Modifications of DNA clamps and their role in DNA damage management

Wit, N.

Link to publication

Citation for published version (APA):
Wit, N. (2013). Modifications of DNA clamps and their role in DNA damage management

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.
Mammalian DNA polymerase Kappa can be activated in the presence or absence of proliferating cell nuclear antigen (PCNA) ubiquitination.

Niek Wit¹, Paul C.M. van den Berk¹, Jacob G. Jansen², 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, Leiden, The Netherlands

Manuscript in preparation
SUMMARY

Translesion synthesis (TLS) provides an important, highly conserved mechanism that enables DNA synthesis on a damaged template. TLS is performed by specialized DNA polymerases of which polymerase (Pol) κ is important for the cellular response to DNA damage induced by benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), ultraviolet (UV) light and the alkylating agent methyl methanesulphonate (MMS). Since TLS polymerases act intrinsically error-prone, tight regulation of these polymerases is required. An important level of control is provided by ubiquitination of the homotrimeric DNA clamp PCNA at lysine residue (K) 164 (PCNA-Ub). We here provide genetic evidence that in mammals Polκ can function independently of PCNA modification. Compared to cell lines established from mice deficient for PCNA modification (Pcna<sup>K164R</sup>) or Polκ, double mutant cell lines display hypersensitivity to methyl methanesulphonate (MMS) but not to BPDE or UV-C. MMS-induced Polκ sub-nuclear foci are completely dependent on PCNA-Ub. However, in response to MMS treatment, double mutant cells display delayed replication fork progression and accumulate higher levels of replication stress than the single mutant cells. This results in an S phase delay proportional to the levels of replication stress. Taken together, in response to MMS-induced DNA damage Polκ can be activated in a PCNA-Ub-dependent and PCNA-Ub-independent manner.
INTRODUCTION

Translesion DNA synthesis (TLS) is an effective DNA damage tolerance (DDT) pathway that is highly conserved and enables cells to cope with replication-blocking DNA lesions [1]. TLS depends on specialized DNA polymerases that are able to accommodate bulky DNA lesions in their flexible active sites [2]. However, this feature also reduces their fidelity on a non-damaged DNA template, as they can accommodate non-Watson-Crick base paring in their active sites. Moreover, these enzymes lack the 3’ to 5’ exonuclease activity associated with the proofreading ability of replicative polymerases. This ability is essential in enabling DDT as otherwise a futile cycle is initiated [3,4]. Because of these characteristics, TLS polymerases require strict regulation.

The TLS polymerases Polκ, Polη, Polι and REV1 belong to the Y-family, the largest family of TLS polymerases [1]. For TLS across bulky guanine N2-lesions, Polκ seems to be most effective. For example, in vitro it is able to incorporate the correct nucleotide opposite a BPDE-dG adduct, while Polη can only bypass BPDE in an error-prone fashion [5-8]. Moreover, Polκ-deficient cells are sensitive to BPDE, emphasizing the in vivo relevance of Polκ-dependent TLS across BPDE lesions [9]. Mice lacking Polκ display a spontaneous mutator phenotype in various tissues. Over a period of two years reduced survival rates compared to WT mice have been observed, but otherwise no overt phenotypes are evident [10,11]. Interestingly, the mutation spectra observed in various tissues of Polκ-deficient mice are dominated by mutations at G/C base pairs, similar to mice treated with BPDE [12]. These observations suggest that the primary in vivo role of Polκ is to prevent mutagenesis by naturally occurring polycyclic hydrocarbon guanine adducts.

The homotrimeric DNA clamp PCNA is a central player in controlling DNA replication and DDT. During unperturbed replication, PCNA acts as a processivity factor for replicative DNA polymerases. In the presence of a replication-blocking lesion, the replication machinery will stall and if not stabilized may collapse, a process that can lead to a cytotoxic double-stranded DNA break (DSB) [13]. To prevent this, the ubiquitin conjugase/ligase dimer Rad6/18 is recruited by Replication Protein A (RPA) that binds single-stranded DNA (ssDNA). This results in the monoubiquitination of PCNA (PCNA-Ub) [14], an event that initiates a polymerase switch from a replicative polymerase to one of the damage-tolerant TLS polymerases. Two possible scenarios are being considered: 1) the presence of ubiquitin binding domain(s) (UBD) in all TLS polymerases facilitates the polymerase switch [15-18]; 2) PCNA-Ub prevents the PCNA binding of a set of proteins that would otherwise inhibit the binding of TLS polymerases to PCNA [19,20]. Alternatively, TLS can occur independently of PCNA-Ub [21,22].

In the budding yeast Saccharomyces cerevisiae, TLS is completely dependent on PCNA-Ub, as cells deficient for a TLS polymerase and PCNA modification (PcnaK164R) are as sensitive to UV irradiation as the single PCNA mutant [20]. Moreover, Polζ-dependent mutagenesis requires PCNA-Ub [20]. Likewise, higher eukaryotic PcnaK164R cells are also sensitive to UV-induced replication-blocking lesions, which implies functional conservation [21-25]. Furthermore, the mammalian Y-family TLS polymerases η, ι and REV1 have a higher affinity for PCNA-Ub than for unmodified PCNA [15-18,26]. However, the relevance of PCNA-Ub in the activation of Polκ is less clear, as some studies report that, for example, Polκ critically needs its UBD for activation [27], while others have reported that Polκ can participate in RAD18-independent TLS of UV lesions [28].
To determine the requirement of PCNA-Ub in the activation of Polκ in a genetically well-defined mammalian system, we here established homozygous Pcna^{K164R};Polk^{-/-} (henceforth double mutant), as well as WT and single mutant cell lines. Although double mutant cells display a similar sensitivity to UV-C and to the polycyclic aromatic compound BPDE as compared to single mutants, our data reveal that double mutant cells are, as compared to the single mutants, more sensitive to the monofunctional alkylating agent methyl methanesulphonate (MMS). This suggests that Polκ can act independently of PCNA-Ub in response to methylated DNA bases. This PCNA-Ub-independent Polκ activation does not rely on the accumulation of Polκ in distinct subnuclear foci, since formation of MMS-induced Polκ foci completely depended on PCNA-Ub. Furthermore, double mutant cells progressed slower through S phase and showed higher levels of DNA damage checkpoint activation, which is consistent with impaired DDT. Also, our data imply that Polκ- and PCNA^{K164} -dependent TLS probably occurs post-replicative. Collectively, our data show that Polκ can be activated both dependently and independently of PCNA-Ub in a lesion-specific fashion.

MATERIALS AND METHODS

Primary cells isolation and cell culture – The generation and genotyping of Pcna^{K164R} and Polk^{-/-} mice have been described elsewhere [29,30]. Polk^{-/-} mice were kindly provided by Claude-Agnès Reynaud, Institut National de la Santé et de la Recherche Médicale Unite, Paris, France. Primary mouse embryonic fibroblasts (MEFs) and fetal livers were isolated from E14.5 embryos derived from intercrosses of heterozygous Pcna^{K164R};Polk^{+/-} mice. Pre-B cells were generated from single cell suspensions of fetal livers grown on lethally γ-irradiated ST2 cells in complete medium (IMDM, supplemented with 8% FCS, pen/strep and β-mercaptoethanol) containing IL-7, according to [31]. Primary MEFs were cultured under low (3%) oxygen tension, with 5% CO₂ at 37°C. To immortalize MEFs, primary MEF cultures were transduced with a lentivirus encoding a p53-specific shRNA [32].

Cell survival – For UV-C treatment, 10^5 pre-B cells were seeded in 24-wells plates containing an ST2 feeder layer and 0.5 ml complete medium and IL-7. After 15 min cells were irradiated (254 nm, UV Stratalinker 2400) and cultured in total 1 ml complete medium and IL-7. For cisplatin, MMS, BPDE, H₂O₂ and γ-irradiation treatment 10^5 pre-B cells were seeded in 24-wells plates containing an ST2 feeder layer and 1 ml complete medium and IL-7 in the continuous presence of different doses of the above mentioned compounds or after different single doses of γ irradiation from a 137Cs source. To determine cell survival, cells were harvested after three days of culture and stained with propidium iodide (PI). The number of PI-negative cells was measured on a FACSArray (Becton Dickinson). Data analysis was performed with FlowJo software.

MMS-induced foci formation – The generation of WT and Pcna^{K164R} MEFs containing eGFP-Polκ is described elsewhere [22]. Per well 1.5*10^3 MEFs were seeded on a glass coverslip in a 6-well plate. One day later, complete medium was added with or without 0.75 mM MMS. The cells were incubated for 6 hours in the presence of MMS, after which the cells were washed with PBS containing 1 mM Ca²⁺ and 0.5 mM Mg²⁺ (PBS⁺⁺) and fixed in 4% paraformaldehyde in PBS⁺⁺ for 5 min. Cells were washed with PBS⁺⁺, after which the coverslips were mounted in Fluoro-Gel
relevance of PCNA-ub in Pol Kappa activation

Microscopy was performed using a fluorescence microscope (Zeiss). At least 250 cells were analyzed per genotype.

**DNA fiber analysis** – Per well 7.5*10⁴ MEFs were seeded in a 6-well plate in 3 ml complete medium. The next day the medium was removed and 1 ml complete medium was added. Next, 1 ml complete medium containing 50 μM 5-Chloro-2'-deoxyuridine (CldU) was added. After exactly 20 min 2 ml complete medium containing 500 μM 5-Iodo-2'-deoxyuridine (IdU) and +/- 6 mM MMS was added. Exactly 20 min later, the cells were washed in PBS++, trypsinized and counted. For each condition a cell suspension of 3.0*10⁵ cells/ml was made. Of this cell suspension 2 μl was added to a microscope slide (Menzel-Gläser Superfrost, Fisher Scientific) and air-dried for 5 min. The cells were lysed by adding 7 μl lysis buffer (200 mM Tris-HCl pH7.4, 50 mM EDTA, 0.5% SDS) and swirled vigorously with a pipet tip. After air-drying for 3 min the slides were raised to an angle of 15 degrees and the drop was allowed a minimum of 2 min to run down the bottom of the slide after which the slide was air-dried completely. Fixation was performed with 3:1 methanol: acetic acid for 10 min. After the slides were air-dried completely, they were stored at 4 °C. For immunostaining, the slides were rehydrated two times in H₂O, washed once in 2.5 M HCl and denatured for 75 min in 2.5 M HCl. The slides were then washed twice with PBS and twice with blocking solution (PBS containing 1% BSA and 0.1% Tween-20). Blocking was performed by incubating the slides for 50 min in blocking solution. Rat-anti-BrdU antibody (BU1/75, AbD Serotec), 1:500 diluted in blocking solution, and Mouse-anti-BrdU (Clone B44, BD), 1:750 diluted in blocking solution, were added for exactly 60 min to detect incorporated CldU and IdU, respectively. Subsequently, the slides were washed three times with PBS and fixed using 4% paraformaldehyde in PBS for 10 min, after which the slides were washed three times in PBS and three times in blocking solution. Secondary antibodies (Goat-anti-Rat Ig-Alexa Fluor-555 (Molecular Probes) and Goat-anti-Mouse Ig-Alexa Fluor-488 (Molecular Probes), both 1:500 diluted in blocking solution, were then added for 90 min in the dark. The slides were washed twice in PBS and three times in blocking solution in the dark. Finally, slides were mounted in Fluoro-Gel (Electron Microscopy Sciences) and stored in the dark at 4 °C [33]. Microscopy was performed using a fluorescence microscope (Zeiss).

**Alkaline DNA unwinding** – Per well 5*10⁴ MEFs were seeded in a 24-well plate and cultured overnight. Cells were either MMS (1.5 mM in serum-free medium) or mock treated for 30 min. MEFs were pulse labeled with [³H]thymidine (2 μCi/ml; 76 Ci/mmol) for 30 min and cultured in medium for up to 6 hours. At different time points, 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 NaH₂PO₄. The cell lysates were sonicated for 30 sec 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, hydroxyl apatite columns were washed with 0.5 M K₂HPO₄ followed by 10 mM NaH₂PO₄ (pH 6.8). After each cell lysate was loaded, the columns were washed twice with 10 mM NaH₂PO₄ (pH 6.8). ssDNA was eluted with 0.1 M K₂HPO₄ (pH 6.8), and double-stranded DNA was eluted with 0.3 M K₂HPO₄ (pH 6.8). Radioactivity was quantified by liquid scintillation counting [34].
Cell cycle and γH2A.X analysis by flow cytometry – Per condition 9 ml of 2×10^5/ml pre-B cells containing IL-7 were exposed to 200 μM MMS for 30 min at 37 °C. Per time point, 0.5 ml of solution was plated in 24-well plates. At indicated time points cells were harvested and fixed immediately with 1.5 ml ice-cold 100% ethanol and stored at 4 °C. Cells were treated for 20 min with RNAses A (0.5 mg/ml, Sigma Chemical Co), before permeabilizing in Tween-20 solution (0.25% Tween-20 in PBS/1%BSA). γH2A.X was detected with 1 μg/ml anti-phospho-histone H2A.X antibody (Ser139, clone JBW301; Millipore). After staining with AlexaFluor 488 anti-mouse IgG antibody (Molecular Probes) the cells were resuspended in PBS containing 0.5 μM TO-PRO®-3 (Invitrogen) and measured on a FACSCalibur (Becton Dickinson). Data were analyzed using FlowJo software.

Sample preparation and Western blotting – To detect pCHK1 S345, 7.5×10^5 MEFs were seeded in a 10 cm dish per condition. One day later, the cells were mock treated or pulse with 5 mM MMS in complete medium for 30 min and harvested at the indicated time points by scraping on ice. The cell pellet was snap frozen in liquid nitrogen and stored at -80 °C. For whole cell extract preparation, cell pellets were thawed on ice and lysed in 50 μl RIPA buffer (25 mM Tris HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS) containing 1 mM PMSF, 1x protease inhibitor cocktail (Roche), 1x PhosStop (Roche), and incubated for 30 min on ice, sonicated for 10 min and centrifuged for 10 min at 20,800 x g (4 °C). Protein concentration in the supernatant was measured using the Bradford method. Western blotting was performed according to standard protocols. NuPAGE 4-12% gels (Invitrogen) were used for protein separation. Antibodies used were: rabbit anti-pChk1 S345, 1:1000 (clone 133D3, Cell Signaling); mouse anti-Actin, 1:10,000 (clone C4 (MAB1501R), Millipore), goat anti-rabbit-IRDye 800CW (Licor) and goat anti-mouse-IRDye 680RD (Licor).

For PCNA-Ub detection after MMS treatment, 1.5×10^6 cells were seeded on a 15 cm dish per condition. One day later cells were mock treated or treated continuously with 0.75 mM MMS in complete medium. After 6 hours, cells were washed in cold PBS containing 2 mM N-ethylmaleimide (NEM), scraped on ice, snap frozen in liquid nitrogen and stored at -80 °C. To isolate nuclei, cells were thawed on ice and lysed in buffer A (50 mM HEPES pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 1 mM EGTA pH 8.0, 0.2% Triton X-100, 1x protease inhibitors cocktail (freshly added, Roche), 2 mM NEM (freshly added), 1 mM PMSF (freshly added) for 10 min. Nuclei were pelleted by centrifugation at 510 x g at 4 °C for 10 min and washed in buffer A. Subsequently, nuclei were lysed in 0.1% SDS buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS), and the lysate was sonicated and centrifuged (20,800 x g at 4 °C for 10 min) to obtain chromatin proteins (supernatant). Western blotting was performed using standard protocols. NuPAGE 12% gels (Invitrogen) were used for protein separation. Antibody used was: mouse anti-PCNA-HRP 1:5,000 (PC-10, Santa Cruz).

RESULTS

Sensitivity of WT, Pcna^K164R, Polk^-^- and double mutant cells to different DNA damaging agents – To investigate the requirement of PCNA-Ub in the activation of Polκ, we intercrossed heterozygous Pcna^K164R; Polk^-^- mice, to generate WT, the respective single mutants and double mutant embryos. Thereof, primary pre-B cell lines were established and assessed for their
sensitivity to a panel of DNA damaging agents (Fig. 1). In line with previous studies, Polκ-deficient cells were sensitive to MMS [35], BPDE [9], and UV irradiation [30], although the sensitivity to UV was rather modest (Fig. 1). Under these conditions the PcnaK164R mutant cells displayed a higher sensitivity than the Polκ-deficient cells, a finding consistent with the known redundancy of TLS polymerases. Apparently, PCNA-Ub is required for efficient bypass of these lesions. Double mutant cells were even more sensitive for MMS than the PcnaK164R single mutant. Remarkably, this was not observed for BPDE and UV. While PcnaK164R cells were hypersensitive to the crosslinking agent cisplatin, Polκ-deficient cells were not. Finally, no significant differential sensitivity was found in all four genotypes when these cells were exposed to either H2O2 or γ-irradiation. Our data indicate the existence of a PCNA-Ub-independent pathway for the activation of Polκ in response to MMS treatment.

MMS-induced eGFP-Polκ foci formation – To further investigate the increased sensitivity of double mutant cells to MMS, we determined the accumulation of Polκ in distinct subnuclear foci after MMS exposure. Previously, we showed the formation of eGFP-Polη foci in a subset of PcnaK164R cells after UV irradiation [22]. We here determined if eGFP-Polκ could also form foci independently of PCNA-Ub after MMS-induced DNA damage, as the increased sensitivity to MMS of these cells would suggest. MMS treatment weakly induced PCNA-Ub in WT (compared to UV irradiation), but not in PcnaK164R cells (Fig. 2A). The relative weak induction of mammalian PCNA-Ub has been observed in previous studies as well [36]. Remarkably, MMS failed to induce

Figure 1. Cell survival in response to different DNA damaging agents. Pre-B cell survival was normalized to the mock treated cells of each condition. Average of two independent experiments with two independent cell lines per genotype in duplo is plotted ±SD.
eGFP-Polκ foci in \( \text{Pcna}^{K164R} \) cells, whereas under the same conditions about 25% of WT cells formed foci (Fig. 2B). This result indicates that, following MMS treatment, Polκ foci formation completely depends on PCNA-Ub.

**Replication fork analysis after MMS treatment** – Given the selective role for Polκ and the critical contribution of PCNA-Ub in the survival of MMS induced lesions as well as S-phase progression, we determined the consequences for replication fork progression by DNA fiber analyses and alkaline DNA unwinding assays (ADU). TLS is thought to act at different time points: 1) an early pathway that probably involves direct bypass of the lesion at the replication fork; and 2) a late pathway that involves post-replicative gap filling, after re-initiation of replication behind the lesion [1]. The DNA fiber analysis allows one to visualize individual replication forks on a single molecule level and thus to determine the frequency of stalled forks that reflect early DNA damage bypass. However, it fails to distinguish elongating forks from reprimed forks, as the gap that is formed upon repriming is too small to be detected (Fig. 4A) [37,38]. On the other hand, in the ADU assay, cells are first exposed to MMS after which newly synthesized DNA is radioactively labeled and chased for the indicated time points. Subsequently, the persistence of radioactivity in the terminal fragments of approximately 30-50 kbp is measured. As this assay monitors the fate of all elongating replicons, it collectively measures all DDT pathways including direct DNA damage bypass and post-replicative gap filling (Fig. 4A). Thus while ADU on its own cannot distinguish between a stalled fork and a reprimed fork, the combination of ADU and DNA fiber analysis enables one to distinguish between these possibilities. For example, after UV irradiation, \( \text{Rev}1^{-/-} \) mouse cells show defects in both the fiber and ADU assay, while REV1 BRCT
mutant cells display defects in the fiber assay but have WT kinetics of replicon progression in the ADU assay [34]. This suggests that REV1 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 [34].

To exclude a function of Polκ or PCNA K164 in unperturbed replication, we first determined the replication speed prior to MMS treatment by DNA fiber analysis. As shown (Fig. 3A, B), there was no significant difference between all four genotypes, indicating that PCNA K164 modification and Polκ do not control unperturbed replication. Next, we analyzed replication fork progression in the presence of MMS (Fig. 3A, C). Irrespective of the genotype, the presence of MMS resulted

Figure 3. Replication fork progression analysis after MMS treatment. A) Scheme of DNA fiber analysis experiment. MEFs were first labeled with 25 μM CldU for 20 min and then 20 min with 250 μM IdU, or 250 μM IdU and 3 mM MMS. The length of CldU and IdU tracks were measured and the replication speed on a non-damaged template of the CldU track was calculated (B), as well as the IdU:CldU ratio (C). Of two experiments, one representative experiment is shown.
in a lower IdU:CldU ratio, indicating replication fork stalling. Of note, this effect was equal among the tested genotypes. As there were no significant differences, it appears that bypass of MMS-induced lesions 1) occurs independently of PCNA-Ub and Polκ or 2) is performed via

**Figure 4. Replication block recovery analysis after MMS treatment.** A) Scheme of ADU experiment. First, cells are treated with 1.5 mM MMS for 30 min. Then, cells are pulse labeled with [³H]thymidine and chased for different time points as indicated. Subsequently, free DNA ends are unwound for approximately 60 kbp by alkaline treatment, after which the DNA is sheared by sonication. Separation on hydroxylapatite columns allows for fractionation of ssDNA and dsDNA. Finally, in the fractions corresponding to either ssDNA or dsDNA radioactive counts are measured. Adapted from [38]. B) ADU of mock treated cells or C) MMS treated cells. Average of 5 independent experiments ± SD.
a late, post-replicative lesion bypass pathway. To test the latter possibility, we applied ADU in MMS treated cells (Fig. 4). As shown in Fig. 4B, untreated cells of all four genotypes displayed a similar, time-dependent, decrease in the amount of radioactive ssDNA, which suggests that replication fork progression at undamaged DNA is independent of PCNA-Ub or Polκ, confirming our DNA fiber analysis data. However, when treated with MMS, double mutant cells show a delayed replication fork progression as compared to WT and single mutant cells. Taken together, these results suggest that Polκ and PCNA-Ub can act in different pathways to bypass MMS-induced DNA damage, possibly in a late DNA damage bypass mechanism.

**ATR/Chk1 activation after MMS treatment** – If post-replicative gaps accumulate in MMS-treated cells, one expects that the ATR/CHK1 checkpoint is activated. CHK1 phosphorylation on serine 345 (pCHK1) is a hallmark of ATR/CHK1 checkpoint. This phosphorylation event occurs upon the activation of the ATR kinase that is induced by regions of persistent ssDNA, a characteristic of replication fork stalling [39]. After MMS treatment, the PcnaK164R and Polκ single mutant cells were found to accumulate slightly more pCHK1, which persisted over a longer period, compared with WT cells (Fig. 5). These effects were more dramatic in the double mutant cells (Fig. 5), suggesting accumulation of ssDNA regions in these cells.

**Replication stress and S-phase progression** – The reduced replication fork progression and the activation of ATR/Chk1 observed in double mutant cells exposed to MMS might result in the activation of an S phase checkpoint and increased levels of replication stress. To test this hypothesis, we analyzed cell cycle progression and γH2A.X formation in S phase pre-B cells after pulse treatment with MMS (Fig. 3). Indeed, cell cycle progression of double mutant cells was most affected by MMS treatment; a gradual decrease in the S-phase progression was observed with regards to the other genotypes: WT > Polκko > PcnaK164R > double mutant cells (Fig. 3A). Upon MMS treatment, a gradual increase in the γH2A.X signal was observed: WT < Polκko < PcnaK164R < double mutant cells (Fig. 3B).
Figure 6. Effect of MMS on γH2A.X formation and cell cycle progression. A) 2*10⁵ pre-B cells per condition were pulse treated with 200 μM MMS for 30 min at 37 °C and fixed after the pulse at indicated time points. DNA content was visualized by TO-PRO®-3 staining and measured by FACS. B) As A, but analysis of γH2A.X in same experiment in early S-phase B cells. C) As B but late S-phase cells. Average of two independent experiments with two independent cell lines per genotype in duplo is plotted ±SD.
Replication stress and S-phase progression after MMS treatment – The reduced replication fork progression and the activation of ATR/CHK1 observed in double mutant cells exposed to MMS might result in a decrease in S phase progression and increased levels of replication stress. To test this hypothesis, we analyzed cell cycle progression and γH2A.X formation in S phase pre-B cells after pulse treatment with MMS (Fig. 6). Indeed, cell cycle progression of double mutant cells was most affected by MMS treatment; a gradual decrease in the S-phase progression was observed with regards to the other genotypes: WT > Polκ−/− > PcnaK164R > double mutant cells (Fig. 6A). Upon MMS treatment a gradual increase in the γH2A.X signal was observed: WT < Polκ−/− < PcnaK164R < double mutant cells (Fig. 6B,C).

Taken together, both Polκ and modification of PCNA at K164 are important for efficient bypass of MMS-induced DNA damage, thereby preventing the accumulation of ssDNA and thus CHK1 activation, suppressing replication stress and preventing S phase checkpoint activation. Furthermore, our data support the notion that Polκ can function independently of PCNA-Ub.

DISCUSSION

Previously, we have shown that Polη can be activated in a PCNA-Ub dependent and independent manner [21,22]. To determine whether this finding applies to other TLS polymerases in the context of specific lesions, we here studied the cellular response to different genotoxic agents by generating genetically defined cell lines deficient for Polκ, PCNA modification (PcnaK164R) or both. Given that double mutant cells were more sensitive and accumulate more replication stress in response to MMS treatment than the respective single mutants, our study provides strong evidence that Polκ can be activated in a PCNA-Ub dependent and independent manner. This feature, however, strongly depends on the type of DNA damage, since Polκ completely depends on PCNA-Ub following exposure to BPDE or UV (Fig. 1).

The observed MMS sensitivity of Polκ- deficient pre-B cells (Fig. 1) is in line with previous studies in mouse embryonic stem cells and MEFs [35]. However, inactivation of POLK in REV3−/− or REV1−/− DT40 cells further sensitizes these cells to MMS [35,40]. This observation suggests that Polκ can play a role in bypassing MMS-induced DNA lesions in avian cells, albeit under conditions where the main players, Rev1 and Polζ, are absent. Thus, while in DT40 cells Polκ seems to serve as a back up TLS polymerase for the Rev1/Polζ pathway, in the murine system Polκ evolved to fulfill a dominant, primary role in bypassing alkylated DNA bases.

Both PCNA-Ub and Polκ seem to play important roles in the bypass of MMS-induced DNA damage. MMS induces a plethora of DNA lesions, among which 3-methyladenine (3meA) and 7-methyladenine (7meG) are the most abundant [41]. So, what specific lesions are bypassed by PCNA-Ub and Polκ-dependent TLS after MMS treatment? In contrast to 7meG, 3meA poses a replication block to replicative polymerases, but not to Y-family TLS polymerases [41,42]. Polη, Polκ and Polι are all able to incorporate a nucleotide opposite or extend from a 3meA analog in primer extension assays, with Polκ being the most accurate [42]. In vivo this might account for the sensitivity of the PcnaK164R mutant cells, as PCNA-Ub is needed for efficient activation of at least Polκ in response to MMS-induced DNA damage. However, both 3meA and 7meG are prone to depurination, thereby leaving a highly mutagenic abasic site [41,42]. Although conflicting data exist [43], almost all TLS polymerases appear to be able to bypass abasic sites
under defined conditions [5,44-46]. Taken together, we suggest that the actual lesions that cause replication fork stalling after MMS treatment might be persistent 3meA and the abasic sites that are generated due the chemical instability of both 3meA and 7meG.

A recent study proposed a role for PCNA-Ub and Polη in MSH2/MSH6-dependent repair synthesis in human cells in G1 phase after H2O2 treatment [47]. Therefore, we tested whether cells deficient for Polκ, PCNA modification (PcnaK164R) or both display sensitivity to the oxidizing compound H2O2 (Fig. 1). We found that in mouse cells all tested genotypes displayed the same sensitivity, suggesting no role for PCNA ubiquitination in the repair of H2O2-induced DNA damage. Future investigations should verify this discrepancy.

Our data indicated that MMS-induced Polκ foci formation completely depends on PCNA-Ub, as no foci were observed in PcnaK164R cells. The additional insight that Polκ can be activated in a PCNA-Ub-independent manner further supports the notion that foci formation is not a prerequisite for TLS activation. In line with this, a truncated mutant of Polκ that lacks the polymerase domain is still capable of foci formation after UV irradiation or hydroxyurea treatment [48].

In human fibroblasts, UV irradiation triggers replication fork restart by repriming, which is followed by Polη-dependent gap-filling [38]. We here determined whether PCNA-Ub and Polκ dependent TLS across MMS-induced lesions follow the same rules. As shown by DNA fiber analysis, there was no effect on early DNA damage bypass. In contrast, as shown by ADU, late DNA damage bypass was found to be delayed in double mutant cells. In line with this observation, pCHK1 S345 levels accumulated higher and persisted longer in PcnaK164R, Polκ and double mutant cells as compared to WT. Our data imply that TLS of MMS-induced lesions by PCNA-Ub and Polκ occurs via a late mechanism. Alternatively, due to redundancy among TLS polymerases, defects in early DNA damage bypass might not be detected in the DNA fiber analysis. Future analyses of mutant cells that lack several TLS polymerases are required to investigate this possibility.

In summary, we have shown that in response to MMS treatment PCNA-Ub-dependent and PCNA-Ub-independent pathways can activate Polκ, a finding similar to Polη activation in response to UV-induced lesions. It remains to be investigated what factors control Polκ in the absence of PCNA-Ub. Potential candidates are unmodified PCNA, the alternative DNA clamp RAD9-RAD1-HUS1, shown in fission yeast to physically interact with Polκ [49], or yet unknown regulators of TLS.

ACKNOWLEDGEMENTS

We thank T. van Harn for technical assistance on the DNA fiber analysis; A. Pfauth and F. van Diepen for cell sorting; L. Oomen and L. Brocks for assistance with fluorescence microscopy; M.A. Hogenbirk for critically reading the manuscript and the animal caretaker team of the NKI-AVL for biotechnical assistance. This work was supported by the Dutch Cancer Society (KWF grant NKI-2008-4112 to HJ and EU IP LSHG-CT-2005-512113 to NdW). The authors declare no competing interests.
REFERENCES


Supplementary figure 1. Complete scans of Western blots of Fig. 2

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Pcnα&lt;sub&gt;K164R&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMS:</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>UV:</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Long exposure

Short exposure
Supplementary figure 2. Complete scans of Western blots of Fig. 5