INTRODUCTION
The ability to integrate stably within target cells has been a distinct advantage of retroviral and lentiviral vectors. However, these vectors have been associated with a risk of insertional mutagenesis.1-3 To address this concern, non-integrating lentiviral vectors (NILV) have been developed for applications where integration is not required.4-7 Such vectors would be most suitable for applications when transient expression of the transgene is desired,8,9 transducing non-proliferating cells,5,10-12 or as donor templates for use in zinc finger nucleases and transcription activator-like effector nucleases.13-16

HIV-1 primarily relies upon integrase (IN) to catalyze the integration of viral DNA into the target cell DNA. The reaction involves an episomal template along with three well-defined catalytic steps: 3′-end processing, DNA strand transfer and a disintegration reaction. This process requires a complete reverse transcription (RT) reaction, an intact IN molecule and available U3 and U5 attachment sites for IN within the viral long terminal repeats (LTRs).17-19 HIV-1-infected cells are known to contain integrated provirus, unintegrated linear virus DNA as well as 1-LTR or 2-LTR circular forms. The 1- and 2-LTR circles arise through homologous recombination or non-homologous end-joining (NHEJ), respectively.20-22 In this paper, insertions of vector DNA into a cell genome by non-IN-mediated methods are referred to as illegitimate integrations.

IN mutations at the highly conserved amino-acid positions D64, D116 and E152 disable the catalytic activity of IN by interfering with 3′-end processing, DNA strand transfer activities and disintegration.23 NILV generated using these mutant IN proteins are both infectious, express the vector transcript, and are associated with a marked reduction in vector integration.4-6,10,24-27 A second approach is to induce aberrant RT by deletion of the 3′ polypurine tract (PPT) within the vector backbone.11 A third, less-well studied approach, is altering the LTR attachment (att) sites by mutagenesis of the CA dinucleotide recognition sequence within the U3 and U5 att sites; these mutations have been shown to inhibit integration while retaining infectivity at ~40 and 10% of wild-type virus, respectively.18

In the current study, a novel non-integrating vector with an ablation of the LTRs entire 12 base-pair U3att site was evaluated, as compared with IN catalytic core mutants, in order to inhibit integration while reducing the risk of reversion mutations. How different mutations alter the processing of vector transcripts, as well as their effect on NHEJ and homologous recombination, was also evaluated. Finally, the rate of illegitimate integration and vector expression was assessed for IN, LTR att and PPT mutations alone or in combination.

RESULTS
Evaluation of IN and LTR attachment site mutant NILV
Three novel NILV were generated; one with an 11 base-pair deletion within the U5 region of the 5′ LTR att site, a second with a 12 base-pair deletion within the U3 region of the 3′ LTR att site, and a third containing an extended mutation in the C-terminus of IN (Figure 1a and b). The novel vectors were compared with previously reported point mutations in IN.23,24,28,29 Although the mutations created were not predicted to significantly alter virion
This was tested experimentally by determining the physical titer (p24) of the vector. As shown in Figure 1c and d, the level of p24 in vector supernatants from mutant vectors was similar to that measured from integration-competent vectors.

To determine whether the mutants were infectious, lentiviral vectors containing eGFP were generated and used to transduce HEK293 cells. As shown in Figure 2a and b, deletions within the C-terminus of IN and at the US-LTR att site did not provide significant gene transfer, whereas the IN mutants IN/D116N and IN/E152V and the novel U3-LTR IN attachment site deletion (U3-LTR att site) mutant demonstrated similar vector expression in HEK293 cells. Based upon these data, subsequent studies utilized these latter three constructs.

In proliferating cell cultures, the average vector copy per cell from non-integrating vectors decreases with time. Therefore, determining infectious titer by traditional titer methods is problematic. To assess whether physical titer (as measured by p24 ELISA) reflects infectious titer, the amount of vector was normalized using p24, then vector DNA was measured four hours after transduction. As shown in Figure 2c, physical titer correlated with vector copy number. Based on this finding, p24 levels were used to normalize the amount of vector product in subsequent experiments.

**Gene expression with non-integrating vectors**

To evaluate transgene expression with non-integrating vectors, firefly luciferase expression was assessed in HEK293 cells. Interestingly, after 3 days the LTR/U3 vector demonstrated higher transgene expression than the IN mutants relative to IN competent vector (Figure 3). In addition, GFP expression was assessed in transduced HEK293 cells over a 21-day period.
was not due to residual GFP protein alone; GFP mRNA was still detectable 21 days after transduction (Figure 4c). Similar to the observations with luciferase, the LTR/U3del vector provided higher and more persistent GFP expression than the IN mutants (Figure 4a) despite similar levels of vector DNA (Figure 4b).

Frequency of illegitimate integration with non-integrating vectors
The disparities observed with the varied levels of transgene expression suggested that there may also be significant variation in the frequency of illegitimate integration by NILV. To determine the frequency of integration, a colony-formation assay was performed using vectors containing an antibiotic resistance transgene and drug selection of transduced HEK293 cells. As shown in Figure 5, the non-integrating vectors significantly reduced the frequency of illegitimate integration at least 2 logs below that of an integration-competent vector. LTR/U3del integrated at a significantly higher frequency than the IN mutant IN/D116N, but the difference is smaller than predicted when considering the differences in transgene expression (Figures 3 and 4a). Combining the IN and U3-LTR att site deletion mutations resulted in a significantly reduced frequency of integration as compared with either mutation independently.

Insertion site analysis of non-integrating vectors
To further evaluate vector integration and expression, vector insertion sites were evaluated. The vector-genome junctions were amplified, sequenced and analyzed for the canonical features associated with IN-mediated insertion as well as other means of integration, including NHEJ and/or homologous recombination. The results of this analysis are depicted in Figure 6. As expected, the integration-competent control vectors insertions demonstrated a 5-bp repeat of genomic DNA flanking the vector and end processing of the IN attachment sites as indicated by the presence of terminal CA dinucleotides. IN mutant vectors (IN/D116N) lack these findings and demonstrate insertions and/or deletions at the vector-genome junctions. Interestingly, LTR/U3del vectors demonstrate insertions and deletions at the 5′ LTR vector-genome junction but display the canonical features associated with IN-mediated insertion at the 3′ LTR. Combining the LTR deletion with the IN mutation was able to recapitulate the insertions or deletions observed with IN/D116N at both LTR junctions, indicative of non-IN-mediated insertions.

The distribution of circular vector DNA transcripts among cells transduced with IN and LTR attachment site mutant vectors
To further evaluate NILV processing, the relative distribution of unintegrated vector was determined by Southern blot analysis of low molecular weight DNA (Figure 7a). Three days after transduction, circular forms were the predominant episomal form for wild-type, IN mutant and LTR att site mutant vectors (Figure 7a). Although 1-LTR circles were detected among all three populations of transduced cells, IN mutants have equal or greater number of 2-LTR circles in comparison with 1-LTR circles. In support of this finding, quantitative PCR analysis found that IN mutant vectors have a fourfold increase in 2-LTR circles compared with the LTR/U3att vector (Figure 7b).

To assess the relative amount of 1-LTR circles, standard PCR was utilized as quantitative PCR methods cannot distinguish 1-LTR circles and linear transcripts. Three days after transduction, 1-LTR circles could be detected in cells transduced with wild-type, IN mutant and LTR att site mutant vectors (Figure 7c). However, 1-LTR circles persisted during the 21-day observation period in cells transduced with wild-type and LTR/U3del vectors but not in the IN mutants.

As would be predicted for NILV in dividing cells, the percentage of GFP-expressing cells and the vector DNA content declined overtime (Figure 4a and b). LTR/U3del-transduced cells maintained a level of gene transfer that was ~20% of integration-competent vector at 21 days, whereas expression from IN mutants was ~5% of integration-competent vector. Fluorescence at day 21

**Figure 3.** Luciferase expression from transduced HEK293 cells. Firefly luciferase activity (relative light units, RLU) was measured 3 days after transduction. Luciferase activity from cells transduced with IN and LTR att site mutant vectors is shown relative to that achieved from integration-competent vector (IN+/LTR+). Data are represented as mean ± s.d.

**Figure 4.** GFP expression from transduced HEK293 cells. (a) The percentage of GFP expressing (% GFP+) cells 3–21 days after transduction. GFP expression was determined at 3, 9, 15 and 21 days by flow cytometry. Data are represented as mean ± s.d. (b) Total vector DNA copies estimated per GFP-expressing (GFP+) cell. Total vector DNA copy number was determined by quantitative PCR 3, 9, 15 and 21 days after transduction. Data are represented as mean ± s.d. (c) GFP mRNA expression from vector DNA 21 days after transduction, as determined by RT-PCR.
The fate of episomal vector DNA in cells deficient for NHEJ process. As 2-LTR circle formation is reported to involve NHEJ, wild-type and DNA-PKcs-deficient MO59J human glioma cell lines were used to evaluate the distribution of unintegrated vector DNA. DNA-PKcs is a necessary component of several DNA damage repair pathways, including NHEJ. As determined by Southern blot analysis and quantitative PCR, wild-type MO59K cells transduced with the LTR/U3del vector had less 2-LTR circles than wild-type and IN/D116N vectors (Figure 8a and b), with a relative distribution similar to that observed in HEK293 cells (Figure 7a and b). In contrast, 2-LTR circles did not predominate in DNA-PKcs-deficient cells regardless of vector type (Figure 8a and b). This suggested that 2-LTR circles in cells transduced with IN mutant vectors are formed, at least in part, by DNA-PKcs-dependent NHEJ activities.

Optimizing NILV vectors for expression while minimizing illegitimate integration

In order to identify clinically useful NILV designs, the integration frequency of various combinations of NILV mutations were compared. As Kantor et al. have shown, there is decreased integration with a deletion in the 3′ PPT. We evaluated pairing LTR/U3att with the 3′ PPT deletion with and without IN/D116N. Similar to results reported by Kantor et al. and Tareen et al., pairing ΔPPT with IN/D116N provided a modest reduction in integration as compared with either mutation individually (Figure 9), indicating that each modification works independently to inhibit integration. Combining the ΔPPT modification with LTR/U3att did not alter the frequency of integration compared with either modification alone. In contrast, combining the IN/D116N mutation with LTR/U3att did decrease integration, suggesting that the mutations work independently. Combining all three mutations (ΔPPT, IN/D116N and LTR/U3att) did not offer any additional improvement to integration frequency reduction.

NILV have also been associated with significantly reduced levels of transgene expression relative to integration-competent vectors. The vectors studied here (ΔPPT, IN/D116N and combined ΔPPT/IN/D116N) show similar levels of expression (Figure 10) consistent with previous reports. Pairing the LTR/U3att and ΔPPT mutations did not affect expression. However, pairing the

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**Figure 5.** Frequency of integration in transduced HEK293 cells. Average antibiotic resistant colony-formation titer assay results from two independent experiments comparing the frequency of integration among an integration-competent vector (wt), a U3-LTR integrase attachment site mutant NILV (LTR/U3att), an integrase-deficient NILV (IN/D116N) and a double mutant NILV (LTR/U3att-IN/D116N). Y axis represents infectious units/ml of vector supernatant. Error bars indicate s.d. of the mean. *P < 0.05; **P < 0.005.

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**Figure 6.** Vector-genome junctions of transduced HEK293 clones. Summary of results obtained from sequencing of Zeocin selected clones transduced with a normal integrating vector (CS-CZW), an integrase catalytic core mutant NILV (IN/D116N), a U3-LTR integrase attachment site deleted NILV (LTR/U3att) and a double mutant (LTR/U3att-IN/D116N). Red highlighted base-pairs indicate flanking 5-bp repeat of genomic DNA by integrase-mediated strand transfer; underlined TG/CA dinucleotides represent end processing by a functional integrase protein; inverted black triangles represent LTR truncations; red highlighted triangle represents deletions of genomic DNA; black squares indicate insertions at the vector-genome junction.
IN/D116N mutation with LTR/U3att modification resulted in a significant decrease in vector expression (Figure 10).

We created a vector containing the ΔPPT, LTR/U3att and IN/D116N mutations to investigate if transgene expression is affected. The triple mutant vector produced levels of transgene expression similar to those observed with all vectors containing the IN/D116N mutation. These findings suggest that the decreased illegitimate integration associated with IN/D116N is at the expense of expression.

DISCUSSION

The goal of this study was to evaluate a novel LTR att site mutation for transgene expression and illegitimate integration. The 12 base-pair deletion in this novel LTR/U3del vector is larger than the point mutants described by Nightingale et al.\(^6\) in order to decrease the chances of reversion mutations. Overall the LTR/U3del had higher and more persistent gene expression compared with IN mutations, but also was associated with a higher rate of illegitimate integration. Differences in processing appeared to contribute to the differences observed in vector expression and integration frequency. A novel mutant consisting of an 11 base-pair deletion of the US-LTR att site was also evaluated that generated vector particles but failed to provide significant expression in transduced cells. This mutation was likely unsuccessful owing to defective RT, attributed to overlap of the US-LTR att site and the RT primer binding site necessary for first strand synthesis.\(^{33-35}\)

The novel LTR/U3att vector was compared with the previously published IN mutation IN/D116N.\(^5,6,26,27\) Vector integration frequency was similar to that reported by Yanez-Munoz et al.\(^6\) using a D64 IN mutant (reversion rate of 1 in 815) but there was a modest 2.7-fold increase in integrations with the LTR/U3att vector versus the IN/D116N. Interestingly, combining the LTR and IN mutations significantly reduced the frequency of integration as compared with either mutation independently, in contrast to previous studies combining IN and LTR att site point mutations.\(^4,10\) The previous LTR/U3att mutations were point mutation and it is possible the relatively large deletion used in our study provided for this difference by reducing the chance of any latent binding of IN to the attachment site.

Insertion site analysis revealed non-IN-mediated insertion at the ablated U3att site of the 3′ LTR-genome junction (Figure 6), similar to what has been observed with IN mutants.\(^4,36-38\) However the intact US att site at the 5′ LTR-genome junction displayed features associated with IN-mediated insertion, consistent with the ability of intact IN to interact with each attachment site independently.\(^18\) This difference in processing LTR/U3att mutants presumably contributes to the increased illegitimate integration observed.

Interestingly, the modest increase in illegitimate integration rates between the mutations were not consistent with the larger differences in vector expression (Figures 4 and 5). To investigate this further, we evaluated the processing of non-integrated vector DNA. Despite similar levels of vector DNA, more GFP-expressing cells were detected upon transduction with LTR/U3del than IN mutants. This observation was associated with differences in the ratio of 1-LTR and 2-LTR vector forms. At the point of maximal
transgene expression, IN/D116N and IN/E152V-transduced cells contained nearly four-to-five times as many copies of 2-LTR circular vector DNA relative to LTR/U3del-transduced cells (Figure 7b). In contrast, 1-LTR circles were the predominate form in cells transduced with LTR/U3del vector (Figure 7a). Given that LTR/U3del maintains higher transgene expression despite similar total vector DNA, further work is warranted to determine whether 1-LTR circles facilitate higher and/or more persistent gene expression than 2-LTR circles. Possible mechanisms meriting further investigation include improved trafficking to the nucleus and resistance to degradation. The presence of a functional IN protein allows for its binding to the wild-type U5-LTR att site for nuclear localization, while providing steric hindrance protecting the viral DNA ends from exonuclease activity. The predominance of 1-LTR episomes can also improve vector viability. 2-LTR circles are derived from linear episomes as products of NHEJ of the viral DNAs 5′ and 3′ ends. Linear episomes are readily degraded by exonuclease activity, which could lend to reduced transgene expression, whereas circular DNA is protected from exonuclease activity. Formation of 2-LTR circles is believed to be dependent upon NHEJ-double strand break DNA repair mechanisms, such as Ku-80, XRCC4, DNA ligase 4 and DNA-PKcs. NILV transduction of DNA-PKcs-deficient cells suggests that a U3-LTR att site deletion may negatively influence NHEJ-mediated vector DNA circularization thereby limiting the number of 2-LTR circles (Figure 8). Another explanation could be due to the proximity of the U3-LTR att site deletion, which is immediately adjacent to the vectors 3′ PPT. The PPT acts as a primer site for second strand synthesis during RT and Kantor et al. have shown that deletion of this element results in aberrant RT and the preferential production of 1-LTR circles. Deletion of the juxtaposed U3 attachment site may also affect

Figure 9. The frequency of integration in NILV with combined modifications for inhibiting integration. Average bleomycin resistant colony-formation assay results from two experiments comparing the frequency of integration among combined mutations to LV design. The horizontal axis designates the independent modification or combining it with the indicated mutation(s). CS-CZW—an integration-competent vector; ΔPPT—an NILV with a deletion of the 3′ polypurine tract; IN/D116N—an integrase-deficient NILV; LTR/U3att—a U3-LTR integrase attachment site mutant NILV. Data are represented as mean ± s.d.

Figure 10. The effect of combining modifications to reduce integration frequency on transgene expression. Average flow cytometry results for GFP expression from two experiments comparing the levels among combined modifications to lentiviral vector design. CS-CGW—normal integrating vector (wild-type); ΔPPT—NILV with a deletion of the 3′ polypurine tract; IN/D116N—an integrase-deficient NILV; LTR/U3att—a U3-LTR integrase attachment site mutant NILV. Data are represented as mean ± s.d.
RT in a similar fashion. The results of this study suggest that IN and LTR att site mutations may differentially influence the circularization of unintegrated vector DNA.

It was observed that combining the IN and LTR mutations led to an improved reduction in integration frequency as compared with either mutation independently (Figure 5). As NILV have been generated by deleting the 3` PPT, we evaluated this mutation in combination with IN and LTR/Usatt mutations. Combining ΔPPT with IN/D116N provided between a two- and threefold reduction in integration (Figure 9), similar to previous observations when combined with a D64E IN mutation. Interestingly, combining the ΔPPT and LTR/Usatt mutation provided little or no reduction in integration frequency (Figure 9). This suggests they evoke similar alterations in the processing of episomal DNA.

Combining ΔPPT and LTR/Usatt had no effect on transgene expression as compared with either mutation alone, however, combining ΔPPT, LTR/Usatt or both together with IN/D116N resulted in a significant reduction in transgene expression (Figure 9). The results show a trend of reduced transgene expression when the vector contains the IN mutation (Figure 10). Hence, many of the vector combinations with the lowest frequencies of integration also demonstrated the lowest levels of transgene expression (Figures 9 and 10). Therefore, design of NILV for clinical applications should balance the risk of insertional mutagenesis and the level of vector expression. The risk of insertional mutagenesis with NILV is dependent on both vector integration rates and vector dose; if a vector with low risk of insertional mutagenesis with NILV is dependent on both levels of transgene expression (Figures 9 and 10). Therefore, lowest frequencies of integration also demonstrated the lowest combining expression as compared with either mutation alone, however, may be defective in DNA repair pathways.

This study suggests that the type of mutation used to generate NILV can influence cellular processing of vector transcripts. Such data will be important when attempting to design non-integrating vectors that maximize vector expression with low frequencies of integration and when considering transduction of target cells that may be defective in DNA repair pathways.

MATERIALS AND METHODS

Cell culture and reagents

HEK293 and HEK293T cells were purchased from American Type Culture Collection (Manassas, VA, USA). MOS9J and MOS9K cell lines were kindly provided by D Gilley (Indiana University School of Medicine, Indianapolis, IN, USA). All cell lines were maintained in DMEM (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO2.

Vector plasmids consisted of the eGFP expressing pCDNA-CS-CGW (kindly provided by P Zoltick, Children’s Hospital of Philadelphia, Philadelphia, PA, USA), the gag-pol expressing pMDL, the VSV-G envelope expressing pMDG, and the rev expressing pRSV-rev plasmid (all kindly provided by Cell Genesys, South San Francisco, CA, USA).

Construction of IN and LTR attachment site mutant vector plasmids

For construction of the IN deletion mutant IN/D174-288, digestion of the pMDL plasmid with BglII and subsequent base fill-in created an early stop codon within the IN coding sequence. Two IN missense mutants, IN/D116N and IN/E152V, were generated by site-directed mutagenesis of pMDL. Site-directed mutagenesis was performed using a Quik Change II XL Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX, USA) according to the manufacturer’s specifications.

For generation of LTR/Usdel, the 3’-LTR from the pcDNA-CS-CGW was subcloned into pZero2 (Invitrogen) at an upstream Asp718 and a downstream ApaI restriction sites. Site-directed mutagenesis was performed on the resulting plasmid, allowing for the deletion of the twelve base-pair IN attachment site. For generation of LTR/Usdel, the 5’-LTR from pcDNA-CS-CGW was subcloned into pcDNA3 (Invitrogen) at an upstream SspI restriction site and a downstream NcoI restriction site. Site-directed mutagenesis was performed deleting the 11 base-pair IN attachment site. Incorporation of a bleomycin resistance transgene in lieu of eGFP was accomplished by insertion using unique AgeI and XhoI restriction enzyme sites. A deletion of the transfer plasmids ΔPPT was incorporated by standard restriction site insertion of the modified region synthesized by GeneArt (Life Technologies, Grand Island, NY, USA). The mutations were confirmed by DNA sequencing.

Vector production and measurement of physical and infectious titer

VSV-G pseudotyped vector particles were produced by a four-plasmid calcium phosphate transient transfection method described previously. All vector supernatants were treated with Benzonase (Novagen, San Diego, CA, USA) to remove residual placcGCGGCAATTCGTA previously described. Triplicates of three dilutions (10⁻³, 10⁻⁴ and 10⁻⁵) of supernatant were analyzed for p24 gag capsid protein production using a p24 gag ELISA kit (Beckman Coulter, Fullerton, CA, USA). The infectious titer of integration-competent vectors was determined in HEK293 cells by a method described previously.

Transduction of cell lines and assessment of GPF or LUC expression

HEK293 cells, seeded at 10⁵ cells per well of a six-well plate or 3 x 10⁵ cells per T25 flask 24 h prior to transduction, were incubated with vector in the presence of 8 μg ml⁻¹ of polybrene for 4 h at 37°C. Transductions, performed in duplicate, utilized vector normalized for p24 (325 ng of p24 corresponding to an estimated multiplicity of infection of five). GFP expression was assessed using a FACScan cytometer and CellQuest analysis software (Becton-Dickinson, San Jose, CA, USA). For measurement of LUC expression, cell lysates were prepared from transduced cells, upon which 10 μg of protein per sample was added per well of a 96-well plate. LUC activity was measured as light intensity upon the addition of the LUC substrate, luciferin (Luciferase Assay System, Promega, Madison, WI, USA). MOS9J and MOS9K cells, seeded at 3 x 10⁵ cells per 100 mm dish 24 h prior to transduction and transduced with vector supernatant containing 2000 ng of p24.

Measurement of integration frequency by antibiotic resistant colony-forming assay

Vectors containing a bleomycin resistance transgene were generated for measurement of integration frequency as described above. HEK293 cells seeded at 10⁵ cells per well, were transduced with vector supernatant normalized by p24 content with 10 replicates per vector. The selection reagent Zeocin (Life Technologies; Carlsbad, CA, USA), at a concentration of 500 μg ml⁻¹, was added to the media 48 h post transduction. The cells were incubated in selection media for 21 days post transduction and the frequencies of integration (demonstrated by infectious titer) were determined by multiplying the number of viable colonies by their respective dilution factor.

Amplification of vector-genome junctions

Illegitimate integration was quantified by isolating drug-resistant clones after transducing HEK293 cells with a Blomycin resistance transgene. Transduced cells were selected for three weeks prior to isolation, after which genomic DNA was extracted using the Puregene DNA Purification Kit (Gentrex Systems, Inc., Minneapolis, MN, USA). The vector-genome junctions were then amplified by linear amplification-mediated PCR (LAM-PCR) off of the 3` LTR as previously described in two separate experiments (CS-CZW n = 12; IN/D116N n = 7; 12; LTR/Usatt n = 8, 11; LTR/Usatt-IN/D116N n = 4, 9) and/or by a modified technique adapted from Ravin et al.40 and Zhou et al. (PPT-IN/D116N n = 12; LTR/Usatt n = 18; LTR/Usatt-IN/D116N n = 22). In brief, to perform the modified technique for surveying insertion sites, the genomic DNA was first sheared to an approximate size of 1000 bp using Covaris sonication (Covaris, Woburn, MA). Starting with 1000 ng of sheared fragments, the ends were repaired using the NEBNext End Repair Module (New England Biolabs, Ipswich, MA) and 3’ a-tailed with the NEBNext a-Tailing Module (New England Biolabs). Products > 300 bp were selected and purified using a 0.8:1 ratio of AMPure XP beads (Beckman Coulter, Brea, CA, USA) to a-tailed product. A partially double stranded linker cassette with a 5’-overhang (LamTinkerL: 5’GACCCGGGAGATCTGAATTCAGTGGCACAG CAGTAGTTG-3’; LamTinkerR: 5’CTAATCTGTGGCCACT3’) was then ligated to the ends of the purified products using the Fast-Link DNA Ligation Kit (Epigenetix, Madison, WI, USA). Exponential PCR was performed using the ligation product as a template with a biotinylated vector-specific

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primer for the 5′ or 3′ end of the vector and a linker-specific primer (LentILAM-LC1: 5′-GACCCGGAGATCTGAATTIC-3′). Vector-specific PCR products were then enriched using Streptavidin-coated M-280 Dynabeads (Invitrogen). Next, nested PCR using a second indexed LTR-specific primer for the 5′ or 3′ end and another linker-specific primer (LentILAM-LC2: 5′-AGTGCCACAGCGTAGG-3′) was performed using the enriched product as the template. The final PCR products were then purified using a 1:8.1 ratio of AMPure XP beads and quantified using a Qubit Fluorometer (Life Technologies).

In order to confirm the insertion sites when LAM-PCR was performed solely off the 3′ LTR, conventional PCR was performed in order to amplify the 5′ vector-genome junction using the insertion site predicted by the 3′ LAM-PCR results.

Vector insertion site analysis
Samples from the first LAM-PCR experimental group were analyzed by pyrosequencing (Roche 454 FLX Titanium) at the Indiana University Center for Genomics and Bioinformatics, Bloomington, IN, USA and analyzed using the SeqMap 2.0 web server platform. Subsequent samples were analyzed using the illumina MiSeq platform at the Genomics Core Facility of the University of Notre Dame. Paired-end reads of 250 base-pair were generated and in-house scripts were used to de-multiplex the reads and to retain paired-end reads that have both perfectly matched viral vector-specific primers and linker primer used in the last round of PCR. MiSeq adapter and linker sequences were trimmed off with Cutadapt version 1.5. Next, paired-end reads were merged with PEAR version 0.9.5-64. For the non-overlapping paired-end reads, the reads containing the viral vector sequence were kept. The viral vector sequences in the merged reads were located with cross-match version 9909329 (http://www.phrap.org). Reads with <30 base-pair viral vector sequence and <10 base-pair remaining genomic sequence were discarded. The remaining reads were further clustered with usearch7.0.1090_i86linux32. Representative reads of each cluster were mapped to human reference genome hg19 using bwa mem. The viral vector-genome junction using the insertion site predicted by the SeqMap 2.0 web server platform.

Non-integrating lentiviral vector processing
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Detection of vector DNA by PCR
Vector and 2-LTR circular DNA was detected using quantitative PCR assay based on a method previously described by Butler et al. Total cellular DNA was prepared using a Puregene DNA Puriﬁcation Kit (Genta Systems, Inc., Minneapolis, MN, USA), from which a 250 or 500 ng sample was used for each reaction. To determine total, integrated and 2-LTR circular vector DNA copy number, standard curves were generated by preparing serial dilutions of pcDNA-CS-CGW, genomic DNA from wild-type vector transduced HEK293 cells, or a 2-LTR circle reference plasmid that was generated upon the introduction of a 2-LTR fragment into a cloning vector, pCR4-TOPO (Invitrogen), respectively. Each sample was run in duplicate and data analysis was performed using Sequence Detection Systems 1.9.1 software (Applied Biosystems, Foster City, CA, USA).

A 250 ng sample of total cellular DNA from transduced HEK293 cells at 3 days post transduction was used for detection of 1-LTR circular vector DNA by standard PCR. 1-LTR circles were amplified using the following primers: 5′-GACCCGGAGATCTGAATTIC-3′ (forward) and 5′-GACCCGGAGATCTGAATTIC-3′ (reverse). The cycling conditions were 95 °C for 10 min, 30 cycles of 92 °C for 1 min, 56 °C for 2 min, 72 °C for 1.5 min and 72 °C for 7 min.

Detection of unintegrated vector DNA Southern blot analysis
Low molecular weight (Hirt) DNA (5–10 μg) purified from wild-type and mutant vector transduced HEK293, MO59J and MO59K cells 3 days post transduction, was digested overnight with BamHI and separated on an agarose gel. In brief, the Hirt DNA extraction protocol was as follows. Transduced cells were resuspended in extraction buffer (0.6% SDS, 0.01 M EDTA and 0.01 m Tris) and 5 M NaCl overnight at 4 °C. The supernatant was collected and the DNA was isolated by phenol-chloroform extraction. Upon transfer to a nylon membrane, the DNA was probed for GFP using a P-labeled eGFP probe prepared with a NEBlot Kit (New England Biolabs).

Detection of GFP mRNA by RT-PCR
Total cellular RNA was prepared from transduced HEK293 cells using a Qiagen RNeasy Micro Kit (Valencia, CA, USA). For first strand complementary DNA synthesis, 200 ng of RNA was used in a RT reaction which included 0.5 μg of oligo(dT)12–18 primer and 2.5 units of HIV-1 reverse transcriptase (Ambion, Austin, TX, USA). The reaction was incubated at 48 °C for 30 min. About 5 μl of complementary DNA was used in a subsequent PCR reaction for amplification of GFP using the following primers: 5′-TGACCTGAAAGCTTCAGACCA-3′ (forward) and 5′-TGACCTGAAAGCTTCAGACCA-3′ (reverse). The cycling conditions were 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and 72 °C for 7 min.

Statistical analysis
Quantitative data are represented as mean ± s.d. of the mean. Data were analyzed using a two-tailed student’s t-test or analysis of variance. Differences were considered statistically significant for P-values < 0.05.

CONFLICT OF INTEREST
Dr Cornetta is a consultant for Cook Regenetics but there are no collaborative or financial relationships with the work contained in this manuscript. Dr Shaw, Dr Joseph, Mrs Jasti, Dr Sastry and Dr Witting declare no potential conflict of interest.

ACKNOWLEDGEMENTS
We thank Teresa Johnson for her assistance with vector production, David Gilley who provided the MO59K and MO59J cell lines and Troy Hawkins and Hongyu Gao for assistance with bioinformatics analysis. The study was funded in part by a grant from the NHLBI (P40HL11621). GJ was supported by Edward T Harper Scholar—funded by NIH NIGMS on 1R2S GM079657-01, Indiana University Initiative for Maximizing Graduate Student Diversity. A5 was supported by the Joe and Shirley Christian Graduate Student Education Fund.

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