Regulation of translesion synthesis polymerases during somatic hypermutation and DNA damage tolerance
Krijger, P.H.L

Citation for published version (APA):
Krijger, P. H. L. (2011). Regulation of translesion synthesis polymerases during somatic hypermutation and DNA damage tolerance

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (http://dare.uva.nl)
PCNA UBIQUITINATION-DEPENDENT AND -INDEPENDENT ACTIVATION OF POLYMERASE η IN MAMMALS

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

¹ Division of Immunology, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands; ² Department of Toxicogenetics, Leiden University Medical Center, 2300 RC Leiden, The Netherlands; ³ Institut 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 second authors

Submitted
ABSTRACT

Replication-blocking DNA lesions induce ubiquitination of PCNA (PCNA-Ub) at lysine residue 164 (PCNA\textsuperscript{K164}) that in \textit{S. cerevisiae} is essential to activate specialized translesion synthesis (TLS) polymerases. To study the regulation of TLS in mammals we established isogenic mice and derived cells that either lack Pol\texteta, carry a PCNA\textsuperscript{K164R} mutation or both. In the absence of PCNA-Ub the replication block recovery and survival after UV treatment were impaired. PCNA\textsuperscript{K164R} cells were far more UV sensitive than Pol\texteta-deficient cells, indicating that PCNA modification regulates more than Pol\texteta alone. Indeed, recruitment of various TLS polymerases was impaired in the PCNA-mutant cells and accumulation of UV-induced $\gamma$H2AX was increased, compared with the Pol\texteta-deficient cells. Surprisingly, compared with the PCNA\textsuperscript{K164R} single mutant cells, the PCNA\textsuperscript{K164R},Pol\texteta double-mutant cells displayed increased UV sensitivity, accumulation of $\gamma$H2AX and failed to recover from UV-induced replication blocks. Together these results indicate the existence of PCNA-Ub-dependent and -independent pathways in activating Pol\texteta in mammalian cells, which is underscored by Pol\texteta-mediated somatic hypermutation independent of PCNA-Ub.
INTRODUCTION

DNA lesions that block the replicative polymerases lead to an arrest of the replication fork. If not relieved, the replication fork may collapse and cause a DNA double strand break (DSB). To maintain genetic integrity and prevent the generation of death signals by secondary lesions, cells are equipped with DNA damage tolerance (DDT) pathways to continue replication without an a priori repair of the initial lesion (1). In eukaryotes DDT has been suggested to be mainly controlled by ubiquitination of the DNA sliding clamp Proliferating Cell Nuclear Antigen (PCNA). Upon replication fork stalling, PCNA is monoubiquitinated (PCNA-Ub) at lysine residue 164 (PCNA$^{K164}$) by the Rad6/Rad18 complex (2, 3). In S. cerevisiae PCNA-Ub triggers a polymerase switch, that enables specialized DNA polymerases to replicate directly across a damaged template in a process known as translesion synthesis (TLS) (4). Also polyubiquitination of PCNA-Ub is observed in yeast and mammals, although in the latter case at much lower levels (3, 5, 6). In yeast, PCNA polyubiquitination is required for template switching in which the DNA lesions are bypassed by using the undamaged sister chromatid as a template. The relevance of this alternative DDT pathway in mammals is currently unclear (4).

In higher eukaryotes, TLS is carried out primarily by the Y family polymerases Pol$\eta$, Pol$\iota$, Pol$\kappa$ and Rev1, and the B family member pol$\zeta$ (7, 8). TLS can either be error-free or error-prone, depending on the TLS polymerase used and the lesion bypassed. For example, Pol$\eta$ is highly efficient and error-free when replicating UV-induced cyclobutane pyrimidine dimers (CPDs), while error-prone when replicating undamaged DNA (9-17). Inactivating mutations of Pol$\eta$ leads to the syndrom Xeroderma Pigmentosum Variant, resulting in hypersensitivity and hypermutability to UV damage, associated with a strong predisposition to skin cancer (9, 18). These observations suggest that at least in the context of UV-induced DNA damage, other TLS polymerases are non-redundant with Pol$\eta$ activity. As mammalian cells and chicken DT40 cells deficient for PCNA ubiquitination are sensitive to replication fork blocking lesions (19, 20), 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 Pol$\eta$, Pol$\iota$ and Rev1 (2, 21-24), which is believed to depend on the Ub-binding domain (UBD) of TLS polymerases (22). 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 (21-25). However, the involvement of PCNA-Ub and the UBD in regulating Pol$\eta$ have recently been questioned, since in human cells: 1) Mutation studies indicate that Ub binding by the UBD of Pol$\eta$ is dispensable for its activation (26, 27), 2) It has been suggested that PCNA-Ub is not required for Pol$\eta$ accumulation into foci, but only increases the residence time within foci (28), and 3) The bypass of a CPD lesion is as efficient in cell extracts of Rad18-deficient cells as in wild-type (WT) cell extracts (29).
To address the role of PCNA-Ub in activating Polh in mammals, we established a unique set of mammalian cell lines that either lack Polh, carry a non-modifiable PCNA^K164R mutation, or both. Upon UV treatment, TLS recruitment and DDT are strongly impaired in the PCNA^K164R mutant cells, resulting in hypersensitivity to UV-induced lesions. Interestingly, compared to single mutants, the PCNA^K164R;Polh double mutant cells were dramatically delayed in the S phase of their cell cycle and far more prone to cell killing following UV exposure. Apparently, in mammals Polh can act independently of PCNA-Ub. These findings are further supported by mutation spectra in hypermutated Ig genes in B cells of PCNA^K164R;Polh double mutant mice. Together these observations, and those made in the companion paper (30), provide strong evidence that in mammals Polh can be activated in PCNA-Ub dependent and independent manners.

RESULTS

Foci-formation of TLS polymerases is impaired in the absence of PCNA-Ub

It has been reported that Polh−, Polκ− and Rev1-deficient cells are hypersensitive to UV-irradiation and that these polymerases accumulate at sites of UV-induced DNA damage, which can be visualized as subnuclear foci (8). As mentioned earlier, the role for PCNA-Ub in the recruitment of these polymerases is heavily debated. To address this question in a genetically defined setting, we here established cell lines from PCNAK164R knock-in mice (31). The PCNA^K164R mutation precludes any posttranslational modification at this residue. To visualize the recruitment of TLS polymerases, GFP-tagged Polh, Rev1 and Polκ were stably introduced into two independent WT and PCNA^K164R mutant cell lines (Fig. 1). In line with previous reports, in WT cells foci formation of Polh, Rev1 and Polκ was induced by UV irradiation (8, 22, 24, 25, 32, 33). 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 and suggests that PCNA-Ub is a central docking station for TLS accumulation. Remarkably, 6% of PCNA^K164R mutant MEFs were still capable of recruiting Polh in the absence of PCNA-Ub, although this recruitment is abolished when the two PCNA-interacting domains (26) were mutated (Fig. S1). These data suggest the existence of two distinct pathways in recruiting Polh: A major, PCNA-Ub-dependent and a minor, PCNA-Ub-independent pathway, both of which appear to depend on Polh/PCNA interaction.

PCNA-Ub independent activation of Polh in damage survival

As the presence or absence of foci formation does not necessarily predict activity of TLS polymerases (34), we intercrossed PCNA^K164R−mutant and Polh−deficient mice (15) and derived independent WT, PCNA^K164R mutant, Polh−deficient and
PCNA\textsuperscript{K164R}; Pol\textsubscript{h} double mutant cell lines to determine the importance of PCNA\textsuperscript{K164} modification in activating DDT and specifically TLS Pol\textsubscript{h}.

To test the relevance of the PCNA\textsuperscript{K164} modification on cell survival in response to DNA damage, pre-B cells were treated with increasing doses of different DNA-damaging agents (Fig. 2). PCNA\textsuperscript{K164R} mutant cells displayed hypersensitivity to

Figure 1. UV inducibility of Pol\textsubscript{h}−, Pol\textsubscript{K}− and Rev1− foci in MEF cells is impaired in the absence of PCNA\textsuperscript{K164} modification. The average of two independent cell lines are shown per genotype. Mean ± SD of two experiments.

Figure 2. Survival of WT, PCNA\textsuperscript{K164R} mutant, Pol\textsubscript{h} deficient and PCNA\textsuperscript{K164R}; Pol\textsubscript{h} double mutant pre-B cells in response to UV, cisplatin, MMS, and \(\gamma\)-irradiation. Cells were normalized to the mock treated cells. The average of two independent cell lines are shown per genotype. Mean ± SD of two experiments.
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 g-irradiation, arguing for a selective defect in DDT and excluding a general survival defect of PCNA-mutant cells. Taken together, our data are consistent with previous observations made in PCNA$^{K164R}$ mutant S. cerevisiae, chicken DT40 cells and in PCNA$^{K164R}$ expressing human cells in which endogenous PCNA was knocked down (3, 4, 19, 20). In addition, it has been reported that in mammalian cells, PCNA is ubiquitinated when exposed to replication blocking lesions, but not to DNA double strand breaks (2). Pol$\eta$-deficiency resulted in a mild sensitivity to UV and cisplatin treatment, consistent with previous in vitro and in vivo findings indicating that Pol$\eta$ bypasses CPD as well as cisplatin-GG, an intra-strand adduct formed by cisplatin (9, 12, 14, 35). Compared to Pol$\eta$-deficient cells, PCNA$^{K164R}$ cells were far more sensitive to UV and cisplatin, arguing that this can only be partly attributed to a defective recruitment and activation of Pol$\eta$. Further evidence for a role of PCNA$^{K164}$ modification in Pol$\eta$-independent DDT is provided by the observation that the failure to modify PCNA$^{K164}$, but not the deficiency of Pol$\eta$ results in hypersensitivity to MMS. Remarkably, PCNA$^{K164R}$;Pol$\eta$ double-mutant cell lines were far more sensitive to UV and cisplatin as compared to the single mutant cell lines. These data reveal for the first time that apart from its major PCNA-Ub dependent activity, Pol$\eta$ also provides a critical survival advantage in the absence of PCNA-Ub.

**Replication block recovery in the absence of PCNA-Ub and Pol$\eta$**

The sensitivity to DNA damage in PCNA$^{K164R}$, Pol$\eta$, and double mutant cell lines likely relates to defective DDT. We therefore determined whether WT, PCNA$^{K164R}$, Pol$\eta$, and double mutant MEFs differ in recovering from UV-induced replication blocks. To address this we used the alkaline DNA unwinding (ADU) assay (36-38). 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 30-50kbp is measured. As this assay monitors the fate of elongating replicons, it collectively measures all DDT pathways, including DNA damage bypass at the replication fork and post-replicative gap-filling. In the mock-treated cells no difference in the recovery of replication fork stalling was observed (Fig. 3, left panel), confirming that PCNA-Ub and Pol$\eta$ are not essential for genomic replication in the absence of exogenous DNA damage. When WT cells were exposed to UV light, the recovery from DNA damage was only transiently delayed (36, 37), (Fig. 3, right panel). At four hours after UV treatment virtually all pulse-labeled DNA ends were present in mature double strand DNA, indicating that all DNA damage was bypassed. In contrast, the recovery of blocked replication forks in Pol$\eta$, PCNA$^{K164R}$, and PCNA$^{K164R}$;Pol$\eta$ cells was delayed. These data argue that both PCNA modification and Pol$\eta$ are necessary for efficient DDT of UV damaged templates. Equally important, in contrast to TLS polymerases Rev1 and Rev3
(36, 37), both Polη and modification of PCNA are not essential as the damage was ultimately bypassed. These observations prove that in mammals PCNA modification-dependent as well as –independent pathways operate to relieve UV-induced replication blocks (see discussion). The tendency that damage bypass is slower in the PCNA\textsuperscript{K164R};Polη double mutant compared to PCNA\textsuperscript{K164R} single mutant cells may indicate a function of Polη in UV damage bypass independent of PCNA modification. However, presumably due to the relative insensitivity of the assay we were unable to detect a statistical significant difference between PCNA\textsuperscript{K164R} and PCNA\textsuperscript{K164R};Polη mutant cells.

**Formation of γH2AX in the absence of PCNA modification and Polη**

Phosphorylation of H2AX at serine 139 (γH2AX) is mediated by the checkpoint kinases ATR in response to single stranded DNA exposed during replication stalling and by ATM in response to DNA double strand breaks that can occur upon fork collapse (39). The delayed recovery of stalled replication forks in Polη, PCNA\textsuperscript{K164R}, and double mutant cells predicts that more single stranded DNA is exposed and DNA breaks may accumulate to higher levels in these cells as compared to WT cells. To investigate whether γH2AX indeed accumulates to higher levels in the mutants we determined the DNA content and the formation of γH2AX in UV-irradiated WT, PCNA\textsuperscript{K164R}, Polη, and PCNA\textsuperscript{K164R};Polη mutant pre-B cells. γH2AX
Figure 4. Phosphorylation of H2AX and cell cycle progression in UV treated WT, PCNA^{K164R} mutant, Pol{eta} deficient and PCNA^{K164R};Pol{eta} double-mutant 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 1,2,3,4,5 to determine the frequency of cells in sub G1, G1, early S, late S, and G2 respectively, are indicated. 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.
was found to accumulate in S phase (Fig. 4A-B), indicating that in this setting the formation of γH2AX depends mainly on replication fork stalling. Consistently, γH2AX staining has been shown to correlate with S phase after UV irradiation (40, 41). In agreement with previous observations (42) and with the defect of Polη-deficient cells in tolerating UV damage, higher levels of γH2AX were found in Polη deficient cells as compared to WT. Over a period of seven hours, the γH2AX levels in Polη-deficient cells decreased to those of WT cells, although they did not reach levels before UV treatment. The induction and persistence of γH2AX levels were much stronger in PCNAK164R-mutant cells compared to Polη deficient cells, suggesting that modification of PCNA regulates other DDT pathways than Polη mediated lesion bypass. PCNAK164R;Polη cells are even more compromised in recovering from stalled replication forks than the single mutant cells as shown by increased levels of γH2AX at 4 hours after UV treatment (Fig. 4B). Together these data indicate that in DNA lesion bypass Polη may act independently of PCNA modification at K164.

**Cell cycle delay in the absence of PCNA modification and Polη**

To determine the impact of UV-induced DNA damage on the cell cycle, 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. To compare the cell cycle of the different genotypes after UV irradiation, the frequencies at 0 hours were normalized (Fig. S2). The number of PCNAK164R, Polη, and PCNAK164R;Polη mutant cells accumulated in early S phase over the first four hours, while at the same time the percentage of cells in late S strongly declined. To visualize this more clearly, we compared the relative frequencies of cells in early and late S (Fig. 4C). While the distribution of WT cells in early and late S phase remained quite stable over 8 hours, indicating relatively unperturbed S phase progression, Polη deficiency resulted in an increase of the frequency of early-S phase cells and a decrease of cells in late S phase during the first four hours, after which the frequencies returned to those of WT. These data are in good agreement with the S-phase delay found in XP-V cells (40, 43, 44), and implicate that after four hours, cells irradiated in G1 or early S ultimately progress to late S. In PCNAK164R cells the S phase delay is even more pronounced, but the cells were still able to enter late S. In contrast, PCNAK164R;Polη double mutant cells were unable to tolerate this damage and virtually all cells died within 24 hours after UV (Fig. 4A). In contrast to the double mutant, the cell cycle distribution in Polη and PCNAK164R cells started to normalize after 24 hours. In summary, replicating PCNAK164R cells are highly sensitive to UV damage, but have the potential to overcome replication blocks. This potential is lacking in the double mutant cells and results in cell death. Thus, in replicating cells Polη provides a survival advantage to UV-induced DNA damage that is independent of PCNA modification.
**PCNA-Ub independent activation of Polη during mutagenesis**

While Polη ensures error-free replication across a template CPD, it is highly error-prone when replicating undamaged DNA, especially when replicating across template T (11). B cells take advantage of the intrinsic error-prone nature of TLS polymerases to introduce point mutations into the variable region of their rearranged immunoglobulin (Ig) genes to generate antibodies of increased affinity, a process known as somatic hypermutation (SHM)(13). B cells lacking Polη or PCNA-Ub are compromised in generating mutation at A/T basepairs (15-17, 31, 45). Given our findings that Polη can act independently of PCNA-Ub during TLS we here determined the contribution of PCNA-Ub in the generation of Polη-dependent A/T mutations in vivo. Using established methods (45, 46) the mutation spectra in hypermutated Ig genes from WT, Polη, PCNA$^{K164R}$ and PCNA$^{K164R}$;Polη mutant B cells were determined. As shown in figure 5 and supplemental figure 3, 50% of point mutations reside at template A and T. Consistent with the role of Polη in A/T mutagenesis (15-17), Polη-deficiency reduced mutations at A/T basepairs to 20%. In the absence of Polη, Polκ has been found to substitute Polη in generating A/T mutations (47). In line with previous publications (31, 45, 48), the frequency of A/T mutations was strongly reduced but not absent in PCNA$^{K164R}$ mutant B cells, indicating that both Polη and κ depend on PCNA-Ub to establish 90% of all A/T mutations. To determine the contribution of Polη in A/T mutagenesis occurring independently of PCNA$^{K164}$ modification, we compared the mutation spectra of PCNA$^{K164R}$ and PCNA$^{K164R}$;Polη double mutant B cells. We found that Polη is responsible for the vast majority (81%) of PCNA-Ub-independent A/T mutagenesis during SHM. Taken together, these data further support the existence of a PCNA-Ub dependent and independent Polη activation pathway.

![Figure 5. Frequency 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 percentage of A/T (black) and G/C (gray) mutations in the different genotypes are displayed.](image)
DISCUSSION

To delineate the role of PCNA-Ub in controlling mammalian DDT in general and in regulating Polθ in particular, we here established a unique set of mice and mammalian cell lines that either lack Polθ, carry a non-modifiable PCNAK164R mutation or both. Our studies indicate that: 1) PCNAK164 modification is important, but not essential for the recovery of DNA damage induced replication blocks, 2) In response to UV-damage as well as during SHM in vivo, Polθ can be activated in a PCNA-Ub dependent and independent manner.

In line with previous studies (3, 4, 19, 20), PCNAK164R mutant cells are highly sensitive to replication-blocking agents. We now show that this sensitivity relates at least partially to a delayed recovery from replication blocks as revealed by ADU and cell cycle analysis. The fact, that PCNAK164R mutant cells are far more sensitive than Polθ deficient cells suggest that PCNA modification does not only regulate Polθ-mediated lesion bypass but also other TLS polymerases and DDT pathways. In fact, UV-induced foci formation of Polθ, Polκ and Rev1 was found to depend strongly on PCNAK164 modification. As the lack of these polymerases results in UV-sensitivity (8), the hypersensitivity of PCNAK164R mutant cells may be caused by an impaired recruitment of these TLS polymerases to sites of UV damage. These data are consistent with previous observations made in Rad18-deficient cells (21), but contrast the work of Sabbioneda et al., which suggested that PCNA-Ub is not required for the formation of Polθ foci, but rather increases the residence time of Polθ in foci (28). The discrepancy may relate to our observation that in the absence of PCNA-Ub a subset of cells is capable of recruiting Polθ into damage-induced foci. In addition, the genetically defined PCNAK164R knock-in system used in the present study excludes PCNA ubiquitination, which is not the case for proteasome inhibition as applied in the latter study. Of note, the failure to recruit TLS polymerases into foci, does not necessarily imply a failure in activating these polymerases (34). Besides TLS activation, alternative PCNA modifications such as polyubiquitination and SUMOylation could regulate additional survival strategies. Our data demonstrate that in the absence of PCNA modifications mammalian cells possess an alternative pathway that allows them to recover from replication blocks. In contrast, PCNA modification appears to be essential for post replication repair in chicken DT40 cells (49). Homologous recombination might be involved in the recovery from replication blocks independent of PCNA modification (50-52). Alternatively, Rev1 may provide a mechanism to recover from replication blocks by recruiting other TLS polymerases independent of PCNA modification (36, 53). PCNA-Ub independent activation of Rev1 is supported by ADU results, as in contrast to our PCNAK164R mutant MEFs, Rev1-deficient as well as Rev3L deficient MEFs are unable to recover from replication blocks (36, 37). Furthermore, Rev1 can act independently of PCNA-Ub in chicken DT40 cells (49, 51) and during SHM in mammals (31, 45, 48).
In addition, we here provide several lines of evidence that Pol\textsubscript{H} can provide DDT independent of PCNA modification. We show, that PCNA\textsuperscript{K164R};Pol\textsubscript{H} double mutant cell lines are far more sensitive to UV and cisplatin than the PCNA\textsuperscript{K164R} cell lines, two DNA damaging agents known to be bypassed effectively by Pol\textsubscript{H} (9, 12, 14, 35). These insights contrast recent findings in which Pol\textsubscript{H} was not found to provide an additional survival advantage in cell lines expressing predominantly exogenous PCNA\textsuperscript{K164R} (20). Residual expression and modification of endogenous PCNA in the latter study may explain this discrepancy. The additional sensitivity of PCNA\textsuperscript{K164R};Pol\textsubscript{H} double mutant cells to UV damage likely relates to more stalled replication forks, as suggested by the higher levels of \gamma\textsuperscript{H2AX} in late S phase as compared to PCNA\textsuperscript{K164R} single mutant cells. PCNA\textsuperscript{K164R};Pol\textsubscript{H} cells ultimately were unable to tolerate this damage and virtually all cells died within 24 hours after UV.

Combining these and previous findings suggest an updated model for the function of PCNA modification in mammalian DDT. In this model the exposure of single-stranded DNA resulting from replication blocks triggers the recruitment and activation of Rad6/Rad18 to ubiquitinate PCNA. Next, PCNA-Ub activates TLS, while PCNA polyubiquitination may activate template switching. Hereby, PCNA modification prevents the collapse of the fork and ensures the completion of replication. In the absence of PCNA modification we now suggest that the recovery of the replication block is delayed, but can be partially relieved by Pol\textsubscript{H}, Rev1, and Pol\textsubscript{Z}.

This model is supported by the data described in the companion paper by Ayal et al. (30). How Pol\textsubscript{H}, Rev1, and Pol\textsubscript{Z} are regulated independent of PCNA-Ub and by which mechanism these polymerases then prevent replication fork collapse and/or post replication gap filling will be exciting to address in future studies. It has been found that Pol\textsubscript{H}, in addition to its role in TLS, may participate in homologous recombination (54, 55). However, given our findings that Pol\textsubscript{H} can only relieve replication blocks induced by UV and cisplatin but not MMS, implies that the PCNA-Ub independent Pol\textsubscript{H} activity relates to TLS.

**MATERIAL AND METHODS**

**Isolation and generation of primary and immortalized cell lines**
The generation and genotyping of PCNA\textsuperscript{K164R} knock-in mice and Pol\textsubscript{H} deficient mice has been described elsewhere (15, 31, 45). All animal experiments were approved by an independent animal ethics committee of the Netherlands Cancer Institute (Amsterdam, Netherlands). Germinal center B cells, pre-B cells and mouse embryonic fibroblasts (MEFs) were isolated from intercrosses of PCNA\textsuperscript{K164R};Pol\textsubscript{H} heterozygous mice using standard procedures as described in SI. MEFs were immortalized (2 per genotype) using lentiviral expression of a p53-specific shRNA (56). Stable cells lines (2 per genotype) expressing GFP-Pol\textsubscript{H}, GFP-Pol\textsubscript{H}-PIP, GFP-Pol\textsubscript{K}, GFP-Rev1 were generated by retroviral transduction and FACS sorting as described in SI.
Characterization of cell lines

ADU and SHM assays were performed using immortalized MEFs and germinal center B cells respectively, as previously described (36, 37, 45, 46). Foci formation was determined in MEFs, PFA fixed 6 hours after UV-C (15J/m²) or mock treatment as described in SI. Survival and cell cycle analysis were performed using primary pre-B cells as described in SI.

ACKNOWLEDGEMENTS

The authors wish to thank E. Friedberg and F. Hanaoka for providing the murine Polη, κ 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). The authors declare that there are no conflicts of interest.

REFERENCE LIST

Derivation and culturing of cell lines: pre-B cells and mouse embryonic fibroblasts

E14.5 embryos were isolated from an intercross of heterozygous PCNA^{K164R};Pol{eta} mice. 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) for the generation of pre-B cell cultures, according to Rolink et al. (1). Germinal center B cells where sorted from 3 WT, 3 Pol{eta}, 2 PCNA^{K164R} and 3 PCNA^{K164R};Pol{eta} mice, as previously described (2). Mouse embryonic fibroblasts (MEFs) were isolated according to Abbondanzo et al. (3). MEFs were immortalized (2 per genotype) using lentiviral expression of a p53-specific shRNA (4). Primary and immortalized MEFs were grown in complete medium. All cells were grown incubated at 37°C in 5% CO2.

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). Using site directed mutagenesis F442A, L443A and F687A, F688A mutations were introduced, inactivating the two PIP domains (5). The pEGFP-Pol{eta} and pEGFP-Rev1 plasmids are described previously (6, 7). To enable virus production, the eGFP-fusions were subcloned into the retroviral pMX vector. 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^{K164R} immortalized MEFs. GFP-positive MEFs were sorted by FACSorter.

Foci formation

Two independent WT and PCNA^{K164R} MEFs expressing the eGFP-Pol{eta} wild-type, eGFP-Pol{eta} PIP mutant, eGFP-Rev1 or eGFP-Pol{kappa} fusion were plated on round 25
mm coverslips. The next day the medium was removed, cells were washed with PBS and irradiated with 15 J/m² UV-C (254 nm, UV Stratalinker® 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-Polη.

**Pre-B cell survival assay**

10⁵ 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® 2400, Stratagene). After 15 minutes, cells were irradiated and cultured in 1 ml complete medium and IL7. For the survival upon cisplatin-, MMS- and γ-irradiation-induced DNA damage, 10⁵ 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 γ irradiation from a ¹³⁷Cs source. For determining the survival, pre-B cells were harvested after three days of culture and live (propidium iodine negative) pre-B cells were counted by on a FACSArray (Becton Dickinson). Data were analyzed using FlowJo software.

**Cell cycle analysis in pre-B cells**

2 x 10⁵ 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/m² UV-C and harvested at the indicated time points, fixed immediately with 1.5ml ice-cold 100% ethanol and kept at 4°C. Cells were treated for 20 minutes with RNase A (0.5 mg/ml, Sigma Chemical Co), before permeabilizing in Tween-20 solution (0.25% Tween-20 in PBS/ 1%BSA). γH2AX was detected with 1 μg/ml anti-phospho-histone H2A.X (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 propidium iodine (PI) and measured on a FACSCalibur (Becton Dickinson). Data were analyzed using FlowJo software.

**Reference List**

Supplementary figure 1: A.) UV inducibility of WT and PIP mutant Polη foci in the presence and absence of PCNA^{K164} modification. The average of two independent cell lines are shown per genotype. Mean ± SD of two experiments. B.) Representative images of cells with or without eGFP-Polη, eGFP-Polη PIP mutant, eGFP-Rev1 or eGFP-Polκ fusion foci in the presence and absence of PCNA^{K164} modification.
Supplementary figure 2: Cell cycle profile. Genotypes and time points (h) after UV treatment are indicated. The progression of UV irradiated WT, Polη deficient, PCNAK164R mutant, and PCNAK164R;Polη double mutant cells in G1, early S, late S, and G2 are shown. Left panel indicate the percentage of total cells, right panel 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 3: Base exchange pattern of point mutations in the JH4 intronic region of hypermutated Ig genes. A.) The specific nucleotide substitutions and genotypes are shown in absolute numbers (left panel) and relative numbers (right panel). B.) The distribution of all G/C (above x axis) and A/T (below x axis) mutations along the JH4 intronic region starting from the splice donor are indicated as percentage of total mutations. The genetic backgrounds are indicated.