Regulation of translesion synthesis polymerases during somatic hypermutation and DNA damage tolerance

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GENERAL INTRODUCTION

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GENERATION OF HIGH AFFINITY ANTIBODIES

In 1890 it was shown that resistance to diphtheria and tetanus results from the formation of substances that circulate in the blood of immunized animals (Silverstein, 1985). These substances are now known as antibodies and are widely used in the diagnostics and treatment of many different diseases (Nelson et al., 2010). Antibodies consist of two identical immunoglobulin heavy chains and light chains and are produced by B cells, a specialized cell of our immune system (Fig. 1). The amino-terminal or variable region of the antibody is responsible for antigen binding, while the carboxy-terminal or constant region determines the effector function and tissue localisation. As each B cell can only produce antibodies with a single specificity, but an organism can generate antibodies against an unlimited range of antigens, Lederberg proposed over 50 years ago that B cells undergo a high frequency of (genome wide) somatic mutation to create a virtual unlimited antibody repertoire with a size-limited genome (Lederberg, 1959). While antibody diversification is now known to occur initially during early B cell development by V(D)J recombination of the immunoglobulin (Ig) genes (Tonegawa, 1983) (Fig. 1), it has been established that the generation of high affinity antibodies in activated mature B cells critically depends on a continuous neo-Darwinian maturation and evolution of antibody specificities by somatic hypermutation (SHM) of the variable region of Ig genes and antigen-mediated selection. (Di Noia and Neuberger, 2007;Neuberger, 2008). Both SHM and selection take place in germinal centers (GC), highly dynamic structures formed within secondary lymphoid organs shortly after activation of B cells by binding of their antibody to an antigen (Rajewsky, 1996;Victora et al., 2010). In addition to SHM, GC B cells can undergo class switch recombination (CSR) to replace the Ig heavy chain constant region for a downstream constant region (i.e. IgG, IgA or IgE), to generate an antibody with a different effector function. (Stavnezer et al., 2008). Thus, over a short period after first encounter with antigen, low-affinity antibodies specific for the antigen can be transformed into high-affinity antibodies, usually of an IgG, IgA, or IgE isotype.

SHM AND CSR: IT ALL STARTS WITH DNA DEAMINATION

SHM and CSR are initiated by the activation-induced cytidine deaminase (AID), an enzyme found to be differentially expressed in B cells of the GC (Muramatsu et al., 2000). AID deaminates cytosine (C) to uracil (U) within single stranded DNA (Pham et al., 2003;Chaudhuri et al., 2003;Dickerson et al., 2003;Ramiro et al., 2003), showing a preference for WRC motifs (W=A/T, R=purine). As AID targets both DNA strands *in vivo* (Dorner et al., 1998;Milstein et al., 1998), the hotspot motif of antibody hypermutation WRCY (Y=pyrimidine), might be explained by deamination of a substrate that contains overlapping WRC motifs

on opposite DNA strands by different subunits of an AID oligomer (Rogozin and Kolchanov, 1992;Beale et al., 2004). While the primary lesion is restricted to cytosine deamination, SHM occurs equally efficient at G/C and A/T basepairs. During the last decennium, humans, mice, and cell lines carrying defined genetic alterations in DNA repair and DNA damage tolerance elements have revealed detailed insights into the molecular pathways controlling the generation of defined point mutations in hypermutating Ig genes. The combination of these pathways enables hypermutating B cells to generate the entire spectrum of nucleotide substitutions at a rate of one per thousand bases per generation, six

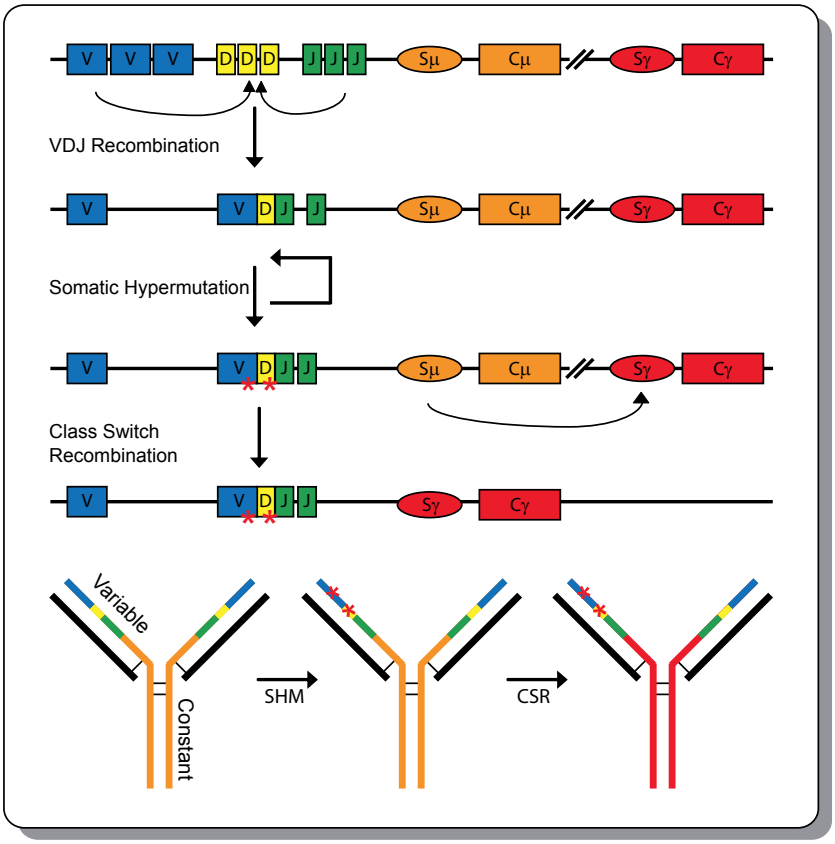

Fig.1. VDJ recombination, SHM and CSR. During early B cell development the primary B cell repertoire is generated by recombination of the V(D)J gene segments encoding for the variable domain of the heavy chain. For simplicity recombination of the VJ genes of the light chain is not depicted. Upon antigen binding, B cells can undergo multiple rounds of somatic hypermutation and selection to increase the affinity of the antibody for the cognate antigen. In addition, B cells can undergo class switch recombination, which involves replacement of the Ig heavy chain constant region for a downstream constant region.

orders of magnitude greater than spontaneous mutagenesis. To establish these point mutations at and around the initial U lesion, B cells proved to be highly creative in transforming established DNA repair pathways into effective mutator pathways. The mutagenic transformation of these faithful repair pathways appears to depend strongly on a family of specialized DNA polymerases with defined error signatures. To date, three main mutation pathways have been identified that contribute to the mutagenic processing of U generated by AID (Figure 2). This chapter focuses on the role of these pathways and present knowledge/models regarding their (in)dependence in establishing specific point mutations. In addition, we here focus on the regulatory aspects controlling the establishment of defined mutations.

DIRECT REPLICATION ACROSS THE URACIL: G/C TRANSITIONS

Besides intentional cytosine deamination of Ig genes by AID in hypermutating B cells, spontaneous cytosine deamination occurs frequently and is one of the most common lesions in our genome. If not removed timely from the genome, a uracil is highly mutagenic. During DNA synthesis a U in the template strand will instruct DNA polymerases to incorporate an Adenine (A), causing G to A and C to T transitions. In agreement with this notion, bacteria defective in removing U from their genome have a high rate of spontaneous G/C to A/T transitions (Duncan and Miller, 1980). First data suggesting a role of this mutation pathway during SHM was provided by the observation that expression of AID in these bacteria resulted in an increase of G/C to A/T transitions (Petersen-Mahrt et al., 2002). The significance of this pathway was further corroborated in mice defective in the removal of U (Shen et al., 2006;Rada et al., 2004). In these mice, the base exchange pattern of mutated Ig genes was extremely compromised, showing only G/C transitions. These data clearly revealed a major pathway in the generation of G/C transition mutations and strongly suggested that the remaining point mutations of the hypermutation spectrum require further modifications of the initial U lesion (Figure 2, left panel). In fact, as outlined below SHM critically depends on two generic DNA repair factors, capable of recognizing U in the DNA, the base excision repair (BER) factor Ung and the mismatch repair (MMR) factor MutSα (Figure 2, middle and right panel, respectively).

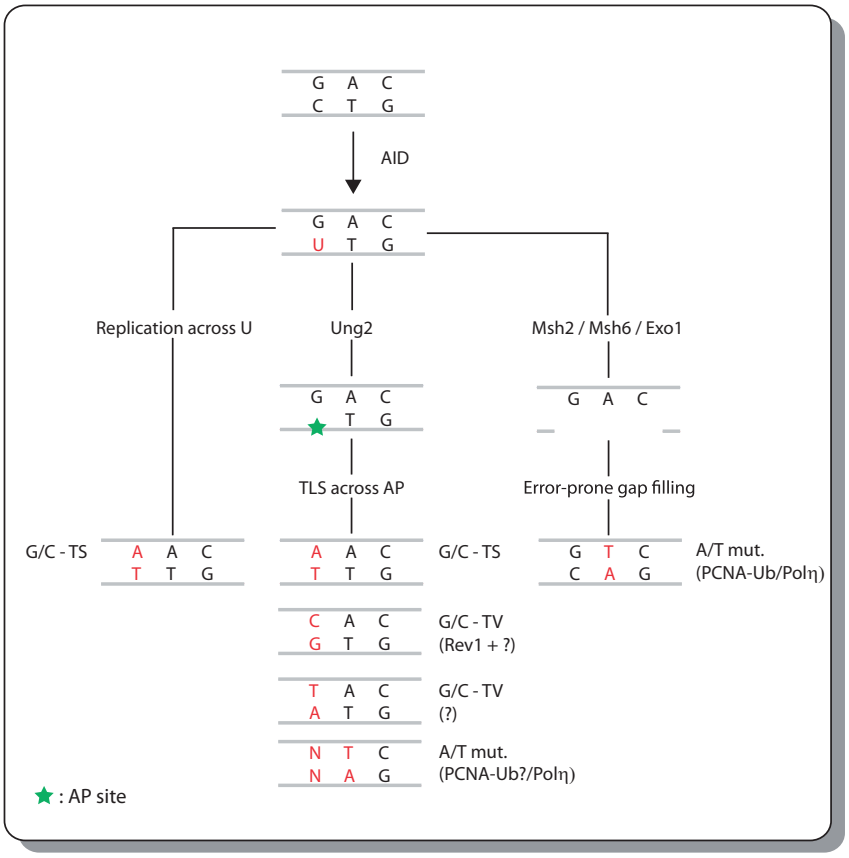

Figure 2. The three pathways of SHM downstream of AID. AID deaminates C to U. Three error prone repair pathways can process the U: I.) Left panel: Direct replication across U; a U in the template strand instructs a template T leading to the generation of G/C to A/T transitions (TS). II.) Middle panel: Ung2 dependent SHM; upon removal of the U by Ung2 an abasic site (AP site, indicated by a star) is generated. Replication across this Ung2-dependent, non-instructive AP site generates G/C transitions and transversions (TV). Rev1 generates G to C transversions. Other unknown polymerases (?) generate G/C transversions and possibly transitions. A minority of A/T mutations (~10%) is generated downstream of Ung2 and depend on Polη and possibly PCNA-Ub. III.) Right panel: MutSα-dependent SHM; the U/G mismatch generated by AID can also be recognized by MutSα. MutSα, an unknown 5' endonuclease and Exo1 generate a large gap. This MutSα/Exo1-dependent gap triggers the Rad6 pathway, leading to monoubiquitination of PCNA (PCNA-Ub), which in turn recruits the A/T mutator Polη to generate A/T mutations.

UNG DEPENDENT SHM ACROSS AP SITES: G/C TRANSVERSIONS AND TRANSITIONS

To maintain the integrity of the genome, U and other highly mutagenic base modifications can effectively be removed by a multistep repair process, known as base excision repair (BER). BER is initiated by a family of highly efficient, partially redundant DNA-glycosylases capable of recognizing and removing modified bases from our genome. DNA-glycosylases catalyze the hydrolysis of the N-glycosidic bond that links the base to the deoxyribose-phosphate backbone. After the base excision step, the DNA duplex now harbors an apurinic or apyrimidinic, i.e. an AP site in its backbone, which is also known as abasic site. The repair of AP sites, the common product of glycosylase action, requires a second class of BER enzymes known as AP endonucleases, APE1 and APE2 in mammals, which generate nicks in the duplex DNA by hydrolysis of the phosphodiester bond immediately 5' to the AP site. In mammalian cells, further processing involves DNA polymerase β (Polβ). Polβ has two enzymatic activities, a large C-terminal DNA polymerase domain and a small N-terminal DNA-deoxy-ribophosphodiesterase (dRpase). While the dRpase activity of Polβ makes a second nick to remove the AP deoxyribose, the polymerase activity fills up the single nucleotide gap. While the lack of an intrinsic proof reading activity renders Polβ error prone (1-2 misinsertions per 10000), accuracy might be gained at the level of DNA ligation and postreplicative MMR (Kunkel, 1985;Friedberg et al., 2006). In addition to this short patch BER pathway (1nt), the nature of the DNA glycosylase and/or lesion may require an alternative pathway of BER, known as long patch BER. Long patch BER involves the flap-endonuclease 1 (FEN1), which after displacement of the strand containing the modified base (flap) makes an incision to generate a long single-strand patch (2-8nt). Repair synthesis of this long patch requires components of the replication machinery such as Polδ and Polε, the DNA sliding clamp Proliferating Cell Nuclear Antigen (PCNA) as well as DNA ligase 1. Given the accuracy of Polδ, and Polε, long patch BER is effective in maintaining genome integrity after base damage. Besides replicative DNA polymerases, Polβ may also contribute to long patch BER, (Podlutsky et al., 2001;Singhal and Wilson, 1993).

In mammals, four DNA glycosylases have been identified that can hydrolyze U from the DNA backbone: Uracil-DNA glycosylase (UNG), SMUG DNA glycosylase (SMUG1), methyl-binding domain glycosylase 4 (MBD4), and thymine DNA glycosylase (TDG) (Krokan et al., 2002). Although redundant in their enzymatic activity *in vitro*, only UNG appears to be essential during SHM (Bardwell et al., 2003;Di Noia et al., 2006;Rada et al., 2004;Rada et al., 2002). Two alternative splice variants of UNG exist, a mitochondrial (UNG1) and a nuclear (UNG2). *Ung* mutant B cells lack most G/C transversions (Rada et al., 2002). These transversions do not depend on Polβ, as Polβ deficient B cells mutate normally (Esposito et al., 2000). The role of the APE endonucleases during SHM is unknown, as APE1

deficiency causes early embryonic lethality (Xanthoudakis et al., 1996). Most likely, during SHM AP sites are not always removed prior to replication or repair synthesis. As AP sites are non-instructive they cause replicative DNA polymerases to stall. To continue DNA synthesis across an AP site specialized polymerases are recruited (see below) that tolerate such blocking lesions and thereby generate G/C transversion as well as transition mutations (Figure 2, middle panel). Besides APE the Mre11/Rad50/Nbs1 (MRN) complex has been proposed to initiate mutagenesis by cleaving AP sites (Larson et al., 2005;Yabuki et al., 2005). As the resulting ends cannot be extended by high-fidelity DNA polymerases, it has now been suggested that error prone DNA polymerases take over to introduce mutations when filling the gap. Future studies in MRN deficient cells should clarify the relevance of this pathway in SHM.

MUTSα DEPENDENT SHM AT MMR GAPS: A/T MUTATIONS

Cytosine deamination in the DNA helix generates a U:G mismatch. Besides BER, the U:G mismatch can be processed by DNA mismatch repair (MMR) (Wilson et al., 2005;Schanz et al., 2009). MMR is an evolutionarily conserved process that normally corrects mismatches that have escaped proofreading during DNA replication. The MMR process involves a complex interplay of MMR-specific proteins with the replication and/or recombination machinery (Jiricny, 1998). MMR is initiated by the binding of the mismatch-recognition factors, MutSα (MSH2/MSH6 complex) to single base mismatches or MutSβ (MSH2/MSH3 complex) to insertion/deletion loops that arise during recombination or from replication. Mammalian MMR is proposed to initiate at strand discontinuities, such as nicks or gaps that are distal to the mispair (Modrich, 2006;Schanz et al., 2009) The recruitment of MutL homologues (MutLα: MLH1-PMS2 complex; MutLβ: MLH1-PMS1 complex) stabilizes the mismatch-bound MutSα complex and appears to prohibit sliding of MutS. Exonuclease-1 (Exo1) mediated degradation of the error-containing strand depends on an incision 5′ of the mismatch. This incision may involve the nuclease activity of PMS2 (Kadyrov et al., 2006) or an alternative nuclease. Once the mismatch is removed, resynthesis of the degraded region by a DNA polymerase, followed by sealing of the remaining nick by DNA ligase, completes the repair process.

Remarkably, given the protective nature of MMR in preventing mutations arising from mismatched non-Watson-Crick base pairs, early studies in mismatch repair mutant mice revealed a selective role of the mismatch recognition complex MutSα as well as Exo1 in establishing somatic mutation at template A/T around the initial U:G mismatch. Interestingly, while MSH2, MSH6 and Exo-1 deficient B cells lack 80-90% of all A/T mutations, the SHM phenotype appears less pronounced

or even normal in B cells lacking other MMR components such as PMS2, MLH1, MLH3 and MSH3 (Rada et al., 1998;Wiesendanger et al., 2000;Bardwell et al., 2004;Martomo et al., 2004;Phung et al., 1998;Phung et al., 1999;Ehrenstein et al., 2001;Winter et al., 1998;Jacobs et al., 1998;Frey et al., 1998). These data suggest that during SHM, selective components of the mismatch repair machinery are required to generate a single strand gap. In contrast to conventional, postreplicative MMR, the gap filling process during SHM appears to employ error prone TLS polymerase(s) that generate predominantly A/T mutations (Figure 2, right panel). At present the identity of the incision maker 5' to the mismatch, which is required for Exo1 is unknown. UNG2/APE has been proposed (Schanz et al., 2009), but given the fact that A/T mutations are mainly unaffected in Ung deficient B cells (Rada et al., 2002;Krijger et al., 2009), alternative uracil glycosylases might take over.

UNG DEPENDENT A/T MUTATIONS AND MUTSα DEPENDENT G/C MUTATIONS

The above mentioned observations have resulted in a model in which there is a strict separation between the MutSα– and Ung-dependent pathways in establishing mutations at template A/T and G/C respectively. However, as Msh2 and Msh6 deficient mice show a more restricted targeting of G/C mutations in the V region (Martomo et al., 2004;Delbos et al., 2007;Frey et al., 1998;Rada et al., 1998), MutSα may also be involved in the generation of G/C mutations. In addition, a significant fraction of A/T mutations (10-20%) are found in MSH2 deficient GC B cells (but not in UNG2/MSH2 double deficient B cells), indicating that UNG2 dependent mutagenesis generates A/T mutations independent of MutSα (Rada et al., 2004). Whether UNG2-dependent A/T mutations are generated during long patch BER, i.e. within the strand containing the AP site, or alternatively during the extension phase of TLS across the AP site is currently unknown. Mice deficient for FEN1 are embryonic lethal, and analysis of B cells from mice expressing a hypomorph variant of FEN1 showed no SHM phenotype (Larsen et al., 2008), conditional knock out mice or deficient cell lines will have to be generated to reveal a function of this pathway in SHM**.**

TRANSLESION SYNTHESIS DNA POLYMERASES

To explain the unusual high mutation rate of SHM, error prone polymerases were postulated about half a century ago (Brenner and Milstein, 1966) . Yet, only during the last two decades the existence of error prone TLS DNA polymerases was revealed. Their characterization *in vitro* and *in vivo* indicated an error rate that

Table 1. DNA polymerases in higher eukaryotes

Family	Pol	Function
A	Polγ	Mitochondrial DNA replication
	Polθ	TLS (?) BER (?)
	Polν	TLS (?)
B	Polα	Replication priming
	Polδ	Replication
	Polε	Replication
	Polζ	TLS SHM (? - Reduced mutations in ko)
X	Polβ	BER
	Polλ	BER NHEJ(V(D)J recombination) TLS (?)
	Polμ	NHEJ (VJ recombination), TLS (?)
	TdT	V(D)J recombination
Y	Polη	TLS SHM (A/T mutations) homologous recombination
	Polι	TLS
	Rev1	TLS SHM (C>G and G>C)
	Polκ	TLS NER SHM (Backup Polη, A/T mutations)

easily matches the one of SHM. In higher eukaryotes, TLS is carried out primarily by the Y family polymerases Polη, Polι, Polκ and Rev1, the B family member polζ (Prakash et al., 2005;Guo et al., 2009). In addition other polymerases have been identified to display TLS activity (Table 1). TLS polymerases share the unique capacity to bypass DNA lesions, i.e. they can continue replication in the presence of noninstructive or misinstructive DNA lesion that otherwise may stall the replicative Polε and Polδ. In general, TLS is thought to proceed in a two-step mode (Shachar et al., 2009;Johnson et al., 2000;Ziv et al., 2009) 1.) Incorporation of nucleotide(s) directly opposite of the lesion. 2.) Elongation from the distorted or bulky non-Watson-Crick base pairs by an extender TLS polymerase. A prerequisite for TLS is the lack of proofreading activity by TLS polymerases. Once extended, proofreading proficient high fidelity DNA polymerase cannot detect the tolerated lesion any longer and resumes DNA synthesis. While the capacity of TLS polymerases to accommodate non Watson-Crick base pairs within their catalytic center is beneficial regarding the accurate replication across specific modified bases, it also makes TLS polymerases highly mutagenic when replicating

across undamaged DNA or other lesions (Jansen et al., 2007;Prakash et al., 2005). Since each polymerase displays its own mutagenic signature, alterations in the mutation spectrum can often be attributed retrospectively to the absence of or failure in activating specific polymerases. This preference has been highly beneficial regarding the identification of DNA polymerases involved in SHM.

Polη

Polη, a polymerase that is absent or hypomorph in patients with the variant form of Xeroderma Pigmentosum (XP-V) (Johnson et al. 1999)(Masutani et al., 1999b), is highly efficient and error-free when replicating UV-induced cyclobutane pyrimidine dimers (CPDs), the oxidative lesion 8-oxodeoxyguanosine (8-oxo-dG) and cisplatin-induced GG intrastrand crosslinks (Johnson et al., 1999;McCulloch et al., 2004a;Matsuda et al., 2000;Shachar et al., 2009;McCulloch et al., 2004b;Vaisman et al., 2000;Haracska et al., 2000). The observations that XP-V patients are hypersensitive and hypermutable to UV damage, associated with a strong predisposition to skin cancer (Masutani et al., 1999a;Johnson et al., 1999) indicate that at least in the context of UV-induced DNA damage, other TLS polymerases are non-redundant with Polη activity *in vivo*. In contrast, Polη is error-prone when replicating undamaged DNA, a feature employed during SHM as indicated by the significant reduction in mutations at A/T base pairs in B cells from XP-V patients (Zeng et al., 2001) and mouse models defective for Polη (Delbos et al., 2005;Martomo et al., 2005). Consistent with these *in vivo* data, Polη has a preference to insert mismatched nucleotides opposite template T (Rogozin et al., 2001). These data suggest that Polη is required to generate A/T mutations downstream of MutSα. In addition to its role downstream of MutSα, Polη is responsible for the remaining A/T mutations downstream of UNG2, as deduced from SHM analysis in MSH2 and MSH2/Polη-deficient mice (Delbos et al., 2007).

Polκ

In vitro experiments have shown that purified Polκ efficiently and accurately bypasses benzo[a]pyrene-guanine (BP-G) adducts (Zhang et al., 2000). The observation that Polκ deficient cells, are sensitive and hypermutable to BP-G suggests that these properties are also employed by Polκ to bypass across BP-G adducts *in vivo* (Ogi et al., 2002). The sensitivity of Polκ deficient cells to UV radiation (Schenten et al., 2002;Ziv et al., 2009), is more difficult to understand as *in vitro* the enzyme does not support TLS across CPD or [6-4] photoproducts (the other main type of UV induced DNA damage). There are however indications, that Polκ might function in extending from bypassed UV lesions (Washington et al., 2002) and removal of UV lesions by Nucleotide Excision Repair (Ogi et al., 2010;Ogi and Lehmann, 2006). Polκ seems not essential for somatic hypermutation in Polκ deficient mice (Schenten et al., 2002; Shimizu et al., 2003). However, the residual A/T mutations found in Polη deficient B cells have been demonstrated to depend on Polκ and at least a third yet unidentified polymerase (Faili et al., 2009). This

observation is compatible with the error-signature of Polκ *in vitro* (Ohashi et al., 2000). Apparently, Polκ can substitute Polη whereas other polymerases of the Y-family, for example Rev1, cannot, as revealed by the normal generation of G to C transversions in Polη deficient mice (see below).

Rev1

Rev1 is selective in its nucleotide incorporation activity as it only incorporates dCMP and therefore in its strictest sense should be regarded as a deoxycytidyl transferase rather than a bona fide DNA polymerase. *In vitro*, Rev1 is able to efficiently and specifically insert dCMP opposite an AP site or a uracil residue, but not opposite a CPD or a [6–4] photoproduct (Nelson et al., 1996;Masuda et al., 2002;Masuda et al., 2001;Lin et al., 1999). The sensitivity of Rev1 mutant cells to UV radiation (Simpson and Sale, 2003;Jansen et al., 2009;Jansen et al., 2006) has therefore been linked to a structural role of Rev1 (Nelson et al., 2000;Ross et al., 2005;Masuda et al., 2009). As the C-terminal region of REV1 can interact with Polη, Polι, Polκ and the noncatalytic REV7 subunit of Polz (Murakumo et al., 2001;Ohashi et al., 2004;Guo et al., 2003), Rev1 have been proposed to direct TLS polymerases for bypass of UV lesions, which would explain the important role of Rev1 in DNA damage-induced mutagenesis (Lawrence, 2002;Jansen et al., 2006;Jansen et al., 2009;Friedberg, 2005). Consistently, the Rev1 C-terminal interaction region is required for resistance to DNA-damaging agents in chicken DT40 cells (Ross et al., 2005). In addition to the C-terminal interaction region Rev1 harbors a BRCA1 C-terminal (BRCT) domain in its N-terminus (Gerlach et al. 1999). This domain is required for (mutagenic) TLS and resistance to UV irradiation and other DNA-damaging agents (Lawrence, 2002;Jansen et al., 2005). BRCT domains have been identified in many cell cycle and DNA repair proteins (Huyton et al., 2000), and are thought to mediate protein interactions by binding to proteins that are phosphorylated by the DNA damage-activated protein kinases ATR and ATM (Yu et al., 2003). It has been shown that Rev1 could bind PCNA via this domain, (Guo et al., 2006a;Jansen et al., 2007). Future studies should reveal whether other proteins, like for example the 9-1-1 complex (see below) are bound by the BRCT domain of Rev1 (Jansen et al., 2007).

B cells derived from Rev1 deficient and catalytic mutant mice as well as chicken DT40 cells indicated a role for the catalytic activity of Rev1 in SHM (Jansen et al., 2006;Ross and Sale, 2006;Masuda et al., 2009;Arakawa et al., 2006). In agreement with the reported *in vitro* ability of Rev1 to bypass AP sites lesion (Nelson et al., 1996), C to G and G to C transversions are reduced in Rev1 mutant B cells. While in chicken DT40 B cells Rev1 is required for the generation of most of the C to G and G to C transversions, Rev1 seems to generate only part of these mutations in mammalian B cells, indicating that other polymerases can make these transversions in the absence of Rev1. While the BRCT domain of Rev1 was shown to regulate TLS of AP sites in yeast (Haracska et al. 2001), the BRCT domain is dispensable during SHM (Jansen et al. 2005). Furthermore, as

A/T mutations are still present in Rev1 deficient B cells (Jansen et al., 2006), the C-terminal polymerase binding region of Rev1 is dispensable for most Polη and Polκ activity during SHM.

Polι

Observations that 'knock-down' of Polι greatly increases the sensitivity of cells to oxidizing agents (Petta et al., 2008), that Polι displays BER activity *in vitro* and *in vivo* (Petta et al., 2008;Bebenek et al., 2001) and accurately bypasses 8-oxo-G (12), have implicated Polι in the protection against oxidative stress. In addition, *in vitro* and *in vivo* studies have indicated a role for Polι in bypassing both CPD and (6–4) PP (Tissier et al., 2000;Ziv et al., 2009;Yoon et al., 2010;Johnson et al., 2000). Polι deficiency does nor result in a survival disadvantage after UV radiation in Polη proficient cells, however it does result in higher sensitivity and hypomutability to UV in the absence of Polη in some (Gueranger et al., 2008;Dumstorf et al., 2006;Ziv et al., 2009), but not all (Ohkumo et al., 2006;Ziv et al., 2009) studies. Moreover, the absence of Polι increased the onset of UV-induced skin cancers in Polη deficient mice (Dumstorf et al., 2006;Ohkumo et al., 2006). So while Polι may protect against UV radiation *in vivo*, the mutagenic bypass of these lesions by Polι and other polymerases may result in cancer as seen in XP-V patients. Given the extreme low-fidelity of Polι when copying undamaged DNA, Polι has been proposed as a candidate TLS polymerase in SHM. In vitro Polι prefers to insert a G rather than an A opposite of T (Zhang et al. 2000)(Johnson et al., 2000;Tissier et al., 2000). In addition Polι has a preference to insert either G or T residues opposite of AP sites (Zhang et al. 2001). While incorporation of G opposite of an AP site will faithfully restore the initial AID induced lesion, the introduction of a T will result in C to A and G to T transversions. Actually, the TLS polymerase(s) involved in establishing these transversions during SHM remain to be identified. No changes in SHM were observed in B cells derived from a *129/J*-mouse strain that carries a spontaneous nonsense mutation in the Polι gene (McDonald et al. 2003). Western blot analysis on testis extracts indeed showed the absence of Polι in this strain. Nevertheless, it has been described that there may be tissue specific and functional alternative splice forms of Polι, and 'Polι activity' seems to be retained in brain extract from this mouse strain (Gening et al. 2006). In this context, *129/J*-derived B cells should be tested for the presence of hypomorph versions of Polι. At present, one cannot formally exclude that Polι is involved in SHM. Analysis of B cells derived from mouse mutants carrying a targeted deletion of Polι will solve this issue.

Polζ

Polζ is a heterodimer composed of a catalytic Rev3 and structural Rev7 protein that extends efficiently from mispaired primer termini on undamaged DNA (Gan et al., 2008), and is the only polymerase who's deficiency in mammals is embryonic lethal (Bemark et al., 2000;Kajiwara et al., 2001;Kawamura et al.,

2001;Van Sloun et al., 2002;Wang et al., 2002). Rev3 deficient chicken DT40 B cells revealed a central role of Rev3 in maintaining genome stability. Beside a defect in TLS, these cells showed reduced gene targeting efficiencies and a significant increase in the level of genomic breaks after ionizing radiation (Sonoda et al., 2003)**.** In mammals Rev3 has been suggested to be essential for the bypass of 6-4 PP during the repair of post replicative gaps, while mostly dispensable for the bypass of CPDs. (Jansen et al 2009). These observations are consistent with results obtained from a plasmid gap assay, in which TLS across 6-4PP strongly depends on Rev3, but not across CPDs (Shachar et al., 2009). Interestingly, TLS across AP site was only 2 fold reduced in Rev3 deficient cells in this assay. While together with Rev1, Polζ is responsible for the majority of DNA damage-induced and spontaneous mutations in yeast and mammals (Lemontt, 1971;Gan et al., 2008) it is currently unknown whether Polζ is involved in SHM. A 'knock down' of the catalytic subunit Rev3 in human B cells or in transgenic mice revealed a decrease in the frequency of somatic hypermutation (Diaz et al., 2001; Zan et al., 2001). Consistently, *in vivo* gene ablation of Rev3 in mature B cells reduced the frequency of somatic mutations (Schenten et al., 2009). As the pattern of SHM was unaffected, these results could imply that Polζ during SHM is involved in all mutations. However as at least G/C transitions do not completely depend on Polζ, the observed phenotype is at least partly caused by the enormous sensitivity of cells to Rev3 ablation.

Other TLS polymerases: Polλ, Polμ, Polθ and Polν

In addition to the above mentioned polymerases, Polλ, Polμ, Polθ and Polν have shown translesion activity *in vitro*. While Polλ and Polμ appear dispensable for SHM (Bertocci et al., 2002) (Lucas et al., 2005), a role for Polθ was initially proposed by the labs of Casali and O-Wang. *In vitro* Polθ is capable of direct catalytic bypass of abasic sites, strongly favoring dAMP (Seki et al., 2004). While the lab of Casali reported a dramatic decrease in the frequency of mutations and an increase in G/C transitions, the lab of O-Wang reported that Polθ deficient mice had only a mild reduction in the number of mutations and an increase in G to C transversions. In addition, the O-Wang group analyzed SHM in mice expressing a catalytically inactive Polθ and found an actual decrease in mutations at template G/C. Given these striking differences, the Gearhart group recently reexamined this issue in Polθ deficient mice and Polθ/Polη double deficient mice. Based on the frequency and spectra of the mutations they observed, Polθ has no major role in somatic hypermutation (Martomo et al., 2008;Masuda et al., 2006;Zan et al., 2005;Masuda et al., 2005). The only non-replicative polymerases not tested for its role in SHM is Polν due to the lack of a of Polν deficient mouse model (Marini et al., 2003). *In vitro* the vast majority of errors made by Polν reflect misincorporation of dTMP opposite template G, while its error signature across AP site is currently unknown.

In summary, only Polη and Rev1 appear to have non-redundant functions in establishing most A/T mutations and G to C transversions, respectively. Other TLS polymerases are likely involved and might compensate the absence of others. The diversity of structurally related TLS polymerases raises a central question: What regulates the activation of TLS polymerases during SHM?

REGULATING TLS BY PCNA UBIQUITINATION

Non-instructive DNA lesions cause problems for high fidelity polymerases and lead to replication fork stalling. If the "stalling" lesion is not repaired, the replication fork may collapse (Tercero & Diffley 2001). Such a collapse can generate double strand breaks, which can in turn trigger cell death (McGlynn & Lloyd 2002). To maintain genomic integrity and prevent the generation of death signals by secondary lesions, eukaryotic cells are equipped with DNA damage tolerance (DDT) pathways to continue replication without an *a priori* repair of the initial lesion (Friedberg, 2005). Cells are equipped with two alternative DNA damage tolerance pathways: TLS (damage bypass) and homology-dependent damage avoidance (HDA; all mechanisms that rely on a homologous donor; this includes strand transfer from the donor (homologous recombination), and template switching, in which the information is copied from the donor) (Friedberg 2005; Haracska et al. 2001; Lawrence 1994; Murli & Walker 1993). HDA uses intact DNA of the sister chromatid or the homologous chromosome to continue replication and is therefore mainly error free (Zhang & Lawrence 2005). As mentioned previously,

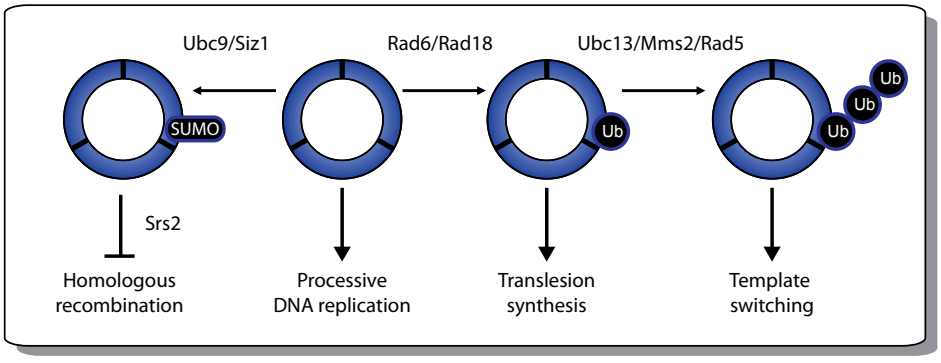

Figure 3. PCNA modification and functions in *S. cerevisiae*. In the absence of DNA damage, S. cerevisiae PCNA is SUMOylated at K164 or K127 (not depicted), which inhibits homologous recombination during replication by recruiting Srs2. Upon fork stalling PCNA is monoubiquitinated at K164 by Rad6/Rad18 to activate direct translesion synthesis across the stalling lesion. Alternatively PCNA can be further polyubiquitinated by Ubc13/Mms2/Rad5 to stimulate template switching.

TLS enables direct replication across the damaged template and depending on the type of damage and the TLS polymerase involved, can be highly error prone.

Studies in *S. cerevisiae* identified that both modes of lesion bypass appear to be controlled by specific posttranslational modifications of the homotrimeric DNA sliding clamp PCNA (Fig.3). PCNA is an essential binding platform for numerous proteins involved in DNA replication, repair, and cell cycle regulation. PCNA tethers DNA polymerases to their substrate and thereby serves as a critical processivity factor for DNA synthesis (Moldovan et al., 2007). During replication, the replicative polymerases bind PCNA through its PIP (PCNA-interacting peptide) box (Warbrick 1998). When the high fidelity replication machinery is stalled upon encountering a lesion, PCNA becomes monoubiquitinated (PCNA-Ub) at lysine residue 164 ($PCNA^{K164}$) by the ubiquitin-conjugating/ligating complex Rad6/Rad18 (E2/E3) (Hoege et al. 2002). In addition, the heterodimeric E2 ubiquitin conjugase consisting of Ubc13 and Mms2 cooperates with the RING finger E3 ligase Rad5 in specific lysine 63-linked polyubiquitination of PCNA-Ub ($PCNA\text{-}Ub^{n}$). Besides ubiquitination, modification of PCNA by the small ubiquitin-like modifier SUMO occurs by Ubc9 and Siz1 at K164 and K127 in *S. cerevisiae* (Hoege et al., 2002). As deletion of Polη, Rev1 and Polζ does further sensitize Rad5- and Siz1-deficient strains, but not Rad6 and $PCNA^{K164R}$ mutant strains to UV radiation (Stelter and Ulrich, 2003;Lawrence and Christensen, 1976;McDonald et al., 1997;Hoege et al., 2002), it is now generally accepted that in *S. cerevisiae* PCNA-Ub is essential for activation of TLS-dependent damage tolerance, while $PCNA\text{-}Ub^{n}$ activates HDA. In agreement, PCNA-Ub is a prerequisite for Polζ– and Rev1-dependent, damage-induced mutagenesis (Lawrence and Christensen, 1976;Stelter and Ulrich, 2003), while $PCNA\text{-}Ub^{n}$ deficient strains show an increased TLS-mediated spontaneous and damage-induced mutagenesis (Broomfield et al., 1998;Brusky et al., 2000;Johnson et al., 1992). PCNA-SUMO is most prominent during S phase and unlike ubiquitination is not markedly enhanced in the presence of DNA-damaging agents. It has been suggested that PCNA-SUMO recruits Srs2 to the replication fork, a helicase that inhibits homologous recombination (Pfander et al., 2005;Papouli et al., 2005;Haracska et al., 2004), and hereby regulates the Rad5 mediated error-free pathway (Branzei et al., 2008) and prevents unwanted recombination during replication stalling. However, in the absence of RAD6 dependent DDT the suppression of homologous recombination makes yeast even more sensitive to replication-stalling agents and replication error-prone due to an increased contribution of Polζ. Thus in contrast to damage-induced mutagenesis, both mono-ubiquitin and SUMO conjugation to PCNA can contribute to the activation of Polζ for spontaneous mutagenesis (Hastings et al., 1976;Stelter and Ulrich, 2003;Quah et al., 1980), although PCNA-SUMO most likely in an independent manner.

PCNA MODIFICATION IN HIGHER EUKARYOTES

Like in *S. cerevisiae*, UV-irradiation in higher eukaryotes lead to PCNA-Ub at the conserved K164 residue (Kannouche et al. 2004). As mammalian cells and chicken DT40 cells impaired for PCNA ubiquitination are sensitive to replication fork blocking lesions (Watanabe et al., 2004;Arakawa et al., 2006;Niimi et al., 2008), it has been suggested that, like in yeast, TLS in higher eukaryotes strongly depends on PCNA-Ub. In agreement, PCNA-Ub in mammals increases its affinity for Polη, Polι and Rev1 (Watanabe et al., 2004;Kannouche et al., 2004;Bienko et al., 2005;Plosky et al., 2006;Guo et al., 2006b), which is believed to depend on the Ub-binding domain (UBD) of TLS polymerases (Bienko et al., 2005). In line with these observations, the recruitment of TLS polymerases to sites of UV damage is impaired in cells lacking Rad18 or when the UBDs are mutated (Watanabe et al., 2004;Bienko et al., 2005;Guo et al., 2008;Guo et al., 2006b;Plosky et al., 2006). In contrast, Rev1 was observed to act mainly independently of PCNA-Ub in chicken DT40 cells to recover from UV induced replication blocks (Edmunds et al., 2008), while the dependence of Rev1 on PCNA-Ub in mammals is currently unknown. Furthermore, the involvement of PCNA-Ub and the UBD in regulating Polη in mammals has recently been questioned. The bypass of a CPD lesion was shown to be as efficient in cell extracts of Rad18-deficient cells as in wild-type (WT) cell extracts (Schmutz et al., 2010). Furthermore, Lehmann, *et al.* suggested that PCNA-Ub is not required for Polη accumulation into foci, but only increases the residence time of Polη within foci (Sabbioneda et al., 2008) and proposed a model in which the UBD domain is targeted to an unknown protein, which results in loading of Polη to PCNA-Ub (Gohler et al., 2011). In contrast, Prakash, *et al.* indicated that not all conserved residues in the UBD domain effect Polη function, suggesting that Ub binding by the UBD of Polη is dispensable for its activation (Acharya et al., 2008;Acharya et al., 2010).

POLYUBIQUITINATION OF PCNA IN HIGHER EUKARYOTES

Like in yeast, damage-inducible PCNA-Ub^n has been observed, although to a lesser extent, in mammals (Chiu et al., 2006), and was found to be mediated by two Rad5 orthologs, HLTF and SHPRH. Like Rad5, both SHPRH and HLTF physically interact with the RAD6/RAD18 and UBC13/MMS2 complexes and promote PCNA polyubiquitination at K164 in a RAD18-dependent manner (Motegi et al., 2006;Motegi et al., 2008;Unk et al., 2006;Unk et al., 2008). The role for PCNA-Ub^n in mammals is currently unknown, however depletion of either SHPRH or HLTF in human cells increases the sensitivity to methyl methanesulfonate (MMS) and enhances genomic instability. These data implicate a role for PCNA-Ub^n in

mammalian DNA damage tolerance. Furthermore, in human fibroblasts the reduced expression of MMS2 and the inhibition of K63 polyubiquitination have been shown to increase the frequency of UV-induced mutations (Li et al., 2002;Chiu et al., 2006), suggesting that like in yeast PCNA-Ub^{n} prevents TLS-mediated mutagenesis.

SUMOYLATION OF PCNA IN HIGHER EUKARYOTES

While the minor SUMO conjugation site of PCNA^{K127} is not conserved in higher eukaryotes, SUMOylation of PCNA has been detected at K164 in chicken DT40 cells (Arakawa et al., 2006). It is currently unknown what the consequences of this modification are in DT40 cells and whether PCNA-SUMO occurs in mammals. Furthermore, no homologs of Srs2 have been identified in higher eukaryotes, although other helicases have been identified that could substitute Srs2 by inhibiting HR in mammalian cells (Marini and Krejci, 2010).

SHM AT TEMPLATE A/T REQUIRES PCNA MODIFICATION IN MAMMALS

To determine whether PCNA modification regulates the generation of somatic mutations in hypermutating B cells, PCNA mutant mice that contain a lysine 164 to arginine mutation (PCNA^{K164R}) have been analyzed for SHM. Analysis of the mutation spectrum of Ig genes in B cells from these knock-in mice revealed a ten-fold reduction in A/T mutations (Langerak et al., 2007). In agreement, PCNA knock-out mice reconstituted with a PCNA^{K164R} transgene showed a reduction of A/T mutations in Ig genes (Roa et al., 2008). These data suggest that most A/T mutations are regulated by PCNA-Ub. As A/T mutations depend mainly on polymerase η (and in it's absence polymerase κ and at least a third yet unidentified polymerase (Faili et al., 2009), these data suggest that during MSH2-dependent SHM, and possibly during Ung2-dependent SHM, both Polη and Polκ depend on PCNA-Ub to establish most A/T mutations. Interestingly, it has been suggested that PCNA-Ub^{n} in B cells suppresses mutagenesis during SHM, similar to PCNA-Ub^{n} in yeast during spontaneous and damage-induced mutagenesis (Motegi et al., 2008). However, as this study was performed in a B cell line in which only a minority of the mutations are known to be at template A/T and the mutation spectrum in this study was not determined, the underlying mechanism is currently unclear.

PCNA-UB INDEPENDENT G/C TRANSVERSIONS DURING SHM

While most A/T mutations depend on PCNA-Ub, the generation of G/C transversions is not impaired in PCNA^{K164R} mutant B cells. Given the role for the TLS polymerase Rev1 in generating G to C transversions during SHM (Jansen et al., 2006;Ross and Sale, 2006), these findings exclude a role of PCNA-Ub in activating Rev1 and all other yet unidentified 'G/C transverters' during SHM in mammals. In agreement, damage tolerance mediated by Rev1 was found to be independent of PCNA-Ub in the chicken DT40 B cell line (Edmunds et al., 2008). In contrast, in DT40 cells, Rev1 depends on PCNA-Ub to generate G to C transversions during SHM (Arakawa et al., 2006). How G to C transversions are controlled during SHM in mammals is currently unknown, however as both the Rad9-Rad1-Hus1 complex (9-1-1) and the Fanconi Anemia pathway have been reported to regulate TLS, analysis of these pathways may reveal the answer to this question.

REGULATING TLS BY 9-1-1

The heterotrimeric 9-1-1 complex is structurally very similar to PCNA (Dore et al., 2009) and is loaded by Rad17 onto DNA in response to replication fork stalling where it triggers the activation of the cell cycle checkpoint and possibly DNA repair in both yeast and higher eukaryotes (Bermudez et al., 2003;Volkmer and Karnitz, 1999;Bai et al., 2010;Parrilla-Castellar et al., 2004). Interestingly, in yeast the 9-1-1 complex is also required for DNA damage-induced mutagenesis, possibly by its direct interaction with Polκ and Polζ (Kai and Wang, 2003;Sabbioneda et al., 2005). Whether the 9-1-1 complex regulates TLS during DDT and SHM in mammals is currently unknown. Deficiency of Rad17, or any member of the 9-1-1 complex is embryonic lethal in mice, and results in cell death in mouse embryonic fibroblast (Hopkins et al., 2004;Weiss et al., 2000;Budzowska et al., 2004). In contrast, mouse embryonic stem cells deficient for Rad9, chicken DT40 B cells deficient for Rad9 or Rad17 and Hus1 mouse embryonic fibroblasts deficient for p21 can be grown in culture, although these cell lines are hypersensitive to UV, MMS and HU (Weiss et al., 2000;Hopkins et al., 2004;Kobayashi et al., 2004). These observations indicate that in higher eukaryotes the 9-1-1 complex is not required for cell survival *per se*, but is essential under replication stalling conditions.

REGULATING TLS BY THE FA PATHWAY

Fanconi anemia (FA) is an autosomal recessive genetic disorder, which at the cellular level is characterized by a hypersensitivity to DNA cross-linking agents such as cisplatin (Kee and D'Andrea, 2010). How the FA pathway mediates

resistance to cross-links is largely unknown. Current models suggest that after replicative DNA polymerases are stalled at a DNA cross-link, FANCD2 and FANCI become monoubiquitinated by the FA core complex. The FA core complex consists of eight essential FA proteins, FANCA, -B, -C, -E, -F, -G, -L, -M, and two FA-Associated Proteins FAAP100 and FAAP24. FANCD2 was shown to stimulate incision of one of the strands containing the cross-link and to recruit TLS polymerases to enable a direct replicative bypass (Knipscheer et al., 2009). In agreement, the TLS polymerases Rev1 and Rev3 have been demonstrated to act synergistically with the FA pathway in cross-link repair in chicken DT40 B cells (Niedzwiedz et al., 2004). Interestingly, it has been reported that chicken DT40 B cells deficient for members of the FA pathway show a decrease in SHM (Yamamoto et al., 2005;Niedzwiedz et al., 2004). Although the precise mechanism for the decrease in the accumulation of non-templated mutations is currently unclear, it is intriguing to speculate that the FA pathway may regulate TLS polymerases like Rev1 during SHM to generate these mutations.

SCOPE OF THIS THESIS

This thesis focuses on the regulation of TLS polymerases during DDT and especially its mutagenic function during SHM of immunoglobulin genes. While the first part (chapters 2 –6) of this thesis addresses the role of specific post-translational modifications of lysine residue 164 in PCNA ($PCNA^{K164}$) in controlling SHM, DDT, and meiosis, the second part (chapter 7-9) addresses $PCNA^{K164}$ independent, regulatory aspects of SHM and DDT. In chapter 2, we first determined where in the previously described SHM pathways (Fig 2) $PCNA^{K164}$ dependent mutations are located and what the contributions of Msh2 and Ung2 are (Chapter 2). We next investigated whether the two known mammalian homologs of Rad5, SHPRH and HLTF, responsible for polyubiquitination of PCNA regulate mutagenesis during SHM, and whether they are essential for survival upon DNA damaging agents in mammals (Chapter 3). In Chapter 4 and 5, we investigated the role of PCNA modification in mammalian DDT. Specifically we questioned whether PCNA modification is essential for polymerase η function upon UV damage, and TLS in general across specific lesions. In addition we determined the role of PCNA modification in regulating polymerase η during SHM. In Chapter 6, we examined why $PCNA^{K164R}$ mutant mice display severe defects in germ cell development by analyzing the function and nature of the PCNA modification in spermatogenesis in more detail. In the last chapters we study the contribution of the Msh2 pathway (Chapter 7), the Fanconi Anermia pathway (Chapter 8) and the proposed topological counterpart of $PCNA^{K164}$ in the 9-1-1 complex (Chapter 9) during SHM. Finally, a general discussion of the preceding chapters and their relation to published literature is presented in Chapter 10.

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