Repriming by PrimPol is critical for DNA replication restart downstream of lesions and chain-terminating nucleosides

Kaori Kobayashi, Thomas A. Guilliam, Masataka Tsuda, Junpei Yamamoto, Laura J. Bailey, Shigenori Iwai, Shunichi Takeda, Aidan J. Doherty, and Kouji Hirota

ABSTRACT
PrimPol is a DNA damage tolerance enzyme possessing both translesion synthesis (TLS) and primase activities. To uncover its potential role in TLS-mediated IgV hypermutation and define its interplay with other TLS polymerases, PrimPol+/- and PrimPol+/PolZ-/-/Polz-/- gene knockouts were generated in avian cells. Loss of PrimPol had no significant impact on the rate of hypermutation or the mutation spectrum of IgV. However, PrimPol+/- cells were sensitive to methylmethane sulfonate, suggesting that it may bypass abasic sites at the IgV segment by repriming DNA synthesis downstream of these sites. PrimPol+/- cells were also sensitive to cisplatin and hydroxyurea, indicating that it assists in maintaining/restart replication at a variety of lesions. To accurately measure the relative contribution of the TLS and primase activities, we examined DNA damage sensitivity in PrimPol-/- cells complemented with polymerase or primase-deficient PrimPol. Polymerase-defective, but not primase-deficient, PrimPol suppresses the hypersensitivity of PrimPol-/- cells. This indicates that its primase, rather than TLS, is pivotal for DNA damage tolerance. Loss of TLS polymerases, Polz and PolZ, has an additive effect on the sensitivity of PrimPol-/- cells. Moreover, we found that PrimPol and PolZ-PolZ redundantly prevented cell death and facilitated unperturbed cell cycle progression. PrimPol-/- cells also exhibited increased sensitivity to a wide variety of chain-terminating nucleoside analogs (CTNAs). PrimPol could perform close-coupled repriming downstream of CTNAs and oxidative damage in vitro. Together, these results indicate that PrimPol’s repriming activity plays a central role in reinitiating replication downstream from CTNAs and other specific DNA lesions.

Introduction

Genome replication is stalled by DNA secondary structures and unrepaird damage, potentially leading to fork collapse, mutagenesis, and genome instability.1 Eukaryotic cells have evolved several mechanisms to complete replication beyond a damaged template. The first is translesion DNA synthesis (TLS), which employs specialized DNA polymerases, including Polymerase η and Polymerase ξ, to permit continued replication beyond the damaged template.2-5 A second mechanism is homologous recombination (HR), which mediates continuous replication using a newly synthesized sister strand.6-8 A third mechanism, in which DNA primases play a role, involves the repriming of DNA synthesis downstream from the lesion or structure.9-13

Primase-Polymerase (PrimPol) is a member of the archaeo-eukaryotic primase (AEP) superfamily,14 which was recently identified as a novel primase-polymerase that possesses both TLS polymerase and primase activities that are involved in DNA damage tolerance in eukaryotic organisms.15-18 PrimPol has two functional domains: an archaeo-eukaryotic primase (AEP) polymerase domain and a DNA-binding, zinc-finger domain.17 The AEP polymerase domain is required for both catalytic activities, while the zinc-finger domain is required only for primase activity,13 with a potential additional role in modulating polymerase fidelity and processivity to limit misincorporation during DNA synthesis. PrimPol’s capacity to repriming appears to be important for maintaining replication fork progression at sites of UV damage12,13 and at structured DNA, such as G quadruplexes.19 Deletion of PrimPol in avian DT40 cells leads to replication fork slowing, damage sensitivity and a pronounced G2/M checkpoint arrest after UV irradiation.20 Deletion of a PrimPol ortholog PPL2 in trypanosomes is lethal.17 PrimPol is a very unprocessive enzyme with low fidelity and a mutation spectrum that is highly biased toward insertion-deletion errors.21 The activity of PrimPol appears to be modulated by its binding to single-stranded binding proteins.

A point mutation (tyrosine 89 to aspartic acid) located in the AEP polymerase domain of PrimPol has been identified in familial high myopia.22 The PrimPolTyr89Asp mutant enzyme is primase-active,
but exhibits significantly reduced DNA polymerase activity.\textsuperscript{23} Mutation or deletion of the zinc-finger domain (PrimPol\textsuperscript{ZF-KO} and PrimPol\textsuperscript{1-354}, respectively) in PrimPol leads to loss of primase activity but does not diminish primer extension synthesis, indicating that the zinc-finger domain plays a key role in the primase activity.\textsuperscript{13}

Recent studies have shown that PrimPol’s enzymatic properties, including its ability to initiate new DNA synthesis beyond several types of replicase stalling lesions and structures, help to maintain active replication.\textsuperscript{12,15,16} One current model of cellular TLS postulates that after replicative polymerases have halted at lesions, the stalled forks are restarted by specialized DNA polymerases, including Pol\textsubscript{h} and Pol\textsubscript{z}, which are capable of TLS.\textsuperscript{4,24} The active sites of these enzymes are less spatially constrained than replicative polymerases and can thus accommodate distorted base-pairing involving damaged bases.\textsuperscript{25,26} Eukaryotic cells have evolved a number of TLS polymerases with distinctive bypass activities, though the division of labor among these various enzymes has not yet been fully elucidated. Likewise, although it is believed that PrimPol contributes to the cellular tolerance of DNA damage by facilitating damage bypass, the relationship between PrimPol and other TLS polymerases remains to be defined.

To better define the roles played by PrimPol in damage tolerance, we generated PrimPol\textsuperscript{\lambda} and PrimPol\textsuperscript{h}/Pol\textsubscript{h}/Pol\textsubscript{z} cells derived from the chicken DT40 B cell line. DT40 cells undergo TLS mediated IgV\textsubscript{j} hypermutation during in vitro passage. Thus, the unique advantage of this cell line for the phenotypic analysis of TLS is that the DNA sequence analysis of IgV\textsubscript{j} allows identification of nucleotides inserted opposite abasic sites by TLS. We show that the loss of PrimPol has no significant impact on the rate of hypermutation and mutation spectrum of IgV\textsubscript{j}. However, PrimPol\textsuperscript{\lambda} cells were sensitive to methylmethane sulfonate (MMS), which generates abasic sites. These data suggest that PrimPol may bypass abasic sites at the IgV\textsubscript{j} segment by repriming DNA synthesis downstream of the abasic sites. Consistently, the expression of a polymerase-deficient PrimPol\textsuperscript{1990} in PrimPol\textsuperscript{\lambda} cells significantly suppressed this hypermutation sensitivity, while the expression of primase-deficient PrimPol exhibited little suppression. Notably, PrimPol\textsuperscript{\lambda}/Pol\textsubscript{h}/Pol\textsubscript{z} cells proliferated very slowly and exhibited increased cell death, although both PrimPol\textsuperscript{\lambda} and Pol\textsubscript{h}/Pol\textsubscript{z} cells were able to proliferate with nearly normal kinetics. These results suggest that repriming by PrimPol and TLS by Pol\textsubscript{h} and Pol\textsubscript{z} are compensatory for each other for facilitating physiological levels of DNA replication. Additionally, we found strong sensitivity of PrimPol\textsuperscript{\lambda} cells to chain-terminating nucleoside analogs (CTNAs). Finally, we demonstrate that PrimPol is able to perform close-coupled repriming downstream of CTNAs and DNA damage lesions. Taken together, these data suggest novel roles for the primase activity of PrimPol in repriming DNA synthesis downstream of CTNAs and lesions incorporated on the primer strand that block 3’ extension by the replicative polymerases.

Results

**DT40 cells lacking PrimPol are viable**

In order to study the possible roles of PrimPol in TLS-dependent IgV hypermutation, we disrupted the PrimPol gene\textsuperscript{15} in CI18, a subclone of DT40.\textsuperscript{27} This subclone has been used for analyzing HR-mediated gene conversion and TLS-dependent hypermutation events in immunoglobulin V\textsubscript{j} (IgV\textsubscript{j}) segments, because this cell line has a uniform IgV\textsubscript{j} sequence and change of sequence can be easily assessed by sequencing analysis.\textsuperscript{23,25} To disrupt the chicken PrimPol locus, we constructed two targeting vectors, PrimPol-\textsubscript{bsr} and PrimPol-\textsubscript{par} (Fig. S1A) and sequentially transfected these constructs into wild-type DT40 cells. Targeted disruption on the PrimPol gene was verified by Southern blot analysis of EcoT22I-digested genomic DNA with the use of an internal 5’ probe (Fig. S1B) and RT-PCR (Fig. S1C). The PrimPol\textsuperscript{\lambda} cells proliferated with normal kinetics (Fig. S1D) but showed a slightly increased G2 population as previously reported (Fig. S1E).\textsuperscript{15,20}

**PrimPol\textsuperscript{\lambda} cells are hypersensitive to UV, MMS and cisplatin**

Previously, we reported that PrimPol\textsuperscript{\lambda} cells exhibit UV, but not ionizing radiation sensitivity.\textsuperscript{15,20} To understand roles played by PrimPol more comprehensively, we measured sensitivity to a wider range of exogenous DNA damaging agents (cisplatin, HU, MMS, ICRF193 and camptothecin). PrimPol\textsuperscript{\lambda} cells were not sensitive to ICRF193, camptothecin or \gamma-rays, confirming that PrimPol is not vital for strand break repair.\textsuperscript{15,20} Survival assays revealed that PrimPol\textsuperscript{\lambda} cells were more sensitive to ultraviolet (UV), methylmethane sulfonate (MMS), cisplatin and hydroxyurea (HU) treatments than wild-type cells (Fig. 1). These data suggest that PrimPol plays roles in the restart of replication at sites of DNA damage. Hypersensitivity to a wide range of DNA replication blocking agents is also observed in RAD18\textsuperscript{\lambda}, Pol\textsubscript{h}/Pol\textsubscript{z} and PCNA K164R/PCNA K164R cells,\textsuperscript{2,28,29} suggesting that lesion bypass is significantly impaired in PrimPol\textsuperscript{\lambda} cells.

**PrimPol plays roles in damage tolerance independently of Pol\textsubscript{h} and Pol\textsubscript{z}**

Notably, we found that PrimPol\textsuperscript{\lambda}/Pol\textsubscript{h}/Pol\textsubscript{z} cells proliferated more slowly than wild-type, PrimPol\textsuperscript{\lambda} or Pol\textsubscript{h}/Pol\textsubscript{z} cells (Fig. 2A). Moreover, PrimPol\textsuperscript{\lambda}/Pol\textsubscript{h}/Pol\textsubscript{z} cells exhibited significantly higher levels of spontaneous cell death than observed with wild-type, PrimPol\textsuperscript{\lambda} or Pol\textsubscript{h}/Pol\textsubscript{z} cells as evidenced by the observations that sub-G1 (dead cells) fraction in the cell cycle distribution was increased in PrimPol\textsuperscript{\lambda}/Pol\textsubscript{h}/Pol\textsubscript{z} cells (Fig. 2B-C). In addition, PrimPol\textsuperscript{\lambda}/Pol\textsubscript{h}/Pol\textsubscript{z} cells tended to exhibit more prominent G2 arrest after exposure to cisplatin than wild-type, PrimPol\textsuperscript{\lambda} or Pol\textsubscript{h}/Pol\textsubscript{z} cells (Fig. 2B-C). These results suggest that the repair kinetics of the DNA damage is critically reduced in PrimPol\textsuperscript{\lambda}/Pol\textsubscript{h}/Pol\textsubscript{z} cells. Furthermore, the loss of Pol\textsubscript{h} and Pol\textsubscript{z} increased sensitivity to DNA damage in PrimPol\textsuperscript{\lambda} cells to the same extent as in the wild-type cells and, critically, the triple mutant was much more sensitive (Fig. 2D). These observations indicate that PrimPol and Pol\textsubscript{h}-Pol\textsubscript{z}-dependent TLS contribute to DNA damage tolerance independently of each other.
PrimPol is dispensable for IgV\(_{\lambda}\) hypermutation

To analyze the roles of PrimPol in TLS in vivo, we analyzed the diversification of the IgV\(_{\lambda}\) region, since its non-templated hypermutation at the C/G pair (hereafter called hypermutation) is caused by TLS past abasic sites. DT40 cells constitutively diversify their IgV\(_{\lambda}\) locus by hypermutation and by gene conversion from pseudo-V\(_{\lambda}\) segments to the downstream V\(_{\lambda}\) segment through HR. Therefore, phenotypic analysis of IgV\(_{\lambda}\) diversification during in vitro passage provides a novel opportunity to functionally analyze the two alternative mechanisms of releasing replication blockage: TLS and HR. Indeed, the rate of TLS dependent IgV\(_{\lambda}\) hypermutation was critically reduced in TLS defective PCNA\(^{K164R}\), RAD18\(^{-/-}\), POLD3\(^{-/-}\) and Pol\(\eta\)^{-/-}/Pol\(\theta\)^{-/-} cells. Notably, the rates of hypermutation and gene-conversion events were similar between PrimPol\(^{-/-}\) and wild-type cells (Fig. 3A-B). Moreover, the mutation spectrum was not significantly changed by the loss of PrimPol in wild-type and Pol\(\eta\)^{-/-}/Pol\(\zeta\)^{-/-} cells (Fig. 3C). Notably, PrimPol\(^{-/-}\)/Pol\(\eta\)^{-/-}/Pol\(\zeta\)^{-/-} cells exhibited only slightly reduced hypermutation levels, demonstrating that TLS is active in the triple mutant (Fig. 3). Taken together, these results indicate that PrimPol is dispensable for TLS dependent IgV\(_{\lambda}\) hypermutation. Considering the serious proliferation defect, as well as the increased sensitivity to damage in the triple mutant, PrimPol might play a critical role in damage tolerance independently of TLS and this role compensates for the TLS defects in Pol\(\eta\)^{-/-}/Pol\(\zeta\)^{-/-} cells.

PrimPol’s repriming activity is required to tolerate replication-stalling lesions

To test whether PrimPol’s repriming activity also contributes to tolerance of other replication-stalling lesions, other than UV, we expressed PrimPol\(^{Y89D}\) (reduced TLS, primase active), PrimPol\(^{ZF-KO}\) (TLS active, primase defective), and...
Figure 2. PrimPol plays roles in damage tolerance independently of Polη and Polζ. (A) Relative growth rate of cells plotted with indicated genotypes. Doubling time for the indicated cells was calculated. Error bars represent standard deviation from independent experiments (n = 3). (B) Indicated cells were treated with 0 or 100 nM of cisplatin for 16 hr. Representative cell-cycle distribution for the indicated genotypes. The top of the box, and the lower left, lower right, and left-most gates correspond to cells in the S, G1, and G2/M phases, and the sub-G1 fraction, respectively. The sub-G1 fraction represents dying and dead cells. The percentage of cells in each gate is indicated. (C) Percentage of the indicated cells in sub-G1 fraction and G2 phase fraction was indicated. Error bar represent standard deviation from independent experiments (n = 3). Statistical significance was determined by a Student’s t-test and p-value was calculated. (*) p < 0.05 (D) Indicated cells were exposed to UV or cisplatin and sensitivities were indicated as in Figure 1.
PrimPol$^{1-354}$ (TLS active, primase defective) in PrimPol$^{-/-}$ cells (Fig. 4A-B). PrimPol$^{Y89D}$ complemented the reduced damage tolerance of PrimPol$^{-/-}$ cells, as did wild-type PrimPol (Fig. 4C). This result is consistent with our previous observation that PrimPol$^{Y89D}$ complements increased fork arrest in PrimPol$^{-/-}$ cells treated with UV light to the same degree as does wild-type PrimPol.$^{23}$ In contrast, neither PrimPol$^{2F-KO}$ nor PrimPol$^{1-354}$ suppressed hypersensitivity to MMS, UV, or cisplatin in PrimPol$^{-/-}$ cells, indicating that the repriming activity of PrimPol, rather than its TLS activity, is pivotal for cellular tolerance to these replication stalling lesions (Fig. 4C). Consistently, the number of chromosome aberrations induced
by cisplatin was increased in PrimPol−/− cells, PrimPol−/− + PrimPol+/− + PrimPol1-354 and PrimPol−/− + PrimPolZF-KO cells, but not in PrimPol−/− + PrimPolY89D cells (Fig. 4D). Moreover, expression of PrimPol1-354 but not PrimPol−/− or PrimPol1-354 rescued hypersensitivity to cisplatin in PrimPol−/−/Polη−/−/Polζ−/− cells (Fig. 4E), indicating that this suppression is independent of Polη and Polζ. Together, these findings suggest that PrimPol-mediated repriming of replication plays a critical role in cellular tolerance to a variety of replication stalling lesions and serves as a critical alternative pathway to complement the potential loss of Polη-Polζ-mediated TLS.

PrimPol’s primase activity is required for cellular tolerance of chain terminating nucleotide analogs (CTNA)

Given the critical requirement of the primase activity of PrimPol for cellular tolerance to replication stalling lesions, we next analyzed the role of this activity in cellular tolerance to CTNAs. CTNAs cause replicase stalling by preventing polymerases from incorporating further nucleotides when CTNAs are added at the 3’-termini of growing DNA polymers.34,35 PrimPol−/− cells exhibited more sensitivity to a wide range of CTNAs including Abacavir (ABC),36-38 Zidovudine (AZT) and
acyclovir \(^{40}\) than observed with wild-type cells (Fig. 5A). Moreover, \(\text{PrimPol}^{Y89D}\) complemented the reduced CTNA tolerance of \(\text{PrimPol}^{-/-}\) cells, while neither \(\text{PrimPol}^{ZF-KO}\) nor \(\text{PrimPol}^{1-354}\) suppressed the increased sensitivity, indicating that the primase activity of PrimPol rather than the TLS activity is pivotal for cellular tolerance to replication stalling induced by incorporation of CTNAs (Fig. 5B).

**PrimPol reprimes replication downstream of CTNA incorporated sites and DNA damage lesions in vitro**

Given the critical role of PrimPol in the cellular tolerance to CTNAs and the apparent requirement of the enzyme’s primase activity for this tolerance, we next tested PrimPol’s capacity to repriming downstream of the 3’ side of an incorporated CTNA. Since the ABC drug is phosphorylated in a unique stepwise anabolism to be converted to the triphosphated guanine analog, carbovir, in cells, \(^{41}\) we assessed repriming downstream of carbovir, in addition to acyclovir, in vitro. In order to analyze repriming downstream of a CTNA incorporation site, a primer containing CTNA (carbovir or acyclovir) at its terminal 3’ end was annealed to a biotinylated DNA template. In addition, we analyzed the ability of PrimPol to repriming downstream of an apurinic/apyrimidinic site (Ap site) and thymine glycol (Tg) lesion located in the template strand, both of which PrimPol is unable to bypass through TLS in the presence of magnesium. \(^{13}\)

In this case, a 3’ dideoxynucleotide primer was annealed upstream of the templating lesion to represent a situation where replication has stalled at the damage site. The 3’ dideoxy moiety also prevents template-independent primer extension that interferes with the evaluation of PrimPol’s repriming activity.

Although PrimPol was unable to synthesize through the lesions or extend from 3’ terminal CTNAs, the enzyme displayed a capacity to perform close-coupled de novo synthesis of primer strands downstream in each case (Fig. 6). The size of the extended products, both with 3’ carbovir and 3’ acyclovir primers, in addition to the templating Ap site and Tg lesion, were consistent with repriming \(\sim 14\) nt downstream of the CTNAs or lesion site. Importantly, in the absence of the CTNA primer or lesion, PrimPol generated longer and more variable synthesis products, indicating that PrimPol is performing close-coupled repriming downstream of a stalled replication fork. Taken together, these results indicate that repriming by PrimPol downstream of an incorporated CTNA or damage site is a potentially important mechanism for maintaining replication in the presence of these potentially lethal chain terminators and DNA lesions.

**Discussion**

In this study, we provide further evidence that PrimPol’s primase activity, rather than its TLS activity, is required for cellular tolerance to a variety of replication blocking lesions. We also show that although PrimPol is required to maintain replication fork progression at sites of UV-damage, \(^{13,15,16}\) we could not detect...
any gross defect in IgV, mutagenesis, which is dependent on TLS bypass of abasic sites. Since abasic sites are the most common spontaneously arising lesions in chromosomal DNA, it is possible that cells have evolved multiple polymerases to continue replication beyond these abundant DNA lesions and any effects due to loss of the PrimPol in IgV, mutagenesis were masked.

Another possibility is that PrimPol facilitates continuous replication beyond lesions via de novo repriming of replication downstream from the damage. In accordance with this hypothesis, expression of mutant PrimPol enzyme (PrimPol\(^{Y89D}\)) possessing reduced TLS activity facilitates the restart of forks arrested at UV lesions in PrimPol\(^{-/-}\) cells, while mutant PrimPol enzymes (PrimPol\(^{ZF-KO}\) or PrimPol\(^{1-354}\)) defective in primase activity do not correct the fork-progression defect observed in UV-treated PrimPol\(^{-/-}\) cells. We recently reported that close-coupled repriming by PrimPol is required for replicative tolerance of G quadruplexes in vertebrate cells. Complementary to these findings, we show here that PrimPol displays a capacity to perform close-coupled repriming downstream of other potential replication fork stalling DNA damage lesions in vitro. Since PrimPol is able to extend RNA primers with ribonucleotides, even when bypassing 8-oxoG lesions, it is possible that TLS by PrimPol also assists transcription at damaged templates.

A point mutation in PrimPol found in familial high myopia (Y89D) is located in the conserved AEP domain. The link between this mutation and high myopia has been a matter of debate. This mutant enzyme exhibits reduced polymerase processivity. Consistently, cells expressing PrimPol\(^{Y89D}\) exhibit reduced fork-progression rates. However, we did not detect any gross defects in chromosome stability or replication-block tolerance (Fig. 4). It is possible that the over-expression of PrimPol\(^{Y89D}\) in our assay system masked the effect of reduced polymerase activity in this mutant enzyme. Another possibility is that multiple polymerases, including TLS polymerases and replicative polymerases, compensate for the reduced polymerase activity of PrimPol. Recent studies consistently show a redundancy in the way multiple TLS polymerases are working, and that even replicative polymerase \(\delta\) compensates for the loss of TLS polymerase.

How the appropriate damage tolerance polymerase is chosen from among the multiple stalled-fork recovery systems is largely unknown. The similar levels of hypersensitivity to DNA damaging agents found in PrimPol\(^{-/-}\) and PrimPol\(^{-/-}\) + PrimPol\(^{ZF-KO}\) cells, in addition to the ability of PrimPol to reprim in DNA damage lesions in vitro, has led us to hypothesize that repriming of replication downstream from a DNA lesion might play a more important role in the recovery of stalled forks than previously thought. The simultaneous loss of Pol\(\gamma\) and Pol\(\zeta\) caused no significant decrease in TLS-dependent IgV, hypermutation but a significant change in the mutation pattern, suggesting that other polymerases carry out TLS in their absence. Moreover, the loss of PrimPol in Pol\(\gamma^{+/+}/Pol\(\zeta^{-/-}\) cells did not significantly further reduce TLS-dependent IgV, hypermutation. Thus, the contribution of PrimPol to TLS-dependent IgV, hypermutation, even in
the absence of Polγ and Polζ might be limited, if it exists at all. In contrast, PrimPol−/−/Polγ−/−Polζ−/− cells exhibited critical defects in cellular proliferation and tolerance to DNA damaging agents, suggesting that PrimPol serves as a critical backup for Polγ and Polζ in stalled-fork recovery. Thus, PrimPol-dependent repriming of replication might provide a complementary mechanism to facilitate fork recovery by TLS. How repriming of DNA replication is regulated is therefore an important issue to be investigated in future studies.

In this study, we also identified a critical role for repriming by PrimPol in the cellular tolerance of CTNAs. Anti-viral CTNAs can lead to strong mitochondrial toxicity due to their incorporation by the less selective mitochondrial Polγ.40 Our findings suggest that repriming by PrimPol might be critical for the completion of DNA replication against CTNAs in both mitochondrial and nuclear replication. The use of chain-terminating nucleoside analogs, in combination with other chemotherapeutic treatments, to kill cancer cells shown to have mutated or low expression levels of PrimPol might be a promising chemotherapy strategy.

Material and methods

Disruption of PrimPol in chicken DT40 cells

DT40 cells were provided by the Takeda lab.27 PrimPol disruption constructs were generated from genomic PCR products combined with puroR and bsrR selection-marker cassettes. Genomic DNA sequences were amplified using primers

5′-CAGTCCAGTAAATAAGAAGGAATCACCTTAC-3′ and 5′-CCCATTTTCTCTTGTCTGCTTAAAGCAA-3′ for the 5′ arm of the targeting construct and 5′-TCAGAGTACCAC-GATCCAGAGATTGATT-3′ and 5′-ATACAGTATTGGCT-TATCAGTAGAAGTTG-3′ for the 3′ arm. PCR was conducted using PrimeStar GXL DNA polymerase (Takara Bio) according to the manufacturer’s instructions. An amplified 1.6 kb 3′ arm and a 1.7 kb 5′ arm were cloned into the XbaI-BamHI and BamHI-SalI sites, respectively, of a pBlueScript SK vector. Marker-gene cassettes, puroR and bsrR selection-marker genes, flanked by loxp sequences, were inserted into the BamHI site to generate PrimPol-puroR and PrimPol-bsrR. To generate PrimPol−/− cells, wild-type DT40 cells were transfected sequentially with PrimPol-puroR and PrimPol-bsrR. A PCR fragment produced from genomic DNA using primers 5′-ATTCTGCTGAATCAAAACCACACA-CAC-3′ and 5′-TACGTTCTCTTTTATGATGTAATGC-3′ was used as a probe for Southern blot analysis to screen gene-targeting events.

Complementation and survival assays in PrimPol−/− DT40 cells

DT40 cells were cultured in RPMI1640 medium (Nakalai Tesque, Kyoto, Japan) supplemented with 10% heat inactivated fetal calf serum (FCS) (Biosera, France, lot No. 10011953), 1% chicken serum (Gibco BRL, Grand Island, HY, USA), 10−5 M mercaptoethanol, 50U/ml penicillin and 50μg/ml streptomycin (Nakalai Tesque) at 39.5°C. PrimPol−/− cells were complemented with transgene as previously described.13 Expression of PrimPol in PrimPol−/− cells was confirmed by western blot analysis using α-CCDC111 (N-13) antibody (Santa Cruz). We measured the amount of ATP in the cellular lysates to determine the number of surviving cells.49,50 Cells were treated with each DNA damaging agent (camptothecin, ICRF193 and HU) in 1 ml of medium using 24-well plates and incubated at 39.5°C for 48 h. To analyze sensitivity to γ-rays, cells were irradiated using a 60Co γ-ray source and diluted to 10⁴ cells/ml in 24-well plates and incubated at 39.5°C for 48 h. For UV light, 1 × 10⁶ cells were suspended in 0.5 ml of PBS (phosphate-buffered saline) containing 1% FCS in 6-well plates and irradiated with UVC (254 nm wavelength) and 10 μl of irradiated cells were transfer to 1 ml of medium using 24-well plates and incubated at 39.5°C for 48 h. For MMS, 1 × 10⁶ cells in PBS containing 1% FCS were exposed to MMS for 1 hr at 39.5°C and 10 μl of exposed cells were transferred to 1 ml of medium using 24-well plates and incubated at 39.5°C for 48 h. Then, we transferred 100 μl of medium containing the cells to 96-well plates and measured the amount of ATP using CellTiter-Glo (Promega) according to the manufacturer’s instructions. Luminescence was measured by Fluoroskan Ascent FL (Thermo Fisher Scientific Inc., Pittsburgh, PA). Camptothecin and ICRF193 interfere with Topoisomerase I and II, respectively, leading to strand breaks. HU inhibits ribonucleotide reductase and thereby prevents replication. MMS, cisplatin and UV induce chemical modifications in base, leading to replication stall at damaged bases.

Measurement of sensitivity to CTNAs

To measure the sensitivity to CTNAs, methylcellulose colony survival assays were performed as described previously.50,51 ABC (Carbosynth, UK), ACV (Sigma, USA), and AZT (Sigma, USA) were mixed in methylcellulose medium.

Flow-cytometric analysis of cell cycle progression

To analyze cell-cycle progression, 1 × 10⁶ cells were exposed for 10 min to 20 μM 5-bromo-2′-deoxyuridine (BrdUrd; Nacalai Tesque, Kyoto, Japan), then harvested and fixed with 70% ethanol. Fixed cells were incubated with 2 M HCl containing 0.5% Triton X-100, treated first with mouse anti-BrdUrd monoclonal antibody (BD PharMingen, San Diego, CA), then with FITC-conjugated anti-mouse IgG antibody (Southern Biotechnology Associates, Birmingham, AL). Cells were resuspended in PBS containing 5 μg/ml propidium iodide (PI) for subsequent analysis using FACS Accuri (BD Biosciences).
AID overexpression by retrovirus infection

Wild-type, PrimPol /−/−, Polγ−/−/Polζ−/− and PrimPol /−/−/Polγ−/−/Polζ−/− cells were inoculated in 96-well plates to obtain single colonies. Single colonies were picked up and their genomic DNA extracted. After the DNA sequence of the V(D)J locus was analyzed, clones without any mutation or gene conversion in the V(D)J locus were obtained. AID overexpression by retrovirus infection was carried out as described previously. The efficiency of infection was about 70%, as assayed by GFP expression. Ig-gene conversion and hypermutation (events/Ig V segment/div) were calculated at dividing times 6.8, 7.0, 7.5 and 11.6 h for wild-type, PrimPol /−/−, Polγ−/−/Polζ−/− and PrimPol /−/−/Polγ−/−/Polζ−/− cells, respectively.

Analysis of Ig Vβ diversification

Genomic DNA was extracted at 14 days post-infection. Using primers 5′-CAGGAGCTCGGGGGGCTACGATTGCC-G′-3′ at the Vβ intron position and 5′-GGCAAGCTTCCTCCCCAGCGTGCCCGAAATCGC-3′ at the back 3′ site of the JC, intron, the rearranged Vβ segments were PCR amplified, cloned into the plasmid, and subjected to sequence analysis. To minimize PCR-introduced mutations, a high-fidelity polymerase, Prime Star GXL (Takara Bio), was used for amplification. The PCR products were cloned in a TOPO Zero Blunt vector (Invitrogen) and sequenced with the M13 forward (−20) primer. Sequencing analyses were done by Takara Bio. Sequence alignment with DNASIS-MAC v3.3 (HITACHI) allowed identification of changes from the parental sequence in each clone. The classification method used was as described previously.

Chromosomal aberrations

For our chromosomal aberration analysis of the DT40 cells, compound-treated wild-type and DNA repair-deficient clones were incubated at 39.5°C for 14.5 h. To arrest cells in the metaphase, 0.06% colcemid (GIBCO-BRL) was added 2.5 h before harvest. Measurement of chromosome aberrations was performed as described previously. Briefly, cells were pelleted by centrifugation, resuspended in 7 ml of 75 mM KCl for 15 min at room temperature, and fixed in 2 ml of a freshly prepared 3:1 mixture of methanol:acetic acid (Carnoy’s solution). The pelleted cells were then resuspended in 7 ml of Carnoy’s solution and pelleted and resuspended in 1 ml of Carnoy’s solution, dropped onto clean glass slides, and air dried. Slides were stained with a 5% HARLECO Giemsa stain solution (Nacalai Tesque) for 20 min, rinsed with water and acetone, and dried. All chromosomes in each mitotic cell were scored at 1000× magnification. Chromosomal aberrations were classified as isochromatid or chromatid gaps, breaks, and exchanges (fusions included triradial, quadriradial, ring, dicentric, and other) according to the ISCN system.

DNA repriming assays

In vitro repriming assays were performed as described previously using DNA templates containing a single lesion and 5′ biotin modification annealed to 3′-dideoxynucleotide primers (sequences listed in Table S1). Oligonucleotides containing carboxyl or acyclovir were chemically synthesized as described previously. Briefly, PrimPol (1 μM) was incubated at 37°C for 15 mins in 20 μl reactions volumes containing 10 mM Bis-Tris-Propane-HCl pH 7.0, 10 mM MgCl2, 1 mM DTT, 250 μM dNTPs or rNTPs, and 2.5 μM FAM dNTPs (dATP, dCTP, dUTP) (Jena-Biosciences). Reactions were quenched with binding-washing buffer (B-W) buffer (10 mM Tris-HCl pH 7.5, 500 mM NaCl, 10 mM EDTA) and supplemented with ~20 μl streptavidin coated beads. Following DNA capture and washing with B-W buffer, beads were resuspended in 20 μL stop buffer (95% formamide solution with 0.25% bromophenol blue and xylene cyanol dyes) and heated to 95°C for 5 minutes. Reaction products were resolved on a 15% polyacrylamide/7M urea gel for 90 minutes at 21 watts and imaged using a FLA-5100 image reader (Fujifilm).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We would like to thank the Radioisotope Research Center of Tokyo Metropolitan University for their support with the use of isotopes. All the data are included in the published version of our paper and supporting supplementary data so there are no other underlying supporting data that is not accessible in the article itself.

Funding

KHz laboratory was supported by grants from the JSPS KAKENHI (26116518 and 25281021) and the Takeda Science Foundation. AIDs group was supported by grants from the Biotechnology and Biological Sciences Research Council (BB/H019723/1 and BB/M008800/1) and a Center grant from the Medical Research Council (G080130). TAG was supported by a University of Sussex funded PhD studentship. Funding for open access charge: Research Councils.UK (RCUK).

References

[1] Aguilera, A., Gomez-Gonzalez, B. Genome instability: a mechanistic view of its causes and consequences. Nat Rev Genet 2008; 9:204-17; PMID:18227811; http://dx.doi.org/10.1038/nrg2268
[2] Hirota K, Sonoda E, Kawamoto T, Motegi A, Masatani C, Hanaoka F, Szüts D, Iwai S, Sale JE, Lehmann A, et al. Simultaneous disruption of two DNA polymerases, Poleta and Polzeta, in Avian DT40 cells unmasks the role of Poleta in cellular response to various DNA lesions. PLoS Genet 2010; 6:e1001151; PMID:20949111; http://dx.doi.org/10.1371/journal.pgen.1001151
[3] Hirota K, Yoshikawa K, Guikhaib G, Tsurimoto T, Murai J, Tsuda M, Phillips LG, Narita T, Nishihara K, Kobayashi K, et al. The POLD3 subunit of DNA polymerase δ can promote translesion synthesis independently of DNA polymerase ζ. Nucleic Acids Res 2015; 43:1671-83; PMID:25628356; http://dx.doi.org/10.1093/nar/gkv023
[4] Sale JE. Competition, collaboration and coordination—determining how cells bypass DNA damage. J Cell Sci 2012; 125:1633-43; PMID:22499669; http://dx.doi.org/10.1242/jcs.094748
[5] Kohzaki M, Nishihara K, Hirota K, Sonoda E, Yoshimura M, Ekino S, Butler JE, Watanabe M, Halazonetis TD, Takeda S. DNA polymerases nu and theta are required for efficient immunoglobulin V gene diversification in chicken. J Cell Biol 2010; 189:1117-27; PMID:20584917; http://dx.doi.org/10.1083/jcb.200912012
[6] Kikuchi K, Narita T, Pham VT, lijima J, Hirota K, Keka IS, Mohiud-din Okawa K, Hori T, Fukagawa T, et al. Structure-specificendonuclases xpf and mus81 play overlapping but essential roles in DNA repair by homologous recombination. Cancer Res 2013; 73:4362-71; PMID:23576554; http://dx.doi.org/10.1158/0008-5472.CAN-12-3154

[7] Kobayashi S, Kasaiishi Y, Nakada S, Takagi T, Era S, Motegi A, Chiu RR, Takada S, Hirota K, Radi8 and Ribn facilitate homologous recombination by two distinct mechanisms, promoting Radi51 focus formation and suppressing the toxic effect of nonhomologous end joining. Oncogene 2015; 34:4403-11; PMID:25417706; http://dx.doi.org/10.1038/onc.2014.371

[8] Li X, Heyer WD. Homologous recombination in DNA repair and DNA damage tolerance. Cell Res 2008; 18:99-113; PMID:18166982; http://dx.doi.org/10.1038/cr.2008.1

[9] Keller RC, Marians KJ. Replication fork reactivation downstream of a blocked nascent leading strand. Nature 2006; 439:557-62; PMID:16452972; http://dx.doi.org/10.1038/nature04329

[10] Yeeles JT, Marions KJ. The Escherichia coli replisome is inherently DNA damage tolerant. Science 2011; 334:235-8; PMID:21998391; http://dx.doi.org/10.1126/science.1209111

[11] Kawabata T, Luebbert SW, Yamaguchi S, Ilves I, Matise I, Buske T, Bianchi J, Rudd SG, Jozwiakowski SK, Bailey LJ, Soura V, Taylor E, Keen BA, Jozwiakowski SK, Bailey LJ, Bianchi J, Doherty AJ. Molecule Schiavone D, Jozwiakowski SK, Romanello M, Guilbaud G, Guilliam Wan L, Lou J, Xia Y, Su B, Liu T, Cui J, Sun Y, Lou H, Huang J. hPrimPol, an archaic primase/polymerase operating in human cells. Nucleic Acids Res 2014; 42:12102-11; PMID:25262353; http://dx.doi.org/10.1093/nar/gku879

[12] Zhao F, Wu J, Xue A, Su Y, Wang X, Lu X, Zhou Z, Qu J, Zhou X. Exome sequencing reveals CCDC111 mutation associated with high myopia. Hum Genet 2013; 132:913-21; PMID:23579484; http://dx.doi.org/10.1007/s00439-013-1303-6

[13] Keen BA, Bailey LJ, Jozwiakowski SK, Doherty AJ. Human PrimPol mutation associated with high myopia has a DNA replication defect. Nucleic Acids Res 2013; 41:421201-11; PMID:25262353; http://dx.doi.org/10.1093/nar/gku879

[14] Lee YS, Gregory MT, Yang W. Human Pol zeta purified with accessory subunits is active in translesion DNA synthesis and complements Pol eta in cisplatin bypass. Proc Natl Acad Sci U S A 2014; 111:2954-9; PMID:24449906; http://dx.doi.org/10.1073/pnas.132001111

[15] Buerstedde JM, Reynaud CA, Humphries EH, Olson W, Ewert DL, Well JC. Light chain gene conversion continues at high rate in an ALV-induced cell line. EMBO J 1990; 9:9217; PMID:2155784

[16] Yamashita YM, Okada T, Matsuoka T, Sonoda E, Zhao GY, Araki K, Tateishi S, Yamaizumi M, Takeda S. RAD18 and RAD54 cooperatively contribute to maintenance of genomic stability in vertebrate cells. EMBO J 2002; 21:5558-66; PMID:12374756; http://dx.doi.org/10.1093/emboj/cdf534

[17] Arakawa H, Moldovan GL, Siribasak H, Saribasak NN, Jentsch S, Buerstedde JM. A role for PCNA ubiquitination in immunoglobulin hypermutation. PLoS Biol 2006; 4:e366; PMID:17105346; http://dx.doi.org/10.1371/journal.pbio.0040366

[18] Di Noia J, Neuberger MS. Altering the pathway of immunoglobulin hypermutation by inhibiting uracil-DNA glycosylase. Nature 2002; 419:43-8; PMID:12214226; http://dx.doi.org/10.1038/nature00981

[19] Saberi A, Nakahara M, Sale JE, Kikuchi K, Arakawa H, Buerstedde JM, Yamamoto K, Takeda S, Sonoda E. The 9-1-1 DNA clamp is required for immunoglobulin gene conversion. Mol Cell Biol 2008; 28:6133-22; PMID:18662998; http://dx.doi.org/10.1093/mcbj/cdf534

[20] Sale JE, Calandrini DM, Takata M, Takeda S, Neuberger MS. Ablation of XRCC2/3 transforms immunoglobulin V gene conversion into somatic hypermutation. Nature 2001; 412:921-6; PMID:11528482; http://dx.doi.org/10.1038/35091100

[21] Nakahara M, Sonoda E, Nojima K, Sale JE, Takenaka K, Kikuchi K, Taniguchi Y, Nakamura K, Sumitomo Y, Bree RT, et al. Genetic evidence for single-strand lesions initiating Nbs1-dependent homologous recombination in diversification of Ig v in chicken B lymphocytes. PLoS Genet 2009; 5:e1000356; PMID:19180185; http://dx.doi.org/10.1371/journal.pgen.1000356

[22] Verdi AJ. DNA polymerases as therapeutic targets. Biochemistry 2008; 47:8253-60; PMID:18642851; http://dx.doi.org/10.1017/bio101179f

[23] De Clercq E, Field HJ. Antiviral prodrugs - the development of successful prodrug strategies for antiviral chemotherapy. Br J Pharmacol 2006; 147:1-11; PMID:16284630; http://dx.doi.org/10.1038/sj.bjp.0706446

[24] Chang ML, Jeng WJ, Liaw YF. Clinical events after cessation of lamivudine therapy in patients recovering from hepatitis B flare with hepatic decompensation. Clin Gastroenterol Hepatol 2015; 13:979-86; PMID:25455774; http://dx.doi.org/10.1016/j.cgh.2014.10.023

[25] Kumar PN, East D, McDowell JA, Symonds W, Lou Y, Hetherington S, Lafon S. Safety and pharmacokinetics of abacavir (1592U89) in combination with zidovudine and lamivudine in CAIDS therapy. Antimicrob Agents Chemother 1999; 43:603-8; PMID:10049274

[26] Tada K, Kobayashi M, Takaku Y, Iwai F, Sakamoto T, Nagata K, Nishohara M, Ito K, Shirakawa K, Hishizawa M, et al. Abacavir, an anti-HIV-1 drug, targets TDP1-deficient adult T cell leukemia. PLoS One 2015; 1:e0140023; PMID:26601161; http://dx.doi.org/10.1371/journal.pone.0140023

[27] Mitusya H, Yachoo I, Bruder S. Molecular targets for AIDS therapy. Science 1999; 249:1533-44; PMID:1699273; http://dx.doi.org/10.1126/science.1699273
[40] Datta AK, Colby BM, Shaw JE, Pagano JS. Acyclovir inhibition of Epstein-Barr virus replication. Proc Natl Acad Sci U S A 1980; 77:5163-6; PMID:6254061; http://dx.doi.org/10.1073/pnas.77.9.5163

[41] Faletto MB, Miller WH, Garvey EP, St Clair MH, Daluge SM, Good SS. Unique intracellular activation of the potent anti-human immunodeficiency virus agent 1592U89. Antimicrob Agents Chemother 1997; 41:1099-107; PMID:9145876

[42] Friedberg EC, Walker GC, Siede W, Schultz RA, Ellenberger T. DNA repair and mutagenesis. 2006.

[43] Martinez-Jimenez MI, Garcia-Gomez S, Bebenek K, Sastre-Moreno G, Calvo PA, Diaz-Talavera A, Kunkel TA, Blanco L. Alternative solutions and new scenarios for translesion DNA synthesis by human PrimPol. DNA Repair (Amst) 2015; 29:127-38; PMID:25746449

[44] Li J, Zhang Q. PRIMPOL mutation: functional study does not always reveal the truth. Invest Ophthalmol Vis Sci 2015; 56:1181-2; PMID:25680973; http://dx.doi.org/10.1167/iovs.14-16072

[45] Keen BA, Bailey LJ, Joziakowski SK, Doherty AJ. Author response: PRIMPOL mutation: functional study does not always reveal the truth. Invest Ophthalmol Vis Sci 2015; 56:1183; PMID:25680976; http://dx.doi.org/10.1167/iovs.14-16123

[46] Ge XQ, Jackson DA, Blow JJ. Dormant origins licensed by excess Mcm2-7 are required for human cells to survive replicative stress. Genes Dev 2007; 21:3331-41; PMID:18079179; http://dx.doi.org/10.1101/gad.457807

[47] Ibarra A, Schwob E, Mendez J. Excess MCM proteins protect human cells from replicative stress by licensing backup origins of replication. Proc Natl Acad Sci U S A 2008; 105:8956-61; PMID:18579179; http://dx.doi.org/10.1073/pnas.0710574105

[48] Johnson AA, Ray AS, Hanes J, Suo Z, Colacino JM, Anderson KS, Johnson KA. Toxicity of antiviral nucleoside analogs and the human mitochondrial DNA polymerase. J Biol Chem 2001; 276:40847-57; PMID:11526116; http://dx.doi.org/10.1074/jbc.M1076743200

[49] Ooka M, Takazawa H, Takeda S, Hirota K. Cytotoxic and genotoxic profiles of benzo[a]pyrene and N-nitrosodimethylamine demonstrated using DNA repair deficient DT40 cells with metabolic activation. Chemosphere 2015; 144:1901-7; PMID:26547024; http://dx.doi.org/10.1016/j.chemosphere.2015.10.085

[50] Shimizu N, Ooka M, Takagi T, Takeda S, Hirota K. Distinct DNA Damage Spectra Induced by Ionizing Radiation in Normoxic and Hypoxic Cells. Radiat Res 2015; 184:442-8; PMID:26430822; http://dx.doi.org/10.1667/RR14117.1

[51] Okada T, Sonoda E, Yamashita YM, Koyoshi S, Tateishi S, Yamazumi M, Takata M, Ogawa O, Takeda S. Involvement of vertebrate polkappa in Rad18-independent postreplication repair of UV damage. J Biol Chem 2002; 277:48690-5; PMID:12356753; http://dx.doi.org/10.1074/jbc.M207957200

[52] Shinkura R, Ito S, Begum NA, Nagaoka H, Muramatsu M, Kinoshita K, Sakakibara Y, Hijikata H, Honjo T. Separate domains of AID are required for somatic hypermutation and class-switch recombination. Nat Immunol 2004; 5:707-12; PMID:15195091; http://dx.doi.org/10.1038/ni1086

[53] Sonoda E, Sasaki MS, Buerstedde JM, Bezzubova O, Shinohara A, Ogawa H, Takata M, Yamaguchi-Iwai Y, Takeda S. Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. EMBO J 1998; 17:598-608; PMID:9430650; http://dx.doi.org/10.1093/emboj/17.2.598

[54] Yamamoto KN, Kobayashi S, Tsuda M, Kurumizaka H, Takata M, Kono K, Jiriniec J, Takeda S, Hirota K. Involvement of SLX4 in interstrand cross-link repair is regulated by the Fanconi anemia pathway. Proc Natl Acad Sci U S A 2011; 108:6492-6; PMID:21464321; http://dx.doi.org/10.1073/pnas.1018487108

[55] Yamamoto KN, Hirota K, Kono K, Takeda S, Sakamura S, Xia M, Huang R, Austin CP, Witt KL, Tice RR. Characterization of environmental chemicals with potential for DNA damage using isogenic DNA repair-deficient chicken DT40 cell lines. Environ Mol Mutagen 2011; 52:546-61; PMID:21385559; http://dx.doi.org/10.1002/em.20656

[56] Fujita M, Sasamuna H, Yamamoto KN, Harada H, Kurosawa A, Adachi N, Omura M, Hiraoka M, Takeda S, Hirota K. Interference in DNA replication can cause mitotic chromosomal breakage unassociated with double-strand breaks. PLoS One 2013; 8:e60043; PMID:23573231; http://dx.doi.org/10.1371/journal.pone.0060043

[57] ISCN1985. An international system for human cytogenetic nomenclature. Report of the Standing Committee on Human Cytogenetic Nomenclature. Karger, Basel 1985.

[58] Yamamoto J, Takahata C, Kuroaka I, Hirota K, Iwai S. Chemical Incorporation of Chain-Terminating Nucleoside Analogs as 3’-Blocking DNA Damage and Their Removal by Human ERCC1-XPF Endonuclease. Molecules 2016; 21; PMID:27294910; http://dx.doi.org/10.3390/molecules21060766