Hybrid Lentivirus-phiC31-int-NLS Vector Allows Site-Specific Recombination in Murine and Human Cells but Induces DNA Damage

Nicolas Grandchamp¹ 2 3, Dorothée Altémir 1 2, Stéphanie Philippe 1 2 3, Suzanna Ursulet 1 2 3, Hélôïse Pilet 1 2 3, Marie-Claude Serre 4, Aude Lenain 5, Che Serguera 6, Jacques Mallet 1, Chamsy Sarkis 1 2 6

1 Unit of Biotechnology and Biotherapy, Centre de recherche de l’Institut du Cerveau et de la Moelle Épinière, Pierre-and-Marie-Curie University/Institut National de la Santé et de la Recherche Médicale, Paris, France, 2 NewVectys, Villebon-sur-Yvette, France, 3 Biosource, Paris, France, 4 Laboratoire de Virologie Moléculaire et Structurale, Gif-sur-Yvette, France, 5 Commissariat à l’Énergie Atomique, Laboratoire de Radiobiologie et Oncologie, Fontenay-aux-Roses, France, 6 Molecular Imaging Research Center - Modélisation des biothérapies, Fontenay-aux-Roses, France

Abstract

Gene transfer allows transient or permanent genetic modifications of cells for experimental or therapeutic purposes. Gene delivery by HIV-derived lentiviral vector (LV) is highly effective but the risk of insertional mutagenesis is important and the random/uncontrollable integration of the DNA vector can deregulate the cell transcriptional activity. Non Integrative Lentiviral Vectors (NILVs) solve this issue in non-dividing cells, but they do not allow long term expression in dividing cells. In this context, obtaining stable expression while avoiding the problems inherent to unpredictable DNA vector integration requires the ability to control the integration site. One possibility is to use the integrase of phage phiC31 (phiC31-int) which catalyzes efficient site-specific recombination between the attP site in the phage genome and the chromosomal attB site of its Streptomyces host. Previous studies showed that phiC31-int is active in many eukaryotic cells, such as murine or human cells, and directs the integration of a DNA substrate into pseudo attP sites (pattP) which are homologous to the native attP site. In this study, we combined the efficiency of NILVs for gene delivery and the specificity of phiC31-int for DNA substrate integration to engineer a hybrid tool for gene transfer with the aim of allowing long term expression in dividing and non-dividing cells preventing genotoxicity. We demonstrated the feasibility to target NILV integration in human and murine pattP sites with a dual NILV vectors system: one which delivers phiC31-int, the other which constitute the substrate containing an attB site in its DNA sequence. These promising results are however alleviated by the occurrence of significant DNA damages. Further improvements are thus required to prevent chromosomal rearrangements for a therapeutic use of the system. However, its use as a tool for experimental applications such as transgenesis is already applicable.

Background

Gene transfer technologies are essential for genetics studies and gene therapies. However, major challenges remain to be addressed. A major issue is the lack of control over the site of DNA integration in the host genome which leads to unpredictable gene expression level and potentially undesirable mutagenesis of important cellular genes [1]. Recent strategies to tackle this challenge are relying on the use of genome editing tools such as ZFNs [2–7], TALENs [8–13] or more recently CRISPR-Cas system [14–17]. However, the vectorization of these tools into viral vectors to optimize their use ex vivo or in vivo raises several problems. Indeed, ZFNs function as dimers and generally require cotransduction of three vectors (one for each dinner and one for the recombining substrate) [18–20]. Moreover ZFNs may induce cellular toxicity due to off target activity [21–23]. TALENs have an important size with repeat domains hampering their vectorization [24]. CRISPR-Cas is a very recent tool and its vectorization has not yet been described. One may however expect its vectorization into viral vectors will be challenging as the system is based on the concomitant use of a chimeric DNA displaying hairpin structures [25] and of Caspase 9 which induces apoptosis when over-expressed [26]. These features will undoubtedly represent challenges for the vectorization of CRISPR-Cas into viral vectors for targeted integration.

Site-specific recombinases such as Cre [27–34] or FLP [35–38] of the tyrosine recombinases family are other genome editing tools more easily vectorizable and widely used for the purpose of site specific integration. However, the use of these recombinases is limited by the absence of endogenous recognition site in

Citation: Grandchamp N, Altémir D, Philippe S, Ursulet S, Pilet H, et al. (2014) Hybrid Lentivirus-phiC31-int-NLS Vector Allows Site-Specific Recombination in Murine and Human Cells but Induces DNA Damage. PLoS ONE 9(6): e99649. doi:10.1371/journal.pone.0099649

Editor: Yuntao Wu, George Mason University, United States of America

Received January 13, 2014; Accepted May 17, 2014; Published June 23, 2014

Copyright: © 2014 Grandchamp et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by grants from European FP6 (INTEGRA NEST-Adventure contract #29025), AFM (Association Française contre les Myopathies), and Rétea France. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Co-authors NG, DA, SP, SU and HP are employed by NewVectys SAS. NewVectys SAS provided support in the form of salaries for authors NG, DA, SP, SU and HP, but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the ‘author contributions’ section.

Competing Interests: NG, DA, SP, SU and HP are employed by NewVectys SAS. CS and JM own shares of NewVectys SAS. There are no patents, products in development or marketed products to declare. This does not alter the authors’ adherence to all the PLoS ONE policies on sharing data and materials.

* Email: chamsy.sarkis@newvectys.com
mammalian cells and by the bidirectionality of the recombination reaction they mediate. Within the superfamily of site-specific recombinases, phage integrases catalyse unidirectional recombination events [39]. Among these the PhiC31 phage integrase (phiC31-int), of the large serine recombinases family, is the most commonly used site-specific integrase for gene transfer purposes [40,41]. In its natural context, phiC31-int mediates efficient recombination between the phage attachment site (attP) and the bacterial attachment site (attB). The recombination of these two sites results in the unidirectional and site-specific integration of the phage genome into the bacterial chromosome (reviewed in [39]) leading to an integrated phage genome flanked by the recombinant attL (left) and attR (right) sites (Figure 1). When used for gene transfer into eukaryotic cells, phiC31-int can catalyse recombination of a plasmid containing an attB sequence into endogenous pseudo attP sites (pattP) displaying a high degree of homology with the wild type attP site [42]. Hence, associated with transfection techniques, phiC31-int has been successfully exploited to stably modify the genome into particular genomic sites of many types of eukaryotic cells in vitro [43–50] for transgenesis [48,51–55] and gene therapy applications [56–65]. The use of phiC31-int presents several advantages. First, the recombacin can be used to generate conservative recombination between attB and pseudo attP sites [42]. Second, Chalberg et al demonstrated that the majority of phiC31-int mediated recombination events in the human genome occur in intergenic regions [66]. However, in most of these studies the vectorization of phiC31-int relied on cotransfection (or nucleofection) of plasmids for both the delivery of phiC31-int and the transgene, thus limiting this technology to in vitro or ex vivo applications.

A strategy to increase the efficiency of DNA delivery in vivo is to use viral-derived vectors. As the expression of the genome editing tool must be transitory to avoid genotoxicity, it could therefore be delivered by a transient viral vector [67]. Even though phiC31-int has already been delivered by an adenoviral vector system [68,69] the use of such vectors is limited by their cell toxicity and immunogenicity [70–72]. In contrast, lentiviral vectors (LV) have the advantage to be non-immunogenic [73,74] and offers the possibility to be pseudotyped by different envelopes, allowing a high degree of flexibility regarding the tropism of the particles and the type of cell(s) they transduce (for review see Cronin J. et al [75]). Most importantly, it was shown that LV integrase can be modified to obtain non integrating lentiviral vectors (NILVs) [76–78], which act as episomal vectors. Hence, NILVs are vectors of choice to deliver genome editing tools and have been successfully used to deliver transposases [79,80], FLP [81] or ZFNs [82,83]. However, the use of NILVs has never been described for the vectorization of a serine recombinase.

In the present study, we combine the unidirectional site-specific recombination capability of phiC31-int with the efficiency of NILVs for gene transfer. For targeted integration, two different NILVs, one delivering the DNA sequence to be integrated and containing the natural attB site, the other expressing the phiC31-int are used. Through a step by step approach, we demonstrate for the first time that phiC31-int can be vectorized in NILV and we provide clues to further improve the system. However, analysis of integration events reveals that significant DNA damages can result from phiC31-int mediated recombination. In conclusion, the vectorization of serine recombinases in NILVs is feasible and constitutes a promising tool for basic research; however, one should remain cautious about the chromosomal aberrations that can be induced by these recombinases, particularly for clinical uses.

**Results and Discussion**

**NILV genomes can be used as a substrate for site-specific integration mediated by phiC31-int into human genome**

We first assessed the ability of a NILV DNA genome to be used as a substrate for phiC31-int. We therefore generated a Hela cell line constitutively expressing phiC31-int thanks to an integrative lentiviral vector expressing the phiC31-int under the control of the CMV promoter (LV-CMV-phiC31-int) to. Hela cells were transduced and clonal populations were isolated and analyzed by RT-PCR to estimate the phiC31-int expression level (Figure 2A). The clone Hi16 was selected for its robust constitutive expression of phiC31-int.

The ability of the constitutively expressed phiC31-int to mediate recombination between a NILV bearing an attB site with genomic pattP sites was then tested. Hi16 cells were transduced with a non-integrative lentiviral vector expressing the Neomycine (Neo) resistance gene under the control of the CMV promoter (NILV attB-CMV-Neo). After two weeks of G418 selection we obtained four cell clones which genomes were analyzed. Theoretically Neo integration is expected to arise from 3 distinct mechanisms: (i) phiC31-int-specific integration, (ii) residual integration mediated by a residual activity of the mutant HIV integrase (review about this field [84]) or (iii) illegitimate integration due to recombination of the episomal DNA molecule with the cellular genome by host cell mechanisms (Figure 3A). If the analysis is realized with clonal populations, these three different mechanisms should be discriminated by performing 2 PCR assays, one amplifying the LTR and the other amplifying the attB site. A positive LTR PCR reveals the presence of a LTR-LTR junction, indicating that the integration has occurred through a LTR-independent mechanism, either involving attB site-specific recombination or illegitimate recombination. In contrast, a positive attB PCR reveals that the attB site is intact, indicating an attB-independent integration, either involving LTR dependent (residual) integration or illegitimate recombination. In summary, cells are analyzed without knowing whether their genome contains one or several integration of NILV. A positive result for LTR PCR only or for attB PCR only allows to identify the mechanism of integration without ambiguities (ie respectively
Although NILV integration also occurred through other means than phiC31-int recombination, our results clearly demonstrate that a NILV is a suitable substrate for phiC31-int mediated recombination in human cells. We therefore investigated whether phiC31-int could function when vectorized in a NILV.

NILVs allow adequate expression of phiC31-int to mediate recombination into a reporter system containing an attP site artificially introduced in human cells

The ability of a NILV to express phiC31-int and mediate recombination between another NILV genome carrying an attB site and a wild-type attP site artificially introduced in a human cell genome was further tested. First, a clonal Hela cell line containing the wild-type attP site inserted in its genome (HDsred line) was generated using an integrative LV (CMV-attP-DsRed2). HDsred cells were then transduced with two NILVs, one allowing expression of phiC31-int (NILV CMV-PhiC31-int), and the other expressing Neo and containing the attB site (NILV attB-CMV-Neo). After cotransduction cells were grown with G418 to select integration events. The genomic DNA extracted from Neo resistant clones was analyzed by PCR to detect attL recombination junction (Figure 5A). Results showed only background signal generated by non-recombined attP site (figure 5B). To prevent this amplification, the genomic DNA was digested by a restriction enzyme that cuts both attP and attR sites but not attL site (Figure 5A) prior to PCR amplification. PCR results obtained after the enzymatic treatment revealed an attL junction in the population transduced with the highest dose of phiC31-int vector (Figure 5C). These results have been further confirmed by nested PCR (Figure 5D) and PCR product sequencing. Taken together, these results demonstrate that a NILV can deliver a functional phiC31-int capable to integrate an episomal lentiviral substrate containing an attB site into an attP site artificially introduced into the human genome.

Modification of the phiC31-int sequence to improve the efficiency of the NILV phiC31-int to allow target integration in pseudosites attP

It was previously shown that C-terminal addition of a nuclear localization system (NLS) to phiC31-Int improves its efficiency in eukaryotic cells [85]. We therefore tested this improved integrase in NILV vectorization strategy. To compare the two versions of phiC31-int, Hela cells were cotransduced with the following NILVs vectors: CMV-Neo with or without attL site and CMV-phiC31-int with or without NLS. Cells were grown with G418 and the resistant clones were quantified. The results from experiments in which CMV-phiC31-int was cotransduced with either CMV-Neo or attB-CMV-Neo are presented in Figure 6A. The CMV-Neo and the attB-CMV-Neo conditions did not display significant differences, indicating that no significant PhiC31-int recombinase activity occurred. In contrast, when the cells are transduced with CMV-phiC31-int-NLS instead of CMV-phiC31-Int (Figure 6B) the presence of the NLS sequence induced significant differences between the CMV-Neo and the attB-CMV-Neo conditions. These
results show that addition of a C-terminal NLS to phiC31-int significantly increased its recombination efficiency with pattP sites. Indeed the phiC31-int-NLS mediated integration was 2 to 2.5 fold above the background level produced by NILV residual integration (Figure 6B).

The use of the phiC31-int-NLS vectorized in a NILV allows to significantly increase the efficiency of recombination. We therefore further tested this hybrid lentivirus phiC31-int-NLS vector to target genomic pattP site into murine and human cells.

The two NILVs system allows site specific recombination in murine and human cells but induced aberrant chromosomal rearrangements.

To target pattP site in the murine and human genome with the two vectors system, Hela cells and NIH-3T3 cells were cotransduced with the NILV attB-CMV-Neo and the hybrid lentivirus phiC31-int-NLS vector. After two weeks of selection, Neo resistant cells were collected and several clonal populations were isolated to facilitate interpretation of PCR analyses. The clones were analyzed with the LTR and attB PCRs assay (Figure 3A) to determine the proportion of specific integration events compared to NILV residual integration and illegitimate recombination events. We categorized clones in the 3 following groups: LTR+/attB− clones (group I, ie phiC31-int-NLS integration), LTR−/attB+ clones (group II, ie residual integration) and LTR+/attB+ clones (group III, ie illegitimate integration or mixed integration profile). The results are presented in Table 1.

We obtained 108 clones for murine cells and 28 for human cells. 7.5% of murine clones are in group I and 20.3% in group III (Table 1A). Consequently the proportion of the hybrid lentivirus phiC31-int-NLS vector specific integration corresponds to the proportion of group I, ie 46.4% (Table 1B).

To determine which integration sites were targeted and confirm the type of integration of clonal groups I and III, we performed an analysis by iPCR as previously described (Figure 3B). The sequencing of iPCR products demonstrates that all human and murine clones tested contain in their genome an integrated vector with a recombinant pattern at the attB site. Therefore, the two
vectors system that we developed allows targeting p\textit{attP} sites in the murine and human genomes (Table 2).

Twelve murine integration sites were isolated, three from group I with the two junctions (\textit{pattL} and \textit{pattR}) and nine from group III with only the \textit{pattR} junction (Table 2). For two of the three group I clones both flanking regions were sequenced but the isolated junctions were too short to allow identification of the integration locus. Surprisingly, these two clones of group I present abnormal \textit{pattL} and \textit{pattR} junctions where several bases were missing, probably due to a deletion mechanism. Similarly, the sequence analysis of group III clones shows that recombination between the \textit{attB} site and \textit{pattP} site is not as precise as expected. Indeed, 6 out of 9 clones have missing bases in the \textit{pattR} junctions. However, because we isolated only one flanking region for clones of group III, we cannot conclude about deletion events, as the missing bases could result from a gap of the \textit{attB} core region involved in recombination.

Five human integration sites were isolated, all from group I. Both junctions were isolated for three sites and only the \textit{pattR} junction for two sites (Table 2). As for the murine integration site analysis, missing bases in the recombed pseudo-sites were detected, including in clones for which both L and R junction could be determined. This further confirms that missing bases indeed reveal a deletion mechanism, probably occurring during the phiC31-int mediated recombination between a natural \textit{attB} site and a \textit{pattP} site. Furthermore, the integration site of the three sites for which the two junctions were isolated could be localized exactly (Table 2). Interestingly, for these three sites chromosomal gaps were observed between the \textit{pattR} site and the \textit{pattL} site. The size gaps are 13 bp, 795 pb and 4.8 kbp. The two first gaps could result from a mechanism of deletion which could occur through NHEJ pathway. Nevertheless, these hypotheses cannot explain the gap of 4.8 kbp. Indeed, in this case, the two flanking regions isolated during iPCR are in the same chromosomal orientation, which is not the case when normal recombination occurs. We hypothesize that the observed aberrant recombination results from two successive recombination events involving two \textit{pattP} sites located 4.8 kb from each other that led to an inversion of the 4.8 kbp sequence (figure 7). This mechanistic model was previously proposed to explain chromosomal rearrangements in mammalian cells resulting from aberrant recombinations mediated by phiC31-int [86].

In conclusion the hybrid lentivirus-phiC31-int-NLS vector that we developed allows targeted integration in \textit{pattP} sites in murine and human genomes but seem to induce frequent deletions of base pairs in the \textit{attB} site present into the vector or into the endogenous \textit{pattP} site. Moreover, it may induce chromosomal deletions and translocations.

**Conclusions**

Our work establishes the ability of the hybrid lentivirus-phiC31-int-NLS to integrate a NILV substrate into \textit{attP} or \textit{pattP} sites in murine and human cell lines. Although the number of integration sites isolated in this study does not allow to determine preferential genome recognition sites for integration mediated by phiC31-int vectorized in NILV, a human \textit{pattP} site already described could be isolated [66]. Most importantly, we demonstrated that the use of hybrid lentiviral phiC31-int-NLS vector can induce DNA damages, probably due to the activity of the recombinase. Indeed, other reports already described similar results using non-viral transfection of PhiC31-int. Anja Ehrhardt et al have shown that 15% of transgenes integrated by phiC31-int were flanked by chromosomal DNA sequence from different chromosomes [86].
Figure 5. Detection of recombination mediated by phiC31-int between an attB site contained into a NILV and a genomic attP site. A) Scheme of the DsRed2 PCR before and after the enzymatic restriction treatment. B) PCR DsRed2 results without restriction enzyme treatment. Lanes 1 to 3: cotransduction with CMV-Neo and CMV-PhiC31 increasing vector input of 50–150–300 ng of p24. Lanes 4 to 6: cotransduction with attB-CMV-Neo and CMV-PhiC31 increasing vector input of 50–150–300 ng of p24. Lane 7: attB-CMV-Neo. Lane 8: positive control generated by triple-transfection (CMV-phiC31-int, attB-CMV-Neo and CMV-attP-DsRed2). Lane 9: negative control without vector. Lane 10: negative control of PCR. C) PCR DsRed2 results after restriction enzyme treatment. Lanes are similar to figure B. D) Nested PCR from the product isolated from lane 6 to confirm the specificity of PCR DsRed2 amplification.

doi:10.1371/journal.pone.0099649.g005
and Ji Liu et al. have shown that phiC31-int induces DNA damages and chromosomal rearrangements in primary and adult human fibroblasts [87,88]. In addition, Ehrard et al. have shown that phiC31-int is competent to integrate linear DNA fragments. They did not characterize the mechanisms and the consequences of this type of event are unknown, but one may hypothesize that such event would induce chromosome break. Considering that the cycle of NILVs involves linear intermediates, it would be of particular interest to further investigate the ability of phiC31-int to integrate these linear forms and the consequences of such events. Taken together, these observations limit the use of the hybrid lentiviral phiC31-int-NLS vector for clinical applications, it remains useful in transgenesis contexts where non aberrant recombination events can be selected. Alternatively, the hybrid lentiviral system may be used to vectorize other recombinases or genome editing tools with higher safety features. Indeed other serine recombinases have been shown to function in human cells [89–91] and could be valuable candidates for vectorization in NILVs. Moreover, the modification of recombinases and other genome editing tools by directed evolution techniques [92,93] could be used to render them hyper-specific and hyper-efficient in order to improve their safety features. For instance, directed evolution has proven efficient to modify the efficiency and/or specificity of a variety of molecular tools, including Sleeping Beauty [94], Cre recombinase [95], FLP recombinase [96], ZFNs [97] or phiC31-int [98].

**Methods**

**Plasmids**

The encapsidation plasmid expressing a functional integrase (p8.91 INWT) has been described previously [77]. The encapsidation plasmid expressing a deficient integrase (p8.91 IND64V) was derived from the plasmid p8.91 INWT and the plasmid pCMVΔAR(int-)8.2 previously described [74] and kindly provided by D.B. Kohn (UCLA, Los Angeles (CA), USA). This plasmid contains a point mutation in the coding region of the integrase catalytic domain, creating a D64V change in the amino acid sequence. The plasmid p8.91 IND64V was generated by replacing the BclI-AflII fragment of p8.91 INWT by the corresponding fragment (containing the substitution) from pCMVΔAR(int-)8.2.

The envelope expression plasmid pMDG(VSV) was used to express the VSV-G from the human CMV immediate early promoter [74].

The vector plasmid pTrip-CMV-phic31-int-WPRE was derived from the plasmid pTrip-CMV-GFP-WPRE previously described [77] and the plasmid pCMV-phic31-int previously described [41] and kindly provided by M.P. Calos (Stanford University, Stanford

**Table 1. Repartition in 3 groups of human and murine clones according to the attB and LTR PCR results.**

|                | Group I (LTR+) Specific integration | Group II (attB+) Residual integration | Group III (LTR+/attB+) Unknow integration |
|----------------|-------------------------------------|--------------------------------------|----------------------------------------|
| **Mouse**      |                                     |                                      |                                        |
| Clone number   | 8                                   | 78                                   | 22                                     |
| %              | 7,5%                                | 72,2%                                | 20,3%                                  |
| **Human**      |                                     |                                      |                                        |
| Clone number   | 13                                  | 15                                   | 0                                      |
| %              | 46,4%                               | 53,6%                                | 0,0%                                   |

doi:10.1371/journal.pone.0099649.t001
Table 2. Mapping and description of pattP sites isolated by iPCR on human and murine cell lines.

| Chromosome | Number of junction isolated | Deletions | Genomic location | If intronic | If intergenic, flanking gene names |
|------------|-----------------------------|-----------|------------------|-------------|----------------------------------|
|            |                             | The half of att site present into the vector | Chromosome | Pseudo site | Context | gene name | 5’ side gene | Distance (kb) | 3’ side gene | Distance (kb) |
| Mouse      |                             | Chromosome |                  |             |         |          |             |              |              |              |
| Groupe I   | 1                           | 2 pattR: 2 bp | ND | 1E4 | repeat sequence |
|            | 2                           | PattR: No pattl: 13 bp | ND | 2H3-H4 | repeat sequence |
|            | 7                           | PattR: 4 bp | ND | 7F2 108099955 | Exonic | MOR204-16 |
| Groupe III | 1                           | PattR: 8 bp | ND | 1H1 159228123 | Intergenic | PAPP-A2 | 277 | AI316802 | 49 |
|            | 5                           | PattR: 16 bp | ND | 5B1 29812070 | Exonic | 4632413E21Rik |
|            | 5                           | PattR: 21 bp | ND | 5C31 58057809 | Intrinsic | Pcdh7 |
|            | 6                           | PattR: No | ND | 651 135251888 | Intergenic | Gag1 | 7 | 1700023A18Rik | 51 |
|            | 7                           | PattR: 4 bp | ND | 7A1 7104238 | Intergenic | AIE1 | 90 | 5730403A18Rik | 10 |
|            | 9                           | PattR: 12 bp | ND | 9A1 3001529 | Intronic | AC131780.5-201 |
|            | 13                          | PattR: No | ND | 13D1 102691720 | Intergenic | AA414921 | 984 | F63010815 | 1.5 |
|            | 17                          | PattR: No | ND | 17A3.3 2398186 | Intergenic | EG622645 | 305 | 4.1B | 53 |
| X          | 1                           | PattR: 10 bp | ND | XA7.3 75331158 | Intronic | Cf8 |
| Human      | Groupe I                     | 13 bp | 1q32.1 20953621/634 | Intergenic | SLC26A9 | 15 | RAB7 | 28 |
|            | 9                           | PattR: 20 bp | ND | 9p21.1 33615936 | Intergenic | BA255A11.3 | 43 | TCRBV2052 | 1.5 |
|            | 2                           | PattR: No pattl: 12 bp | Inversion of 4.8 kb | 17q11.2 26164583/59669 | Intronic | HSD24 |
|            | 2                           | PattR: No pattl: ND | 17q21.3 4506611 | Intronic | C1orf57 |
|            | 2                           | PattR: 10 bp pattl: 34 bp | 795 pb | 17q25.3 82068091/886 | Intergenic | DUS1L | 2559 | FASN | 11080 |

doi:10.1371/journal.pone.0099649.t002
The plasmid pTrip-CMV-phic31-int-WPRE was generated by replacing the BamHI-SnaBI fragment (CMV-GFP) of the pTrip-CMV-GFP-WPRE by the CMV-phic31-int fragment from the plasmid pCMV-phic31-int. The vector plasmid pTrip-CMV-phic31-int-NLS-WPRE was derived from the plasmid pCMV-phic31-int and kindly provided by MP Calos. The C-terminal region of phic31-int was amplified with a primer containing the SV40 NLS sequence (5'-CCCGTTGGCAGGACACCTCAGCAGG-3'/5'-ATTCGCGGATCCGCTAAACCT-TCCTCTCTCTCTAGGCGCCGCACGTCTCCGCGGTCC-3') from the plasmid pCMV-phic31-int. This PCR product was subcloned into the plasmid pCMV-phic31-int in place of the C-terminal region of phic31-int by using EcoRI and BamHI restriction enzymes to generate the plasmid pCMV-phic31-int-NLS. The plasmid pTrip-CMV-phic31-int-NLS-WPRE was finally generated by replacing the SpeI-BamHI fragment (GFP) of the pTrip-CMV-phic31-int-WPRE by the corresponding fragment (phic31-int-NLS) from pCMV-phic31-int-NLS. The vector plasmid pTrip-attB-CMV-Neo-WPRE was derived from the plasmid pTrip-CMV-Neo-WPRE previously described [77] and the plasmid attB previously described [41] and kindly provided by MP Calos. The plasmid pTrip-attB-CMV-Neo-WPRE was generated by inserting the Sall fragment of the attB plasmid (attB sequence) into the linearized plasmid pTrip-CMV-Neo-WPRE. The plasmid pTrip-CMV-attP-DsRed2 was derived from the plasmid pTrip-CMV-DsRed2 kindly provided by P. Ravassard.
Hybridization of 2 single strands DNA fragments (5'-CCCGAAC-TGGGGAACCTTTGAGTCTCTCAGTGGGGG-3'/5'-CCCGAAC-TGGGGAACCTTTGAGTCTCTCAGTGGGGG-3') generated a double stranded DNA fragment corresponding to the adP sequence flanked by BamHI cohesive ends. This fragment was inserted into the linearized plasmid pTrip-CMV-aI-DsRed2 to generate the plasmid pTrip-CMV-aI-DsRed2.

**Lentiviral production**

Lentiviral vectors were generated by the transient transfection of 293T cells by using the calcium phosphate precipitation method previously described [77]. Briefly, cells were cotransfected with the vector plasmid (pTrip-CMV-phiC31-int-WPRE, pTrip-CMV-phiC31-int-NLS-WPRE, pTrip-aI/CAMV-Neo-WPRE or pTrip-CMV-aI/DSRed2-WPRE), the transcomplementation plasmid (p8.9l IN50 for integrative vectors or p8.9l IN64 for non-integrative vectors), and the plasmid encoding the vesicular stomatitis virus envelope glycoprotein (pMD-6). Supernatant was collected 48 hours after transfection, treated with DNaseI and filtered (0.45 μm). Viral particles were then concentrated by ultracentrifugation (90 min, 22,000 rpm, rotor SW28) and resuspended in 0.1M PBS. The HIV p24 Gag antigen was quantified for each stock by ELISA (HIV-1 P24 antigen assay; Beckman Coulter, Fullerton, CA) according to manufacturer's instructions.

**Cell culture**

Human epithelial HeLa, Hii16, HeLa-DsRED2 and 293T cells and murine NIH 3T3 cells were grown in Dulbecco's modified medium (Invitrogen) supplemented with antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) and 10% heat inactivated fetal calf serum (Eurobio). The cells were plated and cultured in a humidified incubator at 37°C in a 5% CO2 and 90% air atmosphere.

**Genomic DNA extraction**

Genomic DNA extractions were performed with a lysis buffer composed of TrisHCl 10 mM (pH 7.5), EDTA 10 mM, SDS 0.6%, RNase A (Qiagen) 100 μg/mL and proteinase K (Eurobio) 100 μg/mL. The lysates were purified by phenol/chloroform and precipitated using sodium acetate and ethanol.

**Generation of a reporter cell line HeLa-DsRED2**

The HeLa-DsRED2 cell line containing an adP site and expressing DsRED2 fluorescent protein was generated using the lentivector CMV-adP/DsRED2. HeLa cells were transduced with LV CMV-adP/DsRED2 (unconcentrated supernatant). Cells were grown for 3 days and seeded at clonal density in 96-well plates (0.3 cell per well) to generate single cell derived colonies. Clones were analyzed for DsRED2 expression by flow cytometry and PCR amplification of the vector genome.

**Transduction**

Cell suspensions were incubated for 3 hours with required vectors in medium supplemented with 1 μM DEAE-dextran. After 3 hours of incubation, cells were seeded at desired density in fresh medium and grown for the purpose of the experiment.

Hii16 cells (2.10^6 cells/mL) were transduced with NILV-phiC31-int (300 ng of p24) and NILV-Neo (100 ng of p24). Cells were seeded in 10 cm plates and grown in medium supplemented with 1 mg/mL of G418 for 12 days. Cells were then seeded in 96 well plates at low density (0.3 cell per well) to generate single cell derived colonies.

HeLa DsRED2 cells (2.10^6 cells/mL) were transduced with NILV CMV-phiC31-int (50, 150, 300 ng of p24) and NILV CMV-Neo (100 ng of p24). Cells were seeded in 10 cm plates and grown in medium supplemented with 1 mg/mL of G418 (renewed every 5 days) for 12 days before extraction and analysis of genomic DNA.

Hela and NIH-3T3 cells (5.10^5 cells/mL) were transduced with NILV CMV-phiC31-int (300 ng of p24) and NILV CMV-Neo (100 ng of p24). Cells were seeded in 10 cm plates and grown in medium supplemented with 1 mg/mL of G418 for 12 days. Cells were then seeded in 96 well plates at low density (0.3 cell per well) to generate single cell derived colonies.

**Evaluation of Recombination Frequency**

HeLa were directly transduced in suspension (8.10^4 cells/mL) with NILV CMV-phiC31-int (3, 6, 18 and 36 ng of p24) and NILV CMV-Neo (12 ng of p24) during 3 hours in 150 μL of medium supplemented with 1 μM DEAE-dextran. Cells were then seeded in 6-wells plates with 2 mL of fresh medium. The day after, medium was removed and replaced with fresh medium supplemented with 1 mg/mL of G418. The medium was replaced every 3 days. Cells were grown 12 days, until clones developed and were then fixed with PFA 4% and stained with trypan blue. Clones on each well were counted. We transduced cells in three replicate tubes for each condition, and results are expressed as the mean of three measurements.

**PCR reactions**

**RT-PCR.** To analyze phiC31-int expression by RT-PCR, total RNAs were extracted from HeLa cells using the RNAeasy minikit (Qiagen), according to the manufacturer’s instructions. Then, RNAs were reverse transcribed using the Superscript First Strand Synthesis kit (Invitrogen), according to the manufacturer’s instructions. PhiC31-int cDNA was ampliﬁed using the primers 5’-GCGAAGATTCTGGCAACG-3’ and 5’-TCGCAAGTACGTTGCC-3’ at the concentration of 10 μM.

**PCR on genomic DNA.** PCR performed on genomic DNA used 500 ng of DNA, 1.5 μM of MgCl2 and 10 μM of each primer. The primers were as follows: amplification of adP region: 5’-CAATTTTGCGAGGGCATTTGAG-3’ and 5’-CTGTCGCCC-TGTAATAAACG-3’; amplification of the LTRs region: 5’-CTCATAAAGGCTGCTTTGAGTTGC-3’ and 5’-TCGAGAT-CTGGGTCTAACAACAGAGACC-3’; amplification of the Ds Red2 region: 5’-AGGCCGACAGATTATTGTCGTG-3’ and 5’-ATGAGTCTGTTCTGTGACAT-3’; amplification of Ds Red2 (nested primers) 5’-AAGAATCTGCGGCTGTAACG-3’ and 5’-AATCCTGGGTAGCTGTTGCC-3’.

**Inverse PCR.** Genomic DNA (10 μg) was primarily submitted to enzymatic restriction. The enzymatic cocktail used was: XbaI (100 U), SfiI (100 U) BsrGI (30 U) and BstXI (30 U). After 16 hours incubation, the enzymes were heat inactivated at 65°C for 30 minutes. Cohesive ends were filled in with Klenow (15 U) and dNTPs (2 mM) at 25°C for 20 minutes. Klenow was inactivated with 1 mM EDTA. The products of digestion were purified by phenol/chloroform and precipitated using sodium acetate and ethanol. The ligation of the digestion products was performed by ligase (1.000 U, NEB) within ligation buffer supplemented with ATP (1 mM). The products of ligation were purified by phenol/chloroform and precipitated using sodium acetate and ethanol. PCR was performed with 100 ng of DNA with primers allowing the amplification of the adP region.

Adapted inverse PCR was performed using the same protocol with the addition of BsmI (30 U) in the enzymatic restriction cocktail.
The products of inverse PCR were visualized on 0.8% agarose gel with ethidium bromide staining. These products were extracted and purified using the Wizard SV Gel and PCR Clean-up System (Promega) according to the manufacturer’s instructions, then cloned in a plasmid using pGEM-T Easy Vector System I according to the manufacturer’s instructions. Next, inverse PCR products were sequenced using T7 and/or Sp6 primers.

**Sequence analysis**

All sequencing was performed by Eurofins genomics. Sequences were aligned with vector and genomic DNA, and recombination junctions were identified by sequence matching to attP. Human and murine blasts were performed using the NCBI and ensemble genome databases. The chromosomal localization of pseudogenes attP has been performed using the Genebank GRCh38.p2.

**References**

1. Hacem-Bey-Ahina S, Von Kalle C, Schmidt M, McCormack MP, Wolffraat N, et al. (2003) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science 302: 415–419. doi:10.1126/science.1088547
2. Urtnow FD, Miller JC, Ler Y-L, Beausejour CM, Rock JM, et al. (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. Nature 435: 464–465. doi:10.1038/nature03556
3. Geurs AM, Cost GJ, Freyvert Y, Zeiler B, Miller JC, et al. (2009) Knockout rats via embryo microinjection of zinc-finger nucleases. Science 325: 433. doi:10.1126/science.1172447
4. Morton J, Davis MG, Hjelmerson EM, Carroll DJ, Becker D (2006) Induction and repair of zinc-finger nuclease-targeted double-strand breaks in Caenorhabditis elegans somatic cells. Proc Natl Acad Sci U S A 103: 16370–16375. doi:10.1073/pnas.0606310103
5. Bozas A, Beumer KJ, Trautman JK, Carroll D (2009) Genetic analysis of zinc-finger nucleic-acid targeting in Drosophila. Genetics 182: 641–651. doi:10.1534/genetics.109.101329
6. Hockemeyer D, Schildkraut I, Aggarwal AK, Bitinaite J, Wah DA (1998) FokI dimerization is required for DNA cleavage. Proc Natl Acad Sci U S A 95: 10570–10575. doi:10.1073/pnas.95.20.10570
7. Tesson L, Usal C, Meñoret S, Leung E, Niles BJ, et al. (2011) Knockout rats for targeted gene inactivation. Proc Natl Acad Sci U S A 108: 11450–11455. doi:10.1073/pnas.10147
8. Boch J, Schacher H, Schornack S, Landgraf A, Hahn S, et al. (2009) Breaking the code of DNA binding specificity of TAL-type III effectors. Science 326: 1501–1505. doi:10.1038/nature03556
9. Mali P, Yang L, Esvelt KM, Aach J, Guell M, et al. (2013) RNA-guided human genome engineering via Cas9. Science 339: 823–827. doi:10.1126/science.1231127
10. Tesson L, Usal C, Meñoret S, Leung E, Niles BJ, et al. (2011) Knockout rats via embryo microinjection of zinc-finger nucleases. Nat Biotechnol 27: 851–857. doi:10.1038/nbt.1936
11. Carlson DF, Tan W, Ilicio SG, Stervakova D, Promfotr C, et al. (2012) TALEN-mediated gene knockout in livestock. Proc Natl Acad Sci U S A 109: 17382–17387. doi:10.1073/pnas.1214461109
12. Sandor JD, Cade L, Khayter C, Reyon D, Peterson RT, et al. (2011) Targeted gene disruption in somatic zebrafish cells using engineered TALENs. Nat Biotechnol 29: 697–698. doi:10.1038/nbt.1940
13. Hockemeyer D, Wang H, Kami S, Lai CS, Gao Q, et al. (2011) Genetic engineering of human pluripotent cells using TALE nucleases. Nat Biotechnol 29: 731–734. doi:10.1038/nbt.1927
14. Cho SW, Kim S, Kim JM, Kim J-S (2013) Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat Biotechnol 31: 230–232. doi:10.1038/nbt.2507
15. Mali P, Yang L, Elovitz KM, Asch J, Guel J, et al. (2013) RNA-guided human genome engineering via Cas9. Science 339: 823–826. doi:10.1126/science.123033
16. Friedland AE, Taur YB, Elovitz KM, Colaiacovo MP, Church GM, et al. (2013) Highly programmable genome editing in C. elegans via a CRISPR-Cas9 system. Nat Methods. doi:10.1038/nmeth.2532
17. Hwang WY, Fu Y, Reyon D, Mader ML, Tsai SQ, et al. (2013) Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat Biotechnol 31: 297–299. doi:10.1038/nbt.2532
18. Bitinaite J, Wah DA, Aggarwal AK, Schildkraut I (1998) FokI dimerization is required for DNA cleavage. Proc Natl Acad Sci U S A 95: 10570–10575. doi:10.1073/pnas.95.20.10570
19. Wah DA, Bitinaite J, Schildkraut I, Aggarwal AK (1998) Structure of FokI has implications for DNA cleavage. Proc Natl Acad Sci U S A 95: 10564–10569. doi:10.1073/pnas.95.20.10564
20. Smith J, Bihovska M, Whithly FG, Reddy AR, Chandrasegaran S, et al. (2000) Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains. Nucleic Acids Res 28: 3361–3369.

**Acknowledgments**

We thank Professor Michele P. Calos for providing us the pCMV-phiC31 (pCMVinit). We also would like to thank Dr. Marie-José Lecomte for critical reading of the manuscript, Delphine Muller and Aurore Berthier for the technical support.

**Author Contributions**

Conceived and designed the experiments: NG C. Sarkis. Performed the experiments: NG DA SU HP AL. Analyzed the data: NG C. Sarkis MCS C. Serguera SP. Contributed reagents/materials/analysis tools: NG AL JM. Wrote the paper: NG SP C. Sarkis C. Serguera.
57. Ortiz-Urda S, Thyagarajan B, Keene DR, Lin Q, Calos MP, et al. (2003) Integration specificity of phiC31 integrase in the human genome. J Mol Biol 357: 28–48. doi:10.1016/j.jmb.2005.11.098
58. Wilson JH (2003) Pointing fingers at the limiting step in gene targeting. Nat Biotechnol 21: 739–760. doi:10.1038/nbt0705-759
59. Elrhardt A, Yant SR, Giering JC, Xu H, Engler JA, et al. (2007) Somatic integration from an adenoviral hybrid vector into a hot spot in mouse liver results in persistent transgene expression levels in vivo. Mol Ther J Am Soc Gene Ther 13: 146–156. doi:10.1038/mt.2006.1001
60. Robert M-A, Zeng Y, Raymond B, Desfosses L, Mairey E, et al. (2012) Efficacy and site-specificity of adenovector vector integration mediated by the phiC31 integrase. Hum Gene Ther Methods 23: 393–407. doi:10.1089/hgtb.2012.122
61. Kaper SE, Chiarello N, Lee FS, Wivel NA, Bagg A, et al. (2003) Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Mol Gene Metab 89: 148–158.
62. Schnell MA, Zhang Y, Tazaal J, Gao GP, Yu QC, et al. (2001) Activation of innate immunity in nonhuman primates following intraportal administration of adeno vectors. Mol Ther J Am Soc Gene Ther 3: 708–722. doi:10.1006/mthe.2001.0330
63. Byrnes AP, Rusby JE, Wood MJ, Charlton HM (1995) Adenovirus gene transfer causes inflammation in the brain. Neuroscience 66: 1015–1024.
64. Kati T, Blömmer U, Peterson DA, Gage FH, Verma IM (1997) Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. Nat Genet 17: 314–317. doi:10.1038/ng1197-314
65. Naldini L, Blömmer U, Gallay P, Ory D, Mulligan R, et al. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272: 263–267.
66. Cronin J, Zhang XY, Reiser J (2005) Altering the tropism of lentiviral vectors through pseudotyping. Curr Gene Ther 5: 387–398.
67. Yáñez-Muñoz RJ, Balaggan KS, MacNeill A, Howe SJ, Schmidt M, et al. (2006) Effective gene therapy with nonintegrating lentiviral vectors. Nat Med 12: 348–353. doi:10.1038/nm.1563
68. Philippe S, Sarkis C, Barkas M, Mammner H, Ladroue C, et al. (2006) Lentiviral vectors with a defective integrase allow efficient and sustained transgene expression in vitro and in vivo. Proc Natl Acad Sci U S A 103: 17604–17609. doi:10.1073/pnas.0606197103
69. Vink CA, Gaspar HB, Gabriel R, Schmidt M, McVors RS, et al. (2009) Sleeping beauty transposition from nonintegrating lentivirus. Mol Ther J Am Soc Gene Ther 17: 1197–1203. doi:10.1038/mt.2009.94
70. Stamminger NH, Volz H, Förster S, Jakobsen M, Montes-Moreno MA, et al. (2010) Effective gene therapy with nonintegrating lentiviral vectors. Mol Ther J Am Soc Gene Ther 18: 1197–1204. doi:10.1038/mt.2010.29
71. Grandchamp N, Henriot D, Philippe S, Amar L, Ursulet S, et al. (2011) Influence of insulators on transgene expression from integrating and non-integrating lentiviral vectors. Gene Vaccines Ther 9: 1. doi:10.1038/1479-1486.2011.2
72. Byrnes AP, Rusby JE, Wood MJ, Charlton HM (1995) Adenovirus gene transfer causes inflammation in the brain. Neuroscience 66: 1015–1024.
93. Tay Y, Ho C, Droge P, Ghadessy FJ. (2010) Selection of bacteriophage lambda integrases with altered recombination specificity by in vitro compartmentalization. Nucleic Acids Res 38: e25. doi:10.1093/nar/gkp1089

94. Zayed H, Izsvák Z, Walisko O, Ivics Z. (2004) Development of hyperactive sleeping beauty transposon vectors by mutational analysis. Mol Ther J Am Soc Gene Ther 9: 292–304. doi:10.1016/j.ymthe.2003.11.024

95. Santoro SW, Schultz PG. (2002) Directed evolution of the site specificity of Cre recombinase. Proc Natl Acad Sci U S A 99: 4185–4190. doi:10.1073/pnas.022039799

96. Bolusani S, Ma C-H, Paek A, Konieczka JH, Jayaram M, et al. (2006) Evolution of variants of yeast site-specific recombinase Flp that utilize native genomic sequences as recombination target sites. Nucleic Acids Res 34: 5259–5269. doi:10.1093/nar/gkl548

97. Guo J, Gaj T, Barbas III CF. (2010) Directed Evolution of an Enhanced and Highly Efficient FokI Cleavage Domain for Zinc Finger Nucleases. J Mol Biol 400: 96–107. doi:10.1016/j.jmb.2010.04.060

98. Sclimenti CR, Thyagarajan B, Calos MP. (2001) Directed evolution of a recombinase for improved genomic integration at a native human sequence. Nucleic Acids Res 29: 5044–5051.