

Redundancy of mammalian Y family DNA polymerases in cellular responses to genomic DNA lesions induced by ultraviolet light

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ABSTRACT

Short-wave ultraviolet light induces both mildly helix-distorting cyclobutane pyrimidine dimers (CPDs) and severely distorting (6–4) pyrimidine pyrimidone photoproducts ((6–4)PPs). The only DNA polymerase (Pol) that is known to replicate efficiently across CPDs is Pol η , a member of the Y family of translesion synthesis (TLS) DNA polymerases. Phenotypes of Pol η deficiency are transient, suggesting redundancy with other DNA damage tolerance pathways. Here we performed a comprehensive analysis of the temporal requirements of Y-family Pols ι and κ as backups for Pol η in (i) bypassing genomic CPD and (6–4)PP lesions *in vivo*, (ii) suppressing DNA damage signaling, (iii) maintaining cell cycle progression and (iv) promoting cell survival, by using mouse embryonic fibroblast lines with single and combined disruptions in these Pols. The contribution of Pol ι is restricted to TLS at a subset of the photolesions. Pol κ plays a dominant role in rescuing stalled replication forks in Pol η -deficient mouse embryonic fibroblasts, both at CPDs and (6–4)PPs. This dampens DNA damage signaling and cell cycle arrest, and results in increased survival. The role of relatively error-prone Pols ι and κ as backups for Pol η contributes to the understanding of the mutator phenotype of xeroderma pigmentosum variant, a syndrome caused by Pol η defects.

INTRODUCTION

Exposure to ultraviolet (UV) light induces both mildly helix-distorting *cis-syn* cyclobutane pyrimidine dimers (CPDs) and strongly helix-distorting (6–4) pyrimidine pyrimidone photoproducts ((6–4)PPs) in the genome (1). Both CPDs and (6–4)PPs form blocks for replicative DNA polymerases (Pols), since their active sites are unable to accommodate these photolesions. The only DNA polymerase known to efficiently replicate across CPDs both *in vitro* and *in vivo* is Pol η , a member of the Y family of DNA polymerases which, in mammalian cells, also includes Pols ι , κ and Rev1 (2,3). Pol η is capable of containing a thymine-thymine CPD, the most frequent photolesion, in its enlarged active site (4). In contrast to CPDs, (6–4)TT lesions form a poor substrate for Pol η *in vitro*, as Pol η frequently inserts a G opposite the 3' T but is unable to carry out the subsequent extension step (5). *In vivo*, TLS at (6–4)PP may involve either Pol η or Pol ι , followed by extension by another polymerase, likely the B family polymerase Pol ζ (6).

The importance of Pol η in DNA damage responses is stressed by patients suffering from the xeroderma pigmentosum-variant syndrome (XP-V), a rare autosomal recessive human disorder, caused by mutations in the gene that encodes Pol η (7,8). Clinically, XP-V is characterized by photosensitivity of the skin and high susceptibility to develop cancer of sunlight-exposed areas of the skin. After exposure to ultraviolet C (UVC) light, the conversion of low molecular weight to high molecular weight nascent DNA is much slower in XP-V cells than in normal cells (9). The TLS defect results in the accumulation of ssDNA regions that activate the ataxia-telangiectasia-mutated and Rad3-related (Atr) kinase (10,11). Activated Atr phosphorylates

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multiple proteins, including Checkpoint kinase 1 (Chk1) that controls S-phase progression by inhibiting origin firing, slowing down replication fork progression, stabilizing stalled replication forks and delaying cell cycle progression (12,13). Nevertheless, XP-V cells are only mildly sensitive to the cytotoxic effects of UVC light and the defect in the progression of replication at damaged DNA is only transient, suggesting that most lesions are ultimately bypassed in these cells. Since XP-V cells display increased mutagenesis upon exposure to UVC light (14), an alternative TLS process presumably operates as a backup to convert ssDNA regions into dsDNA in XP-V cells.

Recently, we have analyzed the *in vivo* roles of individual TLS Pols, including Pol η and other Y family Pols, in the suppression of DNA damage signaling and genome instability in immortalized mouse embryonic fibroblast (MEF) lines upon exposure to UVC light (15). We found that Pol η and Pol κ -deficient MEFs only displayed minor phenotypes in response to UVC light, whereas Rev1 appears to be mainly involved in the bypass of (6–4)PP (15,16). In addition, we observed that, similar to XP-V cells, Pol η -deficient MEFs display a transient defect in TLS, resulting in the accumulation of cells in mid-S phase and activation of DNA damage signaling (15). Mainly TLS across genomic CPDs is affected in these cells (15). Possibly, this transient TLS defect in the absence of Pol η might be due to the Y-family Pols ι and κ that act as backup Pols in bypassing UVC lesions at the genome, and in the suppression of DNA damage responses.

To address this question, here we have used MEF lines with well-defined single, double and triple deficiencies in Pols η , ι and κ . To provide quantitative data on the UV damage responses in these cell lines, the same UVC dose was applied in most experiments. We report that in Pol η -deficient MEFs exposed to UVC light, Pol κ is the predominant TLS polymerase to bypass both genomic CPDs and (6–4)PPs, contributing to (i) alleviating cell cycle arrest, (ii) quenching DNA damage signaling and (iii) promoting cell survival. Pol ι may play a role in TLS of a subset of (6–4)PP. Our results support and greatly extend previous studies of cells in which the expression of multiple TLS polymerases was reduced using siRNA knock-down strategies (6,17–20).

MATERIALS & METHODS

Cell culture

MEFs lacking Pol η , Pol ι or Pol κ were isolated from day 13.5 embryos of Pol η , Pol ι or Pol κ -deficient mice (21,22); Aoufouchi *et al.*, in preparation). Crossings between Pol η , Pol ι and Pol κ -deficient mice produced 13.5-day embryo that were doubly-deficient for Pol η and Pol ι , for Pol η and Pol κ or for all three Pols. From these embryos, MEFs were isolated and immortalized following transfection of SV40 large T antigen. Immortalized MEFs homozygous for a targeted disruption of Rev1 were described previously (16). All MEF lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, Glutamax and pyruvate (Invitrogen) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μ g/ml; MEF medium) at 37°C in a humidified atmosphere containing 5% CO₂.

DNA fiber analysis

Per well of a 6-well plate, 7.5×10^4 MEFs were seeded and cultured overnight in an MEF medium. Prior to UVC exposure (13 J/m²), MEFs were incubated in a medium containing 25 μ M 5-Chloro-2'-deoxyuridine (CldU) for 20 min at 37°C. After UVC exposure, a medium containing 500 μ M 5-Iodo-2'-deoxyuridine (IdU) was added, resulting in a final concentration of 250 μ M IdU and 12.5 μ M CldU. After 20 min at 37°C, cells were trypsinized, 2 μ l of a suspension of 3×10^5 MEFs/ml were spotted onto a microscope slide, incubated for 5 min and lysed with 7 μ l lysis buffer (200 mM Tris-HCl pH7.4, 50 mM EDTA, 0.5% SDS) for 3 min. Slides were tilted to 15°C to allow the DNA to run down the slide. Next, slides were air dried and subsequently fixed in methanol-acetic acid (3:1). After rehydration, fixed DNA fibers were denatured in 2.5 M HCl for 75 min. Incorporation of CldU was detected using rat- α -BrdU antibodies (1:500; BU1/75, AbD Serotec) and Alexafluor-555-labeled goat- α -rat antibodies (1:500; Molecular Probes, Life Technologies Europe BV, Bleiswijk, The Netherlands), whereas incorporated IdU was detected using mouse- α -BrdU antibodies (1:750; Clone B44, BD) and Alexafluor-488-labeled goat- α -mouse antibodies (1:500; Molecular Probes, Life Technologies Europe BV, Bleiswijk, The Netherlands). Finally, slides were mounted in Fluoro-Gel (Electron Microscopy Sciences, Hatfield, USA). Microscopy was performed using a fluorescent microscope (Carl Zeiss BV, Sliedrecht, The Netherlands).

Alkaline DNA unwinding (ADU)

This assay, which measures progression of replicons (23) was performed with minor modifications. The procedure is outlined in Figure 1C. Per well 5×10^4 MEFs were plated in a 24-well plate and cultured overnight in MEF medium. After pulse labeling with [³H]thymidine for 15 min, MEFs were washed once with PBS and subsequently exposed to 5 J/m² UVC or mock treated. Then, at indicated times, DNA at replication forks was locally denatured upon incubation of MEFs with ice-cold denaturation solution (0.15 M NaCl and 0.03 M NaOH) for 30 min. The denaturation of DNA was terminated by adding ice-cold 0.02 M NaH₂PO₄. After sonication, SDS was added to a final concentration of 0.25% and the samples were stored at –20°C for at least 16 h. After thawing, the lysates were loaded onto hydroxyl apatite columns to elute ssDNA using 0.1 M K₂HPO₄ (pH6.8) and dsDNA using 0.3 M K₂HPO₄ (pH6.8), respectively. Radioactivity in each eluate was determined by liquid scintillation counting (PerkinElmer). Replication progression was calculated by determining the ratio of radioactivity in total DNA: ssDNA.

Alkaline sucrose gradients

The replicative bypass of genomic CPDs and the increase in molecular mass of elongating nascent DNA molecules in MEFs exposed to 5 J/m² UVC was determined by a sensitive variant of the alkaline sucrose sedimentation assay as described previously (24). The procedure is outlined in Figure 1E.

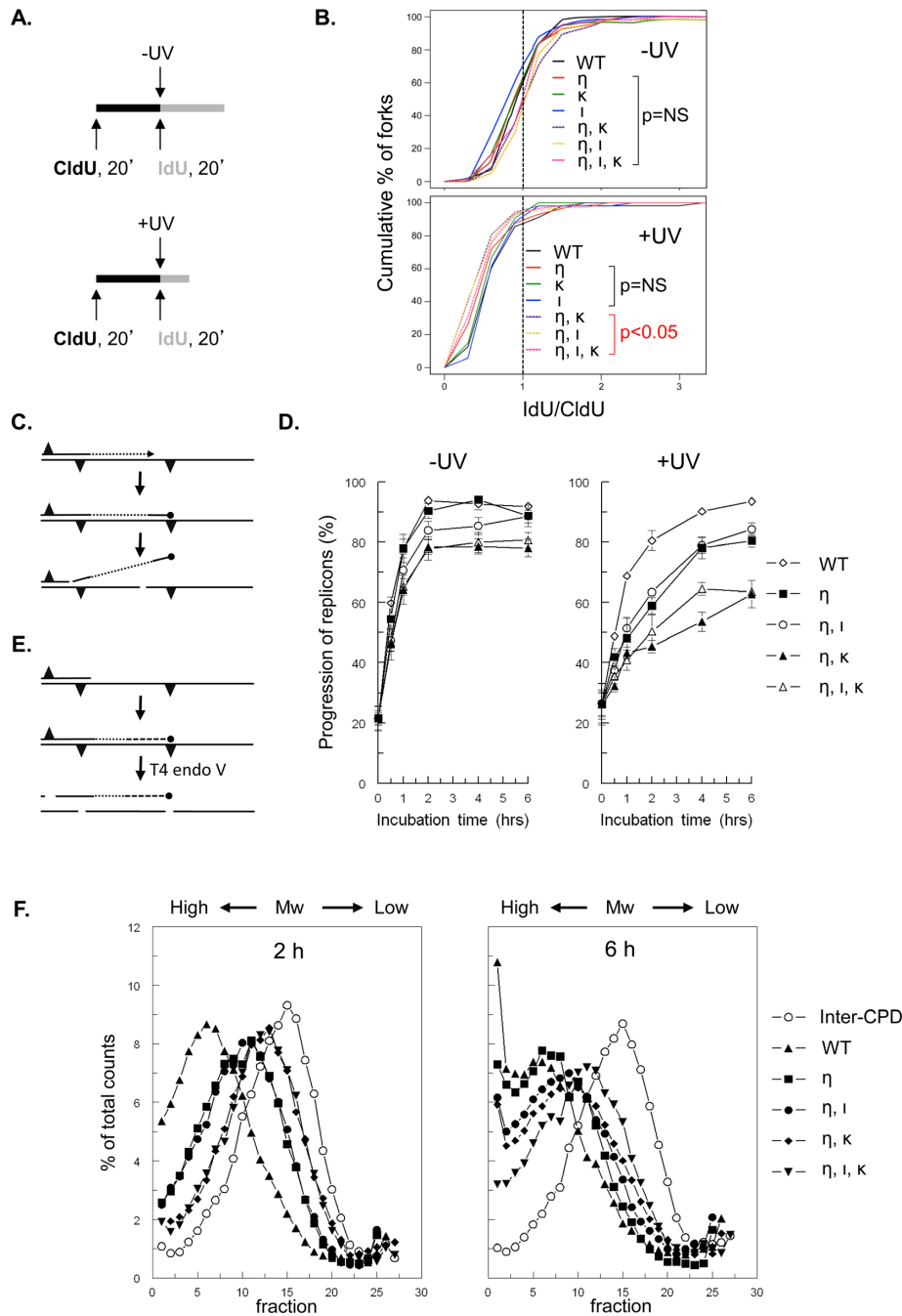


Figure 1. Both Pols ι and κ are required for replicon progression in Pol η -deficient MEFs, late after UVC exposure. (A) Schematic representation of DNA fiber labeling with nucleotide analogs CldU and IdU in MEFs that were mock treated (-UV) or exposed to UVC (+UV). (B) Cumulative percentage of replication forks at ratios of lengths of IdU-labeled tracts to CldU-labeled tracts in wild-type MEFs (WT) or in MEFs with single, double or triple deficiencies in Pol η (η), Pol ι (ι) and Pol κ (κ) exposed to 13 J/m² UVC (+UV). P values are shown of the two-sample Kolmogorov-Smirnov (K-S) test for the ratio distribution of each knock-out genotype compared to wild-type. (C) Scheme of the alkaline DNA unwinding assay. Nascent DNA is pulse labeled with [³H]thymidine (dotted line) immediately before the induction of photolesions (triangles; top). MEFs are then cultured in medium without label (middle). Stalling of a fork at a photolesion results in a DNA end containing [³H]thymidine that is locally denatured using alkaline, followed by sonication and isolation of [³H]thymidine-labeled ssDNA using hydroxyl apatite (bottom). (D) Replication fork progression in mock-treated MEFs (left panel) and in MEFs exposed to 5 J/m² UVC (right panel; n = 4). Error bar, SEM. (E) Scheme of alkaline sucrose gradient sedimentation using T4 endonuclease V. Template DNA was uniformly labeled with [¹⁴C]thymidine (solid line) followed by exposure to UVC inducing CPD and (6-4)PP photolesions (triangles; top). Elongating daughter strands were pulse labeled with [³H]-thymidine for 30 min (dotted line) and cultured in a medium without label (dashed line; middle). At different times, cells were lysed and [¹⁴C]thymidine-containing DNA was cleaved by T4 endonuclease V at a CPD, followed by size fractionation using alkaline sucrose gradients (bottom). The [¹⁴C]thymidine-labeled inter-CPD size distribution serves as an internal standard, since CPDs are not removed in mouse cells. (F) Alkaline sucrose gradient profiles of [³H]thymidine-containing DNA of wild-type MEFs (WT, closed triangle), MEFs deficient in Pol η (η ; closed square) and of Pol η -deficient MEFs containing an additional defect in Pol ι (ι, η ; closed circle), Pol κ (η, κ ; closed diamond) or both TLS polymerases (η, ι, κ ; closed inverted triangle) at 2 and 6 h after exposure to 5 J/m² UVC. Also the profile of [¹⁴C]thymidine labeled, CPD-containing fragments is depicted (open circles). Mw, molecular weight.

Immunostaining

MEFs were cultured overnight on coverslips, incubated with 10 μ M 5-ethynyl-2'-deoxyuridine (EdU; Invitrogen) in an MEF medium for 30 min and subsequently exposed to UVC irradiation (5 J/m²). At indicated times after UVC treatment, cells were fixed and permeabilized as follows: for detection of Rpa, Chk1^{S345-P} and Kap1^{S824-P}, cells were preextracted and permeabilized by 0.3% Triton-X in CSK buffer pH 7.2–7.5 (10 mM HEPES pH7.4, 100 mM NaCl, 3 mM MgCl₂, 0.3% Triton-X100, 300 mM sucrose) for 2 min on ice and immediately fixed with 3.7% paraformaldehyde for 20 min; for detection of Atm^{S1981-P}, cells were fixed and permeabilized with ice-cold methanol:acetone (1:1) for 10 min at –20°C. Cells were blocked with 3% BSA+0.1% Tween-20 for at least 30 min to prevent nonspecific binding, and subsequently incubated overnight with antibodies against Rpa (Cell Signaling), Chk1^{S345-P} (Cell Signaling), Kap1^{S824-P} (Bethyl Laboratory) or Atm^{S1981-P} (Rockland Immunochemicals) at 4°C. Then, appropriate fluorescent dye-conjugated secondary antibodies were applied and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). To visualize EdU-positive cells, which represent the S-phase cells at a time of UVC treatment, Alexafluor 647-conjugated azide was used according to the manufacturer's recommendation (Click-iTTM Edu imaging kit, Invitrogen). Experiments were performed three times. Samples were mounted (Vectashield, Vector laboratories), and images were acquired by wide-field fluorescent microscopy (Axio-plan M2, Carl Zeiss). Fluorescence intensity and numbers of foci were quantified using ImageJ software (National Institutes of Health) as described (25). Between 90 and 135 nuclei per cell line were analyzed for each time point. Detection of CPDs and (6–4)PPs in single-stranded DNA templates was essentially performed as described (16), except that to enable detection of (6–4)PPs the cells were fixed in 3.7% paraformaldehyde for 15 min after extraction with ice-cold 0.3% Triton-X100 in CSK buffer for 2 min.

Bivariate cell cycle analysis

Cell cycle progression of MEFs, exposed to 5 J/m² UVC or mock-treated and pulse-labeled with BrdU, 30 min prior to fixing the cells, was determined by bivariate cell cycle analysis essentially as described previously (15).

Cell proliferation assay

Proliferation of MEFs was determined 3 days after mock treatment or exposure to various doses of UVC light (Philips T UVC lamp, predominantly 254 nm) as described previously (15).

RESULTS

An early role of Pols ι and κ in photolesion bypass

Recently, we have shown that replicative bypass of photolesions was delayed rather than abolished in Pol η -deficient MEFs, suggesting the existence of a backup mechanism that almost completely rescues the Pol η defect (15). Here we tested whether two other Y-family TLS polymerases,

i.e. Pols ι and κ , are involved in this backup pathway. To this aim we compared the responses of Pol η -deficient MEF lines with additional deficiencies in Pol ι , Pol κ or both TLS Pols with wild-type and single-mutant MEF lines. We first determined the progression of replicons in the different MEF lines using DNA fiber labeling. This sensitive assay allows the analysis of replicon progression on single DNA molecules, shortly after exposure to UVC. Thus, cells were incubated with chlorodeoxyuridine (CldU) for 20 min to label replicating DNA, exposed to 0 or to 13 J/m² UVC, and subsequently incubated with Iododeoxyuridine (IdU) for another 20 min. Fibers were generated and stained with specific antibodies for CldU and IdU, visualized by fluorescent microscopy and the lengths of CldU- and IdU-containing tracts were quantified to determine the replication speed and replication fork stalling (Figure 1A). When undamaged templates are replicated, no obvious differences in the replication speed among the cell lines were found (Supplementary Figure S1B), indicating that TLS Pols η , ι and κ are dispensable for replication of undamaged DNA templates. Following UVC exposure, the ratio of IdU to CldU decreased from 1 to approximately 0.5 in wild-type and the single mutant Pol η , Pol ι or Pol κ -deficient MEFs (Figure 1B). This result indicates that (i) UVC-induced DNA damage results in reduced replicon progression and (ii), since the differences in IdU to CldU ratios were statistically not significant, TLS Pols η , ι and κ are seemingly not essential for photolesion bypass at a very early stage after an UVC dose of 13 J/m² (Figure 1B). Nevertheless, it is possible that the DNA fiber analysis is not sensitive enough to visualize discontinuous DNA synthesis by skipping the lesion on UV-damaged DNA as proposed for Pol η -deficient cells (11). At this stage, however, compared with wild-type and single Y Pol mutant MEFs, a significant decrease of the IdU-to-CldU ratio was observed in the double- and triple-mutant MEFs (Kolmogorov-Smirnov test: $P < 0.01$). These results suggest that both Pols ι and κ play a role in alleviating replication fork stalling in Pol η -deficient cells, early after UV exposure, indicating partial redundancy between Pols η , ι and κ (Figure 1B).

A late role of Pols ι and κ in photolesion bypass

To investigate the redundancy between Pols η , ι and κ in TLS at photolesions also at later times after UVC exposure, we employed an alkaline DNA unwinding assay. In this assay, the progression of replicons is determined by measuring the persistence of radioactively labeled ssDNA ends in proliferating cells, pulse-treated with [³H]thymidine immediately prior to mock-treatment or UVC exposure (Figure 1C). Normal replicon progression will result in the relocation of radioactive label from ssDNA ends to dsDNA. In contrast to the DNA fiber assay, the alkaline DNA unwinding assay can be used to detect discontinuous DNA synthesis in TLS-defective cell lines (11,16,26). Previously, we have used this assay to show that MEFs with single defects in Pols η , ι and κ are not measurably defective in replication of undamaged DNA templates (15). Interestingly, Pol η -deficient MEF lines with additional defects in Pol ι , Pol κ or both TLS Pols replicate undamaged DNA templates somewhat less efficiently compared to the Pol η single-mutant cells, in-

dicating a defect in TLS of endogenous DNA lesions or hard-to-replicate DNA sequences (27, 28; Figure 1D, left panel). Upon exposure to 5 J/m² UVC, Pol η single-mutant cells displayed a delay in replication fork progression compared to wild-type cells. This delay was not aggravated by an additional deficiency for Pol ι (Figure 1D, right panel), which at first sight contrasts with the DNA fiber analysis. However, it should be stressed that the UV dose used in the fiber assay is more than 2-fold higher (13 J/m² versus 5 J/m²) and this may increase the frequency of substrates for Pol ι in the absence of Pol η . Thus, Pol ι acts as a backup Pol in Pol η -deficient MEF mainly at early times after UV exposure (Figure 1B). Conversely, as compared with Pol η single-mutant MEFs, the MEF line deficient for both Pols η and κ displayed strongly reduced fork progression following exposure to UVC (Figure 1D, right panel). This defective fork progression was not exacerbated by an additional deficiency for Pol ι in these cells. Together, these data indicate that Pol κ , but not Pol ι , can complement the Pol η defect at later time points after UVC exposure. Nevertheless, in Pol η , Pol κ doubly-deficient MEFs replicons continue to progress, albeit slowly, revealing that Pol κ is not essential for replicative bypass of photolesions in the absence of Pol η .

We wanted to provide an independent approach to study the possible roles of Pols ι and κ as backup polymerases in Pol η -deficient MEFs at later time points after exposure. To this aim, we utilized a sensitive alkaline sucrose gradient-based assay that measures the length of newly synthesized daughter strands, specifically beyond the most prevalent genomic CPD lesions, of which the density is represented by the internal ([¹⁴C]thymidine-labeled) standard (Figure 1E). Consequently, this assay is indicative for both replicon progression and maturation of DNA replication, whereas the alkaline unwinding assay measures only replicon progression. As expected, the generation of nascent DNA molecules was delayed in Pol η -deficient MEFs compared with wild-type cells, especially at 2 h after exposure (Figure 1F). At this time point, maturation of nascent DNA in MEFs deficient for both Pols η and ι was indistinguishable from the single Pol η -deficient MEFs, consistent with the alkaline DNA unwinding assay. The defect of the Pol η -deficient MEFs, however, was aggravated when these cells are also deficient for Pol κ (Figure 1F, left panel). Compared with the MEF line deficient for both Pols η and κ , the triple-mutant MEF line shows a similar deficiency in generating nascent DNA molecules at 2 h after UVC exposure (Figure 1F, left panel). We conclude that, at this time point, Pol κ , but not Pol ι , can complement for the Pol η deficiency. At 6 h post exposure, the maturation of nascent DNA molecules was delayed not only in the MEFs deficient for both Pol η and Pol κ , but also in the MEFs deficient for both Pol η and Pol ι , compared with the Pol η single mutant. In the triple mutant, daughter strand maturation was reduced to an even greater extent (Figure 1F, right panel). The specific defect in the cell lines with a Pol ι defect at 6 h after treatment suggests that, at least in the absence of Pol η , Pol ι is required for TLS at a non-abundant lesion type, whereas Pol κ can complement for the Pol η deficiency in TLS at most photolesions.

Pol κ affects cell cycle progression of Pol η -deficient MEFs

To investigate redundancy in the roles of Pol η , Pol ι and Pol κ in cell cycle progression upon UVC exposure we determined the incorporation of the nucleotide analog bromodeoxyuridine (BrdU) in different cell cycle stages of asynchronously growing MEFs. Thus, at different times after mock treatment or after exposure to 5 J/m² UVC, MEFs were pulse-labeled with BrdU, immediately preceding their fixation. Subsequently, BrdU contents were analyzed by bivariate flow cytometry. Since MEFs deficient for Pol ι or κ display cell cycle progression similar to wild-type MEFs (15), we focused on wild-type MEFs and Pol η -deficient MEFs with and without additional deficiencies for Pols ι and/or κ . No major differences were found between the cell lines after mock treatment, indicating that all tested MEF lines proliferate in a similar fashion in the absence of photolesions (Supplementary Figure S2). Exposure of cells to UVC, however, revealed marked differences in cell cycle distribution between the MEF lines (Figure 2A and B). More specifically, compared with UVC-exposed wild-type MEFs, all mutant MEFs displayed significantly increased levels of early S phase cells and decreased levels of late S phase cells, 12 h after UVC treatment ($P < 0.05$; Student's *t*-test). This suggests an S phase delay in the mutant MEFs. Moreover, at 16 h this phenotype persisted in Pol η -deficient MEFs ($P < 0.05$; Student's *t*-test) and remained unaltered when also Pol ι was disrupted, indicating that Pol ι does not serve as a quantitatively important backup to Pol η (Figure 2A and B). In contrast, the additional deficiency for Pol κ in both Pol η -deficient MEFs and MEFs double deficient for Pol η and Pol ι strongly affects cell cycle progression as the proportion of non-replicating G1 and G2/M phase cells is in the absence of Pol κ significantly increased at later times (Figure 2A and B; $P < 0.05$; Student's *t*-test). Together, these results suggest that S phase progression is perturbed in the absence of Pol η , both early (Figure 1) and late after UVC exposure (Figure 2A and B). The residual S phase progression in these cells strongly depends on Pol κ , rather than on Pol ι .

Low level of double strand DNA breaks in MEFs undergoing replication stress

Persistently stalled replication forks may collapse to double-strand DNA (dsDNA) breaks, underlying genome instability (29–31). To investigate the collapse of replication forks in the mutant cell lines, we assayed for phosphorylation of ataxia-telangiectasia-mutated (Atm) and of heterochromatic KRAB-ZFP-associated protein 1 (Kap1) in MEFs treated with UVC during S phase, as assessed by EdU incorporation, by immunostaining. Phosphorylation of Atm (Atm^{S1981-P}) is an early step in the response to dsDNA breaks (32). Activation of Atm leads to phosphorylation of Kap1 at S824 (Kap1^{S824-P}), although the formation of Kap1^{S824-P} can also be mediated by other phosphatidylinositol-3 kinase-like kinases, including Atr (33). Except for the Rev1-deficient MEFs, we found only a minor UVC-dependent increase of Atm^{1981-P} foci in nuclei of all other MEF lines tested, up to 8 h after UVC exposure (Figure 3A and B), suggesting that only few forks collapse at the UVC dose used (5 J/m²). Interestingly, strong induction

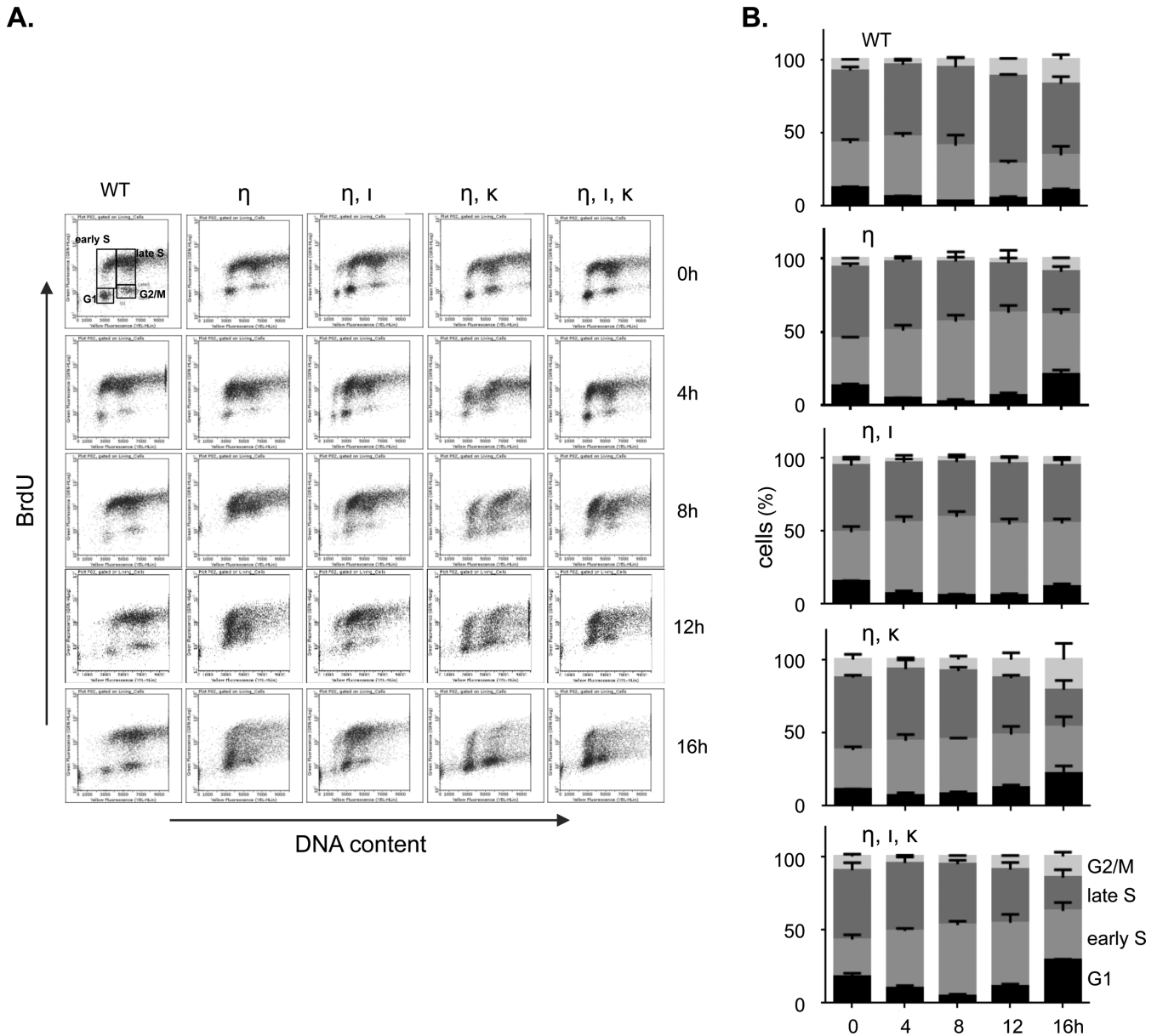


Figure 2. The replicative activity of Pol η -deficient MEFs exposed to UVC relies mainly on Pol κ . (A) FACS profiles showing BrdU content of wild-type MEFs (WT), MEFs deficient in Pol η (η) and Pol η -deficient MEFs containing an additional defect in Pol ι (η , ι), Pol κ (η , κ) or both TLS polymerases (η , ι , κ) after exposure to 5 J/m² UVC. Prior to fixation, MEFs were pulse labeled with BrdU for 30 min, immediately or at 4, 8, 12 and 16 h after UVC treatment. BrdU incorporation was determined by immunostaining and DNA content was measured using propidium iodide. (B) Quantification of MEFs in different cell cycle stages, up to 16 h after UVC exposure ($n = 3$; error bars: SD).

of Kap1^{S824-P} was found in all Pol η -defective MEF lines and in MEFs deficient for Rev1, already 2 h after UVC exposure (see also below). Of note, with the exception of the Rev1-deficient MEFs, Kap1^{S824-P} levels did not increase beyond 2 h after exposure, in agreement with the delay, rather than deficiency, of photolesion bypass in these cell lines (Figure 1).

Quenching of the UV-induced DNA damage response requires Pols η , ι and κ .

We stained the cell lines for phosphorylation of Chk1 (Chk1^{S345-P}), a target for Atr-induced DNA damage sig-

naling at ssDNA tracts (34). Thus, at different time points prior to staining, cells were exposed to 5 J/m² UVC and immediately pulse-labeled with EdU. We included Rev1-deficient MEFs as a positive control, since these cells exhibit strong and persistent Atr/Chk1 signaling following UVC exposure (15,16). At 2h after exposure, all MEF lines displayed Chk1^{S345-P}-positive cells among EdU-positive (replicating) cells. The intensity of Chk1^{S345-P} staining in EdU-positive double deficient MEF lines, and to an even greater extent in the triple-deficient line, was higher than in wild type cells and cells deficient for Pol ι or Pol κ (Figure 4A and B). Furthermore, it should be noted that the extent of

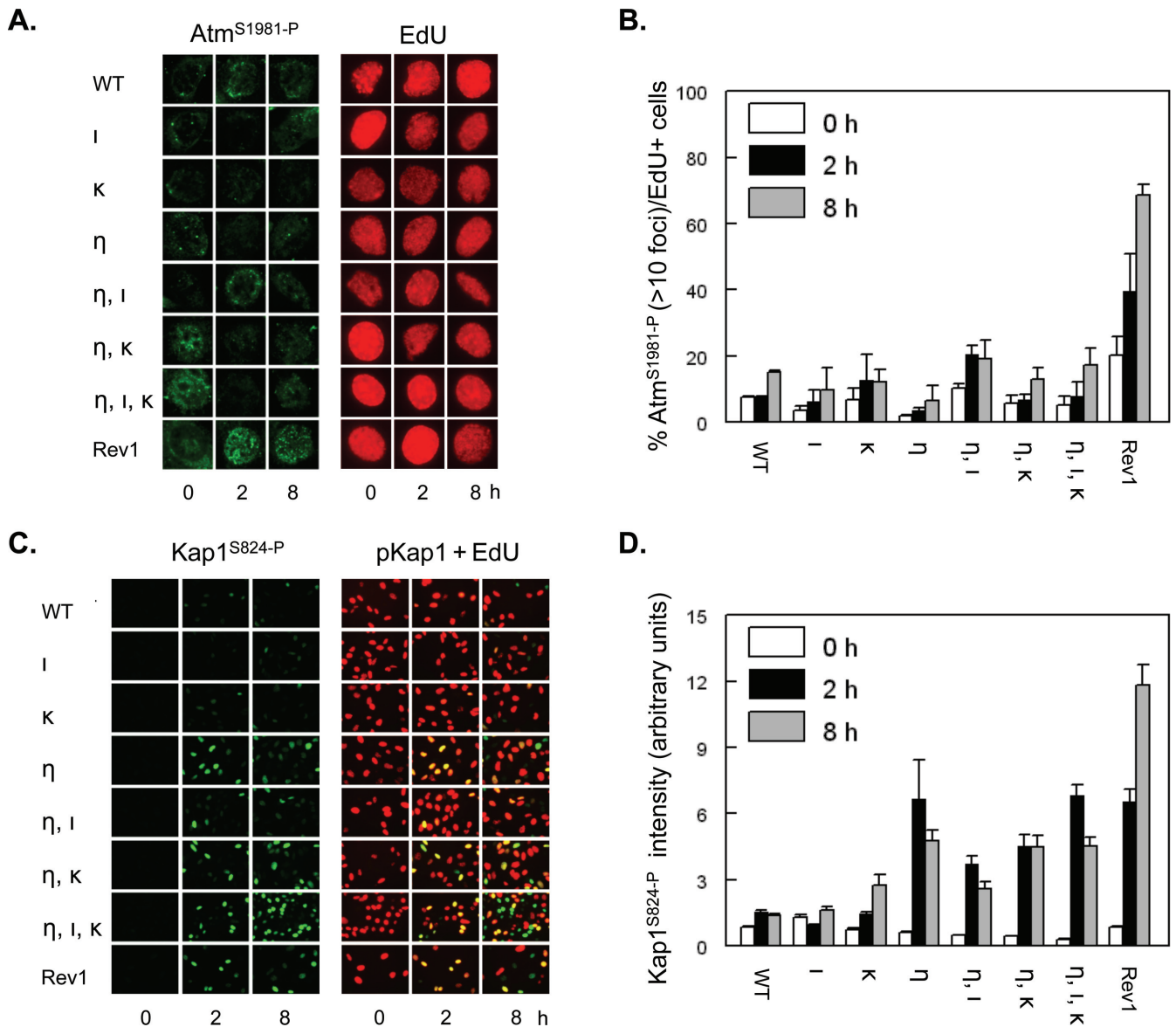


Figure 3. Few UVC-induced dsDNA breaks in Pol η -deficient MEFs with or without additional deficiencies in Pols ι and κ . (A) Wild-type MEFs (WT), MEFs with single, double or triple deficiencies in Pol η (η), Pol ι (ι) and Pol κ (κ), or MEFs deficient in Rev1 (Rev1) were pulse labeled with EdU for 30 min, prior to exposure to 5 J/m² UVC. Then, MEFs were fixed at 0, 2 and 8 h after treatment and immunostained for Atm^{S1981-P} (left panel, online in green) in replicating, EdU-incorporating MEFs (online in red) at the time of UVC exposure. (B) Quantification of EdU-positive MEFs containing at least 10 Atm^{S1981-P} foci. Error bar, SEM. (C) Similar experiment as in (A), except that MEFs were immunostained for Kap1^{S824-P} (left panels, online in green) in replicating, EdU-incorporating MEFs (right panels, merge of staining for Kap1^{S824-P} (online in green) + EdU (online in red)). (D) Quantification of the intensity of Kap1^{S824-P} signal in EdU-positive MEFs. Error bars, SEM.

Chk1^{S345-P} correlated with that of Kap1^{S824-P} in the different MEF lines, although Kap1^{S824-P} is restricted to a subset of EdU-positive cells (compare Figure 4A, B with Figure 3C, D). This result suggests that the formation of Kap1^{S824-P} rather is due to Atr signaling than to the formation of dsDNA breaks. To confirm the presence of ssDNA tracts we assessed the recruitment to chromatin of the heterotrimeric Replication Protein A (Rpa), which binds to ssDNA and recruits Atr. We observed a similar distinction between the MEF lines with respect to the level of Rpa as shown for Chk1^{S345-P} (see Figure 4C and D). These results are in agreement with the pronounced replicon stalling in these MEF

lines as observed in the replication assays (Figure 1). At 8 h after UVC exposure, the intensity of Rpa staining dropped considerably in all MEF lines, except in the Rev1-mutant and in the triple-mutant MEFs (Figure 4C and D). In conclusion, DNA damage signaling in these mutant cell lines qualitatively reflected their defect in TLS, suggesting that ssDNA at stalled replication forks is the primary determinant of DNA damage responses.

Pol κ protects Pol η -deficient MEFs from UVC toxicity

To study the biological consequences of prolonged replication fork stalling, enhanced DNA damage signaling and im-

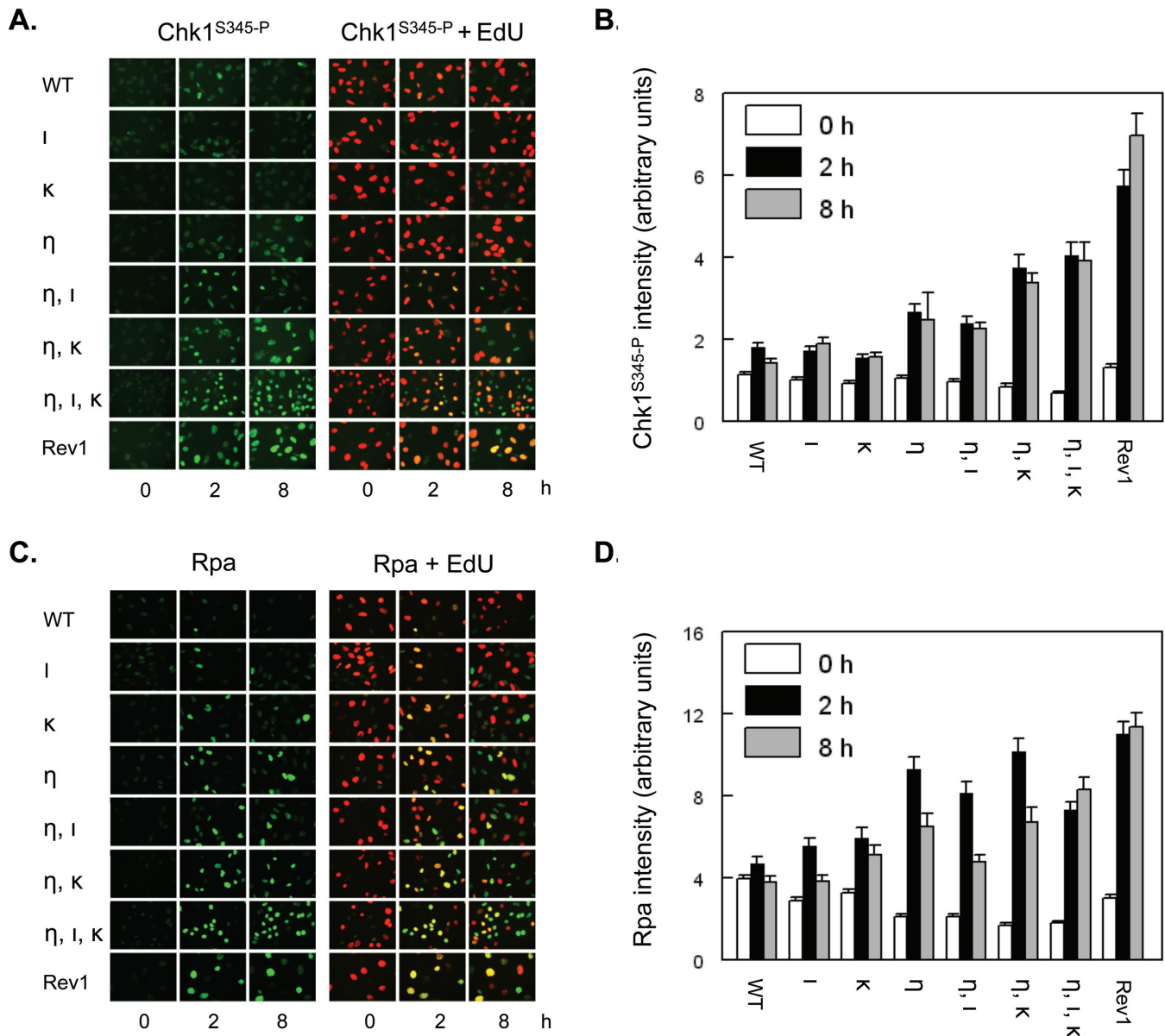


Figure 4. Quenching of DNA damage responses to photolesions requires Pols η , ι and κ . (A) Wild-type MEFs (WT), MEFs with single, double or triple deficiencies in Pol η (η), Pol ι (ι) and Pol κ (κ), or MEFs deficient in Rev1 (Rev1) were pulse labeled with EdU for 30 min, prior to exposure to 5 J/m² UVC. Then, MEFs were fixed at 0, 2 and 8 h after treatment and immunostained for Chk1^{S345-P} (left panels, online in green) in replicating, EdU-incorporating MEFs (right panels, merge of staining for Chk1^{S345-P} (online in green) and EdU (online in red)) at the time of UVC exposure. (B) Quantification of the intensity of Chk1^{S345-P} staining in EdU-positive MEFs. Error bar, SEM. (C) Similar experiment as in (A), except that MEFs were immunostained for Rpa (left panels, online in green). (D) Quantification of the intensity of Rpa staining in EdU-positive MEFs. Error bars, SEM.

paired cell cycle progression, caused by defects in multiple Y family Pols, we analyzed cell proliferation at 3 days after exposure to various doses of UVC. Among the MEF lines tested, MEFs deficient for both Pols η and κ as well as the triple-mutant MEFs displayed the highest sensitivity to UVC light, whereas Pol η -deficient MEFs and MEFs deficient for both Pols η and ι showed an intermediate UVC sensitivity (Figure 5). Confirming previous observations (15), the MEF line deficient for Pol κ was slightly more sensitive to UVC light than wild-type MEFs, whereas Pol ι -deficient MEFs displayed no increased UVC sensitivity (Figure 5).

These results are again consistent with an important role for Pol κ as a backup TLS polymerase for Pol η .

Genomic CPDs are substrates for Pols ι and κ in Pol η -deficient MEFs

By employing a novel immunostaining protocol using monoclonal antibodies that recognize CPDs or (6-4)PPs only when embedded in ssDNA we have previously observed that, in Pol η -deficient MEFs, mainly CPDs cause stalling of replication forks (15). We applied this methodology to the current set of MEF lines to study which genomic photolesions are causing the phenotypes associated with MEFs

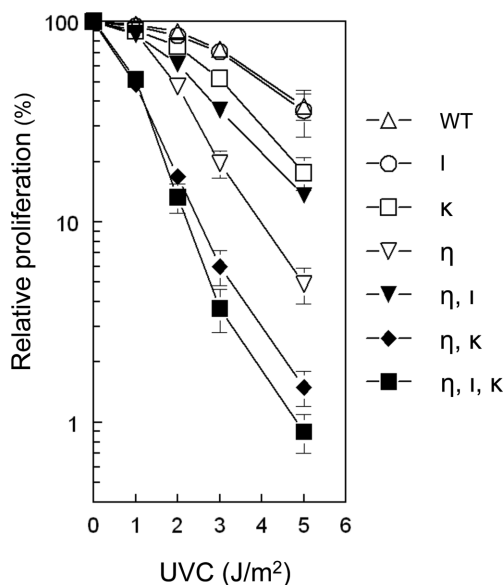


Figure 5. Effect of UVC on proliferation of MEFs with single, double or triple deficiencies in Pols η , ι and κ . MEFs were exposed to different doses of UVC and 3 days later the number of cells was assessed. The number of unexposed MEFs was set at 100%. Error bars, SEM.

deficient for both Pols η and κ and with the triple-mutant MEFs. Thus, cells were pulse-labeled with EdU, to identify the cells that were replicating during UVC exposure, and exposed to 5 J/m² UVC. At 2 or 8 h after UVC exposure, cells were fixed and immunostained for CPDs, or for (6–4)PPs, embedded in ssDNA.

Almost no cells positive for CPDs were detected in wild-type MEFs and MEFs deficient for Pol ι or κ , indicating efficient bypass across genomic CPDs, independent of Pols ι and κ (Figure 6A). As expected, EdU-positive MEFs that are deficient for Pol η displayed unreplicated CPDs at 2 h, and less at 8 h, after UVC exposure (Figure 6A and ref. 15), suggesting transient fork stalling at CPDs. Similar results were observed for Pol η mutant MEFs with an additional deficiency for Pol ι (Figure 6A). MEFs deficient for both Pols η and κ displayed more EdU+CPD positive cells at 8 h upon UVC exposure, indicating that Pol κ does perform TLS at CPDs in the absence of Pol η . Nevertheless, EdU-positive triple-mutant MEFs exhibited the most pronounced staining for unreplicated CPDs at 8 h after UVC exposure (Figure 6A). This result suggests that, in the absence of Pol η , Pol ι can perform TLS at CPDs, but only when also Pol κ is inactive.

All Y family polymerases contribute to TLS of (6–4)PPs

In contrast to CPD lesions, (6–4)PPs impose a strong helical distortion to the DNA, and the 3' pyrimidine base in the pyrimidine dimer is twisted outward and unable to engage in base-pairing (35). Thus far, it has been largely unclear what TLS polymerases are responsible for bypass of these 'severe' lesions at the genome of mammalian cells, although we have previously described a regulatory role for Rev1 (16). Immunostaining of unreplicated (6–4)PPs in EdU-positive cells revealed that TLS of (6–4)PPs is perturbed in MEF

lines defective in Pol η , as judged by the EdU-positive cells staining for single-stranded (6–4)PPs, at 2 h after UVC exposure of these cells (15). Nevertheless, the defect was less pronounced than in MEFs deficient for Rev1 (16 and Figure 6B). Staining for unreplicated (6–4)PPs was also found for double and, to a greater extent, triple-mutant MEFs, at 2 and 8 h after UVC exposure (Figure 6B). These results are the first to demonstrate the involvement of these Y-family polymerases in TLS of genomic (6–4)PP and, moreover, suggest that in the absence of Pol η , both Pols ι and κ act as backup TLS polymerases to replicate across (6–4)PPs.

DISCUSSION

In this study, we have comprehensively analyzed the contributions of the three Y family Pols η , ι and κ in TLS, S phase progression, DNA damage signaling, checkpoint activation and survival in response to genomic CPD and (6–4)PP lesions, using single-, double-, and triple-deficient MEFs. Our results demonstrate that, in the absence of Pol η , Pol κ plays a more important role than Pol ι in responses to genomic photolesions, in agreement with previous studies of cells in which the expression of multiple TLS polymerases was reduced using siRNA knock-down strategies (6,17–20). In support, we and others have shown that Pol κ (but not Pol ι)-deficient mammalian cells are slightly sensitive to UVC light (15,21,36). This sensitivity can be attributed to two not mutually exclusive functions of Pol κ in response to UV light. First, some studies attribute this sensitivity to a defect in NER, at least outside of S phase (37,38). Second, using siRNA strategies, others provide evidence for a role of Pol κ in TLS of a TT CPD on episomal substrates *in vivo* (19,20) while no effect was found on TLS across TT (6–4)PP (6). Indeed, Pol κ can extend from a mismatched nucleotide inserted across 3'Ts of TT CPDs by another DNA polymerase (39). This TLS-related function of Pol κ on abundantly-induced TT CPDs explains the strong defects in replication fork progression, increased staining for CPDs, enhanced DNA damage signaling, slow progression through S phase, and reduced cell proliferation observed in UVC-exposed MEFs deficient for both Pols η and κ . Since Pol κ mainly acts as an extender DNA polymerase in TLS of CPDs, we postulate that in absence of both Pols η and ι another, yet unidentified, DNA polymerase functions as an inserter at these DNA lesions. The role of Pol κ as backup for Pol η is not restricted to UV lesions, since also in somatic hypermutation of immunoglobulin genes, Pol κ acts as a backup in the absence of Pol η (40).

Pol ι plays only a minor role in TLS at genomic photolesions in Pol η -deficient cells, which is apparent from the delayed maturation of nascent strands upon UVC exposure. However, the triple-mutant MEFs displayed the most pronounced phenotypes of all cell lines tested in this study, suggesting that Pol ι is essential for TLS across some UVC-induced DNA lesions in the absence of both Pol η and Pol κ . In support, purified Pol ι can replicate TT (6–4)PPs (41,42) and Pol ι mediates part of the mutagenicity of (6–4)PP *in vivo* (6,43). In addition to a subset of (6–4)PPs, also some CPDs might be candidates for Pol ι -mediated TLS in MEFs deficient for both Pols η and κ . Indeed, Pol ι is capable of inserting nucleotides opposite TT CPDs *in vitro* (41,42,44)

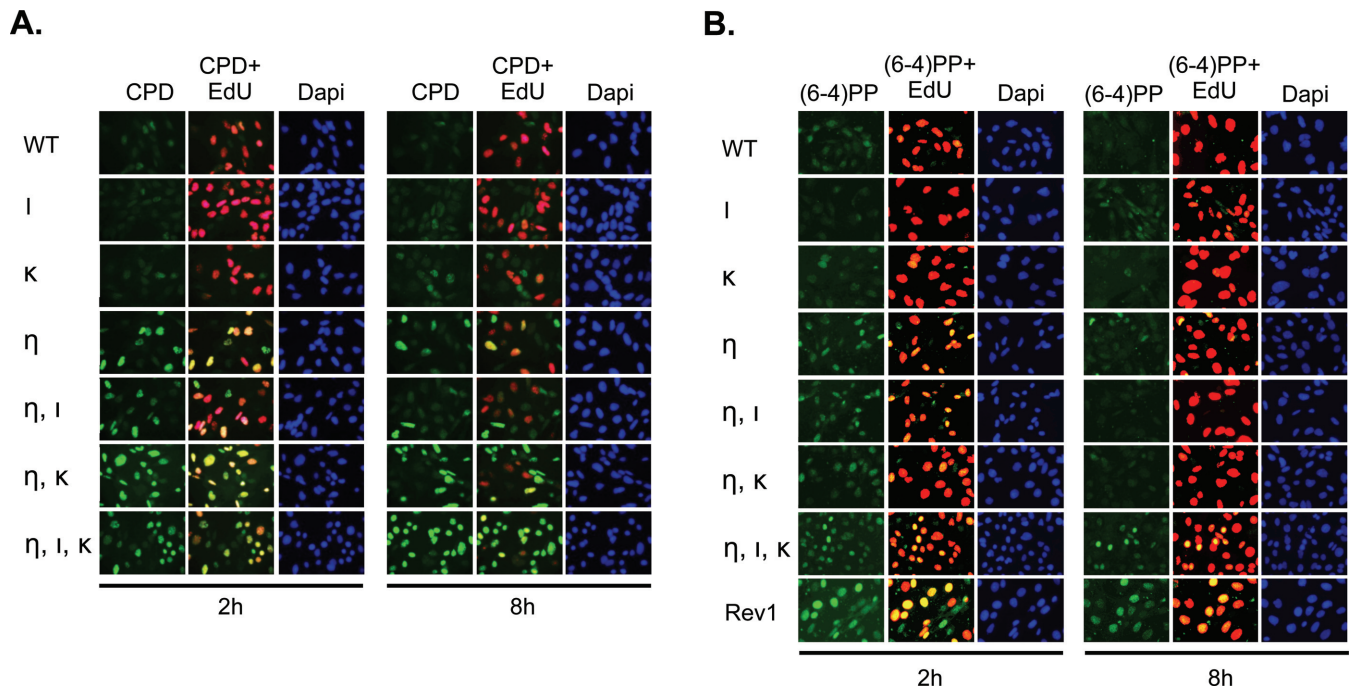


Figure 6. Pols ι and κ are required for efficient bypass of photoproducts in MEFs deficient in Pol η . (A) Wild-type MEFs (WT) or MEFs with single, double or triple deficiencies in Pol η (η), Pol ι (ι) and Pol κ (κ) were pulse labeled with EdU for 30 min, prior to exposure to 5 J/m² UVC, and 2 and 8 h later, MEFs were fixed and immunostained for CPD (online in green; upper panels) in ssDNA of nuclei (online in blue; lower panel) in replicating, EdU-incorporating (online in red, merged with CPD staining; middle panels) and non-replicating MEFs at the time of UVC exposure. (B) Similar as in (A) except that MEFs were immunostained for (6-4)PP (online in green; upper panel). Rev1-deficient MEFs (Rev1) were included as a positive control (15,16).

although CPDs are only poorly bypassed by Pol ι in human (Pol η -deficient) XP-V cells (19,20). Of note, the efficiency by which UV photolesions are induced in the genome strongly depends on the dipyrimidine sequence. Thus, the order of preference for the formation of CPDs is TT > CT = TC > CC, whereas (6-4)PPs are mostly induced at TC and CC dipyrimidines, to a lesser extent at TT dimer sites and not at CT sites (45-49). Moreover, as these lesion types are structurally highly dissimilar (45), they may require different sets of TLS polymerases to allow efficient lesion bypass during DNA replication.

In triple-mutant MEFs, replication forks are permanently stalled only late after UVC exposure. This indicates that some photolesions can be bypassed independently from the three Y family polymerases. Thus, the B family TLS Pol ζ or the recently described archaeal-eukaryotic primase called Primase-Polymerase may play a role in an alternate backup TLS pathway (19,50,51). Nevertheless, as persistent CPDs and (6-4)PPs are observed in the triple-deficient cells, we infer that bypass of some lesions fully depends on the three Y family Pols.

In conclusion, we have unveiled important but redundant roles for the three Y family of TLS polymerases in TLS of genomic CPD and (6-4)PP photolesions. Pol κ appears the most important backup to Pol η although, to a minor extent, Pol ι also functions as a backup. Nevertheless, also in the triple mutant most photolesions are ultimately bypassed, implicating the existence of yet other redundant pathways.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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