

The Base Excision Repair Pathway Is Required for Efficient Lentivirus Integration

Kristine E. Yoder^{1*}, Amy Espeseth³, Xiao-hong Wang⁷, Qingming Fang⁷, Maria Teresa Russo⁴, R. Stephen Lloyd⁵, Daria Hazuda³, Robert W. Sobol^{6,7,8}, Richard Fishel^{1,2*}

1 Molecular Virology, Immunology, and Medical Genetics, Human Cancer Genetics, The Ohio State University Medical Center and Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio, United States of America, **2** Physics Department, The Ohio State University, Columbus, Ohio, United States of America, **3** Department of Antiviral Research, Merck Research Laboratories, West Point, Pennsylvania, United States of America, **4** Department of Environment and Primary Prevention, Istituto Superiore di Sanità, Rome, Italy, **5** Center for Research on Occupational and Environmental Toxicology, Department of Molecular and Medical Genetics, Oregon Health & Science University, Portland, Oregon, United States of America, **6** Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, United States of America, **7** University of Pittsburgh Cancer Institute, Hillman Cancer Center, Pittsburgh, Pennsylvania, United States of America, **8** Department of Human Genetics, University of Pittsburgh Graduate School of Public Health, Pittsburgh, Pennsylvania, United States of America

Abstract

An siRNA screen has identified several proteins throughout the base excision repair (BER) pathway of oxidative DNA damage as important for efficient HIV infection. The proteins identified included early repair factors such as the base damage recognition glycosylases OGG1 and MYH and the late repair factor POL β , implicating the entire BER pathway. Murine cells with deletions of the genes *Ogg1*, *Myh*, *Neil1* and *Pol β* recapitulate the defect of HIV infection in the absence of BER. Defective infection in the absence of BER proteins was also seen with the lentivirus FIV, but not the gammaretrovirus MMLV. BER proteins do not affect HIV infection through its accessory genes nor the central polypurine tract. HIV reverse transcription and nuclear entry appear unaffected by the absence of BER proteins. However, HIV integration to the host chromosome is reduced in the absence of BER proteins. Pre-integration complexes from BER deficient cell lines show reduced integration activity *in vitro*. Integration activity is restored by addition of recombinant BER protein POL β . Lentiviral infection and integration efficiency appears to depend on the presence of BER proteins.

Citation: Yoder KE, Espeseth A, Wang X-h, Fang Q, Russo MT, et al. (2011) The Base Excision Repair Pathway Is Required for Efficient Lentivirus Integration. *PLoS ONE* 6(3): e17862. doi:10.1371/journal.pone.0017862

Editor: Reuben Harris, University of Minnesota, United States of America

Received: November 22, 2010; **Accepted:** February 11, 2011; **Published:** March 23, 2011

Copyright: © 2011 Yoder et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by NIH grants A182422 (KY and RF), DK075974 (RSL), CA148629 (RWS), a grant from Associazione Italiana Ricerca sul Cancro (MTR), and Merck Research Laboratories (AE and DH). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have read the journal's policy and have the following conflict: Authors A. Espeseth and D. Hazuda are employed by Merck Research Laboratories. All other authors declare that no competing interests exist. All authors agree to make freely available any materials and information described in this publication that are reasonably requested by others for the purpose of academic, non-commercial research.

* E-mail: yoder.176@osu.edu (KEY); rfishel@osu.edu (RF)

Introduction

Retroviruses are defined by the enzymatic activities reverse transcription and integration [1]. The RNA genome of a retrovirus is reversed transcribed to a linear cDNA that is part of the poorly understood pre-integration complex (PIC). The PIC enters the nucleus where the viral enzyme integrase catalyzes the covalent attachment of the cDNA to the host genomic DNA. Lentiviruses are a subset of retroviruses that do not require cellular division for PICs to enter the nucleus, traversing the nuclear membrane by an unknown mechanism. The integration event results in the viral cDNA flanked by two single strand DNA gaps of host sequence ranging from 4–6 nucleotides and 5' flaps of two nucleotides of viral sequence. This integration intermediate is repaired to yield the provirus flanked by 4–6 base pair duplications. It is unknown which DNA repair pathway mediates repair of the integration intermediate *in vivo*, but proteins from multiple DNA repair pathways are able to repair a similar substrate *in vitro* [2].

Several siRNA library screens have been performed to identify host factors necessary for HIV infection [3,4,5]. There was little

overlap between the sets of host genes identified in each screen. Although each of the screens identified at least one DNA repair gene, none of these screens conclusively identified a DNA repair pathway that repairs the integration intermediate. A recent siRNA screen targeting DNA repair genes identified several proteins throughout the short patch base excision repair (BER) pathway for oxidative damage that appear to be important for HIV infection [6]. Among the proteins identified in this siRNA study were POLB, LIG3, and XRCC1, which were previously shown to efficiently repair an integration intermediate substrate *in vitro* [2].

The BER pathway begins with recognition of base damage by a DNA glycosylase, which removes the damaged base [7,8]. Each glycosylase recognizes specific base damage; for example, OGG1 recognizes oxidatively damaged guanine as 8-oxo-guanine (8-oxo-G) or formamidopyrimidine-guanine (Fapy-G) [8]. The abasic site left by the glycosylase is recognized by Ape1 to cleave the sugar phosphate backbone 5' to the damaged base site. The resulting 3' hydroxyl is extended by a polymerase, generally Pol β . The remaining nick is sealed by a heterodimer of Lig3 and Xrcc1. Reduced HIV infection was associated with siRNA mediated reduced expression of the oxidative glycosylases OGG1, MYH,

NTH1, NEIL2, NEIL3, the endonuclease APE1, the polymerases POLB, POLI, POLL and the ligase proteins LIG3 and XRCC1 in human cells [6].

Here we have collected mouse embryonic fibroblasts (MEFs) derived from BER gene deletion mutants, including *Ogg1*, *Myh*, *Neil1*, and *Polβ* null cell lines. These cell lines and matched wild type littermate cells were infected with the gammaretrovirus Moloney MLV (MMLV) and the lentiviruses HIV and FIV. Only the lentiviruses showed reduced infection efficiency in the BER deletion cells. Quantitation of the HIV provirus shows that integration to the host genome is reduced in the absence of BER proteins. PICs derived from BER deficient cells showed reduced integration activity compared to PICs from wild type cells. PIC integration activity from Polβ deficient cells was rescued with the addition of recombinant POLB protein. Oxidative damage associated BER proteins appear to affect lentiviral infection efficiency at the integration step.

Results

Deletion of BER genes leads to reduced lentivirus infection

Mouse strains with deletions of DNA glycosylases are viable [9,10]. Deletion of *Polβ* leads to neonatal lethality, allowing for the isolation of *Polβ* null murine embryonic fibroblasts at E10–E14 [11]. It is not possible to isolate cells with deletions of the *Ape1*, *Lig3* or *Xrcc1* genes [10,12,13]. We have collected MEFs from mouse strains with deletions of several oxidative damage BER genes and matched wild type littermates including *Myh*, *Ogg1*, *Neil1*, and *Polβ* [11,14,15,16,17]. This group of cell lines includes proteins from the initiation of BER through the late steps of BER [18].

The DNA damage sensitivity phenotypes of the glycosylase cell lines were confirmed by treatment with varying concentrations of hydrogen peroxide (H₂O₂); viable cells were measured by trypan blue exclusion (Figure 1A). *Myh* null and *Ogg1* null mice were originally crossed to generate *Myh*^{+/-} *Ogg1*^{+/-} mice [16,19]. These mice were subsequently intercrossed to ultimately generate MEFs from wild type, *Myh* null, and *Ogg1* null littermates [16,19]. Compared to matched wild type cells, *Myh* null and *Ogg1* null MEFs were sensitive to the oxidative DNA damaging agent H₂O₂ (Figure 1A, [17,20]. PCR analysis of genomic DNA confirmed the genotypes of the wild type, *Myh* null, and *Ogg1* null cell lines (Figure 1B). Wild type and *Neil1* null MEFs were generated from littermates [15]. *Neil1* null MEFs were more sensitive to increasing concentrations of H₂O₂ compared to matched wild type MEFs (Figure 1C). The genotypes of the *Neil1* wild type and null cell lines were also confirmed by PCR (Figure 1D).

The *Polβ* wild type and null MEFs were derived from littermates and were tested for DNA damage sensitivity. These cells were treated with varying concentrations of H₂O₂ or methyl methane-sulfonate (MMS); viable cells were measured by trypan blue exclusion (Figure 2, A and B). As previously reported, *Polβ* null cells were sensitive to MMS, but not H₂O₂ [11,21]. Immunoblot analysis confirmed expression of the Polβ protein in wild type cells and absence in *Polβ* null MEFs (Figure 2C). PCNA was probed as a loading control (Figure 2C).

Wild type littermate and BER gene deletion MEFs were infected with lentiviral vectors derived from HIV and FIV and a retroviral vector derived from the gammaretrovirus MMLV (Figure 3). These vectors have been shown to faithfully recapitulate the retroviral life cycle from reverse transcription through integration [22]. The HIV, FIV, and MMLV vectors all express GFP following successful integration [23,24,25]. Cells were analyzed by flow cytometry for GFP expression at 72 hours post infection (hpi). Deletion of the

glycosylase genes *Myh* or *Ogg1* led to decreased infection by HIV and FIV (Figure 3A; *Myh* and HIV P=0.004, *Ogg1* and HIV P=0.0011, *Myh* and FIV P=0.013, *Ogg1* and FIV P=0.0001). However, there was no significant difference between MMLV infection of wild type and *Myh* null (P=0.072) or *Ogg1* null cells (P=0.15). Deletion of the *Neil1* glycosylase gene also led to significant decreases in HIV (P<0.0001) and FIV (P=0.0003) infection compared to wild type cells (Figure 3B). *Neil1* null cells did show a significant decrease in MMLV infection (P=0.004), but the infection efficiency was less than 30% different from wild type cells (Figure 3B). Infection of cells with deletion of the DNA polymerase gene *Polβ* was similar to infection of *Myh* and *Ogg1* null cells (Figure 3C, HIV P<0.0001, MMLV P=0.31, FIV P<0.0001), suggesting that the BER pathway significantly affects lentiviral infection but not gammaretroviral infection.

Lentiviral determinants are not associated with the BER pathway

The lentivirus family is a specialized subset of retroviruses [1]. One obvious difference between MMLV and HIV is the presence of accessory genes in the lentivirus, including *vif*, *vpr*, *vpu*, and *nef*. MMLV has no accessory genes. HIV vectors have been engineered to express any subset of accessory genes [26]. BER deletion cell lines were infected with HIV vector particles that did or did not include the accessory genes ($\Delta vif \Delta vpr \Delta vpu \Delta nef$, Figure 4A). Total RNA was purified from HIV vector particle producer cells and analyzed by RT-PCR for accessory gene expression (Figure 4B). Both wild type HIV and HIV ($\Delta vif \Delta vpr \Delta vpu \Delta nef$) vector producer cells express *gag* RNA, but only wild type HIV producer cells appear to produce RNA encoding the accessory genes (Figure 4B). The infection efficiency of HIV without accessory genes was similar to HIV expressing all accessory genes, indicating that the BER proteins are not interacting with lentiviral accessory genes (Figure 4A).

A second notable difference between retroviruses and lentiviruses is the presence of a central polypurine tract (cPPT) [1]. The linear cDNA of lentiviruses has a 5' flap of single stranded DNA resulting from strand displacement synthesis during reverse transcription, which is absent in MMLV retroviral cDNA. This single stranded DNA has been shown to be a substrate for the human DNA repair enzyme FEN1 and has been suggested to mediate nuclear import of the HIV PIC [24,27]. The wild type and BER deletion MEFs were infected with HIV vectors with and without the cPPT and analyzed for GFP expression by flow cytometry (Figure 4B). A similar pattern of infection efficiency was seen in the wild type and matched BER mutant cell lines, indicating that the BER proteins are not interacting with the lentiviral cPPT.

BER proteins do not affect reverse transcription or nuclear import

To determine any effect of BER proteins on reverse transcription efficiency, we evaluated the accumulation of HIV cDNA over time. The BER WT and null cell lines were infected with an HIV vector, DNA fractions were collected at multiple time points, and analyzed by quantitative PCR (qPCR) for late reverse transcripts (Figure 5). The late reverse transcript primer set spans the reverse transcription primer binding site and amplifies all cDNA forms including complete linear cDNA, 1LTR and 2LTR circles, and integrated provirus [28]. The total HIV cDNA accumulation over time was similar for *Myh*, *Ogg1*, *Neil1*, or *Polβ* null and matched wild type cell lines, indicating that reverse transcription is not affected by the BER pathway.

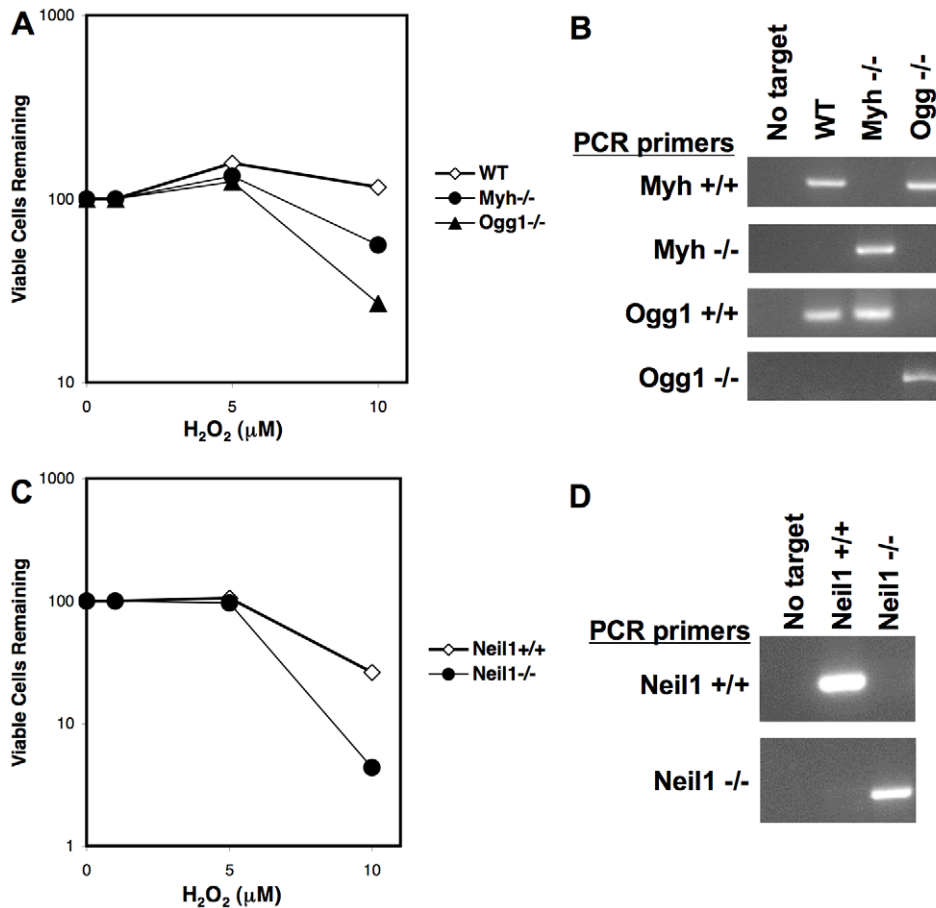


Figure 1. Viability of BER glycosylase deletion cell lines treated with DNA damaging agent H₂O₂. BER glycosylase deletion cells and matched wild type littermate cells were treated with increasing concentrations of H₂O₂. Cells were stained with trypan blue and viable cells counted. The percentage of viable cells remaining is shown. (A) Matched wild type, *Myh* null and *Ogg1* null MEFs treated with H₂O₂. (B) PCR analysis of *Myh* and *Ogg1* genotypes. Primer sets detected wild type *Myh* (*Myh*^{+/+}), the *Myh* deletion construct (*Myh*^{-/-}), wild type *Ogg1* (*Ogg1*^{+/+}) and the *Ogg1* deletion construct (*Ogg1*^{-/-}). PCR targets included water (No target) and genomic DNA from wild type (WT), *Myh*^{-/-}, and *Ogg1*^{-/-} cell lines. (C) Matched wild type and *Neil1* null MEFs treated with H₂O₂. (D) PCR analysis of *Neil1* genotypes. Primer sets detected wild type *Neil1* (*Neil1*^{+/+}) and the *Neil1* deletion construct (*Neil1*^{-/-}). PCR targets included water (No target) and genomic DNA from wild type (*Neil1*^{+/+}) and *Neil1*^{-/-} cell lines. doi:10.1371/journal.pone.0017862.g001

A previous report suggested that the BER protein Ape1, as part of the cytoplasmic SET complex, protects HIV from autointegration [29]. Ape1 also plays an essential role in BER in the nucleus and a likely role in the mitochondria [8,30]. To determine if the BER DNA glycosylases might also act by preventing HIV autointegration, DNA was analyzed by qPCR for autointegration products at 24 hpi (Figure 6A). There was no difference in HIV autointegration products between wild type and BER DNA glycosylase deletion cells.

Retroviral 2LTR circles are only found in the nuclear compartment and are an indicator of successful nuclear import of the retroviral PIC [31]. DNA from infected cells at 24 hpi was analyzed for 2LTR circles by qPCR (Figure 6B). There was no significant difference in the accumulation of 2LTR circles between wild type and BER deletion cells, indicating that the BER proteins do not affect nuclear import of HIV cDNA.

The integrated HIV provirus was also measured by qPCR in BER cell lines (Figure 6C). DNA at 72 hpi was amplified by primers to HIV and host Alu elements and further measured by qPCR [32]. While there appears to be no difference in reverse transcription, autointegration, or nuclear import of HIV cDNA,

the BER mutant cell lines show reduced integrated provirus compared to wild type cells (Figure 6C). This data suggests that the BER proteins specifically affect the integration of HIV.

BER proteins affect HIV PIC integration *in vitro*

The role of BER proteins during integration was further investigated with HIV PICs. HIV vector particles were added to the BER cell lines and infection was allowed to proceed for 6 hours [33]. During this time reverse transcription is completed and PICs are formed [34]. HIV PICs are fully competent to integrate into an exogenous DNA target and integration efficiency is measured by qPCR [35,36]. HIV PICs derived from matched wild type cells were compared to PICs from *Ogg1*, *Neil1*, and *Polβ* null cells (Figure 7A). Integration to purified genomic DNA was significantly reduced when either a DNA glycosylase or Polβ was absent. Increasing concentrations of recombinant Polβ protein was added to integration reactions of the wild type and Polβ null PIC extracts (Figure 7C) [11]. The integration efficiency of HIV PICs from Polβ null cells was rescued by the addition of recombinant protein (Figure 7B). The role of BER proteins during lentiviral infection appears to be associated with the integration reaction.

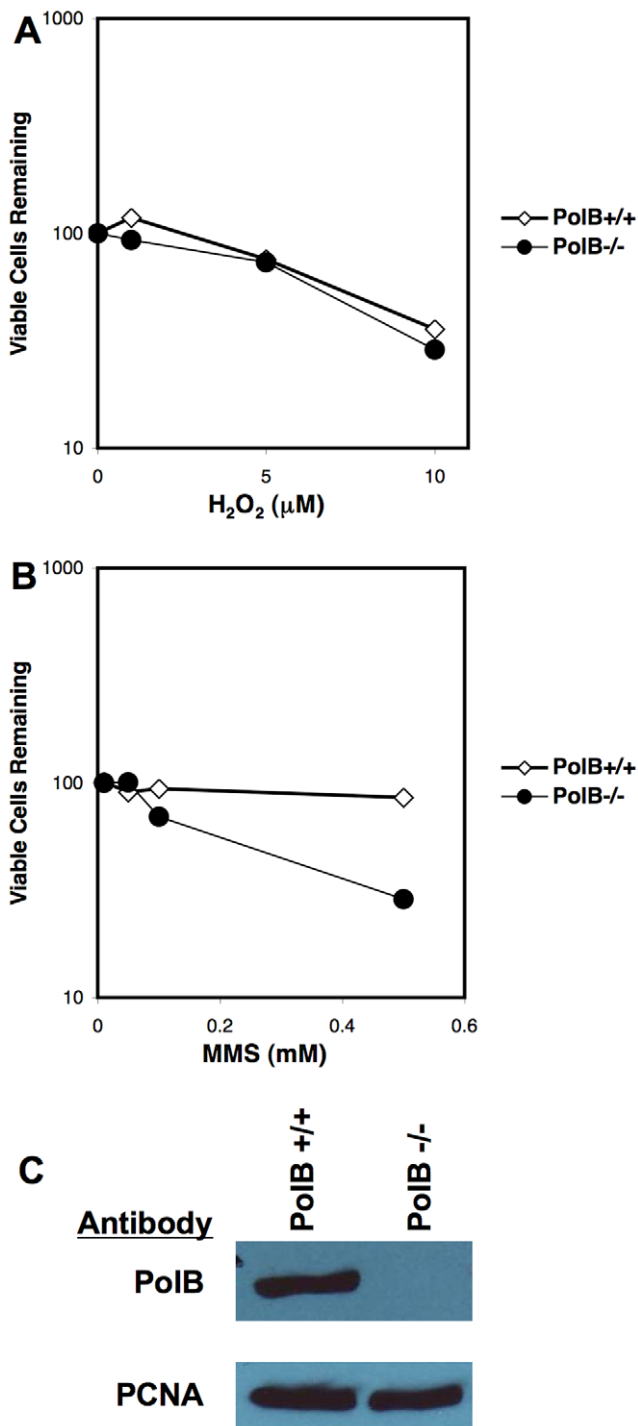


Figure 2. Viability of BER polymerase b cell lines treated with DNA damaging agents. (A) *PolB* deletion cells and matched wild type littermate cells were treated with increasing concentrations of the oxidative base damage inducing agent H₂O₂. (B) *PolB* cell lines were treated with increasing concentrations of the methylation base damage inducing agent MMS. Cells were stained with trypan blue and viable cells counted. The percentage of viable cells remaining is shown. (C) Western analysis of wild type (*PolB*^{+/+}) and *PolB* null (*PolB*^{-/-}) cell lines for *PolB* expression. Blots were stripped and re-probed for PCNA as a loading control. doi:10.1371/journal.pone.0017862.g002

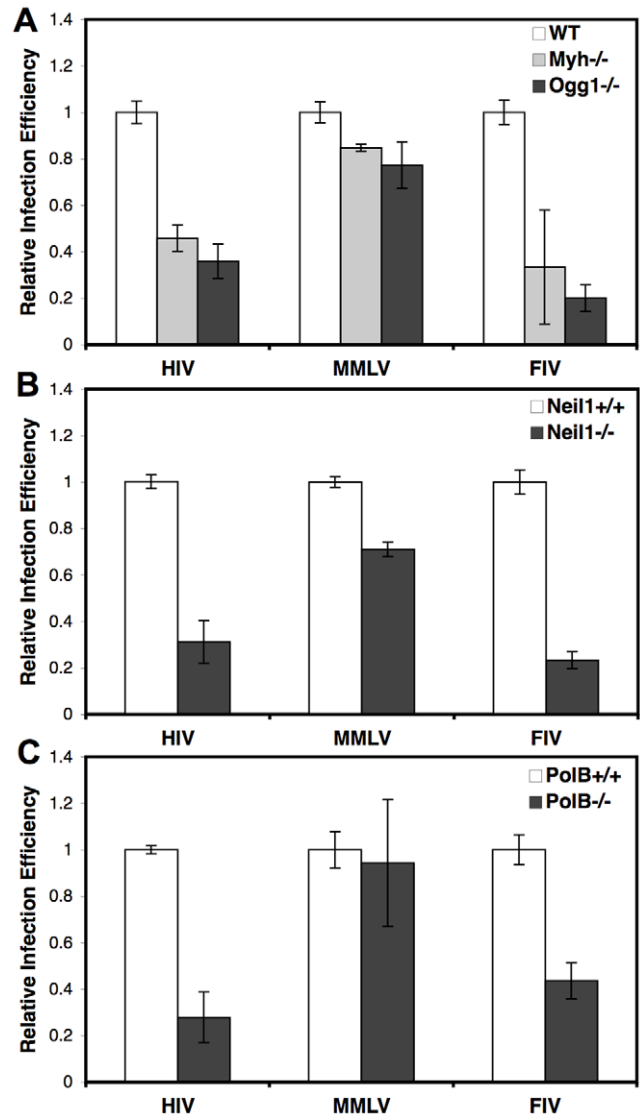


Figure 3. Infection of BER cell lines with HIV, MMLV, and FIV. Cells were infected with HIV, MMLV, and FIV retroviral vectors expressing GFP following integration. Cells were analyzed at 72 hpi by flow cytometry for GFP expression indicating successful infection. Wild type MEFs were from littermates. (A) Wild type (WT), *Myh*^{-/-}, and *Ogg1*^{-/-} cell lines, (B) Wild type (*Neil1*^{+/+}) and *Neil1*^{-/-} cell lines, (C) Wild type (*PolB*^{+/+}) and *PolB*^{-/-} cell lines. Infections were performed at two MOI in duplicate at least three times. Error bars indicate the standard deviation after normalization. doi:10.1371/journal.pone.0017862.g003

Discussion

During the process of retroviral integration, the viral cDNA is covalently joined to the host chromosome but is flanked by 4–6 base pair gaps of host DNA and a 5' dinucleotide flap of viral DNA [1]. The identities of host or viral factors that mediate repair of this integration intermediate are unknown. Three siRNA library screens of host factors that affect HIV infection efficiency failed to conclusively identify DNA repair pathways that might complete repair of the integration intermediate [3,4,5]. Studies of repair with recombinant proteins *in vitro* indicated that any polymerase, endonuclease and ligase could repair the integration intermediate, suggesting that multiple DNA repair pathways may mediate this process *in vivo* [2].

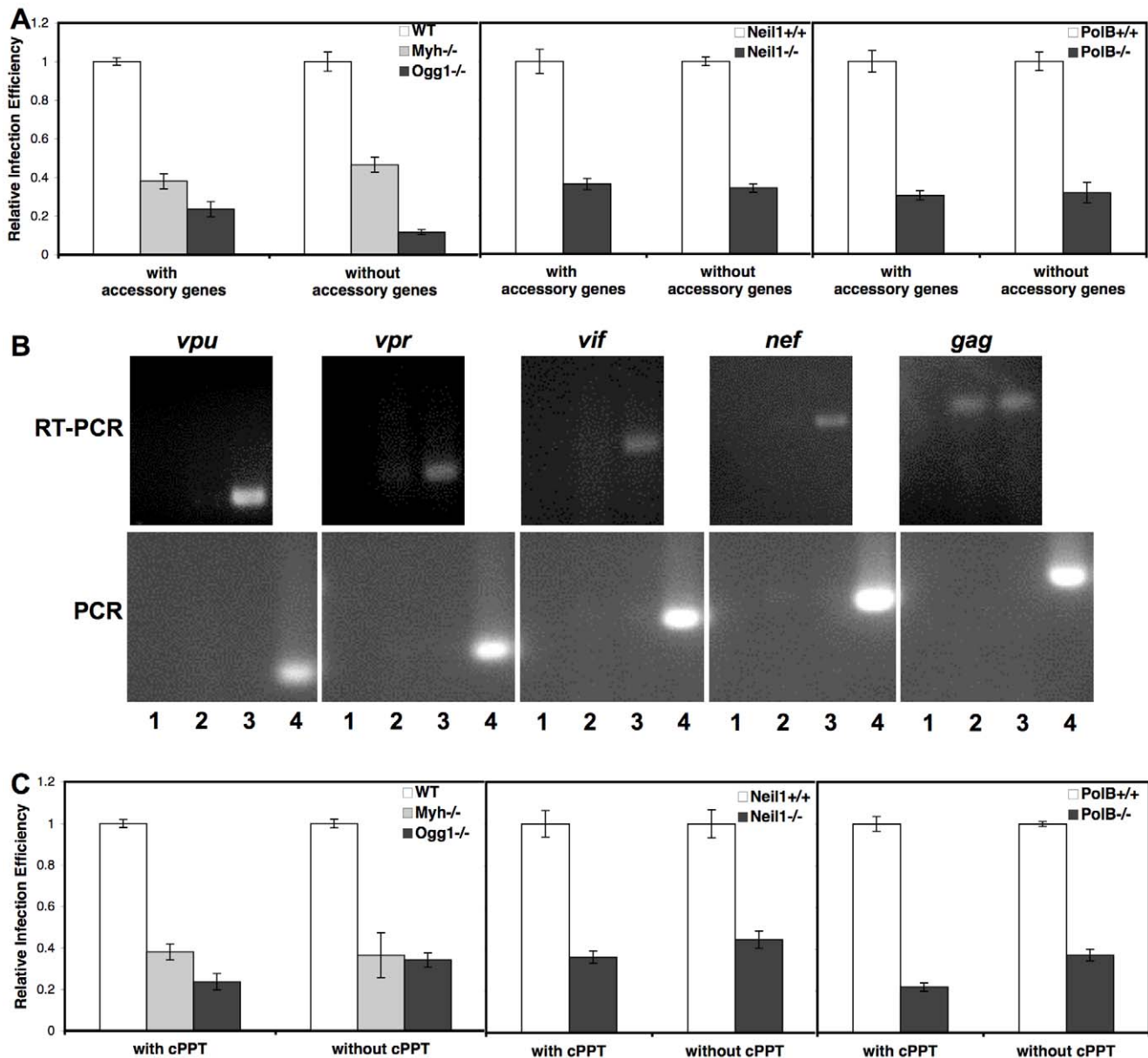


Figure 4. Infection of BER cell lines with HIV with and without accessory genes or the cPPT. Wild type (WT), *Myh*^{-/-}, and *Ogg1*^{-/-} cell lines, wild type (*Neil1*^{+/-}) and *Neil1*^{-/-} cell lines, or wild type (*PolB*^{+/-}) and *PolB*^{-/-} cell lines were infected with HIV retroviral vectors. Wild type murine embryonic fibroblasts were from littermates. Cells were analyzed at 72 hpi by flow cytometry for GFP expression. (A) Cells were infected with an HIV vector with accessory genes or without accessory genes ($\Delta vif\Delta vpr\Delta vpu\Delta nef$). (B) Total RNA was isolated from wild type HIV and HIV($\Delta vif\Delta vpr\Delta vpu\Delta nef$) vector producer cells, treated with DNaseI to digest producer plasmids, and re-isolated. RNA fractions were amplified with the same conditions by RT-PCR or PCR, to confirm the absence of contaminating producer plasmids. RT-PCR and PCR targets were Lane 1 water negative control, Lane 2 HIV($\Delta vif\Delta vpr\Delta vpu\Delta nef$) RNA, Lane 3 wild type HIV RNA, and Lane 4 wild type HIV producer plasmid DNA, positive control for PCR. Primers amplified the accessory genes *vpu*, *vpr*, *vif*, and *nef* as well as the *gag* gene. (C) Cells were infected with an HIV vector with or without the cPPT. Infections were performed at two MOI in duplicate at least three times. Error bars indicate the standard deviation after normalization. doi:10.1371/journal.pone.0017862.g004

A recent study described an siRNA screen targeted to host DNA repair proteins [6]. This study identified multiple host genes throughout the oxidative BER pathway that were required for efficient HIV infection. Using a panel of deletion cell lines, we have found that several BER proteins affect lentiviral infection but not infection by a gamma retrovirus. The role of the BER pathway appears to be at the integration step of the viral life cycle. One obvious mechanism for BER proteins during lentiviral integration is that these proteins complete repair of the integration

intermediate. It is possible that lentiviruses rely largely on BER while retroviruses are less restricted. It is not yet clear how glycosylases might be involved in repair of gapped DNA. It is possible that glycosylases target downstream BER proteins to the integration intermediate.

Other host factors have been identified that play a role during lentiviral but not retroviral infection. Significantly, LEDGF has been shown to enhance lentiviral integration by directly binding to lentiviral integrase and chromatin [37]. Mouse cells with a deletion

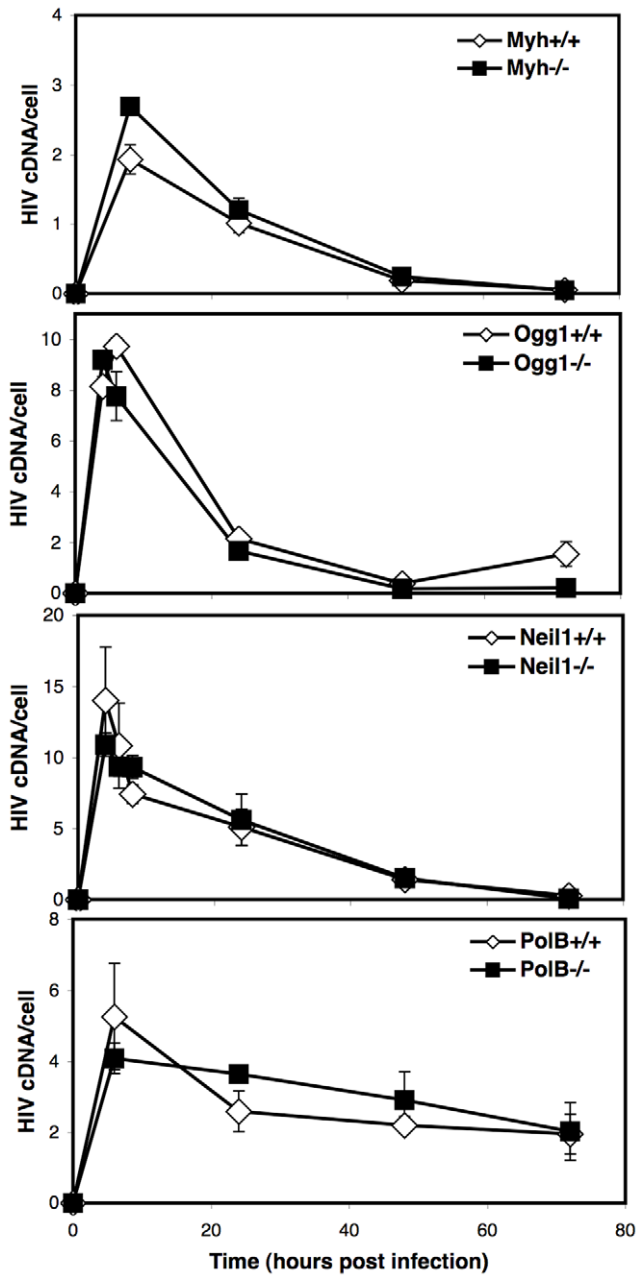


Figure 5. Time course of HIV cDNA accumulation in BER cells. BER wild type and deletion cell lines were infected with HIV. DNA was purified at multiple time points and analyzed by qPCR for HIV late reverse transcripts and the 18S gene. The 18S gene is a measure of the number of genomes present. The late reverse transcript primer set amplifies all complete HIV cDNAs including linear, 1LTR and 2LTR circles, and integrated provirus. The number of late reverse transcripts was divided by the number of genomes to yield HIV cDNA per cell (HIV cDNA/cell). Infections were performed in duplicate three times. Error bars indicate the standard deviation. doi:10.1371/journal.pone.0017862.g005

of the *Ledgf (Pisip1)* gene have been engineered and show a pronounced defect in lentiviral infection and no effect on retroviral infection [36]. While LEDGF is known to affect HIV integration to chromatin DNA targets, HIV PICs generated in *Ledgf* null cells have no integration defect with a naked DNA target [36]. Results with HIV PICs from BER deficient cells indicate that BER affects

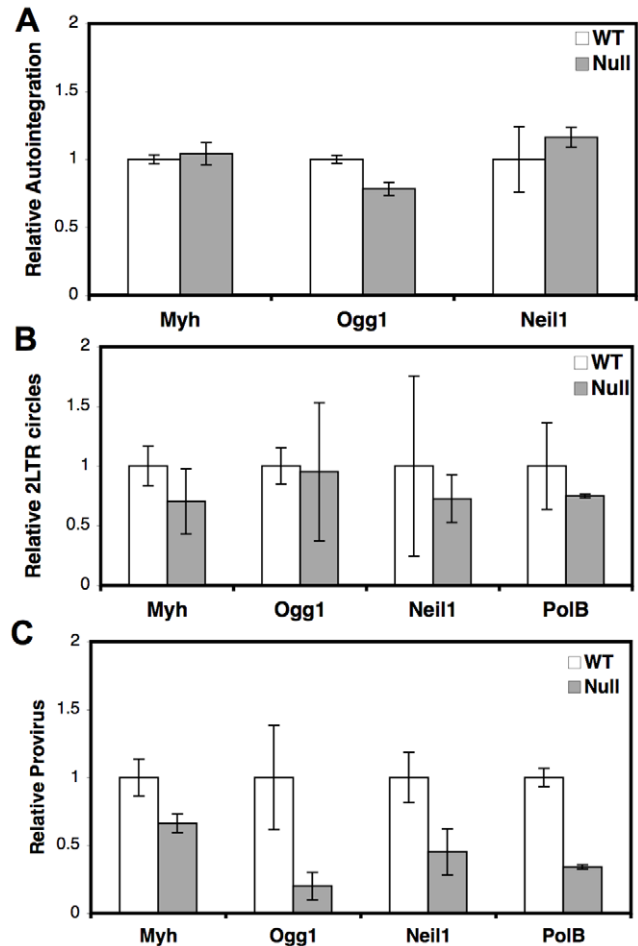


Figure 6. HIV autointegration, 2LTR circles, and integrated provirus in BER cells. BER wild type and deletion cell lines were infected with HIV. DNA was purified at 24 hpi or 72 hpi and analyzed by qPCR for the indicated HIV cDNA and the 18S gene. The number of HIV cDNA was divided by the number of genomes, measured by the 18S gene, to yield the HIV cDNA products per cell. Each HIV cDNA per cell is expressed relative to matched wild type cells. (A) HIV autointegration products at 24 hpi. (B) HIV 2LTR circles at 24 hpi. (C) HIV integrated provirus at 72 hpi. Infections were performed in duplicate three times. Error bars indicate the standard deviation after normalization. doi:10.1371/journal.pone.0017862.g006

integration to naked DNA. The ability of BER to direct integration to chromatin targets remains to be tested. BAF and HMG1A1 proteins were also shown to stimulate HIV PIC integration activity, but reduced expression of these genes showed no effect on HIV infection efficiency [38,39,40,41]. This is the first example of putative HIV integration co-factors that show a difference in the integration efficiency of PICs *in vitro* and infection efficiency *in vivo*.

Retroviral integration sites display a subtle sequence preference unique to each virus [42,43]. The HIV integration site favors G at nucleotides immediately adjacent to the attachment sites. The oxidative DNA glycosylases, with the exception of NTH1, all recognize some form of damaged G [8,30]. Among the most common oxidative base lesions are 8-oxo-G and Fapy-G [7]. It is intriguing that the BER pathway responsible for repair of oxidative damage, largely damaged Gs, appears to be important for HIV integration and that this integration occurs preferentially at Gs. In contrast, BER apparently does not affect MMLV

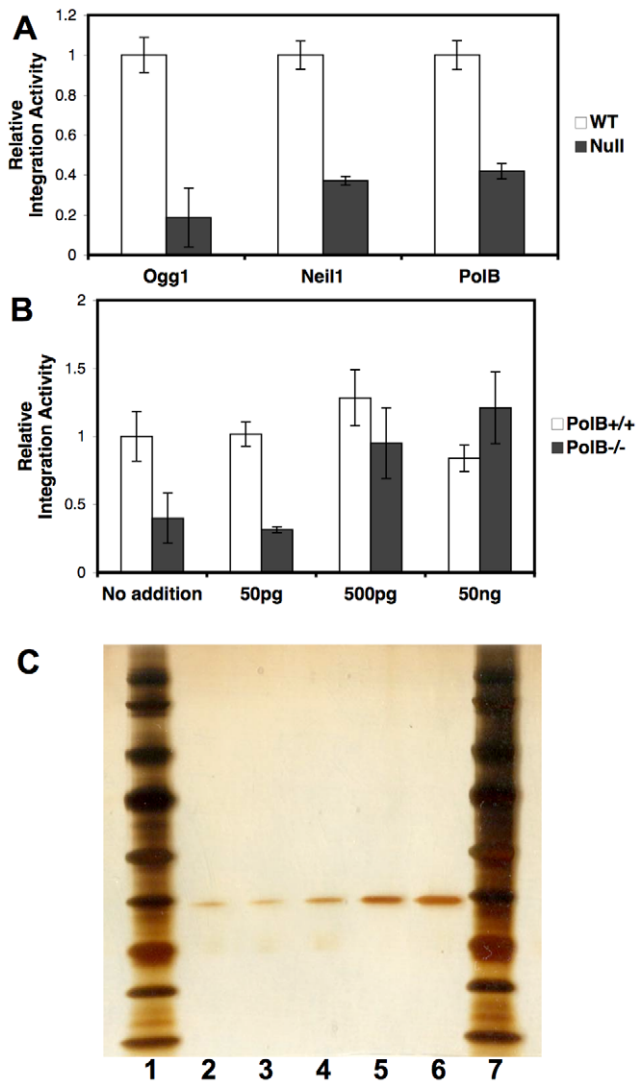


Figure 7. Integration activity of HIV PICs from BER cell lines. HIV PIC extracts were generated at 6 hpi. Purified human genomic DNA was added to HIV PICs. Integration products and total HIV cDNA were measured by qPCR. The number of integration products divided by the total HIV cDNA indicates the integration activity. Integration activity of PICs from mutant cells is expressed relative to PICs from matched wild type cells. (A) HIV PICs from matched wild type and *Ogg1*, *Neil1*, or *PolB* null cells. (B) Increasing concentrations of purified recombinant human POLB protein were added to PICs from wild type and *PolB*^{-/-} cells. PICs were generated from three infections. Integration reactions for each PIC preparation were performed in duplicate. Error bars indicate the standard deviation. (C) Silver stained gel of recombinant human POLB protein. Lanes 1 (left) and 7 (right) show the molecular weight markers. Lanes 2–6 correspond to 20, 40, 80, 120 and 200 ng purified POLB, respectively.
doi:10.1371/journal.pone.0017862.g007

integration and MMLV has no preference for G/C base pairs at integration sites [42,43]. Whether BER proteins affect the integration sites of lentiviruses is under investigation.

Materials and Methods

Cell lines

Murine embryonic fibroblasts used in this study have been previously described [11,14,15,16,17]. Briefly, *Ogg1* null mice were

originally created in 1999 [14]. *Myh* deletion mice were later engineered in 2004 [16]. *Ogg1* null and *Myh* null mice were crossed to generate *Myh*^{+/-} *Ogg1*^{+/-} mice. These mice were subsequently intercrossed generating offspring in predicted Mendelian ratios [19]. The wild type, *Myh* null, and *Ogg1* null MEFs were derived from littermates and spontaneously transformed [17,19]. The wild type *Myh* gene was amplified with previously described primers P1 and P2 generating a 0.26 kb product (Table 1, [16]). Deletion of the *Myh* gene was amplified with primers P1 and *Neo* gene primer P3 generating a 0.38 kb product (Table 1, [16]). The wild type *Ogg1* gene was amplified with previously described primers Ogg1-2 and Ogg1-12 yielding a 0.14 kb product (Table 1, [16]). The deletion of *Ogg1* was confirmed with PCR primers Ogg1-3 and *Neo* gene primer N10 yielding a 1.1 kb product (Table 1, [16]).

The mouse *Neil1* gene was deleted and crosses of heterozygous mice generated offspring in Mendelian ratios of 1:2:1 [15]. Wild type and *Neil1* null MEFs were generated from littermates and immortalized with the adenovirus *E1A* gene [44]. The wild type *Neil1* gene was PCR amplified with previously described primers Neil1 forward and reverse RP-WT yielding a 265 bp product (Table 1, [15]). Deletion of the *Neil1* gene was PCR amplified with the forward primer and reverse RP-KO generating an 804 bp product (Table 1, [15]).

Wild type and *PolB* null MEFs were derived from littermates and immortalized with SV40 large T antigen [11,45]. The *PolB* null cells are wild type for DNA polymerase iota [45,46].

All cell lines were cultured at 37°C in a humidified incubator with 10% CO₂ in DMEM supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamax. All media reagents were from Invitrogen.

Cell extract preparation and immunoblot assays

Nuclear extracts were prepared using the NucBuster nuclear protein extraction reagent (Novagen). Protein concentration was determined using Bio-Rad protein assay reagents according to the manufacturer's instructions. Nuclear protein (30 µg) was separated by 4–12% SDS-PAGE and electrotransferred to a 0.45 mm nitrocellulose membrane (Trans-Blot, Bio-Rad). Antigens were detected using standard protocols. Primary antibodies anti-PolB (NeoMarkers, #MS-1402-P0) and anti-PCNA (Santa Cruz #sc-56) were diluted 1000×. The HRP conjugated secondary antibody (GAM-HRP or GAR-HRP, Bio-Rad) was diluted 10,000× in TBST/5% milk. Each membrane was stripped and re-probed with anti-PCNA antibodies to correct for differences in protein loading.

Retroviral vectors

Retroviral vectors were generated by transfecting 293T cells (ATCC) with three plasmids: a VSV-G envelope protein plasmid, a packaging construct expressing retroviral structural and enzymatic genes, and a genomic RNA plasmid [47]. Media containing the retroviral vector particles was collected, filtered to remove producer cells, and treated with DNaseI to digest producer plasmids.

The HIV packaging construct has a deletion in the *env* gene but expresses all accessory genes [26]. The HIV Δ *vif* Δ *vpr* Δ *vpu* Δ *nef* packaging construct has deletions of the accessory genes but expresses *tat* and *rev* genes [26]. Both packaging constructs were sequenced to confirm the absence of accessory genes in Δ *vif* Δ *vpr* Δ *vpu* Δ *nef* and the presence of complete open reading frames of *vif*, *vpr*, *vpu*, and *nef*. The HIV genomic RNA plasmid p156RRLsinPPTCMVGFPPRE includes the cPPT [24]. The cPPT was removed by digesting with ClaI and HpaI, filling in with

Table 1. Primers used in this study.

Target	Primer name	Primer sequence	Reference
<i>Myh</i>	P1	CAAGTGCTGGGATCAAAGGTG	[16]
<i>Myh</i> wild type	P2	GCTCCTCTTGAGCCGACG	[16]
<i>Myh</i> null	P3	TCCTCGTGCTTTACGGTATCG	[16]
<i>Ogg1</i> wild type	Ogg1-2	GCCTGTGGGCTTTCATA	[16]
<i>Ogg1</i> wild type	Ogg1-12	CACCTGAGGAAGTTGGGCC	[16]
<i>Ogg1</i> null	Ogg1-3	CAAGACCCCACTGAGTGCC	[16]
<i>Ogg1</i> null	N10	GAGAACCTGCGTGCAATCCA	[16]
<i>Neil1</i>	Neil forward	CACCACTGAGCAAGACAGCCAT	[15]
<i>Neil1</i> wild type	RP-WT	GTGGCTGCCAGGTGCAGCTC	[15]
<i>Neil1</i> null	RP-KO	GGGCTGACCGTCTCTCGTGC	[15]
<i>vpu</i>	KY586	GCAATAGTTGTGTGGTCCATAG	this work
<i>vpu</i>	KY587	CAACATCCCAAGGAGCATGG	this work
<i>vpr</i>	KY588	GAGGAGCTTAAGAATGAAGCTG	this work
<i>vpr</i>	KY589	CTACTGGCTCCATTTCTTGC	this work
<i>vif</i>	KY590	CATACAGGAGAAAGAGACTGGC	this work
<i>vif</i>	KY591	TAACACTAGGCAAAGGTGGC	this work
<i>nef</i>	KY592	CAAGGCAGCTGTAGATCTTAGC	this work
<i>nef</i>	KY593	CGGCTGTCAAACCTCCACTC	this work
<i>gag</i>	KY594	ACCCGGCCATAAAGCAAGAG	this work
<i>gag</i>	KY595	CCTGTCTATCGGCTCCTGC	this work

doi:10.1371/journal.pone.0017862.t001

Klenow, and ligating. Both genomic RNA plasmids were sequenced to confirm the presence or absence of the cPPT. The FIV packaging (pFP93) and genomic RNA (pGiNSiN) plasmids have been described [25]. The MMLV retroviral vectors were generated with pHIT60 and pLEGFP-C1 [23] and Clontech).

To determine infection efficiency cells were plated in 6 well dishes to achieve equivalent cell densities and verified by counting. Cells were infected in duplicate with the retroviral vectors at two MOI in the presence of 10 µg/ml DEAE dextran (Sigma Aldrich) and the media was replaced at 2 hpi. The infected cells were incubated for 72 hours then trypsinized, fixed with paraformaldehyde (Sigma Aldrich), and analyzed for GFP expression by flow cytometry (BD FACS Calibur and CellQuest software). The percentage of GFP positive mutant cells was normalized to wild type cells to obtain the relative infection efficiency. Flow cytometry data from figure 2 was analyzed by paired *t* test to generate two-tail P values (GraphPad Prism 4, San Diego). P values were rounded to two significant figures.

To confirm the expression of HIV accessory genes, RNA was isolated from 293T producer cells following transfection (Qiagen RNeasy kit). RNA samples were treated with DNaseI to digest any producer plasmids (Roche). RNA was re-isolated (Qiagen RNeasy kit) and amplified by RT-PCR (Superscript One-Step RT-PCR with Platinum Taq, Invitrogen) or PCR (Platinum Taq, Invitrogen). PCR with Platinum Taq was performed to show that RNA samples did not contain DNA from producer plasmids, a positive control reaction with the HIV packaging construct was included.

References

- Coffin JM, Hughes SH, Varmus HE (1997) Retroviruses. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
- Yoder KE, Bushman FD (2000) Repair of gaps in retroviral DNA integration intermediates. *J Virol* 74: 11191–11200.

25 µl RT-PCR and PCR reactions utilized primers listed in Table 1 with the predicted product sizes of 185 bp for *vpu*, 220 bp for *vpr*, 285 bp for *vif*, 320 bp for *nef*, and 380 bp for *gag*. Reaction products were analyzed by agarose gel stained with ethidium bromide.

Quantitative PCR

At indicated times following infection with HIV, cells were trypsinized and DNA was purified (Qiagen DNeasy Blood and Tissue Kit). The HIV late reverse transcript, 2LTR circle, and provirus primer sets have been described [28,32]. The nested PCR method for detection of HIV autointegration products has been described [29], but primer sets were modified for amplification of the HIV retroviral vector. The first PCR reaction included primers MH532, KY214 5' CCATCTTCTTCAAGGACGAC 3', and KY215 5' GTCGTCCCTTGAAGAAGATGG 3' and the second PCR amplification included primers MH535, SB-76, and probe MH603 [28,48]. The number of cell genomes was determined by qPCR of the 18S gene (Applied Biosystems). The number of HIV cDNA products was divided by the number of cell genomes to yield the number of HIV cDNA forms per cell. Reactions were performed in triplicate. Standards for absolute quantitation were known amounts of plasmid standards or cellular genomes. QPCR was performed with Taqman mastermix in an Applied Biosystems 7900HT Sequence Detection System.

Pre-integration complexes

HIV PICs were generated as previously described [33]. Briefly, BER cell lines were infected with HIV vector particles and incubated for 6 hours. Cells were trypsinized, washed with Buffer K (20 mM HEPES, pH 7.4, 150 mM KCl, 5 mM MgCl₂), and lysed in Buffer K with 0.5% NP-40, 1 mM DTT, and protease inhibitors. Extracts were spun at 3000×g and 10,000×g, supernatants were frozen in liquid nitrogen and stored at –80°C. Integration reactions included PIC extract and 100 ng human genomic DNA at 37°C for 1 hour. DNA was purified following integration (Qiagen DNeasy Blood and Tissue Kit). Recombinant human Polβ was purified as described [49].

Recombinant human Polβ protein

Highly active human Polβ protein was kindly provided to RWS by S.H. Wilson (NIEHS) and was purified as described [50]. The purity of Polβ was determined by SDS-PAGE followed by silver staining. Different amounts of purified Polβ was loaded onto 4–12% precast NuPAGE Tris-glycine gels (2 hrs @ 125V) and stained with a SilverSNAP Stain Kit II (Thermo Scientific, Rockford, IL).

Acknowledgments

Thanks to Eric Poeschla for FIV vector plasmids and Samuel H. Wilson for purified human Polβ protein.

Author Contributions

Conceived and designed the experiments: KEY AE DH RWS RF. Performed the experiments: KEY XW QF. Analyzed the data: KEY AE RSL DH RWS RF. Contributed reagents/materials/analysis tools: MTR RSL RWS. Wrote the paper: KEY AE RSL RWS RF.

3. Brass AL, Dykxhoorn DM, Benita Y, Yan N, Engelman A, et al. (2008) Identification of host proteins required for HIV infection through a functional genomic screen. *Science* 319: 921–926.
4. Konig R, Zhou Y, Elleder D, Diamond TL, Bonamy GM, et al. (2008) Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication. *Cell* 135: 49–60.
5. Zhou H, Xu M, Huang Q, Gates AT, Zhang XD, et al. (2008) Genome-scale RNAi screen for host factors required for HIV replication. *Cell Host Microbe* 4: 495–504.
6. Espeseth AS, Fishel R, Hazuda D, Huang Q, Xu M, et al. (2010) siRNA screening of a targeted library of DNA repair factors in HIV infection reveals a role for base excision repair in HIV integration. submitted.
7. Friedberg EC, Walker GC, Siede W, Wood RD, Schultz RA, et al. (2006) DNA Repair and Mutagenesis. Washington, D.C.: ASM Press.
8. Hegde ML, Hazra TK, Mitra S (2008) Early steps in the DNA base excision/single-strand interruption repair pathway in mammalian cells. *Cell Res* 18: 27–47.
9. Friedberg EC, Meira LB (2006) Database of mouse strains carrying targeted mutations in genes affecting biological responses to DNA damage Version 7. DNA Repair (Amst) 5: 189–209.
10. Puebla-Osorio N, Lacey DB, Alt FW, Zhu C (2006) Early embryonic lethality due to targeted inactivation of DNA ligase III. *Mol Cell Biol* 26: 3935–3941.
11. Sobol RW, Horton JK, Kuhn R, Gu H, Singhal RK, et al. (1996) Requirement of mammalian DNA polymerase-beta in base-excision repair. *Nature* 379: 183–186.
12. Izumi T, Brown DB, Naidu CV, Bhakat KK, Macinnes MA, et al. (2005) Two essential but distinct functions of the mammalian abasic endonuclease. *Proc Natl Acad Sci U S A* 102: 5739–5743.
13. Tebbs RS, Flannery ML, Meneses JJ, Hartmann A, Tucker JD, et al. (1999) Requirement for the Xrcc1 DNA base excision repair gene during early mouse development. *Dev Biol* 208: 513–529.
14. Klungland A, Rosewell I, Hollenbach S, Larsen E, Daly G, et al. (1999) Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage. *Proc Natl Acad Sci U S A* 96: 13300–13305.
15. Vartanian V, Lowell B, Minko IG, Wood TG, Ceci JD, et al. (2006) The metabolic syndrome resulting from a knockout of the NEIL1 DNA glycosylase. *Proc Natl Acad Sci U S A* 103: 1864–1869.
16. Xie Y, Yang H, Cunanan C, Okamoto K, Shibata D, et al. (2004) Deficiencies in mouse Myh and Ogg1 result in tumor predisposition and G to T mutations in codon 12 of the K-ras oncogene in lung tumors. *Cancer Res* 64: 3096–3102.
17. Russo MT, De Luca G, Casorelli I, Degan P, Molatore S, et al. (2009) Role of MUTYH and MSH2 in the control of oxidative DNA damage, genetic instability, and tumorigenesis. *Cancer Res* 69: 4372–4379.
18. Almeida KH, Sobol RW (2007) A unified view of base excision repair: lesion-dependent protein complexes regulated by post-translational modification. *DNA Repair (Amst)* 6: 695–711.
19. Russo MT, De Luca G, Degan P, Parlanti E, Dogliotti E, et al. (2004) Accumulation of the oxidative base lesion 8-hydroxyguanine in DNA of tumor-prone mice defective in both the Myh and Ogg1 DNA glycosylases. *Cancer Res* 64: 4411–4414.
20. Xie Y, Yang H, Miller JH, Shih DM, Hicks GG, et al. (2008) Cells deficient in oxidative DNA damage repair genes Myh and Ogg1 are sensitive to oxidants with increased G2/M arrest and multinucleation. *Carcinogenesis* 29: 722–728.
21. Trivedi RN, Almeida KH, Fornsgaard JL, Schamus S, Sobol RW (2005) The role of base excision repair in the sensitivity and resistance to temozolomide-mediated cell death. *Cancer Res* 65: 6394–6400.
22. Butler SL, Johnson EP, Bushman FD (2002) Human immunodeficiency virus cDNA metabolism: notable stability of two-long terminal repeat circles. *J Virol* 76: 3739–3747.
23. Cannon PM, Kim N, Kingsman SM, Kingsman AJ (1996) Murine leukemia virus-based Tat-inducible long terminal repeat replacement vectors: a new system for anti-human immunodeficiency virus gene therapy. *J Virol* 70: 8234–8240.
24. Follenzi A, Ailles LE, Bakovic S, Geuna M, Naldini L (2000) Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat Genet* 25: 217–222.
25. Poeschla EM, Wong-Staal F, Looney DJ (1998) Efficient transduction of nondividing human cells by feline immunodeficiency virus lentiviral vectors. *Nat Med* 4: 354–357.
26. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D (1997) Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat Biotechnol* 15: 871–875.
27. Rumbaugh JA, Fuentes GM, Bambara RA (1998) Processing of an HIV replication intermediate by the human DNA replication enzyme FEN1. *J Biol Chem* 273: 28740–28745.
28. Butler SL, Hansen MS, Bushman FD (2001) A quantitative assay for HIV DNA integration in vivo. *Nat Med* 7: 631–634.
29. Yan N, Cherepanov P, Daigle JE, Engelman A, Lieberman J (2009) The SET complex acts as a barrier to autointegration of HIV-1. *PLoS Pathog* 5: e1000327.
30. Svilar D, Goellner EM, Almeida KH, Sobol RW (2010) Base Excision Repair and lesion-dependent sub-pathways for repair of oxidative DNA damage. *Antioxid Redox Signal*.
31. Bukrinsky MI, Sharova N, Dempsey MP, Stanwick TL, Bukrinskaya AG, et al. (1992) Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. *Proc Natl Acad Sci U S A* 89: 6580–6584.
32. Brussel A, Sonigo P (2003) Analysis of early human immunodeficiency virus type 1 DNA synthesis by use of a new sensitive assay for quantifying integrated provirus. *J Virol* 77: 10119–10124.
33. Hansen MS, Smith GJ, 3rd, Kafri T, Molteni V, Siegel JS, et al. (1999) Integration complexes derived from HIV vectors for rapid assays in vitro. *Nat Biotechnol* 17: 578–582.
34. Ellison V, Abrams H, Roe T, Lifson J, Brown P (1990) Human immunodeficiency virus integration in a cell-free system. *J Virol* 64: 2711–2715.
35. Farnet CM, Haseltine WA (1990) Integration of human immunodeficiency virus type 1 DNA in vitro. *Proc Natl Acad Sci U S A* 87: 4164–4168.
36. Shun MC, Raghavendra NK, Vandegraaff N, Daigle JE, Hughes S, et al. (2007) LEDGF/p75 functions downstream from preintegration complex formation to effect gene-specific HIV-1 integration. *Genes Dev* 21: 1767–1778.
37. Llano M, Saenz DT, Meehan A, Wongthida P, Peretz M, et al. (2006) An essential role for LEDGF/p75 in HIV integration. *Science* 314: 461–464.
38. Beitzel B, Bushman F (2003) Construction and analysis of cells lacking the HMG A gene family. *Nucleic Acids Res* 31: 5025–5032.
39. Chen H, Engelman A (1998) The barrier-to-autointegration protein is a host factor for HIV type 1 integration. *Proc Natl Acad Sci U S A* 95: 15270–15274.
40. Farnet CM, Bushman FD (1997) HIV-1 cDNA integration: requirement of HMG I(Y) protein for function of preintegration complexes in vitro. *Cell* 88: 483–492.
41. Shun MC, Daigle JE, Vandegraaff N, Engelman A (2007) Wild-type levels of human immunodeficiency virus type 1 infectivity in the absence of cellular emerin protein. *J Virol* 81: 166–172.
42. Holman AG, Coffin JM (2005) Symmetrical base preferences surrounding HIV-1, avian sarcoma/leukosis virus, and murine leukemia virus integration sites. *Proc Natl Acad Sci U S A* 102: 6103–6107.
43. Wu X, Li Y, Crise B, Burgess SM, Munroe DJ (2005) Weak palindromic consensus sequences are a common feature found at the integration target sites of many retroviruses. *J Virol* 79: 5211–5214.
44. McCurrach ME, Lowe SW (2001) Methods for studying pro- and antiapoptotic genes in nonimmortal cells. *Methods Cell Biol* 66: 197–227.
45. Sobol RW, Prasad R, Evenski A, Baker A, Yang XP, et al. (2000) The lyase activity of the DNA repair protein beta-polymerase protects from DNA-damage-induced cytotoxicity. *Nature* 405: 807–810.
46. Sobol RW (2007) DNA polymerase beta null mouse embryonic fibroblasts harbor a homozygous null mutation in DNA polymerase iota. *DNA Repair (Amst)* 6: 3–7.
47. Karolewski BA, Watson DJ, Parente MK, Wolfe JH (2003) Comparison of transfection conditions for a lentivirus vector produced in large volumes. *Hum Gene Ther* 14: 1287–1296.
48. Schroder AR, Shinn P, Chen H, Berry C, Ecker JR, et al. (2002) HIV-1 integration in the human genome favors active genes and local hotspots. *Cell* 110: 521–529.
49. Abbotts J, SenGupta DN, Zmudzka B, Widen SG, Notario V, et al. (1988) Expression of human DNA polymerase beta in *Escherichia coli* and characterization of the recombinant enzyme. *Biochemistry* 27: 901–909.
50. Beard WA, Wilson SH (1995) Purification and domain-mapping of mammalian DNA polymerase beta. *Methods Enzymol* 262: 98–107.