on the surface of the B cell (Gold et al. 1992). The B cell receptor complex contains the B cell receptor in association with the Igα and Igβ polypeptides (DeFranco et al. 1995). The B cell receptor itself is incapable of transmitting a signal and relies instead on the associated Igα and Igβ polypeptides for signal transmission to the cell (Taddie et al. 1994; DeFranco et al. 1995). Igα and Igβ each contain an immunoreceptor tyrosine-based activation motif (ITAM) on their cytosolic end that is phosphorylated by Src-family kinases upon B cell receptor engagement to the antigen (DeFranco et al. 1995). B cell receptor signalling
also relies on the B cell co-receptor complex, which includes CD21, CD19, CD81 and Leu-13 (Sato et al. 1997; Fearon et al. 2000; Mongini et al. 2001; Mongini et al. 2002). CD21, also known as complement receptor 2, binds to complement fragments on the antigen, clustering the co-receptor complex with the B cell receptor, which leads to phosphorylation of CD19 by B cell receptor-associated kinases, thus initiating PI3-kinase signalling cascade (Fearon et al. 2000).

B cell activation requires a second signal in addition to B cell receptor cross-linking. This second signal depends on the type of antigen, and can either be T cell-dependent or T cell-independent. The B cell receptor binds the pathogen and internalizes it by endocytosis, then fragments it into peptides, and some of these peptides are then presented on the B cell surface in the context of MHC class II (Sproul et al. 2000; Clark et al. 2004). An antigen presented in the context of MHC class II on B cells can be recognized by the cognate T cell receptor of a helper T cell, which in turn provides a second signal for B cell activation through CD154-CD40 ligation and cytokine stimulation (Gowthaman et al. 2010). T cell-dependent activation in most cases requires that the helper T cell and activated B cell recognize the same antigen. This leads to the germinal center reaction and the generation of high-affinity antibodies, which bind to and eliminate the pathogen by the mechanisms described above. Alternatively, some antigens lead to T cell-independent B cell activation. Certain microbial antigens can activate an innate immune receptor in addition to the B cell receptor, providing the second signal needed for B cell activation (Ruprecht et al. 2006). A well-known example of this is LPS mediated activation of Toll-like receptor 4 (Hoshino et al. 1999). A microbial antigen with a highly repetitive epitope can also lead to B cell activation, simply by causing enhanced cross-linking of the B cell receptor (Vos et al. 2000). T cell independent B cell activation cannot generate a germinal center
response but can lead to long-lived plasma cells (Taillandet et al. 2009; Defrance et al. 2011; Bortnick et al. 2013).

1.1.3 The germinal center reaction

Upon activation, B cells either enter or produce one *de novo* germinal center, where they undergo high levels of proliferation and secondary antibody diversification. B cells expressing antibodies with a high affinity for antigen are then selected in the germinal center to differentiate either into antibody-secreting plasma cells or memory cells, while B cells expressing low affinity antibodies undergo apoptosis (Victora et al. 2012). The germinal center is organized into a dark zone and light zone. The dark zone is concentrated with hyper-proliferating B cells, while the light zone is characterized by a network of follicular dendritic cells that capture antigen and provides the basis for selecting B cells that produce high-affinity antibodies to the antigen in question (Victora et al. 2012).

Traditionally, the germinal center response was viewed as a simple model in which activated B cells enter the dark zone, where they become centroblasts that undergo hyper-proliferation and somatic hypermutation (Allen et al. 2007). Somatic hypermutation is a process by which antibodies accumulate mutations in the antibody encoding genes (see below), changing the affinity of the antibody for the antigen. The cells then enter the light zone and become centrocytes that compete with each other to bind antigen-immune complexes presented by the follicular dendritic cells (Allen et al. 2007). As antigen engagement provides survival signals to the B cells, the B cells with high affinity for antigen are selected for, while B cells with low affinity for antigen do not receive survival signals and undergo apoptosis (Victora et al. 2012). B cells can receive survival signals from TNF ligand family members, including BAFF and APRIL.
This process of selection is called affinity maturation. B cells with high affinity are selected to survive, proliferate and interact with the cognate helper T cell, initiating differentiation into plasma cells or memory cells. However, two-photon microscopy technology developed recently for real-time imaging of B cells in the germinal center has suggested that B cell proliferation occurs in both dark and light zones, and B cells migrate in both directions between the two compartments suggesting that the traditional view of the germinal center reaction may not be completely accurate (Allen et al. 2007).

1.1.4 Antibody protein structure and isotype function

An antibody is composed of two heavy chains and two light chains held together by disulfide bridges, to form a Y-shaped structure (Figure 1). The two arms of the Y-shaped antibody join at a flexible region called the hinge region. At the N-terminal ends of the two antibody arms lies the antigen binding sites, which is constructed by the variable (V) regions of both the heavy and light chains. The C-terminal end of the antibody is referred to as the constant region, because there is minimal sequence diversity in this region compared to the V-region. The constant region dictates the effector function of the antibody. There are five different constant regions. These five classes or isotypes are IgM, IgD, IgG, IgE and IgA. IgM and IgD are initially expressed by mature B cells, but B cells can then switch to express IgG, IgE or IgA. IgG is the most abundant antibody, IgE is important in allergic responses and IgA is critical for mucosal immunity. There are four subclasses of IgG in humans – IgG1, IgG2, IgG3 and IgG4; and five subclasses in mice - IgG1, IgG2a, IgG2b, IgG2c, and IgG3. Inbred mouse strains including C57BL/6, C57BL/10, SJL and NOD contain the Igh1-b gene produce IgG2c, and contain a
Figure 1: Protein structure of IgM, IgD, IgG, IgE and IgA antibodies. Each antibody consists of 2 heavy chains and 2 light chains, which together form a Y-shaped structure. The antigen binding variable sites are found at the N-terminal ends of the two antibody arms. The constant region is at the C-terminal end of the antibody. There are five different constant regions: IgM, IgD, IgG, IgE and IgA. For IgD, IgG and IgA, the two arms and the leg of the Y-shaped antibody join at a flexible region called the hinge region. IgM and IgE have a third domain replacing the hinge region. IgM can form a pentamer held together by the J chain, while IgA can form a dimer held by the J chain.
deletion of the IgG2a gene (Martin et al. 1998). While most antibodies exist as monomers, IgA can form dimers and IgM can form pentamers/hexamers.

The antibody constant region can be bound by Fc receptors and immunoglobulin receptors found on cells. Different receptors have different affinities for each of the constant regions, thus dictating how each class of antibody functions. Fc receptors can lead to a stimulatory or inhibitory response. For instance, IgG1 binds to FcγRIII and is important for NK cell activation and antibody-dependent cellular cytotoxicity (Sulica 2001). IgM bound by FcμRI, and IgG1 and IgG3 bound by IgG receptors can activate the classical complement pathway, while IgA bound by FcαRI can activate the alternative and lectin complement pathways (Daha 2011). Polymeric Ig receptors (pIgR) bind to and transport IgA dimers and IgM pentamers across epithelial tissues (Raghavan et al. 1996). IgE binds to FcεRI on mast cells, eosinophils and basophils, and leads to the allergic response (MacGlashan 2012).

1.1.5 The antibody loci

The immunoglobulin loci consist of one μ heavy chain locus and two light chain loci, κ and λ. In humans, the μ heavy chain is on chromosome 14, κ on chromosome 2 and λ on chromosome 22. In mice, the μ heavy chain is on chromosome 12, κ on chromosome 6 and λ on chromosome 16 (Honjo 1983). Beginning from the 5’ end, the heavy chain is organized into the variable gene segments, intronic enhancer Eμ, constant gene segments and finally 3’ Regulatory region (3’RR), which contains the distal enhancers (Figure 2). The variable gene segments can be subdivided into variable (V), diversity (D) and joining (J) regions on the heavy chain, and V and J regions on the light chains, which together encode the corresponding variable domain of the antibody. During maturation, a gene rearrangement process termed V(D)J recombination,
Figure 2: Locus structure of immunoglobulin heavy chain in human and mouse. The heavy chain is organized starting from the 5’ end, into the variable gene segments (VDJ), intronic enhancer (Eμ), constant gene segments and finally 3’ Regulatory region (3’RR), which contains the distal enhancers. Each constant gene segment (C) is associated with a promoter and switch region (S). The human heavy chain consists of μ, δ, γ3, γ1, α1, γ2, γ4, ε, and α2 constant regions. The mouse heavy chain consists of μ, δ, γ3, γ1, γ2b, γ2a, ε, and α constant regions. However, C57BL/6, C57BL/10, SJL and NOD mice contain a deletion of the γ2a gene, while expressing the Igh1-b gene that produces antibodies with γ2c constant region.
which is initiated by the Rag1/2 protein complex, recombines one V, one D and one J gene segment together on the μ heavy chain locus, and one V and one J gene segment together on the light chain locus, to form a single coding exon called the variable domain (described in further detail below). Within the variable domain sequence, there are three distinct hypervariable or complementarity-determining regions that come together on the folded protein to make up the antigen-binding hypervariable surface.

1.2 ANTIBODY DIVERSIFICATION

1.2.1 Primary antibody diversification

Humans and mice produce millions of B cells each day. Each B cell produces antibodies that recognize one epitope, but different B cells recognize different epitopes. Combining all the millions of B cells produced every day, the human antibody repertoire is estimated to be $10^{11}$. This antibody repertoire is generated through primary antibody diversification, a mechanism based on somatic gene rearrangement referred to as V(D)J recombination (Hozumi et al. 1976). V(D)J recombination occurs within developing B cells in the bone marrow and involves genetic rearrangement at the immunoglobulin heavy and light chain loci as described above (Bernard et al. 1978; Brack et al. 1978).

Primary antibody diversification uses combinatorial diversity and junctional diversity mechanisms to derive the antibody repertoire. As described above, the light chain is encoded by two gene segments, the V and J segments, while the μ heavy chain locus contains three gene segments, the V, D and J segments. Recombination occurs between one of the multiple V regions and one of the multiple J regions, to assemble one functional light chain. At the heavy chain locus, recombination occurs between one of the multiple D and J regions, followed by
recombination between one of the V regions to the DJ regions to construct a functional heavy chain (Early et al. 1980; Alt et al. 1984). All the different combinations of V to J in the light chain, and all the different combinations of V to D to J in the heavy chain, provide combinatorial diversity. All the possible combinations of μ heavy chain to either the κ or λ light chains, adds a further layer of combinatorial diversity to the primary antibody diversification. Junctional diversity, on the other hand, is generated by nucleotide processing of the two DNA ends involved in generating the joint between V and J in light chain, and the joints between V and D and between D and J in the heavy chain (Boubnov et al. 1993). The addition of nucleotides at these joints during the recombination process also contributes to junctional diversity (Lieber et al. 1988). Together, combinatorial and junctional diversity contribute to the estimated 10^{11} antibody repertoire.

V(D)J recombination requires the lymphoid-specific RAG1 and RAG2 proteins to recognize the recombination signal sequences located directly beside the V, D and J segments (Schatz et al. 1989; Oettinger et al. 1990). In the light chain, the RAG complex binds to the recombination signal sequences and brings the V and J segments into close proximity. RAG1/2 then mediates cleavage of the recombination signal sequences such that the intervening DNA is removed, and the two DNA ends of the V and J segments are combined to form a hairpin structure (Schatz et al. 2011). The hairpin is cleaved by Artemis to generate palindromic or P-nucleotides, and terminal deoxynucleotidyl transferase adds nucleotides to the DNA ends in a non-templated fashion generating N-nucleotides (Komori et al. 1993; Rooney et al. 2003). P and N nucleotides along with nuclease activity that leads to the removal of nucleotides from the DNA ends contribute to the junctional diversity described above. Finally, the two DNA ends are ligated together to form a coding joint through non-homologous end joining, which will be
described below in more detail, to form a complete chain (Pergola et al. 1993; Taccioli et al. 1993). A similar mechanism of recombining D to J and then V to DJ will generate the complete variable portion of the heavy chain. During coding joint formation, nucleolytic processing can cause frame-shifts leading to non-productive rearrangements. Developing B cells bearing these non-functional rearrangements undergo apoptosis.

1.2.2 Overview of Secondary antibody diversification

While primary antibody diversification produces a wide variety of antibodies against the array of antigens that challenge the host, the majority of these antibodies possess low affinity for the target. Antibodies with high affinity for the pathogen are most desirable to mount a strong immune response, while antibodies of different classes are most desirable to fine-tune the type of immune response in order to be most effective against the type of pathogen that has infected the host. Increases in antibody affinity and changes in antibody class are achieved through secondary antibody diversification processes that are triggered by B cell activation. In the initial phase of the adaptive response, B cells produce low affinity antibodies of class μ and δ. Upon interacting with the pathogen, B cells enter the germinal center and undergo secondary antibody diversification processes that generate both high affinity antibodies and antibodies with different classes. While the processes of somatic hypermutation (SHM) and gene conversion (GCV) generate high affinity antibodies, class switch recombination (CSR) generates the different classes (Figure 3). SHM and CSR occur in humans and mice, while GCV occurs in certain animals including chicken and sheep. All three secondary diversification processes absolutely require the B cell specific enzyme, activation-induced cytidine deaminase (AID) (Muramatsu et al. 2000; Arakawa et al. 2002).
Figure 3: **Secondary Antibody diversification.** Secondary antibody diversification processes include somatic hypermutation, gene conversion and class switch recombination. Somatic hypermutation introduces point mutations along the recombined variable region of the heavy and light chains. Gene conversion is the non-reciprocal insertion of gene segments from the upstream pseudo V genes into the rearranged variable region. Class switch recombination is the replacement of the heavy chain constant region by a downstream constant region.
1.2.2.1 *Activation-induced cytidine deaminase (AID)*

AID is a B cell-specific member of the apolipoprotein B mRNA-editing catalytic component (APOBEC) family (Muramatsu *et al.* 1999). It contains a cytidine deaminase domain, a bipartite nuclear localization signal and a nuclear export signal. Abnormalities in AID expression and function have been linked to hyper IgM syndrome, autoimmunity, lymphoid hyperplasia and B cell lymphomas (Minegishi *et al.* 2000; Revy *et al.* 2000; Greeve *et al.* 2003; Xiao *et al.* 2007).

1.2.2.1.1 *AID substrates and targeting:*

AID is generally believed to act on single-stranded DNA to catalyze deamination of deoxycytidines to deoxyuridines (Bransteitter *et al.* 2003; Dickerson *et al.* 2003; Larijani *et al.* 2007). *In vitro* studies have demonstrated that AID acts on deoxycytidine by preferentially targeting a WRC motif (W=A/T, R=A/G) for deamination (Pham *et al.* 2003; Larijani *et al.* 2005). Transcription of the immunoglobulin locus upon B cell activation produces the single-stranded DNA substrates required for AID (Chaudhuri *et al.* 2003; Ramiro *et al.* 2003). AID mutates both template and non-template strands equally, as there is no observed bias in mutation frequencies towards either strand *in vivo* (Xue *et al.* 2006). Recent work has suggested that the molecular substrates of AID are short single-stranded DNA patches that are present equally on the template and nontemplate strands and are linked to transcription (Parsa *et al.* 2012). Specifically, these single-stranded DNA patches are thought to be formed by transcription-induced negative supercoiling (Parsa *et al.* 2012).

Although AID is capable of inducing genome-wide mutations in mammalian cells, it nevertheless preferentially mutates the immunoglobulin locus by orders of magnitude over other
loci, indicating that AID is targeted to the immunoglobulin locus (Wagner et al. 1996; Parsa et al. 2007; Liu et al. 2008; Staszewski et al. 2011). Initial work in the field was focused on identifying AID-targeting *cis* elements, with a particular interest in sequences within the immunoglobulin enhancer regions. However, these enhancers are required for efficient transcription, making it difficult to delineate whether the enhancers were required for AID activity through transcription and/or AID targeting (Pavri et al. 2011). However, recent data shows that the 3’ regulatory region of the μ heavy chain locus is necessary for SHM and CSR, but only partially required to induce transcription of the immunoglobulin μ gene, suggesting that the 3’ regulatory region has AID-targeting *cis* elements (Rouaud et al. 2013). In addition, a region of 4kb found downstream of the distal 3’ enhancer in chicken λ light chain was suggested to possess AID targeting abilities (Kothapalli et al. 2008).

*Trans* acting factors have also been implicated in AID targeting. RNA polymerase II stalling was linked to AID targeting (Rajagopal et al. 2009; Pavri et al. 2010). Spt5 (Suppressor of Ty5 homolog), which associates with stalled RNA polymerase II, was found to bind to AID and recruit it to regions of stalled transcription (Pavri et al. 2010). This is followed by the recruitment of the RNA exosome, an RNA processing complex, to the AID-Spt5-Polymerase II complex at the stalled sites (Basu et al. 2011; Pavri et al. 2011). Stalled RNA polymerase II can often backtrack, uncovering the free 3’ end of the nascent mRNA, thereby allowing the RNA exosome to load and degrade the nascent RNA, in turn exposing the bottom strand in addition to the free top strand, to AID deamination activity (Basu et al. 2011; Pavri et al. 2011). This model also provides a means for targeting AID to the immunoglobulin locus to both the template and coding DNA strands.
Interestingly, N-terminal AID mutations cause reduced SHM but maintain CSR, while C-terminal AID mutations cause reduced CSR and retain SHM, suggesting SHM and CSR may require differential cofactors, possibly for AID targeting (Ta et al. 2003; Shinkura et al. 2004).

1.2.2.1.2 AID Regulation:

Owing to the ability of AID to mutate the genome, it is not surprising that AID has been found to be highly regulated at multiple levels. AID expression is largely restricted to the centroblast stage of germinal center B cells, the stage at which the B cells undergo SHM and CSR (Muramatsu et al. 1999). AID transcript levels are regulated by two miRNAs, miR-155 and miR-181b (de Yebenes et al. 2008; Dorsett et al. 2008; Teng et al. 2008). Moreover, AID expression and activity is largely restricted to the G1 phase of cell cycle (Schrader et al. 2007).

AID protein is restrained to the cytoplasm. Nuclear AID level is limited by two mechanisms. First, AID is actively exported from the nucleus through CRM1-dependent nuclear export (McBride et al. 2004). Second, nuclear AID is less stable than cytoplasmic AID: nuclear AID has a half-life of ~2.5 hours while cytoplasmic AID has a half-life of ~8 hours (Aoufouchi et al. 2008). The decreased stability of nuclear AID is likely due to polyubiquitination and proteasomal degradation within the nucleus (Aoufouchi et al. 2008), while cytoplasmic AID is protected from proteasomal degradation by hsp90 (Orthwein et al. 2010).

AID can be phosphorylated at multiple Ser/Thr residues. In particular Ser38 phosphorylation by PKA plays an important role in SHM and CSR (Cheng et al. 2009). It is believed that PKA and AID are independently recruited to S regions, where PKA phosphorylates AID at Ser38, which allows AID to interact with RPA, a single-stranded DNA binding protein.

Intriguingly, ectopic expression of AID using AID transgenic mice showed frequent development of T cell lymphomas instigated by AID-induced chromosomal translocations (Okazaki et al. 2003). The observation that ectopic AID lead to T cell lymphomas instead of B cell lymphomas suggests that there are other B cell specific regulators that control AID function to prevent AID off-target effects and oncogenesis (Okazaki et al. 2007).

1.2.2.1.3 AID off-target effects:

AID-induced off-target effects can lead to oncogenic mutations, chromosomal translocations, genome instability and promote B cell lymphomas (Perez-Duran et al. 2007; Staszewski et al. 2011; Gazumyan et al. 2012). Chromosomal translocations involving the immunoglobulin locus and the c-myc, Bcl2 and Bcl6 have been associated with Burkitt’s lymphoma, follicular B cell lymphoma and diffuse large B-cell lymphoma respectively (Ramiro et al. 2007). These translocation events have been ascribed to AID (Lu et al. 2013; Robbiani et al. 2013), and this is consistent with the ability of AID to mutate the genome at many sites, albeit at significantly lower levels than immunoglobulin loci (Liu et al. 2008).

1.2.2.2 DNA repair pathways engaged by AID activity

1.2.2.2.1 Base Excision Repair (BER):

As shown in Figure 4, BER generates nicks at the AID-induced dU:dG mismatches (Petersen-Mahrt et al. 2002). Uracil N-Glycosylase (UNG) excises the deoxyuridine formed by AID activity, producing an abasic site (Di Noia et al. 2002; Rada et al. 2002; Kavli et al. 2005).
Figure 4: AID-induced mutations. AID-induced cytidine deamination can be engaged by DNA replication, base-excision repair (BER) and mismatch repair (MMR) pathways. DNA replication through the dU:dG mismatch creates transition mutations. In BER, UNG/APE activity generates an abasic site, and replication through the abasic site produces transition and transversion mutations. Finally, engagement of the MMR pathway, orchestrated by Msh2 and Exo1, in concert with UNG/APE-mediated BER, leads to the recruitment of translesional replicative Polymerase η, producing transversion mutations and mutations at A-T basepairs.
The apurinic/apyrimidinic endonuclease (APE) is then recruited to cleave the abasic site to produce single-stranded DNA breaks (Peled et al. 2008). Translesional replication through the abasic site can generate transversion and transition mutations. On the other hand, if the AID/UNG induced nicks occur in very close proximity on both strands of DNA, these lesions can be converted to staggered double-stranded DNA breaks (DSBs) (Schrader et al. 2005). The DSBs produced during CSR are thought to proceed via the above described mechanism.

1.2.2.2 Mismatch Repair (MMR):

In the context of secondary antibody diversification, MMR paradoxically mutates dA:dT base-pairs as depicted in Figure 4, contrary to its classic role as a DNA repair pathway (Cascalho et al. 1998; Roa et al. 2010). AID-induced dU:dG mismatches can be recognized by the Msh2/Msh6 heterodimer (Martomo et al. 2004; Wilson et al. 2005). Subsequent recruitment of the Mlh1/Pms2 heterodimer leads to the recruitment of the Exo1 exonuclease to a nearby nick, allowing Exo1 to excise a stretch of single-stranded DNA, including the mismatch (Saribasak et al. 2009). This is followed by recruitment of DNA polymerases to fill in the excised strand. However, in secondary antibody diversification, partly due to the concerted action of UNG (in BER pathway) and MMR, the error-prone polymerase Polymerase η can be recruited to repair the excised patch, which fills in the excised strand, while producing mutations at dA:dT basepairs (Zeng et al. 2001). Alternatively, Exo1-mediated single-stranded DNA track excision can cause single-stranded DNA breaks to become double-stranded DNA breaks if a nick is present on the strand opposite to the one being excised (Schrader et al. 2005; Stavnezer et al. 2005; Schrader et al. 2007). As will be discussed below, the MMR system plays a minor but detectable role in producing the pre-requisite DSBs during CSR.
1.2.2.3 Consequences of AID activity

1.2.2.3.1 Somatic hypermutation

SHM is the process by which AID induces point mutations along the rearranged variable region of the immunoglobulin heavy and light chains, creating antibodies with varying affinities to the antigen. SHM of the V-region occurs at a mutation rate of $10^3$ mutations/base-pair/generation (Peled et al. 2008), and both strands of DNA are subject to hypermutation (Milstein et al. 1998). AID-mediated deamination of cytidine to uridine creates dU:dG mismatches in the DNA, which are processed through DNA replication, BER, or error-prone MMR to generate point mutations along the immunoglobulin locus (Figure 4). Replication through the dU:dG mismatch causes transition mutations, engagement by the BER pathway generates transition and transversion mutations, and involvement of the MMR pathway leads to mutations at dA:dT basepairs (Di Noia et al. 2007). More specifically, the joint activity of both BER and MMR results in A/T mutations (Frieder et al. 2009). The UNG-produced abasic site causes MMR to recruit error-prone polymerase η, which causes A/T mutations (Frieder et al. 2009). However, there are other unknown manners by which these A/T mutations arise, since A/T mutations still occur in UNG−/− mice. While mutations can occur at any of the four basepairs, a general bias towards transition mutations over transversion mutations is observed (Peled et al. 2008). The mutations are primarily confined to the region starting ~150 basepairs downstream of the transcription start site to ~2 kilo-basepairs downstream of the transcription start site, although the mutation rate peaks at ~500 basepairs and then drops as the distance increases from the promoter (Lebecque et al. 1990; Rada et al. 2001). Transcription is critical to SHM, given that the promoter is required, and mutation rates have been found to correlate with transcription rates (Fukita et al. 1998; Bachl et al. 2001). These mutations can be neutral,
negative or positive with respect to antigen affinity. B cells harbouring mutations that confer the antibody with higher affinity to the antigen will be selected during affinity maturation of the immune response.

### 1.2.2.3.2 Gene conversion

In some vertebrates including birds, rabbits, cows, pigs, sheep and horses, primary antibody diversification generates an extremely limited antibody repertoire (Tang *et al.* 2007). This is compensated through the secondary antibody process of GCV, which in chickens, occurs in a highly specialized compartment called the Bursa of Fabricius. Upon completing V(D)J recombination, the B cells colonize the bursa to undergo GCV. In this case, the immunoglobulin heavy and light chain loci contain pseudo gene V regions upstream of the rearranged V(D)J segment. The pseudo V genes differ from the rearranged V-region by 10-20% at the nucleotide level (Tang *et al.* 2007). During GCV, parts of the V gene sequence in the rearranged V(D)J gene are replaced with sequences from the upstream pseudo V regions, in a non-reciprocal fashion, to create the antibody repertoire. This process likely proceeds through a two-ended DSB intermediate and requires AID (Tang *et al.* 2006). AID-induced dU:dG mismatches are processed by BER and MMR, as described above, to produce DSBs that are then repaired through a homologous recombination-dependent DNA repair mechanism, to complete GCV. Homologous recombination is discussed below in more detail.

### 1.2.2.3.3 Class switch recombination

CSR is the process by which the µ constant region of the antibody gene is replaced by a downstream heavy chain constant region segment (γ, ε, α), providing the antibody with a different effector function (Figure 5). CSR occurs at the immunoglobulin locus, through a
**Figure 5. Class switch recombination.** CSR can be broadly divided into two phases: 1) generation of DSBs and 2) repair of DSBs. AID-induced cytidine deamination is engaged by BER/MMR, leading to the formation of DSBs at the donor and acceptor switch regions. The DSBs are repaired in the manner that replaces the donor constant region with the acceptor constant region, while deleting the intervening DNA.
process in which the downstream acceptor constant region is joined with the upstream rearranged V(D)J segment while the intervening DNA is deleted. This proceeds via a requisite DSB intermediate, and can be broadly categorized into 2 phases: AID-induced DSB generation and DSB repair (Stavnezer et al. 2008).

Upon activation, AID is expressed in B cells, and transcription is initiated at the donor and acceptor switch regions, providing single-stranded DNA substrates for AID-mediated deamination, as described above. The switch regions are located adjacent and upstream of each constant region, with the exception of δ. However, δ constant region contains a vestigial recombination site allowing for rare switching events to IgD (Kluin et al. 1995). Transcription is initiated from the intronic (I) promoter that is located just upstream of the I exon, which precedes each switch region (Kinoshita et al. 2001). Transcription proceeds through the I exon into the associated switch region and ends downstream of the associated constant region, producing a sterile transcript (also referred to as germline transcripts). Switch regions are highly repetitive sequences that are G-rich on the nontemplate or coding strand and can range from 1-12 kilo-basepairs in length (Chaudhuri et al. 2004). While Sμ, Sε and Sα are made of pentameric repeat sequences, the Sγ subclasses Sγ1, Sγ2a, Sγ2b and Sγ3 are made of repeats of 49-52 basepairs (Chaudhuri et al. 2004). Depending on the type of B cell activation, different acceptor switch regions are transcribed. For example, in ex vivo B cells, LPS stimulation leads to Sγ2b and Sγ3 transcription, LPS with IL4 leads to Sγ1 and Sε transcription, LPS with IFNγ leads to Sγ2a transcription, and LPS with TGFβ and IL5 leads to Sα transcription (Chaudhuri et al. 2004). Hence, the cytokine milieu of the activated B cell dictates the constant region that will be selected for CSR.
As described above, AID induces dU:dG mismatches at the G₁ phase of cell cycle and these mutations are subsequently processed by MMR or BER (Schrader et al. 2005; Stavnezer et al. 2005; Schrader et al. 2007). MMR/BER processing of the AID-induced mutations cause single-stranded DNA breaks, and if these breaks occur on opposite strands and are close enough to one another, staggered DSBs are generated at the switch regions (Schrader et al. 2005; Stavnezer et al. 2005). Consistent with this notion, although single-deficient UNG⁻/⁻ or Msh2⁻/⁻ mice have reduced switching, switching is abolished in double-deficient Msh2⁻/⁻ UNG⁻/⁻ mice (Xue et al. 2006). These DSBs elicit the DNA damage response (DDR), which can initiate DNA repair, leading to long-range end joining between the donor and acceptor switch regions. DNA repair is primarily mediated by non-homologous end joining (NHEJ) or alternatively by microhomology-mediated end joining (MMEJ), thus completing CSR in the G₁ phase of the cell cycle (Boboila et al. 2012). However, AID-induced breaks have been reported to escape cell cycle checkpoints and progress from G₁ into S phase of cell cycle, where they are detected and repaired by homologous recombination (Hasham et al. 2012). DDR and other DNA repair pathways will be discussed in further detail below.

1.3 THE DNA DAMAGE RESPONSE (DDR)

DSBs can be generated by various means, including environmental cues like irradiation or ultraviolet light, external chemical hazards, oxidative stress, errors occurring during DNA replication, and during the process of antibody diversification. These DSBs ubiquitously elicit the DNA Damage Response (DDR) pathway. DDR is an elaborate pathway in which factors are recruited to the DSB site within minutes of DSB induction, in a highly regulated and sequential fashion. This signalling network involves the initial “sensors” that detect the DSB and pass the
signal onto “transducers”, which then activate the “effectors” leading to cell cycle arrest and DNA repair. The DDR pathway activated by DSBs can be broadly divided into three distinct yet inter-connecting cascades, each initiated by one of three different sensor complexes, Mre11-Rad50-Nbs1 (MRN)/ATM, the Ku70/Ku80-DNAPKcs, and ATR-ATRIP (Yang et al. 2003). In each of these 3 pathways, the initial DSB recognition involves a PI3-kinase-like serine/threonine kinase, ATM, DNAPK and ATR respectively (Yang et al. 2003). This recognition triggers a cascade of signalling events, with highly regulated recruitment and/or post-translational modifications of DDR factors. Accumulation of these DDR factors at the ionizing radiation (IR)-induced DSB can be observed as irradiation-induced nuclear foci (IRIF) by confocal microscopy. This pathway culminates in cell cycle arrest and DNA repair, or apoptosis in the case of aberrant repair.

1.3.1 DNA damage response in class switch recombination

During CSR, the AID-induced DSBs activate ATM signalling (Lumsden et al. 2004; Reina-San-Martin et al. 2004). The MRN/ATM complex is recruited to the DNA break (Lee et al. 2004; Lee et al. 2005), where ATM phosphorylates histone H2AX (Stiff et al. 2004). γ-H2AX (phosphorylated H2AX) signals the recruitment of MDC1 (Goldberg et al. 2003; Stewart et al. 2003; Stucki et al. 2005), which recruits even more MRN/ATM, thereby leading to an amplification of the γH2AX signal (Lukas et al. 2004; Lou et al. 2006). ATM also phosphorylates MDC1, allowing for the recruitment of an E3 ubiquitin ligase, RNF8, which acts with the E2-conjugating enzyme Ubc13, to mediate mono-ubiquitination of H2A-type histones (Huen et al. 2007; Kolas et al. 2007; Mailand et al. 2007). Mono-ubiquitinated H2A-type histones signal the recruitment of another E3 ubiquitin ligase, RNF168, which also acts with
Ubc13 to protect and amplify the RNF8-mediated ubiquitination (Doil et al. 2009; Pinato et al. 2009; Stewart et al. 2009). Specifically, RNF168 mediates H2A-K15 mono-ubiquitination at the break site (Mattiroli et al. 2012). This H2A post-translational modification is recognized by the ubiquitination-dependent recruitment motif of 53BP1 leading to the recruitment of 53BP1 (Fradet-Turcotte et al. 2013). 53BP1 is in turn phosphorylated by the master kinase, ATM, which leads to the recruitment of Rif1 (Silverman et al. 2004; Di Virgilio et al. 2013; Escribano-Diaz et al. 2013; Feng et al. 2013). 53BP1-Rif1 control the DNA repair pathway choice in the G1 phase of the cell cycle by preferentially initiating the NHEJ pathway (Chapman et al. 2013; Escribano-Diaz et al. 2013; Feng et al. 2013; Zimmermann et al. 2013). As discussed further below, the DDR factors, ATM, NBS1, γ-H2AX, MDC1, RNF8, RNF168, 53BP1 and Rif1 belong to the 53BP1-dependent DDR network, and have all been demonstrated to play a role in CSR (Figure 6). Identifying that RNF8 and RNF168 play a role in CSR is part of my PhD thesis, and will be discussed further in Chapter 2. This 53BP1 dependent pathway is thought to mediate CSR by facilitating long-range DNA end joining (Difilippantonio et al. 2008; Dimitrova et al. 2008), and by protecting DNA ends from DNA resection, thus promoting NHEJ (Bothmer et al. 2010; Bunting et al. 2010; Bothmer et al. 2011). However, the molecular mechanism by which 53BP1 and associated DDR factors mediate CSR remains to be elucidated.

1.3.2 ATM

Ataxia-telangiectasia (A-T) is an autosomal, recessive disorder in humans and mice that is caused by a deficiency in ATM, and is characterized by radiosensitivity, neurodegeneration, genome instability, cancer predisposition and immune deficiency (Savitsky et al. 1995; Ziv et al. 1997). ATM is a PI3-Kinase-like kinase that phosphorylates multiple targets, preferentially at a
Figure 6. The DNA damage response in class switch recombination. DDR factors are recruited to the break in a sequential fashion, displayed above from left to right. The break is recognized by the MRN/ATM complex, leading to the phosphorylation of H2AX (γH2AX). γH2AX signals the recruitment of MDC1, which is also phosphorylated by ATM. This recruits RNF8, which mediates histone H2A and H2AX ubiquitination, which in turn recruits RNF168. RNF168 adds to the ubiquitination at the DNA break, and specifically leads to H2A-K15-ubiquitin, leading in turn to the recruitment of 53BP1. 53BP1 is also phosphorylated by ATM leading to the recruitment of Rif1. 53BP1-Rif1 block DNA resection and promote NHEJ.
S/TQ consensus motif (Kim et al. 1999). It belongs to the family of PI3-Kinase-like kinases, which includes ATR, DNAPKcs, SMG1, mTOR and TRRAP (Shiloh et al. 2013). ATM kinase activity is important for cell cycle regulation, DNA repair and apoptosis (Lavin et al. 2007). Deficiency in ATM causes aberrant G1/S phase cell cycle checkpoint (Xie et al. 1998). In humans, ATM is maintained in an inactive state as homodimers within the nucleus, but upon DSB induction, ATM dissociates into active monomers, which undergo autophosphorylation at Ser1981 and relocate to the DNA break site (Bakkenist et al. 2003). It has been suggested that ATM Ser1981 autophosphorylation is not required for the recruitment of ATM, but rather for its retention at the DNA break (So et al. 2009). In mice however, ATM autophosphorylation does not affect activation (Pellegrini et al. 2006). Upon activation, ATM phosphorylates a multitude of substrates including H2AX, NBS1, MDC1, 53BP1, Chk2, p53 and MDM2 (Shiloh et al. 2013).

A-T patients and ATM-/- mice develop lymphomas due to translocations resulting from abnormal V(D)J recombination, implicating ATM in primary antibody diversification (Taylor et al. 1996; Xu et al. 1996; Liyanage et al. 2000). Indeed, ATM-/- mice have reduced lymphocyte development in support of this notion (Xu et al. 1996). This result prompted investigators to test whether ATM also acts in CSR. Interestingly, ATM-deficient mice exhibit approximately 70% reduction in CSR without affecting intrinsic B cell growth, germline switch region transcription, SHM or intra-switch recombination (Lumsden et al. 2004; Reina-San-Martin et al. 2004). The CSR defect observed in ATM-/- mice was attributed to defective long-range DNA end joining (Reina-San-Martin et al. 2004).
1.3.3 Mre11-Rad50-Nbs1 (MRN)

ATM alone cannot act as a DNA damage sensor. Rather, to obtain complete activation, ATM must interact with the MRN complex (Uziel et al. 2003; Lee et al. 2004; Lee et al. 2005), as deficiency in either Mre11 or Nbs1 can lead to reduced ATM recruitment to DSBs, reduced ATM autophosphorylation at S1981 and reduced phosphorylation of ATM substrates. Patients with Nbs1 mutations present with Nijmegen breakage syndrome and patients with mutations in Mre11 present with ataxia-telangiectasia-like disorder, both of which display similar clinical symptoms to A-T patients, which include radiosensitivity, neurodegeneration, increased cancer predisposition and immune deficiency, further indicating an epistasis between ATM and MRN (Varon et al. 1998; Stewart et al. 1999). Deletion of any of the three MRN components leads to embryonic lethality in mice (Xiao et al. 1997; Luo et al. 1999; Zhu et al. 2001).

Mre11 forms the central element of the complex, as it binds to Rad50, DNA and Nbs1 (Williams et al. 2007). Nbs1 in turn interacts with ATM, and together it forms the MRN-ATM sensor complex (You et al. 2005). Mre11 can mediate DNA end processing due to its 3’-5’ exonuclease, single-stranded DNA endonuclease and DNA unwinding capabilities. Rad50, on the other hand, forms a homodimer and holds the two DNA ends together. The Rad50 protein contains ATP-Binding cassette (ABC) ATPase, Zn hook, and coiled coil domains. Nbs1 contains BRCA1 carboxy-terminal (BRCT) and Forkhead-associated (FHA) domains, and S/TQ sites; and is linked to cell cycle checkpoint regulation (Kobayashi et al. 2004). BRCT and FHA domains are very common to DDR factors. While BRCT is a protein-protein interaction motif, FHA is a phospho-peptide binding motif. Together, the MRN complex is important for 1) sensing DSBs and forming a complex with ATM to initiate the DDR, 2) aiding in DNA repair via DNA end
processing, and 3) providing stability by tethering the DNA DSB ends together and preventing chromosomal detachment (Williams et al. 2007).

Mouse models have been used to investigate the role of MRN in CSR. Conditional Nbs1 knock-out mice, in which Nbs1 is specifically deleted in the B cell compartment, have decreased B cell proliferation and increased genome instability (Reina-San-Martin et al. 2005). Importantly, CSR is reduced by about 50% in Nbs1−/− mice (Reina-San-Martin et al. 2005). Previous work had demonstrated that Nbs1 foci can form at immunoglobulin heavy chain locus in an AID-dependent manner during CSR, which further implicates Nbs1 in CSR (Petersen et al. 2001). Mre11 was also found to co-localize with immunoglobulin heavy chain locus at a much higher frequency in the presence of AID as compared to AID−/− cells, suggesting that like Nbs1, Mre11 forms AID-dependent foci during CSR (Reina-San-Martin et al. 2005). Mouse B cells that were deficient for the whole MRN complex, generated by deletion of Mre11, showed 80% decrease in CSR ex vivo, while Mre11 nuclease-deficient mouse B cells, which maintained normal levels of MRN, showed a 50% decrease in CSR (Dinkelmann et al. 2009). Persistence of chromosomal breaks at the immunoglobulin locus of MRN-deficient cells demonstrated that it likely plays a role in DNA repair during CSR (Dinkelmann et al. 2009).

1.3.4 H2AX

H2AX is one of three H2A core histone variants, and corresponds to 2-25% of the H2A found within mammalian cells (Yin et al. 2009). Deficiency of H2AX leads to radiation sensitivity, genome instability and G2/M checkpoint defect in mammalian cells (Bassing et al. 2002). H2AX−/− mice display radiation sensitivity, genome instability, retarded growth, reduced spermatogenesis, and immune deficiency (Celeste et al. 2002). Upon DNA damage, H2AX is
immediately phosphorylated, typically within minutes, on its C-terminus at Ser139 by PI3K-like kinases, ATM, ATR and DNAPK (Burma et al. 2001; Hammond et al. 2003; Stiff et al. 2004). Accepted as a classical marker of DNA damage, phosphorylated histone variant H2AX (γH2AX) is part of the initial phase of the DDR. In response to DNA damage, amplified ATM kinase activity can lead to γH2AX formation extending up to 2 mega-basepairs surrounding the DNA break site. This forms a docking site for accumulation of downstream DDR factors necessary for cell cycle checkpoint arrest and DNA repair.

The observation that RAG induces γH2AX formation on the T cell receptor α locus and immunoglobulin κ light chain locus suggested that γH2AX may play a role in V(D)J recombination (Chen et al. 2000). However, H2AX−/− mice do not exhibit reduced lymphocyte development, suggesting that H2AX does not have a direct role in the repair of RAG-induced DSBs. Instead, H2AX+/− mice displayed increased chromosomal translocations originating from RAG-induced DSBs (Bassing et al. 2003). Hence, γH2AX may prevent DSBs from progressing to chromosomal translocations, possibly facilitating the tethering of DNA strands together, so as to prevent their detachment and translocation (Franco et al. 2006; Yin et al. 2009). γH2AX foci were also observed in the constant region of switching cells (Petersen et al. 2001). These γH2AX foci co-localized with Nbs1 foci and were dependent on AID. H2AX deficiency leads to approximately 70-80% reduction of CSR, without affecting germline transcription, B cell proliferation, SHM and intra-switch recombination (Reina-San-Martin et al. 2003). Akin to ATM, γH2AX is thought to be important for the long-range DNA end joining aspect of CSR (Reina-San-Martin et al. 2003).
1.3.5 MDC1

MDC1 acts in conjunction with H2AX to promote cell cycle arrest and recruit DNA repair factors to the DNA break site (Stewart et al. 2003). MDC1-deficient cells display defects in intra-S- and G2/M-phase cell cycle arrest upon IR (Stewart et al. 2003). This protein contains two tandem BRCT domains at its C-terminus, and a FHA domain at its N-terminus (Stewart et al. 2003). As well, MDC1 has a cluster of S/TQ sites near its N-terminus, targeted for phosphorylation by PI3K-like kinases including ATM and ATR (Stewart et al. 2003). MDC1 co-localizes with γH2AX within minutes of IR induced DNA damage, and requires γH2AX to form IR-induced foci, suggesting that MDC1 recruitment follows γH2AX formation (Stewart et al. 2003). MDC1 requires its tandem BRCT domain to bind to the C-terminus of γH2AX (Stucki et al. 2005). This γH2AX-MDC1 interaction is required for effective recruitment and foci formation of factors in the DDR network, including 53BP1, BRCA, Nbs1 and phosphorylated ATM, and for achieving radio-resistance in cells, but is dispensable for regulating intra-S phase checkpoint (Stewart et al. 2003; Stucki et al. 2005). MDC1 may not be required for the initial γH2AX signal, but is required for its maintenance, either by blocking de-phosphorylation of γH2AX by phosphatases or by mediating retention of activated ATM at the break site (Stucki et al. 2005). MDC1 likely facilitates the accumulation of activated ATM at the break, by physically interacting with Nbs1, which is a component of the MRN complex that associates with ATM (Lukas et al. 2004; Chapman et al. 2008). Through phosphorylation-dependent MDC1-Nbs1 interactions, recruitment of MDC1 to the break site leads to further deposition and retention of MRN-ATM complex surrounding the break, which in turn can amplify the ATM-dependent γH2AX signal at the break (Lou et al. 2006). This MDC1-dependent accumulation of active ATM is also important for the ATM-dependent phosphorylation of downstream factors like
Chk1 and Chk2 (Lou et al. 2006). MDC1<sup>−/−</sup> mice, like ATM<sup>−/−</sup> and H2AX<sup>−/−</sup> mice, show radiation sensitivity, growth retardation, male infertility, genome instability and immune deficiency (Lou et al. 2006). Interestingly, MDC1<sup>−/−</sup> mice only display a 25-50% decrease in the ability to undergo CSR (Lou et al. 2006).

1.3.6 RNF8

Upon IR, phosphorylation of MDC1 in an ATM-dependent manner leads to the recruitment of RNF8 (Kolas et al. 2007). In mice, RNF8 deficiency causes defective spermatogenesis, growth retardation, radiosensitivity, genome instability, cancer predisposition and immune deficiency (Li et al. 2010; Santos et al. 2010). RNF8 is an E3 ubiquitin ligase containing a C-terminal RING finger domain and an N-terminal FHA domain (Huen et al. 2007; Mailand et al. 2007). Upon IR, RNF8 co-localizes with γH2AX (Huen et al. 2007; Mailand et al. 2007). This co-localization depends on H2AX, Ser139 residue on H2AX, and MDC1; and requires the RNF8 FHA domain to bind to phosphorylated MDC1 (Huen et al. 2007; Kolas et al. 2007; Mailand et al. 2007). The recruitment of DDR factors downstream of RNF8 requires the RNF8 RING domain to mediate ubiquitination at the DNA break (Huen et al. 2007; Kolas et al. 2007; Mailand et al. 2007). RNF8 acts with the E2 ubiquitin conjugating enzyme, Ubc13, to mediate mono-ubiquitination of histones H2A and H2AX (Huen et al. 2007; Kolas et al. 2007; Mailand et al. 2007). This ubiquitination is a prerequisite for the recruitment of downstream factors, BRCA1 and 53BP1 (Huen et al. 2007; Kolas et al. 2007; Mailand et al. 2007). RNF8<sup>−/−</sup> cells displayed partial 53BP1 IR-induced foci formation, suggesting the possibility of an RNF8-independent mechanism of 53BP1 recruitment (Li et al. 2010). RNF8 deficiency leads to increased IR sensitivity and disruption of G2/M cell cycle checkpoint (Huen et al. 2007; Kolas et
As part of my PhD thesis, I collaborated with Richard Chahwan to demonstrate that RNF8 also plays a role in CSR (Chapter 2 below). Subsequent studies on RNF8\(^{-/-}\) mice showed a 50-70% decrease in CSR (Li et al. 2010; Santos et al. 2010).

1.3.7 RNF168

Following RNF8 recruitment and activity, another E3 ubiquitin ligase, RNF168, binds to the DNA break (Doil et al. 2009). RNF8-mediated ubiquitination of histones H2A at the DNA break site can be bound by the RNF168 Motif Interacting with Ubiquitin (MIU) domain (Stewart et al. 2009). RNF168 contains two MIU domains, an N-terminal and C-terminal, in which the C-terminal MIU is predominantly responsible for binding to ubiquitin at the DNA break (Stewart et al. 2009). RNF168 also contains a RING domain that acts with the E2-conjugated enzyme, Ubc13, in protecting and amplifying the RNF8-mediated ubiquitination, leading to the formation of K63-linked poly-ubiquitin chains at the DNA break site (Doil et al. 2009; Panier et al. 2009; Stewart et al. 2009). Further studies showed that RNF168 mediates mono-ubiquitination of histone H2A on residues K13-15 (Mattiroli et al. 2012). RNF168 is crucial for instigating both arms of DDR, leading to the recruitment of Brca1 on one side and 53BP1 on the other (Doil et al. 2009; Stewart et al. 2009). The Brca1 complex, made up of Bard1, Abraxas and Rap80, binds to the RNF8/168-mediated ubiquitination at the DNA break through the Rap80 ubiquitin-binding motif (Doil et al. 2009). The Brca1 pathway leads to DNA end resection, promoting HR, and is beyond the scope of this thesis and so will not be further discussed. RNF168 is also crucial for the recruitment of the highly characterized DDR factor, 53BP1, which blocks DNA resection, promoting NHEJ, and will be discussed further below.
Along with RNF8, Richard Chahwan and I also published that RNF168 plays a role in CSR (Chapter 2 below). Subsequent studies on RNF168\(^{-/-}\) mice corroborated our work, demonstrating that RNF168\(^{-/-}\) mice have 40-50% reduction in CSR (Bohgaki \textit{et al.} 2011). These mice, akin to all DDR factors, are radiosensitive, have impaired spermatogenesis and are immunodeficient due to deficiencies in both CSR and V(D)J recombination (Bohgaki \textit{et al.} 2011). Interestingly, RNF168 deficiency is linked to RIDDLE syndrome that has been identified in one patient who displays with radiosensitivity, immunodeficiency, dysmorphic features and learning difficulties, similar to the clinical features of A-T, ATLD and NBS patients mentioned above (Stewart \textit{et al.} 2007; Stewart 2009; Stewart \textit{et al.} 2009; Bohgaki \textit{et al.} 2011).

1.3.8 53BP1

53BP1 was initially identified as a p53-interacting factor in yeast 2-hybrid studies (Iwabuchi \textit{et al.} 1994). In comparison to p53\(^{-/-}\) mice, p53\(^{-/-}\) 53BP1\(^{-/-}\) double-deficient mice develop increased incidence of tumors, particularly T cell lymphomas and to a lesser extent, B cell lymphomas, sarcomas and teratomas (Morales \textit{et al.} 2006). While p53\(^{-/-}\) mice present with aneuploidy, the double-deficient mice present with both aneuploidy and chromosomal translocations, suggesting that 53BP1 acts synergistically with p53 to maintain genomic stability, possibly through its functions in cell cycle checkpoint regulation and DNA repair (Morales \textit{et al.} 2006).

53BP1 shares homology with the prototypical cell cycle checkpoint factors Rad9 in budding yeast and Crb2 in fission yeast (Schultz \textit{et al.} 2000). Human cells deficient in 53BP1 are sensitive to IR, and have deregulated G2/M and intra-S phase checkpoints (Wang \textit{et al.} 2002). Upon IR, 53BP1 is itself phosphorylated in an ATM-dependent manner (Anderson \textit{et al.} 2001;
Rappold et al. 2001), and 53BP1 contributes to optimal ATM-mediated phosphorylation of Checkpoint kinase 2 (Chk2), leading to cell cycle arrest and G2/M checkpoint control (Fernandez-Capetillo et al. 2002; Wang et al. 2002; Peng et al. 2003; Wilson et al. 2008).

53BP1 protein contains a glycine-arginine stretch, a tandem BRCT domain that to date has not been identified with a specific function, a tandem tudor domain, and several phosphorylation sites. 53BP1 forms foci that co-localize with γH2AX, Mre11 and Nbs1 upon DNA damage (Schultz et al. 2000). 53BP1 relocates to DSBs upon DNA damage, as its tudor domain tethers to the DNA break site by binding to dimethylated-H4K20 residues that surround the DSB (Botuyan et al. 2006). Dimethylated-H4K20 is a constitutively present histone mark, initially causing a puzzle as to how 53BP1 recruitment to a constitutive histone mark was restricted to DNA damage. Initially, it was believed that dimethylated-H4K20 may be buried in the absence of DNA damage, but DSBs cause chromatin relaxation/modification exposing this constitutive histone mark for 53BP1 access. Additionally, the methyltransferase MMSET was found to increase H2K20 methylation specifically at DSBs, providing additional substrates for 53BP1 binding (Pei et al.). However, further investigations suggested alternative, RNF8/168-dependent mechanisms of exposing this histone mark for 53BP1 binding. One study suggested that KDM4A, also called JMJD2A, constitutively binds to dimethylated-H4K20, preventing 53BP1 binding (Mallette et al.). However, DSB-induced RNF8/168 activity leads to the degradation of KDM4A/JMJD2A at the break site, exposing dimethylated-H4K20 for 53BP1 recruitment (Mallette et al.). Another study suggested that the VCP/p97-UFD1-NPL4 complex is recruited to the DNA break in a RNF8-dependent fashion, and is important for the recruitment of 53BP1 and BRCA1 (Meerang et al.). Acs et al. also showed that VCP/p97 is recruited to DSBs in an RNF8/168-dependent fashion, but they suggest that it is important for the ubiquitination and
removal of a Polycomb protein L3MBTL1 from dimethylated-H4K20, thus exposing this histone mark for 53BP1 (Acs et al. 2011). However, the ground-breaking finding that 53BP1 contains an ubiquitination-dependent recruitment motif, located adjacent to its tandem tudor domain, that binds to RNF168-mediated H2A-K15 mono-ubiquitin, finally solved the puzzle of DNA damage-induced 53BP1 recruitment (Fradet-Turcotte et al. 2013). 53BP1 accumulation upon DNA damage is dependent on RNF168 function, as 53BP1 localizes to the DNA break site by binding to dimethylated-H4K20 via its tandem tudor domain and H2A-K15-mono-ubiquitin via its ubiquitination-dependent recruitment motif (Fradet-Turcotte et al. 2013). However, low levels of 53BP1 can transiently bind at the DNA break in a manner independent of MDC1, RNF8 and RNF168, and this is likely reflective of low levels of 53BP1 binding to dimethylated-H4K20me2 independent of H2A-K15-mono-ubiquitin (Fradet-Turcotte et al. 2013).

Most interestingly, CSR is almost completely ablated in 53BP1-deficient mice (Manis et al. 2004; Ward et al. 2004). The 53BP1 tudor domain, oligomerization domain and N-terminal ATM phosphorylation motifs are all important for CSR (Bothmer et al. 2011). These mice have no abnormality in AID expression, germline switch region transcription, or SHM, suggesting that while the function of AID in CSR is not affected, 53BP1 may be involved in the ligation of DNA ends (Manis et al. 2004; Ward et al. 2004). More specifically, 53BP1 is thought to facilitate the switch-synapse formation by enhancing long-range DNA end joining and by protecting the DNA ends from DNA resection (Details provided below). However, the precise mechanism of 53BP1 function and the downstream events following 53BP1 recruitment, at the molecular level, in CSR remains unknown. Intriguingly, of the DDR factors characterized to date, 53BP1 deficiency leads to the most severe CSR defect.
1.3.8.1 **53BP1 in long-range end joining:**

53BP1 has been suggested to mediate long-range end joining in CSR. In support of this notion, 53BP1-deficient B cells show increased levels of intra-switch recombination (Reina-San-Martin *et al.* 2007). Furthermore, 53BP1 facilitates long-range NHEJ during V(D)J recombination at the TCR locus (Difilippantonio *et al.* 2008), and 53BP1 promotes NHEJ of dysfunctional telomeres by increasing chromatin mobility (Dimitrova *et al.* 2008).

During the DDR to DSBs in heterochromatin, 53BP1 recruitment forms part of an amplification loop involving Mre11 and Nbs1, and this leads to the concentration of active ATM at the DNA break (Noon *et al.* 2010). This amplification of active ATM, in addition to the first MDC1-Nbs1 dependent amplification described above, causes high accumulation of active ATM leading to ATM-mediated phosphorylation of Kap1 on residue S824 at the break site, which can be observed as phosphorylated Kap1 foci (Noon *et al.* 2010). Kap1 phosphorylation is particularly important for heterochromatic DNA repair, as phosphorylation of Kap1 leads to release of chromatin from a repressed state, possibly allowing for increased chromatin mobility (Ziv *et al.* 2006). Although the 53BP1-dependent, ATM-mediated Kap1 foci are crucial to heterochromatic DNA repair, these findings may be extended to CSR in which relaxed chromatin may promote increased DNA end mobility facilitating long-range end joining, although this notion has yet to be investigated (Noon *et al.* 2010).

1.3.8.2 **53BP1 and DNA end resection:**

Emerging evidence indicates that 53BP1 can block DNA ends from resection, thus promoting NHEJ-mediated DNA repair over the homology-based DNA repair mechanisms, including HR and MMEJ (Bothmer *et al.* 2010; Bunting *et al.* 2010). 53BP1-null cells displayed
increased resection of DNA ends leading to MMEJ repair (Bothmer et al. 2010). This DNA resection, which is antagonized by the presence of 53BP1 at the DNA ends, requires ATM activation (Bothmer et al. 2010). The DNA resection and processing are mediated by CtIP, Exo1 and RecQ DNA helicase (Bothmer et al. 2013). The 53BP1 chromatin-binding tudor domain, oligomerization domain and the cluster of ATM phosphorylation sites in the N-terminus are all required for the ability of 53BP1 to protect DNA ends from processing (Bothmer et al. 2011). In agreement with these findings, 53BP1-mediated blockade of DNA end resection inhibited homologous recombination in BRCA1-null cells, leading to error-prone NHEJ-mediated repair in S phase cells, in turn causing genome instability (Bunting et al. 2010). Deleting 53BP1 rescued genomic instability and the translocation phenotype of BRCA1-deficient cells, by alleviating the 53BP1-mediated block on homologous recombination (Bunting et al. 2010). 53BP1 possibly inhibits DNA resection by binding to and blocking DNA ends from access to DNA end processing enzymes. While 53BP1-mediated DNA end protection is advantageous in G1 cells, promoting NHEJ and CSR, it is deleterious in cells that are in S phase, since it would block HR and promote mutagenic repair by NHEJ (Bunting et al. 2010). Parallel studies in the context of V(D)J recombination showed that γH2AX and MDC1, both of which act upstream of 53BP1, inhibited CtIP-mediated DNA resection of RAG-induced DSBs (Helmink et al. 2011). Intriguingly, γH2AX formation and CtIP activation are both dependent on ATM activity (Bothmer et al. 2010; Bothmer et al. 2013). Hence ATM initiates both the pathway that suppresses DNA resection and the pathway that promotes DNA resection (Bothmer et al. 2010). CtIP and DNA resection are largely restricted to S/G2/M cell cycles, where HR dominates, and are blocked in G1 cell cycle to promote NHEJ (Escribano-Diaz et al. 2013). In cells at G1, CtIP
activation would promote error-prone MMEJ, causing chromosomal deletions and genome instability (Helmink et al. 2011).

1.3.8.3 53BP1 recruits Rif1 and PTIP

While the ATM phosphorylation motifs clustered towards the N-terminus of 53BP1 are important for end joining and CSR, they are dispensable for 53BP1 foci formation, suggesting that ATM phosphorylation of 53BP1 is not required for its recruitment, but rather for possibly recruiting downstream factors that promote NHEJ and inhibit HR (Escribano-Diaz et al. 2013). Very recent work has revealed that a key downstream factor is Rif1, which acts in concert with 53BP1 to protect DNA ends from resection (Chapman et al. 2013; Di Virgilio et al. 2013; Escribano-Diaz et al. 2013; Feng et al. 2013; Zimmermann et al. 2013). Rif1 depletion in BRCA1-deficient cells phenocopies 53BP1 depletion in BRCA1-deficient cells, confirming 53BP1-Rif1 epistasis (Chapman et al. 2013; Escribano-Diaz et al. 2013; Feng et al. 2013).

ATM phosphorylation of 53BP1 is absolutely required for the 53BP1-Rif1 interaction, leading to the recruitment of Rif1 to DNA breaks (Silverman et al. 2004; Di Virgilio et al. 2013; Escribano-Diaz et al. 2013; Feng et al. 2013). Rif1 functions to inhibit DNA end processing by CtIP, Exo1 and BLM helicase (Zimmermann et al. 2013). Rif1 and BRCA1 control DNA repair pathway choice as the cell progresses through the cell cycle (Chapman et al. 2013; Escribano-Diaz et al. 2013; Feng et al. 2013). First, 53BP1-Rif-dependent inhibition of DNA resection in G1 antagonizes the BRCA1-CtIP dependent HR pathway and MMEJ, while promoting NHEJ (Chapman et al. 2013; Escribano-Diaz et al. 2013; Feng et al. 2013). Second, the BRCA1-CtIP pathway antagonizes Rif1 in S/G2, promoting DNA end resection and HR, whilst blocking NHEJ (Chapman et al. 2013; Escribano-Diaz et al. 2013; Feng et al. 2013). Importantly, Rif1 depletion
leads to 80-90% reduction in CSR (Chapman et al. 2013; Di Virgilio et al. 2013; Escribano-Diaz et al. 2013). Hence, Rif1 is an important CSR factor acting downstream of 53BP1.

Further investigation revealed that a 53BP1 mutant, with alanine substitution mutations in the first 8 N-terminal phosphorylation sites, maintained Rif1 recruitment and CSR (Callen et al. 2013). However, this mutant was unable to rescue abnormal repair and genome instability in BRCA1-deficient cells, and this is attributed to the inability of the 53BP1 mutant to bind to PTIP (Callen et al. 2013). 53BP1 facilitates deleterious mutagenic repair in BRCA1-deficient cells by promoting NHEJ and blocking HR in S phase cells (Bunting et al. 2010). While both the functions of blocking DNA end resection and HR require 53BP1 N-terminal phosphorylation by ATM, blocking DNA resection relies on 53BP1 interacting with Rif1, while blocking HR and promoting mutagenic NHEJ during S phase rely on 53BP1 recruitment of PTIP (Callen et al. 2013). While CSR requires that 53BP1 interacts with Rif1, the interaction of 53BP1 with PTIP appears to be dispensable for CSR (Callen et al. 2013).

1.3.9 DDR factors within the ATM network not investigated in the context of CSR

1.3.9.1 Deubiquitination in DDR

Multiple deubiquitylases (DUB) have been implicated in suppressing the DDR, including USP3, USP16, BRCC36, POH1, Usp44 and OTUB1 (Jackson et al. 2013). These enzymes antagonize RNF8-RNF168 ubiquitylation (Jackson et al. 2013). USP3 is involved in H2A/H2B-deubiquitination, and has been linked to DDR (Nicassio et al. 2007). USP16 removes RNF8 and RNF168-mediated ubiquitylation (Shanbhag et al. 2010). BRCC36 and the proteasome-associated POH1 have a propensity towards removing K63-linked ubiquitin (Jackson et al. 2013). BRCC36 is part of the Rap80-BRCA1 complex that gets recruited to DSBs downstream
of RNF168. Usp44 was recently demonstrated to oppose the RNF8-RNF168-mediated H2A ubiquitylation (Mosbech et al. 2013). Intriguingly, OTUB1 opposes RNF168 ubiquitylation independent of its isopeptidase enzymatic activity, which displays specificity towards K48-linked chains (Nakada et al. 2010). Instead, OTUB1 binds to and inhibits the RNF168 E2 ubiquitin conjugating enzyme, Ubc13 (Nakada et al. 2010; Blackford et al. 2011). OTUB1 simultaneously binds Ubc13 conjugated to ubiquitin and a free ubiquitin molecule in a manner that places the two ubiquitins in a configuration similar to K48-linked ubiquitin, the inherent product of OTUB1 enzyme function (Juang et al. 2012; Wiener et al. 2012). The authors suggest that by co-opting the recognition of K48-linked chains, OTUB1 inhibits Ubc13 from facilitating RNF168 K63-linked ubiquitylation (Juang et al. 2012).

1.3.9.2 H2B Ubiquitylation in DDR

In addition to H2A ubiquitylation, H2B ubiquitylation also plays a role in DDR (Moyal et al. 2011). The RNF20-RNF40 complex mediates histone H2B mono-ubiquitylation at K120 in humans and K123 in yeast, and has been linked to transcription (Kao et al. 2004; Zhu et al. 2005; Jung et al. 2012). DNA damage leads to the recruitment of a RNF20-RNF40 heterodimer to DSBs, where it locally mono-ubiquitylates histone H2B (Moyal et al. 2011). While ATM is dispensable for the recruitment of RNF20-RNF40, ATM-mediated phosphorylation of RNF20 is required for its retention at DSBs (Moyal et al. 2011). However, this H2B mono-ubiquitylation represents an arm of the ATM network that is independent of the MDC1-RNF8 pathway (Moyal et al. 2011). H2B mono-ubiquitylation at DSBs is thought to facilitate chromatin decondensing, allowing for the recruitment of DNA repair factors (Fierz et al. 2011).
Indeed, the RNF20-RNF40 complex was shown to be required for efficient repair by both the NHEJ and HR repair pathways (Moyal et al. 2011). H2B-ubiquitination is prerequisite to histone H3 methylation at K4 and K79 (Ng et al. 2002; Sun et al. 2002). RNF20 and H2B mono-ubiquitination enables H3K4 methylation and recruitment of chromatin remodeling factor SNF2h to DSBs (Nakamura et al. 2011). RNF20 and SNF2h recruitment to DSBs is critical for CtIP-dependent DNA end resection, and BRCA1 and Rad51 loading for HR (Nakamura et al. 2011). RNF20 acts in HR, in part to promote chromatin remodelling, as the DNA intercalater chloroquine, which induces chromatin relaxation by modifying chromosome structure, can partially bypass the requirement for RNF20 during HR (Nakamura et al. 2011). Similarly, RNF40 is important for cell cycle checkpoint activation, H3K56 acetylation and recruitment of the Facilitates Chromatin Transcription (FACT) complex to chromatin upon DNA damage (Kari et al. 2011). RNF40 and the FACT complex component, SUPT16H, are required for efficient DNA resection and DNA repair, likely by promoting chromatin remodelling (Kari et al. 2011).

1.3.9.3 Sumoylation in DDR

Recent work has identified sumoylation as another post-translation modification relevant to DDR. The PIAS4 Small Ubiquitin-like Modifier (Sumo) E3 ligase was shown to be an important component of DDR, required for RNF168, K63-linked ubiquitin chain, 53BP1 and BRCA1 foci formation, while the PIAS1 Sumo E3 ligase was specifically required for BRCA1-Rap80 recruitment (Galanty et al. 2009). PIAS1 and PIAS4 are themselves recruited to DNA breaks and are responsible for Sumo1/2/3 accumulation at the breaks (Galanty et al. 2009). PIAS1 targets BRCA1 for sumoylation, while PIAS4 can target RNF168, BRCA1 and 53BP1 for sumoylation (Galanty et al. 2009). Both Sumo E3 ligases are important for HR and NHEJ and depleting both
ligases confers the cells with radiation sensitivity (Galanty et al. 2009). Sumoylation is another post-translational modification, in addition to phosphorylation and ubiquitination, utilized by the DDR pathway.

1.3.9.4 RNF4 function

The discovery of RNF4 extended the complexities of the inter-crossing ubiquitination and sumoylation networks in DDR. RNF4 is an E3 ubiquitin ligase and contains both a RING domain and a SUMO-interacting motif (SIM). RNF4 accumulates at DSBs by virtue of its SUMO-interaction motif that binds to the sumoylation added by PIAS1 and PIAS4 activity (Galanty et al. 2012). Upon recruitment to DSBs, RNF4 functions in the protein turnover of MDC1 and RPA (Galanty et al. 2012; Luo et al. 2012; Vyas et al. 2012). By ubiquitinating these factors, RNF4 targets MDC1 and RPA for proteasomal degradation (Galanty et al. 2012; Luo et al. 2012; Vyas et al. 2012). MDC1 turnover may facilitate DNA repair and timely recovery from cell cycle arrest, possibly by providing access to allow for the recruitment of downstream DDR factors, including Rap80-BRCA1 (Galanty et al. 2012; Guzzo et al. 2012; Luo et al. 2012). RPA turnover is necessary for its replacement by BRCA2 and Rad51 on resected DNA in order to complete homologous recombination (Galanty et al. 2012; Luo et al. 2012; Vyas et al. 2012). However, an independent study suggested that RNF4 was required for RPA recruitment rather than turnover (Yin et al. 2012). This was attributed to reduced recruitment of CtIP and DNA resection, and hence fewer single-stranded DNA for RPA loading, in RNF4-depleted cells (Yin et al. 2012). RNF4 was also suggested to regulate BRCA2 protein turnover (Vyas et al. 2012). RNF4 depletion results in the persistence of γH2AX, RNF8, RNF168 and 53BP1 at DSBs and inefficient DNA repair (Galanty et al. 2012; Luo et al. 2012; Yin et al. 2012). RNF4 functions in
both NHEJ and HR (Galanty et al. 2012; Luo et al. 2012; Vyas et al. 2012; Yin et al. 2012). RNF4-deficient cells display increased sensitivity to DNA damage, and RNF-4 deficient mice display increased sensitivity to DNA damage and defective spermatogenesis (Vyas et al. 2012).

1.3.10 DDR leads to cell cycle arrest

Cells can either be at a resting state, referred to as G₀, or undergo cycling through the G₁, S, G₂, and M phases of cell cycle. S phase, during which DNA replication occurs, and M phase, during which mitosis occurs, are separated by the G₁ and G₂ phases of cell cycle. Cell cycle progression is controlled by cyclin and cyclin-dependent protein kinases. G₁/S transition, intra-S-phase and G₂/M transition checkpoints are in place to ensure that the cell cycle progression is regulated. DNA damage-induced DDR will activate cell cycle checkpoints and cause an arrest in cell cycle until the damage is repaired, preventing the cell from progressing to the next phase without repairing the damage (Langerak et al. 2011). This is accomplished by the phosphorylation and activation of the effector kinases, Chk1 and Chk2, by components of DDR, including ATM and ATR (Latif et al. 2004; Kiyokawa et al. 2008; Reinhardt et al. 2009). While replication-induced DNA damage activates the ATR pathway, leading to Chk1 activation, IR-induced DNA damage activates the ATM pathway, leading to Chk2 activation (Matsuoka et al. 1998; Chaturvedi et al. 1999; Heffernan et al. 2002). Both effector kinases lead to the phosphorylation and inactivation of Cdc25 phosphatase family members that regulate cyclin/cyclin-dependent kinases, including Cdc2/Cdk1, involved in cell cycle checkpoint regulation, thus causing cell cycle arrest allowing for DNA repair (Sancar et al. 2004; Kiyokawa et al. 2008; Reinhardt et al. 2009).
1.4 DNA REPAIR

1.4.1 Non-homologous end joining (NHEJ)

Antibody diversification primarily relies on NHEJ for DNA repair (Soulas-Sprauel et al. 2007). Both V(D)J recombination and CSR mechanisms utilize NHEJ (Soulas-Sprauel et al. 2007). NHEJ is active during the entire cell cycle, but is primarily used during the G₀ and G₁-phases of cell cycle (Lieber 2010). Since AID is expressed and prompts DSBs in the G₁-phase of the cell cycle (Schrader et al. 2007), these breaks are engaged by NHEJ to complete CSR.

NHEJ proceeds through a blunt-end ligation type of mechanism, and thus does not rely on regions of homology to dictate repair (Lieber 2010). Hence, NHEJ joints are characterized by very little or no homology (Stavnezer et al. 2010). NHEJ suppresses DNA resection and chromosomal translocations, but can lead to error-prone DNA repair, due to short nucleotide insertions or deletions at the joint (Boboila et al. 2012). As illustrated in Figure 7, the break is recognized by the Ku70/Ku80 complex, which then recruits the catalytic domain, DNAPKcs, to form the DNAPK holoenzyme (Gottlieb et al. 1993; Smider et al. 1998). This is followed by the recruitment of XRCC4 and DNA Ligase IV to mediate blunt end joining (Grawunder et al. 1997; Chen et al. 2000). Artemis and XLF/Cernunnos have also been implicated in NHEJ (Ahnesorg et al. 2006; Buck et al. 2006). However, Ku70, Ku80, XRCC4 and Ligase IV are evolutionarily conserved and make up the core components of NHEJ, while Artemis and DNAPKcs are auxiliary components found in vertebrates (Boboila et al. 2012).

The first phase of NHEJ involves sensing of the break and formation of the break synapsis. Ku70/Ku80 heterodimer recognizes and binds to the break, protecting the ends from resection, although Ku displays lyase activity towards terminal abasic sites at DSBs (Roberts et al. 2010). The Ku heterodimer then recruits DNAPKcs to form DNAPK. DNAPKcs can autophosphorylate
**Figure 7. Non-homologous end joining.** The DSB recruits Ku70/Ku80, which is followed by the recruitment of DNAPKcs. Together, this forms the DNAPK holoenzyme. Subsequent recruitment of the XRCC4-DNA Ligase IV completes repair. XRCC4-DNA Ligase IV function is enhanced by XLF/Cernunnos.
itself and/or phosphorylate other proteins involved in NHEJ (Meek et al. 2008). Indeed, DNAPKcs and ATM have incomplete redundancy, as deficiency of both kinases leads to a more severe effect on end joining compared to single deficiency (Zha et al. 2011). Further, the DNAPKcs autophosphorylation status can affect the protein’s affinity for DNA, and thus regulate its dynamics at the break site (Uematsu et al. 2007). Autophosphorylation leads to release of DNAPKcs from the break, providing the downstream NHEJ factors access to the break (Cui et al. 2005).

The second phase of NHEJ is the ligation of the break, which requires the XRCC4/Ligase IV complex, and the ancillary factor XLF. XRCC4/Ligase IV are recruited to the break, where XRCC4 provides a structural role in stabilizing Ligase IV, which catalyzes the ligation of the break. XLF can directly bind to and enhance XRCC4/Ligase IV-mediated blunt-end ligation (Ahnesorg 2006). Mutations in XLF can cause radiosensitivity and defective V(D)J recombination in humans (Buck et al. 2006).

Artemis and DNAPKcs may be relevant to NHEJ of breaks that require processing before repair, especially if the two ends are incompatible. Artemis endonuclease is activated by DNAPKcs-mediated phosphorylation (Ma et al. 2002). This endonuclease plays a key role in V(D)J recombination, owing to its ability to cleave the coding joint hairpin, a requirement for V(D)J recombination (Ma et al. 2002). Artemis nuclease activity may play a role in DNA end resection in CSR (Franco et al. 2008). Incompatible ends may also require gap-filling by DNA polymerases Polλ and Polμ (Ogiwara et al. 2011). Such nucleotide processing at the DNA breaks can lead to error-prone DNA repair.

_In vivo_ mouse models have been generated to test the role of each factor in CSR. Mice with NHEJ core component deficiencies cannot complete B cell development, due to impaired V(D)J
recombination. The B cell development defect can be bypassed by engineering transgenic mice with prearranged heavy and light chains (Ramiro et al. 2007). Using this technology, Ku70/Ku80-deficient mice were shown to exhibit reduced CSR (Casellas et al. 1998; Manis et al. 1998). This defect was shown to be independent of proliferative defects and was shown to be B cell intrinsic (Casellas et al. 1998; Manis et al. 1998). Ku-deficient cells were able to generate DSBs, but were unable to complete the repair, showing that Ku heterodimer is involved in the DNA repair phase of CSR (Casellas et al. 1998; Manis et al. 1998). DNAPKcs, on the other hand, showed inconsistent results. Using the same technology described above for Ku-deficient mice, DNAPKcs-null mice with rescued B cell development exhibited reduced CSR to all isotypes except IgG1 (Manis et al. 2002). However, severe combined immunodeficient (SCID) mice transgenic for immunoglobulin heavy and light chains, but with a truncated, enzymatically inactive DNAPKcs, maintained similar levels of CSR as control mice, suggesting that DNAPKcs kinase activity may be dispensable to CSR (Bosma et al. 2002). The discrepancy between these two studies could be due to the possibility that the SCID mice expressed a truncated or enzymatically dead protein that plays a structural role in CSR (Ramiro et al. 2007). Furthermore, DNAPKcs-deficient cells displayed impaired repair of a subset of AID-induced breaks at the immunoglobulin heavy chain locus by FISH experiments, implicating DNAPKcs in CSR (Franco et al. 2008). Artemis was considered dispensable for CSR, as Artemis-deficient mice exhibited normal CSR, but fluorescent in situ hybridization assays also demonstrated that a subset of AID-induced breaks relied on Artemis for repair (Franco et al. 2008). XRCC4 and Ligase IV deletion in mice causes embryonic lethality due to neuronal apoptosis. However, embryonic lethality can be rescued by deletion of p53 (Yan et al. 2007). Combining the p53-null mutation with either a deletion of XRCC4 or Ligase IV also exhibited defective but not a
complete abrogation of CSR (Yan et al. 2007). A mouse model with a B-cell specific conditional deletion of XRCC4 has also shown that in the absence of XRCC4 and NHEJ, there is a two-fold reduction in CSR (Soulas-Spraul et al. 2007).

1.4.2 Microhomology-mediated end joining (MMEJ)

MMEJ is an alternate pathway to NHEJ that has emerged recently. Mouse B cells deficient in the core NHEJ factors, Ku, XRCC4, and Ligase IV, displayed ~50% reduced CSR compared to controls (Soula-Spraul et al. 2007; Yan et al. 2007; Han et al. 2008; Boboila et al. 2010; Boboila et al. 2010). Switch joints from these mice demonstrated short regions of homology, suggestive of an alternative end joining mechanism to NHEJ. Indeed, patients with Ligase IV deficiencies also showed marked increase in the use of microhomology at switch junctions (Pan-Hammarstrom et al. 2005). This pathway, which is secondary to NHEJ and likely acts in the absence of NHEJ, is referred to as MMEJ. MMEJ mediates ligation of the two DNA ends but relies on sequence microhomology consisting of only a few nucleotides. MMEJ is more error-prone than NHEJ, more often causing deletions and chromosomal translocations, leading to genome instability (Yan et al. 2007; Boboila et al. 2010). However, the enzymes and factors involved, as well as the mechanism of the MMEJ pathway, largely remain elusive. Msh2, Mlh1, Exo1, Parp1, XRCC1, DNA Ligases I and III, CtIP and Mre11, have all been directly implicated in MMEJ (Zhong et al. 2002; Liang et al. 2008; Robert et al. 2009; Rahal et al. 2010; Eccleston et al. 2011; Saribasak et al. 2011; Zhang et al. 2011; Jia et al. 2013). However, XRCC1 and DNA Ligase III were also shown to be dispensable for MMEJ in CSR (Boboila et al. 2012). CtIP likely mediates DNA end resection, providing regions of microhomology required for MMEJ (Zhang et al. 2011). Recent findings suggest that there may be more than one alternative end-
joining pathway to NHEJ (Boboila et al. 2010). Importantly, MMEJ is an alternate pathway distinct from the core NHEJ factors, Ku70, Ku80, XRCC4 and Ligase IV (Yan et al. 2007; Boboila et al. 2010).

1.4.3 Homologous recombination (HR)

HR relies on the genetic information present on the undamaged duplicate sister chromatid or homologous chromosome to repair DNA damage, and so it is active during the S and G2 phases of cell cycle, after DNA replication has occurred. HR requires large regions of homology, typically greater than 100 bp, to direct the repair (Dudas et al. 2004). Similar to MMEJ, HR begins with DNA end resection at the 5’ ends, forming two 3’ single-stranded DNA ends (Truong et al. 2013). One 3’ end invades the homologous chromosome producing a D loop structure, where the complimentary strand of the intact chromosome is displaced and gets bound by the other 3’ single-stranded end (Dudas et al. 2004). DNA synthesis begins at the two 3’ ends using the undamaged chromosomal strands as templates (Dudas et al. 2004). Subsequent ligation forms Holliday junctions that can be resolved to produce a crossover or non-crossover end product (Dudas et al. 2004). Rad51, Rad52, Rad55, Rad57, Brca1, Brca2, XRCC2, RPA and MRN participate in HR, but the molecular functions have not been completely defined (Sancar et al. 2004).

HR is critical for gene conversion, and was largely considered dispensable for CSR. However, recent evidence suggests that AID-induced DSBs, and in particular off-target DSBs, are engaged by HR (Hasham et al. 2010; Hasham et al. 2012; Lamont et al. 2013). The XRCC2-dependent HR pathway plays a key role in protecting B cells from AID-induced off-target effects, thus maintaining genome stability (Hasham et al. 2010). Although AID induces the off-
target DSBs in G1-phase of cell cycle, these breaks can bypass the G1/S cell cycle checkpoint, progressing into S phase, where they are repaired by HR (Hasham et al. 2012). HR not only protects the B cells from potential AID-induced genome instability, but can also successfully complete CSR in S-phase (Hasham et al. 2012).

1.5 OBJECTIVES

It is well established that the 53BP1-dependent DDR pathway is required to mediate CSR. However, the exact molecular function of this pathway, and the factors acting downstream 53BP1 leading up to DNA repair in CSR, are not fully understood. My objective during my thesis work was to better understand how the DDR pathway functions at the molecular level in CSR. To this end, I first established an in vitro system to study the role of DDR factors in CSR. Using this system, I then screened for novel CSR factors, and from these CSR candidates, identified potential DDR factors for further characterization. By characterizing the molecular functions of novel DDR factors in CSR, we can better understand how the DDR pathway facilitates CSR.
2 THE RNF8/RNF168 UBIQUITIN LIGASE CASCADE FACILITATES CLASS SWITCH RECOMBINATION.

Published Work:

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S. Ramachandran, R.C., R.N., D.F., S.P., S. Roa, and A.Z. performed research;

S.P. and D.D. contributed new reagents/analytic tools;

S. Ramachandran, R.C., S. Roa, D.D., M.D.S., and A.M. analyzed data;

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2.1 ABSTRACT

An effective immune response requires B cells to produce several classes of antibodies through the process of class switch recombination (CSR). Activation-induced cytidine deaminase (AID) initiates CSR by deaminating cytidines at switch regions within the immunoglobulin locus. This activity leads to double-stranded DNA break formation at the donor and recipient switch regions that are subsequently synapsed and ligated in a 53BP1-dependent process that remains poorly understood. The DNA Damage Response E3 ubiquitin ligases, RNF8 and RNF168, were recently shown to facilitate recruitment of 53BP1 to sites of DNA damage. Here, we show that the ubiquitination pathway mediated by RNF8 and RNF168 plays an integral part in CSR. Using the CH12F3-2 mouse B cell line that undergoes CSR to IgA at high rates, we demonstrate that knockdown of RNF8, RNF168, and 53BP1 leads to a significant decrease in CSR. We also show that 53BP1-deficient CH12F3-2 cells are protected from apoptosis mediated by the MDM2 inhibitor, Nutlin-3. In contrast, deficiency in either E3 ubiquitin ligase does not protect cells from Nutlin-3-mediated apoptosis, indicating that RNF8 and RNF168 do not regulate all functions of 53BP1.

2.2 INTRODUCTION

Part of an effective immune response requires the production of antibodies of different classes, each of which mediates a different effector function. This process is initiated by activation-induced cytidine deaminase (AID), which induces class switch recombination (CSR) by deaminating cytidines within immunoglobulin switch regions (Di Noia et al. 2007; Stavnezer et al. 2008). The recognition and subsequent processing of the mutated residues by the base excision repair and/or mismatch repair machineries generates double-stranded DNA breaks.
(DSBs) at switch regions (Saribasak et al. 2009). In an attempt to mend the ensuing breaks, B cells mount a damage response similar to that signalled by irradiated cells (Stavnezer et al. 2008). Ultimately, AID-induced double-strand DNA breaks are repaired predominantly by non-homologous end joining (Pan-Hammarstrom et al. 2005; Franco et al. 2008) and, to a lesser extent, by an alternative end joining pathway(s) (Yan et al. 2007).

Generally, DSBs are readily recognized by sensor-kinase protein complexes. These include the Mre11-Rad50-Nbs1 (MRN)/ATM, the Ku70/Ku80-DNAPKcs, and the ATR-ATRIP complexes (Wang et al. 2004). Upon binding to a DSB site, these factors are activated to trigger a cascade of events culminating in cell cycle delay and/or DNA repair (Wang et al. 2004). During this process, sequential chromatin modifications around the break sites appear crucial for the adequate resolution of the DNA breaks. Active chromatin modification is initiated by ATM-dependent phosphorylation of the histone variant H2AX, to the γH2AX form (Rogakou et al. 1998; Burma et al. 2001; Celeste et al. 2002). γH2AX then preferentially binds the tandem-BRCT motifs of Mediator of DNA Damage Checkpoint 1 (MDC1) (Stucki et al. 2005; Lou et al. 2006), which in turn recruits more MRN/ATM, thereby amplifying the γ-H2AX signal (Stucki et al. 2005; Lou et al. 2006). Recently, it was shown that ATM also phosphorylates MDC1 at a TQxF consensus, allowing for the recruitment of a novel E3 ubiquitin ligase, called RNF8, via its phospho-binding FHA module (Huen et al. 2007; Kolas et al. 2007; Mailand et al. 2007). Once at the break, RNF8 mediates the mono-ubiquitination of H2A-type histones, among other potential proteins. Mono-ubiquitinated H2A-type histones subsequently signal the recruitment of another E3 ubiquitin ligase, RNF168 (Doil et al. 2009; Stewart et al. 2009), which was recently found to be mutated in an immunodeficient and radiosensitive Riddle Syndrome patient (Stewart et al. 2007; Stewart et al. 2009). RNF168 protects and amplifies the RNF8-mediated
ubiquitination by catalyzing the addition of K63-linked poly-ubiquitin chains at DNA damage sites (Doil et al. 2009; Stewart et al. 2009). Whilst low levels of 53BP1 can transiently localize to DNA breaks via direct binding to di-methylated H4K20 (Botuyan et al. 2006), ubiquitination by RNF8 and RNF168 seem crucial for the accumulation and stabilization of 53BP1 at break sites.

Most of the factors required for DNA Damage Response (DDR) also appear to contribute to the process of CSR (Stavnezer et al. 2008). However, one important distinction between general DDR and CSR is the major reliance of the latter on 53BP1 (Ward et al. 2003; Manis et al. 2004; Ward et al. 2004), since 53BP1-deficiency results in a drastic decrease in CSR. It is still unclear whether this marked defect is due to a repair function that 53BP1 fulfills and/or because it is required to bring distal switch regions into close proximity and assist in switch synapse formation (Stavnezer et al. 2008). Recent data seem to focus on the latter aspect, especially since 53BP1-deficient B cells show increased levels of intra-switch region recombination (Reina-San-Martin et al. 2007), which is consistent with 53BP1 promoting and/or stabilizing the synopsis of distant switch regions during CSR. Similarly, 53BP1 facilitates long-range end joining during V(D)J recombination at the TCR locus (Difilippantonio et al. 2008), and also promotes end joining of dysfunctional telomeres by increasing chromatin mobility (Dimitrova et al. 2008). Historically, 53BP1 was first described as one of two proteins to interact with p53 (Iwabuchi et al. 1994). This aspect of 53BP1 function is generally thought to mediate apoptosis and is seen as distinct from its ability to localize to DSB.

We and others have previously shown that the ubiquitination of PCNA is important for both somatic hypermutation and CSR (Langerak et al. 2007; Roa et al. 2008). In a continued effort to delineate the importance of the ubiquitin system in antibody diversification, we examine
here whether the RNF8 and RNF168 members of the E3 ubiquitin ligase cascade, that are upstream of 53BP1 in the DDR pathway, are also required to mediate CSR.

2.3 MATERIALS AND METHODS

2.3.1 In Vitro Cell Culture

CH12F3-2 cells were maintained and CSR assays were performed as previously described (Nakamura et al. 1996). Briefly, cells were stimulated with 1 ng/mL recombinant human TGFβ1 (R&D systems), 10 ng/mL recombinant mouse IL-4 (R&D systems) and 2 μg/mL functional grade purified anti-mouse CD40 (eBiosciences), and analyzed by flow cytometry, as described below. Lentiviral shRNA constructs and protocols for 53BP1 (V2LMM 83391), RNF8 (AAF59E8 and AAF59E12), RNF168 (V2MM 16141), and non-silencing negative control (RHS4346 and SHC002) shRNA were purchased from Open Biosystems. CH12F3-2 cells were transduced with lentivirus by centrifugation at 800xg for 90 min at room temperature in the presence of 5 μg/mL polybrene. Cells were then incubated for 3 days and positively transduced clones were subsequently obtained by limiting dilution cloning and puromycin selection. For growth curve analysis, CH12F3-2 cells were diluted to a concentration of 1x10⁵ cells/ml and aliquoted in duplicate on a 96-well plate. At various time points, the numbers of live-trypan blue excluded cells were counted using a haemocytometer. NIH3T3 cells were treated with an siRNA SMARTpool (from ThermoFisher) targeting murine RNF168. siRNA transfections were performed using Dharmafect 1 (ThermoFisher) in forward transfection mode. CH12F3-2 cells were treated with 25 μM Nutlin-3 (Sigma) or DMSO as a negative control.
2.3.2 Flow Cytometric analyses

CH12F3-2 cells were analysed by intracellular staining with PE conjugated anti-mouse IgA clone 11-44-2 (eBiosciences), requiring the use of Cytofix/Cytoperm and Perm/wash buffers (BD Biosciences). CH12F3-2 cells were surface stained for Annexin V using Annexin V-APC Apoptosis Detection Kit (eBiosciences). Stained cells were analyzed by FACSCalibur (BD Bio) and FlowJo software (Truestar Inc.).

2.3.3 Irradiation sensitivity assays

CH12F3-2 cell lines were irradiated with various doses of X-rays and subsequently plated at various dilutions in duplicate 96 well plates. Survival was determined by counting the number of expanding clones normalized to plating efficiency.

2.3.4 Western blot analyses

53BP1 (Alexis Biochemicals and Novus), RNF8 (Abcam), γH2AX (Upstate), PCNA (Santa Cruz Biotechnology), β-Actin (Abcam), Msh2 (BD Pharmingen) antibodies were used as specified by the manufacturers protocols. The RNF168 polyclonal antibody was raised against a murine GST-RNF168<sup>381-567</sup> fusion protein and affinity purified using a murine HIS<sub>6</sub>-RNF168<sup>381-567</sup> Sepharose column.
2.3.5 Immunofluorescence

Cells were pelleted at 1000 rpm for 3 min in Eppendorf tubes. Cells were then fixed with 4% paraformaldehyde (Sigma), permeabilized with 0.3% Triton X (Roche), and blocked with 10% Fetal Calf Serum (Hyclone) and 0.01% saponin (Sigma) in PBS. The following antibodies were used: mouse FITC-anti-γH2AX (Millipore), rabbit anti-53BP1 (Novus), and anti-rabbit Alexa Fluor 568 (Invitrogen). Stains were done overnight at 4°C, and washes were done with blocking buffer. Cells were pelleted on slides using a Shandon cytospin machine at 400 rpm for 2 min. DAPI stain in mounting dye (Vectashield) was used to detect DNA.

2.3.6 Statistical Analysis

Analyses were performed on GraphPad Prism. For Student’s t tests, two-way analysis of variance (ANOVA), and Mann-Whitney tests, p values of 0.05 or less were considered significant.

2.4 RESULTS

2.4.1 shRNA-mediated RNAi can establish functional knockdown in CH12F3-2 cells.

We first ascertained that the CH12F3-2 cell line is a faithful model of CSR, with respect to the role of DDR factors. To do this, we assessed whether RNA interference (RNAi) by shRNA for 53BP1, which has been shown to be an essential CSR factor (Manis et al. 2004; Ward et al. 2004), can achieve functional knockdowns in CH12F3-2 cells. Cells were transduced with a lentiviral shRNA construct specific for 53BP1 (sh53BP1), and individual clones were isolated by puromycin selection. Compared to clones transduced with a scrambled, non-silencing shRNA
construct (control), 53BP1 knockdown cells showed a ~5 fold reduction in 53BP1 transcript levels, as determined by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, as well as by western blot analysis (Figure 8A). These cells have a slightly reduced rate of growth compared to control cells (Figure 8B) and demonstrate no major differences in apoptosis, before and after stimulation (Figure 8C). Although 53BP1-deficient cells have previously been shown to have a mild sensitivity to irradiation (Nakamura et al. 2006), 53BP1-depleted CH12F3-2 cells did not display an increased sensitivity to irradiation in comparison to control cells (Figure 8D).

We next tested whether 53BP1 knockdown cells were deficient in CSR. While CSR was not affected in control clones relative to the parental cells (Figure 8E), eight individual 53BP1 knockdown clones showed a complete abrogation of CSR at 48 hours after stimulation (Figure 8E), even though both groups of cells expressed similar levels of AID (Figure 9). This defect in CSR persisted even at 4 days post-stimulation (Figure 8F). Although the 53BP1-depleted cells showed a mild proliferation defect, previous findings have established a role for 53BP1 in CSR (Manis et al. 2004; Ward et al. 2004), suggesting that the mild proliferative defect observed in these cells is not responsible for the dramatic lack of CSR. These results confirm the importance of 53BP1 for CSR in the CH12F3-2 cells used in these studies. Furthermore, we show that shRNA-mediated RNAi can achieve a functional knockdown in CH12F3-2 cells, thereby setting the stage for testing whether RNF8 and RNF168 are involved in CSR.
Figure 8. shRNA-mediated RNAi knockdown of 53BP1 in CH12F3-2 cells abrogates CSR. (A) Western blot analysis of control and sh53BP1 CH12F3-2 cells quantifying 53BP1 and Msh2 (loading control). (B) Two clones of each indicated CH12F3-2 cell population were analyzed for growth in duplicate and statistical significance was tested by 2-way ANOVA test. **** = P<0.0001 (C) Percentage of CH12F3-2 populations undergoing apoptosis (surface Annexin V+) with and without stimulation for 2 days. Assay was carried out with at least 2 individual clones for each population. (D) Clonogenic survival assays with asynchronous CH12F3-2 populations exposed to varying doses of X-rays. Assay was carried out with at least 2 individual clones for each population. (E) Six individual control and eight sh53BP1 CH12F3-2 clones were stimulated for 2 days and analyzed for IgA expression. Values were normalized by dividing the % IgA-positive cells in the experimental group to the % IgA-positive cells in the stimulated parental CH12F3-2 cells. CSR assays were performed on each clone in duplicate and statistical significance was tested by one-tailed t-test. (F) Three control and three sh53BP1 CH12F3-2 clones were stimulated for 2, 3 and 4 days and IgA expression was analyzed. CSR assays were performed in duplicate for each clone. Statistical significance tested by 2-way ANOVA. **** = P<0.0001.
Figure 9. AID expression is not affected by RNAi of CH12F3-2 cells. Control, shRNF168, and sh53BP1 CH12F3-2 cells were stimulated (+) and analyzed for AID expression by western blot analysis, with β-actin as the loading control. Unstimulated (-) and stimulated (+) parental CH12F3-2 cells (CH12F3) represent the negative and positive controls, respectively. Molecular weigh markers have been indicated on the right of the figure.
2.4.2 RNF8 is required for CSR in CH12F3-2 cells.

To achieve knockdown of RNF8, we tested five different shRNA lentiviral constructs specific for RNF8. Two of those constructs (shRNA #1 and 2) achieved strong depletion of RNF8 protein following puromycin selection in CH12F3-2 cells, as verified by Western blot analysis (Figure 10A). We then assessed whether RNF8 depletion in those lines affected the response to DNA double-strand breaks. As expected, activated cells had a considerably higher γH2AX signal in comparison to non-activated samples (Figure 10B). However, there was no significant difference in γH2AX protein levels in control and RNF8-depleted CH12F3-2 cells, indicating that both groups of cells responded similarly to AID-induced DNA double-strand breaks.

To test the effects of RNF8 depletion on CSR, we selected eight individual clones from each transduction for subsequent analysis. Although different switching efficiencies were achieved within each group of cells, there was significantly reduced CSR in RNF8-depleted clones (Figure 10C). The extent of the switching defect was greater in the clones (shRNA #2) that were more depleted for RNF8 (Figure 10A and C). In addition, we followed CSR at different time intervals in a representative sample of the clones, and found that CSR in RNF8-depleted cells remained low relative to control cells (Figure 10D). RNF8-depleted cells did not show a defect in proliferation (Figure 8B) or apoptosis, before or after stimulation (Figure 8C). In addition, RNF8-depleted cells and control cells were equally sensitive to irradiation (Figure 8D). These data indicate that the reduced CSR observed in RNF8-depleted cells is not due to proliferation defects, and that RNF8 function is required for efficient CSR.

To gain insight into the role of RNF8 in CSR, we studied the effects of RNF8 knockdown by immunofluorescence. When unstimulated CH12F3-2 cells were fixed and stained with anti-
Figure 10. CSR is impaired in RNF8-deficient CH12F3-2 cells. (A) Western blot analysis quantifying RNF8, 53BP1, and PCNA (loading control) in CH12F3-2 cells with control shRNA, and two different lentiviral vectors targeting RNF8 (1) and (2) (B) Western blot analysis of stimulated and unstimulated control or shRNF8 treated CH12F3-2 cells, quantifying RNF8 and γH2AX protein levels with PCNA as the loading control. (C) Eight individual clones for each of control, shRNF8 (1), shRNF8 (2) CH12F3-2 cells were stimulated for 3 days and analyzed for IgA expression. Values were normalized by dividing the % IgA-positive cells in the experimental group to the % IgA-positive cells in the stimulated parental CH12F3-2 cells. Statistical significance was tested by two-tailed t-test. * = P<0.05 and **** = P<0.0001. (D) Two control, two shRNF8 (1), and two shRNF8 (2) treated CH12F3-2 clones were stimulated and IgA expression was analyzed at 2, 3 and 4 days post-stimulation. Statistical significance tested by two-way ANOVA. *** = P<0.001 and **** = P<0.0001. (E) Stimulated control and shRNF8 CH12F3-2 cells were collected at time 0 h and 48 h, and stained with the denoted antibodies and DAPI. Cells, from two separate experiments (each with n=1000), having single γH2AX foci with 53BP1 (γH2AX + 53BP1 foci) or without 53BP1 (γH2AX + 53BP1 foci) co-localization were counted and analyzed as shown. Greater than 90% of the cells did not exhibit γH2AX foci after cytokine stimulation. Statistical significance was tested by two-tailed t-test between control 48hr and shRNF8(2) 48hr. (* = P=0.014).
γH2AX and anti-53BP1, almost all cells expectedly manifested a diffuse 53BP1 nuclear pattern and a lack of γH2AX signal (Figure 11 - top panel). Upon activation, control cells showed a marked increase in single γH2AX foci that co-localise with single 53BP1 foci, indicative of induced DSBs, and hence active switching events (Figure 10E, Figure 11 bottom panel). Importantly, compared to control cells, shRNF8-transduced cells had a significant increase in γH2AX foci with a diffuse 53BP1 signal (Figure 10E, Figure 11). 53BP1 depletion has no effect on germline transcription ((Manis et al. 2004) or AID expression (Figure 9). In addition, we could show that shRNF8 cells generated similar levels of AID-induced γH2AX, as assayed by immunoblotting and immunofluorescence, as control cells. This suggests that the cascade upstream of AID-induced breaks, which is dependent on germline transcription and AID, is most likely intact. Taken together, this suggests that the inability of shRNF8-treated cells to undergo efficient CSR is due to their inability to signal the accumulation and/or stabilization of 53BP1 at AID-induced breaks.

2.4.3 RNF168 is important for CSR in CH12F3-2 cells.

We next examined the contribution of RNF168 to CSR. To achieve knockdown of RNF168, we tested three different shRNA lentiviral constructs specific for RNF168, one of which led to a ~2-3 fold reduction in RNF168 protein levels (Figure 12A). Akin to the shRNF8 transduced cells, shRNF168-transduced cells and control cells displayed similar growth rates (Figure 8B), apoptosis levels before and after stimulation (Figure 8C), and sensitivity to irradiation (Figure 8D). On the other hand, we observed that RNF168-knockdown cells had 46% lower CSR levels compared to control cells (Figure 12B and C). Like the RNF8-depleted cells, this ~2-fold reduction of CSR correlates with the level of knockdown achieved in the CH12F3-2
**Figure 11.** 53BP1 foci formation is reduced in RNF8-knockdown clones. Control and shRNF8 CH12F3-2 cells were fixed and stained with the denoted antibodies and DAPI, and visualized by confocal microscopy. Stimulated control and shRNF8 CH12F3-2 cells were collected at time 0 h and 48 h. A representative sample of cells is shown in both panels. Apparent DAPI foci are most likely due to the high levels of heterochromatin in B cells.
Figure 12. CSR is impaired in RNF168-deficient CH12F3-2 cells. (A) Western blot analysis of control or shRNF168 CH12F3-2 cells quantifying RNF168, and β-actin as the loading control. NIH3T3 cells treated with control siRNA and siRNF168 were used as negative and positive controls for RNF168 protein expression, respectively. (B) Six individual control and seven individual shRNF168 CH12F3-2 clones were stimulated for 2 days and analyzed for IgA expression. Values were normalized by dividing the % IgA-positive cells in the experimental group to the % IgA-positive cells in the stimulated parental CH12F3-2 cells. CSR assays were performed on each clone in triplicate and statistical significance was tested by two-tailed t-test. ** = P=0.0023 (C) Three control and three shRNF168 CH12F3-2 clones were stimulated for 2, 3 and 4 days and IgA expression was analyzed. CSR assays were performed in duplicate for each clone. Statistical significance tested by two-way ANOVA. (** = P=0.0034).
cells. Thus, the CSR defect observed in the absence of RNF8 can be recapitulated by depleting RNF168. Taken together, these findings indicate that the E3 ubiquitin ligase cascade composed of RNF8 and RNF168 plays a significant role in CSR.

2.4.4 53BP1 deficiency, but not RNF8 or RNF168-deficiency, protects cells from Nutlin-3-induced apoptosis.

Nutlin-3 is a small molecule inhibitor of MDM2 that induces activation of p53 and apoptosis of cancer cells in a process that is dependent on 53BP1 (Brummelkamp et al. 2006). That is, 53BP1-deficient cells are resistant to Nutlin-3-mediated apoptosis. To test whether this activity of 53BP1 is influenced by RNF8 or RNF168, we examined whether deficiency in any of these proteins protects CH12F3-2 cells from the effects of Nutlin-3. In contrast to control cells incubated with Nutlin-3, which exhibited dramatically increased levels of apoptosis, Nutlin-3 did not induce high levels of apoptosis in 53BP1-depleted cells (Figure 13A). Strikingly, RNF8-depleted cells and RNF168-depleted cells remained sensitive to Nutlin-3-mediated apoptosis (Figure 13A). These data indicate that 53BP1 has functions that are independent of RNF8 and RNF168 (Figure 4B).

2.5 DISCUSSION

2.5.1 Role of RNF8 and RNF168 in CSR and its relation to 53BP1 localization.

Although the necessity of 53BP1 for CSR has been shown before (Wang et al. 2002; Manis et al. 2004; Ward et al. 2004), the exact function of the 53BP1-dependent pathway in CSR remains elusive. Recent findings demonstrate that RNF8 and RNF168 play essential roles
Figure 13. 53BP1 knockdown cells, but not RNF8/168 knockdown cells, are resistant to Nutlin-3-mediated apoptosis. (A) Percentage of control, sh53BP1, shRNF8 (2), and shRNF168 CH12F3-2 cells undergoing apoptosis (i.e. surface Annexin V+) after treatment with Nutlin-3 or DMSO for 1 day. Each assay was carried out with 2 different clones of each population. Statistical significance tested by 2-way ANOVA. **** = P<0.0001. (B) Schematic illustrating two different roles for 53BP1. AID indirectly induces a DSB by the actions of the mismatch repair (MMR) and base excision repair (BER) pathways. 53BP1 is recruited to the double-stranded DNA break either by directly binding di-methylated H4K20, or through the γH2AX-RNF8-RNF168 amplification pathway, the latter of which leads to the accumulation and stabilization of 53BP1 at the break site. These pathways ultimately lead to end processing and resolution of the DNA break. 53BP1 stabilizes p53 (independent of RNF8/168) that has been dissociated from the inhibitor MDM2 to induce apoptosis.
in recruiting and stabilizing 53BP1 at sites of DSBs during the irradiation-induced DDR (Huen et al. 2007; Kolas et al. 2007; Mailand et al. 2007; Doil et al. 2009; Stewart et al. 2009). In support of a role for 53BP1 localization to sites of AID-induced DSBs, our data show that knockdown of either RNF8 or RNF168 causes a significant decrease in CSR. These findings support the notion RNF8/RNF168 and is required for the efficient bridging of the two distal DNA ends (Difilippantonio et al. 2008; Dimitrova et al. 2008; Stavnezer et al. 2008).

Since the recruitment of RNF8 and subsequent recruitment of RNF168 to DSB sites requires ATM/MRN, γH2AX and MDC1, it would be reasonable to assume that all these factors have similar effects on CSR (Figure 4B). Yet, in reality the loss of these DDR factors seem to have different effects on CSR. While ATM-, MRN-, and H2AX-deficiency leads to a ~70% reduction of CSR (Reina-San-Martin et al. 2003; Lahdesmaki et al. 2004; Reina-San-Martin et al. 2004; Reina-San-Martin et al. 2005; Dinkelmann et al. 2009), MDC1-deficiency has a milder effect (25%–50% reduction (Lou et al. 2006)), and 53BP1-deficiency leads to the most profound effect (~90% CSR reduction; this study and (Manis et al. 2004; Ward et al. 2004)). A lesser dependence of CSR on MDC1 may be either due to: 1) the residual levels of MDC1 protein in the MDC1 gene-trap knock-out mice that was sufficient to amplify the ATM/MRN-mediated γH2AX signal (Lou et al. 2006); or 2) a redundant MDC1-independent mechanism that allows for recruitment of low levels of RNF8, and subsequently 53BP1, allowing for intermediate levels of CSR. On the other hand, the difference between the low levels of CSR observed in ATM-, MRN-, and γH2AX-deficiency and the near complete lack of CSR in 53BP1-deficient cells might be explained by the potential transient recruitment of 53BP1 to switch regions via direct binding to either di-methylated H4K20 that are exposed at the DSB site (Botuyan et al. 2006) or through other yet to be determined factors. As a result, this transient recruitment of 53BP1 could
be sufficient for the resolution of a subset of DNA breaks mediating low levels of CSR in the absence of the upstream proteins required for 53BP1 accumulation and stabilization (Figure 4B). It remains to be seen whether complete knock-out of RNF8 or RNF168 affect CSR in a manner akin to the loss of 53BP1 or γH2AX. Our data suggest that RNF8/RNF168 have an incomplete effect on CSR. However our shRNA constructs cannot completely deplete these factors. The finding that serum IgG is reduced by ~10 fold in a Riddle syndrome patient deficient in RNF168 (Stewart et al. 2007) suggests that complete ablation of RNF8 or RNF168 would produce a pronounced phenotype.

2.5.2 53BP1 has a unique function that is independent of RNF8 and RNF168.

It was previously shown that Nutlin-3, which inhibits the p53-Mdm2 interaction, induces p53-mediated apoptosis in a 53BP1-dependent manner (Brummelkamp et al. 2006). Consistent with this finding, our results show that 53BP1-deficient CH12F3-2 cells are resistant to Nutlin-3-induced apoptosis. However, we found that RNF8- and RNF168-deficient CH12F3-2 cells were still sensitive to the apoptotic effects of Nutlin-3. 53BP1 was initially discovered as a p53-binding protein (Iwabuchi et al. 1994), interacting with p53 through its C-terminal tandem-BRCT motifs in a phosphorylation-independent manner. This interaction is thought to stabilize p53 and stimulate p53-mediated transcriptional activation (Iwabuchi et al. 1998). It is possible that p53, upon release from MDM2-mediated inhibition, is degraded in 53BP1-deficient cells and as such, apoptosis is not induced, or that 53BP1 functions as a co-factor of p53 to induce apoptosis. Whether this function of 53BP1 is related to its role in localization at sites of DSB remains unclear. However, since the p53-53BP1 interaction is phosphorylation-independent and does not seem to require ATM activation (Iwabuchi et al. 1998), it is plausible that this might be
a function of 53BP1 distinct from foci formation at DSBs. The finding that Nutlin-3-mediated apoptosis is not affected in RNF8- or RNF168-deficient CH12F3-2 cells supports this notion and suggests that 53BP1 has distinct roles in response to genotoxic stress (Figure 13B).
3 IDENTIFYING NOVEL DDR FACTORS FROM A GENOME-WIDE RNA INTERFERENCE SCREEN FOR CSR.

Contributions:

Cell culture, cell sorting and genomic DNA extractions: Shaliny Ramachandran, Rajeev Nepal and Dionne White.

Microarray and sequencing: Troy Ketela, Bohdana Fedyslyn, Dahlia Kasimer and Kaajal Nagar

Microarray and Sequencing analysis: Shaliny Ramachandran and Troy Ketela

Screen Validation analysis: Shaliny Ramachandran, Maribel Berru, Anat Kapelnikov, Alan Jiao, Michael Le

AID expression analysis: Shaliny Ramachandran and Angela Zhang
3.1 ABSTRACT

B cells undergo CSR to generate different classes of antibody, which direct diverse functions, resulting in a humoral immune response that can be fine-tuned to the infection at hand. CSR requires AID-generated DSBs at the antibody locus, and subsequent repair of this lesion by DDR. Using the CH12F3-2 B cell line, we demonstrated that shRNA-mediated knockdown of the DDR factors, RNF8, RNF168 and 53BP1, led to a significant decrease in CSR (Chapter 2). However, the molecular events following 53BP1 and leading up to DNA repair in CSR remain elusive. To identify novel DDR factors in CSR, we conducted a pooled, loss-of-function, genome-wide shRNA screen for CSR factors, using the CH12F3-2/RNA interference system established above in Chapter 2. Using microarray and sequencing-based readouts, hairpins that inhibit CSR were identified with validation frequencies of 63.2% and 61.5%, respectively. The CSR candidates were then screened for AID expression to narrow down the CSR defect relative to AID. Surprisingly, nearly 80% of the 33 tested hairpins showed a significant effect on AID expression. In some instances, the effect on AID expression might be attributed to off-target effects that have an indirect effect on AID protein levels. Given that CSR is tightly linked to AID expression, future RNA interference studies on CSR in this system must ensure that any effects on CSR is specifically due to the gene of interest and not due to off-target effects on AID protein levels.

3.2 INTRODUCTION

B cells undergo CSR to shift from expressing the IgM antibody to IgG, IgE and IgA classes. Each class of antibody is attributed with a different effector function, allowing for a fine-
tuned response that depends on the nature of the infection. During CSR, AID is absolutely required to catalyze the deamination of deoxycytidines within the switch regions (Kracker et al. 2011). This deamination forms dU:dG mismatches, which are subsequently engaged by the MMR and BER pathways leading to the production of nicks and/or stretches of excised DNA strands within the switch regions (Stavnezer et al. 2008). Owing to the highly repetitive nature of the switch regions, BER- and MMR-generated nicks or strand excision tracts formed within close proximity on opposite strands can become staggered DSBs (Schrader et al. 2005; Stavnezer et al. 2005). AID activity produces staggered DSBs at both donor and acceptor switch regions, which can then be repaired in a manner that places the downstream acceptor region immediately adjacent to the rearranged V(D)J region, while deleting the intervening DNA containing the donor switch region (Chaudhuri et al. 2004). This replaces the donor constant region with the acceptor constant region on the antibody, completing CSR.

CSR requires an intricate network of factors that function in a highly regulated manner that lead to expression of AID and its requisite co-factors, switch region transcription, and factors involved in the DNA repair pathways required to process the AID-induced mutations to generate the staggered DSBs, and to repair them (Xu et al. 2007; Stavnezer et al. 2008; Pavri et al. 2011; Stavnezer 2011). AID-induced DSBs activate DDR, leading to cell cycle arrest and repair primarily via NHEJ (Chaudhuri et al. 2007). While much of the CSR process is known, the exact molecular events within the DDR and the repair aspects of CSR have not been completely characterized. In this report, we carried out a genome-wide CSR screen designed to identify genes involved in the all the steps leading up to CSR. We used the mouse B cell line CH12F3-2 that undergoes CSR specifically from IgM to IgA at high levels upon stimulation (Nakamura et al. 1996). We established a CH12F3-2/RNA interference system to enrich for factors involved in
the CSR process. Using this RNA interference approach, we previously demonstrated that RNF8, RNF168 and 53BP1 play a role in CSR, in Chapter 2 above (Ramachandran et al. 2010). We conducted a pooled, loss-of-function screen testing approximately 80,000 lentiviral shRNA hairpins in total and analyzed the results using microarray-based and next-generation sequencing readout methods, preformed validation analyses to determine the confirmation rates, and finally screened a subset of CSR candidates for AID expression to identify possible DDR and DNA repair factors.

3.3 MATERIALS AND METHODS

3.3.1 Pooled shRNA lentiviral library

Lentiviral library targeting the mouse genome was generated by The RNAi Consortium. Containing a total of ~78,000 shRNA hairpins targeting the mouse genome, the virus used for transduction was a pool of 10 sub-pools, each containing ~8000 shRNA hairpins. Lentivirus sub-pools were generated as previously described (Ketela et al. 2011).

3.3.2 In Vitro Cell Culture

CH12F3-2 cells were maintained as previously described in Chapter 2 above (Ramachandran et al. 2010). Lentiviral shRNA constructs and protocols were performed as previously described in Chapter 2 above (Ramachandran et al. 2010). Briefly, for lentiviral transductions, CH12F3-2 cells were incubated with virus at 37°C for 24 hours at an MOI of 0.2-0.3. Positively transduced cells were selected for puromycin resistance, with 1 μg/mL puromycin for 3 days, followed by CSR stimulation. Cells were stimulated with 1 ng/mL TGFβ, 10 ng/mL IL-4 and 2 μg/mL anti-CD40 for 3 days.
3.3.3 Flow cytometry cell sorting

Stimulated CH12F3-2 cells were subjected to extracellular staining with PE conjugated anti-mouse IgA clone (Southern Biotech). Stained cells were sorted by FACS Aria (BD Bio).

3.3.4 Microarray analysis

Genomic DNA was extracted from each of the sorted population using the Blood Maxi prep kit (Qiagen). shRNA hairpin barcodes were PCR-amplified and used as probe in microarray experiments as described previously (Ketela et al. 2011).

3.3.5 Sequencing analysis

Genomic DNA was extracted and subjected to PCR-amplification as described above. PCR product was then subjected to a second PCR reaction in order to be tagged with an Illumina adapter sequence, as previously described (Ketela et al. 2011). Illumina sequencing and analysis was performed as previously described (Ketela et al. 2011).

3.3.6 Western blot analysis

AID (Cell Signalling) and β-Actin (Sigma) antibodies were used as specified by the manufacturers’ protocols.

3.3.7 Statistical Analysis

Analyses were performed on GraphPad Prism. For Student’s t-tests and linear regression analysis, p values of 0.05 or less were considered significant.
3.4 RESULTS

3.4.1 CH12F3-2/shRNA: A system to screen for potential CSR factors.

The CH12F3-2 mouse B cell line undergoes CSR from IgM specifically to IgA at very high levels upon stimulation with anti-CD40, IL4 and TGFβ (CIT) (Figure 14). After 72 hours of stimulation, the system reaches saturation with over 60% of cells typically expressing IgA. CH12F3-2 is a subclone variant isolated from the IgM-expressing CH12.LX mouse B line, a transformed cell line originating from a lymphoma that developed in a B10-2a4b mouse immunized with sheep red blood cells (Kunimoto et al. 1988; Nakamura et al. 1996). We previously established a protocol to individually test for potential CSR factors using an RNA interference approach in CH12F3-2 cells in Chapter 2 above (Ramachandran et al. 2010). Using shRNA, we achieved knock-down of target proteins in CH12F3-2, and ascertained whether the knock-down affected CSR, thus determining whether the target protein is important for CSR. Taking advantage of this system, we developed a screen to identify novel CSR factors. For this screen, we utilized the pLKO.1 vector-based library of shRNA targeting the mouse genome (from The RNAi Consortium), with a total of 77,690 hairpins targeting approximately 16,000 mouse genes, with 4-5 different hairpins targeting each gene (Moffat et al. 2006). These shRNA have been designed to specifically target the gene of interest, and have at least 3 nucleotide mismatches to other related genes so as to prevent off-target effects (Moffat et al. 2006).

3.4.2 Screen methodology

We designed a pooled, loss-of function shRNA screen (Figure 15). CH12F3-2 cells were transduced with the library of 77,690 shRNA at a low MOI of 0.2 - 0.3 to minimize the
Figure 14. **CH12F3-2 in vitro class switch recombination assay.** CH12F3-2 cells were stimulated at a concentration of 100,000 cells/mL with 1 ng/µL TGFβ, 10 ng/µL IL4 and 2 µg/mL α-CD40 antibody, and incubated at 37 °C, 6% CO₂. At 2, 3 and 4 days post-stimulation, cells were stained and analyzed for IgA expression by flow cytometry. Unstimulated cells were assayed in parallel as non-switched negative controls.
Figure 15. CSR Screen methodology. CH12F3-2 cells were transduced with the lentiviral shRNA library at a MOI ~ 0.2-0.3. Positively transduced cells were selected for puromycin resistance and stimulated to undergo CSR (in 3 biological replicates). Switched IgA+ cells were sorted from non-switched IgA- cells. Genomic DNA was extracted from each population for PCR-mediated amplification of the shRNA barcode, which was then subjected to microarray analysis and sequencing analysis, to determine the level of each hairpin in each population. For each hairpin, CSR screen score is determined as fold difference in hairpin representation in IgA- population over IgA+ population.
probability of integrating more than one shRNA hairpin into any one individual cell. Transductions were scaled-up so that each hairpin could have at least 1000 independent genomic representations. Positively transduced cells were selected for puromycin resistance, and subsequently stimulated with the cocktail of TGFβ, IL4 and anti-CD40 in three biological replicates. After 3 days of stimulation, switched IgA+ cells were sorted from non-switched IgA-cells. Genomic DNA was extracted from each population for PCR-mediated amplification of the shRNA sequence, which acts as the unique barcode used to identify the hairpin and the target gene. The PCR product was subjected to microarray-based Gene Modulation Array platform analysis and next-generation sequencing analysis, to determine the representation of each individual hairpin in each of the sorted IgA- and IgA+ populations. Gene Modulation Array platform analysis was performed in 2 technical replicates, while next-generation sequencing analysis was performed in 1 technical replicate. For each hairpin, a CSR screen score was determined as fold difference in hairpin representation in IgA- population over the IgA+ population. Hairpins that are equally-represented in both IgA- and IgA+ populations likely target neutral genes that have no effect on CSR. Hairpins that are under-represented in both IgA- and IgA+ populations, as observed relative to the entire cohort of hairpins analyzed, likely target genes involved in cell survival and proliferation. Hairpins under-represented in the non-switched IgA- population as compared to switched IgA+ population may target genes that inhibit CSR. Hairpins over-represented in the non-switched IgA- population as compared to switched IgA+ population are considered to target potential CSR factors.
3.4.3 **CSR Screen results and analysis.**

A total of 77,690 shRNA hairpins were screened for the ability to affect CSR. The raw results are displayed in Figure 16. ShRNA hairpins with a high coefficient of variation between technical replicates (CV>0.3 for microarray, no technical replicates for sequencing); a low signal (Signal<10 for microarray, Signal<1 for sequencing); and a high coefficient of variation between biological replicates (CV>0.4 for microarray, CV>0.6 for sequencing) were eliminated (Table 1). The analyzed results are displayed in Figure 17. Hairpins with a screen score >= 1.75, that is fold representation of a given hairpin in IgA- population divided by that in the IgA+ population, were considered to be candidate CSR factors. 1070 shRNA that target 1035 genes and 3044 shRNA targeting 2743 genes were identified as hits by the microarray and sequencing analyses, respectively. A comparison between the 1070 hairpins identified by the microarray analysis and 3044 hairpins identified by the sequencing analysis revealed 271 common hairpins identified as strong hits from both analyses. This represents 268 potential CSR factors.

3.4.4 **Validation analysis**

34 hairpins out of the 1070 hairpins identified as a CSR candidate (screen score >= 1.75) in the microarray analysis and 243 of the 57,057 non-candidate hairpins (screen score <1.75) were selected for validation analysis. Similarly, 38 of the 3044 candidates in the sequencing analysis and 239 of the 39,983 non-candidates were selected for validation analysis. Importantly, all of these non-candidate hairpins target the same genes as the candidates selected for validation, but these non-candidates likely could not generate a functional knock-down of the targeted gene. Typically, one of the five hairpins desgined to target a gene appeared as a CSR candidate on the screen. The ability of each hairpin to affect CSR was individually tested and normalized to a
Figure 16. Raw CSR Screen Results. Raw results of the screen are displayed for microarray (A) and sequencing (B) analysis. Each hairpin is represented by a single point. The representation of a hairpin in the IgA- population is graphed on the x-axis, while the representation of the hairpin in the IgA+ population is on the y-axis.
### CSR Screen analysis

A total of 77,690 shRNA hairpins were screened for the ability to affect CSR. ShRNA hairpins with a high coefficient of variation between technical replicates (CV>0.3 for microarray, no technical replicates for sequencing), a low signal (Signal<10 for microarray, Signal<1 for sequencing), and a high coefficient of variation between biological replicates (CV>0.4 for microarray, CV>0.6 for sequencing) were eliminated. Hits were set as hairpins with a screen score => 1.75. Screen score is fold representation of hairpin in IgA- population divided by IgA+ population.

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#### Table 1

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<tr>
<td>Background signal</td>
<td>IgA- OR IgA+ Signal &gt; 10</td>
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</tr>
<tr>
<td>Biological Replicates</td>
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<td>58127</td>
</tr>
<tr>
<td>Hits – Hairpins</td>
<td>Screen score =&gt;1.75</td>
<td>1070</td>
</tr>
<tr>
<td>Hits – Genes</td>
<td>Screen score =&gt;1.75</td>
<td>1035</td>
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</tbody>
</table>
Figure 17. Analyzed CSR Screen Results. Screen results are displayed for microarray (A) and sequencing (B) analysis. Each hairpin is represented by a single point. The representation of a hairpin in the IgA- population is graphed on the x-axis, while the representation of the hairpin in the IgA+ population is on the y-axis.
control shRNA hairpin targeting GFP set in parallel. Validation analysis revealed an 8.7% false-negative frequency, and a 37.8% false-positive frequency, for the microarray analysis; and 13.5% false-negative and 38.5% false-positive frequencies respectively, for the sequencing analysis (Figure 18). This translates into a confirmation rate of 63.2% and 61.5% for microarray and sequencing readouts, respectively. Furthermore, an inverse correlation between the observed CSR, when individually tested in the validation analysis, and the CSR screen score was observed, with $r^2 = 0.4103$ ($P<0.0001$) for microarray and $r^2 = 0.1854$ ($P=0.002$) for sequencing (Figure 19).

Z score and volcano plot analyses was performed on both the microarray and sequencing results (Figure 20 and 21). The microarray analysis revealed 2 independent hairpins that target AID (TRC clone ID: TRCN0000112031, $P=0.0026$; and TRCN0000112033, $P=0.0365$) as top hits of the screen, while sequencing analysis revealed 3 independent hairpins that target AID (TRC clone ID: TRCN0000112031, $P=0.0450$; TRCN0000112032, $P=0.0184$; and TRCN0000112033, $P=0.0598$) as top hits of the screen (Table 2). Indeed, hairpins TRCN0000112031 and TRCN0000112033 have been previously shown to reduce AID protein levels by 5.8- and 2.4-fold, and CSR levels by 5- and 2.5-fold, respectively (Parsa et al. 2012). These results further validated the screen and revealed that CSR in CH12F3-2 cells is highly sensitive to any effects on AID expression.

3.4.5 Secondary screen to identify possible DDR and repair factors

In order to decipher the DDR and repair factors among the CSR candidates identified, a secondary screen was performed on a subset of genes identified to be interest. Genes that have been previously associated with DNA damage, genes with multiple targeting hairpins identified
Figure 18. Validation analysis of CSR screen. Validations of microarray (A) and sequencing (B) analyses are displayed. 34 hairpins identified as a hit (score $\geq 1.75$) and 243 non-hit hairpins (score <1.75) were selected for validation analysis of the microarray results. Similarly, 38 hits and 239 non-hits were selected for validation analysis of the sequencing results. The ability of each hairpin to affect CSR was individually tested and normalized to a control shRNA targeting GFP set in parallel. Validation analysis reveals a 8.7% false-negative rate and a 37.8% false-positive rate for the microarray analysis, and 13.5 % false-negative and 38.5 % false-positive rates for sequencing analysis.
**Figure 19.** Validation results correlate with screen scores. Correlations are displayed for microarray (A) and sequencing (B) analysis. For both the microarray and sequencing analysis, the normalized CSR levels of each hairpin (obtained from the validation analysis) is displayed on the y-axis and the CSR screen score is displayed on the x-axis.
Figure 20. **Z score analysis on the microarray (A) and sequencing (B) results.** Z score values are shown on the y-axis. Each dot represents a hairpin, displayed in order of highest Z score to lowest on the x-axis. A total of 58,127 hairpins and 43,027 hairpins are included for microarray and sequencing analysis, respectively.
Figure 21. Volcano plot analysis on the microarray (A) and sequencing (B) results. For each hairpin, the negative log (to the base 10) of the P-value was calculated and plotted on the y-axis. The P-value was determined an using paired, two-tailed student’s t-test between the hairpin levels in the IgA- population versus the IgA+ population. For each hairpin, the log (to the base 2) of the CSR screen score (fold difference in the hairpin level in IgA- population relative to the IgA+ population) was calculated and plotted on the x-axis. A total of 58,127 hairpins and 43,027 hairpins are included for microarray and sequencing analysis, respectively.
Table 2

<table>
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CSR screen scores for AID-targeting shRNA. AID-shRNA with CSR screen scores above 1.75 for microarray and sequencing analysis are displayed.
as screen hits, or genes that appeared in both microarray and sequencing analyses were selected for the secondary screen to identify hairpins that did not affect AID expression. The hairpins that reduce CSR without affecting AID likely target genes acting downstream of AID, and possibly act in the repair phase of CSR. Surprisingly, a significant proportion of hairpins identified to target potential CSR factors by the screen also affected AID expression in CH12F3-2 cells (Figure 22). Of the 33 hairpins tested by the screen, 26 hairpins reduced AID protein levels. The remaining 7 hairpins had no effect on AID protein levels and hence likely target CSR factors that act downstream of AID expression and may play in role in DDR and DNA repair. These may be strong candidates for detailed characterization (Chapter 4).

3.5 DISCUSSION

We developed and conducted a pooled, loss-of-function, genome-wide screen for CSR using CH12F3-2 cells in conjunction with lentiviral-based RNA interference technology. A previous study used the RNAi approach to identify novel factors involved in CSR, but used an entirely different methodology (Pavri et al. 2010). Pavri et al conducted a limited screen using 8797 shRNA hairpins targeting 1745 genes, that were primarily selected based on expression in CH12F3-2 cells and germinal centre B cells, and factors that could be associated with AID or DNA repair based on molecular functions and literature review (Pavri et al. 2010). They individually tested the effect of each hairpin on CSR, and identified 181 hits, of which 28 have been previously linked to CSR (Pavri et al. 2010). The authors selected Spt5 for in-depth characterization (Pavri et al. 2010). We, however, adopted a different approach by developing a genome-wide screen using a pooled format (Blakely et al. 2011). While our approach tests almost the entire set of mouse genes, owing to our large-scale approach, it is likely that our
Figure 22. A significant number of CSR candidates identified by the screen affect the expression of AID in CH12F3-2 cells. Of the 33 hairpins identified to target potential CSR factors by the screen (either microarray, sequencing or combined analysis), 26 hairpins affect AID expression. 7 hairpins likely target CSR factors that act downstream of AID expression and may play a role in DNA repair.
approach would fail to identify all the factors involved in the CSR process. Indeed, Spt5 did not appear as a CSR candidate in our screen. In the microarray analysis, all 5 hairpins targeting Spt5 had a CSR screen score of ~1, and in the sequencing analysis, all 5 hairpins were eliminated either due to low signal or a high coefficient of variation between replicates. However, our unbiased approach enabled us to uncover novel and possibly unexpected genes that might function in CSR. Since many genes are not differentially expressed in stimulated and unstimulated cells, or are expressed at low levels, the approach conducted by Pavri *et al* might preclude genes that fall in this category but still function in CSR.

Two distinct screen readout methods, relying on microarray-based technology and sequencing technology, were used in parallel to screen for the strongest CSR candidates. Direct comparison of the screen score obtained for each hairpin revealed a low correlation, with $r^2$ of 0.2985 ($P<0.0001$) between the two readouts (Figure 23). Previous studies have also shown a modest correlation between microarray and sequencing methods of analysis (Ketela *et al.* 2011). This difference may be partially explained by a difference in the relationship of readout signal to barcode PCR concentration between the two methods (Ketela *et al.* 2011). The absolute fold-change observed for each hairpin varies between microarray and sequencing analyses (Ketela *et al.* 2011). While sequencing provides a signal that is linearly proportional to the levels of a shRNAs in the two populations, microarray readout demonstrates a nonlinear relationship with shRNA levels, within the reliable signal range (Ketela *et al.* 2011). There is a similar trend observed between the two readout methods, but the absolute fold-changes observed show a low degree of equivalence. Having used only one technical replicate for the sequencing analysis and possibly having a poor quality of genomic DNA in sequencing may have added to the deviation between the sequencing and microarray results. The validation analysis of the screen revealed a
Figure 23. Correlation between CSR results of microarray readout and sequencing readout. Each dot represents a hairpin. Microarray screen scores are displayed on the y-axis and sequencing screen scores are displayed on the x-axis.
false-negative rate of 8.7% for microarray and 13.5% for sequencing. Given the nature of this high-throughput, pooled genomic screen, these false-negative rates are quite minimal, suggesting that most CSR factors may have been identified as hits by the screen (Blakely et al. 2011). Nevertheless, cross-hybridization and signal saturation can confound the true effects of a shRNA, leading to false-negative readout on microarray. Similarly, secondary structures formed by shRNA sequence that affect its binding efficiency to the flow cell of the sequencer can cause false-negative readout in sequencing analysis.

A point of surprise and concern from the screen results was the lack of known CSR factors appearing as hits, and in particular DDR factors involved in CSR like 53BP1. This can be attributed to multiple reasons, including those described above in explaining the causes of false-negative readings. With respect to DDR factors specifically, certain genes may be not appear as hits if the shRNA hairpins targeting these factors are ineffective. With respect to 53BP1, while the pGIPZ vector based 53BP1 shRNA used in Chapter 2 above is highly effective, the pLKO.1 vector 53BP1 shRNA sequence may not be as effective, although this remains to be tested. Alternatively, they could be effective but lead to a reduction in survival and growth or an enhancement in apoptosis, and in both cases these targets will be lost in the pooled format, and not be detected by the screen.

This loss-of-function approach is likely useful in identifying factors that function in CSR at different stages. Included within this group of genes are genes important for the first phase of CSR, which involves AID expression, stability, targeting and other factors important in DSB formation, as well as genes involved in the second phase of CSR requiring DSB-induced DDR and repair. As we are interested in the second phase of CSR involving DDR and repair, we utilized a secondary screen on a subset of genes to find potential DDR and repair factors. By
screening for hairpins that maintained AID levels, we ruled out any genes involved in AID expression and protein stability, and specifically focused on genes that are likely to function downstream of AID, possibly in DDR and repair. Intriguingly, nearly 80% of the hairpins tested affected AID expression. This result emphasizes that AID expression in CH12F3-2 cells either relies on a whole host of factors (involved in cytokine signalling and in AID protein stability) and/or may be highly susceptible to off-target effects. We have previously demonstrated that CSR is highly sensitive to small changes in AID expression in CH12F3-2 cells (Parsa et al. 2012). Even the slightest effect on a single factor, whether on-target or off-target, upstream of AID could affect AID expression. Hence, these results highlight that in the CH12F3-2 system, AID expression is highly sensitive to alterations in the expression of many other factors. And since CSR is highly sensitive to AID expression, future work examining CSR using the CH12F3-2/ RNA interference system should be highly cautious in interpreting results, ensuring that any effects on CSR are due to the gene in question and not due to off-target effects on AID. These results further stress the pertinence to test off-target effects, by AID reconstitution experiments, when using RNA interference-based systems.

Alternative to our secondary screen approach, another approach that utilizes the in vitro system developed by Tasuku Honjo can be useful to focus the efforts on CSR events downstream of AID expression (Kobayashi et al. 2009). In this system, AID is fused to the hormone-binding domain of the estrogen receptor and can be induced by estrogen analogue, 4-hydroxytamoxifen (Kobayashi et al. 2009). For this secondary screen, an AID-deficient CH12F3-2 cell line with an integrated AID-estrogen receptor fusion construct described above would be generated. Only those hairpins that were identified to target CSR factors from the primary screen would be selected for the secondary screen. Hence, using this modified system can specifically identify
those hairpins that target factors acting downstream of AID expression, likely in the DDR and repair phase of CSR.

Although this genome-wide screen has its caveats, the very high confirmation rates of the screens prove advantageous in discovering and characterizing novel factors within the pathway, and can be translated for other genome-wide studies as well. In particular, variations of the established CH12F3-2/shRNA system can be used to answer novel questions. For instance, MMEJ, the alternative end-joining pathway to NHEJ in CSR, remains completely elusive. While a few factors may be implicated in MMEJ, very little is known about this pathway. A parallel CSR screen approach using an NHEJ-deficient CH12F3-2 cell line instead, in which the cells will solely rely on MMEJ to complete CSR, would identify MMEJ candidates. In this context, genome-wide, loss-of-function, shRNA-mediated knock-down approach can be used to identify the genes that completely abrogate CSR in the NHEJ-deficient cell line, as these are potential MMEJ candidates.
4 CLASS SWITCH RECOMBINATION REQUIRES H2B UBIQUITINATION AND DEUBIQUITINATION.

All experiments and data analysis (with the exception of *ex vivo* mouse CSR assay) were performed by Shaliny Ramachandran.

*Ex vivo* mouse experiment was performed by Conglei Li.
4.1 ABSTRACT

B cells produce several classes of antibodies to mediate an effective humoral response. This process, termed class switch recombination (CSR), requires the generation and repair of DSBs at the antibody locus, and relies on the 53BP1-dependent DNA Damage Response (DDR). However, the molecular mechanism and function of DDR in CSR is not fully understood. To identify novel DDR factors in the 53BP1-signalling network of CSR, we developed and conducted a genome-wide screen for CSR factors. In this study, we have characterized one promising CSR candidate identified by the screen, the Drosophila enhancer of yellow 2 homolog (Eny2). Eny2 is a structural component of the deubiquitination module of the SAGA complex, which primarily deubiquitinates histone H2B at K120. We demonstrate that Eny2 likely acts with the SAGA complex to facilitate CSR downstream of AID-induced DSBs, and may be required for optimal DNAPK activation.

4.2 INTRODUCTION

Class switch recombination is the process by which B cells switch from expressing the IgM antibody to IgG, IgE or IgA. Each class of antibody mediates a different effector function. Critical for eliciting an effective immune response, CSR requires the enzyme activation-induced cytidine deaminase (AID), to catalyze the deamination of deoxycytidines at the immunoglobulin switch regions, specifically in the G1 phase of cell cycle (Schrader et al. 2007; Stavnezer et al. 2008). These AID-induced mutations are processed by the mismatch repair and base excision repair pathways, leading to the generation of staggered DSBs at the donor and acceptor switch regions (Schrader et al. 2009). These AID-induced DNA breaks instigate the 53BP1-dependent DDR, a signalling network akin to that elicited by irradiation-induced DNA damage (Manis et al.
DDR in turn can initiate the DNA repair program. Repair of the DSBs in a manner that places the acceptor switch region immediately adjacent to the recombined V(D)J segment, while deleting the intervening DNA, marks successful completion of CSR (Chaudhuri et al. 2004). This repair process is primarily completed during the G1 phase of cell cycle and largely depends on NHEJ, but can also rely on alternative end joining mechanisms (Yan et al. 2007).

DSBs are ubiquitously recognized by DDR factors. This type of DNA break can be recognized by DDR sensor complexes, including the Mre11-Rad50-Nbs1 (MRN)/ataxiatelangiectasia-mutated (ATM) complex; the Ku70/Ku80-DNA-PKcs complex; the ataxiatelangiectasia and Rad3-related (ATR)-ATR-interacting protein complex (Yang et al. 2003); and the more recently characterized Parp1 (Ali et al. 2012). Each sensor can initiate a cascade of signalling events leading to cell cycle arrest and DNA repair, and in the absence of successful repair, apoptosis. Specifically, the MRN/ATM complex has been identified to play a role in CSR (Lumsden et al. 2004; Reina-San-Martin et al. 2004; Reina-San-Martin et al. 2005). Upon recognition of the AID-induced DSB, ATM kinase catalyzes the phosphorylation of the S/TQ motif in an array of substrates, key among these substrates being histone variant, H2AX (Stiff et al. 2004). ATM-mediated phosphorylation of H2AX at S139 produces γH2AX, forming a docking site for MDC1 (Stewart et al. 2003; Stucki et al. 2005). MDC1 in turn binds to and further recruits Nbs1, concentrating the MRN/ATM complex at the break (Lukas et al. 2004; Lou et al. 2006). This leads to an amplification of the γH2AX signal, which can be visualized as distinct nuclear foci by fluorescence microscopy. ATM can also phosphorylate MDC1 on the S/TQ consensus site, leading to the recruitment of the E3 ubiquitin ligase, RNF8 (Huen et al. 2007; Kolas et al. 2007; Mailand et al. 2007). RNF8 mediates mono-ubiquitination of histone
H2A and H2AX leading to the requirement of another E3 ubiquitin ligase, RNF168, which further augments the ubiquitin levels at the break (Doil et al. 2009; Pinato et al. 2009; Stewart et al. 2009). RNF168-mediated H2A-K15 mono-ubiquitination is recognized by the ubiquitination-dependent recruitment motif of 53BP1, leading to the recruitment of 53BP1 (Fradet-Turcotte et al. 2013). 53BP1 localizes to the DNA break site by simultaneously binding the RNF168-mediated H2A-K15 mono-ubiquitin and the constitutively expressed histone H4 dimethylated-K20 (Fradet-Turcotte et al. 2013). 53BP1 is in turn phosphorylated by the master kinase, ATM, which leads to the recruitment of Rif1 (Silverman et al. 2004; Di Virgilio et al. 2013; Escribano-Diaz et al. 2013; Feng et al. 2013). 53BP1-Rif1 control the DNA repair pathway choice in G1 phase of cell cycle by preferentially promoting NHEJ (Chapman et al. 2013; Escribano-Diaz et al. 2013; Feng et al. 2013; Zimmermann et al. 2013). ATM, NBS1, Mre11, γ-H2AX, MDC1, RNF8, RNF168, 53BP1 and Rif1 belong to the 53BP1-dependent DDR pathway, and also play a role in CSR (Reina-San-Martin et al. 2003; Lumsden et al. 2004; Manis et al. 2004; Reina-San-Martin et al. 2004; Ward et al. 2004; Reina-San-Martin et al. 2005; Lou et al. 2006; Li et al. 2010; Ramachandran et al. 2010; Santos et al. 2010; Bohgaki et al. 2011; Chapman et al. 2013; Di Virgilio et al. 2013; Escribano-Diaz et al. 2013). 53BP1 mediates CSR by facilitating long-range DNA end joining (Difilippantonio et al. 2008; Dimitrova et al. 2008), and by blocking the DNA ends from resection, and promoting NHEJ (Bothmer et al. 2010; Bunting et al. 2010).

The established DDR pathway involves modifications of histones, but has been largely focused to histone H2A and variant H2AX. Recently, H2B has also been suggested to participate in the DDR (Moyal et al. 2011). Upon DNA damage, the RNF20/40 E3 ubiquitin ligase heterodimer is recruited to DNA breaks, where it gets retained in an ATM-dependent manner and catalyzes H2B-ubiquitination (Moyal et al. 2011). H2B-ubiquitin is thought to initiate
chromatin disassembly, providing access to DNA repair factors in NHEJ and HR to bind and complete repair (Fierz et al. 2011; Moyal et al. 2011). The RNF20/40 complex primarily mediates H2B-K120-mono-ubiquitination in mammals (and K123 in yeast) (Shiloh et al. 2011). H2B-K120/123-ubiquitination can then be engaged by the SAGA complex, which catalyzes the deubiquitination of H2B-K120/123 (Henry et al. 2003; Daniel et al. 2004). The SAGA complex has two enzymatic activities, deubiquitination and acetylation (Zhang 2003). Acetylation is mediated by the Gcn5-containing module, while deubiquitination is mediated by the Usp22 deubiquitinase, which requires Eny2 and Atnx7 as structural components in order to function (Zhao et al. 2008). RNF20/40-mediated H2B-K120 mono-ubiquitination and Eny2-dependent, SAGA-mediated H2B-K120 deubiquitination are known to play a role in transcription (Weake et al. 2008).

Intriguingly, a component of the SAGA complex, Eny2, was identified as a strong candidate in the microarray and in the sequencing-based analysis of the CSR screen above (Chapter 3). Furthermore, Eny2-shRNA (hairpin 3 in Figure 21) reduced CSR without affecting AID expression, suggesting that Eny2 may play a role in DDR or DNA repair. In this investigation, we demonstrated that Eny2 plays a role in CSR. Knockdown of Eny2 led to reduced CSR in CH12F3-2 and *ex vivo* mouse splenic B cells. We found that Eny2 acts downstream of AID, and a lack of Eny2 leads to G1 cell cycle arrest and apoptosis specifically in cells stimulated to undergo CSR. As expected, Eny2 is necessary for H2B-K120-deubiquitination in the absence of DNA damage, and especially upon irradiation-induced DNA damage. However, Eny2 does not appear to protect cells from irradiation-induced DNA damage, unlike its role in CSR. Eny2 likely functions within the context of H2B-deubiquitination to facilitate CSR, as we found that other factors in the pathway, including RNF20, RNF40 and
Atxn7, also affect CSR. Eny2 and H2B-deubiquitination may be required for optimal DNAPK activation and NHEJ.

### 4.3 MATERIALS AND METHODS

#### 4.3.1 In Vitro Cell Culture

CH12F3-2 cells were maintained and CSR assays were performed as described above in Chapter 2 (Ramachandran et al. 2010). Ligase IV\(^{-/-}\) CH12F3-2 cells were provided by Keifei Yu (Han et al. 2008). Briefly, cells were stimulated with 1 ng/mL recombinant human TGFβ1 (R&D systems), 10 ng/mL recombinant mouse IL-4 (R&D systems) and 2 µg/mL functional grade purified anti-mouse CD40 (eBiosciences), and analyzed by flow cytometry, as described below. Lentiviral shRNA constructs targeting 53BP1 (V2LMM 83391) and non-silencing negative control (RHS4346) were obtained from Open Biosystems and used as described above in Chapter 2 (Ramachandran et al. 2010). Lentiviral shRNA constructs and protocols for shRNA from TRC were provided by Jason Moffat (Moffat et al. 2006). shRNA constructs targeting the negative control of GFP (TRCN0000072181), Eny2 (TRCN0000086039 and TRCN0000086041), RNF40 (TRCN00000041048) and Atxn7 (TRCN0000104973) were used from TRC. CH12F3-2 cells were transduced with lentivirus for 24 hours, on 24 well plates for bulk transductions or diluted and plated on 96 well plates to obtain clones. Positively transduced cells were selected with 1 µg/mL puromycin for 3 days. For growth curve analysis, CH12F3-2 cells were diluted to a concentration of 1x10\(^5\) cells/ml and aliquoted in duplicate on a 96-well plate. At various time points, the numbers of live-trypan blue excluded cells were counted using a haemocytometer. ATM inhibitor (KU 55933) and DNAPK inhibitor (NU 7441) were obtained from Tocris Bioscience.
4.3.2 *Ex vivo* mouse experiments

Splenic B cells were purified from wild-type C57BL/6 mouse, using a negative selection mouse B cell enrichment kit (Stemcell technology). The cells were cultured in complete RPMI media with 25 ng/mL LPS for 24 hours, followed by lentiviral transduction in the presence of 8 ng/mL polybrene and LPS at a final concentration of 25 ng/mL for 24 hours. Positively transduced cells were then selected with 0.6 μg/mL puromycin and stimulated with 25 ng/mL IL4 for 4 days, and then analyzed by flow cytometry as described below.

4.3.3 Cell staining and Flow cytometry

For CSR analysis, CH12F3-2 cells were stained with PE conjugated anti-mouse IgA (Southern Biotech), and *ex vivo* mouse B cells were stained with PE conjugated anti-mouse IgG1 antibody clone A85-1 (BD Bioscience). For cell cycle analysis, cells were incubated with 10 μM BrdU for 1 hour followed by ethanol fixation overnight. Cells were then washed and incubated in 2 M HCl for 30 minutes, washed and stained with FITC conjugated anti-BrdU antibody clone PRB-1 (eBiosciences), and then washed and incubated in 20 μg/mL PI and 10 μg/mL RNAse A for 30 minutes. For apoptosis analysis, CH12F3-2 cells were surface stained for Annexin V using Annexin V-APC Apoptosis Detection Kit (eBiosciences). Stained cells were analyzed by FACSCalibur (BD Bio) and FlowJo software (Truestar Inc.).

4.3.4 RNA extraction, RT and semi-quantitative and qPCR analysis

RNA extraction was performed using Trizol (Invitrogen), followed by DNase treatment (Fermentas) and reverse transcription reactions using a Superscript III kit (Invitrogen) or Maxima kit (Thermo Scientific) to prepare cDNA. cDNA samples were diluted and subject to
PCR reactions for semi-quantitative RT-PCR or qPCR reactions. For semi-quantitative PCR, $\mu$-C$\mu$ and $\alpha$-Ca amplification primers are same as previously described (Muramatsu et al. 2000), Gapdh primers (Gapdh RTPCR For and Rev) can be found in Table 3. For qPCR analysis, primers for Gapdh (Gapdh qPCR For and Rev), Eny2 (Eny2 qPCR For and Rev), RNF20 (Rnf20 qPCR For and Rev), RNF40 (Rnf40 qPCR For and Rev) and Atxn7 (Atxn7 qPCR For and Rev) can be found on Table 3.

4.3.5 Plasmids

pFucci-S/G2/M Green vector (AM-V9016) was obtained from Amalgam. Eny2 was PCR-amplified from CH12F3-2 cDNA using Eny2 Clone For and Rev primers. PCR product was digested with HindIII and NotI restriction enzymes (NEB), and ligated into pCDNA3.1+ vector using T4 DNA ligase (NEB). shRNA-resistant Eny2 vector was generated by site-directed mutagenesis of the Eny2-pCDNA3.1+ plasmid, by PCR with Eny2-SDM For and Rev primers that introduced 7 silent mutations in the shRNA-complimentary sequence of Eny2 cDNA, followed by DpnI (NEB) digestion, ethanol precipitation and bacterial transformation. Primers used are listed in Table 3. All plasmids sequences were verified by the TCAG sequencing facility.

4.3.6 Plasmid Integration assay

Plasmid integration was performed by plasmid linearization and electroporation at 550V and 50 $\mu$F. For Eny2 reconstitution assays, shRNA-resistant Eny2 expression vector and control vector were linearized by BglII (NEB), electroporated, diluted and plated on 96 well plates to obtain clones. For blunt-end plasmid integration assay, pCDNA3.1+ plasmid was linearized with SspI
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</table>

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(NEB), electroporated plated on 96 well plates at various dilutions. Plasmid integration efficiency was determined by counting the number of expanding clones dividing by the total number of cells plated.

4.3.7 Irradiation assays
CH12F3-2 cell lines were irradiated with various doses of γradiation using a gammacell 1000 irradiator. For clonal survival assays, cells were subsequently plated at various dilutions in duplicate on 96 well plates, and survival was determined by counting the number of expanding clones normalized to plating efficiency. For apoptosis assays, cells were plated on 24 well plates and analyzed by Annexin V staining as described above. For western blot analysis, cells were harvested to prepare lysates at various time points.

4.3.8 Western blot analyses
γH2AX (Upstate), H2B-K120-ubiquitin (Millipore), Kap1-S824-phosphorylation (Cell signalling), p53-S15-phosphorylation, βactin (Sigma) antibodies were used as specified by the manufacturers’ protocols.

4.3.9 Statistical Analysis
Analyses were performed on GraphPad Prism. For Mann Whitney tests and linear regression analysis, p values of 0.05 or less were considered significant.
4.4 RESULTS

4.4.1 Eny2 acts downstream of AID in CSR

To understand how DDR facilitates CSR at a molecular level, we developed and conducted a screen for novel CSR factors from which we identified potential DDR and repair factors (Chapter 3). Briefly, we performed a loss-of-function, genome-wide screen to identify CSR factors, and subsequently screened a subset of strong CSR candidates for normal AID expression, to identify possible DDR and repair factors. Eny2 was identified as a strong CSR candidate in the microarray and in the sequencing based screen analysis (Chapter 3), and knock-down of Eny2 did not affect AID expression (Hairpin 3 in Figure 22). We first determined whether Eny2 is indeed a CSR factor, using the CH12F3-2 mouse B cell line. We ascertained whether shRNA-mediated knock-down of Eny2 led to a decrease in CSR. Of the five Eny2-targeting shRNA lentiviral vectors tested, shEny2-39 and shEny2-41 mediated knock-down of Eny2 transcript by 50% and 80%, respectively, as compared to a control hairpin that targets GFP (shGFP-81) (Figure 24A). The shEny2-39 and shEny2-41 hairpins led to a 30% and 70% decrease in CSR to IgA in CD40/II-4/TGFβ (CIT)-stimulated CH12F3-2 cells, respectively (Figure 24B). Given that shEny2-39-mediated knock-down of Eny2 is minimal, we carried out subsequent analysis using the shEny2-41 lentiviral vector. ShEny2-41 maintains knock-down of CSR in CH12F3-2 cells over a 3-day period (Figure 24C). The effect of this hairpin on CSR is independent of off-target effects, as reconstitution of a shEny2-41-knock-down clone with a shRNA-resistant Eny2-cDNA expression vector, rescued both Eny2 transcript levels and CSR (Figures 24D and 24E). On the other hand, reconstitution with an empty vector did not rescue Eny2 transcript levels or CSR (Figure 24D). Furthermore, ex vivo spleen mouse B cells
Figure 24. ShRNA-mediated knock-down of Eny2 reduces CSR. (A) Eny2 transcript levels relative to Gapdh were determined by qPCR analysis from CH12F3-2 cells with Eny2-shRNA hairpins, shEny2-39 and shEny2-41, and compared against GFP-targeting control shRNA, shGFP-81. (B) CSR analysis on CH12F3-2 cells with two Eny2-shRNA, shEny2-39 and shEny2-41, compared to control shGFP-81. (C) CSR time course analysis of CH12F3-2 cells with Eny2-shRNA (shEny2-41) compared to control-shRNA (shGFP-81). Statistical significance was tested by two-tailed, Mann-Whitney test at each time point. (D) CSR analysis of clones derived from a CH12F3-2 shEny2-41-knock-down clone reconstituted with pCDNA3.1 vector control (pCDNA3.1) that maintained low Eny2 transcript levels, or clones derived from the CH12F3-2 shEny2-41-knock-down clone reconstituted with shRNA-resistant Eny2 expression vector (Eny2-pCDNA3.1) that rescued Eny2 transcript levels. CSR was compared to a parental CH12F3-2 clone (clone I). Statistical significance was tested by two-tailed Mann Whitney test. (E) Correlation of CSR and Eny2 transcript levels on clones derived from CH12F3-2 shEny2-41 clone reconstituted with either pCDNA3.1 control vector or shRNA-resistant Eny2 expression vector. Grey circles are clones reconstituted with pCDNA3.1, white circles are clones...
reconstituted with Eny2-pCDNA3.1, and the black circle is parental CH12F3-2 clone I. Statistical significance was tested by linear regression analysis. (F) CSR analysis of ex vivo mouse spleen B cells with Eny2 shRNA (shEny2-41) compared to control-shRNA (shGFP-81).
transduced with shEny2-41 also demonstrated a reduced ability to undergo CSR, further validating Eny2 as a CSR factor (Figure 24F).

To confirm that Eny2 acts downstream of AID (based on observations from secondary screen in Chapter 3), we determined whether AID protein expression or germline transcription, both of which are critical for CSR, were affected by shEny2-41. As shown in Figures 25A and 25B, upon stimulation with the CIT cocktail, there was no significant difference in AID expression or germline transcripts in CH12F3-2 cells containing the shEny-41 or shGFP-81 vectors. Interestingly, we found that while unstimulated shEny2-41 cells grew at a rate similar to control cells, upon stimulation with the CIT cocktail, there was a significant decrease in the ability of shEny2-41 cells to multiply in comparison to the controls (Figures 25C and 25D). To assess whether knock-down of Eny2 affects cell cycle progression, we analysed the cell cycle profile of cells stimulated for 24 hours using BrdU/PI staining, and found that compared to control cells, shEny2-41 cells maintained a higher level of G1 cells and a lower level of S-phase cells, suggesting that cells with reduced Eny2 undergo G1-cell cycle arrest upon stimulation (Figure 25E). Furthermore, we found that stimulation also led to a significant increase in apoptosis of shEny2-41 cells when compared to control cells at 48 hours post-stimulation (Figure 25F). Taken together, these data suggest that upon stimulation, AID-induced DSBs form in G1, but in Eny2-deficient cells this leads to G1 cell cycle arrest and apoptosis most likely due to an inability to repair the DSBs. Collectively, this implicates Eny2 as a factor acting downstream of AID, likely in DDR and/or the DNA repair phase of CSR.
Figure 25. Eny2 likely acts downstream of AID in CSR. (A) Western blot analysis on AID expression relative to β-actin, in CH12F3-2 cells with Eny2-shRNA (shEny2-41) compared to control-shRNA (shGFP-81). Cells were stimulated with the CIT-cocktail for 24 hours. Statistical significance tested by two-tailed, Mann Whitney test. (B) Semi-quantitative RT-PCR analysis on germline transcription through the μ switch region (Iμ-Cμ) and α switch region (Iα-Cα) with Gapdh loading control, on CH12F3-2 cells with Eny2-shRNA (shEny2-41) compared to control-shRNA (shGFP-81). Cells were stimulated with CIT for 24 hours. The blot is representative of two replicates tested at both, 24 hours and 48 hours post-stimulation. Growth of unstimulated (C) and CIT-stimulated (D) CH12F3-2 cells with Eny2-shRNA (shEny2-41) compared to control-shRNA (shGFP-81) over time. (E) Cell cycle profile of CH12F3-2 cells with Eny2-shRNA (shEny2-41) compared to control-shRNA (shGFP-81) determined by BrdU/PI staining at 24 hours post-CIT-stimulation. Statistical significance was tested by two-tailed, Mann Whitney test. (F) Apoptosis levels of CH12F3-2 cells with Eny2-shRNA (shEny2-41) compared to control-shRNA (shGFP-81) as determined by Annexin V staining at 48 hours post-CIT-stimulation. Statistical significance tested by two-tailed, Mann Whitney test.
4.4.2 Eny2 mediates H2B-K120-deubiquitination upon DNA damage, independent of 53BP1

Previous work demonstrated that Eny2 provides a structural role to the SAGA complex, which mediates H2B-K120 deubiquitination during transcription. We first ascertained whether Eny2 carries out a similar function in CH12F3-2 cells. As such, we tested the steady-state levels of H2B-K120-ubiquitin in CH12F3-2 cells. Indeed, shEny2-41 led to a 5-fold increase in steady-state H2B-K120-ubiquitin (Figures 26A and 26B). Moreover, we tested the steady-state H2B-K120-Ub levels in clones expressing different levels of Eny2 transcript, and found that there is a strong inverse correlation between Eny2 transcript levels and steady-state H2B-K120-ubiquitin levels (Figure 26C), suggesting that Eny2 is required for H2B-K120-deubiquitination in CH12F3-2 cells.

To determine whether Eny2 plays a role in DDR, we tested whether Eny2-deficiency causes radiation sensitivity. Irradiation of asynchronous cells did not affect clonal survival or apoptosis of shEny2-41 cells when compared to control cells (Figures 27A and 27B), suggesting that Eny2-knock-down does not affect radiation sensitivity. Given that Eny2-knock-down led to increased G1 cell cycle arrest and apoptosis upon CSR stimulation (Figures 27E and 27F), and given that AID-induced mutations occur in G1 cell cycle, we postulated that Eny2 may confer radiation resistance to cells specifically during the G1 cell cycle. To test this notion, we utilized the mAG1-hGEM S/G2/M-labeling fluorescent ubiquitination-based cell cycle indicator (Fucci), which expresses an Azami-Green1 (mAG1) fluorescent protein fused to part of the human Geminin protein, which accumulates in S/G2/M-phases of cell cycle. By transfected cells with this expression vector and sorting for mAG1-negative cells (i.e. G1 cells), we enriched for G1 cells by up to ~65% G1, which is considerable given only ~30% of unsorted CH12F3-2 cells are in G1 (data not shown). However, irradiation of mAG1-negative CH12F3-2 shEny2-41 cells that
Figure 26. Eny2 is important for H2B-K120-deubiquitination in CH12F3-2 cells.
Representative (A) and compiled (B) western blot analysis on steady-state levels of H2B-K120-ubiquitin relative to β-actin, in CH12F3-2 cells with Eny2-shRNA (shEny2-41) compared to control-shRNA (shGFP-81). Statistical significance tested by two-tailed Mann Whitney test. (C) Correlating H2B-K120-ubiquitin levels to Eny2 transcript levels on clones derived from a CH12F3-2 shEny2-41 clone reconstituted with either pCDNA3.1 control vector or shRNA-resistant Eny2 expression vector from Figure 1D and 1E above. Grey circles are clones reconstituted with pCDNA3.1, white circles are clones reconstituted with Eny2-pCDNA3.1, and the black circle is parental CH12F3-2 clone I. Statistical significance was tested by linear regression analysis.
Figure 27. Eny2 mediates H2B-K120-deubiquitination in DDR, but is independent of the 53BP1-pathway. Clonal survival assays on asynchronous (A), or S/G2/M-Fucci excluded (C), CH12F3-2 cells, with Eny2-shRNA (shEny2-41) or control-shRNA (shGFP-81), exposed to varying doses of γ-radiation. Apoptosis assays, as determined by Annexin V staining, on asynchronous (B) or S/G2/M-Fucci excluded (D) CH12F3-2 cells, with Eny2-shRNA (shEny2-41) or control-shRNA (shGFP-81), exposed to varying doses of γ-radiation. CH12F3-2 cells with Eny2-shRNA (shEny2-41) or control-shRNA (shGFP-81) were exposed to 8 Grays of γ-radiation, and harvested at various time points for western blot analysis of H2B-K120-ubiquitin levels relative to β-actin. Representative blot (E) and compiled data (F) are displayed. A CH12F3-2 clone with 53BP1-shRNA (sh53BP1) and a control clone with non-silencing shRNA (Non-silencing) were exposed to 8 Grays of γ-radiation, and harvested at various time points for western blot analysis of H2B-K120-ubiquitin levels relative to β-actin.
were primarily in the G1 phase of the cell cycle, also did not affect clonal survival and apoptosis as compared to mAG1-negative control cells (Figure 27C and 27D). This suggests that Eny2 may not have a significant role in the cellular response to irradiation-induced DNA damage. Interestingly, shEny2-41 cells acquire very high accumulation of H2B-K120-ubiquitin starting about 2 hours post-irradiation when compared to control cells (Figures 27E and 27F). Together, these data suggest that Eny2 is required for deubiquitination of H2B-K120-ubiquitin upon γ-irradiation, but unlike its role in CSR, Eny2 may not be as important for repair of irradiation-induced DNA damage.

To further characterize H2B-K120-ubiquitin in DDR, we tested the kinetic profile of H2B-K120-ubiquitin in parallel with Kap1-phosphorylation and γH2AX formation. While γH2AX and Kap1-phosphorylation formed immediately upon irradiation (Figures 28A and 28B), H2B-K120-ubiquitin formation lagged behind, with the signal becoming visible at about 1.5 hours post-irradiation and peaking at 2-3 hours (Figure 28C). This suggests that H2B-K120-ubiquitin is part of a later phase of DDR, unlike Kap1-phosphorylation and γH2AX, and may either be dependent on the 53BP1 pathway or be an independent arm of DDR. To test whether H2B-K120-ubiquitin requires the 53BP1 pathway, we determined whether knock-down of 53BP1 affected H2B-K120-ubiquitin accumulation upon irradiation. Although a ~5-fold reduction in 53BP1 protein completely abrogated CSR (Figure 8A, 8E and 8F), there was no difference in H2B-K120-ubiquitin upon irradiation of sh53BP1 cells in comparison to control cells, suggesting that H2B-K120-ubiquitin may be independent of the 53BP1 arm, or upstream of 53BP1 in DDR (Figure 27G).
Figure 28. Time-course analysis of irradiation-induced post-translational modifications involved in DDR. CH12F3-2 control (shGFP-81) cells were exposed to 8 Grays of γ-radiation, and harvested at various time points for western blot analysis of γH2AX (A), Kap1-Ser824-phosphorylation (B) and H2B-K120-ubiquitin (C), all relative to β-actin.
4.4.3 H2B-K120-ubiquitination/deubiquitination is important for CSR.

We hypothesized that Eny2 might also function in H2B-K120-deubiquitination in CSR. To examine this, we first tested whether other factors in the H2B-K120-ubiquitination/dequibitionation pathway are required for CSR. First, we identified a shRNA hairpin targeting Atxn7 that decreased Atxn7 transcript levels (Figure 29A). Interestingly, akin to Eny2 knock-down, Atxn7-shRNA lead to an increased accumulation of irradiation-induced H2B-K120-ubiquitin, and most importantly, also decreased CSR (Figure 29B and 29E). This suggests that Eny2 likely functions with the Atxn7-containing, SAGA complex in DDR and CSR. To compare the extent of H2B-K120-ubiquitin in cells with knock-down of Eny2 relative to control cells, western blot analysis was performed on Eny2-knock-down cell lysates loaded in 10-fold serial dilutions along with GFP-shRNA control cells (Figure 29E). These blots were exposed for short (60 second exposure) in which H2B-K120-ubiquitin is apparent in cells with shEny2-41 but not control cells, and a long (400 second exposure) in which trace amounts of H2B-K120-ubiquitin is visible in the control cells, highlighting that while H2B-K120-ubiquitination occurs in irradiated control cells, the accumulation of H2B-K120-ubiquitin is significantly higher in Eny2-knock-down cells.

The SAGA complex acts downstream of RNF20/40 in the H2B-ubiquitination pathway, and so we tested whether RNF20/40 also plays a role in CSR. We identified a shRNA targeting RNF40 that decreased RNF40 transcript levels (Figure 29C). This RNF40-shRNA also lead to decreased irradiation-induced H2B-K120-ubiquitin accumulation, and most importantly, also reduced CSR, thus implicating RNF20/40 in CSR (Figure 29D and 29F). Together, these data suggest that RNF20/40 and the SAGA complex, which are factors involved in the H2B-ubiquitination/deubiquitination pathway, play a role in CSR.
Figure 29. The H2B-K120-ubiquitination/deubiquitination pathway facilitates CSR. (A) qPCR analysis of CH12F3-2 cells with Atxn7-shRNA (shAtxn7-73) compared to control-shRNA (shGFP-81). (B) CH12F3-2 cells with Atxn7-shRNA (shAtxn7-73), Eny2-shRNA (shEny2-41) as positive control, and negative control-shRNA (shGFP-81), were exposed to 8 Grays of γ-radiation, and harvested at 3 hours for western blot analysis of H2B-K120-ubiquitin levels relative to β-actin. (C) qPCR analysis of CH12F3-2 cells with RNF40-shRNA (shRNF40-48) compared to control-shRNA (shGFP-81). (D) CH12F3-2 cells with RNF40-shRNA (shRNF40-48) and control-shRNA (shGFP-81) were exposed to 8 Grays of γ-radiation, and harvested at various time points for western blot analysis of H2B-K120-ubiquitin levels relative to β-actin. (E) Comparing H2B-K120-ubiquitin levels between CH12F3-2 cells with Eny2-shRNA (shEny2-41) and control-shRNA (shGFP-81) at 3 hours after 8 Gy IR. shEny-41 cell lysates were loaded in 10-fold serial dilutions, and blots were exposed for short (60 seconds) and long (400 second) exposure. (F) CSR analysis on CH12F3-2 cells with Atxn7-shRNA (shAtxn7-73), RNF40-shRNA (shRNF40-48) compared to control-shRNA (shGFP-81). (G) CSR analysis on CH12F3-2 clones with either normal steady-state levels of H2B-K120-ubiquitin or high steady-state levels of H2B-K120-ubiquitin. Grey circles are clones derived from Eny2-knock-down clone reconstituted with pCDNA3.1, white circles are clones reconstituted with Eny2-pCDNA3.1, and the black circle is parental CH12F3-2 clone I from Figure 1D and 1E above. Statistical significance was tested by two-tailed, Mann Whitney test.
To further examine whether H2B-K120-ubiquitin is linked to CSR, we determined whether there was a correlation between H2B-K120-ubiquitin levels and CSR. CH12F3-2 clones with increasing Eny2 transcript levels had an inversely correlating decrease in H2B-K120-ubiquitin steady-state levels (Figure 26C). Taking advantage of these clones, we compared the clones containing wild-type or low steady-state H2B-K120-ubiquitin levels, against clones that contained high steady-state H2B-K120-ubiquitin (at least ~ 2.5-fold above parental levels) for their ability to switch. Indeed clones with wild-type or low steady-state H2B-K120-ubiquitin maintained wild-type levels of CSR, while clones with high steady-state H2B-K120-ubiquitin demonstrated significantly reduced levels of CSR (Figure 29G). Indeed, this inverse correlation between H2B-K120-ubiquitin and CSR suggests that H2B-deubiquitination may be important to CSR.

4.4.4 Accumulation of H2B-K120-ubiquitin may inhibit DNAPK activation

Next, we tested whether Eny2 and H2B-K120-ubiquitin may directly affect the DDR, by examining various DDR signalling events in Eny2-knock-down cells. Specifically, we tested whether shEny2-41 affected the induction of Kap1-Ser824-phosphorylation, p53-Ser15 phosphorylation and γH2AX formation upon irradiation. Interestingly, shEny2-41 did not affect Kap1-Ser824 or p53-Ser15 phosphorylation, both of which are post-translational modifications that rely solely on ATM (Figures 30A and 30B). However, shEny2-41 modestly reduced the formation of γH2AX, a phosphorylation event that can rely on either ATM or DNAPK in irradiation-induced DDR (Figure 30C). This suggests that knock-down of Eny2 may interfere with optimal DNAPK activity. To delineate the Eny2 and DNAPK relationship further, we tested γH2AX induction upon irradiation in cells pre-incubated with either ATM inhibitor (ATMi) or
Figure 30. Accumulation of H2B-K120-ubiquitin may inhibit DNAPK. CH12F3-2 Eny2-shRNA (shEny2-41) cells and control-shRNA (shGFP-81) cells were exposed to 8 Grays of γ-radiation, and harvested at various time points for western blot analysis of p53-Ser15 phosphorylation (A), Kap1-Ser824-phosphorylation (B) and γH2AX formation (C), all relative to β-actin. CH12F3-2 Eny2-shRNA (shEny2-41) cells and control (shGFP-81) cells were exposed to 8 Grays of γ-radiation, and harvested at various time points for western blot analysis of γH2AX formation relative to β-actin, in the presence of either DNAPKi (D) and (E), or ATMi (F) and (G). (D) and (F) are representative blots and (E) and (G) are the compiled data. (H) Blunt-ended plasmid integration efficiencies of CH12F3-2 and Ligase IV-deficient (Ligase IV−) cells containing either Eny2-shRNA (shEny2-41) or control-shRNA (shGFP-81).
DNAPK inhibitor (DNAPKi). Cells pre-incubated with ATMi will largely depend on DNAPK activity to produce γH2AX upon irradiation. Similarly, cells pre-incubated with DNAPKi will largely rely on ATM to produce the γH2AX signal upon irradiation. Intriguingly, in cells with ATMi, shEny2-41 led to reduced γH2AX signal when compared to control cells (Figures 30D and 30E), while DNAPKi showed no difference between the shEny2-41 and control cells after irradiation (Figures 30F and 30G). Together, this suggests that knock-down of Eny2 may be affecting DNAPK recruitment and/or activation, hence affecting NHEJ. Furthermore, we find that while the difference between blunt-ended plasmid integration efficiency in Eny2-knock-down and control cells is not statistically significant, there was a trend in the Eny2-knock-down cells to display a decreased ability to integrate blunt-ended plasmid. Interestingly, there is no difference between shEny2-41 and control cells in Ligase IV-deficient CH12F3-2 cells, suggesting a possible epistasis between Eny2 and NHEJ (Figure 30H).

4.5 DISCUSSION

H2B ubiquitination has been suggested to primarily facilitate chromatin disassembly (Fierz et al. 2011), providing access to downstream DNA repair factors to repair the DSBs (Shiloh et al. 2011). While RNF20/40 functions downstream of ATM, this H2B ubiquitination pathway is independent from the ATM-MDC1-RNF8 pathway, suggesting that H2B-ubiquitination and MDC1-RNF8-mediated DDR are two distinct arms of the ATM-signalling cascade (Moyal et al. 2011). In a similar manner, we showed that H2B-ubiquitination is independent of 53BP1, which acts downstream RNF8. Our findings further demonstrate that ATM orchestrates the DDR by initiating multiple branches of signalling pathways. While the MDC1-RNF8-53BP1 pathway promotes NHEJ, the RNF20/40 and Eny2-SAGA complex-mediated H2B-
ubiquitination/deubiquitination pathway functions distinctly, possibly in disassembling chromatin and providing DSB access to the DNA repair factors recruited by the 53BP1-dependent pathway.

A surprising finding was that while Eny2-knock-down caused increased G1 cell cycle arrest and apoptosis upon CSR stimulation, irradiation of both asynchronous and ~65% G1-sorted cells neither affected clonal survival nor apoptosis. This finding should first be confirmed using a more homogenous population of G1 cells, which may be achieved using the mAG1-hGEM-S/G2/M Fucci marker in conjunction with the mKO2-Cdt1-G1 Fucci marker. Alternatively, it is possible the Eny2 does not confer protection from irradiation-induced apoptosis specifically in CH12F3-2 mouse B cells. Hence, the role of Eny2 in irradiation-induced clonal survival and apoptosis must be tested in other cell lines, including U2OS. If Eny2 is not important for radiation resistance, this suggests that Eny2 may exhibit differential roles in the ubiquitous DDR pathway, versus the DDR pathway in CSR. We speculate that AID expression may cause the CH12F3-2 B cells to be more prone to apoptosis in comparison to the cells cycling in culture, which are used for irradiation. Indeed, it has been previously demonstrated that AID-/- mice show significantly reduced apoptosis of germinal centre B cells (Zaheen et al. 2009). Given the increased tendency to undergo apoptosis, Eny2-knock-down cells that are unable to repair the AID-induced damage may immediately resort to death. However, cells cycling in culture may be able recover from DNA damage, even in Eny2-knock-down.

Previous studies that focused on RNF20/40 demonstrated that H2B-ubiquitination upon DNA damage is important for both the HR and NHEJ repair pathways (Moyal et al. 2011). We observed that Eny2 and H2B-deubiquitination displays epistasis with NHEJ, although we have
not tested whether Eny2-mediated H2B-deubiquitination also plays a role HR. This can be tested using *in vitro* NHEJ and HR substrates.

Intriguingly, we observed that Eny2 knock-down may potentially affect DNAPK activation. It is possible that H2B-deubiquitination is required for DNAPK recruitment. Alternatively, DNAPK enzymatic activity may be affected in the absence of H2B-deubiquitination. DNAPKcs contains two autophosphorylation clusters, an ABCDE cluster associated with increased DNA end processing and a PQR cluster associated with decreased end processing (Meek *et al.* 2008). DNAPKcs autophosphorylation is required for efficient DNA repair, and in the absence of autophosphorylation, the DNAPKcs is retained at the unrepaired break, suggesting that autophosphorylation can cause DNAPKcs dissociation from the break (Uematsu *et al.* 2007). It is thought that autophosphorylation mediates DNAPKcs association and dissociation from the break and that DNAPKcs dynamics may be important for NHEJ (Cui *et al.* 2005). In particular, S2056 autophosphorylation within the PQR cluster occurs upon irradiation-induced DNA damage, is important for NHEJ, and is attenuated in S-phase cells (Chen *et al.* 2005). Investigating S2056 autophosphorylation in Eny2-deficient cells may provide further insights on the link between Eny2 and DNAPKcs. Observing an effect on DNAPKcs S2056 autophosphorylation in Eny2-knock-down cells would suggest that Eny2 may be important for the recruitment and/or activation of DNAPKcs, which in turn can affect NHEJ. Consistent with this notion, we observed a possible epistasis between Eny2 and NHEJ using a plasmid integration assay, implicating that Eny2 is involved in NHEJ and not MMEJ. However, this can be tested using an *in vitro* MMEJ substrate, or by determining the extent of microhomology usage in switch junctions of Eny2-knock-down cells (Stavnezer *et al.* 2010).
H2B-ubiquitination is also considered a prerequisite for histone H3 trimethylation at K4 (H3K4me3) (Chandrasekharan et al. 2010). H3K3me3 has been linked to DDR (Faucher et al. 2010), and to CSR (Daniel et al. 2010; Stanlie et al. 2010). In the context of CSR, H3K4me3 is associated with germline transcripts and is suggested to be important for AID targeting and switch region DSB formation (Daniel et al. 2010; Stanlie et al. 2010; Begum et al. 2012). However, we observed no difference in germline transcripts, an increase in G1 cell cycle arrest and an increase in apoptosis upon stimulation of Eny2-knock-down cells, suggesting that AID-induced DSB is likely unaffected by Eny2 deficiency. H3K4me3 has also been linked to PTIP (Cho et al. 2007), and PTIP has previously been linked to CSR (Daniel et al. 2010; Schwab et al. 2011). This study found that PTIP can act downstream of DSBs, and may be involved in chromatin modification and accessibility (Daniel et al. 2010). Furthermore, PTIP is known to interact with 53BP1 (Jowsey et al. 2004; Wu et al. 2009; Callen et al. 2013), suggesting that the 53BP1-PTIP interaction may be linked to H2B-ubiquitination and H3K4me3. However, recent work suggested that the 53BP1-PTIP interaction is dispensable for CSR (Callen et al. 2013), making it unlikely that H3K4me3-PTIP-53BP1 association is important to CSR. Further investigation is needed to understand whether H2B-ubiquitination directly affects H3K4me3 at AID-induced DSBs and whether this is linked to CSR, either upstream or upstream of AID.
5 DISCUSSION AND FUTURE DIRECTIONS

5.1 RNF8 and RNF168 play a role in CSR.

Using the *in vitro* CH12F3-2/shRNA system that we established, we demonstrated that the E3 ubiquitin ligasae cascade in DDR, composed of RNF8 and RNF168, facilitate CSR. By using a knock-down approach, we showed that reducing RNF8 and RNF168 protein levels caused a correlating reduction in CSR. Subsequent *in vivo* mouse gene knock-out models confirmed our findings. RNF8 knock-out mice displayed a 50-70% decrease CSR, and RNF168 knock-out mice displaye a 40-50% decrease CSR (Li *et al.* 2010; Santos *et al.* 2010; Bohgaki *et al.* 2011), further validating our findings from an *in vitro* system, using an *in vivo* system.

5.2 DDR does not form a linear signalling cascade in CSR.

While factors in the 53BP1-dependent DDR pathway, including ATM, Mre11, NBS1, γ-H2AX, MDC1, RNF8, RNF168, 53BP1 and Rif1, have all been demonstrated to play a role in CSR, in all likelihood, this cascade forms part of a network rather than a linear succession of signalling events. Within this network, the significance and functional contribution of each protein to CSR differs, possibly due to functional redundancy and the ability of other factors within the network to compensate for the lack of certain factors. *In vivo* studies using gene-deletion mouse models, have revealed that each of the DDR factors involved do not equally contribute to CSR. ATM-, MRN-, and H2AX-deficiency leads to a ~70% reduction in CSR (Reina-San-Martin *et al.* 2003; Lumsden *et al.* 2004; Reina-San-Martin *et al.* 2004; Reina-San-Martin *et al.* 2005), MDC1-deficiency leads to 25%-50% reduction (Lou *et al.* 2006), RNF8-deficiency causes 50-70% decrease (Li *et al.* 2010; Santos *et al.* 2010), RNF168-deficiency has
40-50% (Bohgaki et al. 2011), 53BP1-deficiency leads to the most profound effect, reducing CSR by ~90% reduction (Manis et al. 2004; Ward et al. 2004), and Rif1 causes 80-90% reduction (Chapman et al. 2013; Di Virgilio et al. 2013). It is possible that in certain studies, the use of gene-trap knock-out mice allowed for residual levels of protein expression, leading to a less pronounced effect on CSR (Ramachandran et al. 2010). Alternatively, functional redundancy between certain proteins can also explain the reduced effects. For instance, given that 53BP1 and Rif1 exhibit the most drastic effects on CSR, it is likely that these factors are absolutely crucial to the process. As the upstream factors, ATM, MRN, H2AX, MDC1, RNF8 and RNF168 do not exhibit the same degree of effect on CSR, it is plausible that there are alternative methods of 53BP1-Rif1 recruitment and activation leading to CSR. For instance, low levels of 53BP1 can bind to H4K20me2 in a MDC1-RNF8-RNF168-independent fashion (Fradet-Turcotte et al. 2013), and this recruitment may account for the reduced contribution of MDC1-RNF8-RNF168 to CSR. 53BP1-Rif may be critical, either because these proteins have multiple molecular functions in CSR and/or they do not exhibit functional redundancies with any other proteins.

Recent work has shown that 53BP1-Rif1 promote NHEJ, while blocking DNA-resection-dependent pathways (Chapman et al. 2013; Di Virgilio et al. 2013; Escribano-Diaz et al. 2013; Feng et al. 2013; Zimmermann et al. 2013), and so 53BP1 and Rif1 may be considered as a bona fide NHEJ factors. However, while 53BP1-deletion leads to 90% reduction in CSR (Manis et al. 2004; Ward et al. 2004), Ligase IV- or XRCC4- deletion only leads to 50-70% reduction (Yan et al. 2007), suggesting that 53BP1 may also have NHEJ-independent functions. Moreover, switch junctions of 53BP1-deficient B cells do not display the increased use of microhomology that is observed with Ligase IV-deficiency (Manis et al. 2004). While this suggests that 53BP1 could
potentially assist alternative-end joining mechanisms like MMEJ, the observation that 53BP1 blocks DNA resection, and thus microhomology-based repair pathways, provides opposing evidence (Bothmer et al. 2010; Bunting et al. 2010), creating a subject of controversy. Delineating the exact molecular link between 53BP1-Rif and NHEJ and a possible relation with MMEJ would provide a better understanding of the interconnections between these pathways. Specifically, creating a NHEJ- and 53BP1- double-deficient system, and investigating the extent of microhomology usage in the absence of both NHEJ and 53BP1, may provide further insights into the role of 53BP1 in NHEJ.

5.3 H2B-ubiquitination in DDR and repair.

In addition to investigating aspects of the 53BP1-dependent DDR, we have also characterized a 53BP1-independent aspect of DDR, H2B-ubiquitination. While we and others have demonstrated that H2B-ubiquitination occurs independently of MDC1, RNF8 and 53BP1 (Moyal et al. 2011), it is, however, possible that H2B-ubiquitination/deubiquitination may affect aspects of the MDC-RNF8/168-53BP1 pathway. While the recruitment of MDC1, RNF8 and 53BP1 was not affected by depletion of the H2B-ubiquitinase, RNF20, the disappearance of γH2AX and 53BP1 foci was at a slower rate (Moyal et al. 2011). This persistence of γH2AX and 53BP1 is likely due to lack of DNA repair. However, there might be molecular links between H2B-deubiquitination and the 53BP1-dependent DDR pathway. Future work examining 53BP1 recruitment to, and dissociation from DNA breaks, by foci formation assays in the context of persisting H2B-ubiquitination upon irradiation of Eny2-depleted cells, can start underpinning any possible links between these two arms of ATM signalling.
Interestingly, our work has suggested that H2B-deubiquitination may be linked to DNAPKcs recruitment and/or activation. It is possible that either H2B-ubiquitination actively blocks DNAPKcs or H2B-deubiquitination promotes DNAPKcs activation. Firstly, whether H2B-ubiquitin specifically affects DNAPKcs recruitment and/or activation must be investigated by foci-formation analysis on DNAPKcs, and the activated form of DNAPKcs, which is autophosphorylated at S2056 in human cells (Chen et al. 2005). Subsequent studies can focus on the molecular mechanism linking H2B-ubiquitination to DNAPKcs activation, possibly by observing foci formation kinetics of H2B-ubiquitin and phosphorylated DNAPKcs, and by in vitro biochemical studies.

5.4 Generating an in vivo mouse model to investigate H2B-ubiquitination.

The findings from our in vitro studies should be further corroborated using in vivo systems. Generating a mouse model in which Eny2 is deleted at the genetic level would be ideal. However, given the potential role of Eny2 in transcription, it is likely that a complete deletion would cause embryonic lethality. Indeed, Atanassov et al. attempted to create a Gcn5 knock-out mouse (Atanassov et al. 2009). Gcn5 is a component of the histone acetyltransferase module of the SAGA complex (Zhang 2003). Gcn5 deletion leads to embryonic lethality at embryonic day 8.5, due to increased apoptosis (Atanassov et al. 2009). However, embryos with a catalytically-dead Gcn5 not only survive longer, but also do not display the increased apoptosis seen in Gcn5-deletion, suggesting that the embryonic lethality at day 8.5 is not due to histone acetyltransferase function of Gcn5 (Atanassov et al. 2009). It has been shown that in order to maintain the deubiquitination function, both the acetylation and deubiquitination modules need to associate and form a complete SAGA complex (Atanassov et al. 2009). Given that the enzymatic function
requires all the structural components of the complex, it is very likely that akin to Gcn5, deletion of Eny2 will cause embryonic lethality. However, generating conditional knock-out mice using the Cre-LoxP system, in which Eny2 is specifically deleted in either the B cell compartment or germinal center B cells would be a valuable tool. In addition to Eny2-deletion, which would eliminate H2B-deubiquitination, creating a conditional RNF20 knock-out mouse, which would eliminate H2B-ubiquitination, would provide further insights. Briefly, if the mice with complete knock-out of Eny2 are viable, it would be most interesting to test radiation sensitivity, genome stability, cancer predisposition, V(D)J recombination and CSR in these mice. However, if Eny2 is specifically deleted in the B cell compartment then the role of Eny2 in V(D)J recombination and CSR would be investigated.
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