CHAPTER 4

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 (UVC) 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 photolesions, particularly CPDs, is Polη, a member of the evolutionary conserved Y family of translesion synthesis (TLS) DNA polymerases. Polη-deficient mouse embryonic fibroblasts (MEFs) display a defect in TLS at CPDs and (6-4)PPs and consequently an enhanced DNA damage signaling and cell cycle delay upon exposure to UVC light. However, these phenotypes are transient, suggesting redundancy with other DNA damage tolerance pathways. Here we investigated whether Y-family Pols ι and κ may act as backups for Polη in bypassing genomic CPD and (6-4)PP lesions, by using MEF lines with single and combined disruptions in these Pols. Our data demonstrate that Polκ plays a dominant role in alleviating stalling of genomic replication forks in Polη-deficient MEFs, both at CPDs and (6-4)PPs. This dampens DNA damage signaling and cell cycle arrests, and resulted in increased proliferation. Conversely, the contribution of Polι is restricted to a subset of the lesions. This study contributes to understanding the mutator phenotype of Xeroderma Pigmentosum-variant, a syndrome caused by Polη 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) at the genome (Beukers *et al.*, 2008). 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 Polη, a member of the Y family of DNA polymerases, which, in mammalian cells, also includes Pols ι, κ and Rev1 (Guo *et al.*, 2009; Sale *et al.*, 2012; Waters *et al.*, 2009). Polη is capable of containing a thymine-thymine CPD, the most frequent photolesion, in its enlarged active site (Biertumpfel *et al.*, 2010). In contrast to CPDs, (6-4)TT lesions form a poor substrate for Polη *in vitro*, as Polη frequently inserts a G opposite the 3' T but is unable to carry out the subsequent extension step (Johnson *et al.*, 2001). *In vivo*, TLS at (6-4) PP may involve either Polη or Polι, followed by extension by another polymerase, at least at an episomal substrate (Yoon *et al.*, 2010).

The importance of Polη 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 Polη (Johnson *et al.*, 1999; Masutani *et al.*, 1999). Clinically, XP-V is characterized by photosensitivity of the skin and high susceptibility to develop cancer of sunlight-exposed areas of the skin. After UVC exposure, the conversion of low molecular weight to high molecular weight nascent DNA is much slower in XP-V cells than in normal cells (Lehmann *et al.*, 1975). The TLS defect results in the accumulation of ssDNA regions that activate the Ataxia-telangiectasia-mutated and Rad3-related (Atr) kinase (Despras *et al.*, 2010; Elvers *et al.*, 2011). Activated Atr phosphorylates 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 (Feijoo *et al.*, 2001; Zachos *et al.*, 2005). Nevertheless, XP-V cells are only mildly sensitive to 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 (Maher *et al.*, 1975), 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 Polη 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 (Temviriyanukul *et al.*, 2012). We found that Polι and Polκ-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 (Jansen *et al.*, 2009; Temviriyanukul *et al.*, 2012). In addition, we observed that, similar to XP-V cells, Polη-deficient MEFs display a transient defect in TLS, resulting in the accumulation of cells in mid-S phase and

activation of DNA damage signaling (Temviriyanukul *et al.*, 2012). Mainly TLS across genomic CPDs is affected in these cells. Possibly, this transient TLS defect in the absence of Polη 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.

In previous studies the expression of multiple TLS polymerases was reduced using siRNA knock-down strategies while in these cells TLS was investigated only at lesion-containing episomal plasmids (Hendel *et al.*, 2008; Shachar *et al.*, 2009; Yoon *et al.*, 2009; Yoon *et al.*, 2010; Ziv *et al.*, 2009). 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 Polη-deficient MEFs exposed to UVC light, Polκ surprisingly 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. Polι may play a minor role in TLS of a subset of (6-4)PP.

RESULTS

An Early Role of Pols ι and κ in Photolesion Bypass

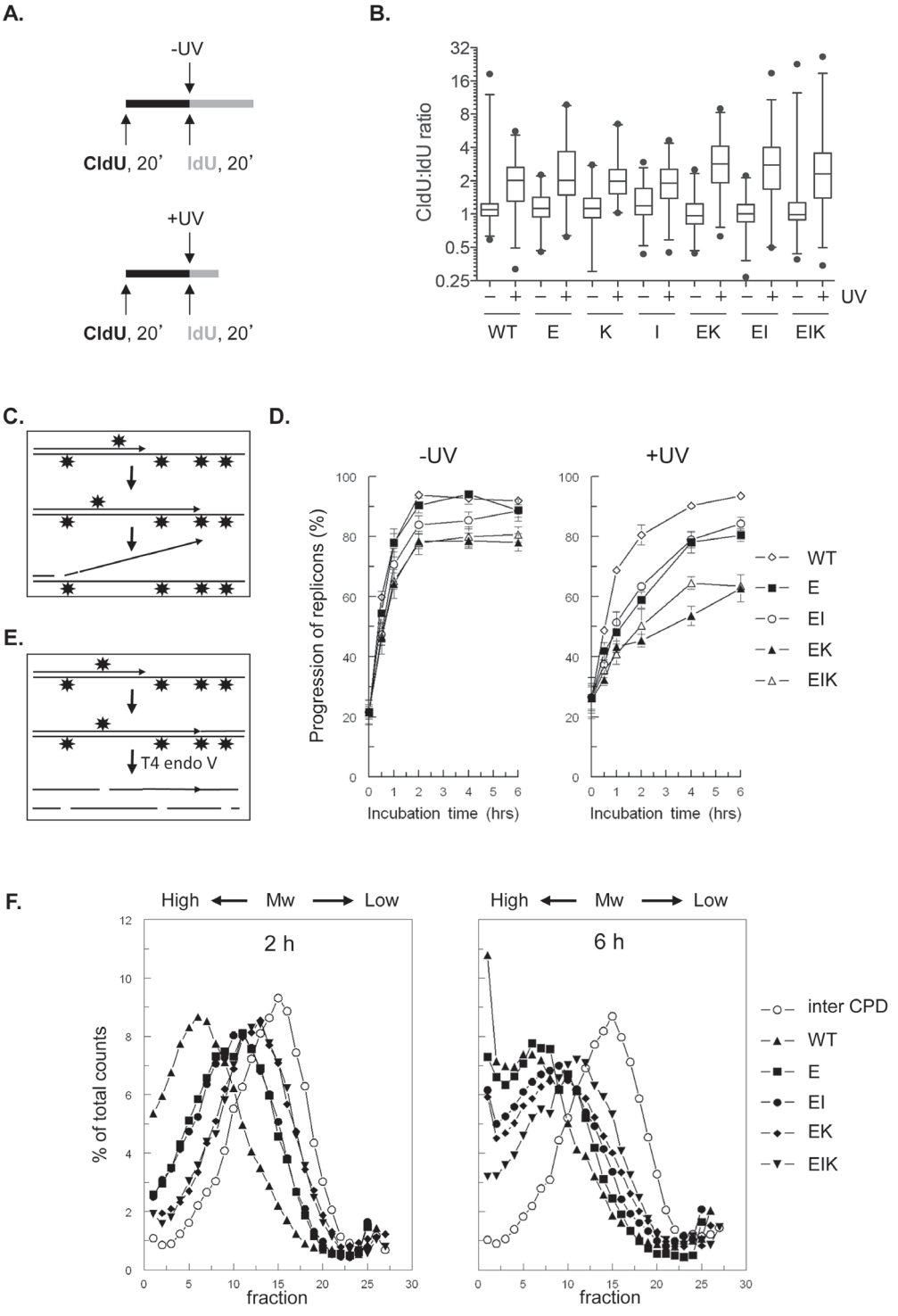
Recently, we have shown that replicative bypass of photolesions was delayed rather than abolished in Polη-deficient MEFs, suggesting the existence of a backup mechanism that almost completely rescues the Polη defect (Temviriyanukul *et al.*, 2012). Here, we tested whether two other Y-family TLS polymerases, *i.e.* Pols ι and κ, are entailed in this backup pathway. To this aim, we compared the responses of Polη-deficient MEF lines with additional deficiencies in Polι, Polκ 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/m^2 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 (Fig. 1A). When undamaged templates are replicated, no obvious differences in the replication speed amongst the cell lines were found (Supplementary Fig. S1). In addition, under mock-treated conditions all cell lines showed the expected ratio of CldU to IdU of 1, indicating that TLS Pols η, ι and κ are dispensable for replication of undamaged DNA templates (Fig. 1B). Following UVC exposure, the ratio of CldU to IdU increased from 1 to approximately 2 in wild type and single mutant Polι or Polκ-deficient MEFs. This result indicates that (i) UVC-induced DNA damage results in reduced replicon progression and (ii) TLS Pols ι and κ are not essential for photolesion

bypass at an early stage after UVC exposure (Fig. 1B). At this stage, compared with wild type and single mutant Polι or Polκ-deficient MEFs, a slightly increased spreading of the CldU to IdU ratio was observed in the Polη-mutant, suggesting reduced fork progression, specifically at damaged forks. Fork progression in the double and triple mutant MEFs was delayed to an even greater extent (Student's *t* test: $p<0.01$). These results suggest partial redundancy between these TLS polymerases, early after UVC exposure (Fig. 1B).

A Late Role of Pols ι and κ in Photolesion Bypass

To investigate redundancy between Pols η, ι and κ in TLS at photolesions more sensitively, and 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 [^{3}H]thymidine immediately prior to mock-treatment or UVC exposure (Fig. 1C). 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 (Temviriyanukul *et al.*, 2012). Interestingly, Polη-deficient MEF lines with additional defects in Polι, Polκ or both TLS Pols replicate undamaged DNA templates somewhat less efficiently compared to the Polη single-mutant cells, indicating a defect in TLS endogenous DNA lesions (Fig. 1D, left panel). Upon exposure to 5 J/m^2 UVC, the MEF line deficient for both Pols η and ι displayed a similar replication fork progression as the Polη single-mutant cells, indicating that fork progression on UV-damaged DNA in Polη-deficient MEFs does not rely on Polι. Conversely, as compared with Polη single-mutant MEFs, the MEF line deficient for both Pols η and κ displayed strongly reduced fork progression following exposure to UVC (Fig. 1D, right panel). This defective fork progression was not exacerbated by an additional deficiency for Polι in these cells. Together, these data strongly suggest that Polκ, but not Polι, can complement the Polη defect also at later time points after UVC exposure. Nevertheless, also in Polη, Polκ doubly-deficient MEFs replicons continue to progress, albeit slowly, revealing that Polκ is important but not essential for replicative bypass of photolesions in the absence of Polη.

We wanted to provide an independent approach to study the possible roles of Pols ι and κ as backup polymerases in Polη-deficient MEFs at later time points after exposure. To this aim, we utilized a sensitive alkaline sucrose gradient-based assay that measures the maturation of newly synthesized daughter strands, specifically beyond the most prevalent genomic CPD lesions, of which the density is represented by the internal ([^{14}C]thymidine-labelled) standard (Fig. 1E). As expected, the generation of nascent DNA molecules was delayed in Polη-deficient MEFs compared with wild type cells, especially at 2h after exposure (Fig. 1F). At this time point, maturation of nascent DNA in MEFs deficient for both Pols η and ι was indistinguishable from the single Polη-deficient MEFs, consistent with the alkaline DNA unwinding assay. The defect of the Polη-deficient MEFs, however, was aggravated when these cells are also deficient


A.
-UV
CldU, 20'
IdU, 20'
+UV
CldU, 20'
IdU, 20'
B.
CldU:IdU ratio
32
16
8
4
2
1
0.5
0.25
UV
WT
E
K
I
EK
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C.
E.
T4 endo V
D.
-UV
+UV
Progression of replicons (%)
Incubation time (hrs)
Incubation time (hrs)
WT
E
EI
EK
EIK
F.
High ← Mw → Low
2 h
6 h
% of total counts
fraction
fraction
inter CPD
WT
E
EI
EK
EIK

for Polκ (Fig. 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 2h after UVC exposure (Fig. 1F, left panel). We conclude that, at 2h after UVC exposure, Polκ, but not Polι, can complement for the Polη deficiency. At 6h post-exposure, the percentage of mature DNA molecules was reduced not only in the Polη+ Polκ double mutant, but also in the Polη+Polι double mutant, compared with the Polη single mutant. In the triple mutant, daughter strand maturation was reduced to an even greater extent (Fig. 1F, right panel). The specific defect in the cell lines with a Polι defect at 6h after treatment suggests that, at least in the absence of Polη, Polι is required for TLS at a non-abundant lesion type, whereas Polκ can complement for the Polη deficiency in TLS at most photolesions.

Polκ Rescues S-phase Progression of Polη-Deficient MEFs

To investigate redundancy in the roles of Polη, Polι and Polκ 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/m^2 UVC, MEFs were pulse-labeled with BrdU, immediately preceding their fixation. Subsequently, BrdU contents were analyzed by bivariate flow cytometry. Since MEFs deficient for Polι or κ display cell cycle progression similar to wild type MEFs (Temviriyanukul *et al.*, 2012), we focused on wild type MEFs and Polη-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

◀ **Figure 1 | Both Pols ι and κ are required for replicon progression in Polη-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)** Box plot depicting the ratio of lengths of CldU-labeled tracts to IdU-labeled tracts in wild type MEFs (WT) or in MEFs with single, double or triple deficiencies in Polη (E), Polι (I) and Polκ (K), mock treated (-) or exposed to 13J/m^2 UVC (+). **(C)** Scheme of the alkaline DNA unwinding assay. Nascent DNA is pulse labelled with [^{3}H]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 [^{3}H] thymidine that is locally denatured using alkaline, followed by sonication and isolation of [^{3}H]thymidine-labelled ssDNA using hydroxyl apatite (bottom). **(D)** Replication fork progression in mock-treated MEFs (left panel) and in MEFs exposed to 5J/m^2 UVC (right panel) (n=4). Error bar, SEM. **(E)** Scheme of alkaline sucrose gradient sedimentation using T4 endonuclease V. Template DNA was uniformly labelled with [^{14}C] thymidine (solid line) followed by exposure to UVC inducing CPD and (6-4)PP photolesions (triangles; top). Elongating daughter strands were pulse labelled with [^{3}H]-thymidine for 30 min (dotted line) and cultured in medium without label (dashed line; middle). At different times, cells were lysed and [^{14}C] thymidine-containing DNA was cleaved by T4 endonuclease V at a CPD, followed by size fractionation using alkaline sucrose gradients (bottom). The [^{14}C]thymidine-labelled inter-CPD size distribution serves as an internal standard, since CPDs are not removed in mouse cells. **(F)** Alkaline sucrose gradient profiles of [^{3}H]thymidine-containing DNA of wild type MEFs (WT, closed triangle), MEFs deficient in Polη (E; closed square) and of Polη-deficient MEFs containing an additional defect in Polι (EI; closed circle), Polκ (EK; closed diamond) or both TLS polymerases (EIK; closed inverted triangle) at 2 and 6h after exposure to 5J/m^2 UVC. Also the profile of [^{14}C]thymidine labelled, CPD-containing fragments is depicted (open circles). Mw, molecular weight.

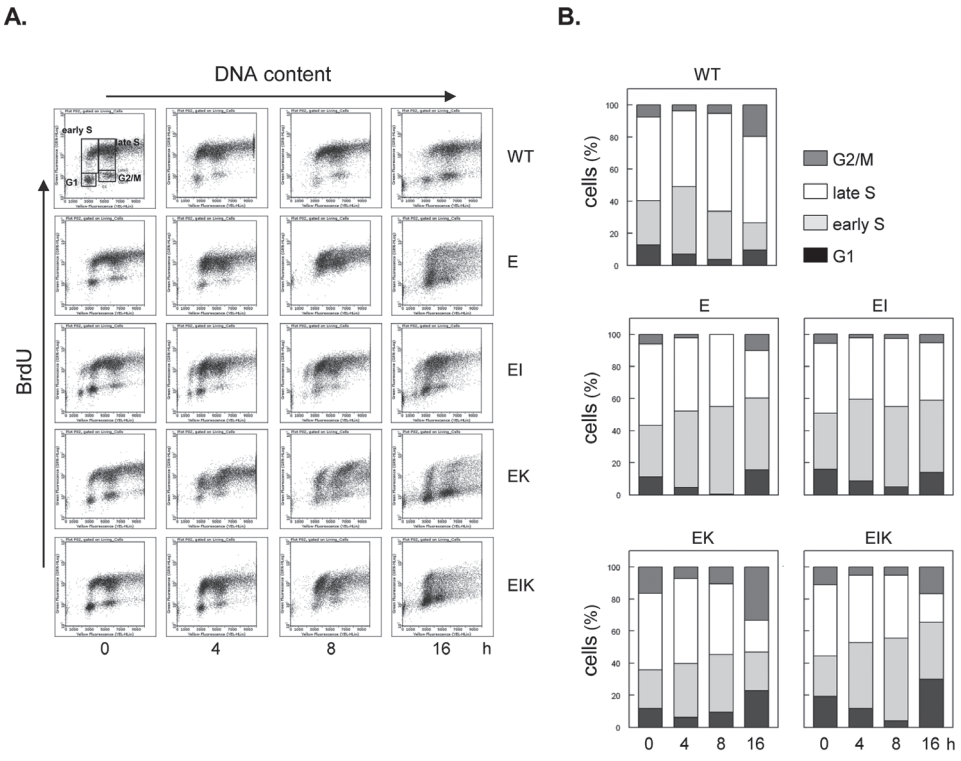

Figure 2 | The replicative activity of Polη-deficient MEFs exposed to UVC relies mainly on Polκ. (A) FACS profiles showing BrdU content of wild type MEFs (WT), MEFs deficient in Polη (E) and Polη-deficient MEFs containing an additional defect in Polι (EI), Polκ (EK) or both TLS polymerases (EIK) after exposure to 5J/m^2 UVC. Prior to fixation, MEFs were pulse labelled with BrdU for 30 min, immediately or at 4, 8 and 16h 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 16h after UVC exposure.

a similar fashion in the absence of photolesions (Supplementary Fig. S2). Exposure of cells to UVC, however, revealed marked differences in cell cycle distribution between the MEF lines (Figs. 2A and B). More specifically, compared with UVC-exposed wild type MEFs, Polη-deficient MEFs displayed increased levels of (early) S phase cells containing low-to-intermediate levels of BrdU (Figs. 2A and B). This phenotype was unaltered when also Polι was disrupted indicating that Polι does not serve as a quantitatively important backup to Polη (Figs. 2A and B). In both Polη-deficient MEFs and MEFs double deficient for Polη and Polι this response was aggravated by the additional deficiency for Polκ (Figs. 2A and B). Together, these results suggest that S phase progression is perturbed in the absence of Polη, both early (Fig. 1) and late after UVC exposure (Figs. 2A and B). The residual S phase progression in these cells strongly depends on Polκ, rather than on Polι.

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 (Lundin *et al.*, 2002; Petermann *et al.*, 2010; Saintigny *et al.*, 2001). 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 ($ATM^{S1981\text{-}P}$) is an early step in the response to dsDNA breaks (Shiloh *et al.*, 2013). Activation of Atm leads to phosphorylation of Kap1 at S824 ($Kap1^{S824\text{-}P}$), although the formation of $Kap1^{S824\text{-}P}$ can also be mediated by other phosphatidylinositol-3 kinase-like kinases, including Atr (White *et al.*, 2006). Except for the Rev1-deficient MEFs, we found only a minor UVC-dependent increase of $ATM^{1981\text{-}P}$ foci in nuclei of all other MEF lines tested, up to 8h after UVC exposure (Figs. 3A and B), suggesting that only few forks collapse at the UVC dose used ($5J/m^2$). Interestingly, strong induction of $Kap1^{S824\text{-}P}$ was found in all Polη-defective MEF lines and in MEFs deficient for Rev1, already 2h after UVC exposure (see also below). Of note, with the exception of the Rev1-deficient MEFs, $Kap1^{S824\text{-}P}$ levels did not increase beyond 2h after exposure, in agreement with the residual photolesion bypass in these cell lines (Fig. 1).

Quenching of The UV-Induced DNA Damage Response Requires Pols η, ι and κ

We stained the cell lines for phosphorylation of Chk1 ($Chk1^{S345\text{-}P}$) a target for Atr-induced DNA damage signaling at ssDNA tracts (Cimprich *et al.*, 2008). Thus, at different time points prior to staining, cells were exposed to 5 J/m^2 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 (Jansen *et al.*, 2009; Temviriyanukul *et al.*, 2012). At 2h after exposure, all MEF lines displayed $Chk1^{S345\text{-}P}$-positive cells among EdU-positive (replicating) cells. The intensity of $Chk1^{S345\text{-}P}$ 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 Polι or Polκ (Figs. 4A and B). Furthermore, it should be noted that the extent of $Chk1^{S345\text{-}P}$ correlated with that of $Kap1^{S824\text{-}P}$ in the different MEF lines, although $Kap1^{S824\text{-}P}$ is restricted to a subset of EdU-positive cells (compare Figs. 4A, B with Figs. 3C, D). This result suggests that the formation of $Kap1^{S824\text{-}P}$ 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 $Chk1^{S345\text{-}P}$ (see Figs. 4C and D). These results are in agreement with the pronounced replicon stalling in these MEF lines as observed in the replication assays (Fig. 1). At 8h 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 (Figs. 4C and D). In

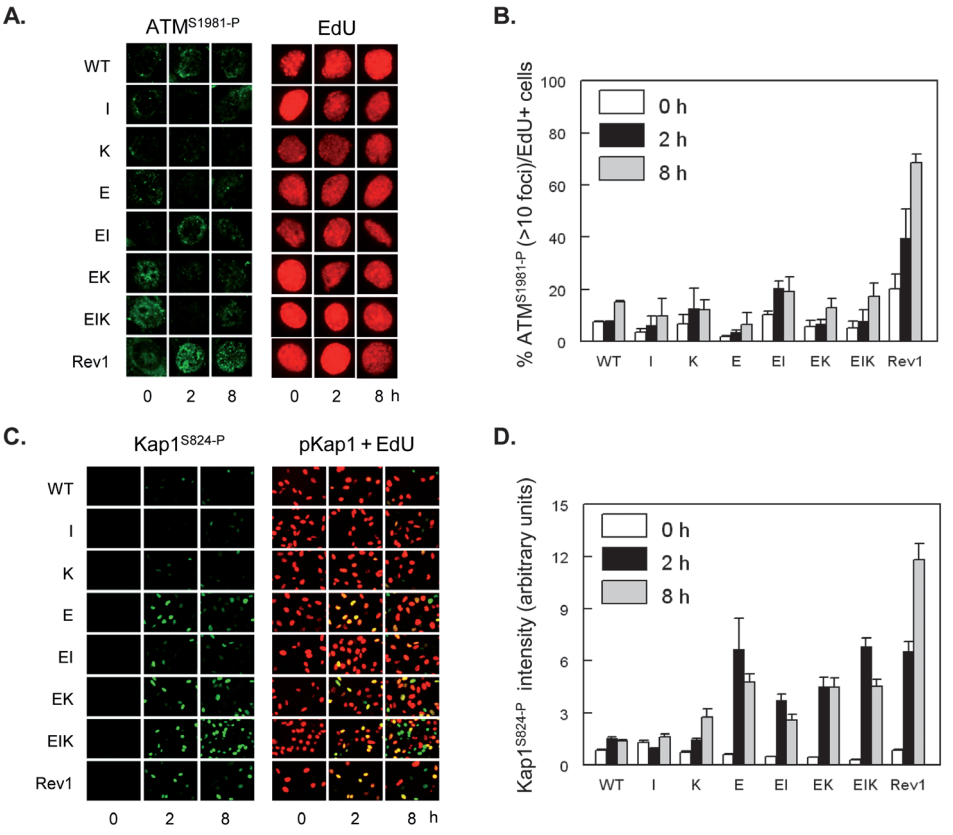

Figure 3 | Few UVC-induced dsDNA breaks in Polη-deficient MEFs with or without additional deficiencies in Pols ι and κ. (A) Wild type MEFs (WT), MEFs with single, double or triple deficiencies in Polη (E), Polι (I) and Polκ (K), or MEFs deficient in Rev1 (Rev1) were pulse labelled with EdU for 30 min, prior to exposure to 5J/m^2 UVC. Then, MEFs were fixed at 0, 2 and 8h after treatment and immunostained for ATM$^{S1981-P}$ (green, left panel) in replicating, EdU-incorporating MEFs (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^{S821-P} (left panels, green) in replicating, EdU-incorporating MEFs (right panels, merge of staining for Kap1^{S821-P} (green) + EdU (red)). **(D)** Quantification of the intensity of Kap1^{S821-P} signal in EdU-positive MEFs. Error bars, SEM.

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.

Polκ Protects Polη-Deficient MEFs from UVC Toxicity

To study the biological consequences of prolonged replication fork stalling, enhanced DNA damage signaling and impaired cell cycle progression, caused by defects in multiple Y family Pols, we analyzed cell proliferation at 3 days after exposure to various

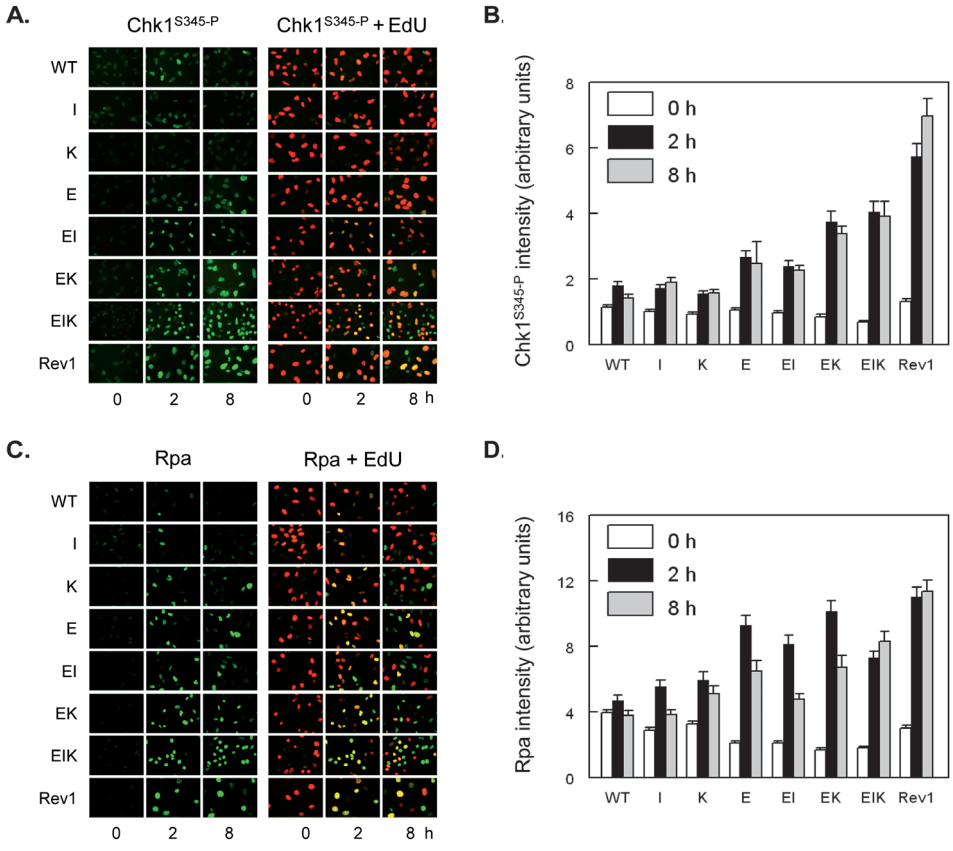

Figure 4 | Quenching of DNA damage responses to photolesions requires Pols η, ι and κ. (A) Wild type MEFs (WT), MEFs with single, double or triple deficiencies in Polη (E), Polι (I) and Polκ (K), or MEFs deficient in Rev1 (Rev1) were pulse labelled with EdU for 30 min, prior to exposure to 5J/m^2 UVC. Then, MEFs were fixed at 0, 2 and 8h after treatment and immunostained for Chk1^{S345-P} (left panels, green) in replicating, EdU-incorporating MEFs (right panels, merge of staining for Chk1^{S345-P} (green) and EdU (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, green). **(D)** Quantification of the intensity of Rpa staining in EdU-positive MEFs. Error bars, SEM.

doses of UVC. Amongst 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 Polη-deficient MEFs and MEFs deficient for both Pols η and ι showed an intermediate UVC sensitivity (Fig. 5). Confirming previous observations (Temviriyanukul *et al.*, 2012), the MEF line deficient for Polκ was slightly more sensitive to UVC light than wild type MEFs, whereas Polι-deficient MEFs displayed no increased UVC sensitivity (Fig. 5). These results are again consistent with an important role for Polκ as a backup TLS polymerase for Polη.

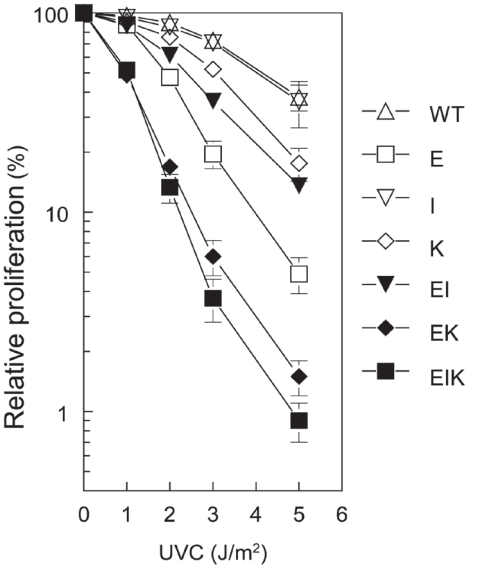

Figure 5 | Effect of UVC on proliferation of MEFs with single, double or triple deficiencies in Pols η, ι and κ. MEFs were exposed to different doses of UVC and 3 days later the number of cells was assessed. The number of unexposed MEFs was set at 100%. Error bars, SEM.

Genomic CPDs are Substrates for Pols ι and κ in Polη-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 Polη-deficient MEFs, mainly CPDs cause stalling of replication forks (Temviriyanukul *et al.*, 2012). We applied this methodology to the current set of MEF lines to study which genomic photolesions are causing the phenotypes associated with MEFs 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/m^2 UVC. At 2h or 8h 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 Polι or k, indicating efficient bypass across genomic CPDs, independent of Pols ι and κ (Fig. 6A). As expected, EdU-positive MEFs that are deficient for Polη displayed unreplicated CPDs at 2h and less at 8h after UVC exposure (Fig. 6A and (Temviriyanukul *et al.*, 2012)), suggesting transient fork stalling at CPDs. Similar results were observed for Polη mutant MEFs with an additional deficiency for Polι (Fig. 6A). MEFs deficient for both Pols η and κ displayed more EdU+CPD positive cells at 8h upon UVC exposure, indicating that Polκ does perform TLS at CPDs in the absence of Polη. Nevertheless, EdU-positive triple-mutant MEFs exhibited the most pronounced staining for unreplicated CPDs at 8h after UVC exposure (Fig. 6A). This

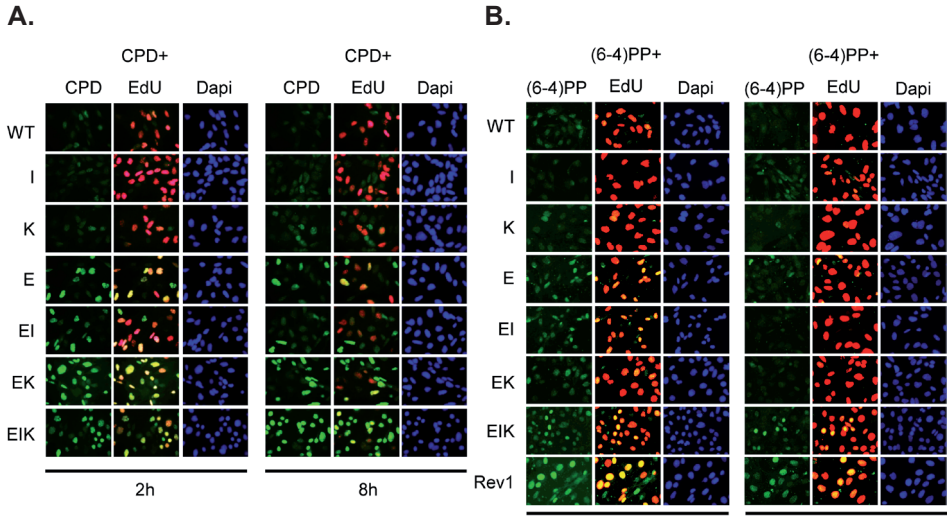

Figure 6 | Pols ι and κ are required for efficient bypass of photoproducts in MEFs deficient in Polη. (A) Wild type MEFs (WT) or MEFs with single, double or triple deficiencies in Polη (E), Polι (I) and Polκ (K) were pulse labelled with EdU for 30 min, prior to exposure to 5J/m^2 UVC. Two and eight hours later, MEFs were fixed and immunostained for CPD (green; upper panels) in ssDNA of nuclei (blue; lower panel) in replicating, EdU-incorporating (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 (green; upper panel). Rev1-deficient MEFs (Rev1) were included as a positive control (Jansen *et al.*, 2009; Temviriyanukul *et al.*, 2012).

result suggests that, in the absence of Polη, Polι can perform TLS at CPDs, but only when also Polκ 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 outwards and unable to engage in base-pairing (Rastogi *et al.*, 2010). 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 (Jansen *et al.*, 2009). Immunostaining of unreplicated (6-4)PPs in EdU-positive cells revealed that TLS of (6-4)PPs appeared perturbed in MEF lines defective in Polη, as judged by the EdU-positive cells staining for single-stranded (6-4)PPs, at 2h after UVC exposure of these cells (Fig. 6B). Nevertheless, the defect was less pronounced than in MEFs deficient for Rev1 [(Jansen *et al.*, 2009) and Fig. 6B)]. Staining for unreplicated (6-4)PPs was also found for double and, to a greater extent, triple mutant MEFs, at 2 and 8 hours after UVC exposure (Fig. 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 Polη, 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 Polη, Polκ plays a more important role than Polι in responses to genomic photolesions. In support, we and others have shown that Polκ (but not Polι)-deficient mammalian cells are slightly sensitive to UVC light (Ogi *et al.*, 2002; Schenten *et al.*, 2002; Temviriyanukul *et al.*, 2012). Although some studies attribute this sensitivity to a defect in NER, at least outside of S phase (Ogi *et al.*, 2006; Ogi *et al.*, 2010), others, using siRNA strategies, provide evidence for a role of Polκ in TLS of a T.T CPD on episomal substrates *in vivo* (Yoon *et al.*, 2009; Ziv *et al.*, 2009) while no effect was found on TLS across TT (6-4)PP (Yoon *et al.*, 2010). Indeed, Polκ can extend from a nucleotide inserted across 3'Ts of TT CPDs by another DNA polymerase (Washington *et al.*, 2002). This TLS-related function of Polκ 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. The role of Polκ as backup for Polη is not restricted to UV lesions, since also in somatic hypermutation of Immunoglobulin genes Polκ acts as backup in the absence of Polη (Faili *et al.*, 2009).

Polι plays only a minor role in TLS at genomic photolesions in Polη-deficient cells, which only 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 Polι is essential for TLS across some UVC-induced DNA lesions in the absence of both Polη and Polκ. In support, purified Polι can replicate TT (6-4)PPs (Haracska *et al.*, 2001; Vaisman *et al.*, 2003; Zhang *et al.*, 2001) and Polι mediates part of the mutagenicity of (6-4)PP *in vivo* (Dumstorf *et al.*, 2006; Yoon *et al.*, 2010). In addition to a subset of (6-4)PPs, also some CPDs might be candidates for Polι-mediated TLS in MEFs deficient for both Pols η and κ. Indeed, Polι is capable to inserting nucleotides opposite TT CPDs *in vitro* (Haracska *et al.*, 2001; Vaisman *et al.*, 2003; Zhang *et al.*, 2001) although CPDs on an episomal substrate are only poorly bypassed by Polι in human (Polη-deficient) XP-V cells (Yoon *et al.*, 2009; Ziv *et al.*, 2009). 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 (Brash, 1988; Lippke *et al.*, 1981; Mitchell *et al.*, 1992; Rastogi *et al.*, 2010; Tornaletti *et al.*, 1993). Moreover, as these lesion types are structurally highly dissimilar (Rastogi *et al.*, 2010), 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 Polζ or the recently described archaeal-eukaryotic primase called Primase-Polymerase may play a role in an alternate backup TLS pathway (Bianchi *et al.*, 2013; Mouron *et al.*, 2013; Yoon *et al.*, 2009). 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. Polκ appears the most important backup to Polη although, to a minor extent, Polι also functions as backup. Nevertheless, also in the triple mutant most photolesions are ultimately bypassed, implicating the existence of yet other redundant pathways.

MATERIALS & METHODS

Cell culture. MEFs lacking Polη, Polι or Polκ were isolated from day 13.5 embryos of Polη, Polι or Polκ-deficient mice (Delbos *et al.*, 2005; Schenten *et al.*, 2002); Aoufouchi *et al.*, in preparation). Crossings between Polη, Polι and Polκ-deficient mice produced 13.5-day embryo that were doubly-deficient for Polη and Polι, for Polη and Polκ 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 (Jansen *et al.*, 2009). All MEF lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4,5 g/liter glucose, Glutamax and pyruvate (Invitrogen) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) (MEF medium) at 37°C in a humidified atmosphere containing 5% CO_2.

DNA fiber analysis. Per well of a 6-well plate, $7.5x10^4$ MEFs were seeded and cultured overnight in MEF medium. Prior to UVC exposure (13 J/m^2), MEFs were incubated in medium containing 25µM 5-Chloro-2'-deoxyuridine (CldU) for 20 min at 37°C. After UVC exposure, medium containing 500µM 5-Iodo-2'-deoxyuridine (IdU) was added, resulting in a final concentration of 250µM IdU and 12.5µM CldU. After 20 min at 37°C, cells were trypsinized, 2µl of a suspension of $3x10^5$ MEFs/ml was spotted onto a microscope slide, incubated for 5 min and lysed with 7µl lysis buffer (200mM Tris-HCl pH7.4, 50mM EDTA, 0.5% SDS) for 3 min. Slides were tilted to 15^0 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 2.5M 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), 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). Finally, slides were mounted in Fluoro-Gel (Electron Microscopy Sciences). Microscopy was performed using a fluorescent microscope (Zeiss).

Alkaline DNA unwinding (ADU). This assay, which measures progression of replicons (Johansson *et al.*, 2004) was performed with minor modifications. The procedure is outlined in Fig. 1C. Per well, $5x10^4$ MEFs were plated in a 24-well plate and cultured overnight in MEF medium. After pulse labeling with [^{3}H]thymidine for 15 min, MEFs were washed once with PBS and subsequently exposed to 5 J/m^2 UVC or mock-treated. Then, at indicated times, DNA at replication forks was locally denatured upon incubation of MEFs with ice-cold denaturation solution (0.15M NaCl and 0.03M NaOH) for 30 min. The denaturation of DNA was terminated by adding ice-cold 0.02M NaH_2PO_4. After sonication, SDS was added to a final concentration

of 0.25% and the samples were stored at -20°C for at least 16h. After thawing, the lysates were loaded onto hydroxyl apatite columns to elute ssDNA using 0.1M K_2HPO_4 (pH6.8) and dsDNA using 0.3M K_2HPO_4 (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/m^2 UVC was determined by a sensitive variant of the alkaline sucrose sedimentation assay as described previously (van Zeeland *et al.*, 1981). The procedure is outlined in Fig. 1E.

Immunostaining. MEFs were cultured overnight on coverslips, incubated with 10μM 5-ethynyl-2'-deoxyuridine (EdU) (Invitrogen) in MEF medium for 30 min and subsequently exposed to UVC irradiation (5 J/m^2). At indicated times after UVC treatment, cells were fixed and permeabilized as follows: for detection of Rpa, Chk1$^{S345\text{-}P}$ and Kap1$^{S824\text{-}P}$, cells were pre-extracted and permeabilized by 0.3% Triton-X in CSK buffer pH7.2-7.5 (10mM HEPES pH7.4, 100mM NaCl, 3mM $MgCl_2$, 0.3% triton-X100, 300mM sucrose) for 2 min on ice and immediately fixed with 3.7% Paraformaldehyde for 20 min; for detection of ATM$^{S1981\text{-}P}$, 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 non-specific binding, and subsequently incubated overnight with antibodies against Rpa (Cell Signaling), Chk1$^{S345\text{-}P}$ (Cell Signaling), Kap1$^{S824\text{-}P}$(Bethyl Laboratory) or ATM$^{S1981\text{-}P}$ (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-conjuagated azide was used according to the manufacturer's recommendation (Click-iT™ Edu imaging kit, Invitrogen). Samples were mounted (Vectashield, Vector laboratories), and images were acquired by wide field fluorescent microscopy (Axioplan M2, Carl Zeiss). Fluorescence intensity and numbers of foci were quantified using ImageJ software (National Institutes of Health). Between 90-135 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 (Jansen *et al.*, 2009), 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/m^2 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 (Temviriyanukul *et al.*, 2012).

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 mm) as described previously (Temviriyanukul *et al.*, 2012).

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SUPPLEMENTARY FIGURES

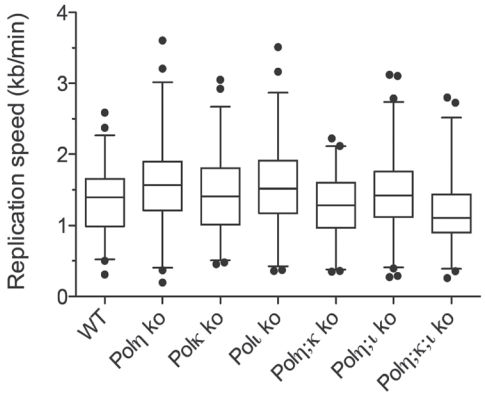

Figure S1 | MEFs with deficiencies in different TLS polymerases display similar replication speed. Lengths of CldU-labeled tracts in DNA fibers of unexposed wild type MEFs (WT) and MEFs with single, double or triple deficiencies in Polη (η), Polι (ι) and Polκ (κ) were determined. Then, the replication speed was calculated and depicted as box plots.

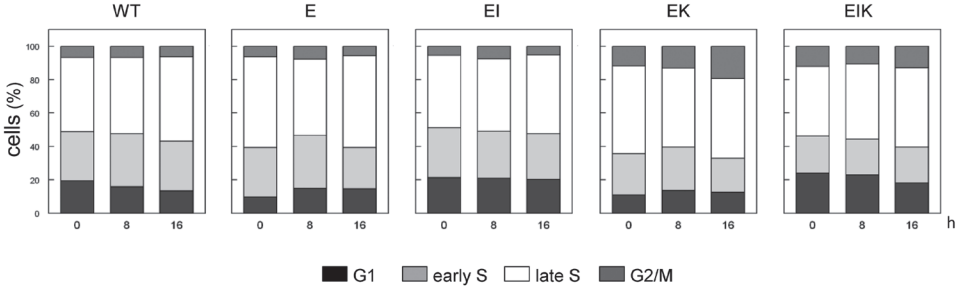

Figure S2 | MEFs with deficiencies in different TLS polymerases proliferate in a similar fashion in the absence of Photolesion. FACS profiles were generated of wild type MEFs (WT), MEFs deficient in Polη (E) and Polη-deficient MEFs containing an additional defect in Polι (EI), Polκ (EK) or both TLS polymerases (EIK) that were pulse labelled with BrdU for 30 min, immediately or at 4, 8 and 16h after mock treatment. BrdU incorporation was determined by immunostaining and DNA content was measured using propidium iodide. The percentage of cells in different cell cycle stages was determined, up to 16h after mock treatment.

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