Characterization of the uracil-DNA glycosylase activity of Epstein-Barr virus BKRF3 and its role in lytic viral DNA replication

Chih-Chung Lu¹, Ho-Ting Huang¹, Jiin-Tarng Wang¹, Geir Slupphaug², Tsai-Kun Li¹, Meng-Chuan Wu¹, Yi-Chun Chen¹, Chung-Pei Lee¹ and Mei-Ru Chen¹*

1. Graduate Institute and Department of Microbiology, College of Medicine, National Taiwan University; 2. Department of Cancer Research and Molecular Medicine, Faculty of Medicine, Norwegian University of Science and Technology, Trondheim, Norway

*Corresponding author: Mei-Ru Chen

e-mail: mrc@ha.mc.ntu.edu.tw

Address: No. 1, Jen-Ai Rd, 1st section, Graduate Institute of Microbiology, College of Medicine, National Taiwan University, Taipei, Taiwan. 100

Tel: 886-2-23123456, ext 8298
Fax: 886-2-23915293

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ABSTRACT

Uracil-DNA glycosylases (UDGs) of the UNG family are the primary DNA repair enzymes responsible for removal of inappropriate uracil from DNA. Recent studies further suggest that the nuclear human UNG2, and the UDGs of large DNA viruses, may coordinate with their DNA polymerase accessory factors to enhance DNA replication. Based on its amino acid sequence, the putative UDG of EBV, BKRF3 belongs to the UNG family of proteins, and was demonstrated previously to enhance oriLyt-dependent DNA replication in a co-transfection replication assay. However, the expression and enzyme activity of EBV BKRF3 have not yet been characterized. In this study, His-BKRF3 was expressed in bacteria and purified for biochemical analysis. Similar to the E. coli and human UNG enzymes, His-BKRF3 excised uracil from single-stranded DNA more efficiently than from double-stranded DNA and was inhibited by the purified bacteriophage PBS1 inhibitor Ugi. In addition, BKRF3 was able to complement an E. coli ung mutant in rifampicin and nalidixic acid resistance mutator assays. The expression kinetics and subcellular localization of BKRF3 products were detected in EBV positive lymphoid and epithelial cells using BKRF3 specific mouse antibodies. Expression of BKRF3 is mainly regulated by the immediate early transcription activator Rta. The efficiency of EBV lytic DNA replication was slightly affected by BKRF3 siRNA, whereas cellular UNG2 siRNA or inhibition of cellular and viral UNG activities by expressing Ugi repressed EBV lytic DNA replication. Taken together, we demonstrate the UNG activity of BKRF3 in vitro and in vivo and suggest that UNGs may participate in DNA replication or repair and thereby promote efficient production of viral DNA.
INTRODUCTION

Uracil in DNA may be generated from misincorporation of dUMP instead of dTMP, by DNA polymerase during replication, resulting in a relatively harmless U:A pair, or from spontaneous deamination of cytosine to uracil, which will create a mutagenic U:G mismatch. The frequency of cytosine deamination is expected to be in the order of 60-500 per mammalian genome per day (36). If such a change is not repaired prior to the next round of replication, an A:T transition will ensue with further rounds of replication. Base excision repair (BER) is the major pathway to remove a damaged or inappropriate base (31). The initial step in BER to remove inappropriate uracil is catalyzed by a uracil-DNA glycosylase (UDG). UDGs hydrolyse the N-glycosidic bond between the uracil and the deoxyribose sugar, leaving an abasic site (AP site) in the DNA. After incision of the AP site by AP endonuclease 1 (APE1), BER may follow two tracks. In short patch BER, the 5’-deoxyribose phosphate is removed by DNA polymerase β, which also inserts a C or T depending on the template base. Finally DNA ligase seals the nick. The alternative long-patch BER pathway largely uses replication proteins. In humans, polymerase δ or ε aided by proliferating cell nuclear antigen (PCNA) and replication factor C (RF-C) insert 2-8 nucleotides. The displaced “flap” containing the 5’-deoxyribose phosphate is removed by the flap endonuclease FEN-1, and the nick is sealed by DNA ligase I (32). The UDG superfamily is divided into four protein families. Although these share a common structural fold, they are surprisingly divergent at the amino acid level (1, 51). The UNG family (family 1) of proteins are the most ubiquitous, share similar biochemical properties and are highly conserved at both the amino acid and structural levels (61, 65, 81). UNG proteins are relatively small monomeric proteins that usually do not require cofactors or ions for their activity. They are furthermore highly specific against uracil in both single-stranded (ss) and double-stranded (ds) DNA, with a preference for ssDNA (64, 75).
The human UNG gene encodes both nuclear (UNG2) and mitochondrial (UNG1) isoforms of the enzyme through a mechanism that comprises transcription from two different promoters and alternative splicing (47). In addition to DNA repair, UNG2 is also involved in the somatic hypermutation and class switch recombination that yield secretory, high affinity antibodies in B lymphocytes. Mutations in both alleles of UNG result in a hyper-IgM syndrome with life-threatening infections (25). Furthermore, UDG has been demonstrated recently to be essential for translocations between c-myc and the IgH locus (Igh), which is a characteristic feature of Burkitt’s lymphoma (57). Notably UNG2 interacts with both proliferating cell nuclear antigen (PCNA) and replication protein A (RPA and colocalises with both proteins in cellular replication foci (29, 50).

Genome replication of DNA viruses is closely linked to the cellular DNA repair machinery. In Herpes simplex virus type 1 (HSV-1), several DNA repair proteins are recruited to the viral replication compartments, presumably for participation in virus DNA replication or repair (70, 80). It is notable that PCNA, RF-C and a series of mismatch repair (MMR) proteins are assembled precisely at viral replication compartments after induction of EBV lytic replication (11). A more recent study demonstrated upregulation of base-excision repair (BER) activities such as UNG2 and APE1 that may be involved in viral replication in HCMV-infected cells (59).

Interestingly, some herpesviruses as well as poxviruses, also encode UDGs belonging to the UNG family. The vaccinia virus D4R gene, which encodes the viral UNG, is essential for replication in tissue culture, although the catalytic activity is dispensable (13), suggesting that vaccinia UNG may participate in the formation of multi-protein DNA replication complexes. Indeed, the interaction of vaccinia UNG with A20 (a stoichiometric component of the viral processivity factor), along with E9 (viral DNA polymerase), is necessary and sufficient for the processive polymerase holoenzyme (67). The UNG encoded by human cytomegalovirus (HCMV)
UL114 also was shown to associate with ppUL44 (viral DNA polymerase processivity factor), and UL114 functions as part of the viral DNA replication complex to increase the efficiency of both early and late phase viral DNA synthesis (53). Moreover, deletion of HCMV UNG delays and diminishes replication of the virus in serum-deprived primary human fibroblasts (10). HSV-1 UNG (UL2 product) was first reported to be dispensable for viral replication in tissue culture (46), but later evidence suggested that the protein is required for virus reactivation from latency and for efficient replication in nerve tissue, which contains very low levels of cellular UNG (19, 55, 74).

Epstein-Barr virus (EBV), a gamma-1-herpesvirus, can establish life-long persistent infections in its natural host and transform B cells in vitro. EBV infection is associated with many lymphoproliferative diseases, such as infectious mononucleosis, Burkitt's lymphoma, Hodgkin's disease, and also tumors of epithelial origin, such as nasopharyngeal and gastric carcinomas (60). According to the genomic organization and sequence homology with the HSV-1 UL2 gene, EBV BKRF3 was recognized as a putative uracil DNA glycosylase. In an earlier study, BKRF3 was shown to enhance oriLyt dependent DNA replication about two fold in a cotransfection-replication assay (18). Despite that, the function of the gene product of EBV BKRF3 has not been investigated. Analogous to the crucial roles of other viral and human UDGs in DNA repair, DNA replication and pathogenesis, we wondered whether BKRF3 might play an important role in the lytic replication of EBV or its pathogenic mechanisms.

Here we demonstrate that EBV BKRF3 encodes a structurally and functionally conserved viral uracil DNA glycosylase of the UNG family, and provide evidence that BKRF3 as well as the cellular UNG2 is involved in DNA replication and/or repair to ensure the replication fidelity of viral DNA and promote higher levels of viral DNA.
MATERIALS AND METHODS

Constructs and strains. To construct BKRF3 expressing plasmids, PCR products were amplified
by 5’ primer: 5’- CGGGATCCATGGCATCGCGGGGC (nt 98065~98080) and 3’ primer: 5’-
CGGGATCCCTACAGCCTCCAATCTATC (nt 98832~98814) using Akata cell lysates as
template, subjected to BamHI digestion and cloned into the BamHI site of pET15a (Novagen, Inc.)
or pCMV-Tag2B (Stratagene) to generate pET15a-BKRF3 or pFlag-BKRF3. The sequences in
bold are derived from GenBank accession number AJ507799. pET15a-BKRF3 has a T7 promoter
for in vitro translation using a TNT reticulocyte lysate (Promega) and with six consecutive
histidine residues at the N-terminus for purification. To generate the pET15a-BKRF3mt (deleted
a.a 84-93, water-activating loop), an improved method for generating an internal deletion (44)
was performed using the single primer 5’
TGCGACCCCTCTGATATTCACGGGGGTCAAGCAAA (nt 98298~98313, nt
98344~98360) and pET15a-BKRF3 as DNA template. To generate pTrc99A-BKRF3 and
pTrc99A-BKRF3mt for expressing BKRF3 and BKRF3mt in an E. coli mutator assay, the BamHI
fragment from pET15a-BKRF3 or pET15a-BKRF3mt was cloned into the E. coli expression
vector pTrc99A (Pharmacia) which has a strong trc promoter that can be induced by addition of
IPTG. The plasmid pTrc99A-UNGΔ84 was described by Slupphaug et al. (64) and encodes the
common catalytic C-terminal domain of UNG1 and UNG2 (49).

The Rta-expressing plasmid RTS15 (pSG5-Rta, a gift from Diane Hayward) was described by
Ragoczy et al. (56). siZta and siGFP plasmids are pSUPER (5) based constructs as described by
Chang et al. (7). siRNA-expressing plasmids were constructed by cloning siRNA sequences into
pSUPER via the BamHI and HindIII sites. An economic and efficient method of siRNA vector
construction (6) was adapted to generate siRNAs targeted against BKRF3 and cellular UNG2 and
using the two top-ranked sequences predicted by an online program
(http://www.sirnawizard.com/siRNA.php). The siRNA sequences were further subjected to a
BLAST search against human genome and EST databases to ensure no other human genes were
targeted. The siBKRF3-1 and siBKRF3-2 are directed against the BKRF3 sequence 5’-
GGAAAGGAGAAACAGGAGAT-3’ (nucleotides 44–62 of the ORF) and 5’-
GGCTAGATTTCCTACAACT-3’ (nucleotides 101–119 of the ORF). The siUNG2-1 and
siUNG2-2 are directed against the UNG2 sequence 5’- GGGACAGGATCCCATATCAT-3’
(nucleotides 452–470 of the ORF) and 5’- GGCAAGAAGCCCATTGACT-3’ (nucleotides 909–
927 of the ORF), respectively.

pUgi-IRES-hrGFP plasmid was constructed by ligating UNG inhibitor encoding gene from
EcoRI-XhoI fragment of pZWtac1 (79) into a EcoRI-XhoI site of pIRES-hrGFP-1a (Stratagene).

E. coli strains NR8051 [\(\Delta\) (pro-lac), \(\text{thi}, \text{ara}\)] and its ung-I derivative NR8052 [\(\Delta\) (pro-lac),
\(\text{thi}, \text{ara}, \text{trpE9777, ungI}\)] (33) were used in the mutator assay.

**Mutator assay.** The mutator assay protocol was modified from that described by Olsen *et al.* (48).
Briefly, *E. coli* strains NR8051 (wt) and NR8052 (ung\(^{-1}\)) were transformed with the plasmids
aliquots of medium were inoculated with 0.5 ml of the overnight cultures of transformants and
grown at 37°C until the \(\text{OD}_{600}\) reached 0.4-0.5. Isopropylthiogalactoside (IPTG) was added to a
final concentration of 1 mM to induce UNG\(\Delta\)84, BKRF3, or BKRFmt expression. Rif\(^R\) and Nal\(^R\)
colonies were selected on LB plates containing 100 \(\mu\)g/ml rifampicin (inhibitor of bacterial RNA
polymerase \(\beta\) subsunit, Sigma Co.) or 40 \(\mu\)g/ml Nalidixic acid (inhibitor of bacterial DNA gyrase,
Sigma Co.), respectively. Mutation to Rif\(^R\) and Nal\(^R\) occurs normally at low frequency by base
substitution in the \(\text{rpoB}\) or \(\text{gyrA}\) gene. Mutation frequencies were measured by determining the
number of colony-forming cells that survived antibiotic selection per 4x10^8 viable cells plated.

DNA glycosylase assay. Single-stranded LMRC-U (5’-AGC TAC CAT GCC TGC ACG AAU TAA GCA ATT CGT AAT CAT GGT CAT), which were 5’-end-labeled with [γ-^32P]ATP followed by purified and quantified as previously described (43) or duplex oligonucleotides, which were prepared by mixing equimolar amounts of the appropriate single-stranded oligonucleotides followed by heating for two minutes at 95°C and slowly cooling to temperature, were incubated with either purified His-BKRF3, _E. coli_ UNG enzyme (NEB), _in vitro_ transcription/translation products or cell lysates at 37°C for 10 min. The standard assay mixture for DNA glycosylase activity containing 0.2 pmol of the labeled single-stranded or duplex oligonucleotides in a 20 μl buffer containing 1 mM EDTA, 1 mM DTT, 20 mM Tris–HCl (pH 8.0). UDG activity was stopped at 95°C for 5 min. After glycosylase cleavage, abasic sites were incised by 0.1 mM NaOH treatment at 95°C for 5 min. Reaction products were analysed by electrophoresis through denaturing 15% (w/v) polyacrylamide gels (7 M urea, 1x TBE), visualized using a PhosphorImager Storm 840 (Molecular Dynamics, Sunnyvale, CA) and quantified using ImageQuant software. For Uracil DNA glycosylase inhibitor (Ugi) assay, experiments were performed with 1U of Ugi (NEB) in each reaction.

Purification of bacterially expressed recombinant BKRF3. To express His-BKRF3 protein, pET15a-BKRF3 was transformed into BL21 (DE3) and the bacteria were induced in the exponential phase with 1 mM IPTG at 37°C for 2 h. The cell extracts were homogenized in lysis buffer (20 mM Tris/HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole) and 1 tablet/10 ml of Complete® mini protease inhibitor mixture (EDTA-free, Roche). The protein sample was then incubated with nickel nitrilotriacetic acid-agarose (Qiagen) at 4°C with rotation for 1 h. The
protein was eluted with a buffer [20 mM Tris/HCl, pH 8.0, 300 mM NaCl, 10 % (v/v) glycerol] containing increasing concentrations of imidazole. The fractions containing His-BKRF3 were pooled together and dialyzed against 20 mM Tris/HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 10 % (v/v) glycerol for uracil DNA glycosylase activity assay.

Antibodies. To generate BKRF3-specific polyclonal antibodies, six-week-old BALB/c mice were immunized with 50 µg His-BKRF3 protein in complete Freund's adjuvant subcutaneously, followed by three boosters with incomplete Freund's adjuvant at 2 week intervals. Other primary antibodies used for immunoblotting detection and indirect immunofluorescence including monoclonal antibodies BRLF1 mAb 467, BZLF1 mAb 4F10 (71), BMRF1 mAb 88A9 (72), and rabbit antiserum against BGLF4 (77) and BALF5 (39) and cellular UDG2 (PU059) rabbit polyclonal antibody (64).

Cell culture and induction of viral lytic cycle. Akata and Raji are EBV-positive Burkitt's lymphoma cell lines (54, 68). NPC-TW01 is an NPC cell line lacking the EBV genome (35) and NA was derived from recombinant Akata EBV converted NPC-TW01 (9). For lytic cycle induction, NA cells were treated with 40 ng 12-O-tetradecanoylphorbol-13-acetate (TPA) ml⁻¹ and 3 mM sodium butyrate (Sigma) or transfected with Rta expressing plasmid RTS15. Akata cells were induced with 0.5% (v/v) goat-anti human IgG antibody (Cappel Inc.)

Indirect immunofluorescence. To detect intracellular expression of BKRF3, HeLa, TW01 or NA cells were slide-cultured, harvested at indicated time points, air-dried and fixed with 4% paraformaldehyde in PBS for 30 min. Immunostaining was conducted with anti-BKRF3 polyclonal antibody (1:50). Rhodamin red-conjugated anti-mouse IgG antibodies (1:200; Jackson)
were used as secondary antibodies. After PBS washing, cells were stained with Hoechst 33258 at room temperature for 1 min, and covered with mounting medium (H1000; Vector) for fluorescence (Axioskop 40 FL; Zeiss) microscopy and confocal laser scanning (Leica).

Subcellular fractionation. The subcellular fractionation protocol was adapted from that described by Krajewski et al. (30). Briefly, cells were covered or incubated with hypotonic buffer (5 mM Tris/HCl, pH 7.4, 5 mM KCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 1 mM DTT and 1 mM PMSF) on ice for 1 h, harvested by scraping and homogenized by passage through 27 gauge needles 15 times. Cell lysates were subjected to centrifugation at 500 X g for 5 min at 4 °C. The resulting pellet was the nuclear fraction. The supernatant was then mixed with 3 volumes of LSB buffer (50 mM Tris/HCl, pH 7.5, 25 mM KCl, 5 mM MgCl₂) and further centrifuged at 150,000 X g at 4 °C for 2 h. The resulting pellet contained heavy membrane (rough endoplasmic reticulum, mitochondria, peroxisomes, Golgi apparatus and lysosomes) and light membrane (plasma membrane) fractions. The cytosolic fraction in the supernatant was further concentrated for gel analysis.

Transfection. Cells were transfected with different plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. To suppress Zta or BKRF3 expression, NA cells were pre-transfected with 4 µg siRNA producing plasmid for 24 h and then co-transfected with 2 µg siRNA producing plasmid and 2 µg Rta-expressing plasmid. For suppression of cellular UNG2 expression, NA cells were pre-transfected with 5 µg siUNG2 plasmid for 24 h, reseeded and transfected again with 5 µg siUNG2 plasmid for 24 h before co-transfection with 3 µg siUNG2 plasmid and 2 µg Rta-expressing plasmid. To identify Rta responsive gene expression, 50 µg of Rta expressing plasmid RTS15, or vector control pSG5
(Stratagene Co.) was transfected into $1 \times 10^7$ Raji cells in 0.2 ml RPMI 1640 medium by electroporation at 200 V, 950 $\mu$F with a ECM630 system (BTX Co.).

Detection and quantification of EBV DNA. Cells were lysed, digested by proteinase K and then subjected to the following PCR analysis as described previously (8). For quantification of EBV DNA, real-time PCR was performed according to the manufacturer's instructions (Applied Biosystems). The detection target of real-time PCR was the EBNA1 region of the EBV genome and details of the primers and probes were provided in a previous study (40). We used H2B4 cells harboring one EBV genome per cell to generate a standard curve for quantification (8) and EBV copy number was calculated by comparison with the standards. All samples were tested in duplicate.
RESULTS

EBV BKRF3 encodes a putative conserved uracil-DNA glycosylase

According to the newly revised EBV sequence (12, AJ507799 in GenBank), the predicted coding sequence of BKRF3 contains 765 nt and encodes a protein of 255 amino acids. The predicted amino acid sequence of BKRF3 was aligned with different eukaryotic and prokaryotic Ung sequences (Fig. 1A) and showed an identity of 48%, 42%, 47% and 52% with UNG enzymes of HCMV, HSV-1, human and E. coli, respectively. The short N-terminus of BKRF3 is distinct from most of the UNG proteins described so far. The BKRF3 sequence is highly conserved through the various UNG domains that are involved in catalysis and binding, such as the conserved motifs GQDPYH (water-activating loop) and HPSPLS (DNA intercalation loop). To explore the possible function of BKRF3, UDG activity was determined using a 5’[^32P]-labeled synthetic uracil containing 45mer-DNA as a substrate (Fig. 1B). Uracil excision by UDG generates an apyrimidinic site in the DNA oligomer, which is sensitive to alkaline conditions and will result into two fragments, one of which ([^32P]-labeled) is detected as a faster migrating band (20-mer) upon gel electrophoresis. Because NaOH treatment alone did not generate fast migrating oligonucleotides, this indicates that there were no detectable AP sites in the substrate prior to UNG treatment (Fig. 1D, lane 2). As for the purified E. coli UNG enzyme (NEB. Co), the incubation of substrate with the in vitro transcription/translation product of BKRF3 resulted in the generation of the cleaved 20-mer. Furthermore, deletion of a.a 84~93 encompassing the conserved catalytic motif of BKRF3 abolished the UDG enzyme activity, indicating that BKRF3 is a highly conserved enzyme (Fig. 1C & D).

EBV BKRF3 complements an E. coli ung mutant in vivo. Uracil-DNA glycosylases are known to be responsible for removing uracil from DNA in the base excision repair (BER) pathway to
ensure DNA integrity. In addition to the UDG activity of BKRF3 observed in vitro, we sought to determine whether BKRF3 may contribute to the maintenance of genome integrity in vivo. To that end, a plasmid (pTrc99A-BKRF3) containing the EBV BKRF3 open reading frame expressed under the control of a trp/lac (tac) hybrid promoter was transformed into the ung mutant E. coli strain NR8052 (ung<sup>-1</sup>), and the effect of BKRF3 on mutation frequency to rifampicin resistance (Rif<sup>R</sup>) was measured (Fig. 2). Mutation to Rif<sup>R</sup> occurs normally at low frequency by base substitutions in the rpoB gene. As expected, in the presence of the transcriptional inducer IPTG, the wild-type E. coli strain NR8051 generated Rif<sup>R</sup> colonies at low frequency (46.9 Rif<sup>R</sup> colonies per 4x10<sup>8</sup> cells, Fig. 2A, C & Table. 1). In contrast, the ung mutant E. coli strain NR8052 and the vector-transformed controls (pTrc99A) resulted in a 9.8 fold increase of Rif<sup>R</sup> colonies (Fig. 2A &Table 1). Similar to the effect of positive control human UNG<sup>△84</sup> (indicated as hUNG), induction of BKRF3 reduced the mutation frequency ten fold.

The mutator assay was also performed with wild type strain NR8051 harboring hUNG, BKRF3, and BKRF3<sub>mt</sub>, giving virtually the same number of resistant colonies as wild type cells, indicating exogenous expression of UDG cannot reduce the mutation frequencies further (Fig. 2A, C & Table 1). The efficiency of plating (EOP) showed that E. coli growth was not influenced by the expression of BKRF3, BKRF3<sub>mt</sub> or hUNG (Fig 2B). These data demonstrate that BKRF3 is able to suppress the mutator phenotype of the E. coli ung mutant in vivo. This complementation was not restricted to the selection applied, being also evident when mutation to the inhibitor of bacterial DNA gyrase, nalidixic acid (Nal) resistance, was monitored (Fig. 2D and Table 1).

Mutation to Nal<sup>R</sup> results from base substitutions in the gyrA gene. The variation in the reduction of mutation frequency observed in different selections may reflect differences in the types and abundances of mutations that allow E. coli growth. Because BKRF3<sup>△a.a 84~93</sup> lost the ability to complement the ung mutant, we conclude that BKRF3 complementation is dependent on UDG.
activity. These results not only demonstrate that BKRF3 may participate in a base-excision repair (BER) pathway in vivo, but also confirm that UNG proteins are highly conserved through prokaryotes, eukaryotes and viruses.

To confirm that BKRF3 encoded UDG activity in the E. coli ung mutant correlated with the mutator assay, cell-free extracts of the wild-type E. coli NR8051 and ung mutant E. coli NR8052 carrying the plasmid expressing BKRF3, BKRF3mt, hUNG, or vector control were assayed for UNG enzyme activities (Fig. 2E). Indeed, the extracts from wild-type E. coli, hUNG, and BKRF3 transformants displayed measurable uracil excision activity in contrast to extracts from ung mutant E. coli NR8052 with vector or the BKRF3mt (Fig. 2E). We then examined the sensitivity of BKRF3 to the natural UNG inhibitor Ugi, which is expressed by phages PBS-1 and -2 and allows these uracil containing DNA phages to survive and replicate in their genotypically ung+ hosts. Ugi can function in DNA mimicry that specifically and irreversibly inhibits E. coli and human UNG (45, 62). As shown in Fig. 2F, addition of Ugi resulted in the inhibition of the UDG activities of E. coli UNG, hUNG and BKRF3 in vitro. These results demonstrate that BKRF3 encodes a conserved uracil-DNA glycosylase that can function in vitro and in vivo.

Expression and purification of BKRF3 UNG. The UNGs characterized so far are relatively small monomeric proteins that do not require cofactors for enzyme activity and have a preference for uracil in single-stranded DNA (ssDNA) over dsDNA in vitro (64). To examine further the biochemical properties of BKRF3 UDG activity, the coding region of the BKRF3 gene was cloned into the expression vector pET-15a, which encodes six consecutive histidine residues at the N-terminal end to facilitate purification. After transformation into BL21(DE3) cells, a 28 kDa protein was induced by IPTG as displayed by SDS-PAGE analysis (Fig. 3A). The soluble fraction of the His-BKRF3 protein was purified using an affinity Ni2+ column and the target protein was...
recovered by elution with buffers containing step gradients of 0–500 mM imidazole. In this way, a homogeneous purified protein was obtained as judged by SDS–PAGE and Coomassie blue staining (Fig. 3A).

**BKRF3 prefers uracil-containing ssDNA substrates.** The substrate specificity of the recombinant His-BKRF3 protein was then characterized. The kinetics of uracil excision was measured in single-stranded and double-stranded oligonucleotides containing a mismatch of U·G, U·C, U·A or U·T (Fig. 3C & Table.2). 5' [32P]-labeled oligonucleotides were incubated with increasing concentrations of either His-BKRF3 or *E. coli* UNG. In these experiments, 0.1 nM His-BKRF3 excised about 50% of the uracils from ssDNA, whereas 1.0 nM His-BKRF3 was needed to achieve a similar degree of excision from dsDNA. Furthermore, the specificity constant ($K_{cat}/K_m$) with single-stranded substrate was about ten times higher than with the U·G, U·C, U·A, or U·T duplex substrates (Table. 2). This result indicates that the His-BKRF3 preferentially excises uracil from ssDNA rather than dsDNA.

Several studies observed that the UDG activity of human UNG2 was stimulated 10- to 27-fold in the presence of 10 mM Mg\(^{2+}\) or Mn\(^{2+}\) (28, 29). To investigate possible effect of Mg\(^{2+}\) and Mn\(^{2+}\), UDG activity of BKRF3 was measured in the absence or presence of 0.5, 1, 2, 4 and 8 mM MgCl\(_2\) or MnCl\(_2\). Possibly because of minor contaminated nuclease activity, total amounts of labeled oligonucleotides were slightly reduced in the presence of 4 or 8 mM MgCl\(_2\) or MnCl\(_2\). However the uracil excision efficiency indicates that the UDG activity of BKRF3 was inhibited about 80% in the presence of 8 mM MgCl\(_2\) (Fig. 3E) or MnCl\(_2\) (Fig. 3F).

According to the sequence alignment (Fig. 1A), the BKRF3 protein showed a 52% sequence identity to *E. coli* UNG. Therefore, the kinetic constants of *E. coli* UNG acting on the same oligonucleotides were measured simultaneously for comparison (Fig. 3D, Table 2). The kinetic
analyses demonstrated that the overall efficiency ($k_{cat}/K_m$) of BKRF3 was lower than that of *E. coli* UNG, and that this primarily was caused by a much higher turnover ($k_{cat}$) of the latter enzyme. Notably, however, BKRF3 showed a markedly higher preference for ssDNA- over the dsDNA substrates (~10-fold) than *E. coli* UNG (<2-fold) and this was mainly caused by a low $K_m$ of BKRF3 against U in ssDNA. Taken together, the single-strand preference of BKRF3 as well as the inhibition of its enzyme activity by Ugi, are characteristics shared by the *E. coli* and human UNG proteins (28), and indicate that the active site of the three enzymes are structurally conserved.

**Expression of BKRF3 in EBV-positive cells upon induction of the lytic cycle.** To determine whether BKRF3 is expressed in EBV-positive cell lines after induction of the lytic cycle, we used the purified His-BKRF3 proteins to generate a BKRF3 specific mouse serum. The serum showing the strongest signal in detecting Flag-BKRF3 in transiently transfected 293T cells (Fig. 4A) was used to detect BKRF3 in chemically induced EBV-positive epithelial NA cells. BKRF3 became detectable at 24 hours post induction (hpi) and reached maximal expression levels at 36 hpi with an apparent molecular weight of 28 kDa, which is in agreement with the molecular weight predicted from the nucleic acid sequence. Simultaneously, two immediate early transactivators BZLF1 (Zta), BRLF1 (Rta) and early antigen BMRF1 (EA-D) showed similar expression kinetics (Fig. 4B). These data also accorded with our and others’ previous observation in microarray analysis that BKRF3 is expressed in the second cluster of EBV early genes (42, 82, 83). We then detected the expression of BKRF3 in anti-IgG-induced Akata cells and Rta transfected Raji cells (Fig. 4C). Because Raji cells harbor an EBV genome with a deletion in the coding region of the major DNA-binding protein and are unable to complete viral DNA replication, the expression of BKRF3 protein in Raji cells indicates that its expression is independent of viral DNA replication.
In addition, because it is known that exogenous expression of Rta in Raji cells does not induce expression of the other immediate early transactivator Zta, the detection of BKRF3 protein in Rta-induced Raji cells suggests that Rta alone is able to induce the expression of BKRF3.

**BKRF3 is expressed in both the nucleus and cytoplasm of EBV positive and EBV negative cells.** The human *UNG* gene has been shown to encode both nuclear (UNG2) and mitochondrial (UNG1) isoforms of the enzyme. In addition to the conserved catalytic domains, the N-terminal extension found in human UNG is involved in its subcellular localization (47). We then determined the subcellular localization of BKRF3 using indirect immunofluorescence. Following transient transfection, BKRF3 was detected both in the nucleus and cytoplasm of HeLa cells (Fig. 5A). BKRF3 also localized in both nucleus and cytoplasm at 36 hpi in chemically induced NA cells. Moreover, BKRF3 associated with viral factors in replication compartments such as BGLF4 (protein kinase) and BALF5 (DNA polymerase), as observed with fluorescence microscopy and confocal laser scanning (Fig. 5B). Subcellular fractionation of NA at 24 hpi further confirmed the localization of BKRF3 in the nucleus and cytoplasm, but not in the heavy membrane fraction (Fig. 5C). A similar staining pattern was observed at 48 hpi, indicating the subcellular localization of BKRF3 does not change with the progression of virus replication (data not shown).

**BKRF3 contributes to EBV lytic replication.** BKRF3 was reported previously to enhance oriLyt dependent DNA replication about two fold in a cotransfection-replication assay (18). Moreover, HSV-1 UNG-minus mutants replicated and spread poorly in mice (55). More recent studies suggested that HCMV UNG excises uracil residues from replicating HCMV DNA to create a single nick for initiation of rolling circle replication at a late stage in the infection process.
(10). To explore the possible biological function of BKRF3, experiments were carried out to
determine whether BKRF3 contributes to EBV lytic DNA replication with a pSUPER-based
system (5) to drive siRNA synthesis in Rta-transduced NA cells. Exogenous expression of Rta
induced the expression of BZLF1, BMRF1 and BKRF3 proteins in NA cells, as detected by
immunoblotting. The amplification of EBV DNA was detected simultaneously using a
quantitative real-time PCR method (Fig. 6). Expression of BKRF3 was inhibited by double
transfection of BKRF3-targeted siRNA (siBKRF3-1 or -2), and Zta expression was inhibited by
siZta but not affected by pSUPER or siGFP controls (Fig. 6A). As a relevant control, Zta-
dependent expression of BMRF1 was blocked in the presence of siZta. Simultaneously, Rta-
induced BKRF3 expression decreased moderately in the presence of siZta (Fig. 6A), indicating
that the lytic induction of BKRF3 could be activated by Rta alone and probably enhanced
synergistically by Zta and Rta. Regarding the Rta-induced amplification of viral genomes,
expression of siZta completely blocked the viral DNA amplification in the lytic cycle (Fig. 6E).
Meanwhile, siBKRF3-2 showed about 20% inhibitory effect on the amplification of viral
genomes at 48 hpi. Compared with RV-pSUPER controls, the UDG activity in a cell extract was
increased by about 10% in Rta-induced NA cells (Fig. 6B & C). In addition, the total UDG
activities of induced-NA cells were inhibited about 10% when the expression of BKRF3 was
knocked down by siRNA (Fig. 6D). This implies that cellular UNG might partially complement
the function of viral UNG when the latter is not expressed.

**Cellular UDG2 contributes to EBV lytic replication.** To investigate if cellular UDG activity
might compensate for BKRF3 during virus replication, NA cells were transfected with UNG2-
targeted siRNA (siUNG2-1 and -2). Expression of UNG2 was specifically inhibited by triple
transfection of siUNG2-1 and slightly inhibited by siUNG2-2 but not siGFP control in Rta-
induced NA cells (Fig. 7A). The total UDG activities of induced-NA cells were inhibited to about 50% by siUNG2-1 (Fig. 7B, C). In agreement with this, Rta-induced amplification of the viral genome was also repressed 50%~60% at 48 hpi (Fig. 7D).

Requirement of UNG activity in EBV lytic replication. To further investigate the contribution of UNG activity to viral lytic replication, NA cells were co-transfected with Rta and Ugi expressing plasmids which can inhibit both viral and cellular UNG activities (Fig. 2F). Indeed, the UDG activity could barely be detected in reactivated NA cells transfected with the Ugi-expressing plasmid (Fig. 8B). The weak residual activity could be caused by incomplete inhibition of UNG proteins, or the presence of other cellular UDG activities such as SMUG1, that are not inhibited by Ugi. Interestingly, the copy numbers of EBV DNA in Rta-induced NA cells were reduced to about half in the absence of detectable UDG activity at various time points (Fig. 8C). However the expression of early lytic genes was not affected by the presence of Ugi (Fig. 8A). This observation suggests that the enzymatic activity of UNG proteins is required for higher efficiency of viral DNA replication.

DISCUSSION

Several lines of evidence from the present study indicate that EBV BKRF3 encodes a structurally and functionally conserved uracil-DNA glycosylase of the UNG-family. First, amino acid sequence alignment demonstrates high degree of homology with other UNG proteins within the C-terminal catalytic domain and absolutely conserved catalytically important motifs. Notably, deletion of the water-activating group renders BKRF3 catalytically dead (Fig. 1). Second, expression BKRF3 in E. coli can restore the normal phenotype of ung mutants to the same extent as human UNG (Fig 2). Third, BKRF3 excises uracil in both dsDNA and ssDNA, but has a
preference for the latter. (Fig. 3). Fourth, BKRF3 is inhibited by Ugi (Fig. 3), which is highly specific towards the UNG-family of proteins and do not inhibit other UDGs (4, 45, 78).

UNG proteins have a well documented function in base excision repair of uracil, and may remove uracil close to the replication fork by association with replicative proteins, or within non-replicating DNA (28). In addition, some virally encoded UNGs may have a more direct function in viral DNA replication. In HSV1, the deletion of UL2, which encodes UNG, affects the ability of the virus to replicate in mice, particularly in the central nervous system (55). Moreover, lack of viral UNG expression in HCMV infection has been demonstrated, leading to a delay in viral DNA synthesis and replication (10, 52). However, the viral UNG activity is dispensable for viral replication in cultured cells, presumably because adequate cellular activity is present. This also suggests that cellular UNG may complement viral UNG mutants in cultured cell lines (10, 46, 52).

The cellular UNG2 activity is cell cycle associated, with maximal activity detected during late G1 and early S phase (16, 66), and is undetectable in adult neurons (19, 75). Thus, a role is implied for viral UNG in the replication of the virus in the host, particularly in non-dividing cells (e.g. terminally differentiated cells), where levels of cellular UNG2 are believed to be low. Such a model is also supported by the present study. By using the Ugi-expressing plasmid to inhibit both viral and cellular UNG2 activities, or siUNG2 to knock down UNG2, EBV DNA replication was suppressed to half of the vector control, whereas EBV lytic DNA replication was only affected slightly when BKRF3 expression was inhibited by siBKRF3. This indicates that the UNG activity is important for EBV lytic DNA replication and that cellular UNG2 may back-up viral UNG in tissue culture cells. We hypothesize that the role of BKRF3 UDG activity in EBV replication is particularly important in terminally differentiated and non-dividing cells. This likelihood is supported by several reports indicating EBV lytic replication occurred in terminal differentiated cells. It is now generally believed that EBV persists in resting memory B cells in which only a
small fraction of the infected cells are dividing in the peripheral blood (2, 24, 27). Moreover,
using limiting-dilution RT-PCR to detect BZLF1 and the early gene BHRF1 demonstrated that
about 10-20% of the EBV infected plasma cells are undergoing lytic replication, compared to less
than 0.1% of EBV-infected germinal center B cells and memory B cells (24). The authors thus
suggested that the differentiated plasma cell is naturally responsible for replicating the virus and
the terminal differentiation of B cells into plasma cells may provide the signal that initiates EBV
replication (24). The promoter of the immediate-early gene BZLF1, is activated only after
differentiation of memory cells into plasma cells (34). In addition, acute infectious
mononucleosis caused by EBV is accompanied by central nervous system (CNS) disorders in 1-
18% of patients (17, 21). It was also reported that cerebrospinal fluid (CSF) samples from IM
patients contained EBV DNA and EBV-specific anti-VCA and anti-EA antibodies in the
neurological stage, but not during convalescence (26). This suggested that EBV-associated
neurological complications may be due to direct virus invasion of the CNS, although the
pathogenesis is not completely understood (14, 26, 76). Considering these observations together,
we postulate that EBV may replicate in terminally differentiated cells or non-dividing cells such
as plasma B cells and neuronal cells. Thus, the BKRF3 UNG appears to play an important role
for replication in fully differentiated non-proliferating cells that probably contain low levels of
cellular UNG2.

In cell culture, BKRF3 was found previously to increase about two-fold the replication
efficiency of an origin of lytic replication (oriLyt) containing plasmid in a transient transfection
system (18). By using siBKRF3, total cellular UDG activities in the extract was barely affected
(Fig.6D), meanwhile the viral DNA replication efficiency in cultured NA cells was reduced about
20% at the late stage by siBKRF3-2 (Fig.6E). Previous studies have shown that UNGs from
human, HCMV, and vaccinia virus interact with proliferating cell nuclear antigen (PCNA) or its
viral homolog and colocalize to the DNA replication foci (50, 53, 67). Moreover, the interaction of UNG with the processivity factor of vaccinia virus increased the processivity of the viral DNA polymerase (50, 53, 67). It is thus possible that cellular UNG2 activity may be sufficient at the early stage of viral DNA replication, whereas the viral enzyme is important in a later stage of viral DNA replication through interaction with viral replication factors thereby enhancing the efficiency of viral replication.

Likewise, three possible mechanisms may account for the role of UNG activity in EBV lytic DNA replication: (i) UNG activity might participate in the DNA repair system to ensure the fidelity of viral DNA replication and therefore promote higher production of viral DNA. (ii) UDG might be involved in the initiation of rolling-circle replication which can produce a burst of virus genomes. It is believed that herpesviral DNA replication is initiated with the origin-specific circular latent genome which leads to an early theta form replication and later undergoes a switch to rolling-circle DNA replication. In the process of switching, a single-strand break needs to be introduced into the double-stranded circular form DNA to generate a free 3’-hydroxyl group, serving as a primer for subsequent rolling circle replication. UDG has been suggested to play a role in producing nicks for the initiation of rolling-circle DNA replication (10, 41, 42). This suggestion is partly supported by the observation in yeast that uracil as a critical source of AP sites in DNA which can be further converted into single-stranded breaks by AP endonuclease (22). (iii) UDG might enhance the interaction of the origin-binding protein and oriLyt by removing uracil residues within the oriLyt region. In both EBV and HSV, the binding of origin binding protein (OBP) to the origin of lytic replication is absolutely required for viral DNA replication. In HSV, it has been demonstrated that uracil replacing either cytosine (mimicking cytosine deamination) or thymine (mimicking dUTP misincorporation) on site I of OriS diminishes the interaction between OBP and OriS in a gel shift assay (20). Thus, experiments to determine the
effects of the binding of Zta to uracil containing Zta responsive element (ZRE) are awaited. No matter which mechanisms are involved, the UDG activity is important in the EBV lytic DNA replication as expression of Ugi suppressed the efficiency of viral replication. Additionally, recent studies in Kaposi sarcoma virus (KSHV) identified human UNG2 as a protein interacting with Latency Associated Nuclear Antigen (LANA). Depletion of UNG2 in KSHV positive cells using siRNA reduced the viral genome copies and produced infection deficient virus (73). Because UNG2 was previously found to interact with several cellular replication proteins such as PCNA, PRA and polymerase α (29, 50, 63), it would be interesting to study whether UNG2 can recruit these cellular machineries to viral replication compartment for enhancing viral replication efficiency.

In addition to its role in viral DNA replication, a possible contribution of BKRF3 UDG activity to EBV pathogenesis is of concern. Cellular UNG2 is known to play a role in the somatic hypermutation (SHM) and class switching recombination (CSR) that can generate high-affinity antibodies of different isotypes. CSR requires the up-regulation of the B cell-specific enzyme, activation-induced cytosine deaminase (AID). CSR is regulated tightly and usually requires up-regulation of CD40 ligand on Ag-activated CD4+ T cells (3). UNG and AID are required for the reciprocal translocation between IgH and the oncogene, c-Myc in vivo, which is the hallmark of B lymphoma (57, 58). Furthermore, combined loss of p53 function and DNA double-strand break (DSB) responses such as ATM or the non-homologous end-joining (NHEJ) pathway would lead to the accelerated appearance of AID-induced c-myc/IgH translocation (57). This places the expression of c-Myc under the control of IgH, which is expressed constitutively in B cells, suggesting its etiologic role in B cell transformation. More than 90% of EBV-associated endemic Burkitt’s lymphomas have undergone a c-myc/IgH chromosome translocation (69). Notably, EBV encoded latent membrane protein 1 (LMP1) induces CD40-independent CSR from Cµ to
multiple downstream Cγ, Cα, and Cε genes in B cells. This induction is associated with up-
regulation of AID (23). Moreover, EBV infection of human B cells resulting in AID expression
has been investigated and increasing numbers of mutations in p53 were also seen in culture with
time (15). It has been observed that expression of EBV LMP1 protein represses p53-mediated
DNA repair and transcriptional activity through the activation of the NF-κB pathway. This
suggests the abrogation of DNA repair by LMP1 may induce genomic instability and contribute
subsequently to tumorigenesis (37, 38). Here we propose that EBV BKRF3 may function in
addition to LMP1 to contribute to genome instability, thus favoring AID/UNG-induced c-
myc/IgH translocations.

In summary, although expression of BKRF3 transcripts has been observed upon induction of
EBV positive B cells and by microarray analysis of NK/T-cell lymphoproliferative cell lines (42,
82, 83), we demonstrated here for the first time that BKRF3 encodes a conserved and functional
uracil DNA glycosylase of the UNG family. Because inhibition of both viral and cellular UDG
activity results in suppression of EBV lytic DNA replication, we suggest that BKRF3 might play
an important role in EBV lytic replication, particularly in terminal differentiated cells where the
cellular counterpart is absent or limited in abundance. Future insights into the mechanism of
BKRF3 function should allow a more complete understanding of EBV lytic cycle replication and
the oncogenesis of EBV associated malignances, and may pave the way for the future design of
specific BKRF3 inhibitors.

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on the composition of viral proteins within prereplicative sites and correlates with

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FIGURE LEGENDS

Fig 1. Sequence alignment of uracil-DNA glycosylases encoded by EBV, HSV-1, HCMV, human and E. coli. (A) The amino acids are numbered according to EBV BKRF3 and the identical residues are depicted in red, whilst similar residues are shown in blue. The conserved motifs are indicated. (B) The flow chart of DNA glycosylase activity assay. 5’ [\(^{32}\)P]-labeled 45mer oligonucleotides containing a uracil residue at position 21 were incubated with UNG at 37°C for 10 min. Reaction products were treated with 0.1 N NaOH to reveal abasic sites generated by DNA glycosylase. Final products were displayed by 15% PAGE containing 7 M urea. (C) Wild type BKRF3 or mutant protein was synthesized in the TNT\(^{\text{\textregistered}}\) Lysate coupled transcription/translation with [\(^{35}\)S]-methionine labeling and resolved by 12% SDS- PAGE. (D) 5’ [\(^{32}\)P]-labeled LMRC-U was incubated with E. coli UNG (used as a positive control, NEB. Co.) or 5 µl of in vitro transcription/translation products of BKRF3 and BKRF3mt. control: LMRC-U probe only. No UNG: LMRC-U probe treated with 0.1 N NaOH.

Fig. 2. EBV BKRF3 can complement a E. coli ung mutant in mutator assays. (A) Mutation frequencies were examined by plating equal amounts of wild-type E. coli NR8051 or ung mutant E. coli NR8052 harboring pTrc99A-hUNG, pTrc99A-BKRF3, pTrc99A-BKRF3mt or vector control pTrc99A. Representative rifampicin plates demonstrated the mutation frequencies relative to the wild-type control as indicated. (B) The serial dilutions of bacteria indicated were spotted on LB agar plate containing ampicilin or not to demonstrate the equal efficiency of plating (EOP). The frequency of rif\(^{R}\) (C) or Nalidixic acid (Nal\(^{R}\)) (D) mutations per 4x10\(^{8}\) cells is indicated. Each point represents the mutation frequency of an independent culture. (E) In vitro UDG activity assay of individual cell lysates with single-stranded uracil containing substrate. Cell
lysates containing 1 μg of total protein were used in this experiment. (F) One unit of UNG inhibitor protein, Ugi was added into DNA glycosylase assay.

Fig. 3. UDG activities of BKRF3 on single- or double-stranded substrates containing uracil.
(A) Recombinant His-BKRF3 recovered by elution with buffer containing step-gradients of 0-500 mM imidazole was displayed on SDS-PAGE. (B) One unit of Ugi was added to a DNA glycosylase assay to inhibit the enzyme activities of purified E. coli UNG or His-BKRF3 protein. Titration of UDG activities of His-BKRF3 (C) or E. coli UNG (D) with single- and double-stranded oligonucleotides containing uracil. Effect of MgCl₂ (E) and MnCl₂ (F) on the activity of the His-BKRF3 proteins. The single-stranded oligonucleotide containing uracil was incubated with 0.5 nM His-BKRF3 in the presence or absence of MgCl₂ and MnCl₂. The radioactivities of the products was scanned by Phosphoimager and quantified by ImageQuant program and indicated as excision %.

Fig. 4. Expression of EBV BKRF3 in EBV-positive cells upon induction of lytic cycle. (A) 293T cells were transfected with the plasmid expressing Flag-BKRF3. Immunoblotting analysis of Flag-BKRF3 using anti-BKRF3 polyclonal antibody. (B) EBV-positive NA cells were induced with TPA/sodium butyrate and harvested at different time points as indicated. Immunoblotting analysis of BRLF1, BMRF1, BZLF1, BKRF3 and β-actin was performed with specific antibodies. (C) Detection of BKRF3 expression in anti-human IgG induced Akata cells at 24 hpi and Rta-induced Raji cells at 48 hpi.

Fig. 5. Expression of BKRF3 in both the nucleus and cytoplasm of transiently transfected and EBV replicating cells. (A) HeLa cells were transfected with plasmids expressing Flag-
BKRF3. The expression of BKRF3 was detected with pre-immune serum, specific mouse serum or Flag Ab. (B) Expression of BKRF3 in NA cells was detected with specific mouse serum at 36 hr post induction and the nuclei were stained with Hoechst 33258. Expression of BGLF4 and BALF5 in NA cells was detected with specific rabbit antiserum and observed with fluorescence microscopy and confocal laser scanning as indicated. (C) Subcellular localization of BKRF3 in NA cells at 24 hpi fractionated into nuclear (N), heavy membrane (H) and cytosolic plus light membrane (C) fractions. After electrophoresis and blotting, BKRF3 was detected with specific mouse serum. PARP and α-tubulin were detected as nuclear and cytoplasmic markers.

Fig. 6. EBV DNA replication in NA cells expressing siBKRF3. NA cells were transfected with plasmids expressing siBKRF3-1, siBKRF3-2, siGFP or siZta for 24 h and then transfected with Rta expressing plasmids or control vectors (RV) for 24 h and 48 h. (A) Immunoblotting analysis of BRLF1, BMRF1, BZLF1, BKRF3 and β-actin. (B) UDG activity assays using 1 µg total protein of cell lysates with single-stranded uracil containing oligonucleotide substrates. (C, D) Serially diluted total extracts were used for UDG activity assay with single-stranded uracil containing oligonucleotide substrates. The radioactivities of the products was scanned by Phosphoimager and quantified by ImageQuant program and indicated as excision %. (D) Quantification of EBV DNA at various time points by real-time PCR to detect EBNA-1 sequences.

Fig. 7. EBV DNA replication in NA cells expressing siUDG2. NA cells were transfected with plasmids expressing siUNG2-1, siUNG2-2 or siGFP twice and then transfected with Rta expressing plasmids or control vectors (RV) for 24 h and 48 h. (A) Immunoblotting analysis of BRLF1, BZLF1, BKRF3, UNG2 and β-actin. (B) UDG activity assays using 1 µg total protein of
cell lysates with single-stranded uracil containing oligonucleotide substrates. (C) Serially diluted total extracts were used for UDG activity assay with single-stranded uracil containing oligonucleotide substrates. The radioactivities of the products was scanned by Phosphoimager and quantified by ImageQuant program and indicated as excision %.

(D) Quantification of EBV DNA at various time points by real-time PCR to detect EBNA-1 sequences.

Fig. 8. EBV DNA replication in NA cells expressing Ugi. NA cells were co-transfected with plasmids expressing UNG inhibitor (Ugi) and Rta. (A) Immunoblotting analysis of BRLF1, BMRF1, BZLF1, BKRF3 and β-actin at 24 or 48 post transfection. (B) UDG activity assays using 1 µg cell lysates with single-stranded uracil containing oligonucleotide substrates. (E) Quantification of EBV DNA at different time points by real-time PCR to detect the EBNA-1 sequence.
Table 1. Mutation frequency of BKRF3-transformed UNG-deficient *Escherichia coli*<sup>a</sup>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Selection</th>
<th>Median mutation frequency (per 4X10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>Vector</td>
</tr>
<tr>
<td>NR8051</td>
<td>ung&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Rifampicin</td>
<td>46.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nalidixic acid</td>
<td>4.3</td>
</tr>
<tr>
<td>NR8052</td>
<td>ung&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>Rifampicin</td>
<td>430.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nalidixic acid</td>
<td>89.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each median mutation frequency was determined from 6-8 independent cultures.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km (µM)</th>
<th>Kcat (S⁻¹)</th>
<th>Kcat/Km (S⁻¹µM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BKRF3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ssDNA</td>
<td>0.065 ± 0.003</td>
<td>0.275 ± 0.015</td>
<td>4.2</td>
</tr>
<tr>
<td>dsDNA (U:A)</td>
<td>0.258 ± 0.006</td>
<td>0.125 ± 0.005</td>
<td>0.48</td>
</tr>
<tr>
<td>dsDNA (U:T)</td>
<td>0.296 ± 0.023</td>
<td>0.133 ± 0.008</td>
<td>0.45</td>
</tr>
<tr>
<td>dsDNA (U:C)</td>
<td>0.388 ± 0.035</td>
<td>0.154 ± 0.006</td>
<td>0.4</td>
</tr>
<tr>
<td>dsDNA (U:G)</td>
<td>0.295 ± 0.023</td>
<td>0.124 ± 0.016</td>
<td>0.42</td>
</tr>
<tr>
<td><strong>E. coli UNG</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ssDNA</td>
<td>0.139 ± 0.005</td>
<td>5.13 ± 0.17</td>
<td>36.8</td>
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<tr>
<td>dsDNA (U:A)</td>
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<td>2.94 ± 0.19</td>
<td>16.9</td>
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<tr>
<td>dsDNA (U:T)</td>
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<td>3.26 ± 0.05</td>
<td>20.9</td>
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<tr>
<td>dsDNA (U:C)</td>
<td>0.194 ± 0.025</td>
<td>3.45 ± 0.18</td>
<td>17.9</td>
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<tr>
<td>dsDNA (U:G)</td>
<td>0.207 ± 0.012</td>
<td>3.65 ± 0.13</td>
<td>17.7</td>
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</table>

*aEach kinetics constants was determined from 4 independent experiments. The Km of the UNG was determined by incubating the respective proteins (0.5 nM BKRF3 for ssDNA, 5 nM BKRF3 for dsDNA, 0.05 nM E. coli UNG for ssDNA, and 0.1 nM E. coli UNG for dsDNA) with increasing concentrations (ranged from 2 nM to 2 µM) of oligonucleotides. The kinetic constants were calculated using Michaelis-Menten fit.*

Table 2. Lu et al.
Figure 1. Lu et al.
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Figure 3. Lu et al.
Figure 4. Lu et al.
Figure 5. Lu et al.
Figure 6. Lu et al.
Figure. 7 Lu et al.
Figure 8. Lu et al.