Shifting Retroviral Vector Integrations Away from Transcriptional Start Sites via DNA-Binding Protein Domain Insertion into Integrase

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INTRODUCTION

Retroviral vectors, which are mostly based on the murine leukemia virus (MLV), are the most widely used vectors, preferentially integrate in genomic regions upstream of genes, mainly transcriptional start sites (TSSs) regions, and within genes, respectively. This feature can cause detrimental effects when retroviral vectors integrate upstream of oncogenes and when lentiviral vectors integrate into tumor-suppressor genes. For example, the strong integration preference of MLV-based gammaretroviral vectors for genomic regions near TSSs can lead to uncontrolled growth of transduced human cells through upregulation of downstream oncogenes. Oncogenic potential may limit the use of gammaretroviral vectors in clinical applications.

Substantial effort has been made to better understand how retroviruses and lentiviruses select integration sites in mammalian genomes. A few cellular proteins with bromodomain and extraterminal domain, and lens epithelium-derived growth factor have been found to tether MLV and HIV-1 to their inherently preferred genomic regions near TSSs and within genes, respectively. Based on these findings, several trials, often involving viral integrase engineering, have been conducted to alter the retroviral integration patterns by interfering with the interactions between the cellular tethering factors and the virus, or by conferring viruses the ability to interact with new host proteins. In particular, MLV integrations around TSSs have been successfully reduced, but still occur at significantly high frequencies, indicating that there might be other, unknown mechanisms underlying the integration process. However, searching the complete MLV integration mechanisms and finding ways to block all the critical mechanisms require substantial time and effort. In this work, instead, we developed a straightforward method that can be generally applied to make safer retroviral and lentiviral vectors. This method involves the perturbation of the structure.

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of integrase, which plays key roles in determining retroviral and lentiviral integration patterns.

RESULTS AND DISCUSSION

Retroviral Vectors with Integrase-Zinc-Finger Domain Fusion Proteins

We aimed to develop safer MLV-based gammaretroviral vectors without significant integration preference for TSSs. To this end, we perturbed the structure of integrase by inserting DNA-binding protein domains into an internal site (in front of the 274th amino acid residue; Figure 1A) of the enzyme, which consists of 408 amino acid residues. We expected the consequent changes in the integrase structure to alter the retroviral integration pattern, as generally observed in enzyme structure-function relationships. In addition, the DNA-binding property of the inserted domains might support association of the integrase with host genomic DNA. As DNA-binding domains, 10 zinc-finger domains (ZFDs) containing between two and five finger units were used (A1–A5 and B1–B5; Figure 1B; Table S1). Because of the short length of the ZFD and the potential alteration of ZFD folding by the flanking viral protein domains, site-specific targeted integration could not be aimed for.

Each ZFD was inserted in the MLV vector, resulting in 10 mutant MLV vectors (Figure 1B). Despite the protein insertion, these mutant vectors could still transduce human cells at significant levels, although the transduction titers were inevitably reduced by these mutations, 45.0- to 1,624.8-fold compared with that of the wild-type vector (Figure 1C). A marker protein, EGFP, was expressed at similar levels by wild-type and mutant vectors when the fractions of transduced cells were equivalent (e.g., wild-type versus two mutant vectors [A4 and A5] in Figure S1). Distinct gene expression patterns among the mutant vectors were not recognized in preliminary experiments (J.-E.L., Y.Y., and K. Lim, unpublished data). In contrast, the insertion of foreign proteins into most other sites within the integrase, except for the sites in front of the 38th, 274th, and 378th amino acid residues, completely abrogated the transduction ability of MLV-based vectors. Thus, it is not easy to establish infectious mutant virus with protein insertion into an internal site of the integrase. The significant reduction in the functional titer (Figure 1C) may limit the use of
mutant vectors for gene delivery in vivo. However, these vectors might be useful for ex-vivo transduction of therapeutic genes into cells, followed by isolation of transduced cells, expansion, and reintroduction of the engineered cells into the patient. Furthermore, the transduction titer of the mutant vectors can be increased by engineering of their genomes and structural proteins. For example, our preliminary independent study revealed that promoter modifications, addition of RNA-stabilizing motifs, and shortening of the genome by omitting the unnecessary parts may increase retroviral transduction titers by several folds.

In this preclinical, basic study, we focused on investigating whether retroviral integration patterns can be altered through perturbation of the integrase structure by using HEK cells, which have been widely used in studies on retroviral integrations,28–30 rather than using primary cells or stem cells, which would be more relevant for clinical applications.

ZFD Insertion Significantly Reduces Retroviral Integration Preference for the TSS Genomic Regions

Next-generation sequencing (NGS) and subsequent bioinformatics analysis of host-virus genome junctions was used to assess which human genomic regions harbored retroviral DNAs. Samples were barcoded to allow for multiplex sequencing to reduce the cost while maximizing data yield. We did obtain sufficient non-redundant genome junction reads (Table S2) to detect statistically significantly different integration patterns (p < 0.05 in many cases; Figures 2 and 8). In addition to the determination of the statistical significance for observed differences, post hoc power analysis using the G*Power 3.1 tool31 was conducted to calculate the achieved power given the significance level (z = 0.05), differences, and sample numbers (here, genome junction read numbers). Power values >0.8 (Figures 2 and 8) indicated that we had a sufficient number of genome junction reads to statistically confirm the integration pattern differences for the corresponding cases.31

In accordance with previous studies,7,9,32 the wild-type MLV vector showed a very strong integration preference for genomic regions near TSSs, which are dangerous spots for gene therapy applications, with an integration frequency of 49.7%, which was 4.8-fold higher than that expected by random chance (10.4%; p = 7.30E−73 in comparison with the random case; Figure 2A). In stark contrast, the mutant A2 (Figure 1B) integrated into the TSS regions at only 9.0%, which was 5.5-fold lower than the TSS integration frequency of the wild-type vector and equivalent to that expected by random chance (Figure 2A). This observation indicates that insertion of ZFDs into MLV integrase can completely abolish the retroviral near-TSS integration preference. All other mutants, except A5, B3, and B5, also integrated into near-TSS genomic regions significantly less frequently than the wild-type vector, but more frequently than expected by random chance. This result implied that insertion of DNA-binding domains into integrase does not always yield retroviral vectors with safe integration profiles comparable with that of random integrations. Depending on the size and type of the inserted ZFD, the extent of perturbation of the integrase structure may vary.

Furthermore, within a window of 5 kb upstream and downstream of TSSs, wild-type MLV strongly preferred genomic locations very near the TSSs; it integrated within 1 kb from TSSs at the frequency of 31.4% (Figure 2B, left and right panels). This integration preference specifically for the TSS-proximal regions was 14.2-fold higher than that expected by random chance (2.2%). However, the mutant A2 had a modest preference for these specific genomic regions, with an integration frequency of 4.0%, which was only 1.8-fold higher than that expected by random chance, but 7.9-fold lower than that of the wild-type vector (Figure 2B, left and right panels). The random and mutant A2 integrations showed similar cumulative frequencies over the distance from the TSSs (Figure 2B, right panel). On the other hand, the mutant vector B2 produced a cumulative integration frequency profile between those of the random and wild-type vector integrations, indicating it retained a certain level of integration preference for the TSS-proximal regions (Figure 2B, right panel).

Two Mutant Vectors Show Significantly Lower Preference for TSS Regions Near Oncogenes Than Wild-Type MLV Vector

The oncogenic potential of retroviral vectors is closely correlated with the frequency of vector integrations upstream of oncogenes. QuickMap, which uses the Wellcome Trust Sanger Institute census of human cancer genes,33 was used to search oncogenes within 5 kb of TSSs that neighbor retroviral integrations. Wild-type MLV vectors integrated into these dangerous regions at the frequency of 3.05%, which was significantly higher (19.3-fold) than that expected by random chance (0.16%; p < 2.22E−16; Figure 2C). More specifically, the wild-type vector integrations hit the TSS regions that were near six oncogenes, HOXA9, HOXA11, CCDC6, Myc, PSIP1, and PTPN11 (Table S3). In contrast, the mutants A1, A2, A4, A5, B2, and B3 did not integrate into these oncogenic regions (Figure 2C). Using these integration frequency data, we tested the hypothesis that some mutants integrate less into the oncogenic regions than wild-type vector. A2 and B2 mutant integration patterns satisfied this hypothesis with statistical significance (p = 0.045 and 0.024, respectively; Figure 2C). Integration patterns of A1, A4, and A5 supported this hypothesis with moderate levels of significance (p = 0.074, 0.087, and 0.077, respectively; Figure 2C). These results suggested that insertion of ZFDs into integrase might be a promising approach to reduce the oncogenic risk of MLV-based vectors.

Mutant Vectors Have Significantly Reduced Preference for cis-Regulatory Elements and CpG Islands Compared with Wild-Type MLV Vector

Wild-type MLV vector strongly preferred genomic sites near cis-regulatory elements, as well as CpG islands, which are often enriched in regulatory DNA components, with integration frequencies, respectively, being 4.7- and 6.8-fold higher than that by random chance (Figure 2D). However, insertion of ZFDs into the integrase
significantly reduced such integration preference by up to 2.4-fold for cis-regulatory elements and 3.0-fold for CpG islands (for the A2 mutant; Figure 2D). While the wild-type vector also showed slightly higher integration preference for genomic regions within genes than that expected by random chance ($p = 2.47 \times 10^{-3}$), the mutants A2 and B2 hit these regions at frequencies similar to that expected by random integrations (Figure 2D). The integration frequencies of the wild-type vector in genomic regions near TSSs, cis-regulatory elements, CpG islands, and within genes (by 39.3%, 51.2%, 41.5%, and 10.7%, respectively, 142.7% in total) were consistently higher than those expected by random integration, whereas the frequency of integrations into repeats (by 14.4%; Figure 2D) was lower than the random frequency. This large mismatch for 142.7% up and 14.4% down compared with the random case in integration preference for multiple genomic regions indicates that the wild-type vector would integrate into the genomic regions having multiple functions at the same time. Such a strong mismatching trend was not observed for the A2 mutant.

Wild-type MLV vector integrations were also strongly concentrated in genomic sites very near to cis-regulatory elements and CpG islands (within a distance of 1 kb; Figures 3A and 3B). These

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**Figure 2. Integration Patterns of Mutant Vectors with Integrase-ZFD Proteins in the Human Genome**

(A) Frequency of retroviral integrations into the genomic regions within 5 kb of TSSs in the human genome. HEK293T cells were transduced with MLV-based retroviral vectors. Random integrations were additionally computationally generated, and the relevant frequencies of random and retroviral integrations into the TSS regions were quantified, all using the QuickMap tool (Gene Therapy Safety Group). Statistical significance for the differences between wild-type and mutant integration patterns is indicated by p values (shown in black) that were obtained by the chi-square test. The corresponding statistical power values (see main text) were calculated using G’Power 3.1. Statistical significance for the difference between the random and wild-type patterns is indicated by p values (shown in blue) that were obtained by the chi-square test. Corresponding statistical power values were calculated using G’Power 3.1. (B) Left panel: experimentally observed retroviral integrations are spatially distributed within a window of 5 kb upstream and downstream of CpG islands. Right panel: cumulative integration frequencies over the indicated distance from cis-regulatory elements. The frequencies based on total integrations (y coordinate of a point) within x kb from cis-regulatory elements (x coordinate of a point) are marked in the figure. Random integrations were additionally computationally generated using the QuickMap tool (Gene Therapy Safety Group). The relevant frequencies of random and experimental retroviral integrations into the genomic regions near the cis-regulatory elements and CpG islands were quantified using the QuickMap tool. The random integrations are also presented over this genomic window.

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**Figure 3. Spatial Distribution of Retroviral Integrations around cis-Regulatory Elements and CpG Islands**

(A) Left panel: experimentally observed retroviral integrations are spatially distributed within a window 5 kb upstream and downstream of cis-regulatory elements (CREs) that were computationally predicted (referring to cisRED, a genome-scale database). Right panel: cumulative integration frequencies over the indicated distance from cis-regulatory elements. The frequencies based on total integrations (y coordinate of a point) within x kb from cis-regulatory elements (x coordinate of a point) are marked in the figure. Random integrations were additionally computationally generated using the QuickMap tool (Gene Therapy Safety Group). The relevant frequencies of random and experimental retroviral integrations into the genomic regions near the cis-regulatory elements and CpG islands were quantified using the QuickMap tool. The random integrations are also presented over this genomic window.
narrowly distributed wild-type retroviral integrations near regulatory element-rich regions may result in the perturbation of inherent gene expression regulation in host cells. This localized integration centered at regulatory regions was also reduced for the mutant A2 (Figures 3A and 3B).

**Proviruses of Mutant Vectors Frequently Have Residual Primer-Binding Site Sequences in the 3' Long Terminal Repeat**

Sequence analysis of the host-virus genome junctions revealed that all the mutant vectors frequently produced proviruses with incompletely processed primer-binding site sequences in their 3' long terminal repeat (LTR) end (Figures 4A and 4B). The formation of primer-binding site sequences at the end of proviruses is thought to be linked to altered activity of the RNase H domain of reverse transcriptase (Figure S2).34,35 Unexpectedly, proviruses generated from the wild-type vector also had these residual primer-binding site sequences in the 3' LTR end (Figure 4B). However, the frequency of occurrence of residual primer-binding site sequences per provirus was higher for mutants (14.0%–41.7%) than for the wild-type (3.6%; Figure 4B). Mutants had short residual primer-binding site sequences of variable length (5–18 bp [the entire primer-binding site sequence is 18 bp]; Figures 4A; Figures S3 and S4). Addition of short duplicated DNA sequences from the host genome at the ends of integrated retroviral DNAs often occurs.36 However, it is unclear whether short DNA sequences (<20 bp) flanking the integrated retroviral vector genomes interfere with the expression of transgenes, which are generally located inside the vector genomes. Notably, perturbation of the integrase structure by ZFD insertion led to reduced function of another enzyme, reverse transcriptase. Therefore, our integrase mutants can be considered as class II mutants that can affect reverse transcription, assembly of progeny particles, and other infection steps.37 Similarly, previous studies have reported that mutations of retroviral integrase affected reverse transcription.38–40

In addition, although the 5' overhang of two bases (AA), generated from the 3' end processing of retroviral genomic DNA (Figure 4A) by integrase,41,42 was mostly removed for wild-type vector (at the frequency of 94.4%; Table S4), this sequence was less frequently removed for the mutants (at frequencies of 35.5%–67.4%; Table S4). However, the fact that mutant integrases strongly maintained the ability to perform the 3' end processing of the retroviral genomic DNA corroborated that MLV integrase was functional even with ZFD insertion.
Mutant and Wild-Type Vectors Integrate into the TSS Regions Less Frequently When Having Residual Primer-Binding Site Sequences

To check whether the presence of residual primer-binding site sequences in the provirus 3' LTR end correlated with the lower tendency of retroviral vectors to integrate into the TSS regions, we divided proviruses into two groups based on the presence or absence of residual primer-binding site sequences and then compared their integration patterns. We first analyzed the mutant vector A2, which showed the lowest integration frequency in the TSS regions (Figure 2A). A2 integrated into genomic regions near TSSs significantly less frequently when having residual primer-binding site sequences than when having no primer-binding site sequence in the 3' LTR end (2.5% versus 13.3%; Figure 4C, right panel). Similarly, this mutant integrated into cis-regulatory elements significantly less frequently when having residual primer-binding site sequences (Figure 4C, right panel). Integration frequencies for the genomic regions of TSSs, cis-regulatory elements, and CpG islands were partly correlated with the presence of residual primer-binding site sequences also for other mutants (Figures S5 and S6). Interestingly, the wild-type vector showed a similar trend: integrations into the TSS regions, cis-regulatory elements, and CpG islands varied in frequency in accordance with the presence or absence of residual primer-binding site sequences in the provirus 3' LTR end (Figure 4C, left panel).

Construction of New Mutants with Modifications of Primer-Binding Site in Their RNA Genome

Formation of residual primer-binding site sequences at the 3' LTR end of retroviral DNA by engineering the MLV RNA genome to have one to eight additional copies of the wild-type primer-binding site sequence in the region flanking the 5' LTR (Figure 6A). Through host tRNA binding to the newly added primer-binding site(s), the chance of formation of residual primer-binding site sequences at the 3' LTR end of the retroviral genomic DNA was predicted to increase (refer to Figure S2). To construct additional genome mutants, the fifth and ninth bases of the primer-binding site were also randomly selected to be replaced with one of the three other bases by point mutation (G [5th] to A, C, or T; C [9th] to A, G, or T; Figure 6A). The frequency of occurrence of residual primer-binding site sequences in the provirus 3' LTR end was slightly increased by 1.7- and 2.1-fold for the PBS2 and SGT mutants, respectively, compared with that in the wild-type vector (Figure S7; p = 0.185 and 0.0635, respectively). Most mutants with additional primer-binding sites or an altered primer-binding site sequence still showed strong integration preference for genomic regions of TSSs, with frequencies equivalent to that of the wild-type vector (Figures S8 and S9). This result indicates that changes in the primer-binding site sequence alone cannot significantly shift retroviral integrations toward safe genomic regions.

In addition, we compared the secondary structures formed by the RNA sequences containing the 5' functional elements (R, U5, primer-binding site, splice donor site) to evaluate whether incorporation of more primer-binding sites into the viral RNA genome (Figure 6A) could disturb the 5' stem loops of the genome. The secondary RNA structures of the wild-type and primer-binding site mutant viruses were predicted with Mfold. The most noticeable change induced by the addition of primer-binding sites was the linear extension of the primer-binding site domain (shown in red in Figure 7). As more primer-binding sites were added, the primer-binding site domain was further extended. In contrast, the secondary structures of other parts were not considerably changed by the addition of primer-binding sites (Figure 7).

Mutants with ZFD Insertion Do Not Produce Safe Integration Patterns When Having Additional Primer-Binding Sites in Their RNA Genome

Reasoning that the modification of both the integrase in the viral Gag-Pol polyprotein and primer-binding site in the viral genome may result in safer retroviral integration patterns, we further engineered the MLV vectors. This combined modification approach might additionally aid in assessing the two above-mentioned mechanistic
scenarios relevant to the safer integration patterns of the mutants (Figure 5). We inserted one of two ZFDs (A2 or B2; Figure 1B) into the integrase and simultaneously introduced three additional primer-binding sites (PBS4) or a point mutation at the fifth or ninth base of the primer-binding site sequence into viral RNA genome (5GA or 9CG; Figures 6A–6C). With the addition of three primer-binding sites into the genome, proviruses of the double-mutant PBS4+A2 (Figure 6C) had residual primer-binding site sequences in the 3′ LTR end at a significantly lower frequency (14.3%) than those of the mutant A2 (40.0%) without primer-binding site addition (the single mutant, A2) (Figure 8A; p = 0.01). The reduced frequency of residual primer-binding site sequences for this double mutant was associated with a significantly increased frequency of integrations into genomic regions near TSSs (from 9.0% to 31.4%; Figure 8B; the corresponding p value = 3.54E−06). This result indicates that if the primer-binding site domain of the viral RNA genome is not intact, the insertion of ZFD into the integrase may not effectively shift the retroviral integration pattern.

The Presence of Residual Primer-Binding Site Sequences in Provirus Is Not Sufficient for a Safe Retroviral Integration Pattern

With a point mutation at the fifth base of the primer-binding site (G to A; Figures 6A–6C), proviruses of the double mutants 5GA+A2 and 5GA+B2 had residual primer-binding site sequences in the 3′ LTR at frequencies of 33.0%, respectively (Figure 8A), which were equivalent to those of the mutants A2 and B2 without primer-binding site point mutation (40.0% and 41.7%, respectively; Figure 8A). Although they maintained a high frequency of residual primer-binding site sequences, 5GA+A2 and 5GA+B2 integrated into the near-TSS genomic regions and 33.0%, respectively (Figure 8A), which were equivalent to those of the mutants A2 and B2 without primer-binding site point mutation (40.0% and 41.7%, respectively; Figure 8A). Although they maintained a high frequency of residual primer-binding site sequences, 5GA+A2 and 5GA+B2 integrated into the near-TSS genomic regions
at frequencies of 34.1% and 30.9%, respectively (Figures 8C and 8D), which were higher than those of the corresponding single mutants A2 and B2 (9.0% and 21.7%; p = 6.48E−23 and 2.69E−2, respectively) (Figures 8C and 8D). This observation indicates that the occurrence of residual primer-binding site sequences in the 3′ LTR end of proviruses is not sufficient to guarantee safe retroviral integration patterns. In other words, the formation of residual primer-binding site sequences likely results from an unknown molecular mechanism that significantly alters retroviral integration patterns (scenario II in Figure 5), as observed for multiple mutants.

On the other hand, with a point mutation at the ninth base of the primer-binding site (C to G; Figures 6A–6C), proviruses of the double mutants 9CG+A2 and 9CG+B2 had residual primer-binding site sequences in the 3′ LTR at even higher frequencies (52.8% and 52.3%, respectively; Figure 8A) than those of the corresponding single mutants A2 and B2 (40.0% and 41.7%; p = 0.067 and 0.093, respectively; Figure 8A). In contrast with 5GA+A2 and 5GA+B2, 9CG+A2 and 9CG+B2 integrated into near-TSS genomic regions at low frequencies (10.4% and 20.7%, respectively; Figures 8E and 8F), equivalent to those of the corresponding single mutants A2 and B2 (9.0% and 21.7%, respectively; Figures 8E and 8F). Comparison of the integration patterns of the double mutants that had a point mutation at the fifth or ninth position of the primer-binding site further indicates that the ZFD insertion-mediated shift in retroviral integrations toward safer genomic regions requires an intact primer-binding site domain, although the ninth position of the primer-binding site is not a critical position that needs to be conserved.

Conclusions
In this study, we showed that perturbation of the integrase structure by insertion of DNA-binding domains is a simple way to obtain safer retroviral integration patterns. This approach obliterates the need to completely understand the molecular mechanisms that affect retroviral integration patterns and to find effective ways to control these mechanisms. Modification of the integrase significantly reduced the inherent retroviral integration preference for the TSS regions of the human genome to a level expected for random integrations. The concept of integrase structure perturbation can be applied to enhance the safety of lentiviral vectors that have strong integration preference for intergenic regions. Results from preliminary trials indicate that insertion of DNA-binding domains into a few internal positions of the integrase can significantly reduce HIV-1-based vector integrations into genes (Y.Y. and K. Lim, unpublished data). Several decades of efforts in the gene therapy field have resulted in the first approved commercial virus-based gene therapy drugs, Glybera® and Strimvelis®. Better control of the safety of retroviral vectors by molecular engineering as shown in this study will allow the production of more effective gene therapy drugs in the near future.

MATERIALS AND METHODS
Construction of Plasmids Encoding Gag-Pol Mutant Proteins
Two to five zinc-finger units were introduced into an internal site (in front of the 274th amino acid residue) within the MLV integrase as part of the Gag-Pol polyprotein (accession numbers GenBank: J02255, J02256, and J02257) to construct 10 integrase mutant proteins (Figure 1). The DNA molecules encoding the zinc-finger units were amplified via PCR using Phusion High-Fidelity Polymerase (New England Biolabs [NEB], Ipswich, MA, USA). Two plasmids that harbor sequences encoding two zinc-finger complexes, each composed of multiple finger units, were used as PCR templates. The amplified DNA molecules were introduced into the sequence encoding the MLV integrase within the pCMV Gag-Pol plasmid.

Prediction of RNA Secondary Structures
RNA secondary structures of the partial 5′ UTR (from R to splice donor site) for wild-type and PBS mutants. The wild-type primer-binding site sequence, newly added primer-binding site sequences, and splice donor site are shown in green, red, and purple, respectively, PBS, primer-binding site.
were introduced into HEK293T cells grown in 10-cm dishes via the calcium phosphate-based transfection method. The cell supernatant containing packaged viral particles was harvested twice (1.5 and 2.5 days post-transfection). The harvested supernatant was first filtered through a 0.45-μm syringe filter and then concentrated in a 20% (w/v) sucrose cushion by ultracentrifugation (Optima Ultracentrifuge LE-80K; Beckman Coulter, Brea, CA, USA) using an SW28 rotor at 4°C and 24,000 rpm for 2 hr or an SW41 rotor at 4°C and 25,000 rpm for 1.5 hr. The pellet, containing the virus particles, was resuspended with cooled PBS. Cell supernatant-containing viral particles were also used directly, without concentration.

Titration of Retroviral Transducing Particles
All of the vector genomes (encoded by pCLPIT GFP or modified versions of pCLPIT GFP with mutated primer-binding site sequences) carried the gene encoding EGFP as a reporter gene. Titration of transducing viral particles was performed by transducing HEK293T cells and counting the EGFP-positive cells. Expression of EGFP in cells was measured by flow cytometry on a FACSCanto II flow cytometer (BD Biosciences, San Diego, CA, USA) 7 days post-transduction.

Host-Viral Genome Junction Cloning
HEK293T cells were transduced with wild-type and mutant retroviral vectors at an MOI of less than 1. Transduced cells were expanded for several days and then genomic DNA was isolated from these cells using the QIAamp DNA Mini kit (QIAGEN, Valencia, CA, USA). The genomic DNA was first fragmented with the endonuclease BamHI (NEB) and then linearly amplified by PCR with Taq DNA polymerase (NEB) and a single biotinylated oligonucleotide that binds to the MLV 3′LTR region (5′-biotin-ATTGTGTTAAAGACAG -3′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGAGGGTCTTCTGGGGCTCGG-3′). The thermal cycling program was as follows: initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 45 s, and extension at 72°C for 2 min, final extension at 72°C for 10 min, and cooling at 4°C for 5 min (C1000 thermal cycler; Bio-Rad, Hercules, CA, USA).

After PCR amplification, the product containing the viral sequence was selectively isolated using Dynabeads M-280 Streptavidin (Thermo Fisher Scientific, Carlsbad, CA, USA). To fill the single-stranded region of the isolated DNA product to produce double-stranded (ds)DNA, we conducted an additional DNA synthesis reaction with random hexamer, dNTP, and Klenow enzyme. The dsDNA product was then digested with MfeI (NEB), linker DNA molecules were pre-assembled with two oligos (linker+: 5′-GAATACAGCTACTATAGGGCTCCGCTTAAGGGCAG-3′, linker−: 5′-TAGTCCCTAAAGCCGAG-NH2-3′). The obtained host-viral genome junctions were amplified by PCR with Phusion High-Fidelity Polymerase (NEB) and primers that bind to the viral 3′ LTR or linker (forward, 5′-biotin-GAATTCGTCCTGCTGTTCCTTGG-3′, and reverse, 5′-GAATACAGCTACTATAGGGCTCCGCTTAAGGGCAG-3′). The thermal cycles were as follows: initial denaturation at 98°C for 2 min, 25 cycles of denaturation at 98°C for 2 min, annealing at 55°C for 90 s, and extension at 72°C for 1 min, final extension at 72°C for 5 min, and cooling at 10°C for 3 min.

NGS of Genome Junctions
The PCR products were analyzed by Illumina NGS. Samples were preporecessed using two consecutive PCRs to add adaptor and index sequences using Phusion High-Fidelity Polymerase (NEB) and the following primers: forward primer for adaptor addition: 5′-TCGTGGGCAAGCCTCTAGATGTGATTGACTACCCGTCAGCGG-3′; reverse primer for adaptor addition: 5′-GTCTCCTGTTGGGTCGGAGATGTGTATAAGAGACAGAC TCACTATAGGGCTCCGCTTAAGGGCAG-3′; forward primer for index addition: 5′-AATGATACGGCGACCAACCGATCTACACFFFFFFFTCTCGCGAGCCTGCTC-3′; reverse primer for index addition: 5′-CAAGCAGAAGACCGCATAGAGATRRRRRTGTCCTGTTGCCG-3′, where “FFFFFFF” and “RRRRRRR” are the index sequences.

The thermal cycling condition for adaptor addition was as follows: initial denaturation at 98°C for 2 min, 25 cycles of denaturation at 98°C for 2 min, annealing and extension as a single step at 72°C for 90 s, final extension at 72°C for 5 min, and cooling at 10°C for 3 min. The thermal cycling condition for index addition was as follows: initial denaturation at 98°C for 2 min, 8 cycles of denaturation at 98°C for 12 s, annealing and extension as a single step at 72°C for 90 s, final extension at 72°C for 5 min, and cooling at 10°C for 3 min.

Sequencing using Illumina MiSeq and raw read processing were carried out by Macrogen, a sequencing service provider. To consider only high-quality sequence data, reads with a mean quality score below 20 were filtered out with PRINSEQ-lite (v 0.20.4). Only host-virus genome junction reads containing the viral 3′ LTR sequence (5′-GGAGGGTGTCCTCTGAGTAGTATGACTCAAGCCTACGGGGGTCTTTCA-3′, 48 bp) were captured with total 2-bp mismatch
allowance using EMBOSS Needle (v 6.6.0.0) and in-house scripts for downstream analysis. Redundant sequences were also removed during this process. Host sequences from junction reads were mapped to the human genome using the QuickMap tool (Gene Therapy Safety Group”).

SUPPLEMENTAL INFORMATION
Supplemental Information includes four tables and nine figures and can be found with this article online at https://doi.org/10.1016/j.ommen.2018.11.001.

AUTHOR CONTRIBUTIONS
J.-S.N., J.-E.L., K.-H.L., and K.-I.L. designed the experiments; J.-S.N., J.-E.L., and Y.Y. conducted the experiments; all of the authors were involved in analyzing the experimental data; J.-E.L., Y.Y., and K.-I.L. wrote the paper.

CONFLICTS OF INTEREST
The authors declare no competing interests.

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