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Molecular Mechanism of Immunoglobulin Gene Conversion In Chicken DT40 Cells

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Summary

Immunoglobulin (Ig) gene conversion is one of three B cell specific processes which create the repertoire of antigen receptors in B cells. The process involves the unidirectional transfer of sequences from pseudogene V segments into the rearranged V gene. There are general and lymphoid-specific trans-acting factors as well as cis-acting elements involved in this process.

Gene conversion is most likely initiated by AID mediated cytosine deamination. If the resulting uracils need to be further processed by uracil glycosylase (UNG), UNG inactivation should block gene conversion and induce transition mutations. We report in this thesis that is indeed the phenotype in the B cell line DT40. Ig gene conversion is almost completely extinguished in the $UNG^{-/-}$ mutant and large numbers of transition mutations at C/G bases accumulate within the rearranged Ig light chain gene. The mutation rate of $UNG^{-/-}$ cells is about seven times higher than of pseudo V gene deleted (ψV^{-}) cells in which mutations arise presumably after uracil excision. In addition, $UNG^{-/-}$ cells show relatively more mutations upstream and downstream of the VJ segment. This suggests that hypermutating B cells process AID-induced uracils with approximately one seventh of uracils giving rise to mutations depending on their position.

Besides gene conversion DT40 is also a good tool to analyze somatic hypermutation. Whereas in human and mice all of the nucleotides are target for mutations, in DT40 C/G bases are the main targets. Proliferating cell nuclear antigen (PCNA) coordinates DNA synthesis during replication. Mono-ubiquination of PCNA by RAD18 is induced by DNA damage and leads to the engagement of translesion DNA polymerases in *S. cerevisiae*. In this thesis we showed that RAD18 is sensitive to DNA damaging agents and somatic mutations are reduced in the absence of RAD18. The results demonstrate that PCNA ubiquitination by RAD18 as well as by some other factors in vertebrate cells are needed, most likely by recruitment of error-prone translesion polymerases.

Zusammenfassung

Immunglobulin (Ig) Genkonversion ist einer der drei B-Zellspezifischen Prozesse, welche für die große Variabilität der Antigenrezeptoren in den B-Zellen verantwortlich sind. Maßgebend für den Prozess ist der unidirektionale Transfer von Sequenzen eines strangaufwärts gelegenen V-Segment-Pseudogens in das exprimierte, umgebaute V-Region-Gen. Der Prozess läuft unter der Beteiligung allgemeiner und lymphoid-spezifischer trans-wirkender Faktoren, sowie cis-wirkender Elemente ab.

Genkonversion wird wahrscheinlich durch die AID-Vermittelte Desaminierung von Cytosin eingeleitet. Wird das resultierende Uracil im weiteren Verlauf durch Uracil Glykosylase (UNG) modifiziert, sollte die Inaktivierung von UNG die Genkonversion stoppen und Transition-Mutationen einführen. In dieser Doktorarbeit konnte nachgewiesen werden, dass in einer $UNG^{-/-}$ Mutante der B-Zelllinie DT40 die Genkonversion fast komplett zum Erliegen kommt und sich Transitionen an C/G Basen im umgebauten Ig Gen der leichten Kette häufen. Die Mutationsrate von $UNG^{-/-}$ Zellen ist ungefähr siebenmal höher als in Zellen mit einer Deletion der V-Pseudogene (ψV^-), bei welchen Mutationen vermutlich nach dem Ausschneiden des Uracils auftreten. Darüber hinaus zeigen $UNG^{-/-}$ Zellen relativ mehr Mutationen stromaufwärts und stromabwärts des VJ-Segments. Das weist darauf hin, dass in Hypermutierenden B-Zellen etwa ein Siebtel des durch AID eingeführten Uracils in Abhängigkeit von seiner Position zu Mutationen führt.

Neben der Genkonversion lässt sich in DT40 auch die somatische Hypermutation untersuchen. Während beim Menschen und in der Maus alle Nukleotide Ziel für Mutationen sind, werden in DT40 hauptsächlich C/G Basen mutiert. „Proliferating cell nuclear antigen“ (PCNA) koordiniert die DNA-Synthese während der Replikation. Mono-Ubiquitinierung von PCNA durch RAD18 wird durch Schädigung der DNA eingeleitet und führt zur Rekrutierung der Translesion DNA Polymerasen in *S. cerevisiae*. In der vorliegenden Arbeit haben wir gezeigt, dass RAD18 sensitiv für DNA schädigende Agenzien ist und somatische Mutationen in Abwesenheit von RAD18 abnehmen. Die Ergebnisse veranschaulichen, dass die Ubiquitinierung von PCNA durch RAD18 und einige andere Faktoren in Zellen von Wirbeltieren für die somatische Hypermutation notwendig ist, wahrscheinlich weil es Error-Prone Translesion Polymerasen rekrutiert.

Abbreviations:

AID	Activation Induced Cytidine Deaminase
AP	Apurinic/ apyrimidinic
APOBEC-1	Apolipoprotein B mRNA Editing Catalytic Polypeptide 1
BDT	Big Dye Terminator
BSR	Blasticidine S Resitance gene
CIP	Calf Intestine Phosphatase
CSR	Class Switch Recombination
C region	Immunoglobulin Constant region
dNTP	Deoxynucleotide Triple Phosphate
DMSO	Dimethyl sulfoxide
D region	Immunoglobulin Diversity region
DSB	Double Strand Break
EDTA	Ethylene di-Amine Tetra Acetic Acid
EF	Elongation Factor
EST	Expressed Sequence Tag
EtBr	Ethidium Bromide
FBS	Fetal Bovine Serum
GC	Gene Conversion
GPT	Guanine Phosphoribosyl Transferase
HR	Homologues Recombination
4-HT	4 Hydroxy Tamoxifen
Ig	Immunoglubulin
IRES	Internal Ribosome Entry Site
J region	Immunoglobulin Joining region
LB	Luria Broth
NHEJ	Non Homologues End Joining

PBS	Phosphate Buffer Saline
pKS (+)	pBluescript
PCR	Polymerase Chain Reaction
Pol	Polimerase
PRR	Post-Replication Repair
SDS	Sodium Dodecyl Sulphate
sIgM	Surface Immunoglobulin M
SHM	Somatic Hypermutation
S region	Switch region
SOB	Hanahan's Broth
TAE	Tris Acetic Acid ETDA
TE	Tris EDTA
TLS	Trans Lesion Synthesis
UNG	Urasil DNA Glycosylase
V region	Immunoglobulin Variable region
2YT	2 x Yeast Extract Tryptone

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METHODS

INTRODUCTION:

I. Immunity and Generation of Antibody Diversity:

The function of the immune system is to protect vertebrates from foreign substances. Two types of responses have evolved to accomplish this task: Innate and adaptive (acquired) immunity. Innate immunity involves hereditary components which provide an immediate "first-line" of defense to frequently encountered pathogens whereas acquired immunity involves particular type of cells and proteins which provide a protection to particular type of agents (Tortora et al., 1995).

A component of the blood, white blood cells are produced in the bone marrow and help to defend the body against infectious disease and foreign materials as part of the immune system. Lymphocytes are a type of white blood cells and they are the primary effectors in the adaptive immune system. There are two broad categories of lymphocytes, namely T cells and B cells. T cells are chiefly responsible for cell-mediated immunity whereas B cells are primarily responsible for humoral immunity (antibody related). Antibodies (immunoglobulins-Ig) are proteins synthesized and secreted by B cells during immune response within processes like phagocytosis or inflammation. Each antibody recognizes a specific antigen unique to its target (Janeaway et al., 2001).

To fight against such a variety of invaders, the immune system needs to generate vast numbers of receptors. To do this, immune system has evolved different strategies like V(D)J recombination, somatic hypermutation (SHM) and class switch recombination (CSR).

A. V(D)J Recombination:

As a primary immune repertoire, V(D)J recombination (Fig. 1) involves combinatorial gene rearrangement of functional immunoglobulin genes (Tonegawa et al, 1983). It is antigen independent and occurs in the bone marrows. Initiated by lymphoid and sequence specific recombination activating genes (RAG1 and RAG2) (Schatz et al., 1988; Schatz et al., 1989), it recruits Non Homologous End Joining (NHEJ) mechanism for resolution (Chaudhuri et al., 2004). B cells then migrate to secondary lymphoid organs like spleen and lymph node, where they can undergo further antigen driven Ig gene diversification through Somatic Hypermutation and Class Switch Recombination.

B. Somatic Hypermutation (SHM):

Immunoglobulin molecules from the primary immune repertoire generally bind antigens with only modest affinity and specificity; so a fine-tuning of the antibody response is necessary for complete recognition and destruction of the antigen (Papavasiliou et al., 2002). This is provided by another lymphocyte specific process that is known as Somatic Hypermutation (Fig. 1) (Lederberg, 1959). After Brenner and Milstein proposed a model in which nucleotide point mutations arise as a consequence of DNA synthesis errors that are generated during repair of localized breaks (Brenner et al., 1966), Weigert and his colleagues, were the first to observe hypermutations in a study using mouse λ light chains at the protein level (Weigert et al., 1970).

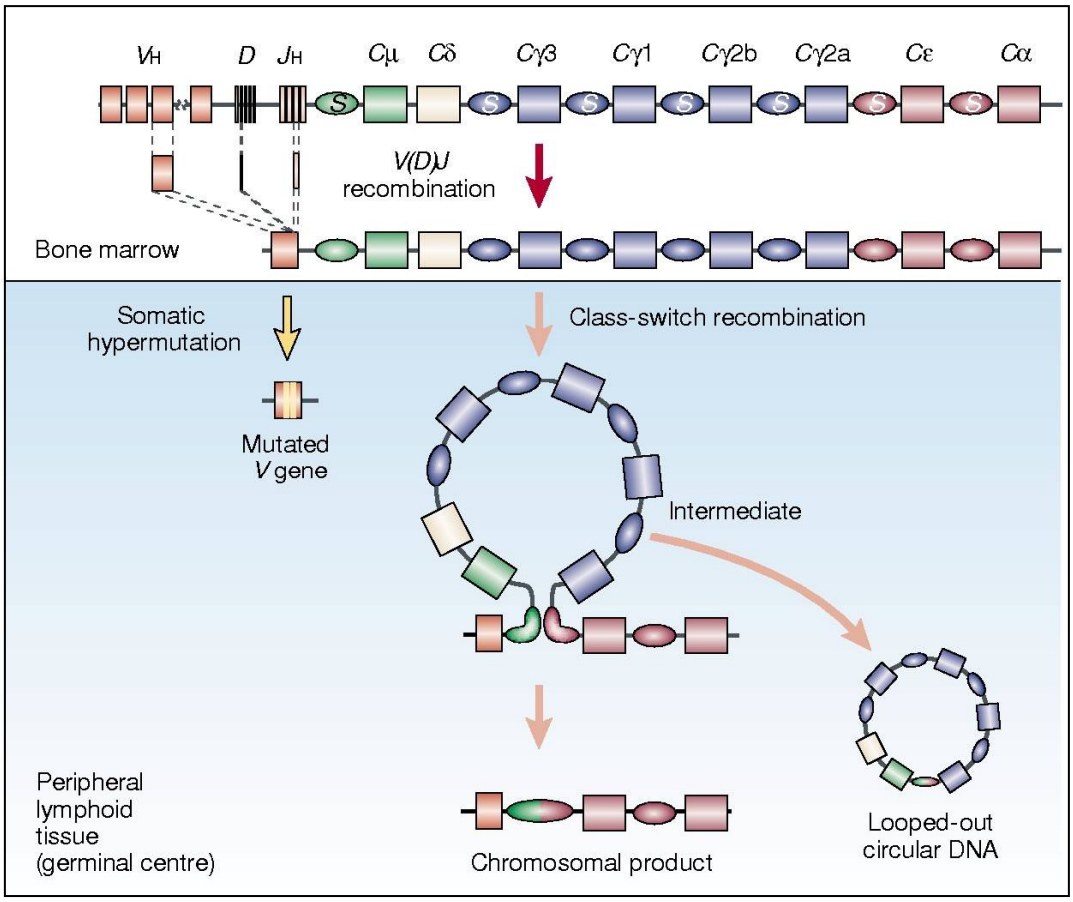


Figure 1. Immunoglobulin heavy chain gene locus and generation of antibody diversity. Solid boxed represent coding regions, and ovals switch regions. In bone marrow mainly V(D)J recombination occurs as a primary immune repertoire. Further diversification is introduced in peripheral lymphoid tissues by mecahisms of Somatic Hypermutation and Class-Switch Recombination (Modified from Kinoshita et al., 2001).

Somatic Hypermutation is triggered when the surface Ig of B cell encounters with an antigen. Following this, point mutations are introduced at a high rate specifically into immunoglobulin V genes of activated B cells (Kinoshita et al., 2001). In the germinal centers, successive cycle of these mutations and selection lead B cells produce antibodies with higher affinity which is called as affinity maturation (Fig. 2) (Siskind et al., 1969). Owing to the strong effects of selection, the true rates of SHM in vivo are unclear. However it is thought generally that SHM occurs at an extraordinarily high frequency (10^{-3} bases per generation). This frequency is almost one million times higher than the level of mutation in housekeeping genes (Martin et al., 2002). Transition mutations occur more frequently than transversions (Golding et al., 1987) and using a huge collection of mutation data it is found out that there is a hotspot motif for SHM (Rogozin et al., 1992). That is, mutations of G-C base pairs that are in RGYW (R=A or G, Y=C or T, W=A or T) and the complementary WRCY motifs occur at a high frequency. These mutational properties are probably the result of the biochemical specificities of the mutator enzymes (Martin et al., 2002).

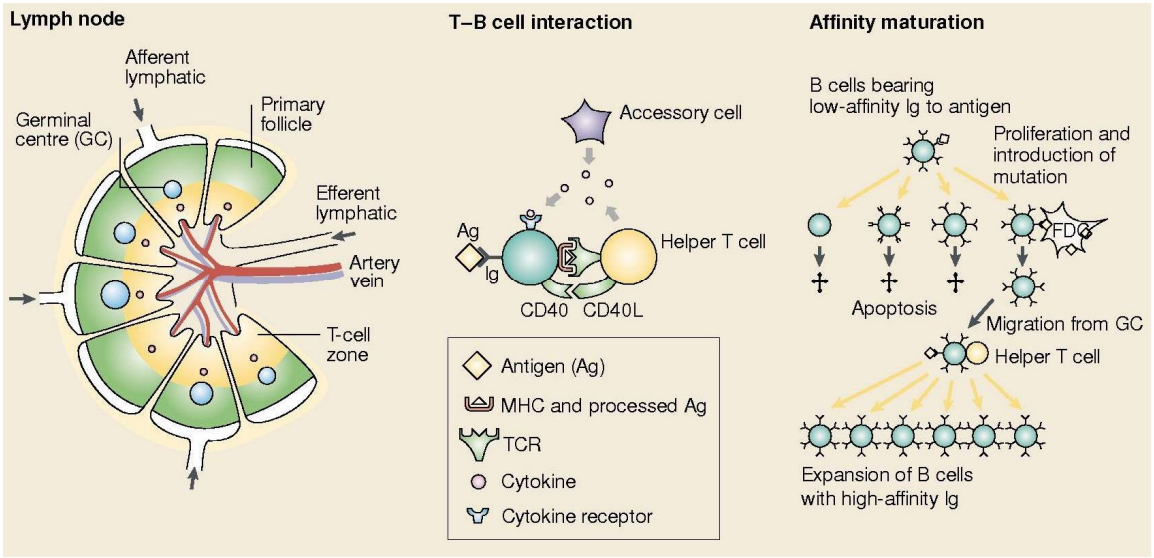


Figure 2. Immune response in a lymph node. When B cells in the primary follicle are activated by invading antigens and helper T cells , they begin to proliferate rapidly to form a germinal center. In germinal centers, T-B cell interaction mainly occurs through T cell receptor (TCR), processed antigen on major histocompatibillty complex (MHC) on B cells, and with ligand and receptor of CD40. This interaction activates T cells and accessory cells to secrete several cytokines to modulate B-cell response. These signals induce the expression of AID and thereafter SHM and CSR starts. B cells expressing Ig with a high affinity to given antigens are selected by follicular dendritic cells (FDC), which hold non processed antigens on the surface. Low affinity immunoglobulins die by apoptosis (Modified from Kinoshita et al., 2001).

One of the questions for SHM is how the target genes are selected for mutations. In several studies, it has been shown that transcription of the target locus is essential for somatic hypermutation (Peters et al., 1996; Fukita et al., 1998), and that the frequency of mutation directly correlated to the rate of transcription (Goyenechea et al., 1997; Bachl et al., 2001). Mutations start downstream of the promoter and lie around 1.5kb to 2 kb along Ig locus (Lebecque et al., 1990). In addition, Ig enhancer as well as transcription regulatory elements were also shown to be important for SHM (Betz et al., 1994; Klix et al., 1998; Michael et al., 2003).

It could well be that SHM start with a single strand (Brenner et al., 1966) or a double strand DNA breaks (Bross et al., 2000; Papavasiliou et al., 2000) or both (Sale et al., 1998), although the association of double strand breaks are controversial (Bross et al., 2002; Chua et al., 2002).

Mutations can arise actively from the error-prone processing of DNA breaks or passively from the absence of normal repair machineries (Papavasiliou et al., 2002). It has been documented in many articles that part of the Base Excision Repair and Mismatch Repair System is involved in SHM (reviewed in Martin et al., 2002; Neuberger et al., 2005). The proteins shown to be important for SHM up to now are including a lymphoid specific factor Activation Induced Cytidine Deamniase (AID) (Muramatsu et al., 2000), Uracil DNA Glycosylase (UNG) (Di Noia et al., 2002; Rada et al., 2002), MSH2 (Rada et al., 1998; Rada et al., 2004) as well as error-prone polymerases like pol η (Zeng et al., 2001) and REV1 (Simpson et al. 2003; Jansen et al., 2006).

C. Class Switch Recombination:

Class Switch Recombination (Fig. 1) allows the expression of antibodies that have the same antigen specificity but are of a secondary isotype like (IgG, IgA, or IgE). The immunoglobulin locus contains C_H genes, which encode proteins that are capable of different effector functions (Chaudhuri et al., 2004). CSR involves recombination between switch regions, which are highly repetitive GC-rich sequences of 1-10 kb in length that lie upstream of Ig C-region genes (Martin et al., 2002). Signal transduction through surface IgM, CD40 and cytokine receptors of a B cell induce the reaction of CSR and the repair step is mediated by non-homologues end-joining repair system (Kinoshita et al., 2001).

Mechanistically, CSR is a deletional recombination event like V(D)J recombination, however because neither consensus nor homologues sequence is generally found around junctions, it is different from it. Despite being distinct processes targeted to distinct Ig regions, CSR and SHM are similar in that they both occur in antigen-simulated B cells, require transcription as well as AID for initiation (Chaudhuri et al., 2004). The comparison between SHM and CSR is summarized in Table 1.

Table 1. Comparison between SHM and CSR

	SHM	CSR
Target	V region and its flanks	S region and its flanks
Transcription	Required	Required
DNA Cleavage	Required	Required
Ig Enhancer	Required	Dispensable
Repair Phase	Translesion Synthesis	NHEJ

II. Chicken B-Cell Repertoire Formation and Bursal B-Cell Line DT40:

A. Immunoglobulin Gene Conversion:

Immunoglobulin gene diversification and the ontogeny of B lymphocytes differ species to species (Reynaud et al., 1994). Although humans and mice develop B lymphocytes in the bone marrow by V(D)J recombination, in most of the species like chicken, cow, and rabbit since there is only one single functional V and J segments it is only to ensure the assembly and expression of a single functional gene (Arakawa et al., 2004). Therefore, the main diversity as a primary immune repertoire is introduced into the rearranged V(D)J segments by the mechanism so called gene conversion (GC) in the organ bursa of Fabricious in chicken (Fig. 3) (Reynaud et al., 1987). It is a kind of homologues recombination event and it uses pseudo V genes (pseudogene) upstream of the functional V gene. Chicken light chain includes 25 pseudogenes whereas heavy chain includes 80 pseudogenes. All pseudogenes lack promoter, leader exon or V(D)J recombination signal sequences. Only a few of the pseudogenes contain stop codons or frameshift mutations, but quite a few are

truncated in their 5' or 3' ends (Reynaud et al., 1989). Unlike V(D)J recombination or CSR, after gene conversion pseudogenes do not disappear that is, it is not a cut & paste mechanism but a copy & paste mechanism (Reynaud et al., 1987; Carlson et al., 1990). Only the pseudogenes on the same chromosome are used as donors (Carlson *et al*., 1990) and pseudogenes that are either more homologous, closer or in the opposite orientation to the rearranged V segment are preferred (McCormack et al., 1990; Sayegh et al., 1999). Conversion tracts range from 8 bp to around 200 bp (McCormack et al., 1990). The 5' ends of the gene conversion tracts always begin in regions of homology between the pseudogene donor and recipient V segment, whereas the 3' ends can occur in regions of non-homology and often encompass nucleotide insertions or deletions (McCormack et al., 1990).

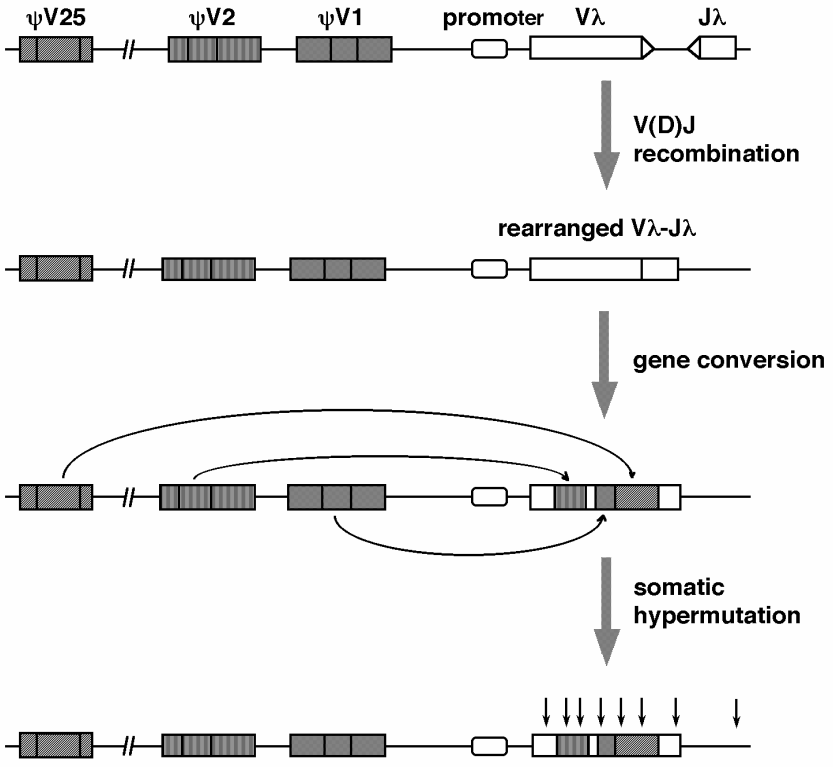


Figure 3. Chicken Ig gene diversification. Antibody diversity in chicken mainly involves gene conversion and somatic hypermutation. Chicken has only a single V and J segment in the light chain locus which can be functionally rearranged by V(D)J recombination. The rearranged VJ segment is diversified by segmental gene conversion using pseudo V genes as donors. Rearranged VJ segments also undergoes somatic hypermutation in the bursa and spleen.

B. Chicken B Cell Development:

The bursa of Fabricius plays a central role for chicken B cell development which can be classified into pre-bursal, bursal and post-bursal stages (Arakawa et al., 2004). In the pre-bursal stage the first DH-JH joints in the heavy chain locus can be detected in the yolk sac on embryonic day 5 or 6 and V(D)J recombination at the locus is virtually complete by embryonic day 15 (Reynaud et al., 1992). Cells which have already completed V(D)J recombination at the heavy and light chain loci, can be detected in the blood, spleen, thymus and even in non-lymphoid organs. During the bursal stage, the mature bursa of Fabricius is composed of approximately 10 000 lymphoid follicles and presents the major site for B cell proliferation and repertoire formation. Between embryonic days 10 and 15 each follicle is seeded by oligoclonal B stem cells (Pink *et al*., 1985). Gene conversion in bursal B cells is initiated around embryonic day 15 even in the absence of environmental antigen (Mansikka *et al*., 1990) and most likely it continues until the bursa involuted 4-6 months after hatching. In addition, SHM at low frequency also contributes to bursal Ig gene diversification (Arakawa *et al*., 2002a). In the post busal stage adult chicken generate germinal centers in the spleen in response to antigen. Germinal center formation is clearly detectable by day 7 after antigen stimulation and begins to wane 14 days after immunization. The Ig genes of antigen-activated B cells are diversified both by gene conversion and somatic hypermutation in the very early phase of the germinal center reaction (Arakawa *et al*., 1996). In the later stage, gene conversion is down-regulated, and most modifications are somatic hypermutations. That means antibody maturation in chicken involves first GC and then subsequently SHM (Arakawa *et al*., 1998).

C. DT40:

Chicken immunoglobulin gene conversion is difficult to study using primary bursal B cells, as these cells survive only a short time during in vitro culture (Bezzubova et al., 1994). It was, however found out that, the ALV induced lymphoma line DT40 (Baba et al., 1985) seems to be arrested at the stage of bursal B cells and continues Ig light chain gene conversion during in-vitro cell culture (Buerstedde et al., 1990; Kim et al., 1990).

Although wild-type DT40 cells are dominantly surface (s) IgM(+), spontaneously arising sIg(-) subclones can be isolated which contain frameshifts in the rearranged light

chain V segment. Repair of these frameshifts by pseudogene templated gene conversion events lead to re-expression of sIg (Buerstedde et al., 1990). This reversion from sIg(-) status to sIg(+) status can be used to quantify gene conversion efficiency by Ig reversion assay (Arakawa et al., 2004a).

In a remarkable study done by Buerstedde and Takeda in 1991 it was realized that transfected gene constructs can be integrated into their endogenous loci at higher ratios (Buersttede et al., 1991). This was surprising, since transfection of mammalian or murine cell lines leads to integration of the gene constructs pedominantly at random chromosamal positions (Smithies et al., 1985; Thomas et al., 1986). This efficient gene targeting appears to reflect an intrinsic character of chicken B cells, which is shared by most analyzed chicken B cell lines and not by any non-B cell lines (Yamazoe et al., 2004). It is still not understood whether the increased ratio of gene targeting in chicken B cell lines is related to gene conversion activity of bursal B cells. It would be highly attractive to find out a factor which enhances the integration of the recombining substrates (Bezzubova et al., 1994).

Besides efficient targeting, DT40 cells possess a number features that make it elegant. First, DT40 cells exhibit relatively a stable character in both karyotype and phenotype even during longer periods of cell culture (Sale et al., 2004). This stable character is a great advantage over murine ES cells. Thus the analysis of genetic networks on a cellular level by targeting of multiple genes is possible. Second, since DT40 has a rapid growth rate (doubling time ~10 h) cell culture assays like colony formation is greatly facilitated. Third, since the cloning efficiency of wild type cells is nearly 100%, isolation of stably transfected cells as well as subcloning of cells is easily done (Yamazoe et al., 2004). And lastly, not only for analysis of Ig gene conversion but many researchers in the fields of B cell antigen receptor signaling, cell cycle regulation, apoptosis, histon acetylation, homologues recombination as well as DNA repair nowadays use DT40 efficiently (Winding et al., 2001).

III. Molecular Mechanism of Gene Conversion:

The studies published in recent years have shed light on the mechanism of Ig gene conversion greatly. We can categorize the mechanism into several stages for simplicity:

A. Factors involved in initiation:

1. AID:

Immunoglobulin gene diversification mechanisms SHM, CSR and GC were always thought to be different until one AID comes out. In 1999, Honjo and his colleagues have identified a novel protein, Activation Induced Cytidine Deaminase (AID) that is specifically expressed in CSR active cells (Muramatsu et al., 1999). In one later study, the same group has shown that not only CSR requires AID but also SHM depends on it in mouse (Muramatsu et al., 2000). In the same year another study showed that mutations in AID cause an immunological disorder hyper IgM syndrome due to lack of CSR and SHM (Revy et al., 2000). These findings have aroused big excitement between the scientists. Because this was the first evidence that two distinct processes were under the control of one single protein. Later our group has shown that GC requires AID too (Arakawa et al., 2002b). That means one AID was enough to unite and rule them all (Fugmann et al., 2002; Nussenzweig et al., 2004).

AID is a rather small protein (~200 aa) that makes cytidine (C) to uracil (U) change either on DNA or RNA. At first, it was suggested that AID is a RNA-editing enzyme since there is a homology between AID and APOBEC-1 (Muramatsu et al., 2000). However, although still indirect, most experimental evidence support a role of AID in the deamination of single-stranded DNA in the immunoglobulin V and switch regions but not double-stranded DNA (Bransteitter et al., 2003; Chaudhuri et al., 2003; Dickerson et al., 2003; Ramiro et al., 2003; Pham et al., 2003; Peterson-Mahrt et al., 2002). It is under intense research to identify the target and action of AID. The two models of AID was described in Fig. 4 for gene conversion.

AID has the ability to mutate all the genes and it must therefore be regulated to avoid widespread genomic damage. The controls that target AID specifically to Ig genes include tissue and stage-specific expression, restricted nuclear residence, interactions with specific factors (Schotz et al., submitted) and accessibility of the target sequence (Barreto et al., 2005). Therefore AID must be tightly controlled particularly in the nucleus. Several studies

showed that AID localizes predominantly in the cytoplasm (Rada et al., 2002b; Ito et al., 2004) and AID has a functional C-terminal nuclear export signal (NES) that actively exports AID from nucleus (McBride et al., 2004; Ito et al., 2004; Brar et al, 2004) and it has an nuclear import signal (NLS) in the N-terminus (Ito et al., 2004). Reynaud and colleagues have reported that some chaperones keep AID in the cytoplasm until B receptor stimulation actively translocates AID to the nucleus (Reynaud et al. 2003).

In order to identify the minimal requirements of AID function and to understand the hypermutation activity in non-lymphoid cells, AID was ectopically expressed in fibroblasts (Yoshikawa et al., 2002) and hybridomas (Martin et al., 2002b). The results showed that AID is the only B-cell specific factor required to generate hypermutations.

Although there are many factors involved in SHM, CSR and GC identified, only Replication Protein A (RPA) has shown to be physically interacting with AID (Chaudhuri et al., 2004). To be able characterize partners of AID, a series of mutant analysis have been done. These studies showed that C terminal of AID is essential for CSR but not for GC and SHM (Ta et al., 2003; Barreto et al., 2003). Vice versa N-terminal has been shown to be important for SHM (Shinkura et al., 2004).

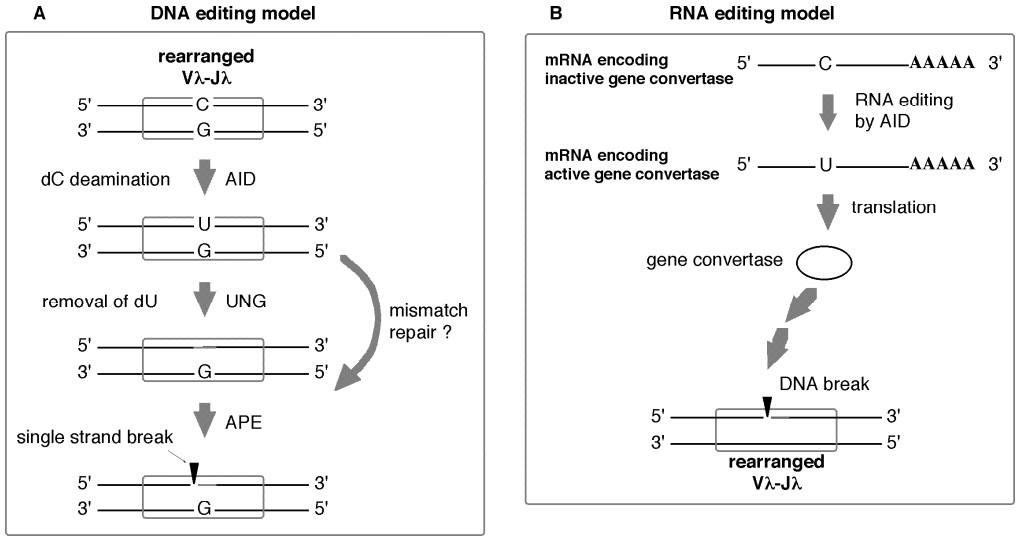


Figure 4. DNA editing and RNA editing models of AID action. In the DNA editing model, AID changes C to U by DNA deamination. A single strand break is then generated after uracil base excision by uracil DNA glycosylase (UNG) and APE endonuclease or through a mismatch repair reaction. In the RNA editing model, AID edits a codon in a mRNA which encodes a DNA modifying enzyme. This change leads to the translation of an active protein which then introduces a DNA alteration in the rearranged V segment.

2. UNG:

Uracil may arise as an aberrant base in DNA either through misincorporation of dUMP opposite adenine during DNA replication (Brynolf et al., 1978; Tye et al., 1978) or by spontaneous deamination of cytosine residues in DNA generating a U:G mispair (Lindahl, 1993; Nilsen et al., 2000). If left not repaired, these uracils may give rise to transition mutations C to T or G to A, in a subsequent replication (Duncan et al., 1982; Impellizzeri et al., 1991) or may result in mutant proteins after transcriptional bypass by RNA polymerase (Viswanathan et al., 1999).

Uracil residues in DNA are substrates for base excision repair (Lindahl, 2000). In this repair, uracil DNA glycosylase (UNG - in all organisms) as well as SMUG1, TDG and MBD4 (in human and mice) are the responsible enzymes to remove uracils from DNA (Lindahl, 1974; Pearl, 2000). Mammalian *UNG* gene encodes two isoforms, nuclear (UNG1) and mitochondrial (UNG2), which are generated via transcription from different promoters and alternative RNA splicing (Nilsen et al., 1997).

Although UNG has been identified more than 30 years since now, a link between UNG and Ig gene diversification has never been thought. It was just 4 years ago Neuberger and his colleagues have published three papers and discovered uracils as the main intermediates of antibody diversity (Petersen-Mahrt et al., 2002; DiNoia et al., 2002; Rada et al., 2002). Later, it was also showed in the human patients that are deficient for UNG that CSR is decreased and SHM is perturbed (Imai et al., 2003). However, direct evidence for the presence of uracils in DNA that were produced by AID, came just recently in the cells that are induced to express AID (Martomo et al., 2005).

The DNA deamination model of antibody diversification predicts that recruitment of AID to the Ig locus causes localized deamination of dC $\rightarrow$ dU; repair of this uracil in DNA then leads to SHM, GC or CSR depending on the pathway used for resolving the dU lesion, as well as on the precise location of the lesion (either the IgV domain or in the vicinity of the switch region) (Neuberger et al., 2003). In the case for gene conversion (Fig. 4), these uracils are removed by UNG, and the abasic sites are repaired in a recombination mediated manner and pseudogenes as donors.

B. The effect of homologous recombination (HR):

Studies in yeast *S. cerevisiae* have shown that gene conversion and targeted integration depends on the RAD52 pathway and these genes mediate the repair of double-strand break (DSB) repair by homologous recombination (Haber et al., 1999). RAD52 epistasis group encode proteins which function either in the recognition of the double-strand break (RAD50, MRE11 and XRS2) or in the promotion of homology search and strand invasion (RAD51, RAD52, RAD54, RAD55, RAD57) (Symington, 2002).

The mechanism of chicken Ig gene conversion and HR might be related, because both processes occur at high levels in DT40 and other chicken B-cell lines (Buerstedde et al., 1990). Analysis of HR in vertebrates was initiated by the discovery of mammalian orthologs of the yeast recombination gene *RAD51* (Shinohara et al., 1993). Homologues of the RAD51, RAD52 and RAD54 genes were first cloned from chicken bursal cells by reverse PCR using degenerate primers derived for conservative sequence motifs of the yeast proteins. Gene disruptions in DT40 revealed that RAD51 deficient cells do not survive, most likely because they cannot repair replication induced DSBs (Sonoda *et al*., 1998). In contrast to the severe recombination and repair defect of the *S. cereviseae* RAD52 mutants, the DT40 RAD52 deficient cells have only modestly reduced targeted integration frequencies and are not hyper-sensitive to DNA damage (Yamaguchi-Iwai *et al*., 1998). However, RAD54 deficient DT40 cells are highly X-ray sensitive compared to wild type cells, have 100 fold decreased targeted integration ratios and also show reduced Ig light chain gene conversion activity (Bezzubova *et al*., 1997). The disruption of the Nbs1 gene which is the counterpart of the yeast XRS2 gene produces a phenotype very similar to the RAD54 phenotype in DT40 (Tauchi *et al*., 2002). Vertebrates have five RAD51 paralogues (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3) and DT40 mutants of each of these genes have reduced targeted integration frequencies and DSB repair deficiencies (Takata *et al*., 2001). Interestingly, some of the functions of the XRCC3 may be compensated by RAD52, because inactivation of XRCC3 in cells lacking RAD52 results in chromosomal instability and cell death (Fujimori *et al*., 2001).

The studies of the RAD52 pathway in DT40 prove that DSB repair is well conserved during eukaryotic evolution and that targeted integration in vertebrate cells is a side effect of DSB repair. The high ratios of targeted integration in DT40 most likely reflects the up-

regulation of the RAD52 pathway in bursal B cells compared to other chicken and mammalian cells. This increased general homologous recombination activation is required for efficient Ig gene conversion, but it cannot explain how gene conversion events are specifically initiated in the Ig loci.

C. Somatic Hypermutation Besides Gene Conversion in DT40:

In wild type DT40 cells besides gene conversion there seems to occur also single point mutations close to GC tracts. These non templated mutations were first suggested as they may arise in consequence of errors in recombination (Buersttedde et al., 1991; Reynaud et al., 1987). However, they were also suggested in several studies that these are not related with gene conversions (Sale, 2004; Carlson et al., 1990). This led the idea that SHM and GC might be mechanistically related since both of the processes occur mainly in immunoglobulin V gene (Reynaud et al., 1987; Maizels, 1995; Weill et al., 1996). Direct evidence for the relationship between SHM and GC came with the inactivation of the RAD51 paralogues in DT40 cells. Normally, these proteins involved in HR but knockout study revealed a reduction in frequency of the GC events along with increase in the frequency of SHM (Sale et al., 2001). This finding showed exactly that both GC and SHM can occur in the same cell line and that the initiating events of GC and SHM are related but they differ in the manner by which most likely U:G mispairs are resolved.

- **Pseudogene (ψV) Knockout:**

In order to get further insight into how SHM and GC is being regulated and to get intermediates of gene conversion reaction, we deleted ψV genes in DT40 (Arakawa et al., 2004b). The results demonstrated that the deletion of the nearby pseudogene donors abolishes Ig gene conversion in DT40 and activates a mutation activity which closely resembles Ig hypermutation (SHM). The features shared between the new activity and SHM include 1) AID dependence, 2) a predominance of single nucleotide substitutions, 3) distribution of the mutations within the 5' transcribed region, 4) a preference for hotspots and 5) Ig gene specificity. The only difference with regard to Ig hypermutation *in vivo* is the relative lack of mutations in A/T bases and a predominance of transversion mutations in the ψV knock-out clones. However, this difference is also seen in

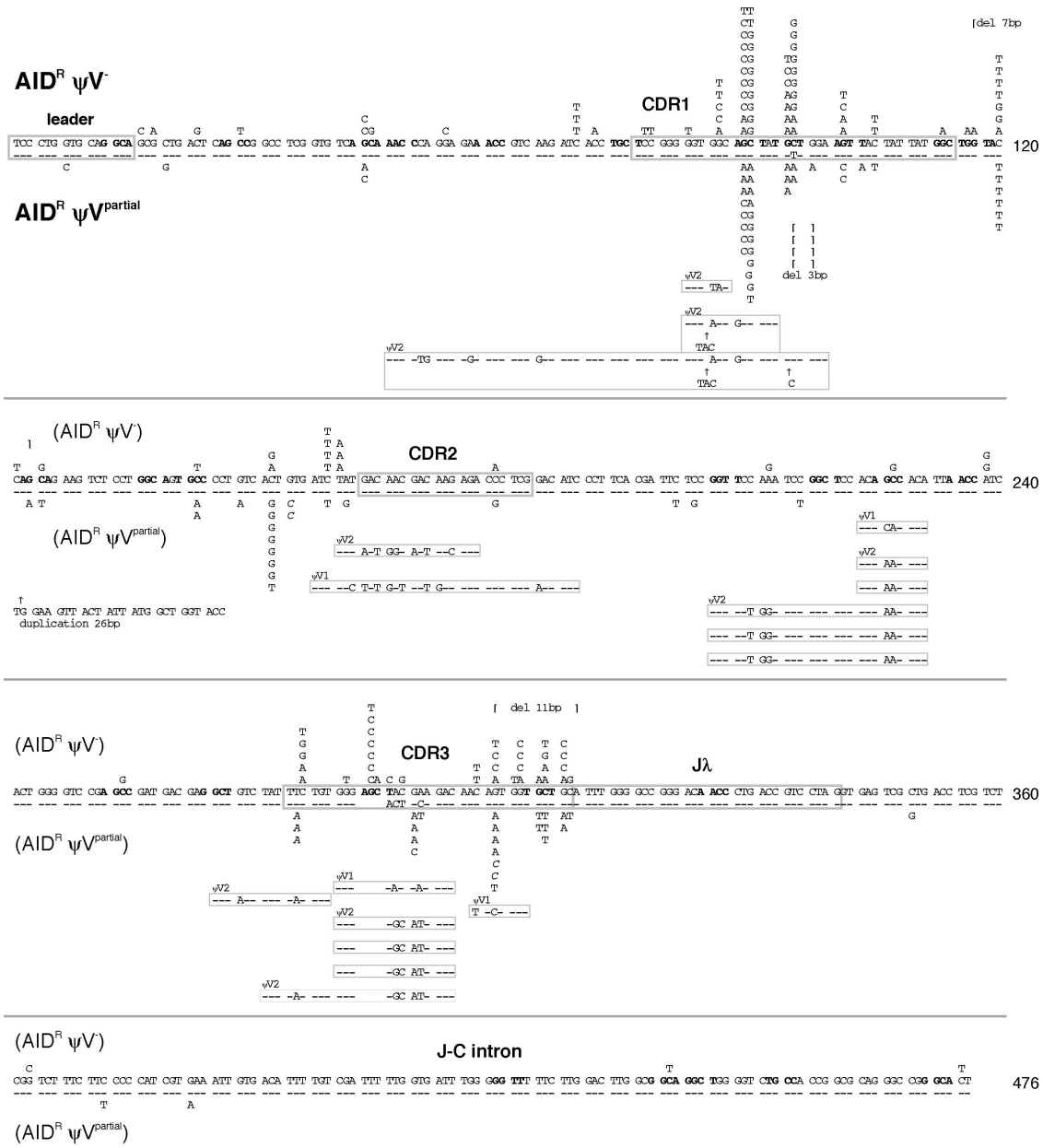


Figure 5. Ig light chain sequence analysis of the ψV knock-out clones Mutation profiles of the AID^{R}ψV^{-} and AID^{R}ψV^{partial} clones. All nucleotide substitutions identified in different sequences in the region from the leader sequence to the J-C intron are mapped onto the rearranged light chain sequence present in the AID^{R} precursor clone. Mutations of the AID^{R}ψV^{-} and the AID^{R}ψV^{partial} clones are shown above and below the reference sequence, respectively. Deletions, insertions and gene conversion events are also indicated. Hotspot motifs (RGYW and its complement WRCY) are highlighted by bold letters.

hypermutating EBV transformed B cell lines (Bachl et al., 1996; Faili et al., 2002) and DT40 mutants of RAD51-paralogues (Sale et al., 2001) indicating that part of the Ig hypermutator activity is missing in transformed B cell lines.

The induction of Ig hypermutation by the blockage of Ig gene conversions supports a simple model explaining how hypermutation and recombination is initiated and regulated (Fig. 6). At the top of the events is a modification of the rearranged V(D)J segment which is either directly or indirectly induced by AID. The default processing of this lesion in the absence of nearby donors or in the absence of high homologous recombination activity leads to Ig hypermutation in form of a single nucleotide substitution (Fig. 6, right side). However, if donor sequences are available, processing of the AID induced lesion can be divided into a stage before strand exchange, when a shift to Ig hypermutation is still possible and a stage after strand exchange when the commitment toward Ig gene conversion has been made (Fig. 6, left side). Whereas completion of the first stage requires the participation of the RAD51 paralogues, the second stage involves other recombination factors like the RAD54 protein.

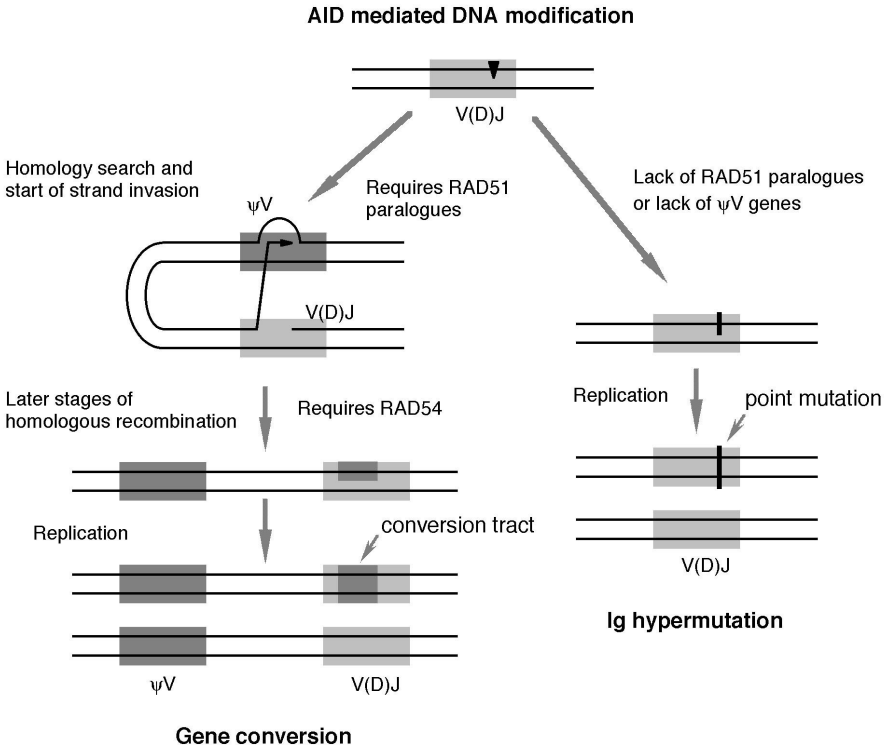


Figure 6. A model explaining the regulation of Ig gene conversion and Ig hypermutation. AID is tightly regulating both GC and SHM tightly in DT40 cells.

D. Translesion Synthesis (TLS):

DNA damage that stalls replication poses a major threat to genomic stability and cellular viability. Several strategies of post-replication repair (PRR) have evolved to allow a cell to tolerate or repair such damage, thereby allowing replication to be completed (Simpson et al., 2003). These strategies can be broadly divided into two groups: translesion DNA synthesis and homologues recombination. We tried to explain HR in the previous section. In this section we will focus on the relationship between TLS and antibody diversity.

Studies from yeast have shown that TLS as well as error free damage avoidance are controlled by a complex system known as the RAD6 pathway (Ulrich, 2004). RAD6 itself encodes a ubiquitin conjugating enzyme (E2) (Jentsch et al., 1987) that is recruited to DNA by the ubiquitin ligase (E3) RAD18 (Bailly et al., 1997). Ubiquitination by RAD6-RAD18 stimulates PCNA switch from replicative to TLS (Jonsson et al., 1997; Hoege et al., 2002). PCNA forms a homo-trimeric sliding clamp around the DNA of the replication fork controlling the access and processivity of replicative polymerases (Fig. 7) (Stelter et al., 2003). Whereas lysine 164 of yeast PCNA can be modified either by mono- or poly-ubiquitin or by SUMO, only mono-ubiquitination of PCNA was observed following MMS treatment or UV irradiation of a human cell line (Hoege et al., 2002; Kannouche et al., 2004; Watanabe et al., 2004). Mono-ubiquitination of human PCNA requires the human RAD18 homologue and increases the affinity of PCNA for the translesion DNA polymerase, Polη (Kannouche et al., 2004; Watanabe et al., 2004) and REV1 (Garg et al., 2005).

A link between SHM and error-prone repair has been first suggested by Brenner and Milstein (Brenner at al., 1966). However a possible answer for the question how error prone repair could induce SHM came just recently with the idea of DNA deamination model of AID. As we have discussed earlier in this model AID converts C to U within Ig V gene. The resulting uracils are then recognized either by UNG or mismatch repair system leading to mutations at G/C and A/T bases, respectively (Di Noia et al., 2002; Rada et al., 2004). During and after the recognition process or uracils, it seems TLS is somehow engaged to the system. Several studies about low fidelity polymerases and SHM, done in recent years showed remarkable results. Polη deficient human and murine B cells (Zeng et

al., 2001; Delbos et al., 2005; Martomo et al., 2005) and a REV1 disrupted mouse and DT40 mutant showed an altered spectrum and a decreased frequency of Ig mutations (Jansen et al., 2006; Simpson et al., 2003). To study TLS in DT40, AID^{R} ψV^{-} cells are the best candidates, because these cells diversify their Ig genes mainly by somatic hypermutation (Arakawa et al., 2004b).

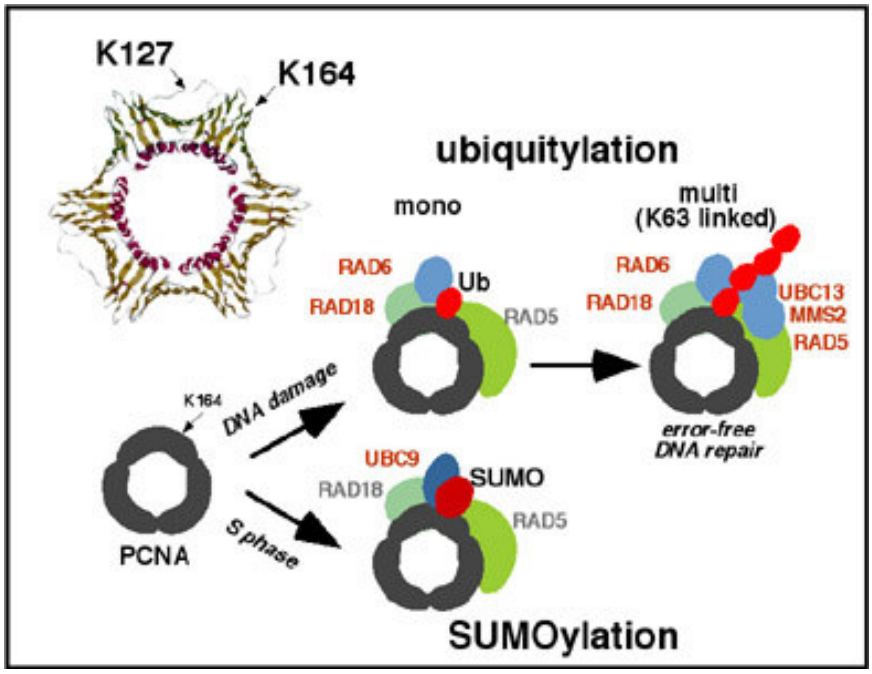


Figure 7. Model for ubiquitination and SUMO modification of PCNA. Two sites for modifications are amino acids at K127 and K164. DNA-damage induces mono-ubiquitination of PCNA at K164 which is catalyzed by RAD6 and RAD18. In the absence of DNA damaging agents PCNA is modified by SUMO in S phase. (Modified from Hoege et al., 2002)

IV. Objectives:

Many studies suggest that hypermutation and switch recombination is initiated by AID-induced cytosine deamination in the rearranged V(D)J segments, and the resulting uracils are removed by UNG (Muramatsu et al., 2000; Di Noia et al., 2002; Imai et al., 2003). It is an interesting question whether gene conversion, in analogy to hypermutation and switch recombination, requires the processing of uracils by UNG. It was reported that expression of an Ugi transgene in DT40 reduced gene conversion to 30% of the wild-type level (Di Noia et al., 2004), suggesting that uracil glycosylase activity enhances gene conversion. However this phenotype remains difficult to interpret, because contrary to expectation no evidence for an increased mutation rate was found and it was unclear how efficiently and specifically Ugi transgene expression inhibited UNG activity. Therefore, our first objective includes disruption of the UNG gene in DT40 and analysis of the phenotype with regard to gene conversion.

PCNA mono-ubiquitination by RAD18 has been shown to be important for DNA damage tolerance in yeast (Ulrich et al., 2004). Although it has been reported RAD18 is dispensable for hypermutation in DT40 (Simpson et al., 2005), we wondered this also holds true in a background which mainly diversifies V region by hypermutations (Arakawa et al., 2004b). Thus our second objective includes knockout of RAD18 in DT40 ($AID^{R}\psi V^{-}$) cells.

As an experimental overview, the targeting constructs were prepared, transfections were done and phenotypes were analyzed by either Ig reversion and or Ig mutation assay as well as by sequencing of the immunoglobulin V genes.

MATERIALS :

I. Instruments:

- PCR machines: GeneAmp PCR System 9700 [Applied Biosystems, CA; USA]
- Centrifuges: Heraeus [Kendro Lab. Products, Osterode, GERMANY]
- Electrophoresis chambers: Horizontal & Vertical [Bio-Rad Lab., CA; USA]
- Gel Visualization: Gel Doc 2000^{TM} [Bio-Rad Lab., California; USA]
- Spectrophotometer: BioPhotometer [Eppendorf GmbH, Hamburg; GERMANY]
- Incubators: Heraeus [Kendro Lab. Products, Osterode; GERMANY]
- Culture Shaker: Innova 4430 [New Brunswick Scientific, Nürtingen; GERMANY]
- Electroporator: Gene Pulse Xcell^{TM} [Bio-Rad Lab., CA; USA]
- Vacuum Pump: [Vacuubrand GmbH, Wertheim; GERMANY]
- Vacuum Manifold: [Millipore GmbH, Schwalbach; GERMANY]
- Analytical FACS: BD^{TM} LSRII Flow Cytometer [Becton Dickinson, CA; USA]
- Quantitative FACS: MoFlo^{TM} Cell Sorter [Dako Colorado Inc., Colorado; USA]
- Light Microscope: Axiovert 25 [Zeiss, Göttingen; GERMANY]
- Cell Viability Analyzer: Vi-Cell^{TM} [Beckman Coulter GmbH, Krefeld; GERMANY]
- Phosphoimager: Fuji, FLA-3000 [Fuji Photo Film Corp., Tokyo; JAPAN]
- DNA Sequencer: ABI 3730 DNA Analyzer [Applied Biosystems & Hitachi; Hitachi High Tech. Corp., Tokyo; JAPAN.

II. Experimental Kits:

- PCR Purification: [Qiagen GmbH, Hilden; GERMANY]
- Gel Extraction: [Qiagen GmbH, Hilden; GERMANY]
- DNA Ligation: Version 2.1 [Takara Bio Inc., Shiga; JAPAN]
- Plasmid Isolation (Mini): [Qiagen GmbH, Hilden; GERMANY]
- Plasmid Isolation (Maxi): [Qiagen GmbH, Hilden; GERMANY]
- First strand cDNA Synthesis: Super Script III^{TM} [Invirogen GmbH, Karlsruhe; GERMAY]

III. Oligonucleotides:

Oligomers (5' to 3') used throughout the study were summarized in Table 2. [Invitrogene GmbH, Karlsruhe; GERMANY].

Table 2. The primers used in the study.

1. Cloning:

UNG 5' target arm	UR15	GGG CTC GAG GCT ATG ATC GGG CAG AAG ACG CTG CAT TGC *Xho*I site
	UR16	GGG GGA TCC ACC TCT TAG TTC CTG CGC ATC CGC TCC AGC *Bam*HI site
UNG 3' target arm	UR17	GGG GGA TCC GTC TTC ACT TGG ACA CAG ATG TGC GAC ATC *Bam*HI site
	UR18	GGG TCT AGA AAC CGT CTG CAG GAC GTG GTG GCG TTT CCT *Xba*I site
UNG cDNA	UR21	GGG GCT AGC GCC ACC ATG ATC GGG CAG AAG ACG CTG CAT T *Nhe*I site
	UR22	GGG AGA TCT GGG CTG TTT GCA CCC ATC AGG CCC CAG AGC *Bgl*II site
Ig Gene	VL51	GGG AAG CTT TGG GAA ATA CTG GTG ATA GGT GGA T *Hin*dIII site
	VL50	GGG TCT AGA CCT CTC AGC TTT TTC AGC AGA ATA ACC TCC *Xba*I site
Ig Gene cDNA	VL31	TCC GCC AAA GCT TGG GCT CCT CTC CTC CTG *Hin*dIII site
	VL36	GGG TCT AGA GTC AGC ACT AGT TCA GTG TCG TGT TT *Xba*I site
EF1α	EF6	GGG AAG CTT CGG AAG AAA GAA GCT AAA GAC CAT C *Hin*dIII site
	EF7	GGG ACT AGT AGA AGA GCG TGC TCA CGG GTC TGC C *Spe*I site

2. Target Screening:

UNG	UR1	CCA GTC CCG CCG CCG CTG CCT TCC CTC CGG
	UR7	GAT GTC GCA CAT CTG TGT CCA AGT GAA GAC
	UR23	AGC GTT CCT CCG GGC TTC GGG GAG AGC TGG
	UR26	ACA CCG TGT ACC TCT TCC TCT CCT CGG CCA
RAD18	18RA2	CAG TGA ATG AGG GGA AAG CCT GGC GGA AAG
	18RA3	AGC AGC AGT TCT CCA GAG GCT GGT AGC AAA
	18RA5	TGA AAC CAT GAC CAT TTT GGT TGT GGG TGC
Marker Genes (Bsr & Gpt)	BS1	CGA TTG AAG AAC TCA TTC CAC TCA AAT ATA CCC
	GP1	TGT TGA TAT CCC GCA AGA TAC CTG GAT TGA

3. Sequencing:

Ig Gene	VL56	GAC TGG AGG ATC TTA GAG GTC TTT TCC AAC
	VL154	GTG CGT GCG GGG CCG TCA CTG ATT GCC GTT
Universal Primers	UC1	AGC GGA TAA CAA TTT CAC ACA GGA
	UC2	CGC CAG GGT TTT CCC AGT CAC GAC

4. UNG activity detection oligomer:

	VL207	*[FITC]* – TCA CCT GCT CCG GGG GTG GCA GUT ACG GCT GGA AGT TAC TAT TAT GGC TG

IV. Enzymes:

- **DNA Polymerases:**

  Expand Long System [Roche Diagnostics GmbH, Mannheim; GERMANY]

  Pfu Ultra Hotstart [Stratagene, California; USA]

- **Restriction Enzymes:** [New England Biolabs GmbH, Frankfurt; GERMANY]
- **Proteinase K:** [Qiagen, Hilden; GERMANY]

- **Calf Intestinal Phosphatase:** [New England Biolabs GmbH, Frankfurt; GERMANY]
- **RNaseA:** [Qiagen, Hilden; GERMANY]

V. Immuno-staining antibodies & anti-antibodies:

In order to quantify the surface IgM on B cells, Mouse Anti Chicken IgM (Clone M-1) and Goat Anti Mouse IgG (H+L)-RPE Human Adsorbed were used. [Southern Biotech. Ass. Inc., Biozol, Eching, Germany]

VI. DNA Size Marker:

A DNA marker which was manually prepared from φX174 DNA-*Hae*III digest and λ DNA-*Hin*dIII digest was used throughout the study [New England Biolabs GmbH, Frankfurt; GERMANY]. It was prepared as follows:

λ DNA (500 ng/µl)	400 µl
φX174 DNA (1000 ng/µl)	100 µl
6X Loading buffer	334 µl
MiliQ water	1166 µl

The mixture was incubated for 15 min at 70^{o}C without mixing and then it was taken into ice for 5 min.

VII. Bacterial Strain:

During cloning of the constructs *E. coli*-DH5α cells were used as competent cells.

VIII. Mammalian Cell Line:

A chicken B-cell line DT40 was used to transfect constructs.

IX. Plasmids:

UNG: In order to construct vectors for knockout and complementation for UNG study, pBluescript II KS (+) phagemid vector [Stratagene, California; USA] was used. In this vector blue/white selection is possible and it has ampicillin resitance gene (amp^{R}).

RAD18: RAD18 targeting vectors were requested from group of Prof. Dr. Shunichi Takeda, Kyoto University, Japan (Yamashita et al., 2002).

METHODS :

I. Molecular Biology:

A. Culture of *E.coli*:

Luria Broth (LB)* agar plates and 2YT Broth* medium were used for culture of *E.coli*. The plates were kept at 4°C for about 1 month. For longer storages glycerol stocks were prepared in a following way: (2 volumes (v.) of cells + 1 v. of 50% glycerol). Stocks were frozen at -80°C deep freezer.

2YT Broth: The mixture was autoclaved and 1ml ampicillin (250mg/ml) was added to the mixture before use.

Bacto tryptone	16 g
Yeast extract	10 g
NaCl	5 g
10N NaOH	250 µl
Distilled water	1000 ml

LB Agar Plates: The mixture was autoclaved. Before pouring onto petri plates, 1 ml ampicillin (250mg/ml) and 1 ml 4% X-gal was added to the mixture.

LB Broth Base	20 g
Bacto^{TM} Agar	15 g
Distilled water	1000 ml

B. *E.coli* DH5α competent cell preparation:

1) A streak of bacterial strain DH5α was made on a LB agar plate without antibiotics for single colony isolation. The plate was incubated over night at 37^{o}C.

2) A single colony was picked up and was cultured in 5 ml L broth or SOB broth* medium at 37^{o}C for over night.

3) 1 ml of SOB broth was taken and the background was measured at OD600.

4) 2.5 ml of overnight culture was transferred into 500 ml of SOB broth which had been kept at 25^{o}C and bacteria were cultured at 25^{o}C.

5) OD600 was measured every 30 min or every 1 h.

6) When the OD600 was 0.4, the bacterial culture was cooled down immediately by ice and was kept on ice for 10 min.

7) The culture was transferred into the large centrifuge tubes and was centrifuged at 3000 rpm at 4^{o}C for 10 min.

8) Supernatant was discarded and the pellet was resuspended into 330 ml of ice-cold FTB*. Vortex should not be used during this step.

9) The culture was kept on ice for 10 min, and was centrifuged at 3000 rpm at 4^{o}C for 10 min.

10) Supernatant was discarded and the pellet was resuspended into 50 ml of ice-cold FTB. Vortex should not be used. 3.5 ml of DMSO was added, mixed gently.

11) 400 µl of aliquots was dispensed into 1.5 ml tubes. Tubes were then frozen immediately by liquid nitrogen and the competent cells were kept at -80^{o}C.

12) The efficiency of competent cells was tested by using 1 ng of Bluescript plasmid for transformation to one competent cell tube (400 µl). After heat shock, 600 ml of L broth was added to the tube. Before spreading, 10 µl (1/100) and 100 µl (1/10) of bacterial liquid was taken onto agar plates. The left (89/100) was centrifuged for 1 min at 13000 rpm. Then, the bacteria were spreaded by a loop.

13) The number of colonies was counted. The titer of competent cells was calculated (Titer of competent cells is the number of colonies which would be produced by transfection of 1 µg plasmid).

* SOB Broth: $MgCl_2$ and $MgSO_4$ were added after autoclave of mixture.

Bacto tryptone	20 g
Yeast extract	5 g
NaCl	0.585 g
KCl	0.l86 g
Distilled water	1000 ml
$MgCl_2$	Final 10 mM
$MgSO_4$	Final 10 mM

* FTB: The pH was adjusted to 6.7 after KCl and $MnCl_2.4H_2O$ treatments respectively.

0,5 M PIPES	20 ml
$CaCl_2.2H_2O$	2.2 g
Distilled Water	1000 ml
KCl	18.64 g
$MnCl_2.4H_2O$	10,88 g

C. Polymerase Chain Reaction (PCR):

For the amplification of genomic DNA, hot start was preferred in order to prevent unspecific bands. For this reason, upper and lower mixes were prepared separately and upper mix was added onto lower mix at 80°C after pre-incubation on the machine. In the case for high fidelity polymerases, since pfu Ultra was inhibited by a taq-directed antibody no upper and lower mixes were prepared.

Lower Mix:	
DNA	1 – 100 ng
dNTP mix	0.2 mM
Primer forward	5 pmol
Primer reverse	5 pmol
10x Expand buffer	0.5 µl
MiliQ water	rest
Total	5 µl

Upper Mix:	
10x Expand buffer	0.5 µl
Expand System	0.5 U
10X Crezol Red	1 µl
MiliQ water	rest
Total	5 µl

Amplifications were done by using Long-range PCR protocol as follows:

Pre-denaturation	93°C, 2 min	
Denaturation	93°C, 10 sec	} 35 cycles
Annealing	65°C, 30 sec	
Extension	68°C, 5 min*	
Final extension	68°C, 7 min	

* 20 sec. increases after each cycle

D. Analysis of DNA by electrophoresis:

Up to the purpose, 0.8% to 2% agarose gel which contains 0.1 µg/ml EtBr was used to visualize DNA by Gel Doc 2000™ [Bio-Rad Lab., California; USA]. DNA samples were run for 35 min at 120 V. For gel purification, they were kept around 1 h in electrophoresis. In the case for UNG activity test 15% TBE-urea polyacrylamide gel was used and the DNA samples were run at 200 V for 40 min.

E. Purification & Gel Purification of DNA:

After PCR or restriction enzyme digestion, DNA was purified by Qiagen PCR Purification kit [Qiagen GmbH, Hilden; GERMANY Qiagen] according to manufacturer's instructions.

F. DNA Ligation:

Ligation was done by using a kit [Takara Bio Inc., Shiga; JAPAN] according to manufacturer. In principle, around 1 volume of vector (100ng/µl), 9 volume of insert (100ng/µl) and 10 volume of kit were used.

G. Transformation:

A heat shock protocol was preferred during transformation of plasmids into bacterial cells:

1) The plasmid (>1ng) was added into 50-400 µl of competent cells, depending on the efficiency of the cells.

2) The tube was incubated on ice for 20 (-30) min and then was heat shocked at 42^{o}C for 45 sec on a heat block, and was incubated again on ice for 2 min.

3) 2YT Broth was added onto tube until the mixture reaches 1 ml and the tube was incubated at 37^{o}C for at least 10 min.

4) After mixing, 100 µl (1/10) was taken from bacterial liquid and was put onto half of LB agar plate. The rest (9 /10) was centrifuged at 13.000 rpm for 1 min, the supernatant was discarded and the rest (~50 µl) was put onto another half of the plate. The cells were then spreaded by using L-loop.

7) The plates were incubated overnight at 37^{o}C.

H. Colony PCR:

The ligation of the DNA was first checked by colony PCR. Single bacterial colonies were taken into U-Bottom 96 well plates which contains 20 µl 2YT Broth. From this, 1 µl was used as a DNA template for PCR.

Mix:	
DNA	1 µl
10X Expand buffer	1 µl
dNTP mix	0.2 mM
Primer forward	5 pmol
Primer reverse	5 pmol
Expand System	0.5 U
10X Crezol red	1 µl
MiliQ water	rest
Total	10 µl

PCR Programme:	
Pre-denaturation	94^{o}C, 1 min
Denaturation	94^{o}C, 20 sec
Annealing	55^{o}C, 1 min
Extension	72^{o}C, 2 min
Final extension	72^{o}C, 6 min

*Amplifications were done 30 cycles.

I. Plasmid preparation:

During construction of the vectors for small number of samples, plasmid extraction was done by using a kit according to manufacturer's protocol [Qiagen GmbH, Hilden; GERMANY]. After constructs were confirmed by enzyme digest, a maxi-prep was done [Qiagen GmbH, Hilden; GERMANY]. For higher number of samples or during cloning of *Ig* gene PCR products for sequencing, large-scale miniprep preparation was used by using deep-well blocks and plates as follows:

1) The bacterial colonies were picked up into a 96 well U-Bottom plate each well containing 70µl of 2YT Broth. 25µl of each individual sample were transferred into two 96 well blocks containing 1.3 ml 2YT Broth.

2) The blocks were incubated overnight at 37 ^{o}C at 220 rpm.

3) The next day, the blocks were centrifuged at 3300 rpm for 10 min.

4) Supernatant was discarded by tapping and 80 µl of Solution-I* was added into each well. The pellet was resuspended, vortexed and incubated for 1 min.

5) 80 µl of Solution-II* was added and vortexed immediately. The samples were incubated for an additional 5 min at room temperature.

6) 80 µl Solution-III* was added and vortexed.

7) After this, the deep well block was centrifuged at 3.300 rpm for 5 min. (During this time Millipore FB plate was placed in the bottom, and NA lysate clearing plate was put on top of the manifold.

8) 200 µl of supernatants were transferred onto the clearing plate.

9) The vacuum was adjusted to not exceed 8 inches Hg in order to ensure uniform filtration. The vacuum was applied for 5 min until the lysate is drawn.

10) The FB plate was taken from inside the manifold and placed on top of the empty manifold. A plastic tray was placed in the bottom of the manifold for waste.

11) 150 µl of Binding Solution* was added to wells and mixed immediately by pipetting. Vacuum was applied at full strength for 2 min.

12) 200µl of 80% ethanol was added to each well. The vacuum was applied at full strength for 2 min. The waste accumulated in the plastic tray at the bottom of the manifold was discarded and the FB plate was centrifuged at 1.000 rpm for 30sec. (This step was repeated two times)

13) The FB plate was put on top of a new 96-well microtiter plate and 100µl of 1x TE Buffer was added to each well. The plate was incubated for 5 min and centrifuged at 3500 rpm for 5 min. The plasmid was kept at -20^{O}C.

Solution-I*	
50X TE	1 ml
Distilled Water	49 ml
RNaseA (100mg/ml)	30 µl

Solution-II*	
10 N NaOH	10 ml
Distilled Water	500 ml
20 % SDS	2,5 µl

Solution-III*	
Glacial asetik asit	70 ml
Distilled Water	70 ml
5 M Potassium Acetate	360 ml

Binding Solution*	
Potassium Iodide	100 g
Distilled Water	70 ml

J. Restriction Enzyme Digestion:

Before ligation: To prepare vector and insert fragments, DNA samples were incubated at 37^{o}C for at least 3 h. After ligation: DNA samples were incubated for 1 h to check cloning. In general 1-5 U of enzyme was enough to digest 1µg of DNA in 1 h.

Mix, prior to ligation:	
DNA	3-5 µg
10X Buffer	4 µl
100X BSA	0.4 µl
Enzyme 1 (and 2)*	2 (-4) µl
MiliQ water	rest
Total	40 µl

Mix, for test digestion:	
DNA	0.5 µg
10X Buffer	2 µl
100X BSA	0.2 µl
Enzyme 1 (and 2)*	0.5 (-1) µl
MiliQ water	rest
Total	20 µl

K. First strand cDNA synthesis:

Super Script III (Invitrogen) was used to synthesize first strand cDNA according to manufacturer's intructions.

L. Determination of DNA and RNA concentration:

The purity and the concentration of the DNA were checked by a spectrophotometer BioPhotometer [Eppendorf GmbH, Hamburg; GERMANY]. Generally, the intensity of absorbance at the wavelength 260 nm was used for calculating concentration and the ratio of 260/280 nm was used to estimate the purity. For pure DNA samples this value should be between 1.8, 2.0.

M.Genomic DNA Isolation:

1) 50 ml of cells (~ $50x10^6$ cells) in good condition were centrifuged at 1500 rpm for 5 min. The supernatant was discarded, the pellet was washed with 1-2 ml 1X PBS and again was centrifuged at 1500 rpm and for 5 min.

2) The supernatant was discarded, the pellet was resuspended in 500µl proteinase K Buffer* containing 0.1 mg/ml of proteinase K. The mixture was transferred into a 2 ml tube and 12.5 µl of SDS (20%) was added, mixed by inverting and spinned down shortly. Protein degradation was carried out overnight at 56°C.

3) The next day, 1 volume of phenol was added onto the DNA extract and mixed carefully. After this the tube was centrifuged at 13000 rpm for 5 min.

4) The upper phase was transferred into a new tube and 1 volume of phenol/ chloroform was added and mixed. Again it was centrifuged at 13000 rpm for 5 min.

5) The upper phase was transferred onto a new tube and 1 volume of chloroform was added and mixed. It was centrifuged again at 13.000 rpm for 5 min.

6) The upper phase was transferred onto a new tube and 2 µl of RNAseA (100mg/ml) was added to digest RNA. And then, it was incubated for 2 h at 37°C.

7) DNA was transferred into dialysis membrane and the ends were closed with clamps. The membrane was put into a glass of cold 1X TE and kept at 4°C for 2 h. (Alternatively, the reaction can be stopped by adding 50µl of 0,5M EDTA pH 8.0).
TE was changed at least three times with 2-4 h intervals.

8) DNA was transferred to a new tube and concentration was measured.

***Proteinase K Buffer:**

5 M NaCl	2 ml
1 M Tris-HCl (pH 8,0)	1 ml
0.5 M EDTA	5 ml
MiliQ water	92 ml
Total	100 ml

N. Total RNA Isolation:

1) 20 ml cells (~ $20x10^6$ cells) were centrifuged at 1500 rpm for 5 min.

2) The supernatant was discarded and the pellet was resuspended in 1.5 ml of TRIzol reagent. After that, it was transferred into a 2 ml eppendorf tube and was incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes.

3) 0.3 ml of chloroform was added onto it. The tube was mixed for 15 sec and incubated for 3 min at room temperature, centrifuged at 13000 rpm, 4^{o}C for 15 min.

5) The upper phase was transferred into a new 2 ml eppendorf tube 750 µl of propanol was added and centrifuged at 13000 rpm, 4^{o}C for 10 min.

6) RNA pellet was washed with 1 ml of 75% ethanol, and centrifuged at 13000 rpm for 5 min. The supernatant was discarded, the sample was air droed for ~10 min and dissolved in 100 µl of DEPC water. The quality was checked using 1% agarose gel after 50 min run.

O. Cell Extract Preparation:

1) Exponentially growing cells (10^6 cells) were washed two times with PBS and resuspended at a concentration of about 10^3 cells/µl in a UNG activity buffer* containing a cocktail of protease inhibitors.

2) The cells were then freeze-fractured by liquid nitrogen and centrifuged at 6500 rpm for 10 min at 4°C.

3) The supernatant was transferred to a new tube and then centrifuged again at 5000 rpm for 5 min at 4°C. The supernatant of this second centrifugation was stored as cell extract at -20°C.

3) UNG activity was measured by incubating 5 µl of cell extract with 10 pmol of a 5' FITC–labelled oligonucleotide containing a single uracil at a position 23 in a final volume of 10 µl for 2 h at 37°C.

4) Because AP endonuclease activity in the extracts was found to be insufficient for complete cleavage of the abasic site after uracil excision, 5 µl of 0.5 M NaOH was added to the reaction and incubation was continued for one hour at 37°C.

5) The DNA was then precipitated by ethanol and resuspended in 10 µl formamide loading buffer before being analysed on a 15% TBE-urea PAGE gel.

6) After electrophoresis, gel images were visualised using a FUJIFILM (FLA-3000) phosphor imager.

***UNG Activity Buffer:**

HEPES	25 mM
DTT	1 mM
EDTA	5 mM
Glycerol	10%
Total	100 ml

II. Cell Culture:

A. Basic Cell Culture Conditions:

DT40 cells can be cultured in cultures flasks, petri dishes, or in 24 well plates. Microtiter plates are suitable for transfection or subcloning. The optimum culture condition for the cells is 41°C with 5% CO_2. Chicken medium* can be used to culture DT40 cells and Freezing medium* can be used to free down.

Chicken Medium:	
RPMI	500 ml
FBS	50 ml
Penicillin / Streptoycin	10 ml
L-Glutamine	5 ml
Chicken Serum	5 ml
1 M β-mercaptoethanol	50 µl

Freezing Medium:	
RPMI	70 ml
FBS	20 ml
DMSO	10 ml

B. Thawing of the cells:

1) The tube which was taken from nitrogen tank was melted at 41 ^{O}C and centrifuged at 1.500 rpm for 5 min.

2) The supernatant was discarded; the pellet was resuspended with chicken medium and transferred to a flask or plate.

C. Freeze down of cells:

1) The cells were transferred to a flask and centrifuged at 1.500 rpm for 5 min.

2) The supernatant was discarded, the cells were resuspended with freezing medium, first stored in - 80 ^{O}C for overnight afterwards transferred to nitrogen tank.

D. Transfection:

Plasmid Preparation:

1) The plasmid DNA was linearized outside of the genes of interest by an appropriate restriction enzyme within 500 µl total reaction volume. Following day the digestion was checked by using 0.5 µg of DNA on an agarose gel.

2) The DNA was purified first with phenol/chloroform, and then with chloroform, precipitated with propanol and rinced with 70% ethanol.

3) The pellet was dried up naturally for 10 min inside the laminar and resuspended in distilled water for final 1 µg/ml concentration.

Electroporation:

1) The concentration of the cells was determined (10 million cells were used per each electroporation). Calculated amount was transferred into a 50 ml tube and centrifuged for 5 min at 1500 rpm, 4^{o}C.

2) The supernatant was removed, the pellet was resuspended in 800 µl of chicken medium and transferred into electroporation cuvette together with DNA.

3) Electroporation was done using 25 µF and 700 V.

4) The mixture was put onto the tube containing 9.5 ml of chicken medium and the solution was distributed into the wells (100µl) of a flat-bottom microtiter plates.

5) The following day (12-24 h after electroporation), 100 µl of selective medium (containing twice the final concentration of the drug) was added to each well.

6) The plates were left for about seven to ten days in the incubator without changing the medium. Drug resistant colonies should be visible by then.

E. Identifying Targeted Events By PCR:

After 7-10 days of electroporation, the visible colonies were picked up into 300 µl of chicken medium in flat-bottom 96-well plates and incubated for 3 days.

Crude extract preparation:

1) The cells were transferred to 96 well PCR plate, washed with PBS and centrifuged 5 min at 1500 rpm.

2) The supernatant was discarded, the pellet was resuspended into 10 µl of K buffer [1 x PCR buffer, 0.1 mg/ml proteinase K and 0.5% Tween 20] for proteinase K-mediated proteolysis .

3) The cells were incubated for 45 min at 56˚C and to inactivate the proteinase K, they were incubated at 95˚C for 10 min.

4) 1 µl of the crude extract was used for targeting screening PCR.

F. Subcloning of DT40 cells:

1) The viability of the cells was counted using cell viability analyzer.

2) Four wells of a six well plate were prepared to contain 15, 50, 150 and 500 viable cells in 5 ml chicken medium and each well is mixed well.

3) Using a stepper, 100 µl of the cells were separated into the 96 well flat bottom microtiter plate (Half plate for one dilution).

4) The plates were incubated for 7-8 days without changing medium. Subclones should be visible by then as round colonies.

5) 10µl of colonies were started to be picked up from lowest dilutions into 1 ml of chicken medium in 24-well flat-bottom plates.

G. Analysis of IgM expression by FACS:

In order to quantify the amounts of surface IgM (sIgM) expression as well as for sIgM loss of DT40 mutant cell lines, antibody staining for FACS analysis was done (The antibodies were prepared by diluting 1/100 – 1/500 folds).

1) The cells were transferred into round-bottom 96-well plates, washed with PBS.

2) For first staining, the cells were incubated with anti-chicken Cµ monoclonal antibody-M1 on ice for 30 min and then twice washed with PBS.

3) For second staining, R-PE-conjugated goat anti-mouse IgG polyclonal antibody was used with same conditions. Then, the cells were washed with PBS three times.

4) The sIgM expression of cell lines were measured and analyzed by flow cytomer.

H. Drug Resistance Marker Recycling:

Up to do purpose, partial or complete excision of the marker genes is possible in DT40 cells.

1) Around 10^5 cells were cultured with fresh chicken medium containing 0.01 mM 4-hydroxt tamoxifen (1 day for partial and 2 days for complete excision).

2) The cells were subcloned by limiting dilution for final concentration of 0.3, 1, 3 and 10 cells / well in 96-well flat-bottom plates.

3) 7 – 8 days after subcloning, 10 µl of the visible subclones were transferred into 1 ml of chicken medium.

4) In order to test the excision of the drug-resistance genes, the cells were incubated with drug-containing mediums. The cells deficient for markers were selected for further studies.

I. Sorting:

1) Enough amounts of cells were stained, filtered, washed and collected in 1-2 ml of PBS.

2) During sorting, for storage of the sorted cells, 50% chicken medium and 50% FBS was used. The tubes were prepared beforehand.

J. Colony Survival Assay:

Preparation of methylcellulose medium:

1) 11.9g D-MEM powder and 2.44g sodium bicarbonate was dissolved in 500ml water and sterilized by 0.2µm filtration.

2) 10g methylcellulose powder and a stir bar were autoclaved in a 1L bottle.

3) Approximately 220mls warm (50 – 65˚C) sterile water was added to the methylcellulose powder. Well mixed, ensuring an even suspension.

4) When temperature of methylcellulose solution is ≤50˚C, DMEM/HAM solution from (1) was added the little by little while mixing well. The bottle was mixed vigorously.

5) This solution was mixed overnight on a magnetic stirrer in a cold room.

6) The 0.2µm-filtered serum/antibiotic/2-mercaptoethanol cocktail was added.

7) The pH was adjusted 7.0 to 7.4 with NaOH (4N solution) as necessary.

8) The mix was stirred for at least 3 hours (preferably overnight) in a cold room and was kept at 4˚C for up to 1 month.

Preparing the methylcellulose plates:

1) Appropriate number of 6 well plates was labeled. One 6 well plate will be required for each dose for every cell line e.g. testing 4 cell lines at 5 different UV doses will require 20 6 well plates.

2) 5 ml of methylcellulose was put into each well. The methylcellulose was stirred for 30 minutes at room temperature before use.

3) The central gaps were half filled between the wells with sterile water.

4) The plates were placed in a 37˚C incubator until ready for use.

Plating out samples:

1) 100µl of sample was pipetted onto the appropriate well in a figure of 8 pattern.

2) The plate was swirled when the all the samples have been pipetted on the plate to ensure that all the cells are evenly distributed across the methylcellulose.

3) The plates are then incubated in a humid box in a CO_2 incubator until the colonies are clearly visible and countable. This usually takes 10 days to 2 weeks depending on the growth characteristics of the cell line. For consistency it is generally best to take the count from the pair of wells with between 0 and 100 colonies. Counting above 100 is unlikely to be accurate and colony formation is likely to be compromised at high densities. The whole experiment should be repeated three times. Survival is plotted as a percentage of the cells surviving on the untreated control.

RESULTS – 1: 'UNG'

I. Genomic Identification of UNG Locus:

Using bursal EST database (Abdrakhmanov et al. 2000), an EST dkfz426_17p6r1 was identified which shows a significant homology to murine and human UNG cDNA. This clone was later sequenced and found to contain a 1096 bp cDNA insert including the full length UNG open reading frame of 299 amino acids. The amino acid sequence of the chicken UNG was found to be 79% and 75% identical to the human and murine nuclear UNG2, respectively (Figure R1). The exact intron-exon structure of UNG genomic locus was tried to be deduced from the PCR products using forward and reverse primers with 100 bp intervals (Figure R1). After chicken genome sequence including the *UNG* locus on chromosome 15 was released, this observation was confirmed (International Chicken Genome Seq. Consortium 2004).

II. Vector Construction:

B. Knockout Vectors: pUngBsr, pUngGpt

The 5' and 3' arms of the *UNG* knockout constructs were designed to insert and early in-frame stop codon at codon 66 and delete the region between the codons 67-120 (Fig. 8A). Two upstream (UR15-UR16) and two downstream primers (UR17-UR18) were selected to amplify 5' and 3' target arms respectively using DT40 genomic DNA as a template. After PCR amplification, 5' target arm was digested with restriction enzymes *Xho*I and *Bam*HI whereas 3' target arm was digested with *Bam*HI and *Xba*I to be able to clone them into pBluescript KS (+) basic vector. All of the clonings were first confirmed by colony PCR using primers from insert and vector backbones and second by miniprep plasmid digest (Fig. 9). During digest, apart from the relevant restriction enzymes to distinguish vector and insert, one more restriction enzyme *Not*I was used to linearize the plasmids in order to see the size difference between the cloned and previous vector. During cloning, at first 5' target arm was cloned into the vector. Cloning was confirmed and clone was named as pUng5'Arm. For second cloning, pUng5'Arm was digested with *Xho*I and *Bam*HI to clone 3' target arm. The cloned vector, pUngArms, was prepared for last cloning by digesting only with *Bam*HI. Drug resistance marker genes, blasticidine S resistance gene (Bsr) and guanine phosphoribosyl transferase gene (Gpt) as floxed *Bam*HI cassettes, were cloned into pUngArms (Arakawa et al. 2001). The resultant clones were confirmed and named as pUngBsr and pUngGpt (Fig. 9A). Finally, plasmids were amplified by maxi-prep and linearized by *Not*I for transfection.

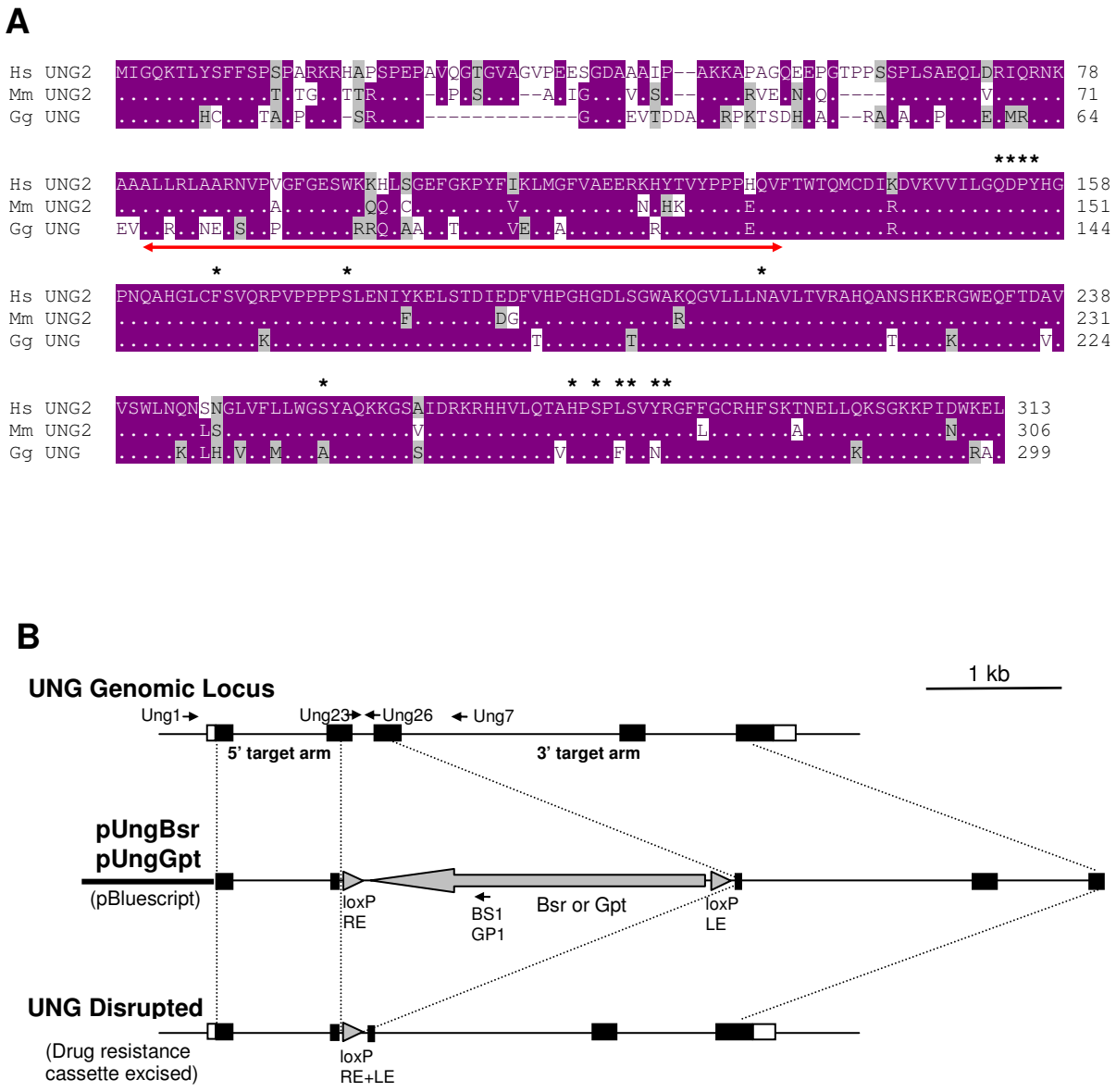
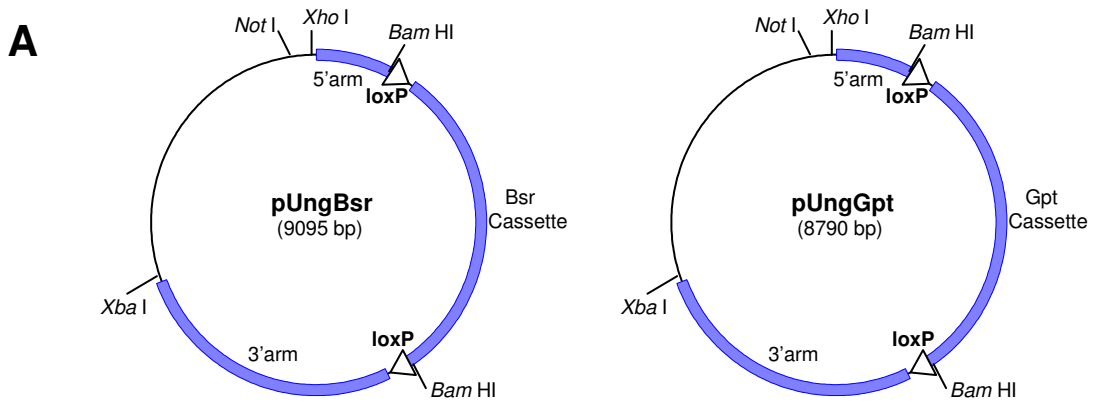
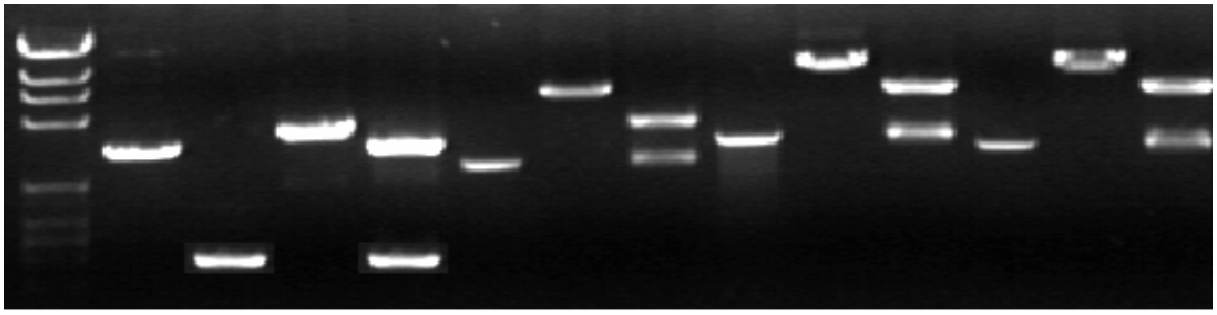
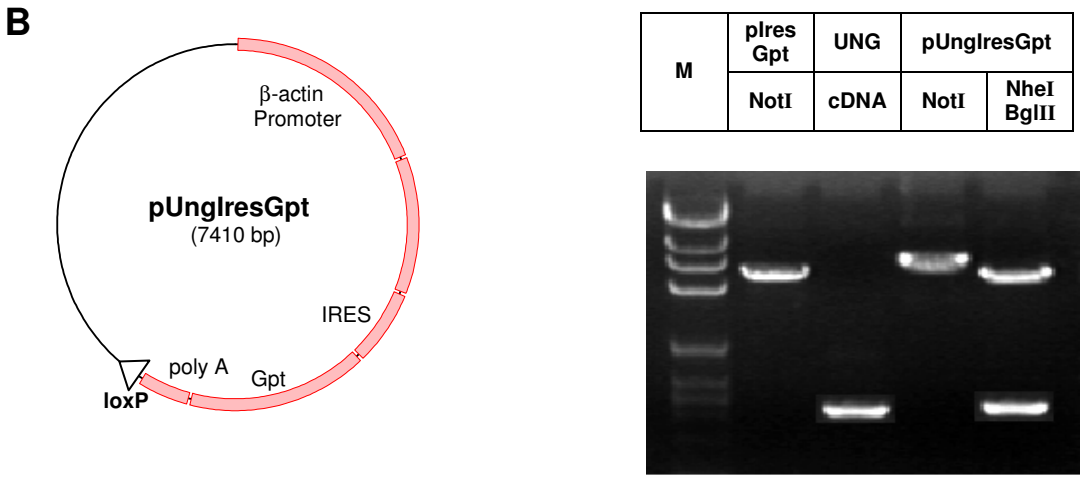


Figure 8. UNG sequence conservation and knockout strategy. A) Alignment of the nuclear isoform of UNG in human (Hs), mouse (Mm) and chicken (Gg) amino acid sequences. Purple and grey shaded backgrounds indicate identities and similarities, respectively. Asterisks above alignment mark residues that are known to be important in catalytic activity. Underlined red region will be deleted after knockout. B) Aligned map of the chicken UNG locus, the targeting plasmid constructs, and the targeted loci after marker excision. Open boxes represent untranslated whereas solid boxes represent coding regions. The locations of the primers used to confirm knockout are indicated by arrowheads.



M	KS(+)	5'arm	pUng 5'Arm		3'arm	pUng Arms		Bsr Cassette	pUngBsr		Gpt Cassette	pUngGpt	
	NotI	PCR band	NotI	BamHI XhoI	PCR band	NotI	BamHI XbaI	BamHI	NotI	BamHI	BamHI	NotI	BamHI





M	pIres Gpt	UNG	pUngIresGpt	
	NotI	cDNA	NotI	NheI BglII

Figure 9. Design of the UNG knockout and expression constructs. A) Map of knockout constructs, pUngBsr and pUngGpt, used in the study and their confirmation by restriction enzyme digestion during cloning shown collectively. B) Map of complementation vector pUngIresGpt and its check by restriction ezyme digestion. In all maps loxP sites required for marker cassette excision were shown as triangles. Relevant restriction enzymes were depicted on the maps.

C. Complementation Vector: pUngIresGpt

To clone UNG cDNA, two primers (UR21-UR22) which contains *Nhe*I and *Bgl*II restriction sites respectively, were used. Using EST sequence of UNG cDNA, PCR was done, the product was confirmed by sequence reaction and the product was cloned into the vector pIresGpt. In this vector since cloned cDNA fragments and Gpt is under the control of one β-*actin* promoter, it allows to express both UNG and marker cassette Gpt via Internal Ribosome Entry Site (IRES) (Jang et al. 1988, Pelletier and Sonenberg 1988). The resultant clone was named as pUngIresGpt (Fig. 9B). A maxi-prep was done and the vector was linearized by *Not*I for stable transfection.

III. Knockout and Complementation of UNG:

UNG Inactivation Does Not Affect Cell Viability:

As a general rule, following all transfections 7-10 days later, the colonies were picked up and a crude extract was prepared to check for targeted events using long range PCR. The positive candidates were then stained for FACS analysis and 3 different clones were decided. Finally the clones were frozen down into liquid nitrogen and genomic DNA was prepared.

For knockout of UNG, sIgM(-) variant of the DT40 clone AID^R cells were used. In this cell line AID is knocked out and the phenotype was complemented with AID overexpression. One of the key features of this cell line, AID cDNA which is between loxP sites can be removed upon induction of 4-HT and by this way gene conversion can be inactivated (Arakawa et al. 2002). Transfection of the first knockout construct, pUngBsr, was done and the result was confirmed by long-range PCR using a primer located upstream of the 5' targeting arm of the construct along with a primer from the resistance marker (Fig. 8B; Fig. 10B). From the positive clones 12 were stained for FACS analysis and after analysis 3 clones were decided as AID^R $UNG^{+/-}$ Cl1, Cl2 and Cl3. Out of these, Clone 1 was taken and second construct, pUngGpt, was transfected and knockout was confirmed as described above. The resultant knockout clone was named as AID^R $UNG^{-/-}$ Cl1 (Fig. 10A; Fig. 10B).

Targeting of the first construct was observed ~1 in 10 events whereas for the second construct it is dropped to ~1 in 30 clones. Upon screening from over 300 clones, 10 clones were found to be targeted into the already targeted *UNG* allele but one clone was identified with two targeting into two different alleles. The low frequency of targeting efficiency observed during

disruption of UNG as compare to knockout of other genes in DT40 could be first due to the length of the target arms as a technical approach. Since genomic locus information was not available when we started the study we were unable to take longer arms. Targeting efficiency increases when the length of the target arms is between 1.5 kb to 2.5 kb. In our case 5' arm is 0.8kb and 3' arm is 2.4kb. A second probability could be due to a polymorphism between two alleles. The identified UNG-deficient clone showed normal cell viability and cloning efficiency, suggesting that the low recovery of homozygous clones was not related to a growth defect of UNG knockout cells.

Recycling of Marker Genes and Complementation:

Since the number of drug marker cassettes is limited, they need to be recycled for further transfections. Considering this, the cells were exposed to 4-HT to activate MerCreMer. Following subcloning, the colonies were picked up, and a drug test was done in order to identify the clones which are resistant to puromycin and sensitive to blasticidine and mycophelolic acid. We want puromycin resistant clones because in overexpression construct of AID^R cells puromycin exists. The positive candidates were checked by PCR, stained for FACS analysis and 3 of them were selected for further analysis (Fig. 10C).

To one of the *bsr* and *gpt* negative clone, UNG expression construct pUngIresGpt was transfected. This construct allows expression of UNG cDNA as a bicistronic message along with the gpt transgene. The next day as a selective drug, different concentrations of mycophenolic acid was spread. As usual, ~7-10 days later, colonies were picked up and stained for FACS analysis. 3 of the complemented clones were decided and named as AID^RUNG^R Cl1, Cl2, and Cl3 (Fig. 10A).

IV. UNG Activity Test:

UNG activity is strongly reduced in UNG-deficient cells:

To determine how disruption of UNG affects overall activity of uracyl glycosylases within cells, one sophisticated method was used. For this, cell extracts of the AID^R, $AID^RUNG^{-/-}$ and AID^RUNG^R clones were incubated with an uracil containing oligonucleotide. Excision of the uracil by uracil glycosylase leads to an abasic site which can be detected by the appearance of a shorter fragment after NaOH or endonuclease mediated strand cleavage (Figure 10C). In the absence of *in vitro* UNG treatment, the amount of cleavage product was strongly reduced in extracts of $AID^RUNG^{-/-}$ cells compared to extracts of wild-type and reconstituted AID^RUNG^R

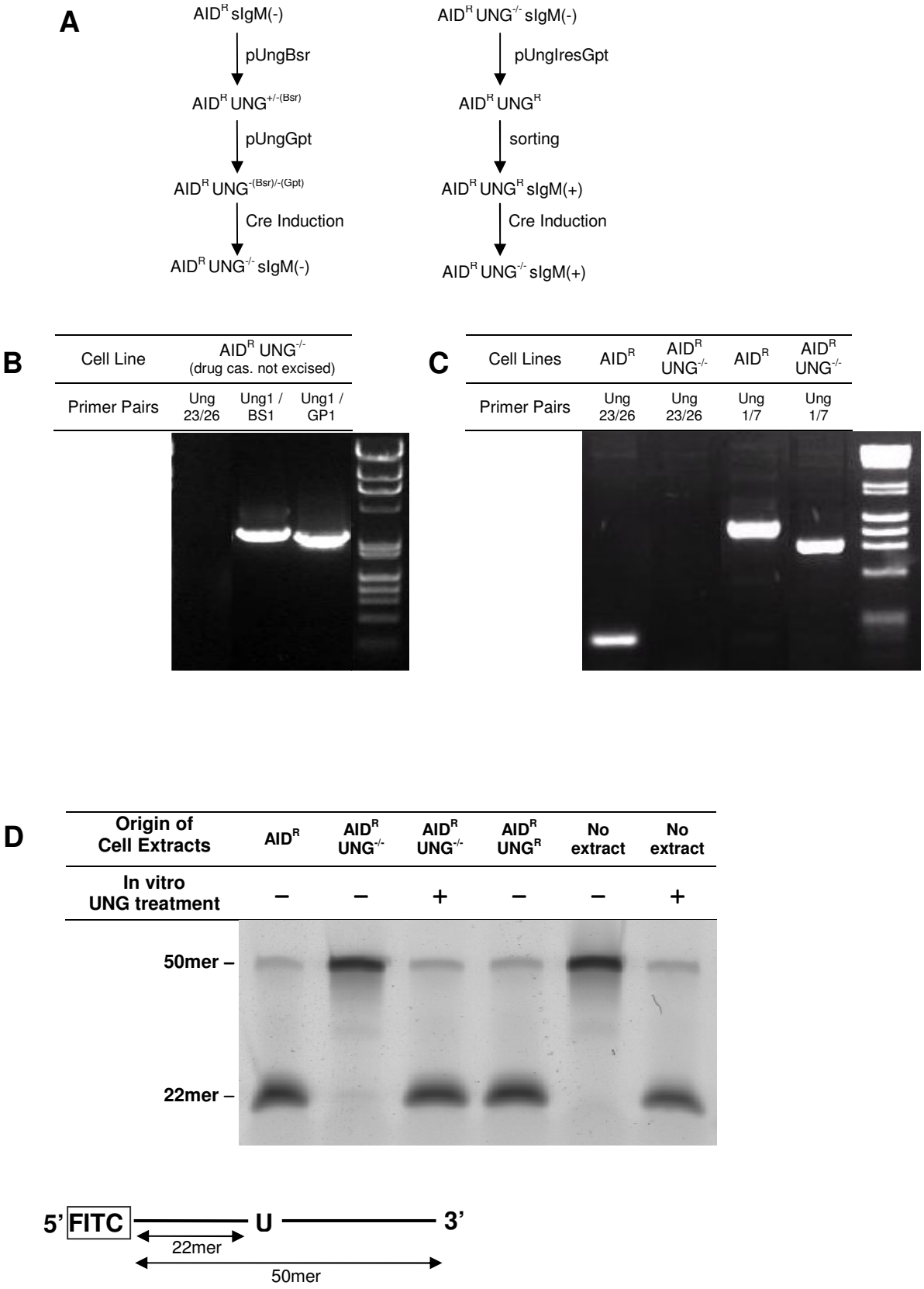


Figure 10. UNG knockout strategy and confirmation. A) Knockout and complementation scheme step by step. B) Detection of marker cassettes after transfections by PCR. C) Complete knockout of UNG (Removal of marker cassettes). In this PCR, AID^R precursor cell line is compared with $AID^R\ UNG^{-/-}$. D) UNG Assay using cell extracts. The structure of the oligonucleotide substrate is shown below the image.

cells. This confirms that UNG function is severely compromised by the gene disruptions and that most of the uracil glycosylase activity in the cell extracts is linked to UNG. Nevertheless, a low level of cleavage is still detectable after incubation with $AID^{R}UNG^{-/-}$ extract (Fig. 10D, lane 2), but not in the 'No extract' control (Fig. 10D, lane 5) upon prolonged gel exposure or incubation time. As the *UNG* gene disruption most likely behaves like a null mutation, the residual activity may reflect the presence of one or more backup uracil glycosylases.

V. Quantification of Surface IgM Levels:

A. Ig Reversion Assay:

Gene conversion is blocked in the absence of UNG

The three clones, AID^{R}, $AID^{R}UNG^{-/-}$ and $AID^{R}UNG^{R}$, carry a frameshift mutation in their rearranged light chain V segment, which can be corrected by gene conversion giving rise to sIgM(+) revertants. To determine how inactivation of the *UNG* gene disruption affects this frameshift repair by gene conversion, sIgM(+) subpopulations were measured using 18-24 subclones of each of the three clones and the stable sIgM(-) control clone, DT40 IgL^{-}. Representative FACS profiles and the calculation of the average percentages of gated events reveal a striking reduction of sIgM(+) cells for $AID^{R}UNG^{-/-}$ (Table 3, Fig. 11). It remains uncertain whether sIgM reversion still occurs at a low level in $UNG^{-/-}$ cells or whether it is entirely blocked, as the average of sIgM(+) events in $AID^{R}UNG^{-/-}$ subclones (0.35%) is only slightly above the background noise seen in DT40 IgL^{-} (0.24%). The lack of sIgM reversion is partially complemented in the $AID^{R}UNG^{R}$ clone and sequence analysis of sIgM(+) subpopulations confirmed that the light chain frameshift was indeed repaired by gene conversions (data not shown). These results show that the inactivation of UNG severely compromises Ig gene conversion.

Table 3. FACS Analysis results(1)

DT40 IgL-	AID^{R}	AID^{R} $UNG^{-/-}$	AID^{R} UNG^{R}
0.10%	7.00%	0.40%	0.60%
0.20%	3.90%	0.20%	0.40%
0.20%	1.80%	0.50%	0.80%
0.10%	3.20%	0.60%	0.30%
0.30%	3.10%	0.30%	0.80%
0.20%	0.70%	0.20%	0.50%
0.10%	48.20%	0.20%	36.40%
0.00%	1.10%	0.10%	0.30%
0.30%	1.80%	0.50%	0.30%
0.30%	4.80%	0.10%	1.00%
0.10%	6.20%	0.40%	0.60%
0.70%	2.50%	0.10%	0.30%
0.10%	13.00%	0.40%	1.10%
0.20%	73.80%	0.40%	0.70%
0.30%	1.40%	0.30%	0.80%
0.70%	0.10%	0.40%	0.80%
0.30%	3.90%	0.50%	0.70%
0.30%	2.50%	0.40%	30.00%
0.20%		0.50%	0.70%
0.10%		0.20%	0.60%
		0.50%	0.60%
		0.50%	18.80%
		0.30%	0.70%
0.24%	9.94%	0.35%	4.25%
(0.00%)	(9.70%)	(0.11%)	(4.01%)

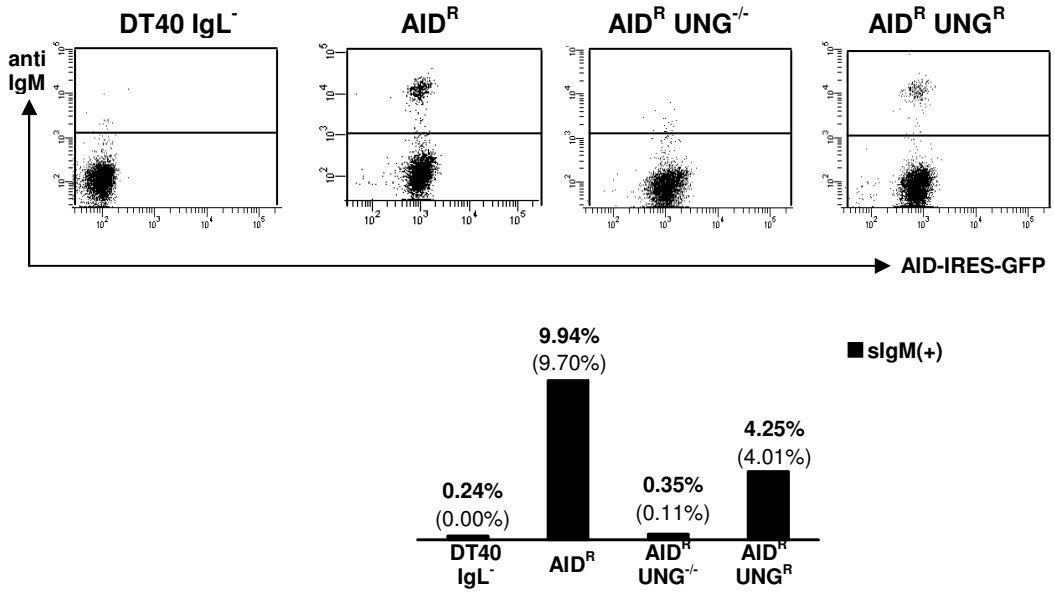


Figure 11. sIgM expression analysis of UNG-deficient and control clones for Ig gene conversion. Average percentages of events falling into sIgM(+) gates were shown as solid bars below the profiles.

B. Ig Mutation Assay:

UNG blockage causes cells lose sIgM

The Ig mutation rate is most conveniently quantified in sIgM(+) clones by measuring the appearance of sIgM(-) cells due to deleterious mutations (Arakawa et al. 2004). To study whether the UNG mutation induces Ig hypermutation, sIgM(+) cells of a $AID^{R}UNG^{R}$ subclone were sorted, shortly exposed to tamoxifen and subcloned. This procedure allowed the isolation of the following sIgM(+) clones: i) $AID^{R}UNG^{R}$ retaining both the AID and UNG cassettes, ii) $AID^{-/-}UNG^{-/-}$ lacking both the AID and UNG cassettes and iii) $AID^{R}UNG^{/-}$ clone1-3 retaining the AID, but lacking the UNG cassette. The appearance of sIgM(-) subpopulations was measured in parallel in subclones of these clones as well as in subclones of the sIgM(+) AID^{R} and $AID^{R}\psi V^{-}$ control clones and also $AID^{R}\ \psi V^{-}\ UNG^{-/-}$ clones. This last clone will give us a clue about UNG whether it works upstream of psudogenes or not. The results obtained during FACS analysis were summaried in Table 4 and Figure 12. Overall, analysis revealed dramatically increased percentages of sIgM(-) cells for all three $AID^{R}UNG^{/-}$ clones (49.00%, 53.93%, 34.57%) as well as $AID^{R}\ \psi V^{-}\ UNG^{-/-}$ clones (55.83%, 37.38%) compared to the AID^{R} precursor clone (1.50%). On average both the $AID^{R}UNG^{-/-}$ and $AID^{R}\ \psi V^{-}\ UNG^{-/-}$ subclones showed twice more sIgM(-) cells than $AID^{R}\psi V^{-}$ clones (23,84%), which were previously shown to rapidly accumulate light chain hypermutations due to the loss of ψV gene conversion donors (Arakawa et al. 2004). The rise of sIgM(-) was significantly reduced in the $AID^{R}UNG^{R}$ clone (7.18%) upon UNG reconstitution. The $AID^{-/-}$

$UNG^{-/-}$ clone remained stably sIgM(+) demonstrating that UNG inactivation causes sIgM(-) loss only in the presence of AID expression.

Table 4. FACS Analysis Results (2)

$AID^{-/-}$ $UNG^{-/-}$	AID^{R}	AID^{R} ψV^{-}	AID^{R} $UNG^{-/-}$ (Subclone 1)	AID^{R} $UNG^{-/-}$ (Subclone 1)	AID^{R} $UNG^{-/-}$ (Subclone 3)	AID^{R} UNG^{R}	AID^{R} $UNG^{-/-}$ ψV^{-} (Subclone 1)	AID^{R} $UNG^{-/-}$ ψV^{-} (Subclone 2)
0.30%	1.30%	13.60%	64.10%	64.10%	21.30%	6.30%	46.60%	40.60%
0.20%	1.20%	11.80%	50.10%	50.10%	26.90%	10.20%	45.80%	30.10%
0.50%	0.50%	68.30%	68.40%	68.40%	37.10%	5.80%	42.30%	66.70%
0.20%	6.80%	24.70%	40.00%	40.00%	56.90%	3.10%	79.20%	28.30%
0.30%	0.70%	11.20%	30.30%	30.30%	14.30%	11.70%	39.00%	22.70%
0.10%	1.40%	67.60%	32.40%	32.40%	13.40%	4.00%	44.00%	56.60%
0.10%	0.70%	19.40%	51.10%	51.10%	47.70%	1.50%	43.80%	56.70%
0.10%	1.00%	10.40%	49.00%	49.00%	37.30%	8.00%	38.70%	28.70%
0.10%	0.20%	20.00%	43.00%	43.00%	38.80%	3.10%	56.50%	56.70%
0.20%	1.40%	58.10%	57.60%	57.60%	21.40%	2.10%	52.50%	22.70%
0.10%	0.40%	21.60%	61.40%	61.40%	18.50%	2.30%	39.00%	21.20%
0.10%	1.40%	15.10%	56.10%	56.10%	57.10%	2.10%	60.10%	28.70%
0.40%	1.90%	15.90%	41.70%	41.70%	20.00%	6.50%	72.40%	59.40%
0.20%	3.50%	26.20%	68.30%	68.30%	40.00%	12.60%	40.30%	47.20%
0.10%	0.70%	20.50%	55.30%	55.30%	41.50%	12.90%	54.90%	57.60%
0.00%	0.80%	19.10%	44.90%	44.90%	57.70%	13.20%	37.10%	57.60%
0.00%	4.30%	23.90%	43.00%	43.00%	18.20%	21.40%	52.70%	25.30%
0.40%	0.40%	14.40%	26.20%	26.20%	19.40%	8.30%	79.50%	43.70%
0.20%	1.40%	10.00%	63.00%	63.00%	26.40%	8.50%	61.20%	11.20%
0.10%	0.60%	5.00%	76.20%	76.20%	30.20%	4.60%	49.50%	47.20%
0.20%	1.10%		30.90%	30.90%	34.60%	2.80%	52.60%	32.20%
0.20%	1.00%		25.40%	25.40%	36.90%		44.50%	25.90%
0.20%	1.70%		54.10%	54.10%	78.30%		49.00%	59.40%
0.30%			22.50%	22.50%	37.10%		58.30%	18.70%
			47.50%	47.50%	16.10%		59.10%	40.60%
			16.90%	16.90%	49.90%		49.30%	30.10%
			78.50%	78.50%	47.60%		53.70%	66.70%
			42.00%	42.00%	23.90%		84.40%	28.30%
			30.90%	30.90%	65.90%		38.80%	21.20%
			45.00%	45.00%	36.30%		34.90%	56.60%
			31.70%	31.70%	14.00%		65.20%	
			42.10%	42.10%	34.50%		78.30%	
			62.10%	62.10%	25.50%		79.50%	
			81.90%	81.90%	25.00%		50.60%	
			65.70%	65.70%	18.80%		70.50%	
			44.70%	44.70%	21.60%		59.50%	
			69.00%	69.00%	77.00%		66.10%	
					26.40%		68.30%	
					24.10%		79.80%	
					54.00%			
					25.80%			
0.19%	**1.50%**	**23.84%**	**49.00%**	**49.00%**	**34.57%**	**7.19%**	**55.83%**	**37.38%**

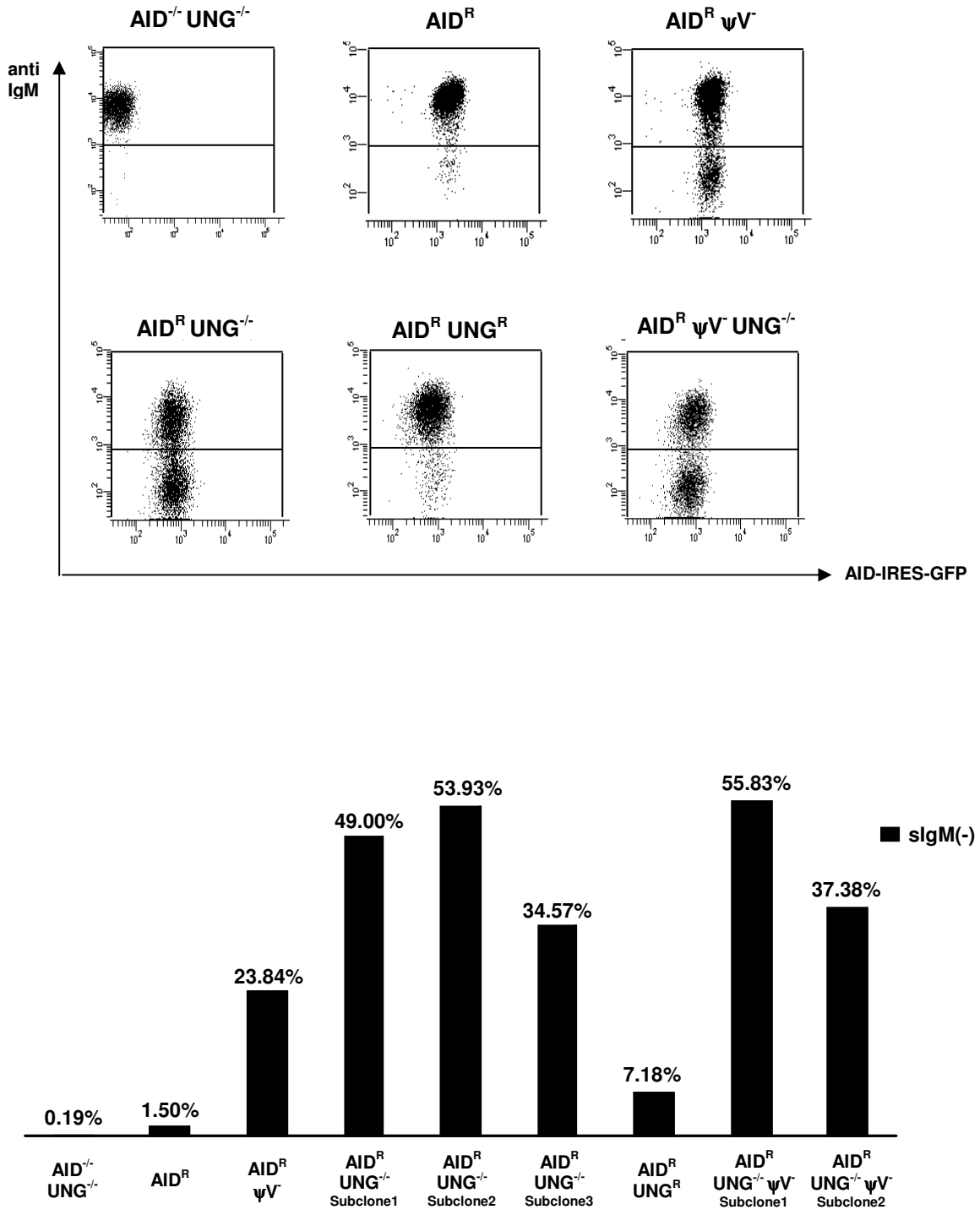


Figure 12. sIgM expression analysis of UNG-deficient and control clones for somatic hypermutation. Average percentages of events falling into sIgM(-) gates were shown as solid bars below the profiles.

VII. Ig Light Chain Sequencing:

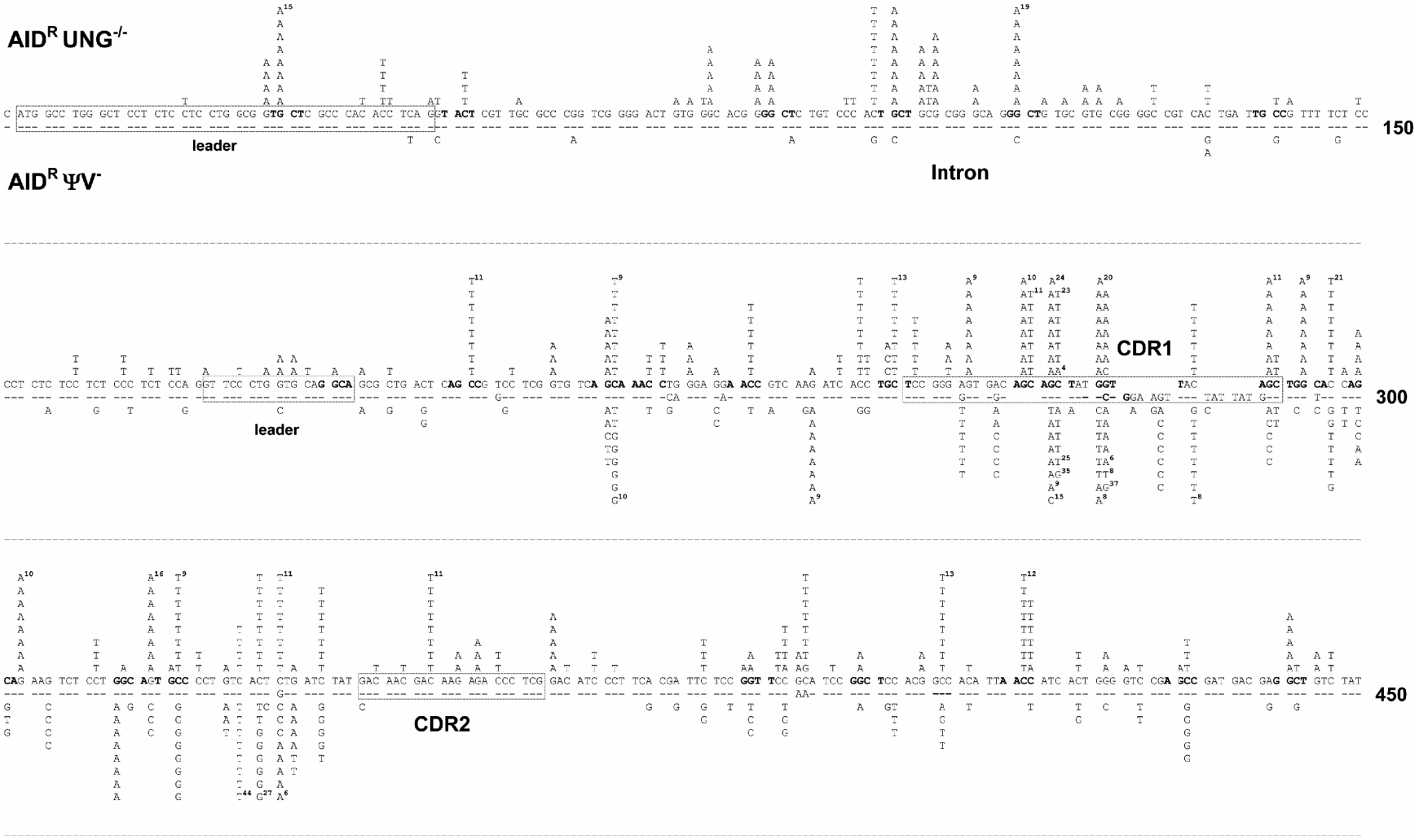
UNG inactivation induces a high rate of Ig transition mutations:

To determine the cause of the rapid sIgM loss in $AID^{R}UNG^{-/-}$ cells, the rearranged Ig light chain genes were sequenced for two subclones of $AID^{R}UNG^{-/-}$, together with $AID^{R}\psi V^{-}$ subclones six weeks after subcloning. As expected from the results of the sIgM analysis sequences of $AID^{R}UNG^{-/-}$ cells showed an extraordinary high number of single nucleotide substitutions at G/C bases and only few gene conversion tracts (Fig. 13). The average number of mutations per sequence was approximately seven fold higher in sequences of $AID^{R}UNG^{-/-}$ than of $AID^{R}\psi V^{-}$ cells (Table 5). Almost all mutations of $AID^{R}UNG^{-/-}$ cells (98%), but only about a third of the mutations of $AID^{R}\psi V^{-}$ cells (40%) was transitions (Fig. 14A). Mutations in both cell populations occurred preferentially at the known hypermutation hot spots (40% for $AID^{R}UNG^{-/-}$, and 49% for $AID^{R}\psi V^{-}$), almost exclusively at G/C bases and in about equal numbers at G and C bases (Fig. 14B). The number of mutations at A and T is so low that it is difficult to rule out artifacts due to PCR amplification. Likewise, very few mutations were detected in sequences of rearranged VJ regions from $AID^{-/-}UNG^{-/-}$ cells and sequences of the EF1α gene from $AID^{R}UNG^{-/-}$ cells (Table 5) confirming that the mutation activity induced by UNG disruption is AID dependent and Ig locus specific.

Table 5. Mutation Comparisons

Cell Line	Culture Time	Region	Mutation Analysis			Type of Mutation				
			Total Mutations	Total Sequences	Mut. / bp.[a] (10^{-3})	PM[b]	GC[c]	Del[d]	Dup[e]	I[f]
$AID^{R}UNG^{-/-}$	1 week	LVJ-intron (1360bp)	610	374	1.15	583	24[g]	2	-	1
	6 weeks	LVJ-intron (1360bp)	963	95	7.38	954	5	4	-	-
	6 weeks	EF1α (500bp)	2	78	0.05	2	-	-	-	-
	6 weeks	LVJ-cDNA (844bp)	484	93	6.10	479	5	-	-	-
$AID^{-/-}UNG^{-/-}$	6 weeks	VJ-intron (480bp)	1	95	0.02	1	-	-	-	-
$AID^{R}\psi V^{-}$	6 weeks	LVJ-intron (1372bp)	603	376	1.14	592	-	7	4	-
	6 weeks	LVJ-cDNA (844bp)	189	95	2.34	188	-	-	-	1

a. Mutation / Basepair (Only point mutations were counted), b. PM: Point Mutation, c. GC: Gene Coversion,
d. Del: Deletion, e. Dup: Duplication, f. I:Insertion,
g. Conversion events seem to have occurred early during clone expansion from one subclone


AID^R UNG^-/-
leader
Intron
150
AID^R ΨV^-
leader
CDR1
300
CDR2
450

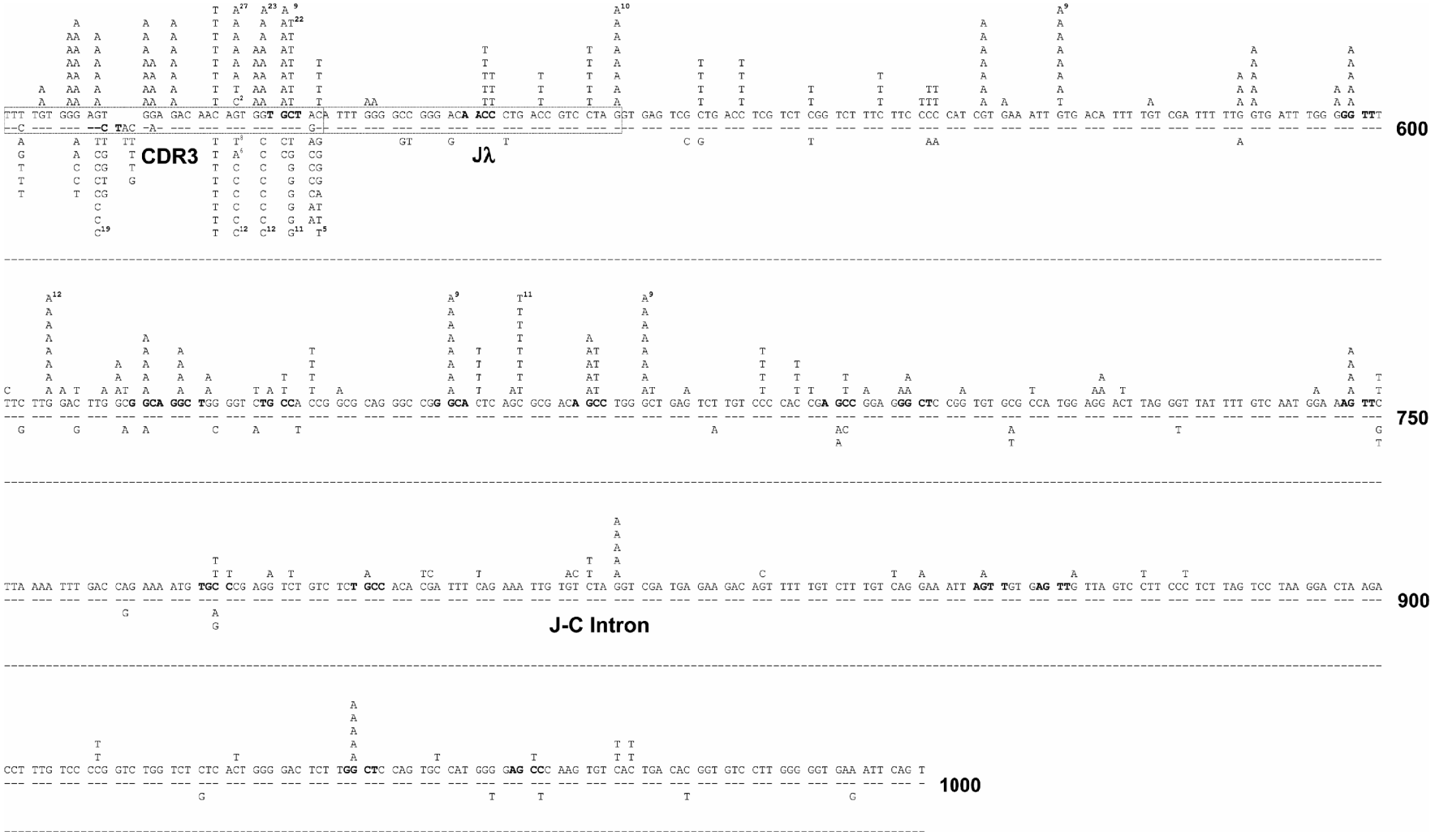


Figure 13. Mapping of mutations to the consensus sequence of the rearranged Ig light gene.
Nucleotide substitutions identified in the region from the leader sequence to the J-C intron were mapped onto the likely sequence of the precursor cell for the subclone. All point mutations identified in the $AID^{R}UNG^{-/-}$ and $AID^{R}\psi V^{-}$ subclones after six weeks culture are shown above and below the precursor sequence, respectively. Hot spot motifs (RGYW and its complement WRCY) are highlighted in bold letters. In the case that mutations at the same position are more than 8, they are shown with their numbers.

A

From \ To	A	C	G	T
A		0	*15*	3
C	25		182	*147*
G	*75*	108		31
T	3	*2*	1	

Cell line
trs:trv

AID^{R} ψV^{-}
239:353
(*40%*:60%)

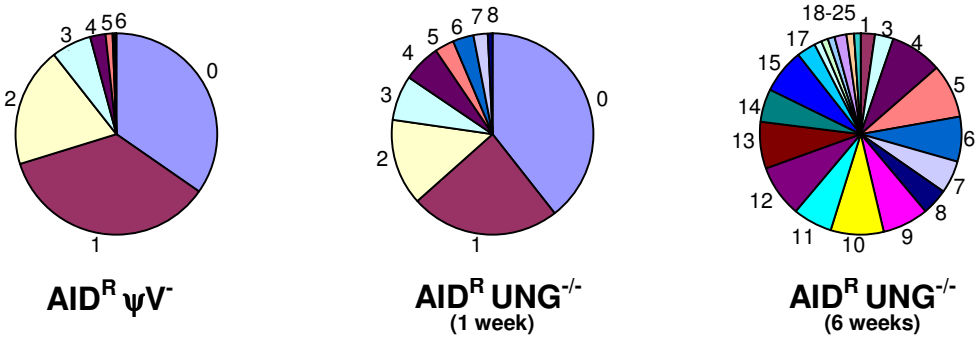
From \ To	A	C	G	T
A		0	*6*	0
C	5		1	*415*
G	*510*	10		5
T	0	*2*	0	

AID^{R} UNG$^{-/-}$
933:21
(*98%*:2%)

B

Cell line	Point Mutations	
	Total	at RGYW/WRCY
AID^{R} ψV^{-}	562	275 (49%)
AID^{R} UNG$^{-/-}$	949	377 (40%)

C



AID^{R} ψV^{-}

AID^{R} UNG$^{-/-}$
(1 week)

AID^{R} UNG$^{-/-}$
(6 weeks)

Figure 14. Mutation profiles of AID^{R} ψV^{-} vs. AID^{R} UNG$^{-/-}$. A) Analysis of the mutation patterns of Ig λ chain sequences 6 weeks after subcloning. B) Hotspot preferences of nucleotide substitution mutations. Mutations occurring within a hotspot motif (RGYW or its complement WRCY) were shown as percentages. C) Percentages of sequences carrying a certain number of mutations (Only point mutations were counted).

Mutations from AID^{R}UNG$^{-/}$ and AID^{R}ψV^{-} cells are differently distributed:

If uracils are not removed in the absence of UNG until the next replication cycle, transition mutations of UNG negative cells will mark the positions of all AID-induced uracils. In contrast, hypermutations of UNG positive cells indicate the positions of only those uracils which escaped error free repair after UNG processing. To compare the location of AID^{R}UNG$^{-/-}$ and AID^{R}ψV^{-} mutations, their respective numbers within windows of 10 bases were plotted against the rearranged Ig light chain gene sequence (Figure 15). This display shows that AID^{R}UNG$^{-/-}$ mutations are more scattered and less focused on the VJ regions than AID^{R}ψV^{-} mutations. To rule out that the difference is an artefact due to the divergent mutation load, AID^{R}UNG$^{-/-}$ sequences were determined one week after subcloning. The average number of mutations per sequence in this culture is similar to the average in the six weeks AID^{R}ψV^{-} culture (Table 5). Nevertheless, AID^{R}UNG$^{-/-}$ mutations from the two cultures are similarly spread along the light chain gene (Figure 15) indicating that the distribution is not influenced by the mutation average. The different distribution of AID^{R}UNG$^{-/-}$ and AID^{R}ψV^{-} mutations is also evident from the relative number of mutations located upstream of the V segment's start, within the VJ coding region and downstream of the J segment's end (Table 6). Whereas the VJ coding region encompasses 91% of the AID^{R}ψV^{-} mutations, this region contains only 65% and 64% of the AID^{R}UNG$^{-/-}$ mutations from the one and six weeks cultures, respectively (Table 6). Because AID^{R}UNG$^{-/-}$ cells show relatively more mutations in downstream J region, Ig Constant region (C) of the two cells AID^{R}UNG$^{-/-}$ and AID^{R}ψV^{-} were sequenced to understand mutational activity in C region using cDNA of two subclones (Fig. 16; Table 5). We saw almost no mutations in Cλ, which means AID can not access to this region Fig. 15B).

Table 6. Mutation Distributions

Cell Line	Sequences	Point Mutations						
		Upstream V (560bp)		VJ (313bp)		Downstream J (487bp)		Total (1360bp)
AID^{R} UNG$^{-/-}$ (1 week)	374	141[a]	(24%)[b]	381	(65%)	61	(11%)	583
AID^{R} UNG$^{-/-}$ (6 weeks)	95	140	(15%)	609	(64%)	205	(21%)	954
AID^{R} ΨV^{-} (6 weeks)	376	22	(4%)	540	(91%)	30	(5%)	592

a. Number of mutations, b. Percentage of mutations out of total.

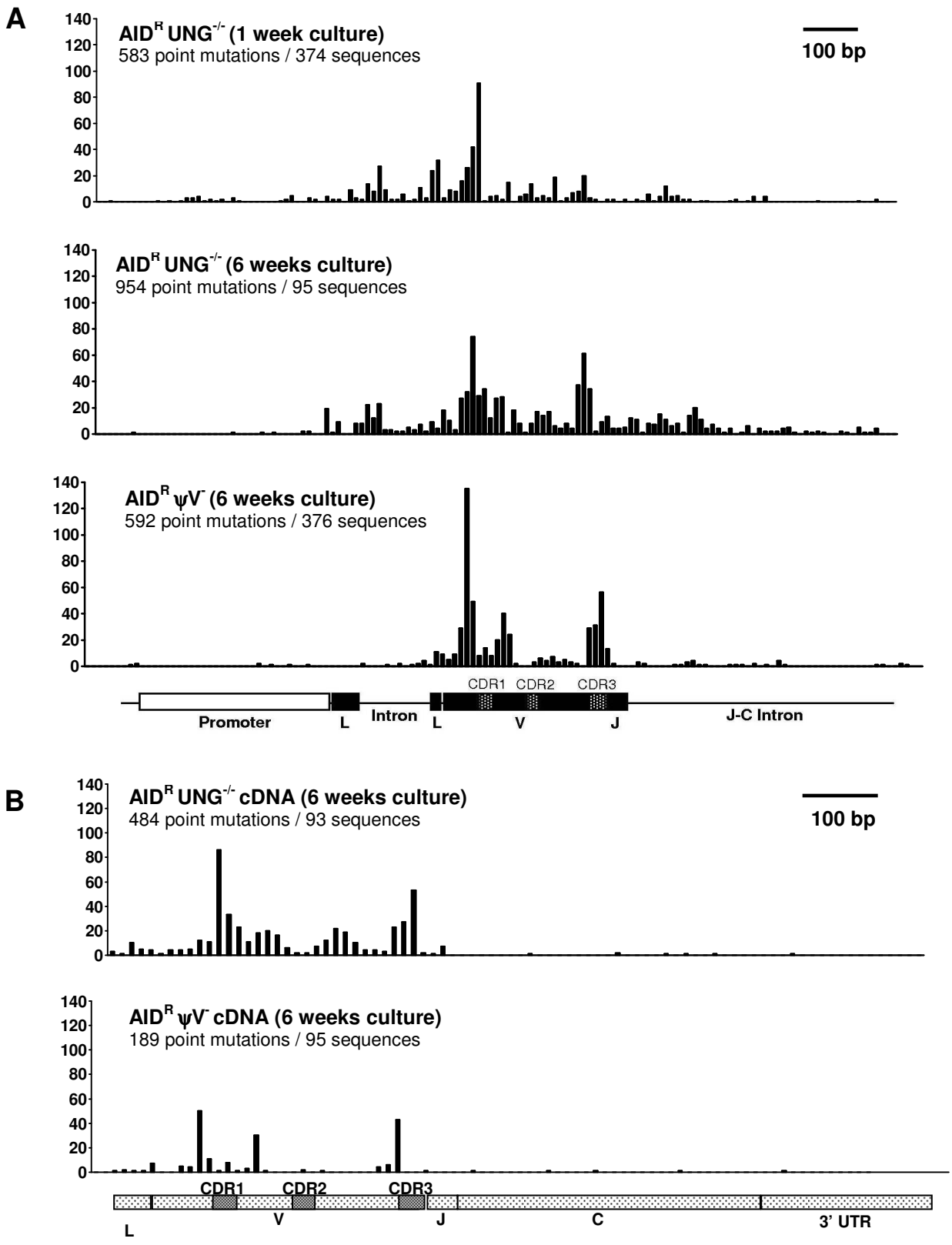


Figure 15. Distribution of the mutations from cell lines $AID^{R}UNG^{-/-}$ and $AID^{R}\psi V$. A) Mutations plotted within adjacent 10-bp intervals along the rearranged L chain locus. B) Mutation analysis with the same criteria along cDNA of Ig L chain.

RESULTS – 2: ‘RAD18’

I. Genomic locus of RAD18 in DT40:

The chicken RAD18 protein shows ~50% identity to the human and mouse RAD18 proteins, and ~25% identity to the yeast RAD18 proteins. Sequence comparison between the homologs shows great divergence. Even mouse and human homologs show only 65% identity. However sequence conservation is highly conserved within the RING-finger domain located at the N-terminus. All the cystein and histidine residues that constitute the RING-finger motifs (C-C-C-H-C-C-C-C) were perfectly conserved at precise intervals from yeast to human (Figure 16, asterisks) suggesting an important role of the RING-finger for RAD18 function.

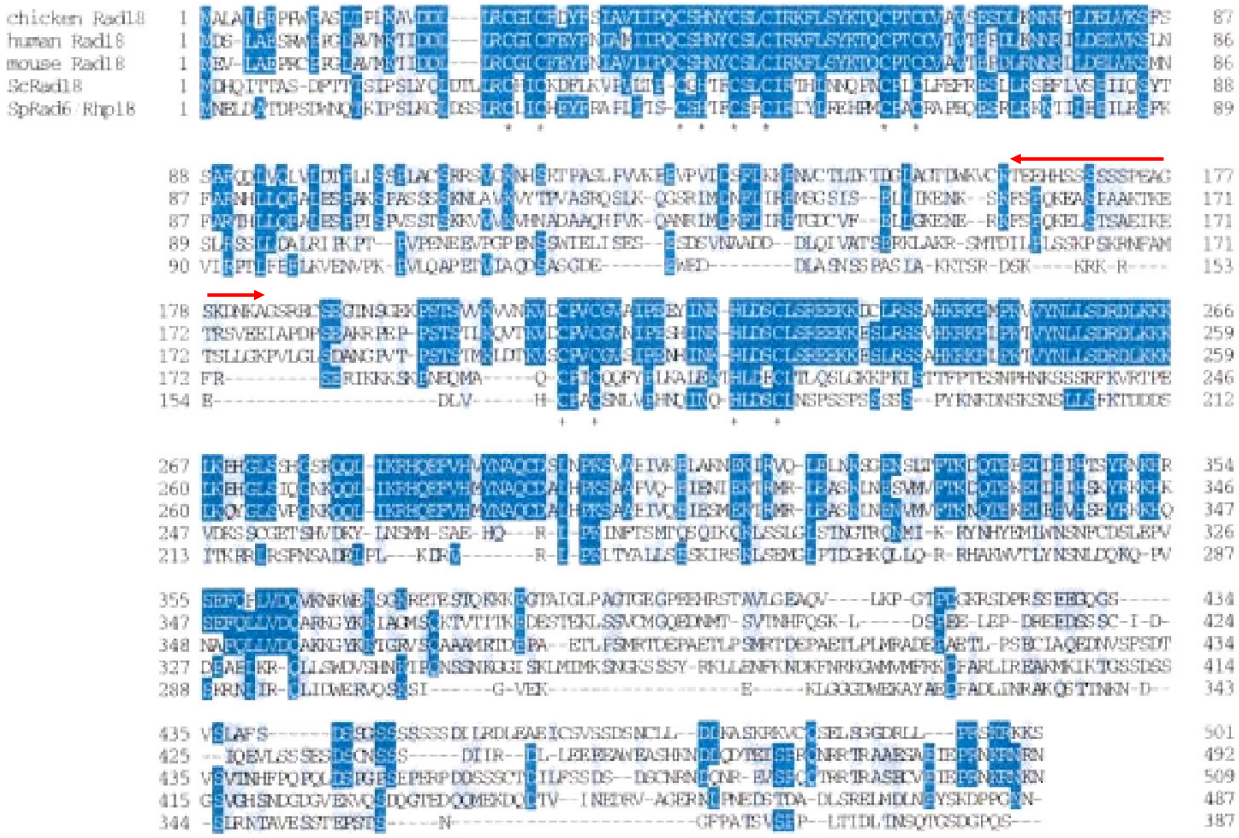


Figure 16. RAD18 amino acid conservation between the species. Alignment of the RAD18 amino acid sequences in chicken, human, mouse, budding yeast (Sc) and fission yeast (Sp). Asterisks below alignment mark residues for RING-finger motif. Highlighted letters represent identical amino acids among more than three species. Shaded letters represent similar amino acids conserved among more than three species. Red line above chicken denotes the region for knockout.

II. Knockout of RAD18 in DT40 cells:

Rad18 knockout constructs pRad18Bsr and pRad18Gpt was designed to introduce a premature stop codon after amino acid 162 and to delete the coding amino acids from 163-182 upon gene targeting (Yamashita et al., 2002). The DT40 variant $AID^{R}\psi V^{-}$ (Arakawa et al., 2004b) was chosen as the progenitor clone of the study, because it, *i)* diversifies its rearranged Ig light chain locus by hypermutation due to the deletion of the nearby pseudo V (ψV) gene conversion donors, *ii)* expresses the AID gene as a floxed cDNA cassette and *iii)* can be induced by tamoxifen to express Cre recombinase.

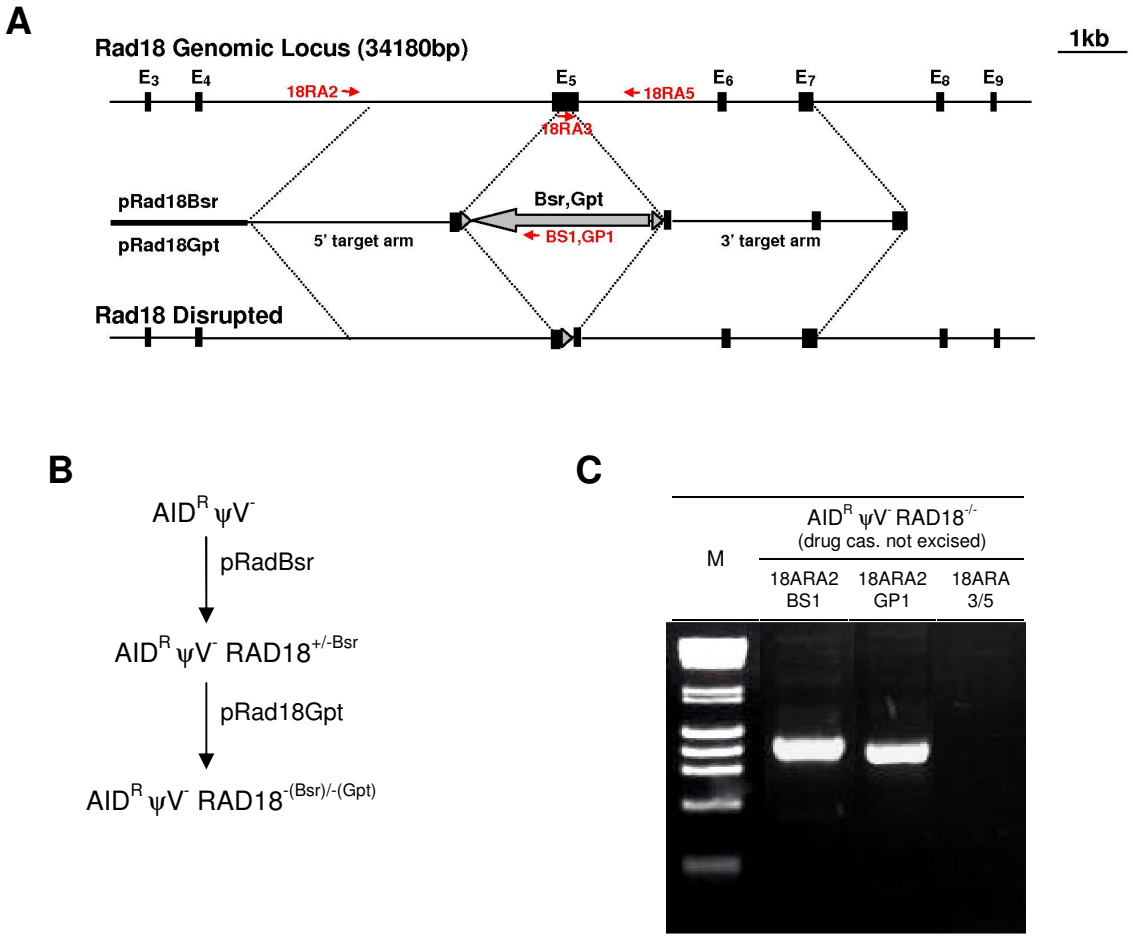


Figure 17. RAD18 knockout strategy. A) Aligned map of the chicken RAD18 locus, the targeting plasmid constructs, and the targeted loci after marker excision. Open boxes represent untranslated whereas solid boxes represent coding regions. The locations of the primers used to confirm knockout are indicated by arrowheads. B) Knockout scheme. C) Confirmation of the knockout by targeting screening PCR. The primers used for PCR were depicted on the gel picture.

At first pRad18Bsr was transfected and then to one of the isolated clone pRad18Gpt was transfected (Fig. 16B). The results were confirmed by long-range PCR using a primer located upstream of the 5' targeting arm along with a primer from the resistance marker (Fig. 16A). For genomic locus we used primer pair 18RA3-18RA5. From each of the transfections, 12 were stained for FACS analysis and after analysis 3 clones were decided. The resultant knockout clone was named as $AID^{R}\ \psi V^{-}\ RAD18^{-/-}$.

Targeting of the first construct was observed in 22 of the 24 events whereas for the second construct it was observed in 21 out of 36 clones. The relatively high frequency of targeting efficiency is reflecting typical feature of DT40 cells. The identified RAD18-deficient clone showed normal cell viability and cloning efficiency suggesting that RAD18 is not essential for cell viability.

III. Mutagen Sensitivity Test:

$RAD18^{-/-}$ cells are sensitive to DNA damaging agents:

To investigate the role of DNA damage tolerance, the survival of the different clones was determined after exposure to the DNA alkylating agent MMS and the DNA interstrand cross-linking agent cisplatin (Fig. 18). As it is reported previously (Yamashita et al., 2002) we found $RAD18^{-/-}$ cells are sensitive to MMS and cisplatin as compare to the wild type cells (Fig. 18).

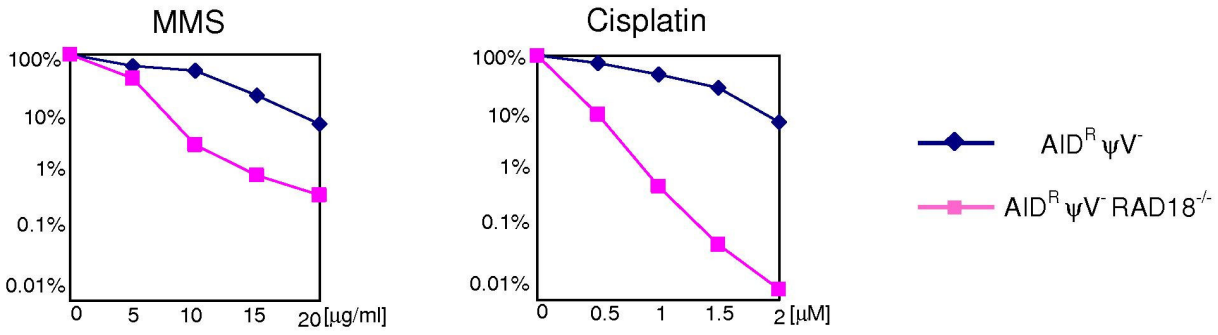
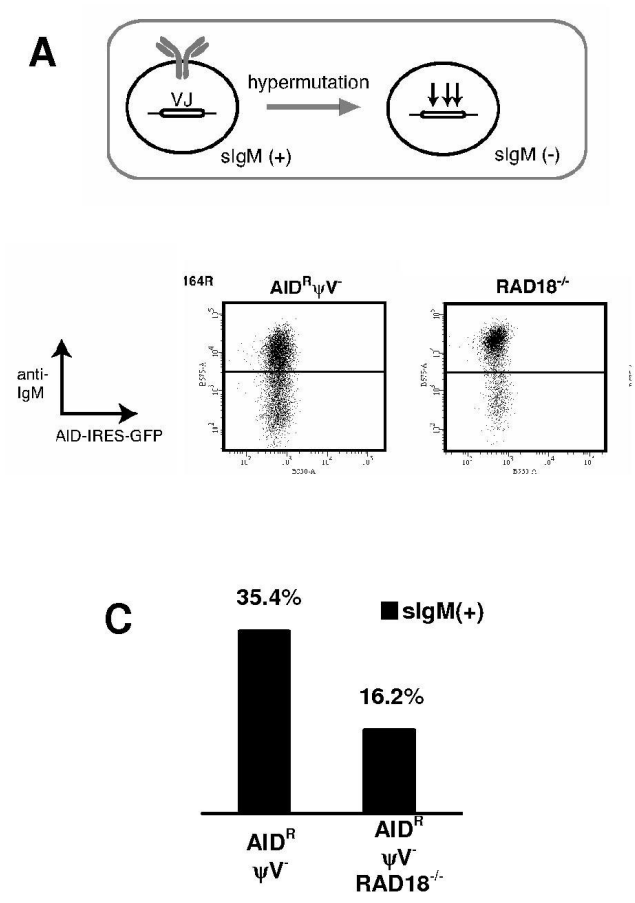


Figure 18. Analysis of mutagen sensitivity. Colony survival curves after exposure to DNA damaging agents. (y-axis represents percentage of surviving and x-axis represents the dose of the mutagen tested).

IV. Quantification of Surface IgM Levels:

Ig Mutation Assay suggests a decrease in surface Ig loss:

Deleterious Ig light chain mutations can be best detected and quantified by loss of sIg expression (Ig Mutation Assay) (Arakawa et al., 2004b). To compare the mutation rates from knockout clones, FACS was performed for 24 subclones of each of the cell lines two weeks after subcloning (Fig. 19). Subclones of the $AID^{R}\psi V^{-}$ progenitor cells showed 35.4% sIg(-) as an average in consistent with previous results. However, the average percentages of sIg(-) events for subclones of the $RAD18^{-/-}$ was only 16.2%. This analysis indicates that the *RAD18* knockout decreases the frequency of deleterious Ig mutations about 2 fold.



B

AID^{R} ψV^{-}	AID^{R} ψV^{-} $RAD18^{-/-}$
11.80%	10.60%
38.40%	6.20%
82.10%	16.60%
74.40%	18.40%
37.20%	12.60%
49.20%	7.50%
43.70%	40.30%
13.40%	20.00%
37.00%	7.20%
12.00%	31.30%
10.20%	32.50%
40.10%	48.30%
37.70%	11.10%
9.50%	15.10%
26.90%	5.30%
42.40%	18.10%
4.80%	9.90%
82.20%	11.70%
18.10%	8.20%
14.10%	16.50%
81.90%	11.50%
11.00%	13.00%
	12.50%

Figure 19. Analysis of Ig hypermutation activity**.** A) FACS profiles of representative subclones derived from a sIgM (+) cell after staining with an anti-IgM monoclonal antibody. B) The percentages of individual subclones falling into sIgM (-) gates. C) Average percentages of 24 subclones are shown by graph.

V. Ig Light Chain Sequencing:

To analyze the mutation activity from $AID^{R}\psi V^{-}RAD18^{-/-}$ cells, the rearranged light chain VJ segments (~0.6 kb) of the 2 subclones were sequenced 6 weeks after subcloning and compared with the sequences of $AID^{R}\psi V^{-}$ cells. As expected from the analysis of sIg loss rates, $RAD18^{-/-}$ cells yielded about 2 fold fewer of mutations per sequence than $AID^{R}\psi V^{-}$ cells (Fig. 20A). A decrease in mutations is seen for type-I transition mutations (G to A and C to T) but more prominently in type-II trasnversion mutations (C to G and G to C) (Fig. 20B).

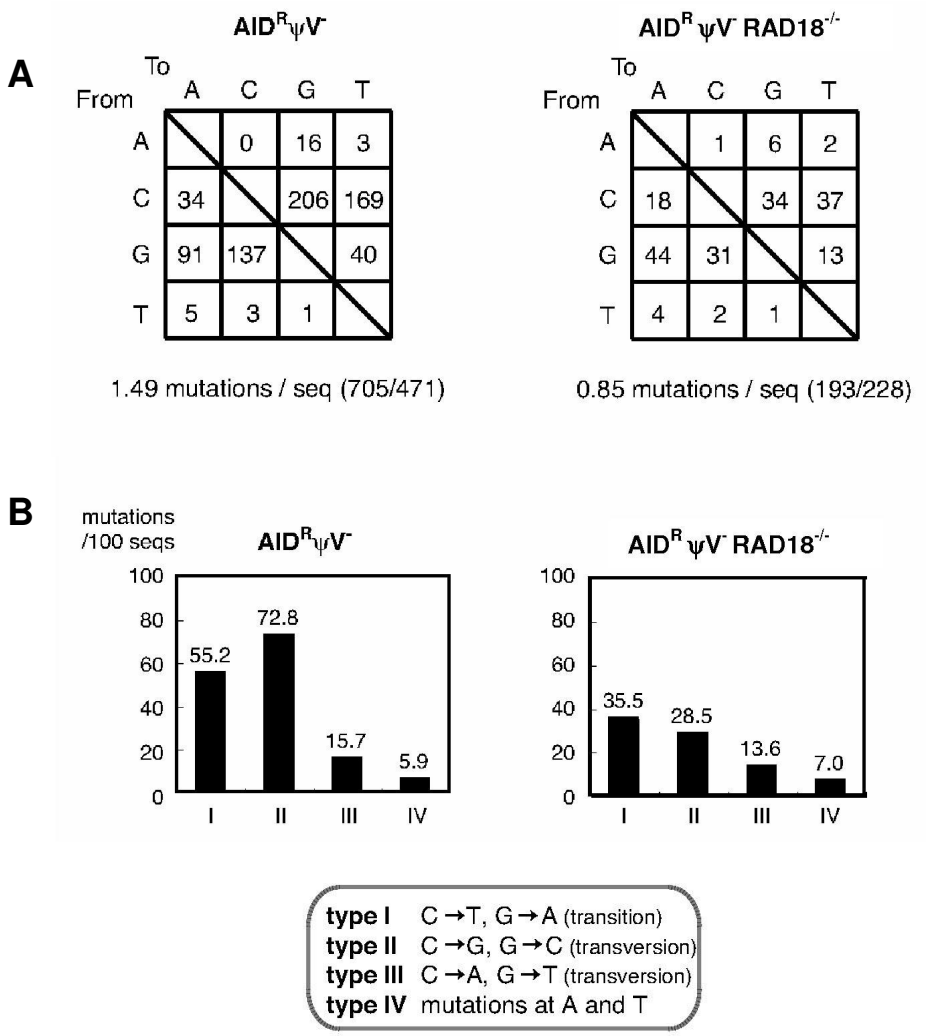


Figure 20. Mutation spectrum. A) Frequencies of nucleotide substitutions within light chain gene. **(B)** A graphical view showing the frequencies of particular types of mutations per hundred sequences.

DISCUSSION:

DNA deamination model of AID suggests a role for UNG downstream of AID to remove uracils that are introduced by AID in immunoglobulin V genes or switch regions (Petersen-Mahrt et al., 2002). Several studies have clarified this and showed that SHM and CSR indeed need UNG (Di Noia et al., 2002; Rada et al., 2002a; Imai et al., 2003).

In the present study we could show that inactivation of the *UNG* gene in AID expressing DT40 almost completely abolishes Ig gene conversions and activates a very high rate of transition mutations at C/G bases in the rearranged light chain VJ region. This demonstrates conclusively that Ig gene conversion like previously shown from hypermutation and switch recombination requires UNG downstream of AID.

The results strongly support a model in which AID first deaminates cytosine within the rearranged V(D)J segments and the resulting uracils are then processed by UNG to give rise to first repair in an error free manner, second to gene conversions and third to hypermutations in the absence of pseudogenes (Fig. 21). Inactivation of the *UNG* gene blocks the processing of uracils and leads exclusively to transition mutations when uracil pairs with adenine in the next replication cycle. So far, this model had been based on results showing that *Ugi* (uracil DNA glycosylase inhibitor) expression shifts Ig hypermutations toward transitions in a XRCC2$^{-/-}$ mutant (Di Noia et al., 2002) and inhibits Ig gene conversion in wild-type DT40 (Di Noia et al., 2004). However, the phenotype of the *Ugi* gene transfectants differs from the phenotype of the UNG$^{-/-}$ mutant in some features at odds with the model. For example, *Ugi* expression increased transition mutations only to 86% in the XRCC2$^{-/-}$ mutant whereas this number was 98% from the UNG$^{-/-}$ mutant (Di Noia et al., 2002). Similarly, *Ugi* expression reduced Ig gene conversion to 30% of wild-type levels (Di Noia et al., 2004), whereas the *UNG* gene disruption decreased gene conversion almost 100 fold based on the light chain frameshift repair assay. The most striking discrepancy to the model is however the lack of detectable transition mutations after Ugi expression in wild-type DT40. The difference between the results obtained after Ugi transfection and UNG disruption could be due to the fact that UNG-deficient cells express a transfected AID, which may differ both in abundance and cell cycle regulation from endogenous AID. However, the most straightforward interpretation seems to be that *Ugi* expression in DT40

only partially inhibited UNG activity and it is of open question whether Ugi inhibits all the activity of uracil DNA glycosylases.

Although our results suggest a major role for UNG to remove uracils in DT40, this does not exclude the possibility of a back up activity due to few gene conversion tracts still seen in UNG deficient cells. This means a minor alternative pathway seems to exist for U/G mismatches. As mentioned earlier in introduction there are totally four uracil DNA glycosylases identified so far. The exact physiological roles of these enzymes are not still understood but current studies suggest that UNG and SMUG1 are responsible for U/G repairs (Nilsen et al., 2000; Kavli et al., 2002; Kavli et al., 2005). In a recent study it was shown that overexpressed SMUG1 is able to excise uracil from immunoglobulin genes both in DT40 cells and in mice (Di Noia et al., 2006). However, what they concluded SMUG1 plays little natural role in antibody diversification because it is diminishingly expressed during B-cell activation and it repairs uracils more in conventional way. U/G mismatches might well be recognized by mismatch repair system (Rada et al., 2004). Which of these genes and or pathways involved in antibody diversification besides UNG as a back up can further be identified by obtaining double or triple mutants.

Murine and human B cells do not use gene conversion for Ig repertoire development and hypermutating chicken DT40 mutants differ from primary B cells, as they do not mutate A/T bases. Nevertheless, the initial steps leading to Ig gene conversion and hypermutations at C/G bases seem to be identical in all three cell systems. Thus, hypermutations at C/G bases are shifted toward transitions in UNG deficient mice and human patients (Rada et al., 2002; Imai et al., 2003) and B cells of the murine UNG/MSH2 double knock-out which lack the A/T mutator due to the MSH2 defect show a mutation spectrum very similar to the DT40 $UNG^{-/-}$ mutant (Rada et al., 2004).

If AID-induced uracils are not repaired in the absence of UNG, hypermutations in $UNG^{-/-}$DT40 cells will offer a unique glimpse at the *in vivo* cytosine deamination activity of AID without subsequent selection or repair bias. The similar numbers of mutations at C and G bases from $UNG^{-/-}$ cells imply for example that the transcribed and the non-transcribed DNA strand are equally accessible to AID.

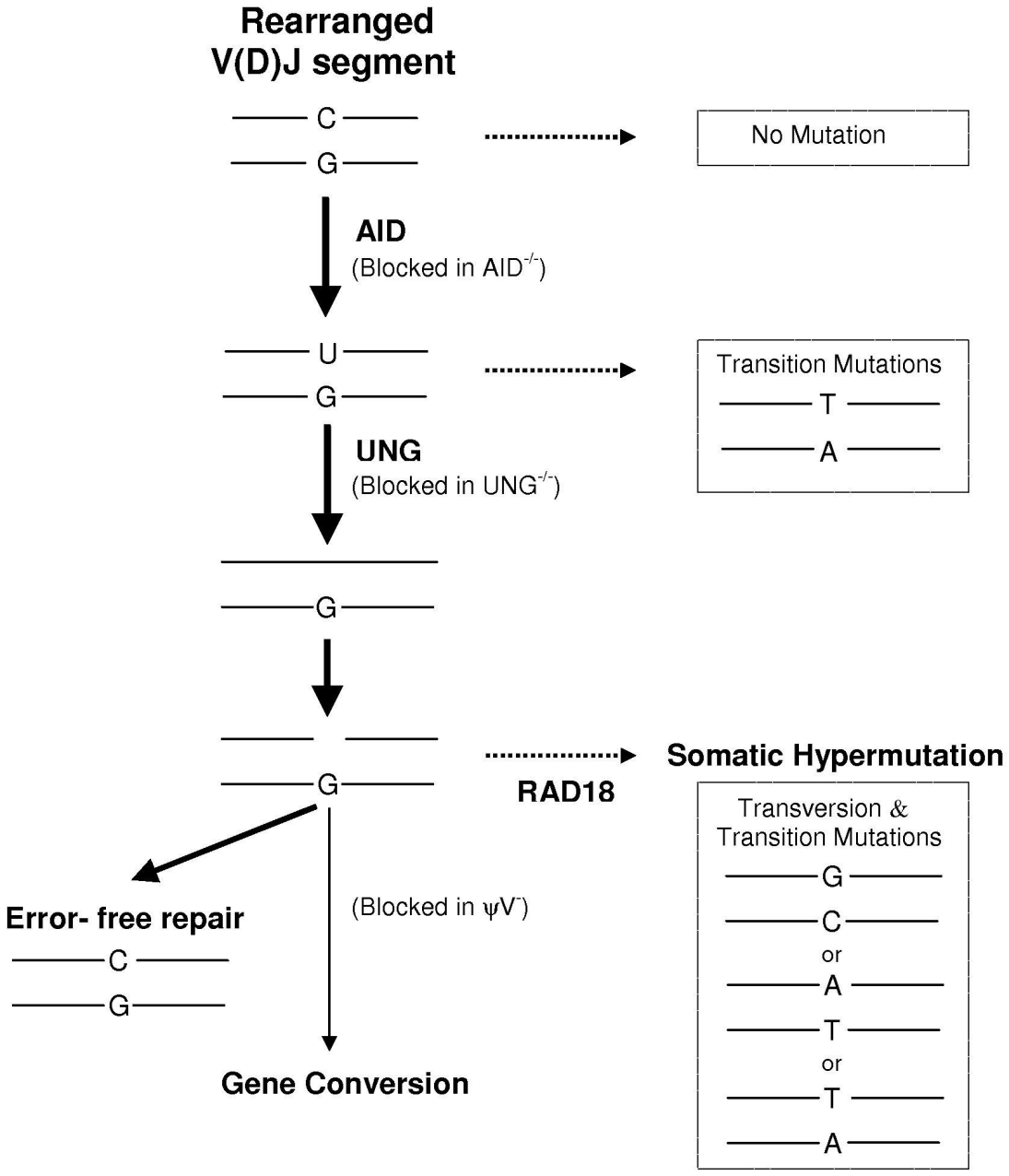


Figure 21. A model explaining the regulation of chicken Ig gene conversion and Ig hypermutation by AID, UNG and RAD18. In this model AID converts C to U in rearranged VDJ segment and uracils are removed by UNG. Normally, most of the uracils are repaired in an error-free manner and little is fixed as gene conversions. In the absence of pseudogenes, uracils which are fixed as hypermutations require not all but little role of RAD18.

The original hypothesis about SHM claims that a mutator factor, i.e., AID, travels with the RNA pol during Ig gene transcription (Peters et al., 1996). In search for the footprint of AID, transition mutations in $UNG^{-/-}$ cells give us a clue about this. Mutations in our study just start at the transcription start site. That implies AID is being loaded to the transcription initiation complex and elongates with it. A recent study suggested there is no evidence of AID footprint in the first 100 bp of transcription (Longerich et al., 2005). They explain this mainly by suggesting AID travels with the elongating transcription complex. It could be that regulation of SHM in mice and chicken is different. Whereas in mice both mismatch repair and UNG can be recruited to repair uracils, in chicken UNG seems to work alone. A critical point which needs to be further clarified.

Comparison of the transition mutations of $UNG^{-/-}$ cells with hypermutations that accumulate in ψV^{-} cells after the UNG mediated processing of uracils provides further insight how the hypermutation process is initiated. $UNG^{-/-}$ cells accumulate light chain mutation seven times faster than ψV^{-} cells, indicating that about one of seven AID-induced uracils is processed into a mutation. This relative frequent conversion of uracils into mutations suggests the presence of an unusual error-prone repair pathway which specifically recognises AID-induced uracils. Interestingly, the distribution of mutations within the rearranged light chain gene varies between $UNG^{-/-}$ and ψV^{-} deleted cells. Although the majority of mutations arise within the VJ coding region in both cell types, relatively more mutations in $UNG^{-/-}$ cells are located upstream of the V and downstream of the J coding regions. The most plausible explanation for this phenomenon is, that UNG mediated processing of uracils is more error prone within the VJ segment than in the flanking regions. Nevertheless, an alternative explanation could be related with a DNA error-free repair capacity of the B-cells. This means the purpose of the AID could be to introduce uracils more than cell can tolerate error-freely. Therefore, the remaining uracils which occur in more V gene, then repaired in an error-prone way leading to gene conversions (Fig. 22). In any case, the comparison of $UNG^{-/-}$ and ψV^{-} mutations provides the first evidence that the characteristic distribution of Ig hypermutation over the V(D)J regions reflects not only AID mediated cytosine deamination, but also UNG mediated permutation of the resulting uracils in a position dependent fashion.

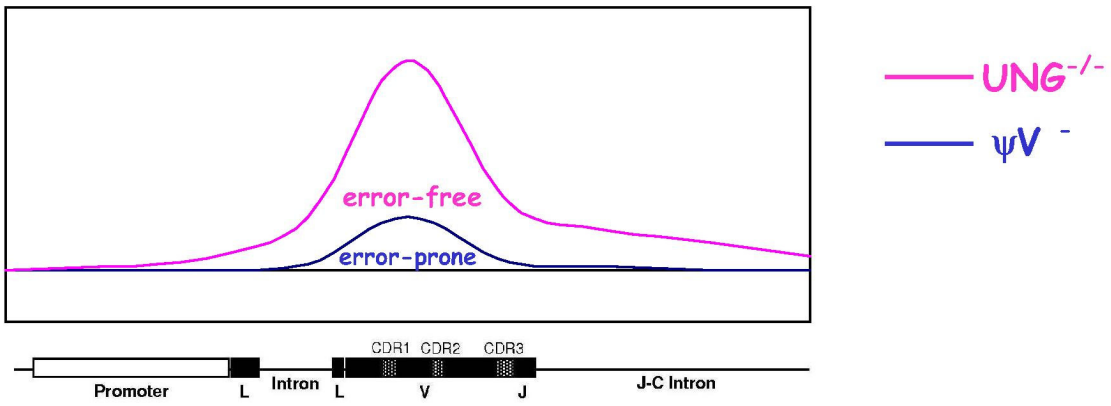


Figure 22. A possible model for the initiation of gene conversion and or hypermutation.

Studies in yeast suggest that PCNA mono-ubiquitination by RAD6/RAD18 induces a switch from replicative to translesion DNA synthesis (Hoege et al., 2002; Ulrich et al., 2004). It is of interesting question whether SHM in DT40 requires this pathway or not?

Our results show that at least partially SHM needs RAD18 induced monoubiquitination (Fig. 21). RAD18$^{-/-}$ cells are sensitive to DNA damaging agents MMS and cisplatin and in RAD18$^{-/-}$ cells only type II (majority) and type I mutations were decreased. The decrease in type II mutations were also seen before in REV1$^{-/-}$ cells (our unpublished data; Simpson et al., 2003). The selective decrease of this type of hypermutation may reflect the deoxycytidyl transferase activity of REV1, which would add cytosine opposite to an abasic site in the template strand (Nelson et al., 1996). This means that ubiquitinated PCNA recruits REV1 as an error-prone polymerase.

Why only partial of the mutations decreased has to be clarified. RAD18 is the only E3 ligase known to mono-ubiquitinate PCNA at lysine 164 in *S. cerevisiae*. The RAD18 knockout construct used in our study most likely generates a RAD18 null mutation (Yamashita et al., 2002), although it does not delete the RAD18 RING finger coding sequence. To rule out that the analysed RAD18 mutant still possessed enzymatic activity, we generated a second RAD18 mutant in which the RING finger coding region is deleted. Analysis of this new mutant confirmed the persistence of low-level PCNA ubiqitination seen in the first RAD18 mutant (data not shown). These data point to the presence of a RAD18 independent back-up pathway of PCNA ubiquitination in vertebrate cells. Most

likely, PCNA ubiquitination in RAD18$^{-/-}$ cells is mediated by another E3 ligase like, for example, the one encoded by the FANCC or FANCL gene (Gurtan et al., 2006). It was previously reported that RAD18 disruption in DT40 does not affect Ig hypermutation (Simpson et al., 2005). However, we see an about 2 fold reduction of Ig hypermutation in RAD18$^{-/-}$ cells. We believe this discrepancy may be caused by the difficulty to accurately measure Ig hypermutation in wild-type DT40, which diversifies its Ig genes predominantly by gene conversion.

If ubiquitinated PCNA by RAD18 functions as a link to the recruitment of error prone polymerases during Ig hypermutation, it remains an intriguing question how it is coupled to upstream events in the hypermutation process. The DNA editing model assumes that AID first deaminates cytosine to uracil and that the resulting uracil is then excised by UNG-2 (Di Noia et al., 2002). We showed in thesis that, comparison of the mutation frequencies in UNG disrupted and ψV deleted DT40 suggests about one in seven AID-induced uracils is converted into a mutation. This high mutation rate indicates that the abasic sites produced by uracil excision are not repaired by the standard base excision repair, but are deliberately channeled into error-prone translesion synthesis. One of the possibilities is that UNG-2 is recruited by PCNA (Otterlei et., 1999) and excises AID-induced uracils shortly before DNA synthesis thereby precluding the possibility of base excision repair. Another possibility is that the DNA lesions produced by the combined action of AID and UNG are for some reason, perhaps by protein attachment or by another type of modification, guarded from faithful repair until they encounter the PCNA clamp.

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