DNA damage enhances integration of HIV-1 into macrophages by overcoming integrase inhibition

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Abstract

Background: The prevention of persistent human immunodeficiency virus type 1 (HIV-1) infection requires the clarification of the mode of viral transduction into resting macrophages. Recently, DNA double-strand breaks (DSBs) were shown to enhance infection by D64A virus, which has a defective integrase catalytic activity (IN-CA). However, the mechanism by which DSBs upregulate viral transduction was unclear. Here we analyzed the roles of DSBs during IN-CA-independent viral transduction into macrophages.

Results: We used cellular systems with rare-cutting endonucleases and found that D64A virus integrated efficiently into the sites of artificially induced DSBs. This IN-CA-independent viral transduction was blocked by an inhibitor of ataxia telangiectasia mutated protein (ATM) but was resistant to raltegravir (RAL), an inhibitor of integrase activity during strand transfer. Moreover, Vpr, an accessory gene product of HIV-1, induced DSBs in resting macrophages and significantly enhanced the rate of IN-CA-independent viral transduction into macrophages with concomitant production of secondary viruses.

Conclusion: DSBs contribute to the IN-CA-independent viral infection of macrophages, which is resistant to RAL. Thus, the ATM-dependent cellular pathway and Vpr-induced DNA damage are novel targets for preventing persistent HIV-1 infection.

Keywords: DNA damage, HIV-1, Integrase inhibitor, Integration, Resting macrophages, Vpr

Background

The prognosis of individuals infected with human immunodeficiency virus type 1 (HIV-1) has improved due to the development of combination antiretroviral therapy (cART) [1]. However, several lines of evidence revealed that the current regimen does not block viral replication completely [2], which promotes the emergence of drug-resistant mutant viruses. Recently, new anti-retroviral drugs that target viral entry or the integration of viral DNA into the host genome have been applied clinically [3,4], which allows the possibility of overcoming viruses that are resistant to conventional cART. Moreover, an advanced study directed at the development of novel anti-HIV-1 compounds attempted to identify the cellular proteins that associate with HIV-1 proteins [5]. Macrophages are less sensitive to the toxic effects of HIV-1 and they function as persistent producers of the virus [2]; therefore, it is important to develop novel anti-HIV-1 compounds that target viral transduction into resting macrophages.

Integrase, a 288-amino-acid and 32-kDa HIV-1 protein, promotes strand-transfer reaction [6], where the reverse-transcribed double-stranded viral DNA is integrated into the host genome. The integrase catalytic activity (IN-CA) excises two nucleotides from the 3'-end of the viral DNA and the CA-3'-OH is ligated to the 5'-O-phosphate end of the genomic DNA [6]. All these strand transfer steps depend on the presence of a D,D(35)E motif in the central domain and any mutations in this motif abrogate the activity required for the strand-transfer process [7]. Notably, single-strand gaps are produced in both regions flanking the viral DNA and it was postulated that cellular factors repair these gaps because viral proteins have a low DNA damage repair activity [8].

Initially, Daniel et al. proposed that DNA-dependent protein kinase was a cellular factor involved in gap-repair [9], and then ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR), Nijmegen
breakage syndrome 1 (NBS1), and poly(ADP-ribose) polymerase 1 (PARP1) have also been nominated as cellular proteins involved in efficient viral transduction [10-13]. Using KU55933, a specific ATM inhibitor, Lau et al. proposed that ATM is also involved in HIV-1 transduction [14], whereas Sakurai et al. demonstrated that DNA damage repair enzymes are involved in multiple steps of retroviral infection [15]. These observations support the importance of DNA double-strand breaks (DSBs) in viral transduction, although their roles are controversial [16-19]. A possible explanation for discrepancies in reported observations is that the single-strand gaps are repaired in a redundant fashion by DNA damage repair enzymes, the expression of which varies among cells [20]. It is also possible that DSBs have modest effects on viral transduction, which may be overwhelmed by the infectivity of the wild-type (WT) virus. This suggests that it is important to evaluate the effects of DSBs using more sophisticated experimental approaches.

Here we focused on the role of DNA damage (DSBs), particularly in integration of viral DNA. Interestingly, HIV-1 DNA integrated into artificially induced DSBs in an IN-CA–independent manner and DNA damaging agents upregulated the infectivity of IN-CA–defective virus. The positive effects of DSBs on viral integration were resistant to raltegravir (RAL), an IN-CA inhibitor. Moreover, Vpr, an accessory gene product of HIV-1, mimicked DNA damaging agents and increased IN-CA–independent viral transduction into monocyte-derived macrophages (MDMs). Even when the catalytic activity of IN was impaired, infectious secondary virus was generated without any mutations that yielded phenotypes resistant to RAL.

Based on these observations, we propose that the ATM-dependent mode of DSB-specific integration of viral DNA and the Vpr-induced DSBs are novel targets for anti-HIV compounds that inhibit viral transduction into MDMs, a persistent reservoir of HIV-1 infection.

**Results**

**HIV-1 integrates into the sites of artificially induced DSBs**

To understand the roles of DSBs in integration of viral DNA into macrophages, we established a system using THP-1 cells, a human monocytic leukemia cell line that differentiates into macrophage-like cells after treatment with phorbol myristate acetate (PMA) (Figure 1A) [21]. We transfected THP-1 cells with plasmid DNA that contained the recognition sequence for I-SceI, a rare-cutting endonuclease [22] and obtained clones with the I-SceI site after drug selection. Using the experimental procedures outlined in Figure 1A, the frequency of viral DNA integration into I-SceI sites was evaluated. After PMA-treated cells were infected with VSVG-pseudotyped WT virus (NL-Luc-E(−)R(−)) together with adenovirus-expressing I-SceI, provirus DNA was detected in the I-SceI provirus (Ad-I-SceI) site or its vicinity (Figure 1B, Additional file 1: Figure S1A). PCR amplification targeting the junction of the I-SceI site and the 5’-end of the integrated proviral DNA (Figure 1A) selectively generated PCR amplicons from the Ad-I-SceI-infected samples (compare the upper and lower panels of Figure 1B).

Sequence analysis of several independent clones detected the presence of provirus DNA in the I-SceI site (Figure 1C, each arrowhead indicates the integration site of individual clones analyzed). Notably, KU55933 blocked I-SceI site-targeted integration (Figure 1D, lower panel) [14].

Similar results were obtained using a different system with another rare-cutting endonuclease, I-PpoI (Figures 1-E–H and Additional file 1: Figure S1B). The recognition sites of I-PpoI are present in the human genome, although the mammalian genome has no gene that encodes the enzyme [23]. In this experiment, we used a lentiviral vector to ensure the generality of our observations (Figure 1E). As shown in Figure 1F, the viral DNA reproducibly integrated into the I-PpoI site, which was confirmed by PCR amplification and sequence analysis (Figure 1G and H). The data clearly indicated that the viral DNA was inserted in the DSB sites.

**Integration into DSB sites was independent of the catalytic activity of integrase**

Interestingly, analysis of the nucleotide sequence of the viral DNA inserted in the I-SceI site revealed that both the 5’- and 3’-long terminal repeat (LTR) ends of the provirus DNA had adenine and cytidine (pAC) dinucleotides (Figure 1I) [6], suggesting that the viral DNA integrated into DSBs in an IN-CA–independent manner (Additional file 1: Figure S2). To confirm this, similar experiments were performed using D64A mutant virus, which is defective in integrase, co-infected with Ad-I-SceI (Figure 2A). PCR amplification followed by sequence analysis consistently detected the presence of pAC in the 5′-ends of the integrated viral LTR (Figure 2B).

We then estimated the frequency of viral integration into the DSB sites in the total number of provirus DNA. Intriguingly, we observed that more than half of the integrated D64V lentiviruses were present in the I-PpoI site (approximately 53%) when viral infection was conducted using HT1080 cells that had been cultured in 0.1% FBS (Figure 2C, Additional file 1: Figure S3A). In contrast, the DSB-specific integration of the viral DNA was reduced to approximately 18% in a similar experiment performed in the presence of 10% FBS. FACS analysis of HT1080 cells that had been pulse-labeled with BrdU revealed that the population of cycling cells decreased from 43% to 18% when cells were cultured in
Figure 1 HIV-1 DNA integrates into a DSB site. (A) An experimental procedures for (B)–(D) and (I). Red arrows indicate the primers used in (B) and (D). (B) PCR amplification of WT provirus DNA integrated in the I-Sce site. Each lane depicts each result of twelve samples independently infected with WT virus and Ad-I-SceI (upper panel) or Ad-LacZ (lower panel). M, molecular marker. (C) Upper panel, representative sequencing chromatogram of the PCR amplicon in samples, which were shown in upper panel of (B). Lower panel, summary of viral DNA integration sites. The 18-bp recognition sequence of the I-SceI site is shown. When digested with I-SceI, a 3′-protruding end of 4 nucleotides is formed (dotted red line). Each arrowhead indicates an actual integration site of viral DNA in samples shown in (B). Integration sites were identified on most of clones except for two clones, which are indicated by arrowheads with a horizontal bar. (D) Effect of KU55933 on viral DNA integration into the I-SceI site. (E) Schematic outline of the I-Ppol-PCR experimental design in (F)–(H) (Top panel). The lentiviral vector was used in this study (bottom panel). (F) PCR amplification of lentiviral vector inserted in the I-Ppol site. Primers are shown by red arrows (G) A representative result of sequence analysis of proviral DNA integrated in the I-Ppol site. (H) Summary of integration sites of the lentiviral vector. Each arrowhead depicts each result of independent clones. The dotted line indicates I-Ppol site with a 3′-protruding end of 4 nucleotides. (I) Summary of the I-SceI-PCR sequence data. A representative nucleotide sequence was shown at the top of each panel. Asterisks indicate the pAC that would be normally removed during IN-mediated integration (see Additional file 1: Figure S2). Dots indicate identical sequence to that of the representative sequence. Dashes indicate deleted nucleotides.
To accurately determine the exact rate of DSB-specific integration of viral DNA, we developed a system for quantitative I-SceI-PCR (I-SceI-qPCR) analysis of the provirus DNA and investigated whether viral DNA integration into the I-SceI site was influenced by RAL (see experimental procedures in Methods section). As shown in Figure 2D, RAL did not attenuate the DSB-specific integration of WT viruses in PMA-treated THP-1 cells (a single dot indicates each result of 11 samples in three independent experimental groups). In contrast, KU55933 efficiently blocked the DSB-specific integration of WT and D64A viruses (Figure 2D). These data suggest that capture of viral DNA in the DSB sites was selectively induced in an IN-CA–independent manner, which was ATM-dependent.

DNA damaging agents upregulate IN-CA–independent viral integration

Next, we examined the effects of the DNA damaging agents etoposide and bleomycin on viral infection. As shown in Figure 3A, both compounds increased the infectivity of D64A virus in all cells examined, which included MDMs and various human cell lines. However, the positive effects of these compounds were not consistently observed in WT virus, although they ectopically enhanced the frequency of viral transduction (Figure 3B, Additional file 1, Figure S4), i.e., etoposide enhanced the infectivity of WT virus in serum-starved HT1080 cells and nocodazole-treated human primary fibroblasts (TIG-3) (Figure 3B, upper panels, Additional file 1, Figure S4) [24]. However, it had no positive effects when cells were cultured in the presence of 10% FBS (Figure 3B, upper panels, Additional file 1, Figure S4). In addition, bleomycin had no positive effects on the infectivity of WT virus under any culture conditions (Figure 3B, lower panels, Additional file 1, Figure S4). These data indicate that the effects of DNA damage on viral transduction are only observable when combined with the IN-CA–defective virus, or they are obscured by the infectivity of the WT virus.

**DSBs enhanced viral transduction at the integration step of viral infection**

We quantified the integrated DNA copy numbers to clarify the roles of DSBs in IN-CA–independent viral transduction in greater detail. We used serum-starved HT1080 cells to minimize the possible effects of DSBs generated 10% and 0.1% FBS, respectively (Additional file 1, Figure S3B). The data indicated that the cellular conditions had a large influence on the rate of viral integration into DSB sites. Of note, no remarkable integration of WT virus into the DSB site was detected under any conditions of cell culture with different concentrations of FBS (Figure 2C). These data suggested that the IN-CA–defective virus was the main target of capture by the DSB sites.
spontaneously during DNA replication. A quantitative PCR (qPCR)-based assay demonstrated that treatment with 1.25–20 μM etoposide or bleomycin significantly increased the number of integrated viral DNA copies (Figure 4A). We performed a colony formation assay to further demonstrate the effects of DNA damaging agents on viral transduction. As shown in Figure 4B, treatment with DNA damaging agents significantly increased the number of drug-resistant colonies, indicating that DSBs promoted the integration of D64A virus (Figure 4B). In contrast, these compounds had no obvious effects on the integration of WT virus (Figure 4C and D). Although it has been reported that DSBs augment viral replication during multiple steps [15], our observations suggested that they enhance the integration step of viral DNA, which is a pivotal step in viral transduction.

DSB-dependent viral integration induced minor structural alterations in provirus DNA but generated infectious progeny viruses

It has been proposed that a non-homologous end-joining (NHEJ) pathway is involved in the repair of the gaps formed during viral integration [25] and that the DSB-specific integration of provirus DNA is susceptible to structural alterations [26]. To evaluate this, we quantified the frequency of structural modifications with provirus DNA using linear amplification mediated-PCR (LAM-PCR), followed by nucleotide sequence analysis [27]. When cells were infected with the virus in the presence of RAL, insertions and deletions in the 5'-LTR region were detected in 70.6% and 35.3% of cells, respectively (Table 1). In contrast, only 5% of the integrants were positive for structural alterations when infected in the presence of dimethyl sulfoxide (DMSO, solvent control) (Table 1 and Additional file 1: Figure S5). The data implicated that viral integration in the presence of RAL is susceptible to disruption of provirus DNA structures, which abrogated the production of secondary viruses.

To clarify this possibility, we investigated the effects of RAL on single-round viral infection using several cell lines. As shown in Figure 5A, we found that the infectivity of the WT virus was significantly attenuated by RAL, i.e., viral infection was reduced to 0.2% and 3.8% when 10 μM RAL was used to treat MAGIC5 cells and MT-4
However, these values were the same with D64A virus, which suggests that restricting IN-CA could not block viral infection completely. This suggestion was supported by tests using azidothymidine (AZT, an RT inhibitor), which further blocked the infectivity of D64A virus. Importantly, the same results were obtained using elvitegravir (EVG, another IN-strand transfer inhibitor) in PMA-treated THP-1 cells (Figure 5A).

These observations strongly suggest that the WT virus can replicate in the presence of RAL, although the potential for viral replication is low and at similar level to IN-CA–defective virus. To test this possibility, we infected MT-4 cells with a replication-competent virus in the presence of RAL and examined the production of the progeny virus using MAGIC5 cells (HeLa/CD4, CCR5, LTR-β-gal) [28]. As shown in Figure 5B, we observed viral replication with the WT virus, although RAL was continuously added in the culture medium (one representative result from three independent experiments is shown with other experimental data in Additional file 1: Figure S6A). To exclude the possibility that the secondary virus possessed mutations that could overcome the inhibitory effects of RAL, we tested the viral RNA recovered from the culture supernatants. Analysis of the nucleotide sequences of 10

Figure 4 DNA damage enhances the integration rate of HIV-1. Serum-starved HT1080 cells were infected with D64A (A) or WT (C) viruses in the presence of etoposide or bleomycin from 0–24 hpi. After 48 h, genomic DNA was extracted and subjected to qPCR. Relative copy numbers of HIV-1 DNA to β-globin were estimated (top) and the fold increase of HIV-1 DNA copy number compared to control infection that was conducted without DNA damaging agents (bottom) were calculated. For colony formation assay, VSVG-pseudotyped D64A (NL-Neo-IN-D64A-E(−)R(−)) (B) or WT (NL-Neo-E(−)R(−)) (D) viruses, which had the neomycin resistant gene (NeoR), were used. HT1080 cells were treated with various doses of etoposide or bleomycin for 24 h, which were added at the same time of viral infection. After selection with 600 μg/mL of G418, numbers of NeoR colonies were counted. Numbers of NeoR colonies were normalized by plating efficiency. Error bars, s.d. of triplicate assays. *P < 0.05; **P < 0.01.
progeny viruses revealed that all clones had no reported mutations related to RAL-resistant phenotypes (Table 2, Additional file 1, Table S1 and Figure S7) [29-32]. A similar experiment was performed using D64A virus. Again, we observed reproducible viral replication in the presence or absence of RAL (Figure 5C, Additional file 1: Figure S6B). Analysis of the nucleotide sequence of the progeny virus RNA revealed that a single clone of the 10 viruses analyzed was positive for a reported mutation linked to a RAL-resistant phenotype (M154I in Table 2, Additional file 1, Table S1). However, the other nine clones were free of such mutations. In addition, no WT virus revertants

**Table 1 HIV-1 DNA integration sites and host-virus junction sequence**

| Seq ID | Inhibitor | Chr | Locus | Strand | RefSeq gene | Insertion | Deletion |
|--------|-----------|-----|-------|--------|-------------|-----------|----------|
| 100902 | DMSO      | 20  | 33371451 | plus   | NCOA6       | -         | -        |
| 100903 | DMSO      | 17  | 35993714 | minus  | DDX52       | -         | -        |
| 100904 | DMSO      | 3   | 156651874 | minus  | LKR1        | -         | -        |
| 100906 | DMSO      | 20  | 60900963 | minus  | LAMA5       | -         | -        |
| 100907 | DMSO      | 3   | 19680267 | minus  | DLG1        | -         | -        |
| 100910 | DMSO      | 4   | 150767744 | plus   | -           | -         | -        |
| 100912 | DMSO      | 9   | 131400460 | plus   | WDR34       | -         | -        |
| 100914 | DMSO      | 1   | 156020423 | minus  | UBQLN4      | 11bp (ACAGCAGTTAG) | 37 bp |
| 100916 | DMSO      | 17  | 43551920 | minus  | PLEKH1M1    | -         | -        |
| 100919 | DMSO      | 20  | 2467413 | minus  | ZNF343      | -         | -        |
| 100921 | DMSO      | 10  | 114725221 | minus | TCF7L2      | -         | -        |
| 106501 | DMSO      | 4   | 87017147 | plus   | MAPK10      | -         | -        |
| 106502 | DMSO      | 2   | 98458616 | minus  | TMEM131     | -         | -        |
| 106505 | DMSO      | 15  | 44399565 | minus  | FRMD10      | -         | -        |
| 106506 | DMSO      | 2   | 197544261 | plus   | CDDC150     | -         | -        |
| 106510 | DMSO      | 7   | 333212296 | minus  | BBS9        | -         | -        |
| 106513 | DMSO      | 2   | 230499770 | plus   | DNER        | -         | -        |
| 106520 | DMSO      | 17  | 38682989 | plus   | CR597260    | -         | -        |
| 106521 | DMSO      | 19  | 17213409 | minus  | MY09B       | -         | -        |
| 106524 | DMSO      | 6   | 33219360 | plus   | VP552       | -         | -        |
| 100925 | RAL       | 9   | 33033919 | plus   | DNAJ1       | 9 bp      |          |
| 100931 | RAL       | 14  | 44024820 | minus  | -           | -         | 20 bp    |
| 100937 | RAL       | 2   | 102387409 | plus   | MAPK4K      | 9 bp (GACACCTAG0) | -       |
| 100940 | RAL       | 5   | 176735496 | plus   | MXD3        | 2 bp (AC) |          |
| 100944 | RAL       | 3   | 105267040 | plus   | -           | 21 bp (AATAAAAAGGTACAAATAGAC) | -       |
| 106525 | RAL       | 14  | 87606685 | minus  | -           | 6 bp (TCATAA) | 3 bp    |
| 106530 | RAL       | 22  | 36006496 | minus  | MB          | 1 bp (A)  | 9 bp     |
| 106534 | RAL       | 7   | 130544996 | minus  | CR618431    | 10 bp (TTGTAATTAC) | -       |
| 106537 | RAL       | 1   | 63779999 | minus  | BC040309    | 16 bp (AAAGAAAAAGGGGGAC) | -       |
| 106538 | RAL       | 3   | 190624485 | minus  | -           | -         | 4 bp     |
| 106540 | RAL       | 3   | 85084717 | minus  | CADM2       | -         | -        |
| 106542 | RAL       | 18  | 40381428 | minus  | RIT2        | 2 bp (CA) | 1 bp     |
| 106558 | RAL       | 6   | 52427867 | minus  | TRAM2       | -         | -        |
| 106574 | RAL       | 9   | 18460635 | plus   | -           | 9 bp (ACACCTAT) | -       |
| 106582 | RAL       | 1   | 6482403 | minus  | HES2        | 4 bp (GGAC) | -        |
| 106588 | RAL       | 11  | 28054901 | plus   | -           | 4 bp (GGAC) | -        |
| 106596 | RAL       | 7   | 92394729 | plus   | CDK6        | 4 bp (GGAC) | -        |

HIV-1 DNA integration sites were determined by LAM-PCR. LAM-PCR sequence were aligned to the human genome reference (GRCh37/hg19, Feb. 2009, UCSC). Chr, chromosome.
were detected. It is interesting to note that MT-4, a cell line infected with human T cell leukemia virus, expresses Tax, a viral protein. One possible explanation for the efficient IN-CA independent viral infection is due to DNA damage that is induced by the biological activity of Tax [33,34].

After establishing that RAL-resistant viral replication could be induced in MT-4 cells, we investigated whether the same mode of viral infection can occur in MDMs. We detected no apparent replication of infectious secondary virus in MDMs, which were infected in the presence of RAL. However, viral replication was detected when DNA damaging agents were treated at the same time as the viral infection (Figure 5D). Importantly, the addition of enfuvirtide (ENF), a fusion inhibitor,
completely abolished the detection of the viral RNA, which indicated that the detected virus was not a remnant of the initially infected virus and that it was a progeny virus. Similar results were obtained in independent experiments using MDMs prepared from a different donor. These data and the absence of reported mutations in these viral RNA showed that DSBs promoted productive viral transduction even in the presence of RAL.

Based on these experiments, we expected that DSB site may capture and incorporate virus DNA as a structurally intact form. To obtain direct evidence for this possibility, we analyzed the nucleotide sequences of the provirus DNA integrated in the DSB site. In these experiments, serum-starved HT1080 cells were co-infected with an Ad-I-PpoI and an IN-defective lentiviral vector (Lenti6-EGFP-D64V), which contained a blasticidin-resistant gene. After infection, the blasticidin-resistant cells were selected and cloned, and the lentivirus-infected cell clones were screened using I-PpoI-qPCR. We isolated a total of 74 clones and obtained 10 (13.5%), five (6.8%), and five (6.8%) clones, which contained proviral DNA at the I-PpoI site in direct, inverted, or both direct and inverted orientations, respectively (Figure 6A). Of these, five clones were EGFP-positive (Figure 6B) and the proviral DNA was integrated only into the I-PpoI site in one of these clones (Figure 6C, D, clone #2413). This was further confirmed by fluorescent in situ hybridization (FISH) analysis, which detected provirus DNA in a single locus in the genome (Figure 6E). Sequence analysis of the provirus DNA of clone #2413 finally identified an intact viral DNA structure with the flanking nucleotide sequence of the I-PpoI site (Figure 6F). The data indicated clearly that the structurally intact viral DNA could integrate into the DSB site.

Vpr mimicked DSBs and enhanced the IN-CA–independent viral transduction into resting macrophages

Vpr, an accessory gene of HIV-1, encodes a 96-amino acid virion-associated nuclear protein with pleiotropic activities, including a cell cycle abnormality during the G2/M phase, enhanced promoter activity and apoptosis. It has also been proposed that Vpr is important for macrophage infection through the nuclear trafficking of a preintegration complex [35]. Previously, it has been reported that Vpr elicits cellular signals triggered by DNA damage [36-40], which suggests that Vpr promotes IN-CA–independent viral transduction. To test this hypothesis, we checked whether infection with R+ virus induced the DNA damage response in MDMs (Figure 7A). In agreement with our previous observations, infection with R+ virus evoked the cellular response triggered by DNA damage [36,37,41,42]. We investigated the infectivity of R+ virus and observed that Vpr enhanced viral transduction in the presence of RAL, which was blocked by AZT (Figure 7B). We investigated the infectivity of R+ virus and observed that Vpr enhanced viral transduction in the presence of RAL, which was blocked by AZT (Figure 7B). Similar to the effect of DSBs, Vpr enhanced the viral infectivity during the integration step (Figure 7C). Moreover, Vpr enhanced the infection of MDMs by D64A virus (Figure 7D and Additional file 1: Figure S8).

To further elucidate the effects of Vpr on the infection of MDMs, we compared the efficiency of viral transduction

| Virus          | Clone # | RNA            | Amino acid     |
|---------------|---------|----------------|----------------|
| WT/RAL        | #1      | -              | -              |
|               | #2      | A144G          | E48E (Silent)  |
|               | #3      | A87G           | P29P (Silent)  |
|               | #4      | A731G          | R244K          |
| D64A/DMSO     | #1      | A191C          | D64A           |
|               | #2      | A191C          | D64A           |
|               | #3      | A191C          | D64A           |
|               | #4      | A191C          | D64A           |
|               | #5      | A191C, A772G   | D64A, K258E    |
|               | #6      | A191C, U842A   | D64A, V281E    |
|               | #8      | A38G, A191C, G62A | E13G, D64A, M154I |
|               | #10     | A191C, A279, A809G | D64A, T93T (Silent), D270G |
| D64A/RAL      | #1      | A191C          | D64A           |
|               | #4      | A191C          | D64A           |
|               | #8      | A191C          | D64A           |
|               | #10     | A191C          | D64A           |
|               | #2      | A191C, G806C   | D64A, R269T    |
|               | #3      | A191C, A718G   | D64A, K240E    |
|               | #5      | A191C, G829U   | D64A, G277C    |
|               | #6      | A191C, U598C   | D54A, L200T    |
|               | #9      | A191C, G462A   | D64A, M154I    |
|               | #7      | G7A, G39A, G103A, A191C | D35N, E13E (Silent), E35K, D64A |
Figure 6 (See legend on next page.)
into MDMs, peripheral blood mononuclear cells (PBMCs), and human cell lines by calculating the fold-increase in the luciferase activity, which reflected the infectivity of each virus (Figure 7E, Additional file 1, Figure S9). As summarized in Figure 7F, the positive effects of Vpr were the most striking when MDMs were infected with D64A virus (D64A/R+ virus). The infectivity of D64A/R+ virus in MDMs was 37.0–265.1-fold higher than that of D64A/R− virus. In contrast, these positive effects were not detected with WT/R+ virus. Moreover, the positive effects of Vpr were less conspicuous in PBMCs, consistent with previous observations that Vpr functions as a positive factor during viral transduction into MDMs [43]. Combined with previous reports that Vpr activates ATM [36,37] and ATR [38], our observations suggest that the enhanced infectivity of D64A/R+ virus in MDMs is attributable to Vpr-induced DSBs [36,37].

Discussion
Since it was first postulated that the cellular proteins responsible for DNA damage repair are positively involved in HIV-1 infection [9], roles of DSBs and DNA damage repair enzymes in viral infection have remained controversial [10-14,16-19,44]. However, several lines of evidence have suggested that DSBs have at least two roles in viral infectivity, i.e., direct upregulation of the rate of viral DNA integration into the host genome and the activation of DNA damage repair enzymes, which contribute to multiple steps in HIV-1 infection including repair of the gaps formed during the integration of viral DNA into the host genome [15]. Here we focused on the first possibility and provided experimental data, which showed that DNA damage increased the frequency of viral integration into the host genome.

In particular, we found that DSBs promoted the transduction of D64A virus, which was defective with respect to the catalytic activity of integrase (IN-CA). Moreover, DSBs upregulated the infectivity of WT virus by overcoming the inhibitory effects of RAL, an IN-CA inhibitor. Furthermore, infectious secondary viruses were generated from the provirus DNA formed through IN-CA–independent viral transduction. Our observations were highly consistent with previous reports that the IN-CA–defective virus can integrate into the host genome [45-47]. Ebina et al. reported that the integration rate of the IN-CA–defective virus was enhanced by DNA damaging agents such as x-ray irradiation or hydrogen peroxide [48], whereas we showed that DSBs upregulated IN-CA–independent viral integration and promoted the production of secondary viruses, which were competent for subsequent viral infection. Importantly, analysis of the nucleotide sequences of the viral RNA from the secondary viruses showed that there were no revertants to WT virus. Most of the viruses analyzed also had no reported mutations linked to RAL-resistant phenotypes [29-32]. Taken together with observation that RAL could reduce the infectivity of WT virus at a similar level to D64A virus, our data also suggest that currently available IN inhibitors cannot completely block productive viral infection, which is possibly enhanced by DSBs.

The mechanism of DSB-induced upregulation of viral transduction remains elusive but our data suggest that DSB sites provide a platform where viral DNA integrates in an IN-CA–independent manner. When cells were co-infected with HIV-1 virus and an adenovirus that expressed rare-cutting endonucleases such as I-SceI or I-Ppol, we reproducibly observed that the viral DNA was integrated into the corresponding DSB sites. However, interestingly, DSB-site specific viral integration was influenced by viral and cellular factors. First, we observed that targeting of viral DNA to the DSB site was observed mainly during IN-CA–independent viral transduction, although its frequency was low compared with WT virus. Second, it was influenced by the cellular conditions of the target cells, i.e., the frequency of IN-CA–independent viral transduction into DSB sites decreased from approximately 53% to 18% when the concentration of FBS was changed from 0.1% to 10% (Figure 2C). These results and the FACS analysis suggest that this difference may be because the spontaneous DSBs generated during DNA replication also captured viral
DNA, which resulted in a decrease in the relative rate of viral integration into artificially induced DSBs.

Interestingly, the DSB-specific integration of DNA fragments has been reported for hepatitis B virus DNA, an adeno-associated viral vector (AAV) [49,50], and Ty1 [51], a DNA retrotransposon of Saccharomyces cerevisiae. These observations suggest that the DSB site-specific integration of exogenous DNA fragments is not lentivirus-specific, which also indicates that DSB site-specific integration is dependent on the cellular response to DNA damage. We observed that KU55933, a specific ATM inhibitor, consistently blocked DSB-specific viral integration (Figures 1D and 2D). Interestingly, x-ray irradiation triggers the retrotransposition of long interspersed element 1 in human cells, which is also dependent on ATM [52], implying that a conserved cellular response to DNA damage is functionally involved in the capture of viral DNA in the DSB site.

We detected minor nucleotide deletions of approximately <9 bp in five of six clones of the provirus DNA, which were derived from cells infected with virus in the presence of RAL (Table 1). Such structural alternations would be due to the NHEJ repair system that is involved in viral integration in the presence of RAL. Because it has been reported that provirus DNA with 10-bp deletions from nucleotides +3 to +12 in the 5′-LTR remained functional [53], such provirus DNA is likely to be replication competent, although minor modifications in the 5′-LTR may be related to reduced expression of viral mRNA, as reported by Ebina et al. [48].
Several researchers have proposed that viral mRNA is expressed from non-integrated viral DNA of the IN-CA-defective virus [54,55], whereas Vpr was shown to promote Nef mRNA expression from such an extrachromosomal viral DNA [56]. However, our study clearly indicated that Vpr upregulates integration of IN-CA–viral DNA [56]. However, our study clearly indicated that Vpr functions as a positive factor during viral transduction into MDMs. Combined with observations that Vpr activates ATM [36,37] and ATR [38] and that macrophages are resistant to DSBs compared with monocytes [20], our data suggest that the enhancement of IN-CA–independent viral transduction into MDMs may be a pivotal role of Vpr in HIV-1 infection.

In summary, our observations may have major importance in the debate on the involvement of cellular factors in viral integration. It has been postulated that DNA damage sensor molecules are involved in the efficient integration of viral DNA. It has also been claimed that DNA damage sensor proteins have no involvement in DNA damage-dependent viral integration. Here we showed that DSBs are particularly important for IN-CA–independent viral transduction and that the effects of DSBs should be analyzed in carefully designed experimental conditions or else their effects are obscured. Collectively, our data suggest that complete prevention of viral integration will require the development of novel compounds that can protect cells from IN-CA–independent viral integration.

Conclusion
The ATM-dependent mode of the DSB-specific viral DNA integration and Vpr-induced DSBs may be novel targets for anti-HIV compounds that inhibit viral transduction into MDMs, which are a persistent focus of HIV-1 infection.

Methods
Plasmid constructs
The vesicular stomatitis virus glycoprotein (VSVG) expression vector pHIT/G [57], the HIV-1 proviral construct pNL4-3 [58], pNL-ADA [59], and the HIV-1 proviral indicator constructs pNL-Luc-E(−) and pNL-Luc-E(−)R(−) [37] have been described previously. To introduce D64A mutation into IN (adenine of nucleotide 4420 to cytosine) to create pNL-IN-D64A, site-directed mutagenesis (QuikChange; Stratagene) was performed using pNL4-3 as a template. To create pNL-ADA-IN-D64A and pNL-Luc-IN-D64A-E(−) that contained D64A mutants, the SpeI-PfuMI fragment (nucleotides 5297–5785) of pNL-Luc-E(−)R(−) was replaced with those of pNL-ADA, pNL-ADA-IN-D64A, and pNL-Luc-IN-D64A-E(−), respectively.

The neomycin-resistant marker expressing vector pNL-Neo-E(−)R(−) was created by inserting a PCR-amplified neomycin-resistant gene into the NotI-Xhol site of pNL-Luc-E(−)R(−). To create a neomycin-resistant marker expressing D64A, the mutant pNL-Neo-IN-D64A-E(−)R(−) was created by the SpeI-PfuMI fragment (nucleotides 1507–5297) of pNL-IN-D64A and replaced with that of pNL-Neo-E(−)R(−). To create pRES2-EGFP-I-SceI, a pIRES2-EGFP (Clontech)-based plasmid with an I-SceI recognition site, a synthetic double-stranded oligonucleotide (I-SceI-sense and 1-SceI-antisense oligonucleotides; see Additional file 1: Table S2) was inserted into the EcoRI and BamHI sites of pIRES2-EGFP.

To make the adenoviral vector Ad-1-ppol, p-pol cDNA was amplified from pBabe-HA-ER-I-ppol using the Adeno-Ppol-Dral-F and Adeno-Ppol-Dral-R primers (Additional file 1: Table S2) and cloned into the SpeI site of the pAxCALNLwtit2 cosmid vector (NIPPON GENE). To generate the EGFP-expressing lentiviral vector (pLentiv6-EGFP), EGFP cDNA from pENTR1a-EGFP was cloned into pLentiv6/V5-DEST (Invitrogen) using LR Clonase (Invitrogen). The IN D64V mutation of the gag/pol-expressing plasmid pLP1 (Invitrogen; pLP1-IN-D64V) was introduced using pLP1 as a template with site-directed mutagenesis (QuikChange; Stratagene).

Cell culture
THP-1, HT1080, HEK293, and HEK293T cell lines were obtained from the RIKEN Cell Bank. TIG-3 (primary human fibroblast cells) and MT-4 cells were obtained from the Health Science Research Resources Bank (Osaka, Japan). HT1080, HEK293, HEK293T, MAGIC5, and TIG-3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). MT-4 cell was maintained in RPMI 1640 supplemented with 10% FBS. To obtain macrophage-like cells, THP-1 cells, maintained in Iscove’s modified Dulbecco’s medium supplemented with 10% FBS, were treated for 2 d with 5.0 × 10−8 M PMA. As described previously [60], PMA-treated THP-1 cells were positive for Mac-1, a specific marker of macrophages. Peripheral blood was derived from healthy donors who worked within the institute and gave informed consent. Experimental procedures were approved by the internal review board. PBMCs and MDMs were prepared and cultured as previously described [61]. MDMs were prepared from healthy volunteers who gave informed consents. The experimental protocol was approved by the internal review board.
HIV-1 and lentiviral vector preparation

The preparation and titration of replication-competent and VSV-G-pseudotyped viruses are described elsewhere [36,37,62,63]. The lentiviral vectors were prepared using pLenti6-EGFP and the ViraPower Lentiviral Packaging Mix (Invitrogen) according to the manufacturer’s protocol. Viral supernatants were centrifuged at 120 × g for 5 min, filtered through a 0.2-μm filter, and stored at −80°C. To exchange the medium for DMEM supplemented with 0.1% FBS, the viruses were ultracentrifuged at 86,000 × g for 1 h.

Quantitative PCR of provirus DNA

For the quantification of early RT, late RT, 2-LTR circle, and integrated DNA, qPCR was performed as described elsewhere [64,65]. Briefly, cells were harvested at 48 hpi, and genomic DNA was prepared by QuickGene (FujiFilm). For the quantification of early RT, late RT, and 2-LTR circle products, the primers and probe sets M667/AA55/R-U5, M667/M661/R-U5, and MH535/2-LTR-AS/NL4-3-U3 were used, respectively. TaqMan Universal PCR Master Mix with UNG (Applied Biosystems) were used according to the manufacturer’s instructions. For Alu-PCR (quantification of integrated DNA), the primer and probe sets first-Alu-F/first-Alu-R/first-gag-R and second-tag-R/2-LTR-S/probe-2 were used for the first and second rounds of qPCR, respectively. The amplification conditions for the first round of PCR, using ExTag polymerase (TaKaRa), were as follows: 30 cycles of 98°C for 10 s, 60°C for 30 s, and 72°C for 30 s. The second round of qPCR was conducted using SYBR Premix ExTag polymerase (TaKaRa) according to the manufacturer’s instructions. For the second round PCR template, 1/25 the volume of the first PCR ampiclon was used. To prepare a standard sample for the first-qPCR, the 5′-LTR DNA fragment of HIV-1 was amplified using the pNL4-3+9074F-Sce-I and pNL4-3+9423R-BamHI and cloned into the EcoRI and BamHI sites of pIRES2-EGFP (pIRES2-EGFP-5′-LTR). Then, HT1080 cells were transfected with pIRES2-EGFP-5′-LTR and HT1080/pIRES2-EGFP-5′-LTR cell was obtained. By Southern blot and sequence analyses we confirmed that two copies of the DNA fragment of pIRES2-EGFP-5′-LTR vector were present in HT1080/pIRES2-EGFP-5′-LTR diploid cells. Sequence information for primers and probes is listed in Additional file 1: Table S2.

Cleavage of I-SceI and I-Ppol sites

Ad-I-SceI and Ad-LacZ were prepared as described previously [36]. PMA-treated THP-1 cells were infected with Ad-I-SceI or Ad-LacZ at 1 h post–HIV-1 infection for 1 h at a multiplicity of infection (MOI) of 100. In Figure 1E–1H, HT1080 cells were transfected with pLenti6-EGFP that encoded a chimeric protein of estrogen receptor-I-Ppol (ER-I-Ppol), and then 4-hydroxytamoxifen (4-OHT) was added to activate the endonuclease and induce DSB. The pAXCALNLwtit2 cosmid vector harboring I-Ppol cDNA was digested with BspTI041 and transduced into HEK293 cells to produce Ad-I-Ppol. The adenoviral vector encoding Cre recombinase, AxCANCRe (TaKaRa), was co-infected with Ad-I-Ppol at an MOI of 30 to remove the floxed stuffer between the CAG promoter and I-Ppol cDNA.

Quantification of the I-SceI site specific viral integration

PMA-treated THP-1 cells were co-infected with WT virus and Ad-I-SceI or Ad-LacZ, and then extracted genomic DNA was subjected to I-SceI-qPCR analysis. I-SceI site targeting integration rate of HIV-1 DNA was estimated by PCR amplification using primer sets pIRES2eGFP+543F/pNL4-3+9207R and pIRES2eGFP+574F/pNL4-3+98R+9173R for the first and second rounds of qPCR, respectively. The amplification conditions for the first round of PCR, using ExTag polymerase (TaKaRa), were as follows: 30 cycles of 98°C for 10 s, 60°C for 30 s, and 72°C for 30 s. The second round of qPCR was conducted using SYBR Premix ExTag polymerase (TaKaRa) according to the manufacturer’s instructions. For the second round PCR template, 1/25 the volume of the first PCR ampiclon was used. To prepare a standard sample for the I-SceI-qPCR, the 5′-LTR DNA fragment of HIV-1 was amplified using the pNL4-3+9074F-Sce-I and pNL4-3+9423R-BamHI and cloned into the EcoRI and BamHI sites of pIRES2-EGFP (pIRES2-EGFP-5′-LTR). Then, HT1080 cells were transfected with pIRES2-EGFP-5′-LTR and HT1080/pIRES2-EGFP-5′-LTR cell was obtained. By Southern blot and sequence analyses we confirmed that two copies of the DNA fragment of pIRES2-EGFP-5′-LTR vector were present in HT1080/pIRES2-EGFP-5′-LTR diploid cells. Sequence information for primers and probes is listed in Additional file 1: Table S2.

Quantification of the I-Ppol site specific viral integration

Serum starved HT1080 cells were co-infected with Ad-I-Ppol and lentiviruses, which were generated by pLenti6-EGFP or pL1-IN-D64V. To estimate I-Ppol site targeting or total integration of the lentiviral vector, I-Ppol-qPCR or EGFP-qPCR was conducted using the TaqMan Universal PCR Master Mix (Applied Biosystems). For I-Ppol-qPCR in the direct or inverted repeat orientation, the primer sets rDNA+11725R/pLenti6+5237F or rDNA+11645F/pLenti6+5237F were used, respectively; pLenti6-LTR was used as the TaqMan probe. For EGFP-qPCR, the primers EGFP-F/EGFP-R and TaqMan EGFP-probe were used. As a standard sample for estimating copy numbers of viral DNA integrated in the I-Ppol site, genomic DNA of HT1080/Lenti6-EGFP-std cells was used. We have confirmed by Southern blot and sequence analyses that HT1080/Lenti6-EGFP-std cells harbored two copies of Lenti6-EGFP proviral DNA in both orientations in the I-Ppol site. On the other hand, as a standard sample for total provirus DNA, genomic DNA of HT1080/pIRES2-EGFP-5′-LTR cells, which possessed two copies of EGFP, were
used. Sequence information for primers and probes is listed in Additional file 1: Table S2.

**PCR and sequence analysis**

To amplify the host DNA/5'-LTR junction at the I-SceI site, the primer sets pIRES2eGFP+11612F/pLenti6+5232F were used for the first and second rounds of PCR, respectively. To amplify the host DNA/3'-LTR junction at the I-SceI site, the primer sets prIRES2eGFP+1910F/L-M667 and prIRES2eGFP+887R/ LambdaT were used for the first and second rounds of PCR, respectively. The amplification conditions for the host DNA/5'-LTR and host DNA/3'-LTR were as follows: 40 cycles for the first round of PCR or 30 cycles for the second round of PCR at 98°C for 10 s, 60°C for 30 s, and 72°C for 30 s and 35 cycles at 98°C for 10 s, 60°C for 30 s, and 72°C for 60 s for the first round of PCR and 30 s for the second round of PCR, respectively. ExTaq polymerase (TaKaRa) was used for the PCR. PCR amplicons were used directly or cloned into pCR2.1-TOPO (Invitrogen) as a template for sequence analysis. To amplify the rDNA/lentiviral vector at the I- pole site in the direct repeat orientation, the primer sets rDNA+11784R/pLenti6+5208F and rDNA+11747R/pLenti6+5232F were used for the first and second rounds of PCR, respectively. To amplify the rDNA/lentiviral vector at the I-poli site in the inverted repeat orientation, the primer sets rDNA+11589F/pLenti6+5208F and rDNA+11612F/pLenti6+5232F were used for the first and second rounds of PCR, respectively. The amplification conditions for the rDNA/lentiviral vector at the I- pole site were as follows: 40 cycles for the first and second rounds of PCR at 98°C for 10 s, 60°C for 30 s, and 72°C for 30 s. ExTaq polymerase (TaKaRa) was used for PCR. PCR amplicons were used directly or cloned into pCR2.1-TOPO (Invitrogen) as a template for sequence analysis. To analyze the IN mutations of NL-ADA and NL-IN-D64A (Invitrogen) as a template for sequence analysis. To analyze the IN mutations of NL-ADA and NL-IN-D64A-ADA viruses in Figure 5B and 5C, viral RNAs were isolated from the IN mutations of NL-ADA and NL-IN-D64A (Invitrogen) as a template for sequence analysis. To analyze

**LAM-PCR**

To estimate colony formation rate (D64A mutant virus), serum-starved HT1080 cells (5 × 10^5 cells) in DMEM with 0.1% FBS were infected with a neomycin-resistant marker expressing VSVG-pseudotyped NL-Neo-IN-D64A-E(-) R(-) virus (100 ng p24) in the presence of 0 (DMSO), 0.625, 1.25, or 2.5 μM etoposide and 0 (water), 0.313, 0.625, or 1.25 μM bleomycin. Cells were selected with G418 from 2 dpi, then stained with Giemsa at 12 dpi. The G418-resistant colony numbers were normalized by plating efficiency, which represented the cytotoxicity of etoposide and bleomycin. The plating efficiencies after treatment of etoposide and bleomycin at 0, 0.625, 1.25, 2.5 μM were 100, 57.2, 26.0 and 19.5%, and 100, 60.4, 68.8 and 60.4%, respectively.

**Replication assay**

To estimate the production of functional virion from RAL-treated cells, 1 × 10^5 MT-4 cells were infected with replication-competent NL4-3 or NL-IN-D64A (20 ng p24). After 2 h of infection, cells were washed with phosphate buffered saline (PBS) twice and suspended in 1.0 mL of medium. To prepare the culture supernatant, three-quarter of the cultures were harvested every 2 d, and the culture was continued by adding 750 μL of the complete medium into each well. From −1 dpi to harvest, MT-4 cells were treated with 10 μM RAL or DMSO. Conditioned medium (100 μL) was added to 1 × 10^4 MAGIC5 cells [28], and at 48 hpi, cells were stained by X-gal to estimate the number of transduced cells. To estimate HIV-1 RNA copy numbers, 1 × 10^5 MDMs were infected with NL-ADA, NL-ADA-R(-), NL-ADA-IN-D64A, or NL-ADA-IN-D64A-R(-) (20 ng p24) for 2 h, then washed with medium four times. Three-quarters of the conditioned medium was harvested and replaced with fresh medium every 2 d. From −1 dpi to harvest, MDMs were treated with 10 μM RAL or DMSO. HIV-1 RNA of conditioned medium was purified and subjected to RT-qPCR using the Lenti-X qRT-PCR Titration Kit (TaKaRa). To evaluate the effect of DNA damaging agents, 2.5 μM etoposide or 1.25 μM bleomycin were added to MDMs from 0–2 dpi. To exclude a possibility that detected HIV RNA merely reflect the RNA from carry-over virion, fusion inhibitor ENF, dissolved in phosphate buffer saline PBS, was added from 0 hpi to harvest as a negative control.

**Colony formation assay**

To evaluate the effect of DNA damaging agents on the integration rate of D64A mutant virus, serum-starved HT1080 cells (5 × 10^5 cells) in DMEM with 0.1% FBS were infected with a neomycin-resistant marker expressing VSVG-pseudotyped NL-Neo-IN-D64A-E(-) R(-) virus (100 ng p24) in the presence of 0 (DMSO), 0.625, 1.25, or 2.5 μM etoposide and 0 (water), 0.313, 0.625, or 1.25 μM bleomycin. Cells were selected with G418 from 2 dpi, then stained with Giemsa at 12 dpi. The G418-resistant colony numbers were normalized by plating efficiency, which represented the cytotoxicity of etoposide and bleomycin. The plating efficiencies after treatment of etoposide and bleomycin at 0, 0.625, 1.25, 2.5 μM were 100, 57.2, 26.0 and 19.5%, and 100, 60.4, 68.8 and 60.4%, respectively.

**Immunohistochemical analysis**

Detection of phosphorylated histone H2AX (γH2AX) and phosphorylated ATM (pATM) was done, according to the reported method using antibodies against γH2AX (phosphorylated at Ser 139, Millipore, cat no. 05–636) and pATM (Ser 1981, Millipore, cat. no. 05–740) [37].
Briefly, the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS. After treatment with PBS supplemented with 10% goat serum for 30 min, the cells were incubated with antibodies. After 1 h of incubation at 37°C, secondary antibodies conjugated with Alexa 546 (Molecular Probes) were added for 1 h at 37°C. Nuclei were stained by Hoechst33258.

Luciferase assay
To evaluate the infectivity of viruses, 1.0 × 10⁴ cells were infected with VSVG-pseudotyped luciferase viruses (2 ng p24) for 48 h, then subjected to luciferase assay by using One-Glo (Promega) and a Veritas Microplate luminometer (TURNER BIOSYSTEMS).

Flow cytometry
To analyze the status of cell cycle, HT1080 cells were labeled with 5 μM BrdU for 30 min and fixed in ice-cold 70% ethanol. Anti-BrdU-fluorescein (Roche Diagnostics) was used to stain the BrdU-labeled cells, according to the manufacturer’s instructions. BrdU-labeled cells were analyzed by BD FACSCalibur flow cytometer (BD Biosciences). To analyze the percentages of EGFP-positive cells, Flow cytometry analysis was performed using a BD FACSCalibur.

FISH analysis
Metaphase FISH analysis was performed to estimate the proviral DNA copy number and co-localization of proviral DNA and rDNA using lentiviral vector and rDNA specific probes (Chromosome Science Labo Inc.).

Statistical analysis
Statistical significance was determined by Student’s t-test, and values of P < 0.05 were considered statistically significant.

Additional file

Additional file 1: Figure S1. Southern blot analysis to verify the cleavage of the I-Sce and I-Pol site. Figure S2. A schematic of the strand transfer of HIV-1 DNA to genomic DNA. Figure S3. Evaluation of lentiviral infectivity and cell cycle status of serum starved HT1080 cells by BrdU incorporation. Figure S4. Raw data in Figure 3. Figure S5. The percentage of insertion and/or deletion (InDel) mutations at the host/ viral junction. Figure S6. Two additional independent data sets in Figure S8 and SC. Figure S7. RAL in 2d cultured conditioned medium is active. Figure S8. Additional data sets with another donor in Figure 7D. Figure S9. Raw data in Figure 7E. Table S1. RAL and EVG resistant mutations reported in literature. Table S2. List of primers and probes used in this study. Supplementary methods. Southern blot analysis.

Competing interests
The authors declare no competing financial interests.

Authors’ contributions
TK and BS designed and performed the work, analyzed the data and wrote the paper. KT and MT contributed reagents and technical advice for the work, and edited the manuscript. YI conceived of the study and supervised the work and wrote the paper. All authors read and approved the final manuscript.

Acknowledgements
We thank Dr. F. Graham (McMaster University, Canada) for the I-Sce adenovirus vector, Dr. I. Saito (Tokyo University, Japan) for ArCaLacβ, Dr. M. O’Connor (KuDOS Pharmaceuticals, England) for KUSS933, and Dr. M. B. Kastan (Duke Cancer Institute, USA) for pBABI-HA-ER-I-Pol. This work was supported by a Grant-in-Aid for Research on HIV/AIDS from the Ministry of Health, Labor, and Welfare of Japan.

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Received: 26 June 2012 Accepted: 11 February 2013
Published: 21 February 2013

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doi:10.1186/1742-4690-10-21
Cite this article as: Koyama et al.: DNA damage enhances integration of HIV-1 into macrophages by overcoming integrase inhibition. Retrovirology 2013 10:21.