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ROLE OF DNA REPAIR IN CLASS SWITCH RECOMBINATION AND SOMATIC HYPERMUTATION

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Stockholm 2011

Published by Karolinska Institutet. Printed by Repro Print AB / Perssons Offsettryck AB.

ISBN 978-91-7457-521-7

To my family

ABSTRACT

Class switch recombination (CSR) and somatic hypermutation (SHM), occurring in the germinal center, are two important processes for B cell development. Both are initiated by activation-induced cytidine deaminase (AID), through deamination of the C residues in the variable and switch regions of the immunoglobulin locus, resulting in either in single stranded or double stranded DNA breaks. At least three pathways (nonhomologous end joining (NHEJ), base excision repair (BER), and mismatch repair (MMR)) have been implicated in processing, repair and ligation of the DNA breaks during CSR and SHM. However, the way in which these pathways are regulated and coordinated to mediate CSR and /or SHM is still not well understood.

To explore the potential role of several proteins in CSR and SHM, including Ataxia-telangiectasia and Rad3-related (ATR), Artemis, Cernunnos and the Mre11-Rad50-NBS1 (MRN) complex, CSR junctions and SHM patterns in the immunoglobulin variable region gene were analyzed in patients with deficiency in these factors.

We first studied the role of ATR during CSR and SHM. CSR junctions obtained from ATR deficiency (ATRD) patients showed significantly increased usage of microhomology, but impaired end joining with partially complementary (1-3 bp) DNA ends. The SHM pattern was also altered, with fewer mutations occurring at A but more at T residues, representing a loss of strand bias in targeting A/T pairs within certain hotspots. The function of ATR and ATM in CSR and SHM was also compared. Our data suggest that the role of ATR is partially overlapping with ATM, whereas ATR is also endowed with unique functional properties in the repair processes during CSR and SHM.

We further studied the CSR junctions in Artemis deficient patients. A significantly increased usage of microhomology of ≥10 bp and an absolute absence of blunt-end joining were observed in Sμ-Sα junctions in the patients. However, the Sμ-Sγ junctions obtained from a patient who carried “hypomorphic” mutations appeared to be largely normal in their usage of microhomology, although an unusual type of sequential switching was observed more frequently than expected. These findings suggest that varying modes of CSR junction resolution were used for different S regions, when the

function of Artemis is impaired. The altered pattern of CSR junctions also strongly link Artemis to the predominant NHEJ pathway during CSR.

CSR junctions were also studied in B cells from Cernunnos deficient patients. These junctions were characterized by a significantly increased usage of microhomology of ≥10 bp and a significantly decreased usage of "direct end joining". This pattern has previously been observed in B cells deficient for DNA Ligase IV (Lig4), XRCC4, Artemis and ATM, suggesting that Cernunnos is likely to be involved in the DNA Lig4 dependent NHEJ pathway during CSR.

One somatically acquired missense mutation (p.Q227R) was also observed in the Cernunnos encoding gene in a germinal B cell like (GCB) diffuse large B cell lymphoma (DLBCL) sample. Two types of translocations (*IGH/BCL2* and *MYC/IGH*) were detected in this tumor sample and one of the switch γ regions appeared to be disrupted during translocation. Clonal-like, dynamic IgA switching activities were also observed, suggesting a link between defects in the Cernunnos dependent NHEJ pathway and aberrant CSR/switch translocations during the development of B cell malignancies.

Mutations in *Mre11* and *NBS1* gene can cause Ataxia-telangiectasia-like disorder (ATLD) and Nijmegen breakage syndrome (NBS) respectively. SHM patterns in cells from these patients were furthermore analyzed. The frequency and distribution of mutations obtained from both patient groups were largely similar to that observed in controls. The mutation pattern from ATLD patients was only slightly changed, with a small increase of the frequency of A to C transversions, suggesting that Mre11 is unlikely to be the major nuclease involved in cleavage of the abasic sites during SHM. The mutation pattern from NBS patients was however, altered with a significantly increased number of G transversions (G→C and G→T), which mainly occurred in AID and/or SHM hotspot motifs. NBS1 might thus have a specific role in regulating the strand-biased repair during phase Ib mutagenesis.

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I. Pan-Hammarström Q, Lähdesmäki A, Zhao Y, **Du L**, Zhao Z, Wen S, Ruiz-Perez VL, Dunn-Walters DK, Goodship JA, Hammarström L. Disparate roles of ATR and ATM in immunoglobulin class switch recombination and somatic hypermutation. *J Exp Med. 2006; 203(1):99-110.*

II. **Du L**, van der Burg M, Popov SW, Kotnis A, van Dongen JJ, Gennery AR, Pan-Hammarström Q. Involvement of Artemis in nonhomologous end-joining during immunoglobulin class switch recombination. *J Exp Med. 2008; 205(13):3031-40.*

III. **Du L***, Peng R*, Björkman A, de Miranda NF, Rosner C, Kotnis A, Berglund M, Liu C, Rosenquist R, Enblad G, Sundström C, Hojjat-Farsangi M, Rabbani H, Revy P, Durandy A, Zeng Y, Gennery AR, Villartay J, Pan-Hammarström Q. Role for Cernunnos (XLF) in regulation of immunoglobulin class switch recombination and in B cell lymphomagenesis. *J Exp Med. Resubmitted*. *these authors contributed equally to this study.

IV. **Du L**, Dunn-Walters DK, Chrzanowska KH, Stankovic T, Kotnis A, Li X, Lu J, Eggertsen G, Brittain C, Popov SW, Gennery AR, Taylor AM, Pan-Hammarström Q. A regulatory role for NBS1 in strand-specific mutagenesis during somatic hypermutation. *PLoS One. 2008; 3(6):e2482*.

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CONTENTS

LIST OF ABBREVIATIONS

A-EJ	Alternative end joining
AID	Activation-induced cytidine deaminase
A-T	Ataxia-telangiectasia
ATLD	Ataxia-telangiectasia-like disorder
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia-telangiectasia and Rad3-related
ATRD	ATR deficiency
AP	Apurinic/apyrimidinic
BER	Base excision repair
CID	Combined immunodeficiency
CSR	Class switch recombination
DLBCL	Diffuse large B-cell lymphoma
DSB	DNA double-strand break
ERCC	Excision repair cross-complementing protein
HR	Homologous recombination
Ig	Immunoglobulin
KO	Knockout
Lig4	DNA ligase IV
MHC	Major histocompatibility complex
MMR	Mismatch repair
MRN	Mre11-Rad50-Nbs1 complex
NBS	Nijmegen breakage syndrome
NER	Nucleotide excision repair
NHEJ	Nonhomologous end joining
NHL	Non-Hodgkin lymphomas
RPA	Replication protein A
PBL	Peripheral blood lymphocytes
PCNA	Proliferating cellular nuclear antigen
PIKKs	Phosphoinositol 3-kinase-like kinases
PMS	Postmeiotic segregation
Pol	Polymerase
RAG	Recombination activating gene
RSS	Recombination signal sequences
RS-SCID	Radiosensitive severe combined immunodeficiency
SCID	Severe combined immunodeficiency
SHM	Somatic hypermutation
TCR	T cell receptors
TdT	Terminal deoxynucleotidyl transferase
TFIIH	Transcription factor IIH
UNG	Uracil-DNA glycosylase
V(D)J	Variable, diversity and joining gene segments
XRCC	X-ray cross-complementing protein

1 INTRODUCTION

1.1 DNA REPAIR

About $1x10^4$ to $1x10^6$ DNA lesions occur in every human cell per day. These damages can be generated not only by endogenous agents, such as free radicals produced during metabolic processes, but also by exogenous agents such as UV light, ionizing radiation and industrial chemicals. Without repair, these damages may translate into mutations or other DNA structure alterations and eventually lead to cell death, or malignant transformation. To maintain genomic integrity, the DNA repair system is constantly active to respond to different forms of DNA damage.

1.1.1 Single-strand break repair

When one of the strands of a double helix is damaged, the other strand can be used as a template for repair. There are three major DNA repair mechanisms for single-strand breaks: base excision repair (BER), nucleotide excision (NER) and mismatch repair (MMR).

The BER pathway usually removes and replaces a base lesion that has been generated by endogenous or exogenous agents. It is initiated by DNA glycosylases, which specifically recognize the modified base, cleaves the N- glycosidic bond and gives rise to an apurinic/apyrimidinic (AP) site (Demple and Sung, 2005; Tell et al., 2009; Wilson and Barsky, 2001). The abasic site is subsequently incised by an AP endonuclease, APE1. The 5′-deoxyribose phosphate strand break product is removed and the missing nucleotide gap is filled most frequently by DNA polymerase β (pol β) (Beard and Wilson, 2006). The remaining nick is sealed by a DNA ligase, which in mammals is typically ligase3α (Lig3α) in complex with the X-ray cross-complementing protein 1 (XRCC1) (Ellenberger and Tomkinson, 2008).

The NER pathway recognizes and removes large loops, bulky and helix-distorting lesions from DNA (Huang and Sancar, 1994). In contrast to BER, which only removes shorter patches of damaged DNA, NER can remove a total of 25-30 nucleotides. After DNA lesion recognition, the DNA is unwound and incised on either side of the aberrant base. DNA polymerases then fill the gap and DNA ligases (DNA Lig1 or Lig3) seal it. A large number of proteins are involved in the NER pathway such as the seven

xeroderma pigmentosum proteins (XPA – XPG), excision repair cross-complementing protein1 (ERCC1), ERCC6, ERCC8, replication protein A (RPA) and transcription factor IIH (TFIIH) (Rouillon and White, 2011).

MMR is a strand-specific DNA repair pathway, involved in the response to certain types of DNA damage, which recognizes and repairs base-base mismatches and insertion/deletion mispairs generated during DNA replication and recombination (Kolodner and Marsischky, 1999). Several human MMR proteins have been identified including MSH1-MSH6, MLH1, MLH3, postmeiotic segregation (PMS)1, PMS2, exonuclease 1 (Exo1), single-strand DNA-binding protein RPA, proliferating cellular nuclear antigen (PCNA), DNA polymerase δ (pol δ) and DNA Lig1 (Buermeyer et al., 1999; Iyer et al., 2006). MSH proteins recognize DNA mismatches and bind to DNA, forming an active repair complex. MSH2/MSH6 complex binds base mispairs and small loop-outs, whereas MSH2/MSH3 does not bind base-mismatches but a wide range of loop-outs (Antony and Hingorani, 2003; Wu et al., 2003). Following mismatch recognition, MLH and PMS proteins such as MLH1/PMS2 bind to the MSH complex, recruit other potential downstream proteins with endonuclease and exonuclease activities and complete the repair process (Mazurek et al., 2002).

1.1.2 Double-strand break repair

DNA double-strand break (DSB), in which both strands in the double helix are damaged, is the most deleterious form of DNA damage. It can arise from oxidative stress, exposure to ionizing radiation and certain chemicals. DSBs are also intermediates in normal endogenous processes such as DNA replication, meiosis and immunoglobulin gene diversification including V(D)J recombination and class switch recombination (CSR) (Lieber and Karanjawala, 2004). Unrepaired or misrepaired DSBs may lead to genomic instability resulting in genetic aberrations or cell death. There are two major pathways to repair DSBs in eukaryotes: homologous recombination (HR) and nonhomologous end joining (NHEJ).

1.1.2.1 Homologous recombination

The HR repair pathway requires a long stretch of sequence homology and occurs during the late S/G2 phase of the cell cycle where the sister chromatids are located in close proximity (Sonoda et al., 2006). When a DSB occurs, the Mre11-Rad50-NBS1 (MRN) complex can remove oligonucleotides from the 5′ prime and recruit Exo1and

Bloom syndrome protein (BLM) to the break site, making extensive resection, resulting in single-stranded DNA tails (Mimitou and Symington, 2009; Shim et al., 2010). Subsequently, the RPA binds to the 3′ end of the tails, then RPA is displaced by RAD51, forming the nucleoprotein filament. Once formed, the complex of Rad51 and single-stranded DNA starts to search for a homologous sequence in the double-stranded DNA and then promotes invasion of the single-stranded DNA into donor double-stranded DNA to form a displaced strand (D loop). After strand invasion, a DNA polymerase extends the end of the invading 3′ strand by synthesizing new DNA, resulting in a change from D-loop to a Holliday junction (HJ) with a cross-shaped structure. Finally the HJs are dissolved by BLM/Sgs1 helicase or resolved by one of the four resolvases (Mus81-Mms4, Slx1-Slx4, Yen1, and Rad1-Rad10) to yield separate intact duplex molecules (Mazon et al., 2010; San Filippo et al., 2008; Svendsen and Harper, 2010).

The Ataxia-telangiectasia mutated (ATM) protein may also be linked to HR, as ATM-deficient mice show severe meiotic disruption (Barlow et al., 1998). Furthermore, the c-Abl protein-tyrosine kinase which is activated by ATM can modulate Rad51 activity in response to DNA damage (Khanna, 2000; Rotman and Shiloh, 1999). ATM also phosphorylates selected proteins, including RPA and Brca1, that are linked to HR.

1.1.2.2 Non-homologous end-joining

NHEJ utilizes little, or no, sequence homology, is often imprecise, functions throughout the cell cycle and is considered to be the major pathway for the repair of DSB in vertebrate cells (Moore and Haber, 1996; Sonoda et al., 2006). Furthermore, the NHEJ pathway is considered as the principle mechanism used during V(D)J recombination and CSR (Chaudhuri and Alt, 2004; Jung et al., 2006; Kotnis et al., 2009). The classical NHEJ machinery requires a set of proteins, including Ku70, Ku80, DNA-PKcs, DNA Lig4, XRCC4, Artemis and the XRCC4-like factor (XLF), also known as Cernunnos.

Once DSBs are introduced, Ku70 and Ku80 form a heterodimer that binds to DNA ends and recruit the DNA-PKcs, forming a triad complex termed DNA-PK. DNA-PKcs binds and phosphorylates Artemis, activating its DNA end-processing activities. The modified DNA ends are then ligated by a complex of DNA Lig4, XRCC4 and Cernunnos/XLF (Lieber, 2010; Lieber et al., 2008; Lieber et al., 2003).

When one of the NHEJ components is lacking, the damaged DNA ends can still be joined via the alternative end joining (A-EJ) pathway, which, however, is not as efficient as NHEJ. The Lig4-independent joining pathway and the Ku-independent joining pathway that are among the A-EJ pathways that have been described, often use a longer microhomology during the repair process (Han and Yu, 2008; Pan-Hammarstrom et al., 2005; Soulas-Sprauel et al., 2007; Weinstock et al., 2007).

1.2 ANTIBODY DIVERSITY

The immune system is the body's defense against infectious organisms such as viruses, bacteria, pathogenic fungi and parasites. It is a made up of a complex network of cells, tissues and organs that protect the body. The innate immune system provides an immediate, but non-specific response, whereas the adaptive immune system has the ability to recognize pathogens specifically and to provide protection against reinfection (Janeway et al., 2005).

There are two major types of lymphocytes in the adaptive immune system: B lymphocytes and T lymphocytes. B cells carry cell-surface immunoglobulin molecules as receptors for antigens, secrete antibodies that bind antigens, act as antigen-presenting cells (APCs) and eventually develop into memory B cells after activation by interaction with antigens. T cells have receptors that recognize peptide fragments of intracellular pathogens transported to the cell surface by the glycoproteins of the major histocompatibility complex (MHC). There are two subsets of T cells (CD8 and CD4) that recognize the two classes of MHC molecules (Class 1 and Class 2), respectively (Swain, 1983). CD8 T cells kill infected target cells, whereas CD4 T cells mainly activate macrophages and B cells (Zamoyska, 1998).

During early T and B lymphocyte development, V(D)J recombination takes place to assemble the variable (V) regions of the T cell receptor and Ig genes, respectively, giving rise to a large repertoire of antibody specificities (Jung et al., 2006). In B cells, two additional mechanisms, which are activated after antigen recognition, further diversify the antibody response: class switch recombination and somatic hypermutation (Manis et al., 2002b; Papavasiliou and Schatz, 2002). Mechanisms involved in those processes will be discussed below.

1.2.1 V(D)J recombination

V(D)J recombination assembles antigen receptor variable region genes from germline variable (V), diversity (D) and joining (J) gene segments (Jung et al., 2006). V(D)J recombination is initiated by recombination activating gene 1(RAG1) and RAG2 proteins that are expressed in early B and T cells. RAG1 and RAG2 form a complex that bind specifically at recombination signal sequences (RSS). RSS are composed of a highly conserved heptamer that resides next to a spacer containing either 12 or 23 unconserved nucleotides followed by a conserved nonamer (Fugmann et al., 2000). RAG proteins recognize the RSS sequences and induce DNA cleavage, following the 12/23 rule (Eastman et al., 1996; Sawchuk et al., 1997). Then the DNA ends are processed and repaired by NHEJ pathway as described above. Terminal deoxynucleotidyl transferase (TdT), is another factor involved in V(D)J recombination. It can add nucleotides randomly to the DSBs ends, providing additional junctional diversities.

The rearrangements occuring in different antigen receptor loci are well-defined, as ordered processes. The Ig heavy chain and TCR β-chain loci are the first to be assembled, with DH to JH joining occurring on both alleles before the initiation of VH to DJH joining. Then, recombination of the Ig light chain locus (Igκ or Igλ) or the TCR α-chain locus is initiated (Alt et al., 1984). The intergenic control region 1 (IGCR1), located between the VH and D clusters, containing two transcriptional repressor CTCF-binding elements (CBEs), has recently been reported as a key regulatory region controlling V(D)J rearrangement (Guo et al., 2011). IGCR1 promotes normal B cell development and balances antibody repertoires by inhibiting transcription and rearrangement of DH-proximal VH gene segments and promoting rearrangement of the distal VH segments (Guo et al., 2011). IGCR1 maintains an ordered and lineage-specific VH(D)JH recombination by suppressing VH joining to D segments not joined to JH segments, and VH to DJH joins in thymocytes, respectively (Guo et al., 2011).

1.2.2 Class switch recombination

IgM is the first antibody produced by naïve mature B cells. After activation by an antigen, these B cells are activated and can switch to produce different antibody isotypes. The change in antibody class is effectuated by a deletional recombination event called CSR. During CSR, the constant region gene of the μ heavy chain ($C\mu$) is

replaced by a downstream CH gene (*Cα, Cγ* or *Cε*) resulting in a change from IgM to IgA, IgG or IgE production, without changing the antibody specificity (Fig. 1) (Iwasato et al., 1990; Matsuoka et al., 1990; von Schwedler et al., 1990).

CSR is initiated by activation-induced cytidine deaminase (AID) (Muramatsu et al., 2000; Revy et al., 2000), which deaminates cytosines to uracils within the switch (S) regions (Petersen-Mahrt et al., 2002). Deoxyuridine is removed by uracil-DNA glycosylase (UNG), a BER enzyme that generates an abasic site. The latter is then recognized by APE1 that creates a nick (Christmann et al., 2003; Rada et al., 2002).

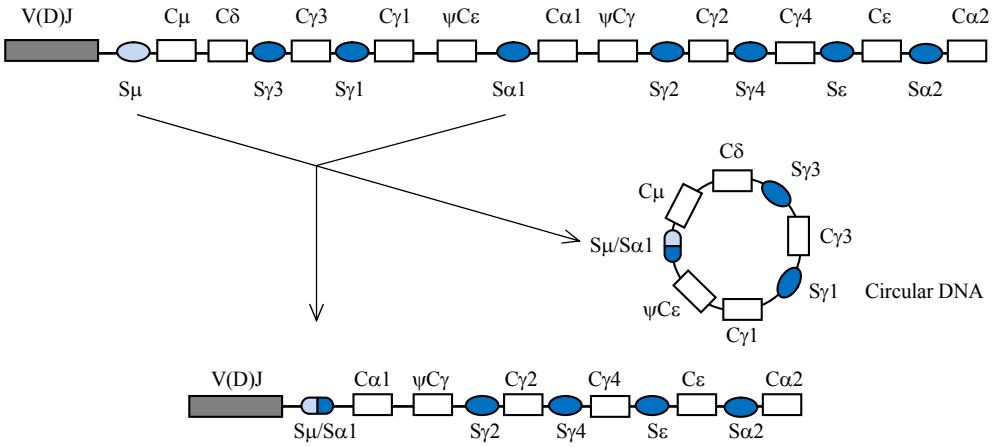


Figure 1. Deletion model of CSR. In this figure, the *Cμ* gene is replaced by a *Cα1* gene, resulting in a change from IgM to IgA1 production. The intervening sequence is excised as circular DNA and, the Sμ and Sα1 regions are recombined, resulting in a Sμ-Sα1 recombination junction and a switch to IgA1.

Participation of other enzymes, such as Exo1, may assist in converting the closely positioned nicks on both strands into large overlapping gaps, resulting in DSBs. In addition, the dU:dG mismatches can be recognized by the MMR proteins and single-strand nicks may be introduced which eventually leads to the formation of DSBs (Stavnezer and Schrader, 2006). Based on the different consequences of UNG deficiency, MSH2 deficiency, and UNG-MSH2 double deficiency, the UNG-dependent pathway has been suggested to be the major pathway for CSR, whereas the MSH2-dependent pathway may serve as a backup (Pan-Hammarstrom et al., 2007; Stavnezer et al., 2010).

NHEJ is considered to be the principal mechanism used in CSR to repair DSBs, as AID-dependent DSBs in S region are introduced and repaired mainly in the G1 phase

of the cell cycle (Schrader et al., 2007). Furthermore, the nature of S region sequences, which lacks long stretches of perfect homology between the different S regions, would theoretically not support HR (Kotnis et al., 2009). Besides NHEJ factors, there are also other DNA repair/damage response factors that have been associated with CSR, such as ATM, ataxia-telangiectasia and Rad3-related (ATR), 53BP1 and the MRN complex.

1.2.2.1 ATM and ATR

ATM and ATR are serine/threonine protein kinase, belonging to the phosphoinositol 3-kinase-like kinases (PIKKs) superfamily that is involved in cell-cycle control and DNA damage responses. ATM phosphorylates several key proteins including NBS1, Mre11, H2AX, MDC1 and 53BP1 that initiate activation of the DNA damage checkpoint control, leading to cell cycle arrest, DNA repair or apoptosis (Lavin and Kozlov, 2007; Shiloh, 2003).

ATM deficiency in humans results in ataxia-telangiectasia (A-T), a rare, multi-system disorder which is characterized by cerebellar degeneration with ataxia, telangiectasia, chromosomal instability, radiosensitivity and cancer predisposition (Chun and Gatti, 2004). Most A-T patients have reduced serum levels of IgA, IgE and IgG subclasses (Shiloh, 1997). The Sμ-Sα junctions in A-T patients are aberrant, with increased microhomology and lack of mutations around the breakpoint, suggesting that ATM is involved in the final step(s) in CSR (Pan et al., 2002). ATM-deficient mice have also been generated in several laboratories and these mice are characterized by growth retardation, infertility, radiosensitivity, defects in T cell maturation and development of thymic lymphomas (Barlow et al., 1996; Elson et al., 1996; Xu et al., 1996). Reduced levels of switching and increased length of sequence homology in Sμ-Sγ1 junctions from ATM-deficient mice further support a role for ATM during CSR (Lumsden et al., 2004; Reina-San-Martin et al., 2004).

The exact function of ATM during CSR remains unclear. One possibility is that ATM recruits and/or activates other DNA-damage response factors, such as γH2AX, 53BP1, MDC1 and possibly the MRN complex, configuring the DNA termini for subsequent repair steps, and/or slowing down cell cycle progression until the repair is complete. A second possibility is that ATM may have a more direct role in the end processing step through phosphorylation of a proposed nuclease that participates in NHEJ, i.e. Mre11 or Artemis (Kotnis et al., 2009; Stavnezer et al., 2010).

ATR is another member in the PIKKs family, which shares several substrates with ATM including H2AX and 53BP1 (Abraham, 2001). Mutations in the *ATR* gene can result in Seckel syndrome, a rare autosomal recessive disorder characterized by intrauterine growth retardation, dwarfism, microcephaly, "bird-like" facial features and mental retardation (O'Driscoll et al., 2003). Disruption of the *ATR* gene in mice leads to early embryonic lethality (Brown and Baltimore, 2000; de Klein et al., 2000). The role of ATR in CSR will be discussed in paper I.

1.2.2.2 NHEJ machinery

The classical NHEJ machinery requires a number of factors, including Ku70/Ku80, DNA-PKcs, DNA Lig4, XRCC4, Artemis and Cernunnos/XLF. All of them have been shown to be involved in CSR and will be discussed below.

Ku70/Ku80

Ku is a heterodimer of Ku70 and Ku80, the first proteins that bind to the DNA ends at a DSB. Ku70 or Ku80 knockout mice have severe immunodeficiency and do not develop B cells due to defective V(D)J recombination (Gu et al., 1997; Nussenzweig et al., 1996). Switching in Ku70- or Ku80-deficient B cells from transgenic mice with recombined heavy and light chain genes is severely impaired, suggesting that the Ku heterodimer is required for CSR (Casellas et al., 1998; Manis et al., 1998). However, proliferative defects in the cells make it unclear if Ku has a direct role in end joining during CSR through NHEJ (Casellas et al., 1998; Manis et al., 1998; Reina-San-Martin et al., 2003). With more optimal activation conditions, Ku70- and Ku80-deficient cells have recently been shown to be able to switch, but at a level of 20-50% of wild type (WT) cells. The switch junctions obtained from the Ku70-deficient cells presented increased microhomolgy and decreased direct joining (Boboila et al., 2010b). Moreover, junctions in cells lacking both Ku70 and DNA Lig4 have more direct joins than cells lacking DNA Lig4 only, suggesting that Ku still functions at DSBs in DNA Lig4-deficient cells and seems to promote microhomology mediated A-EJ at switch junctions in the absence of DNA Lig4 (Boboila et al., 2010a; Boboila et al., 2010b).

DNA-PKcs

Similar to Ku knockout (KO) mice, DNA-PKcs KO mice also require transgenic IgH and IgL chains for B cell development. Switching to all Ig classes, except for IgG1, is severely impaired in these mice, providing convincing evidence that DNA-PKcs is

required for CSR (Manis et al., 2002a). Severe combined immunodeficiency (SCID) mice with a natural mutation in DNA-PKcs, lacking kinase activity or the C terminus of DNA-PKcs, have also been used in several studies. CSR efficiency was either close to normal (Kiefer et al., 2007) or reduced approximately two- to threefold (Bosma et al., 2002; Cook et al., 2003). The difference between the DNA-PKcs-null and SCID mice may suggest that the kinase activity of DNA-PKcs is not essential and might potentially be replaced by ATM during CSR (Callen et al., 2009; Shrivastav et al., 2009). In addition, DNA-PKcs may have a non-catalytic role in CSR, for instance as a synaptic factor, mediating synapse complex formation (DeFazio et al., 2002).

Recently, the first human Radiosensitive (RS)-SCID patient with a DNA-PKcs missense mutation (L3062R) was identified (van der Burg et al., 2009). Although the patient's cells show a normal DNA-PK kinase activity, Artemis activation is insufficient and B and T cells are almost absent in peripheral blood. CSR from B cells in this patient is aberrant and shows a significant increased usage of microhomology at Sμ-Sα junctions (our unpublished data). Although the Sμ-Sγ junctions appear to be normal, a significant increase of an unusual type of sequential switching from IgM, through one IgG subclass, to a different IgG subclass was observed and the Sγ-Sγ junctions show long microhomologies (our unpublished data). The altered patterns of switch recombination junctions from DNA-PKcs deficient patient further suggest that DNA-PKcs may have a non-catalytic role in the NHEJ pathway during CSR.

Artemis

Artemis can be phosphorylated by several kinases including DNA-PKcs, ATM and ATR (Ma et al., 2002; Zhang et al., 2004). It exhibits endonuclease activity on 5′ and 3′ overhangs and hairpins when complexed with DNA-PKcs (Ma et al., 2002). It also plays an essential role in V(D)J recombination through its activity on opening the hairpin structures at the coding ends. In humans, mutations in Artemis result in T^- B^- NK^+ SCID associated with increased radiosensitivity, a genetic disorder that is characterized by a severe defect in V(D)J recombination leading to an early arrest of both B and T cell maturation (Moshous et al., 2001). Hypomorphic mutations in the gene have also been described and are associated either with Omenn's syndrome or combined immunodeficiency (CID) (Ege et al., 2005; Evans et al., 2006; Ijspeert et al., 2011; Moshous et al., 2003). Artemis-deficient B cells that harbored preassembled Ig heavy chain and κ-light chain knock-in alleles undergo robust CSR, with normal serum

immunoglobulin levels and normal Sμ-Sγ1 junctions, suggesting that Artemis may not be involved in CSR (Rooney et al., 2005). However, subsequent studies have shown that Artemis is involved: conditional Artemis KO mice show decreased levels of IgA expression and reduced CSR to IgG3 accompanied by an increased microhomology usage at CSR junctions (Rivera-Munoz et al., 2009). Moreover, B cells from Artemis-deficient mice generated more AID-dependent *IgH* locus chromosomal breaks and translocations, suggesting that Artemis is necessary for joining AID-dependent DSBs during CSR (Franco et al., 2008). The CSR junctions from Artemis-deficient patients were also analyzed, and the role of Artemis during CSR is discussed in paper II.

DNA ligase IV and XRCC4

The X-ray repair cross-complementing protein 4 (XRCC4)-DNA Lig4 complex is responsible for catalyzing the ligation step of NHEJ (Lieber, 2010; Lieber et al., 2008). Disruption of Lig4 or XRCC4 in mice results in embryonic lethality (Barnes et al., 1998; Frank et al., 1998; Gao et al., 1998). Patients with hypomorphic mutations have been described and they are characterized by microcephaly, growth retardation, radiosensitivity and mild to severe immunodeficiency (Buck et al., 2006b; Enders et al., 2006; O'Driscoll et al., 2001; van der Burg et al., 2006). An altered pattern of CSR junctions in B cells was observed in patients with hypomorphic Lig4 mutations, characterized by the absence of direct end joining and greatly increased lengths of microhomology at the Sμ-Sα junctions (Pan-Hammarstrom et al., 2005). $Xrcc4^{-/-}$ mice, produced by mating $xrcc4^{+/-}$ mice with $p53^{+/-}$ mice, only show 20-50% of normal levels of CSR (Soulas-Sprauel et al., 2007; Yan et al., 2007), and the CSR junctions shift towards microhomology usage with an absence of direct end joining (Yan et al., 2007). Similarly, lack of direct joining and a bias towards a microhomology-based joining were observed in DNA Lig4-deficient mouse B cells (Boboila et al., 2010b; Han and Yu, 2008). Thus far, XRCC4 and DNA Lig4 are the only proteins required for all NHEJ reactions that have no reported roles outside NHEJ (Chaudhuri and Alt, 2004). Taken together, the data demonstrate that the classical NHEJ machinery is required for CSR, and in its absence, an A-EJ mechanism is operating.

Cernunnos/XLF

Cernunnos/XLF is a recently identified factor involved in the NHEJ process during DNA DSB repair, which stimulates incompatible DNA end ligation by the XRCC4-

DNA Lig4 complex (Gu et al., 2007; Lu et al., 2007b; Tsai et al., 2007). In humans, mutations in the gene encoding Cernunnos result in a rare, autosomal recessive disorder with growth retardation, microcephaly, radiosensitivity and CID characterized by a profound T and B lymphocytopenia (Buck et al., 2006a). Serum levels of IgG and IgA from Cernunnos-deficient patients are low or absent, whereas the level of IgM is normal or even high, suggesting a possible role of Cernunnos in CSR (Buck et al., 2006a). Switching to IgG1 and IgG3 in cultured B cells from Cernunnos deficient mice is reduced. The frequency of Sμ-Sγ1 direct joints is reduced, along with a small increase in the average length of junctional microhomology, confirming the possible role of Cernunnos during CSR (Li et al., 2008). However, only a modestly decreased number of mature lymphocytes was observed in those mice and Cernunnos deficient pro-B cell lines can support nearly normal levels of V(D)J recombination (Li et al., 2008). The redundant functional properties of ATM and Cernunnos in joining DNA breaks might explain this modest defect of lymphocyte development (Zha et al., 2011). If this indeed reflects a lymphocyte-specific compensation for Cernunnos deficiency in V(D)J recombination, it is unclear why such a mechanism would not rescue the development of T and B lymphocytes in humans. The CSR junctions from Cernunnos-deficient patients were analyzed, and the potential role of Cernunnos during CSR is discussed in paper III.

1.2.2.3 Alternative end-joining pathway

As discussed above, in the absence of the NHEJ pathway, CSR was still observed at low levels suggesting that the A-EJ is operating during CSR. In contrast to NHEJ, the mechanism underlying A-EJ is less well-understood. For instance, we still do not know the kinetics of the repair, during which part of the cell cycle it is operational, the length of the microhomology that is required and whether there is one or multiple pathways involved in A-EJ. Several factors have been suggested to be involved in A-EJ during CSR, including the MRN complex, Poly (ADP-ribose) polymerase 1 (PARP-1), XRCC1 and DNA Lig3 (Kotnis et al., 2009; Robert et al., 2009; Saribasak et al., 2011). In a more recent study, cells from a mouse B cell line CH12, with C-terminal binding protein-interacting protein (CtIP) knocked down, showed a 40-60% reduction in CSR as compared to the control cells, and the Sμ-Sα junctions were markedly altered, characterized by decreased microhomology length and lower frequency of junctions with blunt joining (Lee-Theilen et al., 2011). CtIP is thus required for the microhomology-dependent A-EJ pathway during CSR (Lee-Theilen et al., 2011).

1.2.3 Somatic hypermutation

Somatic hypermutation (SHM) occurs in the germinal center and is a process where point mutations are introduced at a high rate (10^{-3}/basepair/generation (Rajewsky et al., 1987)) into the Ig variable (V) region genes and which helps to shape the Ig repertoire. SHM makes a major contribution to antibody affinity maturation and diversification of the antibody repertoire in humans and mice (Milstein and Neuberger, 1996; Rajewsky, 1996). The generation of mutations starts about 150 bp downstream of the *IgV* promoter and extends over 2 kb, exhibiting decreasing mutation frequency with increasing distance from the promoter (Both et al., 1990; Neuberger and Milstein, 1995; Rajewsky, 1996). During SHM, mutations can occur at all four bases and C/G and A/T base pairs are mutated at roughly equal frequencies. However, more mutations occur at the A base compared to T base within A/T pairs, whereas, C and G bases within C/G pairs are mutated with an almost equal frequency (Golding et al., 1987).

Similar to CSR, SHM is initiated by AID (Muramatsu et al., 2000), which converts cytosines to uracils on DNA. AID preferentially deaminates dC in WRCY (where W = A/T, R = A/G, and Y = C/T) and the complement RGYW hot-spot motifs during SHM (Pham et al., 2003). After deamination by AID, SHM occurs in two phases depending on different repair pathways where phase I targets C/G pairs and phase II targets A/T pairs. Phase I can be further divided into Ia and Ib (Fig. 2) (Di Noia and Neuberger, 2007; Liu and Schatz, 2009).

In phase Ia, replication over the uracil results in C to T or G to A transition mutations. In phase Ib, uracil is recognized by UNG, generating an abasic site (AP), which can be replicated over or repaired in an error-prone manner (possibly by Rev1 or other translesion synthesis polymerases (TLS pol)) to give rise to transition/tranversion mutations at C/G nucleotides. In phase II, mutations are introduced at adjacent positions, predominantly at nearby A/T pairs, mainly through a MSH2/MSH6 triggered, error-prone, patch repair process. DNA polymerase η clearly plays a major role in the introduction of A/T pairs during SHM (Delbos et al., 2005; Martomo et al., 2005). UNG and Msh2/Msh6 can also act as parts of the normal BER and MMR pathways, respectively, resulting in high-fidelity repair of the uracil without mutations (Di Noia and Neuberger, 2007; Liu and Schatz, 2009).

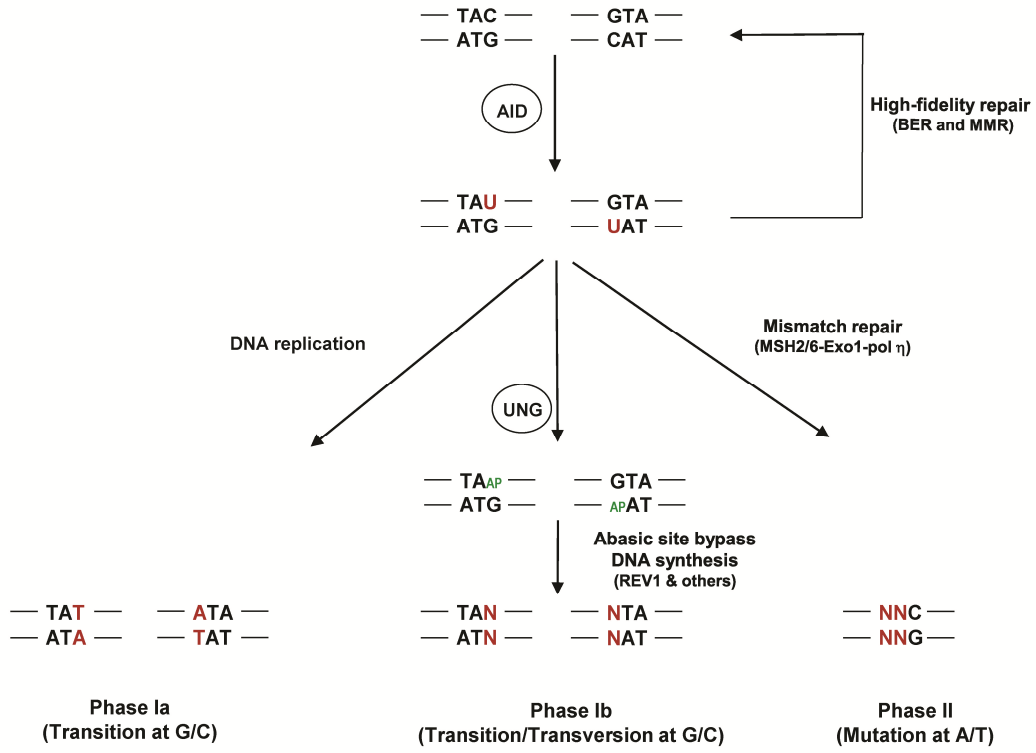


Figure 2. Repair model for SHM.

It is evident that the BER pathway, operating in SHM, is largely dependent on UNG through its uracil excision activity. It is not known, however, which endonuclease acts in the step immediately downstream of UNG i.e. that cleaves at the abasic sites generated by the latter. Two candidates have been proposed, an AP endonuclease (APE) and the MRN complex. The role of the MRN complex during SHM is discussed in paper IV.

1.3 GENOMIC INSTABILITY SYNDROMES

As discussed above, the maintenance of genomic integrity depends on the coordination of DNA repair, cell cycle progression, transcriptional and post-transcriptional regulation and apoptosis and it requires a variety of different damage response proteins. Mutations in the genes that encode these proteins can result in a number of genomic instability syndromes or disorders, that often result in a heightened predisposition to cancer (Duker, 2002). Immunodeficiency is a hallmark of these disorders, as the ability to repair DNA breaks that occur during V(D)J recombination, CSR and SHM are critical for the development of the immune system. Several syndromes with defects in the DNA DSB response will be discussed below (Table I).

1.3.1 Ataxia-Telangiectasia

Mutations in the gene encoding ATM results in ataxia-telangiectasia (A-T, OMIM 208900), a rare, multi-system disorder that is characterized by early-onset progressive cerebellar ataxia, oculo-cutaneous telangiectasia, immunodeficiency, chromosomal instability, radiosensitivity and cancer predisposition (Chun and Gatti, 2004). The incidence of the disorder is estimated to be around 1/100.000 live births (Savitsky et al., 1995). A-T patients are prone to bacterial sinopulmonary infections and pulmonary disease is the most common cause of death in A-T patients (Morrell et al., 1986). A-T patients show reduced levels of immunoglobulins, where approximately 80% of them suffer from IgG2 deficiency and 60% of them have IgA deficiency (Shiloh, 1997). Approximately one-third of A-T patients develop malignancies, with lymphomas and leukemias of T cell origin being predominant (Peterson et al., 1992).

1.3.2 Seckel syndrome

Mutations in the gene encoding ATR results in Seckel syndrome (OMIM 210600), an autosomal recessive disorder characterized by intrauterine growth retardation and postnatal dwarfism with a small head, narrow bird-like face with a beak-like nose, large eyes with an antimongoloid slant, receding mandible and mental retardation (Goodship et al., 2000; O'Driscoll et al., 2003). Mutations in the genes encoding pericentrin (PCNT), Centromere protein J (CENPJ), or Centrosomal protein of 152 kDa (CEP152) can also result in Seckel syndrome (Al-Dosari et al., 2010; Griffith et al., 2008; Kalay et al., 2011). Thus far, 5 patients from two consanguineous families with Seckel syndrome were found to carry mutations in the ATR gene, including a silent 2101A→G transition in exon 9 that results in increased aberrant splicing, either by skipping of exon 9 or by using a cryptic splice donor site within exon 9, both of which introduce a frameshift and a stop codon in exon 10 (O'Driscoll et al., 2003). Overt immune deficiency is not observed in ATR-Seckel patients.

1.3.3 Severe combined immunodeficiency

SCID is a severe form of heritable immunodeficiency. Chronic diarrhea, ear infections, recurrent *Pneumocystis jirovecii* pneumonia and profuse oral candidiasis commonly occur in SCID patients (Buckley, 2000). There are several forms of SCID. The most common type is X-linked SCID which is due to mutations in the gene encoding for the common gamma chain of several cytokine receptors, including those for the interleukins IL2, IL4, IL7, IL9 and IL15. Other forms of SCID usually follow an

autosomal recessive inheritance pattern or are the result of spontaneous mutations in the genes including *RAG1, RAG2, IL7R, CD45, ADA, JAK3* and *Artemis* (Haq et al., 2007; Kalman et al., 2004; Moshous et al., 2001; Pesu et al., 2005; Sanchez et al., 2007). SCID has also classically been divided into patients with residual B cells (T^-B^+ phenotype) and patients who produce neither T nor B cells (T^-B^- phenotype). A subtype of the T^-B^- phenotype SCID (RS $T^-B^-NK^+$SCID), has also been described with heightened sensitivity to ionizing radiation, where mutations have been observed in genes encoding the NHEJ proteins (Nicolas et al., 1998; Schwarz et al., 1996).

Artemis deficiency

Mutations in the *Artemis* gene represent the most common cause of RS-SCID, characterized by a severe defect in V(D)J recombination leading to an early arrest of both B and T cell maturation (Moshous et al., 2001). As of today, more than 100 patients have been described with various null or hypomorphic mutations in *Artemis* (Darroudi et al., 2007; de Villartay et al., 2009; Ege et al., 2005; Evans et al., 2006; Ijspeert et al., 2011; Kobayashi et al., 2003a; Kobayashi et al., 2003b; Lagresle-Peyrou et al., 2008; Le Deist et al., 2004; Li et al., 2002; Moshous et al., 2001; Moshous et al., 2003; Musio et al., 2005; Noordzij et al., 2003; Pannicke et al., 2010; van der Burg et al., 2007; van Zelm et al., 2008).

Cernunnos/XLF deficiency

Mutations in the gene encoding Cernunnos also result in RS-SCID and patients are, characterized by growth retardation, microcephaly, radiosensitivity and CID where serum levels of IgA and IgG are extremely low or absent, but IgM levels may be normal or even high (Buck et al., 2006a). To date, about 10 patients with Cernunnos-deficiency have been identified, including 3 new patients described in paper III (Ahnesorg et al., 2006; Buck et al., 2006a; Turul et al., 2011).

1.3.4 Ataxia-telangiectasia-like disorder

Defects in MRE11 cause ATLD, another rare autosomal recessive disorder that shares a variety of phenotypic similarities with A-T patients, including chromosomal instability and radiation sensitivity but no telangiectasia or immunodeficiency (Hernandez et al., 1993; Klein et al., 1996; Stewart et al., 1999). In contrast to A-T, ATLD is characterized by a late onset of the neurological features and a slower progression of

Table.1 Genomic instability syndromes

Defective proteins	Syndrome	Estimated Prevalence/ No. of patients	Phenotype	Immunodeficiency	References
ATM	Ataxia-Telangiectasia	1:100.000	Ataxia, telangiectasia, immunodeficiency, chromosomal instability, radiosensitivity, cancer.	Reduced levels of IgA, IgE and IgG subclass, Or HIgM	Chun, 2004 Shiloh, 1997
ATR	Seckel syndrome	NA[a]/5	Intrauterine growth retardation, dwarfism with a small head, narrow bird-like face, mental retardation.	No	Goodship, 2000 O'Driscoll, 2003
Artemis	RS-SCID CID	NA/>100	Immunodeficiency, radiosensitivity, cancer	Absent or low level of IgA and IgG, normal or even high level of IgM.	Moshous, 2001 et al [b].
Cernunnos	RS-SCID HIgM CID	NA/10	Growth retardation, microcephaly, radiosensitivity and combined immunodeficiency.	Absent or low level of IgA and IgG, normal or even high level of IgM.	Ahnesorg, 2006 Buck, 2006a Turul, 2011 Paper III
Mre11	ATLD	NA/16	Chromosomal instability, radiosensitivity, cancer.	No	Delia, 2004 Fernet, 2005 Klein, 1996 Pitts, 2001
NBS1	Nijmegen breakage syndrome	1:100,000[c]	Microcephaly, “bird-like” face, growth retardation, mental retardation, immunodeficiency, cancer.	IgA, IgG2, IgG4 deficiency, HIgM	Van der Burgt, 1996 et al [b].

[a] NA, Prevalence is not analyzed.

[b] Additional references are given in the text.

[c] In Eastern European populations.

the disorder, to give the overall appearance of a milder condition than A-T. At present, 16 ATLD patients have been described (Delia et al., 2004; Fernet et al., 2005; Klein et al., 1996; Pitts et al., 2001).

1.3.5 Nijmegen breakage syndrome

Mutations in the *NBS1* gene can cause Nijmegen breakage syndrome (NBS), an autosomal recessive chromosomal instability syndrome characterized by microcephaly, "bird-like" face, growth retardation, mental retardation, immunodeficiency and predisposition to cancer (Carney et al., 1998; Varon et al., 1998). More than 90% of the NBS patients showed a 5-bp deletion (657de/ACAAA) in exon 6 of the *NBS1* gene. Five additional mutations, C>T at 976, 698delAACA, l142delC, 835del-CAGA and 842insT have been identified in NBS patients (Der Kaloustian et al., 1996; Maraschio et al., 2003; Meyer et al., 2004; Saar et al., 1997; Tekin et al., 2002; Tupler et al., 1997; van der Burgt et al., 1996; Varon et al., 2000). The frequency of heterozygous carriers was estimated at 1/177 in three Slavic populations: Poland, Czech Republic, and Ukraine (Varon et al., 2000).

1.4 B-CELL LYMPHOMA

Lymphoma is a cancer in the lymphatic cells of the immune system. Lymphomas are classified into Hodgkin and non-Hodgkin lymphomas (NHL). NHL constitutes around 90% and Hodgkin lymphoma accounts for the remaining 10% of all lymphomas. Most NHL are derived from B (21 sub-types) or T-cells (15 sub-types). The most common types of mature B cell lymphomas are follicular lymphoma (FL), diffuse large B cell lymphoma (DLBCL), B cell chronic lymphocytic leukaemia/small lymphocytic lymphoma (B-CLL/SLL), mucosa-associated lymphoid tissue (MALT) lymphoma, mantle cell lymphoma (MCL), marginal zone lymphoma (MZL) and Burkitt lymphoma (BL).

A hallmark of mature B cell lymphomas is the presence of chromosomal translocations involving the *Ig* genes and a variety of partner genes. As a result of these translocations, oncogenes come under control of *Ig* gene enhancers and become deregulated (Kuppers and Dalla-Favera, 2001; Willis and Dyer, 2000). Translocations may occur at three stages in B cell development: V(D)J recombination, CSR and SHM (Kuppers, 2005; Kuppers and Dalla-Favera, 2001). Although chromosome translocations are important

in B cell lymphomagenesis, they are not sufficient to induce lymphomagenesis. Other genetic events are also necessary for lymphoma development and progression.

DLBCL is the most common type of NHL and accounts for 40% of all NHL malignancies (Campo et al., 2011). According to gene-expression profiles, overall survival rates and response to therapy, DLBCL is classified into three different types: germinal B cell like (GCB) DLBCL, activated B cell like (ABC) DLBCL, and primary mediastinal B cell lymphoma (PMBL) (Hans et al., 2004; Savage et al., 2003). However, 15-30% of DLBCL cannot be classified into any of the above subgroups (Alizadeh et al., 2000; Wright et al., 2003). Genetic alterations reported in NHL and in DLBCL include chromosomal translocations, mutations caused by aberrant somatic hypermutation (ASHM), sporadic somatic mutations and gene copy number alterations denoted by deletions and amplifications (Schneider et al., 2011). Known genes that are mutated in GCB-DLBCL include *BCL2, MYC, EZH2, MLL2, MEF2B, CREBBP/EP30, PTEN, PIK3CA, BCL6* and *REL*. ABC-DLBCL is associated with alteration in genes such as *TNFAIP3(A20), CARD11, CD79A/CD79B, MYD88, BCL6, PRDM1, BCL2* and *INK4a/ARF*. PMBCL accounts for less than 3% of NHLs and genetic alteration in genes such as *JAK2, PDL1, PDL2, SOCS1, TNFAIP3* and *REL* have been observed (Schneider et al., 2011).

AID is essential for *MYC/IGH* translocations in primary B cells and mouse plasmacytomas (Kovalchuk et al., 2007; Potter, 2003; Ramiro et al., 2004; Takizawa et al., 2008). Knocking out one of the NHEJ factors (DNA Lig4, XRCC4, Ku70, Ku80, DNA-PKcs, Artemis or Cernunnos) in mice in a p53 deficiency background leads to development of thymic lymphomas or pro-B cell lymphomas associated with complex *IgH* translocations (Difilippantonio et al., 2002; Gladdy et al., 2003; Rooney et al., 2004; Zhu et al., 2002). These translocations share similar features with the AID dependent IgH translocations observed in IL6-induced murine plasmacytomas and are likely to be associated with aberrant repair of DSBs during CSR, suggesting a link between the NHEJ pathway and aberrant CSR/switch translocations during development of B cell malignancies.

2 AIMS OF THE PRESENT STUDY

2.1 GENERAL AIM

The overall aim of this thesis work is to try to understand the mechanisms involved in DNA repair during immunoglobulin class switch recombination and somatic hypermutation and how these events are involved in the pathophysiological processes resulting in immunodeficiency and cancer development in humans.

2.2 SPECIFIC AIMS

1. Study the CSR and SHM processes in patients with Ataxia-telangiectasia related deficiency (*paper I*).
2. Study the CSR processes in Artemis deficient patients (*paper II*).
3. Study the CSR processes in Cernunnos deficient patients, and its relation to B cell lymphomagenesis (*paper III*).
4. Study the SHM pattern in patients with Nijmegen breakage syndrome and Ataxia-telangiectasia like disorders (*paper IV*).

3 MATERIALS AND METHODS

The materials and methods are described in detail in each paper. Selected methods are discussed below.

3.1 AMPLIFICATION OF SWITCH FRAGMENTS AND ANALYSIS OF THE SWITCH JUNCTIONS

Genomic DNA was purified from peripheral blood lymphocyte (PBL) from patients and controls. The amplification of Sμ-Sα or Sμ-Sγ fragments from switched cells was performed using a nested PCR assay (Pan et al., 2002). Briefly, for amplification of Sμ-Sα fragments, the Sμ1 and Sα-common-1 primers were used in a first PCR run, the PCR product was then used as template to perform a second run of PCR with Sμ2 and Sα-common-2 primers (Fig. 3). The number of switch fragments was determined

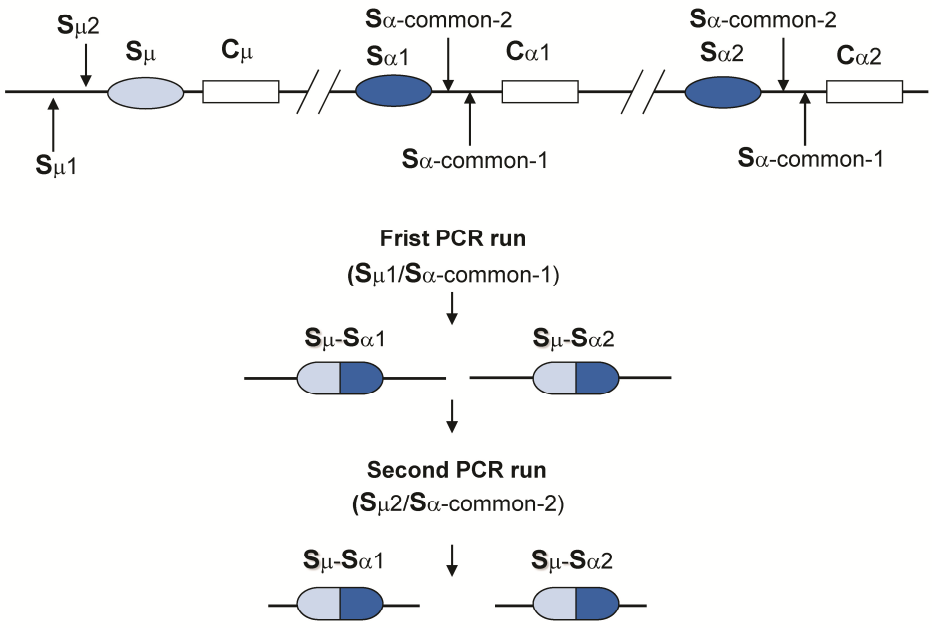


Figure 3. Amplification of Sμ-Sα fragment. The upper part shows the position of primers used, the lower part illustrates the nested PCR strategy.

from 10 reactions run in parallel using DNA from one individual and represents random amplification of switched clones. PCR products were purified, ligated into the pGEM-T vector, transformed into DH5α competent cells and subsequently sequenced. The breakpoints were determined by aligning the switch fragment sequences with the Sμ (X54713)/Sα1 (L19121)/Sα2 (AF030305) or Sμ/Sγ1 (U39737)/Sγ2 (U39934)/ Sγ3 (U39935) germline sequences. Microhomology was defined as successive nucleotides

that were shared by both the Sμ and the downstream S regions (without mismatches). Imperfect repeats were determined by identifying the longest overlap region at the S junction by allowing one mismatch on either side of the breakpoint. Insertion was defined as a nucleotide at the breakpoint that was identical to neither of the S regions. Mutation at or close to CSR junctions was defined as a nucleotide change within 15bp on both sides of the S junction (Fig. 4).

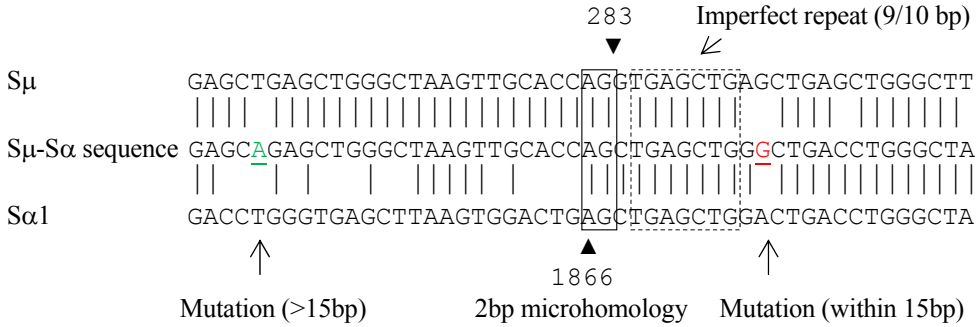


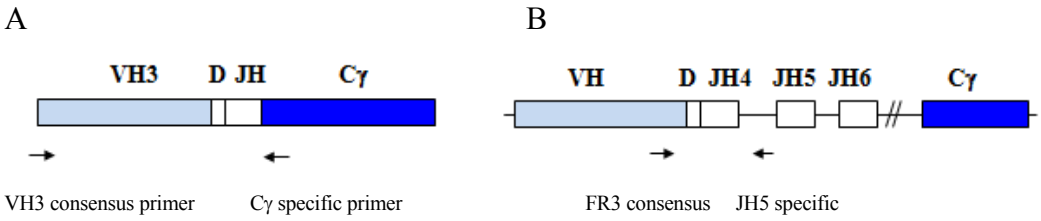
Figure 4. Determination of switch junctions. The Sμ and Sα1 sequences are aligned above and below the recombined switch junctional sequences. 2 bp microhomology was indicated by solid-line boxes and the imperfect repeats were indicated by dotted-line boxes. The Sμ and Sα breakpoints for each S fragment are indicated by a downward arrowhead and an upward arrowhead, respectively, and their positions in the germline sequences are indicated above or below the arrowhead. A nucleotide with green color indicates a mutation away from the junction (>15bp) and the red G indicates a mutation close to junction (±15bp).

3.2 ANALYSIS OF MUTATION PATTERN IN THE VH TRANSCRIPTS OR JH INTRONIC REGIONS

Total RNA was extracted from patients PBL and first-strand cDNA synthesis was performed using a *Cγ* specific primer. Regions composed of the full V region sequence and the V(D)J junctions were PCR amplified using VH3 consensus and *Cγ* specific primers (Fig. 5A). The JH4 intronic regions were amplified from genomic DNA prepared from $CD27^+$ B cells using an FR3 consensus primer and a primer upstream of JH5 (Fig. 5B) (Faili et al., 2004).

The PCR products were cloned, and sequenced using standard methods. The immunoglobulin V(D)J junctional sequences were analyzed by the IMGT/Junction Analysis tool (http://imgt.cines.fr) (Fig. 5C) (Giudicelli et al., 2004). The analysis of JH4 intronic sequences was performed using the LASERGENE software package. Mutations were imported into a Microsoft Excel spreadsheet and computations of the

number of each type of nucleotide substitution and the composition of the flanking sequences around these substitutions were performed using macros in Excel (methods developed by our collaborator Deborah K. Dunn-Walters at the Department of Immunobiology, King's College London School of Medicine, Guy's Hospital, UK).



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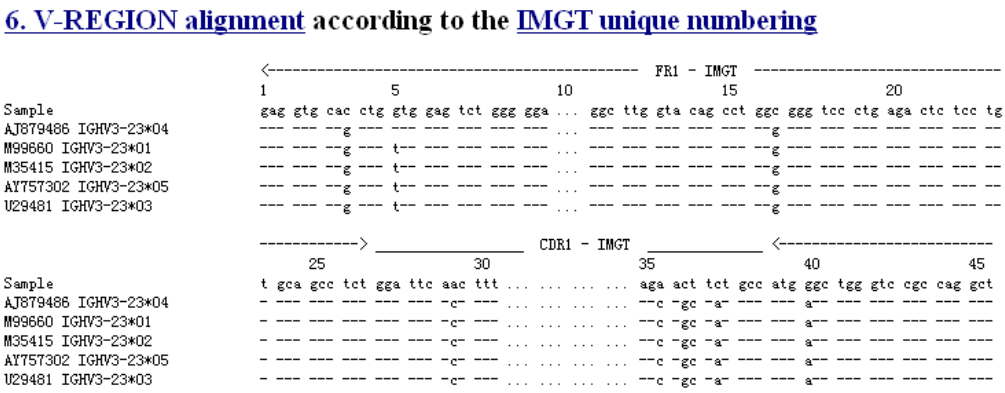

Figure 5. Amplification the VH transcripts and JH intronic regions and analysis of the mutation pattern. (A) PCR strategy for amplification of VH3-Cγ transcripts. (B) PCR strategy for amplification of JH4 intronic regions. The arrows indicated the rough positions of the PCR primers used. (C) Screen snap shot for results from the IMGT Analysis tool.

3.3 *IN VITRO* NHEJ PLASMID ASSAY

The assay was performed as previously described (Verkaik et al., 2002). Briefly, one μg of the pDVG94 construct was digested by EcoRV and EcoR47III, resulting in a blunt-ended linear joining substrate with a 6-bp direct repeat (ATCAGC) at both ends. The linearized plasmids were transiently transfected into fibroblast cell lines using TurboFect (Fermentas, Burlington, Ontario, Canada) according to the manufacturer's instruction. During cell culture, the DNA ends could be joined either via direct joining or via 6bp-microhomology or other types of joining. Joining via 6bp-microhomology can results in the generation of a BstXI restriction site (Fig. 6). Forty-eight hours after transfection, plasmid DNA was recovered from the cell lines using the DNeasy blood & tissue kit. Recombination junctions were PCR amplified using the primers FM30 and DAR5 (Verkaik et al., 2002). The resulting PCR products were gel purified, cloned into

pGEM-T vector and sequenced. The percentage of each type of joining used can be determined after aligning the sequence with the vector sequences.

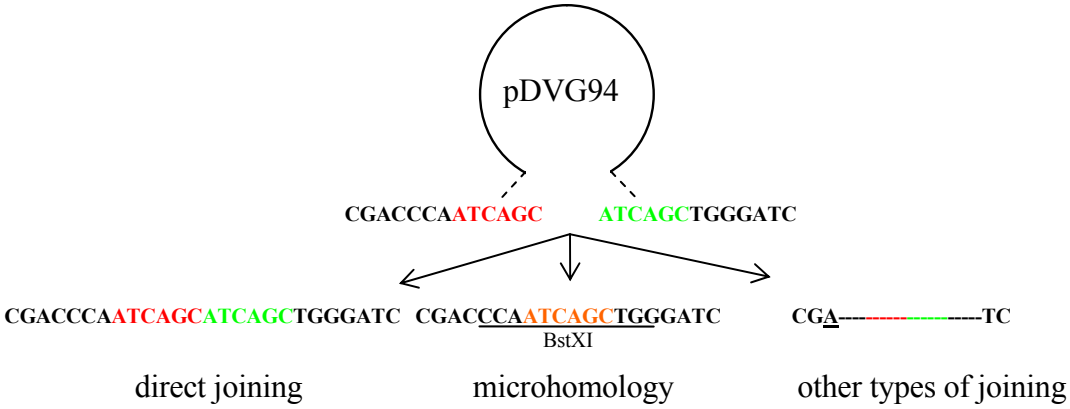

Figure 6. The NHEJ plasmid assay. The 6-bp direct repeats at the blunt ends are marked with red or green color.

In Verkaik et al's paper, the rejoined fragment was amplified and cut by BstXI. The resulted restriction fragments were separated in a 6% polyacrylamide gel and the gel was dried on DE-81 paper (Whatman) after electrophoresis and the products were visualized by phosphor imaging. Subsequently, the amounts of the undigested and digested fragments were quantified using the Image Quant software. However, after aligning the recombination junctional sequences, we found that some patient (Cernunnos deficient patients) samples show a interesting type of "other types of joining", with a 2-100 bp deletion that are associated with 1-7 bp microhomology (exemplified in Fig. 6), which cannot be digested by BstXI (Verkaik et al would consider these as "direct joining"). Our data suggest that direct cloning and sequencing of the joined fragments may give a better picture of the usage of different types of joining (paper III).

4 RESULTS AND DISCUSSION

4.1 DISPARATE ROLES OF ATR AND ATM IN CSR AND SHM (*PAPER I*)

In this paper, we studied the role of ATR during CSR and SHM, showing that ATR has a partially overlapping role with ATM during CSR, but also unique functional properties in the repair processes during CSR and SHM.

Analysis of CSR in ATRD patients

DNA from two ATRD patients was used in this study. Thirty nine unique Sμ-Sα junctions were generated and analyzed. A significantly increased microhomology usage was observed, where the average length of overlap was 3.0 ± 3.8 bp, as compared to 1.8 ± 3.2 bp in controls (Lahdesmaki et al., 2004; Pan et al., 2002). Interestingly, this was mainly because of increased usage of homologies encompassing 4-6 bp, and to a much lesser degree due to 1-3 bp homologies. Forty eight Sμ-Sγ junctions were also generated and analyzed and again the length of microhomology was slightly increased (1.8 ± 2.0 bp vs. 1.2 ± 1.2 bp in controls). The number of S junctions with mutations was less than in controls in both Sμ-Sα and Sμ-Sγ junctions, whereas insertions were observed at a similar rate.

During analysis of CSR junctions, four end joining pathways have been previously proposed (Pan-Hammarstrom et al., 2005; Pan et al., 2002). The first is “blunt end” joining, where DNA ends are either joined precisely (we later refer that as “direct joining”) or where 1-3 bp (mostly 1bp) insertions are introduced. The second pathway involves joining of partially complementary “staggered” DNA ends in an imprecise process, where mutations are frequently introduced at, or close to, the switch junctions. The third pathway exhibits precise joining of complementary DNA ends with a short sequence homology (1-3 bp), and no mutations or insertions being introduced at, or close to, the junctions. The fourth pathway is an alternative joining where at least 4 bp of microhomology is observed at the S junction. The pattern of CSR junctions from ATRD patients suggest that ATR is required for processing of partially complementary ends as the second pathway is less often used in ATRD patients. The increased usage of microhomology of 4-6 bp, but not ⩾10 bp, at ATRD CSR junctions also suggest that

there may be yet another pathway or subpathway, requiring longer microhomologies (⩾10 bp), that may be dependent on ATR but not ATM.

Analysis of SHM in ATRD patients

We subsequently analyzed the SHM pattern in RNA from peripheral blood from three ATRD patients. The frequency of mutations in the *VH3-23* genes derived from ATRD patients varied from 6.8 to 11.9%, which is similar to age-matched control donors. The distribution of mutations was also largely similar to the one found in normal controls. However, there was a significant difference in the targeting of the A or T nucleotides in TA motifs, where the T and A are equally targeted in patients (70 T vs. 69 A mutations, respectively), but in controls, A is clearly more targeted (51 T vs. 99 A mutations). Interestingly, this biased targeting was most striking in TA motifs within four short DNA sequences, which partially overlap with the RGYW motif (underlined) (i.e., two are TAGCA and the remaining two are AGCTAT). This suggests that the loss of the A preference on the coding strand in ATRD patients might be a result of differences in the mechanism generating A/T mutations within these sequences.

As discussed in the SHM section, phase II in SHM is mainly characterized by mutations in A-T pairs. The altered SHM pattern in ATRD patients suggests that ATR may interact with, or recruit, members of the MMR pathway, such as MSH2, MSH6 or polymerase η. However, cells deficient in these proteins show reduced rates of mutations at both A and T nucleotides (Delbos et al., 2007; Martomo et al., 2004; Neuberger et al., 2005), whereas in ATRD cells, the mutation load on A and T nucleotides is normal but an increased ratio of T/A targeting is observed. This may suggest that ATR is not an "A/T mutator," but rather, a "recruitor" for "A/T mutators," preferentially to the transcribed strand at specific sequences. ATR and AID can both associate with RPA. This may raise the possibility that ATR could be acting as early as at the initial AID targeting stage by phosphorylating RPA or by regulating its intranuclear translocation (Barr et al., 2003; Bartrand et al., 2004). Alternatively, RPA recruits the ATR-ATRIP complex to sites of DNA damage (Zou and Elledge, 2003) and ATR involvement could thus be post-initiation. As ATR may be linked to AID via RPA and RPA targets AID to SHM hotspots motif (Chaudhuri et al., 2004), it is thus logical that the ATR effects are more noticeable near the AID target motifs (WRC/GYW).

Functional comparison of ATR and ATM in CSR and SHM

Both ATM and ATR belong to the PIKKs family. The functional roles of ATM and ATR have been extensively studied in the general DNA damage response. *In vivo* patterns of CSR junctions and mutations in the VH regions in A-T patients have been analyzed previously by our group using a similar experimental design (Pan-Hammarstrom et al., 2003; Pan et al., 2002). In this study, we have compared the role of ATR in CSR and SHM with ATM (Table IV in paper I). In CSR, although they all show aberrant S junctions with an increased usage of microhomology and a reduced number of mutations/insertions at the Sμ-Sα junctions, the two characterizations for A-T patients, i.e., an absence of 0 bp homologies (4.5% in A-T vs. 41.0% in ATRD) and an increased proportion of Sμ-Sα fragments exhibiting ≥10 bp homology (30% in A-T vs. 3% in ATRD), were not found in ATRD patients. Another notable difference is that the proportion of junctions with insertions was reduced in patients with A-T (2 vs. 25% in controls), but not in those with ATRD (23%). In SHM, A-T patients show a normal frequency and a largely normal pattern of SHM in the VH regions, whereas in ATRD patients, the mutation pattern was clearly altered. These data indicate that the ATM-dependent pathway is mainly used in the end joining process in CSR, whereas the ATR-dependent pathway appears to be involved in both CSR and SHM.

4.2 INVOLVEMENT OF ARTEMIS IN NHEJ DURING CSR (*PAPER II*)

Artemis is one of components of the NHEJ pathway. It is essential for V(D)J recombination due to its function in opening the hairpin structures at the coding ends (Kurosawa and Adachi, 2010; Riballo et al., 2004). It was first thought that Artemis is not involved in CSR, as both immunoglobulin levels and Sμ-Sγ1 junctions are normal in Artemis deficient mice (Rooney et al., 2005). By analyzing the recombination junctions from Artemis-deficient patients, however, our study provides direct evidence that Artemis is also required for CSR.

Three RS-SCID patients, who carried different mutations in the *Artemis* gene, and one progressive CID patient with hypomorphic mutations in the *Artemis* gene were used in this study. Lower levels of IgA switching were observed in these patients. Fifty four unique Sμ-Sα junctions were obtained from the patients and were characterized by a significantly decreased proportion of junctions with no microhomology (11% vs. 42% in controls) and a significantly increased proportion of junctions exhibiting a long

microhomology of ≥10 bp (39% vs. 16%). The direct end-joining pathway (no microhomology, no insertion) was lacking in these patients, in contrast to the 18% observed in age-matched controls. This suggests that in the absence of Artemis, recombination of the Sμ and Sα regions is heavily dependent on the alternative end-joining pathways that require long microhomologies and the ability of cells to join these two S regions by a direct end-joining mechanism appears to be totally lost. The 1-bp insertion usage and frequency of mutations around the junctions (±15 bp) was significantly reduced in Artemis deficient patients, with an altered pattern of insertions or mutations, where mainly A/T mutations occurred. These dominant A/T mutations/insertions are a unique feature in Artemis-deficient patients as the few mutations observed in ATM- or DNA Lig4-deficient patients were mainly located at G/C sites (Pan-Hammarstrom et al., 2005; Pan et al., 2002).

Sμ-Sγ junctions could only be amplified from the patient who carried "hypomorphic" mutations in *Artemis*. The junctions appeared to be largely normal, however an unusual type of sequential switching was observed (from IgM, through one IgG subclass, to a different IgG subclass). The $S\gamma_x$-$S\gamma_y$ junctions within the Sμ-$S\gamma_x$-$S\gamma_y$ fragments showed long microhomologies. Thus, switching to IgG is more affected than switching to IgA when the function of Artemis is impaired, as the microhomology-based A-EJ pathway cannot be efficiently used as a backup to recombine the Sμ and Sγ regions. This could be explained by the fact that the homology between Sμ and Sγ is much lower than the one observed between Sμ and Sα regions. However, the different Sγ regions share a very high degree of homology and once the switching to IgG3 or IgG1 has occurred, the alternative pathway can be used to drive a switch to a downstream IgG subclass, for instance IgG2, through a recombination of the two Sγ regions.

Unlike the DH-JH junctions from Artemis deficient cells, where long stretches of palindromic nucleotides have been observed (van der Burg et al., 2007), CSR junctions in the Artemis-deficient patients showed a normal frequency and length of the potential palindromic sequences, suggesting that the hairpin-opening activity of Artemis is unlikely to be required for CSR and a hairpin structure is probably not a major intermediate during CSR. Based on the pattern of CSR junctions from Artemis deficient patients, a possible role of Artemis in direct end joining during CSR is proposed. One hypothetical model (Fig. 7, as well as Fig. 3 in paper II) is that Artemis

can process a pair of noncompatible ends to one blunt end and one end with 3′ overhang. Ku, DNA Lig4/XRCC4, and XLF subsequently promote ligation of such modified ends while retaining the 3′ overhang sequence, resulting in no terminal microhomology during end joining. A second possibility could be that processing by Artemis results in two blunt ends and, subsequently, be directly ligated. Yet another possibility could be that Artemis may convert terminally blocked blunt ends with short overhangs and ligatable termini (Yannone et al., 2008). It is unclear, however, if such blocked blunt ends exist during the CSR reaction.

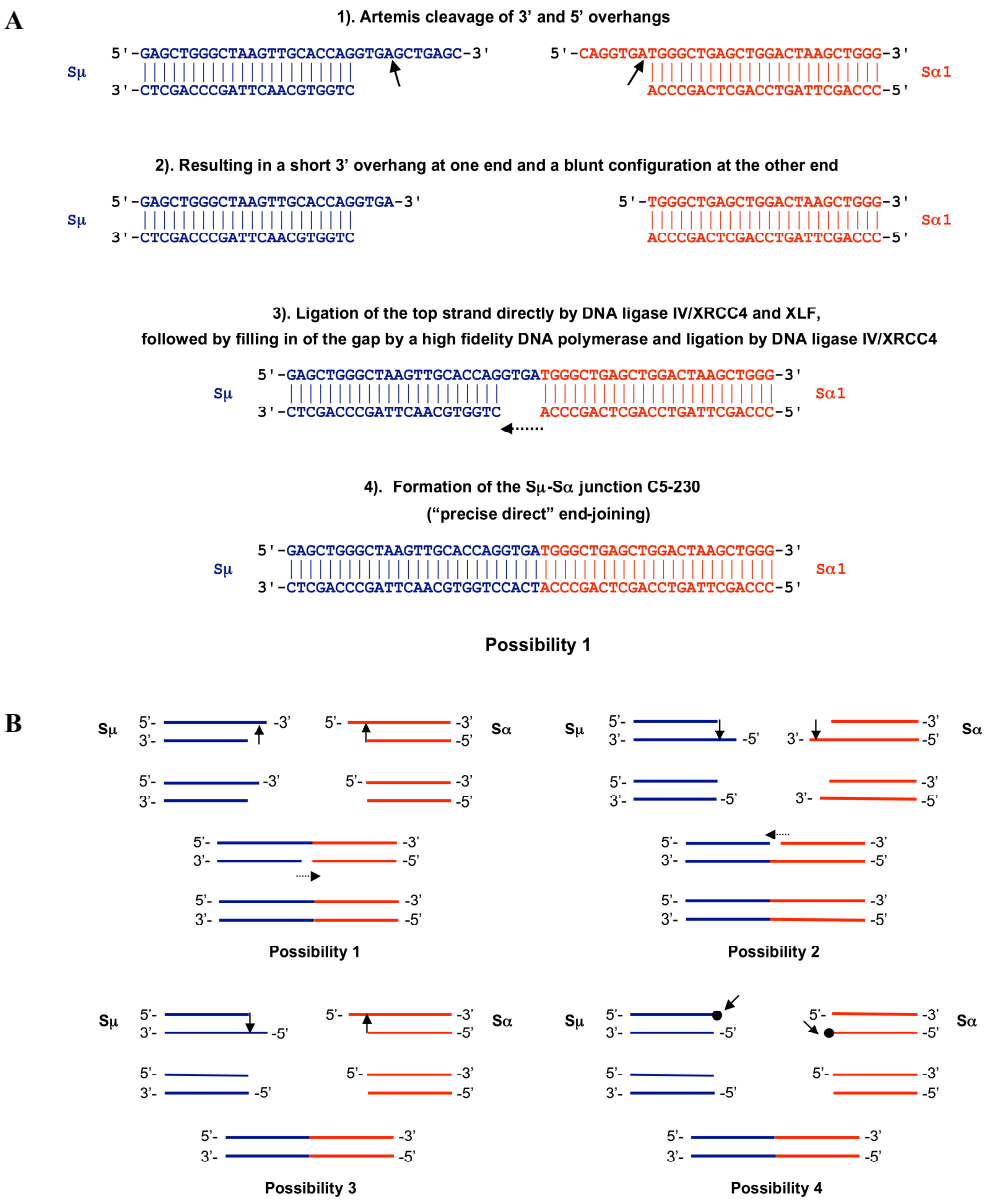

Figure 7. Hypothetical model for Artemis-dependent direct end-joining during CSR. The figure shows several possible ways of end processing by Artemis that may lead to the precise direct end-joining. (A) Possibility 1 is illustrated using the actual sequence of the junction. (B) Schematic models for possibilities 1- 4.

4.3 ROLE OF CERNUNNOS/XLF DURING CSR AND B CELL LYMPHOMAGENESIS (*PAPER III*)

Cernunnos is the latest addition to the NHEJ machinery (Ahnesorg et al., 2006; Buck et al., 2006a). To investigate whether Cernunnos is required for processing of DSBs during CSR, we characterized the S recombinational junctions from Cernunnos deficient human B cells. We also screened for somatic mutations in the *Cernunnos* gene in a large cohort of mature B cell lymphomas to address the question whether Cernunnos deficiency and aberrant CSR events are associated with development of malignancies in mature B cells.

Analysis of CSR in Cernunnos/XLF deficient patients

Seven Cernunnos deficient patients were used in our study. Serum levels of IgA and IgG were markedly reduced in all patients, whereas the levels of IgM were quite variable (Table I in paper III) (Buck et al., 2006a; Verloes et al., 2001). Eighty four unique Sμ-Sα junctions were analyzed from Cernunnos deficient patients. The average overlap is significantly increased in patients (8.5 ± 6.9 vs.3.9 ± 5.1 in controls), mainly due to an increased usage of microhomology of $\geq$10 bp and a significantly decreased usage of "direct end joining". A similar pattern has previously been observed in B cells deficient in DNA Lig4, XRCC4, Artemis (paper II), and ATM (Pan-Hammarstrom et al., 2005; Pan et al., 2002; Rivera-Munoz et al., 2009; Yan et al., 2007). The frequency of mutations around and away from the junctions (±15 bp) was also significantly reduced in patients, with an altered pattern occurring mainly at A/T residues (Table III in paper III).

In contrast to IgA switching, switching to IgG was more difficult to detect in Cernunnos deficient patients. As only 6 Sμ-Sγ junctions could be amplified in spite of multiple attempts, a proper comparison with controls was not possible.

The pattern of CSR junctions from Cernunnos deficient patients largely resembles those from DNA Lig4, Artemis and ATM deficient patients, suggesting that Cernunnos is likely to be involved in the DNA Lig4 dependent NHEJ pathway during CSR. Probably, Cernunnos provides end bridging or stability when terminal microhomology is absent, and facilitates alignment-based gap filling for partially complementary ends. Interestingly, the CSR junctions from patients also show a significantly decreased microhomology usage at 1-3 bp, which was not observed in DNA Lig4 deficient cells

(Pan-Hammarstrom et al., 2005), suggesting that Cernunnos may have a DNA Lig4 independent role for ligation of cohesive ends during CSR using few bp microhomologies. Recently, Cernunnos has also been suggested to have additional roles in the early stages of NHEJ as it quickly responds to DSB induction, can bind to DNA (Lu et al., 2007a) and interacts with Ku in a DNA dependent manner (Yano and Chen, 2008; Yano et al., 2009; Yano et al., 2008). Taken together, Cernunnos may have a number of functions in CSR, both in the early and late steps of DNA Lig4 dependent NHEJ, and in some of the DNA Lig4 independent reactions, such as simple re-ligation of cohesive ends (the third end-joining pathway discussed in the ATR study).

Role of Cernunnos in B cell lymphomagenesis

Through screening the Cernunnos encoding gene in 269 mature B cell lymphoma samples, a novel missense mutation (p.Q227R), was identified in a Chinese GCB-DLBCL patient (DL8). The mutation was not identified in 429 ethnically matched healthy individuals, suggesting that it does not represent a rare polymorphism in the population. Furthermore, by analyzing the microdissected tumor and non-tumor tissues, we could confirm that this mutation was somatically acquired in the tumor cells. The NHEJ plasmid assay was subsequently used to evaluate if the mutation would affect the function of the Cernunnos protein. Our results show that the p.Q227R mutation may have a dominant negative effect, where joining of the resected DNA ends, reflected by deletions at the junctions, is affected. Translocations in *IGH/BCL2* t(14;18)(q32;q21) and *MYC/IGH* t(8;14)(q24;q32) were also identified in 26% and 50% of the cells respectively from DL8 based on FISH analysis (Fig. 3C in paper III). A long distance PCR assay was subsequently carried out to map the *MYC/IGH* translocation breakpoints, and the results show that the switch γ4 regions may be disrupted, although the exact translocation breakpoint could not be mapped. Furthermore, clonal-like, dynamic IgA switching activities were observed in these lymphoma cells (Fig. 4 in paper III).

Although many studies have provided evidence suggesting a link between NHEJ deficiency and B cell lymphomagenesis (Buck et al., 2006b; Gostissa et al., 2011; Moshous et al., 2003; Riballo et al., 2001; Zhang et al., 2010), the mutation (p.Q227R) observed in our study, represents the first somatically occurring mutation identified in a gene encoding a NHEJ factor in human lymphomas. Our study thus suggests a link

between Cernunnos deficiency and aberrant CSR/switch translocations during the development of B cell malignancies.

4.4 ROLE OF THE MRN COMPLEX DURING SHM (*PAPER IV*)

The MRN complex appears to be the major sensor of the DNA breaks and subsequently recruits and activates ATM to phosphorylate other proteins which are involved in cell-cycle control and DNA repair (D'Amours and Jackson, 2002; Lavin and Kozlov, 2007). We have previously shown that the MRN complex is involved in CSR (Lahdesmaki et al., 2004). To test whether the MRN complex is involved in SHM, we analyzed the *in vivo* SHM patterns in cells from ATLD (Mre11 deficient) and NBS (NBS1 deficient) patients.

The SHM pattern in $VH_{3\text{-}23}$-$C\gamma$ transcripts was first analyzed in 4 ATLD and 6 NBS patients. The frequency and distribution of mutations obtained in both patients groups were largely similar to that observed in controls. The mutation pattern observed in cells from ATLD patients is largely normal, although a small, but significant, increase in the frequency of A to C transversions was observed. These mutations mainly occurred in AGA, CAA and CGA, whereas decreased in CTA trinucleotide sequences. These trinucleotides are neither related to AID targeting motif nor the SHM hotspot motif. In NBS patients, the mutation pattern was altered, with a significantly increased number of G transversions (Fig. 1 in paper IV). Unlike ATLD, these G mutations mainly occurred in AID hotspot motifs (WRC/GYW) and/or in SHM hotspot motifs for G/C mutations (RGYW/WRCY) (Fig. 2 in paper IV). The altered pattern of base pair substitutions in NBS patients may thus arise from asymmetric targeting of AID, or linked repair steps.

To exclude the possibility that the altered mutation pattern in NBS cells was a result of selection bias introduced by studying the expressed *VH* genes only, the *JH4* intronic sequences in NBS patients were subsequently analyzed. The mutation pattern in the *JH4* intronic sequences largely recaptures the features in the VH regions, where an increased number of G, but not C, mutations are observed in SHM and/or AID hotspots in NBS patients. Although the mutation pattern is altered in NBS patients, the largely normal frequency of mutation in both patients groups suggest that the MRN complex is not essential for SHM, and Mre11 is thus not likely to be the major nuclear involved in cleavage of abasic sites during SHM.

The increased frequency of mutations with proportionally more C than G mutations was also observed in a human B cell line where the *NBS1* gene is overexpressed, suggesting that NBS1 may facilitate generation of mutations in the C residues in the top strand (Yabuki et al., 2005). However, the results in our study showing a largely normal mutation frequency, normal frequency of mutations at the C residues in the top-strand hotspot (WRC) and increased number of G mutations in the GYW motifs (AID hotspots in the bottom strand), suggest that NBS1 is more likely to influence the outcome of deaminated C residues in the bottom strand, rather than to promote generation of mutations in the top strand. A hypothetical model for NBS's function in SHM is depicted below (Fig. 8, as well as Fig. 4 in paper IV).

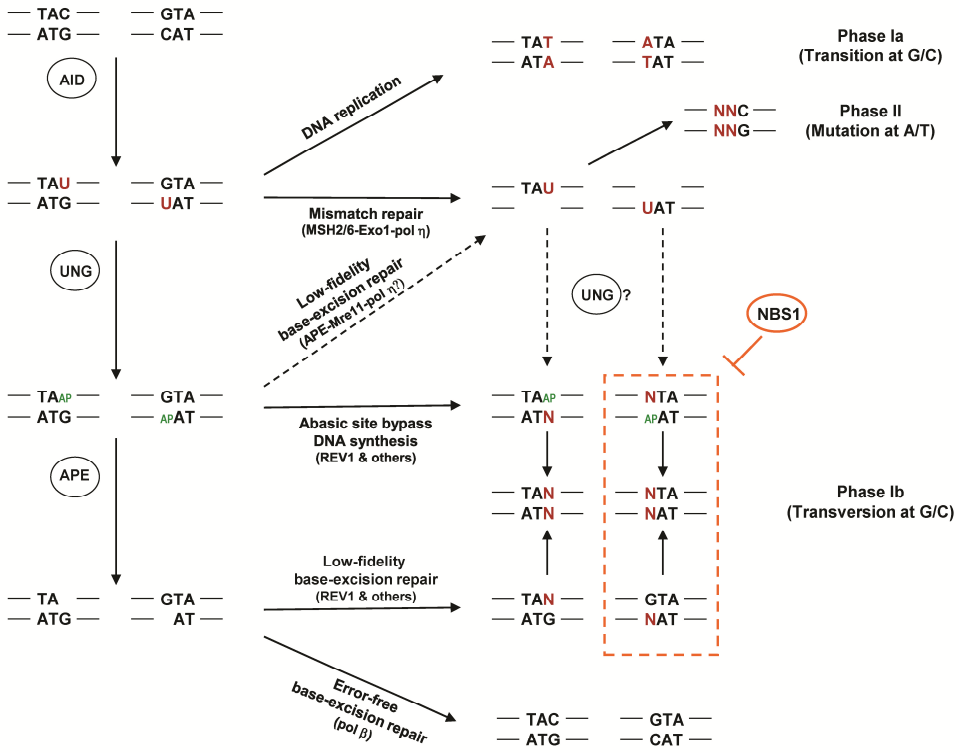

Figure 8. Hypothetical model for strand-biased mutagenic repair during SHM.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

My thesis work was mainly focused on understanding the role of ATR, Artemis and Cenunnous during CSR and the function of ATR and the MRN complex during SHM process. The main conclusions are:

I. ATR has a partially overlapping role with ATM during CSR. However, ATR also has unique functions in the repair process during CSR and SHM.

II. Artemis is required in the predominant NHEJ pathway during CSR. The functional role of Artemis in CSR probably relies on its endonuclease activity on 3′ or 5′ overhangs, rather than on hairpins.

III. Cernunnos is required in the NHEJ pathway during CSR. The somatic mutation identified in the *Cernunnos* gene in a double hit B cell lymphoma suggests linkage of Cernunnos deficiency to aberrant CSR/switch translocations during the development of B cell malignancies.

IV. Mre11 is unlikely to be the major nuclease involved in cleavage of abasic sites, whereas NBS1 might have an Mre11-independent role in regulating the strand-specific mutagenic repair process during SHM.

Our present study demonstrates that ATR, Artemis, Cernunnos and the MRN complex are involved in the CSR and/or SHM process respectively. However, there are still many questions that need to be answered including the components and the mechanism of the A-EJ repair pathways. We have established an *in vitro* cell culture assay that allows the analysis of CSR junctions as well as visual identification of class switched cells by GFP expression. The naturally CSR deficient Ramos B cell line has been stably transfected with plasmids that carry a Sμ region either together with a Sα2 or a Sγ3 region (Fig. 9) (constructed in cooperation with Dr. K. Zhang's group (UCLA, USA). After cytokine stimulation, switching to IgA2 or IgG3 can be visualized by the expression of the excised circular GFP sequence (inverted in the construct). Switch junctions also can be analyzed and preliminary results shows the usage of the A-EJ with long microhomologies. That makes the transfected Ramos cell lines an ideal system to study the factors involved in the A-EJ pathway.

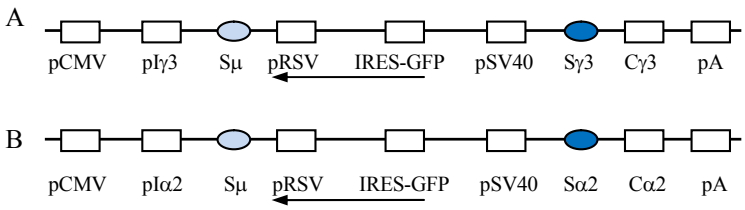

Figure 9. Structure of switch plasmid. Inverted GFP sequences in plasmid can be expressed with promoter SV40 in circular after switch recombination.

The link between defects in the NHEJ pathway and B cell lymphomagenesis also requires further experimental support. We are currently performing next-generation sequencing in different types of B-cell derived malignancies, to investigate the occurrence of genetic alterations in the NHEJ genes. Discovery of such alterations could help us understand the pathogenesis of these malignancies as well as aid the development of novel and targeted therapeutic strategies. Hopefully, our studies will provide a better understanding of B cell development and benefit patients with immunodeficiency and/or cancer.

6 ACKNOWLEDGEMENTS

From the bottom of my heart, I would like to express my sincere gratitude to all of them who have helped and encouraged me through these years. Particularly, I would like to acknowledge:

Professor **Qiang Pan-Hammarström**, my main supervisor, for giving me the opportunity to work with you and your group and with exciting projects. For guiding me through these years with your enormous experience and knowledge, for believing in me and entrusting me with elaborate and costly studies, I am sure I will never get so many excited papers if I did not come to here. Also for inviting my family to your house many times, enjoying gorgeous and delicious foods and watching the great opening ceremony of Beijing Olympic Games. Again, thank you for all the things.

Professor **Lennart Hammarström**, my co-supervisor, for all the support and encouragement during the years, for wise advice in my studies, for revising applications, manuscripts and thesis. Also for the "red bag" you and Pan gave to me, which was the first time I got in my life. And for the wonderful introductions of the Swedish culture.

Xun Xie, the first teacher and friend after I came to Sweden, for inviting me to come to Sweden and introducing me to research, for unforgettable memories of listening seminar together, attending conference together, and going to bar together. Envied by the God, wish you all well in heaven!

Kenneth Xian, my external mentor in science, for sharing your enormous experience to study and live in Sweden, for encouragement and telling me how to hold the slides and lectures during my registration seminar. As well as for the nice English courses.

Yaofeng Zhao and **Sicheng Wen**, the tutors in our laboratory, for sharing enormous experience on experiments, for always being enthusiastic and, for your friendship.

All my colleagues in Pan's and Lennart's group: **Kerstin Bergman** and **Ingegerd Löfving Arvholm**, for ordering things and all the kind help in the lab, for making our lab as home (quotation from Lennart) and for always doing Swedish-English

translation. **Naradja Wissmar,** for being such a warm-hearted person, for always helping out with all kinds of forms and administration issues. **Harold Marcotte** and **Kasper Krogh-Andersen**, for always offering support whatever and whenever I have met problems with equipments and chemicals. **Cornelia Rosner** and **Noel De Miranda**, for sharing with me your vast knowledge and experience, for fruitful discussion and collaboration with study III, as well as for helping to proofread the thesis. **Andrea Björkman**, for always being so nice to help with lots of experiments and doing Swedish-English translation and proofreading of the thesis. **Chenglin Wu,** for technical support within Word. **Ning Wang, Stephan Borte, Beatriz Alvarez, Marcel Frankowiack, Gökce Gunaydin, Sonal Pendharkar, Georgia Kokaraki, Yin Lin, Konstantinos Georgiou, Omar Alkhairy, Renee Engqvist, Carin Johansson, Annica Lindkvist, Kerstin Trumstedt,** for good advice and help, for nice parties and fika together. Past members in Pan's and Lennart's group: **Alberto Cagigi, Giuseppe Cappellano, Chonghai Liu, Roujun Peng, Miaoli Lin, Xiaoying Zhang, Ashwin Kotnis, Sergey W. Popov, Jiayi Lu, Xin Li, Shuyang Yu, Ryan Ramanujam, Magdalena Janzi, Ran Zhang, Anna Hultberg, Neha Pant, Frida Berg, Shafiqul Sarker, Javad Mohammadi, Alexej Schmidt,** for your help and good times in and out the lab.

Professor **Andrej Weintraub**, for guidance and revising kinds of forms and thesis application.

Fredrik Tingstedt and Thore Karlsson, for computer assistance.

All my friends who are present and have been in Sweden: especially **F4** (family 1 of **Yaofeng Zhao/Beibei Zhao**, family 2 of **Sicheng Wen/Jing Ding**, family 3 of **Xinjun Li/Qianren Jin**, family 4 of **Xiaolei Zhou/Pu Zhang**), for inviting me to the nice dinners so many many times, for enjoying the time together. **Shuyang Yu**, for working together during day and night, for the nice foods you cooked. **Yu Huang /Yongmei He,** for the nice BBQ, and good memory as "farmer" together. **Zhiyang Song, Xingmei Zhang, Yutong Song, Chunjiao Ning, Xiaoda Wang, Lin Zheng, Xiaoshan Zhou, Dongnan Zheng, Yunjian Xu, Zhihong Liu, Xingqi Chen, Miao Zhao, Jinling Huang, Lili Li, Hongliang Zhang, Tianyan Zhao, Ping Liu, Mo Lin, Haihui Huang, Xiaoping Wu, Hui Gao, Wen Cai, Wei Liu, Qinyang Wu, Wenpeng Niu, Zhiwen Liu, Chunyan Zhao, Jining Liu, Bo Xu, Xiaohong Wang,**

Xiaoyun Guo, Huilan Gan, Zhene Lin, and many more Chinese friends, for friendship, help and sharing the good times in Sweden.

My friends in China: **Heqiu Shi, Yuntao Zhang, Hui Li, Yuan Liu, Jianwei Liu, Yanyan Zhong,** for being close friends and pleasant moments.

Xianzhi Niu, for introducing Sweden and Karolinska to me, for the support and encouragement, together with younger brother, **Yang Lei**, for all the nice moments and fun during Christmas and New Year.

My grandfather, **Rujun Du**, forgive your grandson who cannot go back to see you for the last time, he miss you so much. My grandmother, **Xiaolan Zhang**, for your love, care, support and encouragement.

My parents, **Xianbin Du** and **Xianlan Niu**, my parents-in-law, **Hongde Jia** and **Xiumei Yan**, my younger sister, **Meili Du** and her husband **Guanghua Yao**, my lovely nephew, **Sen Yao,** my brothers in law, **Haiqing Jia**, **Haigang Jia, Haijun Jia** and his wife **Yan Yang** and cute niece **Beibei**, for your unconditional love, support forever, encouragement forever, and for everything that you have done for us. I'm so grateful for everything. And, the rest of my family: my uncles and aunts, brothers and sisters, for always support and help us in different ways, for making our big family happiness.

Finally, my dear wife, **Haiyan Jia**, for your endless love, for always being there for me, for being an excellent mom to our children. There are no words that can describe what you mean to me. Also, our wonderful beloved children, **Jiarui Du**, my cute son, and **Jiaqi Du**, my beautiful and sweet daughter, for being lovely, for making my life more colorful. I love you all more than life! Thank you for everything, and for everyday!

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