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Simultaneous Disruption of Two DNA Polymerases, Polη and Polζ, in Avian DT40 Cells Unmasks the Role of Polη in Cellular Response to Various DNA Lesions

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Abstract

Replicative DNA polymerases are frequently stalled by DNA lesions. The resulting replication blockage is released by homologous recombination (HR) and translesion DNA synthesis (TLS). TLS employs specialized TLS polymerases to bypass DNA lesions. We provide striking in vivo evidence of the cooperation between DNA polymerase η, which is mutated in the variant form of the cancer predisposition disorder xeroderma pigmentosum (XP-V), and DNA polymerase ζ by generating POLη−/−/POLζ−/− cells from the chicken DT40 cell line. POLζ−/− cells are hyperpersensitive to a very wide range of DNA damaging agents, whereas XP-V cells exhibit moderate sensitivity to ultraviolet light (UV) only in the presence of caffeine treatment and exhibit no significant sensitivity to any other damaging agents. It is therefore widely believed that Polζ plays a very specific role in cellular tolerance to UV-induced DNA damage. The evidence we present challenges this assumption. The phenotypic analysis of POLη−/−/POLζ−/− cells shows that, unexpectedly, the loss of Polη significantly rescued all mutant phenotypes of POLζ−/− cells and results in the restoration of the DNA damage tolerance by a backup pathway including HR. Taken together, Polη contributes to a much wide range of TLS events than had been predicted by the phenotype of XP-V cells.

Introduction

DNA replication involves a rapid but fragile enzymatic mechanism that is frequently stalled by damage in the DNA template. To complete DNA replication, DNA lesions are bypassed by specialized DNA polymerases, a process called translesion synthesis (TLS) (reviewed in [1,2]). A number of TLS polymerases, including Polη and Polζ, that are conserved throughout eukaryotic evolution, have been identified in yeast and mammals. Polη deficiency is responsible for a variant form of xeroderma pigmentosum (XP-V) [3,4] that is characterized by UV photosensitivity and a predisposition to skin cancer (reviewed in [5]). Deficiency of Rev3, the catalytic subunit of Polζ, results in a considerably more severe phenotype, compared with Polη. In fact, Rev3 disruption is lethal to mouse embryogenesis [6]. Chicken DT40 cells deficient in Rev3 exhibit significant chromosome instability and hypersensitivity to a wide variety of DNA-damaging agents [7–10]. In addition to their role in TLS, both Polη and Polζ can contribute to homologous DNA recombination (HR) in DT40 cells [9,11,12].

Exposure to UV induces cyclobutane pyrimidine dimers (CPDs) and 6-4 UV photoproducts in DNA. While Polη can efficiently and accurately bypass CPDs [4,13–15], no single DNA polymerase has been shown to be capable of effectively bypassing 6-4 UV photoproducts in vitro. This suggests that the coordinate use of more than one polymerase is required to bypass such damage in vitro. In support of this notion, several biochemical studies have suggested that lesion bypass can be effected by two sequential nucleotide incorporation events [16,17]. For example, to bypass the 6-4 UV photoproduct, Polη inserts a nucleotide opposite the damage as a first step, followed by extension from the inserted nucleotide as a second step. This extension process has been shown to be catalyzed by yeast Polζ and by human Polκ [1,2,18]. The contribution of mammalian Polζ to the extension step remains elusive, because functional Polζ has not been purified [19,20]. Recently, replication of episomal plasmid DNA carrying various lesions was analyzed in mammalian cell lines to define the role of each DNA polymerase in TLS past individual DNA lesions [15,21,22]. Shachar et al. suggest the sequential usage of Polη and Polζ in TLS past the cisPt-GG
Author Summary

DNA replication is a fragile biochemical reaction, as the replicative DNA polymerases are readily stalled by DNA lesions. The resulting replication blockage is released by translesion DNA synthesis (TLS), which employs specialized TLS polymerases to bypass DNA lesions. There are at least seven TLS polymerases known in vertebrates, Polη and Polζ in the unique genetic model organism, chicken DT40 cells. Currently, it is widely believed that Polη plays a very specific role in cellular tolerance to ultraviolet light–induced DNA damage. Polζ, on the other hand, plays a key role in cellular tolerance to a very wide range of DNA–damaging agents, as Polζ and Polζ cells are hypersensitivity to a number of DNA damaging agents. Our phenotypic analysis of Polζ/Polζ cells shows that, unexpectedly, the loss of Polη significantly rescued all mutant phenotypes of Polζ/Polζ cells. The genetic interaction shown here reveals a previously unappreciated role of human Polη in cellular response to a wide variety of DNA lesions and two-step collaborative action of Polymerase η and ζ.

Results

Deletion of Polη reversed the hypersensitivity of the polζ mutant to UV, ionizing radiation, MMS, and cisplatin

We generated polζ/polζ cells by inactivating both REV3 alleles of the polζ DT40 cells using a previously published gene-targeting strategy [Figure 1A] [9,12]. The growth properties of the mutant cells were examined by measuring their growth rate and cell-cycle profile. As reported previously, the polζ cells had a normal growth rate, whereas the polζ cells proliferated more slowly, exhibiting an increase in the sub-G1 fraction, indicative of spontaneous cell death during the cell cycle (Figure 1C). The loss of Polζ caused a significant increase in the number of spontaneous arising γH2AX foci, which represent replication collapse (Figure S1). Interestingly, deletion of Polη in the polζ cells reversed their growth retardation and reduced the rate of cell death (Figure 1B and 1C). Similarly, the number of spontaneous chromosomal aberrations was significantly reduced in polζ/polζ cells, compared with polζ cells (Table 1). Ectopic expression of human Polη in polζ/polζ cells diminished their growth rate to the level of polζ cells. This observation does not reflect general toxicity of the overexpressed human Polη, since its ectopic expression caused pronounced growth retardation only in the polζ/polζ double mutant but not in wild-type cells (Figure S2). These observations indicate that the growth defect of polζ cells is dependent on the presence of Polη.

The sensitivity of polζ/polζ cells to genotoxic stresses was evaluated using a colony formation assay. polζ cells showed a mild sensitivity to UV but not to the other genotoxic stresses, in agreement with the phenotype of mammalian XP-V cells [29,30]. In contrast, polζ cells showed a marked sensitivity to UV, ionizing radiation, cis-diaminedichloroplatinum-II (cisplatin), and methylmethane sulfonate (MMS) (Figure 2), as previously described [9]. The polζ/polζ mutant cells were less sensitive to UV than were the polζ cells. Furthermore, this double mutant showed significantly increased tolerance to ionizing radiation, cisplatin, and MMS, compared with the polζ cells. This increased tolerance of the polζ/polζ cells was reversed by ectopic expression of human Polη. To investigate whether this reversion depended on the polymerase activity of human Polη, we expressed human Polη cDNA carrying D115A/E116A mutations in the polζ/polζ cells. These mutations in the catalytic site abolish polymerase activity (data not shown), as previously reported in yeast [31]. The expression of the mutant Polη had no effect on the sensitivity of the polζ/polζ cells to the DNA-damaging agents (Figure 2), indicating that Polη–dependent DNA synthesis sensitizes polζ cells to these DNA-damaging agents.

Polζ cells have a more prominent defect in TLS past abasic site than polζ/polζ cells

We wished to investigate if Polη and Polζ could collaborate in TLS past specific types of DNA damage. To this end, we performed two sets of experiments: analysis of immunoglobulin hypermutation, which in DT40 provides a readout of the bypass of abasic sites [24], and analysis of the replication of a T-T (6-4) photoproduct on an episomal plasmid [28].

To induce Ig hypermutation, we overproduced AID in DT40 cells using a retrovirus vector [32,33]. This vector drives the monocistronic expression of AID and green fluorescent protein (GFP), allowing a comparative assessment of the level of ectopic AID expression. At 24 hours after infection with the AID retrovirus, virtually all cells from each line displayed a strong GFP signal, indicating that the deficiency of Polη and Polζ did not affect the expression of AID (Figure 3A). However, a substantial fraction of the polζ cells died at day 3, and with the surviving polζ cells
cells at day 10 showed a decrease level of GFP signals (Figure 3A and 3B). Furthermore, pol\textsubscript{c} cells, but not pol\textsubscript{h} or pol\textsubscript{h}/pol\textsubscript{c} cells, displayed prominent chromosomal breaks at day 3 post-infection (Figure 3C). Since the break sites on the chromosome were randomly distributed, the overexpressed AID protein may be targeting a number of different loci in addition to the Ig locus, in DT40 cells. These observations suggest that TLS past abasic sites created by the combined action of AID and uracil glycosylase may...
Table 1. Frequency of spontaneous chromosomal aberrations in wild-type, pol\(\zeta\), pol\(\eta\), and pol\(\eta\)/pol\(\zeta\) double mutants.*

| Cell          | No. of cells analyzed | Chromatid Gaps | Chromatid Breaks | Isochromatid Gaps | Isochromatid Breaks | Total (per cell ± SE) |
|---------------|-----------------------|----------------|-------------------|-------------------|---------------------|-----------------------|
| Wild-type     | 100                   | 0              | 1                 | 1                 | 0                   | 2 (0.02 ± 0.01)       |
| pol\(\zeta\)  | 100                   | 0              | 2                 | 2                 | 8                   | 12 (0.12 ± 0.03)      |
| pol\(\eta\)   | 100                   | 1              | 0                 | 0                 | 0                   | 1 (0.01 ± 0.01)       |
| pol\(\eta\)/pol\(\zeta\) | 100       | 0              | 0                 | 0                 | 2                   | 2 (0.02 ± 0.01)       |
| pol\(\eta\)/pol\(\zeta\) + wild-type Pol\(\eta\) | 100 | 0 | 6 | 1 | 7 | 14 (0.14 ± 0.04) |
| pol\(\eta\)/pol\(\zeta\) + mutant Pol\(\eta\) | 100 | 0 | 2 | 2 | 0 | 4 (0.04 ± 0.02) |

*For each preparation, 100 metaphase spreads were analyzed. The number of aberrations per cell ± SE was calculated as \(x/N\), where \(x\) is the number of cells analyzed and \(N\) is the number of total aberrations, based on the Poisson distribution of chromosomal aberrations. The number of cells analyzed and total aberrations were defined as \(N\) and \(x\), respectively.

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Figure 2. Increased tolerance of pol\(\eta\)/pol\(\zeta\) cells to various genotoxic stresses, compared with pol\(\zeta\) cells. Colony survival of the indicated genotypes following the indicated genotoxic stresses. Data for the pol\(\eta\)/pol\(\zeta\) #1 clone are shown in the other figures. Error bars show the SD of the mean for three independent assays.

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pol\_f cells, but not pol\_g or pol\_g/pol\_f cells, are hypersensitive to AID overexpression. (A) Reduced AID transgene expression over time in pol\_f cells. The expression of AID was estimated by measuring the intensity of the GFP signal. Filled and open distributions represent GFP intensity at days 1 and 10 post-infection, respectively. (B) Accumulation of dead cells in the pol\_f mutant population 3 days after the AID virus infection. The dot plot shows cells stained with PI. The percentage of dead cells falling into the indicated gate is shown in the histogram. (C) Chromosomal aberrations are induced by AID overexpression in pol\_f mutants. At day 3 post-infection, chromosomal aberrations in mitotic cells of the indicated genotype were analyzed. Fifty metaphase spreads were measured. The number of aberrations per cell ± SE was calculated as x/N ± \sqrt{x/N}, where N and x are the numbers of cells analyzed and total aberrations, respectively.

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be performed less effectively in polδ cells, compared with polδ/polε or polγ cells, resulting in chromosome breakage and cell death.

To verify that Polη-dependent DNA synthesis was toxic to the AID-overproducing polδ cells, we reconstituted polδ/polε cells with either wild-type POLη or the catalytically inactive mutant polδ(D115A/E116A). Wild-type POLη expression sensitized the polδ/polε cells to the overexpression of AID, whereas the mutant POLη had no impact on cell survival (Figure 3A and 3B). We thus conclude that, in polδ cells, TLS past abasic sites may be less effective because neither Polη nor any other polymerase can extend DNA synthesis following the Polη-mediated insertion of nucleotides opposite the abasic site.

We have shown that AID overexpression results in increased TLS-mediated hypermutation at G/C base pairs in Ig V segments [32]. To define the role of Polη and Polε in this TLS process, we determined the Ig V<sub>δ</sub> nucleotide sequences of AID-overexpressing wild-type, polδ, and polδ/polε cells. polε cells were not analyzed because of the difficulty of ectopically expressing AID to the same extent as in the other lines. The number of non-templated point mutations (PM, Figure 4A) was somewhat lower in polδ cells than in wild-type cells (This slight reduction is not significant (p = 0.15, t-test) [32]). Surprisingly, the level of Ig V mutations in polδ/polε cells was comparable to that of wild-type cells. This observation is in marked contrast with the fact that Rev1, an essential factor for the function of Polε [20], plays a critical role in non-templated point mutations at the abasic site [34]. Moreover, Polε played the critical role in cellular tolerance to AID-mediated abasic sites (Figure 3). These observations indicate that in the absence of both Polη and Polε, other unidentified DNA polymerase(s) can participate in TLS past the abasic site. As the polδ/polε cells displayed a significant increase in the proportion of G/C to A/T transitions in the non-templated Ig V mutations (12/25; 48%), compared with wild-type cells (2/18; 11%) (Figure 4B), this unidentified DNA polymerase(s) may preferentially incorporate adenine opposite the abasic site in the absence of both Polη and Polε.

**Lack of TLS past the T-T (6-4) UV photoproduct in polε cells is reversed by the inactivation of Polη**

T-T (6-4) UV photoproducts represent the most formidable challenge to DNA replication, as they potently arrest replicative polymerases [35]. To analyze TLS past a T-T (6-4) photoproduct, we transfected two plasmids, pQTs and pQTo, [28] (Figure 5) carrying T-T (6-4) UV photoproducts into DT40 cells. At two days after transfection we recovered only replicated copies, and were thus able to analyze the replication of site-specific T-T (6-4) UV photoproducts in vivo. This photoproduct can be arranged in one of two ways. In the staggered conformation (pQTs) (Figure 5A), the lesions are separated by 28 intervening nucleotides and placed opposite a GpC mismatch. Replicated copies can thus result from TLS on the top or bottom strand. Error-free template switching should result in GpC at the site of the photoproduct, while TLS

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**Figure 4. TLS-dependent point mutations in Ig V occur with comparable frequency in wild-type and polδ/polε cells.** (A) Rate of non-templated single-base substitutions (PM), ambiguous single base substitutions (Amb) (which are single base changes that have a potential donor sequence in the pseudogene array), and bona fide gene conversion tracts (GC) in the AID-virus-infected wild-type, polδ, and polδ/polε DT40 cells shown in Figure 3. Cells were clonally expanded for two weeks. Three clones were analyzed in each data set. Note that the Amb mutation at A/T pairs represents short-tract gene conversion [32]. (B) Nucleotide substitution preferences (as a percentage of the indicated number in the total mutations) deduced from the V<sub>δ</sub> sequence analysis of the clones shown in (A). The preferences are shown for mutations categorized as non-templated base substitutions (PM), which are caused by TLS past abasic sites [32].

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Figure 5. TLS past a T-T (6-4) UV photoproduct on episomal plasmid DNA. (A) Schematic of the staggered arrangement of T–T (6–4) UV photoproducts in the construct pQTs. The dinucleotide GC is placed opposite each lesion, and there are 28 bp between the lesions, with the possible outcomes of DNA replication over the area. TLS may occur on either the top or the bottom strand, with the most common base insertion shown as AA. Alternatively, the nascent strand of the sister chromatid may be used as an alternative undamaged template; one possible mechanism for such a template switching illustrated. (B) Example sequences of replicated pQTs plasmids recovered from xpa/pol, xpa/pol/g, and xpa/pol/g/f clones, aligned with a schematic drawing of pQTs. TLS on the top strand (inserting mostly AA in the reverse direction, top set), TLS on the bottom strand (middle set), and error-free bypass (bottom set) are shown. Proportions are not representative. (C) The proportion of TLS versus error-free bypass in pQTs sequences recovered from xpa/pol, xpa/pol/g, and xpa/pol/g/f cells, shown as a percentage of the total. Data shown with asterisks are taken from Szüts et al. [28]. A total of 23 and 29 sequences from xpa/pol, and xpa/pol/g, respectively, are shown as the sum of three independent experiments. (D) The pattern of nucleotide incorporation opposite the T-T (6-4) photoproduct in xpa/pol, and xpa/pol/g, cells. Error-free pQTs sequences were excluded from analysis. The percentage of each nucleotide incorporated at each position is indicated by the size of the letter of the nucleotide in the column. del = deletion. The incorporation positions indicated are at the 3' T and the 5' T of the lesion, followed by the next two bases in the template,
past this photoproduct may insert ApA (accurate TLS) or other nucleotides (inaccurate TLS) at this site. Note that our experiment was done in a nucleotide-excision repair-deficient (xpa-deficient) background, and thus excluded the recovery of replicated copies generated by error-free nucleotide-excision repair. In the second, unphysiological, replication template, the lesions are placed opposite to each other (pQTo) (Figure 5E). Using this conformation, replicated DNA copies can be recovered as a consequence of TLS, but not by template switching.

To assess the mode of bypass used when generating replicated copies of pQTo, we analyzed the nucleotide sequences of replicated copies of plasmids recovered from DT40 cells (Figure 5B) and determined the proportion of TLS relative to error-free template switching (Figure 5C). Overall replication efficiency was comparable among cells carrying the various genotypes used in the previous study and this study. Previous study found that 45% and 55% of the recovered plasmid copies resulted from TLS in xpa and xpa/polμ cells, respectively [28]. In comparison, the efficiency of TLS in xpa/polκ cells was significantly reduced, with less than 10% of the recovered plasmid generated as a consequence of TLS. All TLS events observed in the xpa/polκ cells were associated with deletion at damaged sites (Figure 5D).

Thus, as found previously [28] (Figure 5C), we conclude that Polκ is required for the successful bypass of T-T (6-4) UV photoproducts by TLS. Remarkably, the xpa/polν/polκ cells displayed a normal TLS efficiency, indicating that the failure of TLS in polκ cells is dependent on the presence of Polη.

We also classified the replication products obtained from the pQTo plasmid, where bypass can be effected only by TLS or deletion. As found in the previous study [28], the loss of Polκ was frequently associated with the deletion of two or more nucleotides covering the site of the T-T (6-4) UV photoproduct (Figure 5E and 5F). The loss of Polκ did not impair TLS in the absence of Polη, but severely compromised it in the presence of Polη. A possible explanation, discussed below, is that the Polη-dependent insertion of nucleotides opposite the T-T (6-4) UV photoproduct inhibits the completion of TLS in the absence of Polκ (presumably due to defective extension from inserted nucleotides), while other unidentified DNA polymerases can perform the complete TLS reaction in polη/polκ cells.

**Polk plays only a minor role in the damage tolerance of polη/polκ cells**

The milder phenotype of the polη/polκ cells, compared with polκ single mutants, led us to investigate the contribution of other TLS polymerases to TLS in polη/polκ cells. We previously showed that polκ/polκ cells show a higher sensitivity to mono-alkylating agents, compared with polκ cells, though polκ cells show normal sensitivity [36], and thereby suggested that Polκ can partially substitute for the loss of Polκ. We therefore sought to determine whether Polk contributes to damage tolerance in polη/polκ cells. To this end, we deleted the Polk gene in polη/polκ cells and analyzed the phenotype of the resulting triple knockout polη/polk/polκ clones (Figure 6A). Deletion of the Polκ gene tended to reduce growth kinetics. However, the polη/polk/polκ clones exhibited only limited increase in DNA-damage sensitivity, compared with polη/polκ cells (Figure 6B). Likewise, overexpression of chicken Polk did not increase cisplatin tolerance in polκ or polη/polκ cells (data not shown). These observations imply that Polk does not play an important role in TLS in polη/polκ cells.

**polη/polκ cells, but not polκ cells, displayed increased numbers of UV-induced sister chromatid exchange events**

Replication arrest can be released by two major mechanisms: HR and TLS [37]. HR-dependent release is initiated by homologous pairing between the 3’ end of the arrested strand and the sister chromatid, followed by strand invasion and DNA synthesis to extend the invading 3’ strand (Figure 7D). To analyze the efficiency of HR-mediated release from replication blockage, we analyzed sister chromatid exchange (SCE) (Figure 6C and 6D). The level of SCE is likely to be determined by two factors: the number of DNA lesions that cause a replication block and the efficiency of HR-dependent release from replication blockage. As previously reported [9,39], the level of spontaneous SCE was slightly increased (1.5 to 2-fold) in all TLS mutants compared with wild-type cells, presumably because lesions are more frequently channeled to HR. The polη cells displayed a markedly greater increase in the level of UV-induced SCE (the number of spontaneous SCE subtracted from the number of SCE following UV irradiation shown in Figure 6D), which phenotype is attributable to defective TLS over UV-induced damage. In contrast, in the polκ cells, the UV-induced SCE level was similar to that of wild-type cells, suggesting that the defective TLS may not be adequately compensated by HR [9].

SCE was induced more efficiently in the polη/polκ cells than in the polκ cells, which is consistent with the increased UV tolerance of polη/polκ cells, compared with polκ cells. Reconstitution of the polη/polκ cells with wild-type Polη, but not with the catalytically inactive Polη, significantly reduced the number of UV-induced SCE events. Thus, the degree of UV tolerance correlated with the number of UV-induced SCE events at least in polκ and polη/polκ cells. This observation suggests that, in addition to TLS, HR-mediated release from replication blockages contributes to a significantly higher UV tolerance in polη/polκ cells than in polκ cells.

**Discussion**

Polη is required for TLS across a much wider range of DNA lesions than indicated by the sensitivity of Polη-deficient cells. Analysis of XP-V cells indicates that Polη plays a major role in TLS past cyclobutane dimers and certain bulky adducts, but few other lesions. We demonstrate here that, in DT40 cells, Polη is also involved in the interaction of the replication machinery with different types of damage, such as those induced by chemical cross-linking agents (Figure 2), abasic sites (Figure 3 and Figure 4), and T-T(6-4) UV photoproduct (Figure 5). Thus, the deletion of polη in the polκ cells reversed their hypersensitivity to UV, MMS, cisplatin, and the ectopic expression of AID. Our finding that polη/polκ cells were significantly more tolerant to all tested DNA-damaging agents compared with polκ cells, reveals that neither of these polymerases is absolutely required for the tolerance of these types of damage during DNA replication. However, when Polη is present, Polκ is also required for efficient recovery from the effects of these damaging agents.
Figure 6. HR, but not Polk, contributes to increased cellular tolerance to DNA damage in polg/polc cells, compared with polc cells. (A) Growth of polg/polc and polg/polc/polk clones. Cells were cultured for 3 days. The doubling time of each mutant is shown. Error bars show the SD of the mean for three independent assays. (B) Sensitivity profiles of indicated mutant cells to UV and cisplatin. Error bars show the SD of the mean for three independent assays. (C) Induction of SCE in polg, polc, and polg/polc cells by UV. Cells were labeled with BrdU during over cell-cycles with or without UV irradiation (0.25 J/m²) before labeling. Spontaneous and induced SCEs in the macrochromosomes of 50 metaphase cells were counted. Histograms show the frequency of cells with the indicated numbers of SCEs per cell. The mean number of SCEs per cell ± SE is shown. Statistical significance was further calculated by the non-parametric Mann-Whitney U test. (D) The number of UV-induced SCE was calculated by subtracting spontaneous SCE from SCE following UV irradiation.

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XP-V cells show a modest phenotype, including moderate sensitivity to UV and cisplatin [40], only in the presence of caffeine, and do not show significant sensitivity to MMS. Consequently, it was once believed that Polγ does not actually have a significant role when Polε is present. However, this possibility may be unlikely, since physical interaction between Polγ and Rev1, and Polγ dependent recruitment of Rev1 to the DNA damage site support the sequential actions of Polγ followed by Polε rather than inhibitory action of Polε on Polγ [44–47]. Thus, more likely possibility is that, Polγ generates a replicative intermediate in an attempt to bypass the lesion, but cannot complete an effective bypass reaction without Polε (Figure 7A). We suggest that the abortive intermediates generated by Polγ in the absence of Polε lead to a difficult-to-rescue replication collapse, thereby accounting for the hypersensitivity of the single polε mutant (Figure 7B). The modest phenotype of XP-V cells indicates that Polα may efficiently mediate TLS past a variety of DNA lesions, either in collaboration with other polymerases or possibly on its own.

This situation can be explored further in the light of current TLS models. In the canonical model for TLS replication, arrest by agents such as UV, MMS, and cisplatin, leads to PCNA becoming mono-ubiquitinated [40,48–50]. This increases the affinity of PCNA for Polγ and other Y-family TLS polymerases by virtue of their UBM and UBZ ubiquitin-binding motif [44,48,50–52] and likely contributes to the accumulation of Y-family polymerases at the sites of blocked replication forks. It has been suggested that these polymerases can compete with each other by mass action to attempt to carry out TLS. In the case of CPDs, if Polε wins this competition; bypass can occur without the need for a second polymerase. However, with other lesions such as a T-T (6-4) photoproduct, there is currently no evidence to show that any single polymerase can complete bypass. Polγ may be able to start the bypass by incorporating opposite at least the 5’ base of the lesion, but it cannot extend from the resulting mismatch (Figure 7A). This would explain the abortive intermediate referred to above. To complete TLS, Polγ is required to extend from the inserted nucleotides (Figure 7A), an explanation that is consistent with the sequential action of Polγ and Polε demonstrated in in vitro studies [2]. A further implication of this model is that no other polymerase can effectively substitute for Polε in this extension step (Figure 7B).

The successive action of Polγ and Polε might be mediated by the association of the two polymerases with Rev1 [45,53]. The idea of a Rev1-mediated switch from Polγ to Polε is supported by the fact that Polγ tightly interacts with Rev1 [1,46,47,53,54]. Moreover, DNA-damage-induced Rev1 focus formation appears to be dependent on Polγ [46,47]. Adding to these

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**Figure 7. Model for the sequential action of Polι and Polζ in TLS**

(A) The two step-model for TLS. In wild-type cells, Polι is efficiently recruited to a replication blockage at a DNA lesion (green circle) on a template strand and inserts 1-2 base(s) (green box) to bypass the lesion (filled triangle). Polζ further extends from these inserted base(s) (red arrow) before replicative DNA polymerases can take over from Polζ. (B) In polf cells, after Polι inserts nucleotides opposite to DNA lesions, no other polymerases can effectively extend DNA synthesis for subsequent DNA synthesis by replicative polymerases. (C) In polf/polf cells, other TLS polymerases carry out TLS. (D) The replication blockage may be released by a template switch to the intact sister chromatid using the HR machinery. doi:10.1371/journal.pgen.1001151.g007
findings, the present work establishes a role for Polι in the bypass of a wider range of DNA damage than previously thought and demonstrates the in vivo importance of the two-step bypass of many lesions.

The effect on TLS of depleting both Polι and Polζ is distinctly different in DT40 cells than in human cells. Zhu et al. showed that depletion of Rev3 sensitized cells to UV more severely at two days after irradiation in Polζ-deficient XPV fibroblasts than in Polζ-proficient control cells [22], although its impact on Polζ-proficient DT40 cells was considerably stronger than on Polζ-deficient DT40 cells. There are several explanations for this apparent difference. First, we favor the idea that DT40 may be a more reliable cell line than others to evaluate TLS by measuring cellular survival due to following reasons. The cell cycle distribution is distinctly different between DT40 and other mammalian cell lines. In DT40 cells, ~70% of the cells are in the S phase, and the G1/S checkpoint does not function at all [55]. In most of the mammalian cell lines, on the other hand, more than 50% of the cells are in the G1 phase, and G1/S checkpoint works at least partially. Therefore, environmental DNA damage interferes with DNA replication more significantly in DT40 cells than in mammalian cell lines. Accordingly, TLS contributes to the cellular survival of the colony formation assay to a considerably higher extent in DT40 cells than in mammalian cell lines. Zhu et al., on the other hand, evaluated TLS by measuring the number of living cells at 48 hours after UV irradiation [22]. Since a majority of UV irradiated cells may have stayed outside the S phase at 48 hours, it is unclear whether this survival reflects the efficiency of TLS. The same laboratory also analyzed TLS past a cisplatin G-G intrastrand crosslink located in a gapped episomal plasmid [21]. Depletion of Rev3 with or without codepletion of Polι in U2OS cells resulted in an 80% reduction in TLS past the lesion, irrespective of the presence or absence of Polι. The relevance of this finding to TLS during the replication of chromosomal DNA remains elusive. Yoon et al. investigated TLS in a double-stranded plasmid containing a single 6-4 photoproduct as well as replication origins derived from the SV40 virus [23]. Depletion of Rev3 or Rev7 in NER-deficient XP-A fibroblasts reduced the efficiency of TLS in the episomal plasmid by approximately 50%, with a similar reduction obtained in XP-V fibroblasts. This result using human fibroblasts is clearly different from the data we obtained using DT40 cells. Given the close sequence similarity between the two polymerases in human and chicken cells, we consider it unlikely that the mechanisms of lesion bypass are fundamentally different between the two organisms. The apparent difference between mammalian cells and DT40 may be caused by the incomplete si-RNA mediated inhibition in human cells versus the null mutation we have used in DT40. Another possible reason to explain this difference is the active HR system in DT40 cells, and the different usage order of TLS DNA polymerases because the DT40 B lymphocyte line undergoes Ig V hypermutation through TLS [32]. The usefulness of the three episomal plasmids system to the analysis of TLS occurring during replication of chromosomal DNAs should be further investigated [15,21,22,29]. Irrespective of the reason for this apparent difference, our data clearly indicate that, as discussed above, under some conditions Polι can hinder the efficient progress of the replication fork past lesions mediated by Polζ.

**Rescue of failed translesion synthesis by homologous recombination**

It is remarkable that deletion of the three major TLS polymerase genes, Polι, Polξ, and Polζ, results in only a mild reduction in the growth kinetics of DT40 cells (Figure 6A). This is in marked contrast with the immediate cell death associated with the massive chromosomal breaks generated upon deletion of Rad51 [56]. These observations imply that during DNA replication, if replication blocks are encountered, HR can at least partially compensate for defective TLS. The significant functional redundancy between TLS and HR is supported by our previous report, which concludes that the deletion of both RAD18 and RAD54, a gene involved in HR, as well as the deletion of both Rev3 and RAD54, are synthetically lethal to cells [9,39]. We show here that depletion of Polι irrespective of the status of Polζ markedly increases the level of UV-induced SCE (Figure 6C), suggesting that DNA damage that cannot be resolved by TLS because of the absence of Polι may be resolved by HR, leading to increased SCE levels (Figure 7D). However, SCE is not induced to the same level in polζ cells (Figure 6C). Thus, nucleotide incorporation by Polι appears to represent a point of commitment in the TLS reaction beyond which rescue by HR is problematic. This is likely to be explained by the creation of an intermediate, possibly the mismatched primer terminus, which can be efficiently extended by Polζ, but which cannot readily initiate HR.

In summary, the significant increase in the cellular tolerance of polι/polζ cells to DNA-damaging agents, compared with polι cells, can be partially attributable to more efficient HR in polι/polζ cells than in polζ cells. However, the normal level of TLS-dependent Ig V mutation and restoration of TLS during 6-4 photoproduct bypass in polζ/polζ cells (Figure 4B) suggests that one or more other unidentified TLS polymerases can act as a substitute and carry out TLS when both Polι and Polζ are missing (Figure 7C).

**Materials and Methods**

**Cell lines and cell culture**

Generation of polζ (rev3)- and polζ-deficient DT40 cells was described previously [9,12]. To generate polι/polζ cells, we sequentially introduced two rev3 gene-disruption constructs (rev3-hyg and rev3-His) [9] into polι (PuroK/Blk) cells. A puromycin-resistant XP4 disruption construct was used to disrupt the single XP4 allele and recreate the xpa/rev3 cell line. After removal of the BlkK marker gene from the polι/polζ cells by the transient expression of CRE recombinase, a blasticidin-resistant XP4 disruption construct was used to generate the xpa/polζ/polζ cell line. A puromycin-resistant POLX disruption construct was used to generate polι/polζ/polζ cells. To make a POLX expression plasmid, we inserted human POLX cDNA into the multi-cloning sites (MCS) of an expression vector, pCR3-loxP-MCS-ires-GFP-loxP [57]. A mutant Polι that lacks polymerase activity (Mutant POLι) was generated by inserting D115A/E116A mutations into human Polι cDNA. The conditions for cell culture, selection, and DNA transfection were described previously [58]. The growth properties of cells were analyzed as described previously [58].

**AID overexpression by retrovirus infection**

For the retrovirus infection, the pMSCV-ires-GFP recombinant plasmid was constructed by ligating the 5.2 kb BamHI-Nol fragment from pMSCVhyg (Clontech) with the 1.2 kb BamHI-Nol fragment from pRES2-EGFP (Clontech). Mouse AID cDNA [33] was inserted between the BglII and EcoRI sites of pMSCV-ires-GFP. The preparation and infection of the retrovirus were carried out as previously described [33]. Expression of the GFP was confirmed by flow cytometry. The efficiency of infection was more than 90% as assayed by GFP expression.
Assay of TLS past a T-T (6-4) photoproduct on episomal plasmid

pQTs and pQTo plasmids containing a T-T (6-4) photoproduct were generated and transfected into DT40 cells as previously described [28].

Chromosome aberration analysis

Karyotype analysis was performed as described previously [56]. Cells were treated with colcemid for 3 hours to enrich mitotic cells.

Measurement of SCE level

Measurement of SCE level was performed as described previously [38]. For UV-induced SCE, cells were suspended in PBS and irradiated with 0.25 J/m² UV followed by BrdU labeling.

Ig Vκ mutation analysis

Genomic DNA was extracted at 14 days after subcloning. The rearranged Vκ segments were PCR amplified using 5′-CAG-GAICTGGGGGGGCTACTGATGCCGAAG-3′ as the forward primer in the leader-Vκ intron and 5′-GGCCAGATTTCCCCAGCCTGCCGCCAAGTCCAAG-3′ as the reverse primer in the 3′ of the JC3 intron. To minimize PCR-introduced mutations, the high-fidelity polymerase, Phusion (Finnzymes) was used for amplification (30 cycles at 94°C for 30 s; 60°C for 1 min; 72°C for 1 min). PCR products were cloned using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen) and subjected to sequence analysis.

As described previously [59], all sequence changes were assigned to one of three categories: point mutation, gene conversion, or ambiguous. This discrimination is based on the published sequences of Vκ pseudogenes that can act as donors for gene conversion. For each mutation, the database of Vκ pseudogenes was searched for a potential donor. If no pseudogene donor containing a string >9 bp could be found, the mutation was categorized as a non-templated point mutation. If such a string was identified and there were further mutations that could be explained by the same donor, then all these mutations were categorized as a single long-tract gene conversion event. If there were no further mutations, indicating that the isolated mutation could have arisen through a conversion mechanism or could have been non-templated, it was categorized as ambiguous.

Supporting Information

**Figure S1** Increased phosphorylation of histone H2AX in polη-deficient cells. (A) Fluorescence image of fixed DT40 cells with indicated genotype. Cells were stained with antibody to phospho-Histone H2AX (red) and with DAPI (Blue). (B) Percentage of the cells with over 10 phospho histone H2AX signals was calculated. Found at: doi:10.1371/journal.pgen.1001151.s001 (1.94 MB PDF)

**Figure S2** Proliferation of wild-type DT 40 cells carrying human polη transgene or control expression vector. (A) Expression of the human polη transgene was examined by Northern blot analysis. The level of endogenously expressed human polη in 293T cells was below the detection limit, while human polη expression was detected in DT40 cells carrying the human polη transgene, indicating that human polη was overexpressed in DT40 cells. (B) Proliferation of cells was examined for 3 days. Ecotopic expression of human polη in wild-type DT40 cells has no impact on cell proliferation. Found at: doi:10.1371/journal.pgen.1001151.s002 (1.13 MB PDF)

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Author Contributions

Conceived and designed the experiments: KH ES TK AM ST. Performed the experiments: KH ES TK AM ST. Analyzed the data: KH ES TK AM ST. Wrote the paper: KH ES TK AM ST.

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