Modifications of DNA clamps and their role in DNA damage management

Wit, N.

Citation for published version (APA):
Wit, N. (2013). Modifications of DNA clamps and their role in DNA damage management
PCNA UBIQUITINATION-INDEPENDENT ACTIVATION OF POLYMERASE ETA DURING SOMATIC HYPERMUTATION AND DNA DAMAGE TOLERANCE

Peter H.L. Krijger¹, Niek Wit¹*, Paul C.M. van den Berk¹*, Petra Langerak¹, Jacob G. Jansen², Claude-Agnès Reynaud³, Niels de Wind² and Heinz Jacobs¹

¹Division of Biological Stress Responses, The Netherlands Cancer Institute, Amsterdam, The Netherlands  
²Department of Toxicogenetics, Leiden University Medical Center, 2300 RC Leiden, The Netherlands  
³Institut National de la Santé et de la Recherche Médicale U783 'Développement du système immunitaire', Université Paris Descartes, Faculté de Médecine, Site Necker-Enfants Malades, Paris, France.  
*Shared authors

DNA Repair. 2011 Aug 8; 10: 1051-1059
SUMMARY

The generation of high affinity antibodies in B cells critically depends on translesion synthesis (TLS) polymerases that introduce mutations into immunoglobulin genes during somatic hypermutation (SHM). The majority of mutations at A/T base pairs during SHM require ubiquitination of PCNA at lysine 164 (PCNA-Ub), which activates TLS polymerases. By comparing the mutation spectra in B cells of WT, TLS polymerase η (Polη)-deficient, PCNA^K164R-mutant, and PCNA^K164R;Polη double-mutant mice, we now find that most PCNA-Ub-independent A/T mutagenesis during SHM is mediated by Polη. In addition, upon exposure to various DNA damaging agents, PCNA^K164R mutant cells display strongly impaired recruitment of TLS polymerases, reduced daughter strand maturation and hypersensitivity. Interestingly, compared to the single mutants, PCNA^K164R;Polη double-mutant cells are dramatically delayed in S phase progression and far more prone to cell death following UV exposure. Taken together, these data support the existence of PCNA ubiquitination-dependent and -independent activation pathways of Polη during SHM and DNA damage tolerance.
INTRODUCTION

The generation of high affinity antibodies by germinal center (GC) B cells critically depends on neo-Darwinian evolution of antibody specificities by somatic hypermutation (SHM) of the variable region of Immunoglobulin (Ig) genes and antigen-mediated selection [1,2]. SHM is initiated by activation-induced cytidine deaminase (AID), an enzyme that deaminates cytosine (C) to uracil (U) on both DNA strands of the Ig locus. While direct replication across U generates G/C to A/T transitions [3], further processing of U and the subsequent activation of error-prone translesion synthesis (TLS) polymerases are required to generate the entire spectrum of nucleotide substitutions [1].

Recognition of the U:G mismatch by the mismatch repair complex Msh2-Msh6 results in activation of exonuclease 1, which creates a single-stranded gap around the initial U:G mismatch [4-7]. The subsequent gap-filling process mainly employs TLS polymerase η (Polη), and in its absence Polk, which predominantly generates A/T mutations [5,6,8-11]. Alternatively, TLS across apurinic/apyrimidinic (AP) sites, generated by the excision of U by uracil-DNA glycosylase (Ung2), generates G/C transversions and may contribute to G/C transitions [12]. Although multiple mammalian TLS polymerases are able to bypass AP sites in vitro [13], until now only the TLS polymerases Rev1 has been identified to act downstream of Ung2. Consistent with the specific dCMP transferase activity of Rev1, Rev1 mutant B cells selectively lack G/C to C/G transversions [14-16]. Furthermore, mutagenesis downstream of Ung2 generates A/T mutations, as a significant proportion of A/T mutations are found in Msh2-deficient GC B cells, but not in Ung2/Msh2 double-deficient GC B cells [3]. While it has been established that these A/T mutations depend on Polη [17], it remains unclear whether Ung2-dependent A/T mutations are generated during the extension phase of TLS across the AP site, or alternatively during long-patch BER.

Although B cells take advantage of the error-prone nature of TLS polymerases and the ability to bypass AP sites during SHM, TLS polymerases are normally employed during DNA damage tolerance (DDT) to continue replication across a variety of lesions that block replicative polymerases [18]. For example, Polη is highly efficient and error-free when replicating UV-induced cyclobutane pyrimidine dimers (CPDs). The importance of Polη is illustrated by the hypersensitivity to UV damage and strong predisposition to skin cancer in Xeroderma Pigmentosum-Variant patients, which results from germline mutations in Polη [19,20].

Over the last decade it has become clear that eukaryotic TLS is regulated by ubiquitination of the DNA sliding clamp Proliferating Cell Nuclear Antigen (PCNA-Ub), at lysine residue 164 (PCNA^K164), by Rad6/Rad18 [21,22]. However, while in S. cerevisiae PCNA-Ub is essential for the activation of TLS polymerases during DDT, the regulation of TLS polymerases during DDT and SHM in mammals is less clear. As mammalian cells and chicken DT40 cells deficient for PCNA ubiquitination are sensitive to replication fork-blocking lesions [23,24], 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 TLS polymerases [25,26]. Furthermore, during SHM, PCNA-Ub is required for the majority of A/T mutations downstream of both Msh2 and Ung2, indicating that during SHM PCNA-Ub regulates Polη [7,27,28]. The residual A/T mutations found in PCNA^K164 mutant B cells, however, suggest that in mammals TLS polymerases can
also become activated independently of PCNA-Ub. In agreement, Rev1-dependent G/C
transversions are generated independently of PCNA-Ub during SHM in mammals [7,27,28].
Furthermore, DDT mediated by Rev1 was found to act independent of PCNA-Ub in the chicken
DT40 B cell line [29].

To address whether Pol\_\eta can be activated independently of PCNA-Ub, we here
compared the mutation spectra in B cells of WT, Pol\_\eta-deficient, PCNA^{K164R}-mutant and PCNA^{K164R};Pol\_\eta
double-mutant mice. We found that Pol\_\eta is responsible for the vast majority of PCNA-Ub-
independent A/T mutagenesis during SHM. We subsequently determined whether Pol\_\eta can
also act independently of PCNA-Ub in mammalian DDT. Upon UV treatment, recruitment
of TLS polymerases and DDT are strongly impaired in PCNA^{K164R} mutant cells, resulting in
hypersensitivity to UV. Interestingly, compared to single mutants, the PCNA^{K164R};Pol\_\eta double-
mutant cells were dramatically delayed in the S phase of their cell cycle and far more prone to
cell death following UV exposure. Taken together, these data support the existence of PCNA
ubiquitination-dependent and -independent activation of Pol\_\eta during both SHM and DDT.

MATERIAL AND METHODS

Isolation and generation of primary and immortalized cell lines – All animal experiments
were approved by the independent animal ethics committee of the Netherlands Cancer
Institute (Amsterdam, Netherlands). The generation and genotyping of PCNA^{K164R} knock-in
mice and Pol\_\eta-deficient mice has been described elsewhere [7,8,27]. PCNA^{K164R} knock-in
mice were backcrossed six generations to C57BL/6J and intercrossed with Pol\_\eta-deficient
mice (F1 C57BL/6J;129/OLA) to generate PCNA^{K164R};Pol\_\eta heterozygous mice. Germinal center
B cells, pre-B cells and mouse embryonic fibroblasts (MEFs) were isolated from intercrosses
of PCNA^{K164R};Pol\_\eta heterozygous mice using standard procedures. For the generation of pre-B
cell cultures, E14.5 embryos were isolated and single cell suspensions were generated from
fetal livers and subsequently cultured on ST2 feeder cells in IL7-containing complete medium
(Iscoves, 8% FCS and penicillin/streptomycin), according to Rolink et al. [30]. MEFs were isolated
according to Abbondanzo et al. [31]. MEFs were immortalized (two cell lines per genotype)
using lentiviral expression of a p53-specific shRNA [32]. Primary and immortalized MEFs were
grown in complete medium. Cells were grown at 37\(^\circ\)C in 5% CO\_2. Germinal center B cells were
sorted from 3 WT, 3 Pol\_\eta, 2 PCNA^{K164R} and 3 PCNA^{K164R};Pol\_\eta mice, as previously described [7]

Somatic hypermutation analysis – DNA was extracted from GC B cells using proteinase K
treatment and ethanol precipitation. The \(\mathrm{J}_{\text{\eta}}^4\) 3’flanking intronic sequence of endogenous
rearrangements of \(V_{\\text{\eta}}\) J558 family members were amplified during 40 cycles of PCR using PFU Ultra
polymerase (Stratagene) (Jolly et al., 1997). PCR products were purified using the QIAquick Gel
Extraction kit (Qiagen) and cloned into the TOPO II blunt vector (Invitrogen Life Technologies)
and sequenced on a 3730 DNA analyzer (Applied Biosystems). Sequence alignment was
performed using Seqman software (DNAsStar). Calculations exclude non-mutated sequences,
insertions and deletions. Clonally related sequences were counted only once.

Generation of GFP-Pol\_\eta, GFP-Pol\_\kappa and GFP-Rev1 MEFs – Mouse Pol\_\eta cDNA was amplified
and cloned in pEGFP-C1 (BD Biosciences Clontech). To generate the Pol\_\eta\_PIP mutant, site-
directed mutagenesis was applied to introduce F442A, L443A and F687A, F688A mutations,
inactivating the two PIP domains [33]. The pEGFP-Polκ and pEGFP-Rev1 plasmids have been described previously [34,35]. To enable virus production, the eGFP-fusions were subcloned in the retroviral vector pMX. Recombinant virus was isolated from Phoenix-eco cells transfected with the pMX-eGFP-fusion plasmids. Harvested virus particles were used to transduce two WT and PCNA\textsuperscript{\textit{K164R}} immortalized MEFs. eGFP-positive MEFs were isolated by FACSorting.

\textbf{Foci formation} – Two independent WT and PCNA\textsuperscript{\textit{K164R}} MEF lines expressing the eGFP-Polκ wild-type, eGFP-Polκ\textsuperscript{\textit{PIP mutant}} eGFP-Rev1 or eGFP-Polκ fusion protein were plated on round 25 mm coverslips. The next day the medium was removed, cells were washed with PBS and irradiated with 15 J/m\textsuperscript{2} UV-C (254 nm, UV Stratalinker\textsuperscript{\textregistered} 2400, Stratagene) or mock treated. After six hours, cells were fixed in 4\% paraformaldehyde and embedded in Fluoro-Gel (Electron Microscopy Sciences) before analysis on a CCD microscope. 200 cells per genotype were counted for foci-formation of eGFP-polymerase fusions.

\textbf{Pre-B cell survival assay} – 10\textsuperscript{5} pre-B cells were seeded on ST2 feeder cells in 24 well-plates in 0.5 ml complete medium and IL7 prior to UV-C irradiation (254 nm, UV Stratalinker\textsuperscript{\textregistered} 2400, Stratagene). After 15 minutes, cells were irradiated with increasing doses and cultured in 1 ml complete medium and IL7. For the survival upon cisplatin-\textsuperscript{\textit{γ}}, MMS- and \textsuperscript{\textit{γ}} irradiation-induced DNA damage, 10\textsuperscript{5} pre-B cells were seeded on ST2 feeder cells in 24 well-plates in 1 ml complete medium and IL7 in the continuous presence of different doses of cisplatin and MMS or after a single dose of \textsuperscript{\textit{γ}} irradiation from a \textsuperscript{137}Cs source. For determining the survival, pre-B cells were harvested after three days of culture and live (PI negative) pre-B cells were counted on a FACSArray (Becton Dickinson). Data were analyzed using FlowJo software.

\textbf{Cell cycle analysis in pre-B cells} – 2 x 10\textsuperscript{5} pre-B cells were seeded in 24-well plates in 0.5 ml complete medium and IL7. After 15 minutes, cells were irradiated with 2J/m\textsuperscript{2} UV-C and harvested at the indicated time points, fixed immediately with 1.5 ml ice-cold 100\% ethanol and kept at 4 °C. Cells were treated for 20 minutes with RNAs A (0.5 mg/ml, Sigma Chemical Co), before permeabilizing in Tween-20 solution (0.25\% Tween-20 in PBS/ 1\%BSA). \textsuperscript{\textit{γ}}H2AX was detected with 1 μg/ml anti-phospho-histone H2AX (Ser139, clone JBW301; Millipore). After staining with FITC-conjugated anti-mouse IgG antibody (25 μg/ml) (DAKO Cytomation) the cells were resuspended in PBS containing 5 μg/ml PI and measured on a FACSCalibur (Becton Dickinson). Data were analyzed using FlowJo software.

\textbf{Alkaline DNA unwinding} – ADU assays were performed using immortalized MEFs as previously described [36,37]. Briefly, 10\textsuperscript{5} MEFs were seeded per well of a 24-well plate and cultured overnight. Subsequently, MEFs were pulse labeled with [3H] thymidine (2 μCi/ml; 76 Ci/mmol) for 30 min, washed with PBS, immediately exposed to 10 J/m\textsuperscript{2} UV-C, and cultured in medium for up to 6 h. The cells were washed twice with 0.15 M NaCl and incubated in the dark on ice in 0.5 ml of ice-cold unwinding solution containing 0.15 M NaCl and 0.03 M NaOH for 30 min. Unwinding was terminated by forceful injection of 1 ml of 0.02 M NaH\textsubscript{2}PO\textsubscript{4}. The cell lysates were sonicated for 15 s using a Sonifier 250 apparatus (Branson); SDS was added up to 0.25\%, and the plates were stored at –20°C. To separate single-stranded DNA (ssDNA) from double-stranded DNA, hydroxylapatite columns were washed with 0.5 M K\textsubscript{2}HPO\textsubscript{4} followed by 10 mM NaH\textsubscript{2}PO\textsubscript{4} (pH 6.8). After each cell lysate was loaded, the columns were washed twice with 10 mM NaH\textsubscript{2}PO\textsubscript{4} (pH 6.8). ssDNA was eluted with 0.13 M K\textsubscript{2}HPO\textsubscript{4} (pH 6.8), and double-stranded DNA was eluted with 0.25 M K\textsubscript{2}HPO\textsubscript{4} (pH 6.8). Radioactivity was quantified by liquid scintillation counting.
RESULTS

PCNA-Ub independent activation of Polη during SHM – To determine the contribution of Polη in establishing A/T mutations during SHM independent of PCNA-Ub, we here studied non-selected somatic mutations in rearranged J_{\text{H},4} intronic regions of wild-type (WT), single and double-mutant B cells.

![Figure 1](image)

Figure 1. Mutations in rearranged J_{\text{H},4} intronic sequences from WT and PCNA^{K164R}, Polη-deficient and PCNA^{K164R};Polη double-mutant B cells. (A) Pattern of nucleotide substitution. Values are expressed as the total numbers of mutations and percentage of total mutations, the latter corrected for the nucleotide content in the J_{\text{H},4} intron. (B) Relative contribution of A/T and G/C mutations in the immunoglobulin heavy chain locus of hypermutated WT, PCNA^{K164R} mutant, Polη-deficient and PCNA^{K164R};Polη double-mutant B cells. The corrected percentage of A/T (black) and G/C (gray) mutations in the different genotypes are displayed. C.) The distribution of all G/C (above X-axis) and A/T (below X-axis) mutations along the J_{\text{H},4} intronic region starting from the splice donor are indicated as percentage of total mutations. The genetic backgrounds are indicated. 3 WT, 3 Polη-deficient, 2 PCNA^{K164R} and 3 PCNA^{K164R};Polη double-mutant mice were analyzed.
double-mutant GC B cells from intercrosses of PCNA K164R knock-in mice [27] and Polη-deficient mice (Fig 1 and suppl. Fig 1). As shown in figure 1, after correction for base compensation 45% of all point mutations resided at template A and T in WT B cells. Consistent with the role of Polη in A/T mutagenesis [8,11,38], Polη-deficiency reduced the frequency of mutations at A/T base pairs to 16%. These remaining A/T mutations presumably are generated by PolK and an unidentified polymerase [10]. In line with previous studies [7,27,28], the frequency of A/T mutations was strongly reduced in PCNA K164R-mutant B cells, although 9% of these mutations remained. These data indicates that Polη, Polκ and the unidentified polymerase depend on PCNA-Ub to establish 91% of all A/T mutations. To determine whether this residual, PCNA-K164-independent, A/T mutagenesis depended on Polη, we compared the mutation spectra of PCNA K164R and PCNA K164R;Polη double-mutant GC B cells. While 5% of all mutations reside at template A/T (15 A/T mutations / 291 total mutations) in hypermutated sequences of PCNA K164R mutant GC B cells, only 1% (4 A/T mutations / 418 total mutations) were found in PCNA K164R;Polη double-mutant GC B cells (p value < 10^-3). This five-fold reduction indicates that Polη is responsible for the vast majority (78%) of PCNA-Ub-independent A/T mutagenesis during SHM. Apparently, Polη can operate independently of PCNA-Ub during SHM.

Foci-formation of TLS polymerases is impaired in the absence of PCNA-Ub – We asked whether TLS polymerases can also act independently of PCNA-Ub during mammalian DDT. To test this we visualized the recruitment of eGFP-tagged Polη, Rev1 and Polκ to DNA damage in WT and PCNA K164R mutant cell lines (Fig. 2 and Suppl. Fig. 2). These polymerases accumulate at sites of UV-induced DNA damage, which can be visualized as subnuclear foci [39]. In line with previous reports, foci formation of Polη, Rev1 and Polκ was induced in WT cells by UV irradiation [34,35,39-42]. However, foci formation of these TLS polymerases was strongly reduced in the PCNA K164R mutant cells, indicating that PCNA-Ub is required for effective recruitment of these polymerases into subnuclear foci. Remarkably, 6% of PCNA K164R mutant MEFs were still capable of recruiting Polη in the absence of PCNA-Ub, although this recruitment is abolished when the two PCNA-interacting domains (PIP boxes) in Polη [33] were mutated (Fig. 2 and Suppl. Fig. 2). These data suggest the existence of two distinct pathways in recruiting Polη: a major, PCNA-Ub-dependent and a minor, PCNA-Ub-independent pathway, both of which appear to depend on Polη/PCNA interaction.

PCNA-Ub independent activation of Polη in DNA damage survival – As foci formation does not necessarily predict activity of TLS polymerases [43], we isolated independent WT, PCNA K164R mutant, Polη-deficient and PCNA K164R;Polη double-mutant pre-B cells to investigate the role of PCNA K164 modification in the activation of Polη. To test the relevance of the PCNA K164 modification on Polη-mediated cell survival in response to DNA damage, cells were treated with increasing doses of different DNA-damaging agents (Fig. 3 and suppl. Fig. 3). PCNA K164R-mutant cells displayed hypersensitivity to UV, cisplatin and MMS, genotoxic agents that block DNA replication. In contrast, all cell lines were equally sensitive to DNA double-strand breaks, induced by γ-irradiation, excluding a general survival defect of PCNA-mutant cells. Compared to Polη-deficient cells, PCNA K164R cells were far more sensitive to UV, cisplatin and MMS, consistent with a role of PCNA K164 modification in Polη-independent DDT. Remarkably, PCNA K164R;Polη double-mutant cell lines were far more sensitive to UV and cisplatin as compared with the single-mutant cell lines. These data indicate for the first time that, apart from its major
Figure 2. UV inducibility of Polη-, Polκ- and Rev1- foci in MEF cells is impaired in the absence of PCNAK164 modification. The average of two independent cell lines are shown per genotype. Mean ± SD of at least two experiments. B.) Representative images of cells with or without eGFP-Polη and eGFP-PolηPIP mutant foci in the presence and absence of PCNA K164 modification.

PCNA-Ub-dependent activity, Polη also provides a critical survival advantage in the absence of PCNA-Ub during DDT in mammals.

Bypass of UV induced DNA damage in the absence of PCNA-Ub and Polη – To further investigate PCNA-Ub independent Polη activation, we determined whether WT, PCNAK164R, Polη, and double-mutant MEFs differ in recovering from UV-induced replication blocks. We used the alkaline DNA unwinding (ADU) assay to measure (post)replicative bypass of UV lesions [36,37,44]. In this assay, newly synthesized DNA is radioactively labeled, after which the cells are exposed to UV irradiation and chased for the indicated time. Hereafter, the persistence of radioactivity in the terminal fragments of approximately 30-50 kbp is measured. In all mock-treated genotypes, the terminal label was reduced over time to the same extent (Fig. 4 left panel), confirming that PCNA-Ub and Polη are not essential for genomic replication in the absence of exogenous DNA damage. In contrast, following exposure to UV light, the loss of radioactivity in terminal fragments of newly synthesized DNA in Polη, PCNAK164R, and PCNAK164R;Polη double-mutant cells was delayed, compared to WT cells (Fig. 4 right panel), indicating impeded bypass of UV lesions. Since, in contrast to TLS polymerases Rev1 and Rev3 [36,37] the UV lesions were
ultimately bypassed, both PCNA modification and Polη are important, but not essential, for efficient DDT of UV damaged templates. Due to the relative insensitivity of the assay we were unable to detect a statistically significant difference between PCNA K164R and PCNA K164R;Polη mutant cells. Nevertheless, the tendency that damage bypass is slower in the PCNA K164R;Polη double-mutant compared to PCNA K164R single mutant cells is in agreement with a function of Polη in UV damage-bypass independent of PCNA modification.

Formation of γH2AX in the absence of PCNA modification and Polη – Bypass of UV lesions can lead to the formation of single stranded DNA at stalled forks or behind the progressing fork and may trigger the formation of single- and double-stranded DNA breaks. Both single stranded DNA and DNA breaks are known to induce phosphorylation of H2AX at serine 139 (γH2AX) [45,46]. To determine whether the delayed DNA damage bypass in Polη, PCNA K164R, and double-mutant cells is accompanied by enhanced γH2AX, we measured the formation of γH2AX [45] in UV-irradiated WT, PCNA K164R, Polη, and PCNA K164R;Polη mutant pre-B cells. Consistent with previous reports [47,48], γH2AX was found to accumulate in S phase (Fig. 5A-B), indicating that in this setting the formation of γH2AX depends on replication. Compared to WT cells, Polη-deficient cells displayed

Figure 3. Survival of WT, Polη deficient, PCNA K164R mutant, and PCNA K164R;Polη double-mutant pre-B cells in response to UV, cisplatin, MMS, and γ-irradiation. Survival was normalized to mock treated controls and is shown as percentage. The average of two independent cell lines is shown per genotype. Mean ± SD of two experiments.
higher levels of γH2AX, shortly after UV exposure, in agreement with previous observations [49] and with the defect of Polη-deficient cells in tolerating UV damage. Over a period of seven hours after treatment, the γH2AX levels in Polη-deficient cells decreased to those of WT cells, although they did not reach pre-treatment levels. Compared to Polη-deficient cells, the induction and persistence of γH2AX levels were much stronger in PCNA<sup>K164R</sup>-mutant cells. This result supports the notion that modification of PCNA also regulates other TLS polymerases or DDT pathways such as template-switching mediated lesion bypass. PCNA<sup>K164R</sup>;Polη double-mutant cells accumulate DNA strand interruptions to an even stronger extent than the single mutant cells, as shown by increased levels of γH2AX at 4 hours after UV treatment (Fig. 5B). Together these data provide further evidence that in DNA lesion bypass Polη may act independently of modification at PCNA<sup>K164</sup>.

Cell cycle delay in the absence of PCNA modification and Polη – To determine the impact of UV-induced DNA damage on cell cycle progression, we compared the cell cycle profiles in the experiment mentioned above. In addition to DNA content, we used γH2AX to further distinguish G1 cells from early S phase cells, as argued above (Fig. 5C; S3). While the distribution of WT cells in early and late S phase remained quite stable over eight hours after UV treatment, indicating relatively unperturbed S phase progression, PCNA<sup>K164R</sup>;Polη and PCNA<sup>K164R</sup>;Polη double-mutant cells accumulated in early S phase during the first four hours after treatment. At the same time the percentage of cells in late S phase strongly declined in these mutant cells. At later time points, however, both Polη- and PCNA<sup>K164R</sup>-mutant cells progressed to late S phase, although PCNA<sup>K164R</sup> mutant cells were somewhat slower. The cell cycle distribution in Polη- and PCNA<sup>K164R</sup>-mutant cells started to normalize after 24 hours. These data are in good agreement with the S-phase
Figure 5. Phosphorylation of H2AX and cell cycle progression in UV treated WT, PCNA^{K164R} mutant, Pol\eta deficient and PCNA^{K164R};Pol\eta double-mutant pre B cells. Data are representative of two experiments with two independent cell lines per genotype. (A) Flow cytometric analysis of γH2AX formation and cell cycle progression. Genotypes and time points (h) after UV treatment are indicated. The gates 1-5 are indicated to determine the frequency of cells in sub G1 (1), G1 (2), early S (3), late S (4), and G2 (5). Please note, we cannot distinguish G1 from very early S cells at time point zero and from some sub G1 cells at later time points. This fact does however not influence the interpretation of our data. (B) Mean fluorescence intensity (MFI) of γH2AX in early S and late S at the indicated time points after UV treatment are plotted. (C) S phase progression of WT, Pol\eta-deficient, PCNA^{K164R} mutant, and PCNA^{K164R};Pol\eta double-mutant cells in response to UV irradiation at the indicated time points after UV treatment are plotted.
delay found in XP-V cells [47,50,51]. In contrast, PCNA$^{K164R}$;Pol$\eta$ double-mutant cells were unable to tolerate UV damage and virtually all cells died within 24 hours after UV (Fig. 5A). In summary, replicating PCNA$^{K164R}$ cells are highly sensitive to UV damage, but have the potential to bypass UV-induced DNA lesions. This potential is lacking in the double-mutant cells and results in cell death. Thus, in replicating cells, Pol$\eta$ provides a survival advantage to UV-induced DNA damage that is independent of PCNA modification.

**DISCUSSION**

To delineate the role of PCNA-Ub in controlling the activity of Pol$\eta$ during SHM and DDT in mammals, we here analyzed SHM in mice and DDT in cells derived from intercrosses between Pol$\eta$-deficient and PCNA$^{K164R}$-mutant mice. The analysis of SHM in the mice with the different genotypes revealed PCNA$^{K164}$-independent functions for Pol$\eta$ in A/T mutagenesis. Conversely, our data also confirm that part of the A/T mutagenesis, while depending on PCNA$^{K164}$, is independent of Pol$\eta$. Moreover we present evidence for a PCNA-Ub independent activation of Pol$\eta$ during DDT in mammalian cells.

In line with previous studies [21-24], PCNA$^{K164R}$-mutant cells are highly sensitive to replication-blocking agents. We now show that this sensitivity relates at least partially to a delayed bypass of UV-induced DNA lesions, as revealed by the alkaline DNA unwinding assay and cell cycle analysis. The fact that PCNA$^{K164R}$ mutant cells are significantly more sensitive than Pol$\eta$-deficient cells, particularly to MMS, suggest that during DDT PCNA modification does not only regulate Pol$\eta$-mediated lesion bypass but also other TLS polymerases or DDT pathways. In fact, UV-induced foci formation of Pol$\eta$, Pol$\kappa$ and Rev1 was found to depend to a large extent (but not completely, see below) on PCNA$^{K164}$ modification. As the lack of these polymerases results in UV-sensitivity (this work, [36,39]), the hypersensitivity of PCNA$^{K164R}$ mutant cells may be caused by an impaired recruitment of these TLS polymerases to sites of UV damage. These data contrast the work of Sabbioneda et al., which suggests that PCNA-Ub is not required for the formation of Pol$\eta$ foci, but rather increases the residence time of Pol$\eta$ in foci [52]. The genetically defined PCNA$^{K164R}$ knock-in system used in the present study excludes all PCNA ubiquitination, which is not the case for proteasome inhibition as applied previously [52]. Of note, a defect in recruitment of TLS polymerases into foci does not necessarily imply a complete loss in activating these polymerases [43]. In agreement, we here provide several lines of evidence that Pol$\eta$ can provide DDT, independent of PCNA modification. We show that PCNA$^{K164R}$;Pol$\eta$ double-mutant cell lines are far more sensitive to UV and cisplatin than the PCNA$^{K164R}$ cell lines, two DNA damaging agents known to be bypassed effectively by Pol$\eta$ [20,53-55]. These insights contrast recent findings in which Pol$\eta$ was not found to provide an additional survival advantage in cell lines expressing predominantly exogenous PCNA$^{K164R}$ [24]. Residual expression and modification of endogenous PCNA in the latter study may explain this discrepancy. Alternatively this discrepancy may relate to differences between mouse and human cells. Furthermore, in the absence of PCNA modification, damage-induced Pol$\eta$ foci are normal in a small fraction of cells (Figure 2). Therefore, Pol$\eta$ and possibly other TLS polymerases can be recruited and activated independently of PCNA modification. Nevertheless, since disrupting the PIP boxes of Pol$\eta$ completely abolished all Pol$\eta$ foci (Figure 2 and S2), we infer that (unmodified) PCNA is essential for all modes of Pol$\eta$ recruitment.
The increased sensitivity of PCNA$^{K164R}$;Pol$\eta$ double-mutant cells to UV damage likely relates to higher numbers of stalled replication forks or impaired bypass of UV-induced DNA lesions behind the replication fork [45], as supported by the higher levels of $\gamma$H2AX in late S phase as compared to PCNA$^{K164R}$ single-mutant cells. As judged from the data of the alkaline DNA unwinding assay (Figure 4), however, also the PCNA$^{K164R}$;Pol$\eta$ double-mutant cell lines eventually do bypass the UV-induced DNA lesions. Combined with the complete inability of Rev1 and Rev3-deficient MEFs to bypass UV-induced DNA lesions [36,37] these data suggest that, in the absence of PCNA$^{K164}$ modifications and Pol$\eta$, mammalian cells possess an alternative pathway that allows them to bypass DNA lesions and that may depend on Rev1 and Rev3. Indeed, Rev1 can act independently of PCNA-Ub in chicken DT40 cells [29,56] and during SHM in mammals [7,27,28]. Thus, Rev1 may provide a mechanism to bypass UV-induced DNA lesions independent of PCNA modification and of Pol$\eta$ [36,57]. Alternatively, homologous recombination might be involved in the recovery of collapsed forks, independent of PCNA modification and of Pol$\eta$ [56,58,59].

In agreement with the above-mentioned existence of PCNA-Ub-dependent and -independent activation of Pol$\eta$ during DDT, we here provide direct evidence that the same principal holds for SHM. Previous analysis of SHM in B cells from Ung2/PCNA$^{K164R}$ double-mutant mice revealed that the combined failure in removing uracils by Ung2 and loss of PCNA ubiquitination resulted in a mutation spectrum in which almost all mutations were transitions at G/C, indicating that almost all A/T mutations downstream of Msh2 depend on PCNA-Ub. In contrast, analysis of Msh2/PCNA$^{K164R}$ double-mutant mice revealed that only two thirds of the A/T mutations downstream of Ung2 depend on PCNA-Ub [7]. These and the present data argue that, during SHM, PCNA-Ub acts downstream of both Msh2 and Ung2 to ensure that Pol$\eta$ is recruited to introduce mutations at template A/T. We now suggest that, in the absence of PCNA-Ub, high-fidelity polymerases act downstream of Msh2 to perform error-free replication of intact A/T templates. In contrast, TLS polymerases are still required to bypass AP sites, generated by Ung2. Our data also show that during this process Pol$\eta$ may be activated independently of PCNA-Ub, explaining the remaining one third of A/T mutations downstream of Ung2.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENTS

The authors wish to thank E. Friedberg and F. Hanaoka for providing the murine Pol$\eta$, Pol$\kappa$, and Rev1 cDNA. A. Pfauth and F. van Diepen for cell sorting, L. Oomen, L. Brocks and U. Geumann for help with CCD and confocal imaging and the animal caretaker team of the Netherlands Cancer Institute-Antoni van Leeuwenhoek hospital for biotechnical assistance. We also thank T. Sixma and V-L. van Zuylen for critically reading the manuscript. We apologize to everybody whose work could only be cited indirectly due to space limitations. This research was supported by the Netherlands Organisation for Scientific Research and the Dutch cancer foundation (VIDI program NWO 917.56.328 and KWF grant NKI-2008-4112 to HJ, and EU IP LSHG-CT-2005-512113 to NdW).
REFERENCES


**SUPPORTING INFORMATION**

### A

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Polη</th>
<th>PCNA&lt;sup&gt;K164R&lt;/sup&gt;</th>
<th>PCNA&lt;sup&gt;K164R&lt;/sup&gt; Polη</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of mice</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>number of mut. sequences</td>
<td>144</td>
<td>142</td>
<td>46</td>
<td>136</td>
</tr>
<tr>
<td>total bp sequenced</td>
<td>71920</td>
<td>70906</td>
<td>22994</td>
<td>67940</td>
</tr>
<tr>
<td>total mutations</td>
<td>828</td>
<td>489</td>
<td>291</td>
<td>418</td>
</tr>
<tr>
<td>mutation freq mut. Sequences (%)</td>
<td>1.2 (0.8-1.4)</td>
<td>0.7 (0.5-1.0)</td>
<td>1.3 (1.0-1.9)</td>
<td>0.6 (0.5-0.8)</td>
</tr>
</tbody>
</table>

### B

**Supplementary figure 1.** Point mutations in the J<sub>H4</sub> intronic region of hypermutated Ig genes.

A.) Number of mice, mutated sequences, base pairs sequenced, mutations and average mutation frequency per genotype are indicated. Note that the higher mutation frequency found in the PCNA<sup>K164R</sup> mice likely relates to environmental differences as no littermates were used in this study. B.) Pattern of nucleotide substitution. Values are expressed as the total numbers of mutations, percentage and corrected percentage of total mutations.
Supplementary figure 3. Cell cycle progression in UV-treated WT, PCNA<sup>K164R</sup> mutant, Pol<sub>η</sub> deficient and PCNA<sup>K164R</sup>,Pol<sub>η</sub> double-mutant pre B cells. Genotypes and time points (h) after UV treatment are indicated. The progression of UV irradiated WT, Pol<sub>η</sub> deficient, PCNA<sup>K164R</sup> mutant, and PCNA<sup>K164R</sup>,Pol<sub>η</sub> double-mutant cells in G1, early S, late S, and G2 are shown. The left panel indicates the percentage of total cells. The right panel indicates the percentage of total cells after normalization to WT at t=0. Data are representative of two experiments with two independent cell lines per genotype.

Supplementary figure 2. UV inducibility of Pol<sub>η</sub>- , Pol<sub>κ</sub> and Rev1- foci in MEF cells is impaired in the absence of PCNA<sup>K164</sup> modification. A.) UV inducibility of WT and PIP-mutant Pol<sub>η</sub> foci in the presence and absence of PCNA<sup>K164</sup> modification. The average of two independent cell lines is shown per genotype. Mean ± SD of two experiments. B.) Representative images of cells with or without eGFP-Rev1 and eGFP-Pol<sub>κ</sub> foci in the presence and absence of PCNA<sup>K164</sup> modification.
Activation of Polymerase η during SHM and ddt

Figure S3
ADDENDUM

In Chapter 2 we established that, using the ADU assay, Polη-deficient, PcnaK164R and double mutant cells display delayed replication fork recovery after UV irradiation. However, setting does not discriminate between different modes of DNA damage bypass, i.e. direct DNA damage vs. post-replicative gap filling, as ADU measures discontinuities in the daughter strand that include both direct DNA damage bypass and post-replicative gap filling. To investigate which pathway is used by Polη/PCNAK164-dependent TLS we employed DNA fiber labeling experiments (Fig. 1A). DNA fiber analysis enables one to measure replication fork stalling at a single molecule level. However, it fails to distinguish elongating forks from reprimed forks, as the gap that is formed upon repriming is too small to be detected. By combining experimental data from ADU (or related experimental techniques such as sedimentation velocity analysis) and DNA fiber analysis one is able discriminate between the two modes of TLS. If one observes no defect in the DNA fiber analysis this either means that the investigated protein has no function in direct DNA damage bypass or that DNA replication had reprimed behind the lesion, which is undetectable in the DNA fiber assay. If the same cells at the same time do display defects in the ADU assay, this would mean that the relative amount of ssDNA in the genome had increased. This can be caused by defects in either direct DNA damage or late, post-replicative gap filling. However, one can exclude in this situation direct DNA damage bypass as this was option was eliminated by the DNA fiber assay. For example, after UV irradiation, Rev1-/ mouse cells show defects in both the fiber and ADU assay, while REVI BRCT mutant cells display defects in the fiber assay but have WT kinetics of replicon progression in the ADU assay. These results suggest that REVI has two functions in DNA damage bypass: 1) DNA damage bypass at an early stage that is dependent on its BRCT domain and 2) DNA damage bypass at a later stage that involves the filling of post-replicative gaps independently of its BRCT domain [1].

Fig. 1B shows that WT and PcnaK164R cells have equal replication speeds on a non-damaged template, indicating that PCNA modification has no major role in unperturbed replication. UV irradiation-induced replication fork stalling in both WT and PcnaK164R cells at equal levels (Fig. 1C). These results indicate that PCNAK164-dependent TLS does not occur by direct DNA damage bypass. Similar results were obtained with Polη-deficient cells (data not shown). We argue that, based on DNA fiber analysis and ADU data, similar to chicken DT40 PcnaK164R cells and XPV cells [2,3], Polη and PCNAK164-dependent TLS across methylated DNA bases occurs post-replicative.

REFERENCES

Figure 1. Replication fork progression analysis after UV irradiation. (A) Scheme of DNA fiber analysis experiment. MEFs were first labeled with 25 μM CldU for 20’ and then 20’ with 250 μM IdU. UV irradiation occurred immediately after labeling with CldU. The length of CldU and IdU tracks were measured and the replication speed on the non-damaged the CldU track was calculated (B), as well as the IdU:CldU ratio (C). Of two experiments, one representative experiment is shown.