

DNA transposition by protein transduction of the *piggyBac* transposase from lentiviral Gag precursors

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ABSTRACT

DNA transposon-based vectors have emerged as gene vehicles with a wide biomedical and therapeutic potential. So far, genomic insertion of such vectors has relied on the co-delivery of genetic material encoding the gene-inserting transposase protein, raising concerns related to persistent expression, insertional mutagenesis and cytotoxicity. This report describes potent DNA transposition achieved by direct delivery of transposase protein. By adapting integrase-deficient lentiviral particles (LPs) as carriers of the hyperactive *piggyBac* transposase protein (hyPBBase), we demonstrate rates of DNA transposition that are comparable with the efficiency of a conventional plasmid-based strategy. Embedded in the Gag polypeptide, hyPBBase is robustly incorporated into LPs and liberated from the viral proteins by the viral protease during particle maturation. We demonstrate lentiviral co-delivery of the transposase protein and vector RNA carrying the transposon sequence, allowing robust DNA transposition in a variety of cell types. Importantly, this novel delivery method facilitates a balanced cellular uptake of hyPBBase, as shown by confocal microscopy, and allows high-efficiency production of clones harboring a single transposon insertion. Our findings establish engineered LPs as a new tool for transposase delivery. We believe that protein transduction methods will increase applicability and safety of DNA transposon-based vector technologies.

INTRODUCTION

Integrating non-viral vectors based on the *Sleeping Beauty* (*SB*) and *piggyBac* (*PB*) DNA transposons have emerged as attractive alternatives to integrating viral vectors derived from gammaretroviruses and lentiviruses (1,2). DNA transposons are small genetic entities that can relocate within the genome. However, they tend to accumulate mutations and become inactive during evolution. The use of transposable elements in mammalian cells was fueled by mutagenesis-based re-awakening of the *SB* transposon from the genome of salmonid fish (3). Also, *PB*, derived from the cabbage looper moth *Trichoplusia ni*, proved to be potently mobilized in mammalian cells and is characterized, in addition, by its ability to carry large genetic cargo and by leaving no genetic trace at the excision site after re-mobilization (4,5). Yet other transposons, like recently identified *TcBuster* (6), *SPIN* (7) and *piggyBat* (8) elements, may offer alternative functional properties and new possibilities. The biomedical applicability of *SB* and *PB* is documented in cancer gene discovery (9–11), production of transgenic animals including large animal models (12,13), generation of induced pluripotent stem cells (14,15) and gene therapy (16,17). With the engineering of new hyperactive transposases [SB100X (18) and hyPBBase (19) for *SB* and *PB* systems, respectively], DNA transposon systems have gained levels of activity that may in some cellular systems compare with the activity of viral vectors.

Unlike viruses, DNA transposons cannot move between cells and need assistance to reach the nucleus. Transposon-based vectors are two-component systems featuring (i) the transposon carrying the transgene expression cassette and (ii) the transposase. Ideally, genomic insertion of the transposon vector is facilitated by a short

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boost of transposase activity. So far, DNA transposition has relied on the expression of the transposase, e.g. from plasmid DNA. To ease transposase delivery, viral vectors have been adapted to carry the transposase gene (20–23). In gene transfer applications, the delivery of transposase-encoding DNA constitutes an inherent risk of inserting the transposase gene under a strong promoter in the genome of the target cells, expectedly leading to cytotoxicity (24). Thus, a transient and dose-controllable approach to deliver transposase is of particular importance. Transient expression of the transposase has been achieved by delivery of transposase-encoding RNA, either as *in vitro*-transcribed RNA (25) or as engineered retroviral vector RNA that is unable to undergo reverse transcription (26). Both these methods allow short-term expression and reduced cytotoxicity, but they rely on transfer of genetic material and may suffer from reduced efficiency depending on the specific application. Direct delivery of the transposase protein may offer several advantages. However, although production of recombinant protein may be easily adaptable to large-scale production, major limitations include protein trapping in the endosomes (27) and difficulties related to production and/or purification (28). Only few attempts to produce recombinant *SB* or *PB* transposase protein have been reported (29,30), and this strategy seems challenged by difficulties related to purification of functional transposases and/or cellular uptake.

In this report, we demonstrate high levels of DNA transposition achieved by direct delivery of transposase protein incorporated in virus-derived particles. Lentiviral particles (LPs) have previously been adapted as carriers of non-viral proteins fused to the Gag polypeptide (31,32). Also, recombinases and meganucleases have been delivered in retroviral particles (33–35). Here, hyPBBase embedded in Gag precursors is incorporated in LPs, leading by transduction to efficiencies of transposition that are comparable with state-of-the-art plasmid-based transposase delivery. We show the feasibility of co-delivering hyPBBase protein and the transposon donor as RNA in viral particles and describe the unique property of this system to insert a single copy of the transposon even at conditions that allow high-efficiency transposition. This solves an inherent problem with current plasmid-based transposon vector systems, which often form heterogeneous and multicopy clones due to difficulties of controlling the level and time frame of active gene insertion.

MATERIALS AND METHODS

Plasmid construction

Constructs expressing the hyperactive *PB* transposase (pCMV-hyPBBase and pCMV-HAhyPBBase) have been described previously (19) and were provided by Allan Bradley (Wellcome Trust Sanger Institute, Cambridge, UK) and Nancy Craig (The Johns Hopkins University School of Medicine, Baltimore, MD, USA). pCMV-hyPBmut, expressing a mutated inactive version of the hyPBBase, was generated by fusing (by overlap polymerase chain reaction [PCR]) PCR fragments generated with primer pairs YJ087F-YJ076R and YJ075F-YJ088R (see

Supplementary Table S1), thereby leading to introduction of the D447N mutation. The resulting PCR product was digested with HindIII and NotI and inserted into HindIII/NotI-digested pCMV-hyPBBase. pCMV-HAhyPBmut was generated by replacing a PmlI-NotI fragment of pCMV-HAhyPBBase with the corresponding region of pCMV-hyPBmut.

To introduce the *PB* transposase coding sequence into the context of the lentiviral GagPol polypeptide, the various versions of the hyPBBase sequence were introduced into plynMyGFP-gag-pol (31), which was provided by Jun Komano (National Institute of Infectious Diseases, Tokyo, Japan). The hyPBBase and HA-hyPBBase sequences were amplified from pCMV-HAhyPBBase using primer pairs YJ067F-YJ097R and YJ069F-YJ097R, respectively. Mutated hyPBBase was amplified from pCMV-hyPBmut with primer pair YJ067F-YJ097R, whereas mutated HA-hyPBBase was amplified from pCMV-HAhyPBmut with primer pair YJ069F-YJ097R. For generation of a variant that did not include a human immunodeficiency virus-1 (HIV-1) protease cleavage site at the junction site between transposase and GagPol, HA-hyPBBase was amplified from pCMV-HAhyPBBase using primer pair YJ069F-YJ077R. In all cases, PCR products were cut with AgeI/MfeI and inserted into AgeI/EcoRI-digested plynMyGFP-gag-pol, resulting in phyPBBase-gagpol, phyPBmut-gagpol, pHAhyPBBase-gagpol, pHAhyPBmut-gagpol and pHAhyPBBase Δ PC-gagpol.

To generate vectors based on the *SB* system, a codon-optimized (CO) version of SB100X was synthesized by GenScript and cloned into the SacII/SacII sites of pCMV-SB100X (36) to generate pCMV-SB100XCO. Variants of SB100XCO with an N- or a C-terminal extension of five amino acid residues (pCMV-5aa-SB100XCO and pCMV-SB100XCO-5aa, respectively) were constructed by inserting SacII-digested PCR products (generated with primer sets YJ018F-YJ029R and YJ020F-YJ030R) into SacII-digested pCMV-SB100XCO. To create a construct expressing a transposition-defective version of the SB100XCO transposase (pCMV-SB100XCOmut), a D244A mutation was introduced into pCMV-SB100XCO (digested by SacII) by inserting an overlap PCR product (digested by SacII) generated by fusing fragments created with primer pairs 4684-YJ046R and YJ045F-0721.

To create an integrase-defective packaging construct (plynMyGFP-gagpol-D64V) based on the plynMyGFP-gag-pol construct, the D64V mutation was introduced into the integrase gene of plynMyGFP-gag-pol by inserting an EcoRV/XbaI-digested overlap PCR product containing the D64V mutation (fusing the PCR fragments created with primer pairs YJ114F-YJ115R and YJ116F-YJ117R) into EcoRV/XbaI-digested plynMyGFP-gag-pol. To create pNanoLuc-gagpol-D64V, the NanoLuc gene was amplified from pLN1.1 (Promega) with primers NanoLuc(s) and YJ154R and cloned into AgeI/EcoRI-digested plynMyGFP-gagpol-D64V. To create the corresponding constructs harboring variants of the hyPBBase (phyPBBase-gagpol-D64V, phyPBmut-gagpol-D64V, pHAhyPBBase-gagpol-D64V, pHAhyPBmut-gagpol-D64V and pHAhyPB Δ PC-gagpol-D64V), the EcoRV/XbaI-fragment containing the D64V mutation

was isolated from plynMyGFP-gagpol-D64V and used to replace the corresponding fragment in packaging constructs without the D64V mutation (see earlier). Fragments containing the transposase sequence, amplified from pCMV-SB100XCO and pCMV-SB100XCOMut with the primer pair YJ118F-YJ109R, were digested with AgeI and EcoRI and inserted to replace GFP in plynMyGFPgagpol-D64V, resulting in pSB100XCO-gagpol-D64V and pSB100XCOMut-gagpol-D64V.

We have previously described the construction and functionality of a lentiviral vector, pLV/puro-PGK-SBT2, in which the *SB*-derived SBT2/PGK-puro transposon is oriented opposite the transcriptional orientation of the vector genome to avoid premature termination of transcription due to the polyA-sequence in the transposon (37). By adapting a similar design, a lentiviral vector harboring the *PB* transposon, pLV/puro-PGK-PBT, was generated by PCR-amplifying the PBT/PGK-puro transposon from pPBT/PGK-puro (38) and inserting the resulting fragment into MluI/AscI-digested pLV/MCS (37). pLV/Fluc-PGK-PBT, carrying the PBT/PGK-Fluc transposon, was generated by replacing the *SB* IRs of pLV/Fluc-PGK-SBT2 with the *PB* TRs in two cloning steps. First, the 5' TR of *PB* was amplified from pLV/puro-PGK-PBT with primers PB5'TR(s) and PB5'TR(as) and inserted into PacI/AscI-digested pLV/Fluc-PGK-SBT2, resulting in the construct pLV/Fluc-PGK-5'PBT/3'SBT2. Next, the 3'TR of *PB* was amplified from pLV/puro-PGK-PBT with primers YJ152F and YJ153R and the resulting fragment (digested by AvrII and BstXI) was cloned into the XbaI and BstXI sites of pLV/Fluc-PGK-5'PBT/3'SBT2 to reach the final construct pLV/Fluc-PGK-PBT.

Production of LPs and integrase-defective lentiviral vectors

Throughout this work, the term 'lentiviral particle' (LP) was used to designate a particle that does not carry a vector genome and is produced only as a carrier of a foreign protein (e.g. as a carrier of the hypBase transposase, as indicated by the designation 'LP-hypBase'). In addition, integrase-defective lentiviral vectors (IDLVs) carrying the mutated D64V integrase as well as a foreign protein and a vector genome were produced for co-delivery of transposase and transposon-carrying RNA. Names of these vectors indicate the vector composition; IDLV-hypBase/puro-PGK-PBT, e.g. designates an IDLV that contains the hypBase protein and a vector carrying the PBT/PGK-puro transposon.

LP and IDLV vector preps were produced as follows. On day 1, 293T cells were plated at a density of 6×10^4 /cm². On day 2, cells were transfected with calcium phosphate precipitates. To produce IDLVs that did not incorporate foreign protein, cells in 15-cm dishes were transfected with 9.07 μ g pMD.2G, 7.26 μ g pRSV-Rev, 31.46 μ g pMDlg/pRRE-D64V and 31.46 μ g pLV/puro-PGK-PBT. To produce IDLVs incorporating one of the variants of the hypBase, 293T cells plated in 15-cm dishes were transfected with 9.07 μ g pMD.2G, 7.26 μ g pRSV-Rev, 15.73 μ g pMDlg/pRRE-D64V, 15.73 μ g phyPBase-gagpol-D64V (or one of the other

corresponding constructs) and 31.46 μ g pLV/puro-PGK-PBT (or pLV/Fluc-PGK-PBT). The 'hybrid' IDLV was produced with a 1:1 ratio (w/w) of phyPBase-gagpol-D64V and pMDlg/pRRE-D64V, unless otherwise mentioned in the text. To produce LPs, 293T cells in 15-cm dishes were transfected with 10 μ g pMD.2G and 60 μ g pPOI-gagpol-D64V. IDLVs and LPs carrying the SB100XCO transposase (IDLV-SB100XCO/puro-PGK-SBT2 and LP-SB100XCO) were produced in a similar manner, using pSB100XCO-gagpol-D64V and pLV/puro-PGK-SBT2 instead of phyPBase-gagpol-D64V and pLV/puro-PGK-PBT, respectively. After transfection, the medium was replaced on day 3, and supernatants were harvested on day 4 and day 5, passed through a 0.45- μ m filter (Millipore) and centrifuged through a sucrose cushion at 25000 rpm for 2 h. Pellets were re-suspended in phosphate-buffered saline (PBS) and stored at -80°C . Concentrations of HIV-1 p24 were measured by enzyme-linked immunosorbent assay (ZeptoMetrix) according to the manufacturer's protocol.

Cells and culture conditions

HeLa (human cervical cancer cells), 293T, HEK293 (human embryonic kidney cells), HaCaT (human keratinocytes), HT1080 (fibrosarcoma cells), F9 (murine embryonic carcinoma cells) and normal human dermal fibroblasts (NHDFs) were cultured in Dulbecco's modified Eagle's medium (Lonza). CHANG and HepG2 (human liver hepatocellular carcinoma cells) were maintained in RPMI (Lonza). Culture medium was supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μ g/ml streptomycin and 250 μ g/ml L-glutamine. Human retina pigment epithelium ARPE-19 cells were maintained in culture medium containing 50% Ham's F-12 Nutrient Mixture (Invitrogen) and 50% Dulbecco's modified Eagle's medium with serum, glutamine, penicillin and streptomycin, as described earlier. Human primary keratinocytes were grown in serum-free keratinocyte medium (Gibco BRL-Life Technologies) supplemented with bovine pituitary extract (25 μ g/ml) and recombinant epidermal growth factor (0.1–0.2 ng/ml). All cells were cultured at 37°C and 5% (v/v) CO₂.

Colony formation assays

To study the transfer and transposition of the PBT/PGK-puro transposon vector—either carried by plasmid DNA or in lentiviral vectors—we performed colony formation assays in a series of cell types. Cells were seeded in 6-well plates at a density of 10^5 cells/well in 2 ml medium 1 day before transduction with LPs or IDLVs in the presence of polybrene (8 μ g/ml; Sigma-Aldrich). In studies of protein transduction with LP-hypBase in HeLa cells, cells were transfected with 1.95 μ g pPBT-PGK-puro using X-tremeGENE 9 (Roche) 1 day before transduction. Co-transfection of 50 ng pCMV-hypBase and 1.95 μ g pPBT-PGK-puro served as positive control. One day after transduction, the cells were trypsinized and re-seeded in 10-cm dishes at different dilutions. Two days later, cells were subjected to selection with 1 μ g/ml puromycin (Sigma-Aldrich) for 8 days. Drug-resistant colonies were

stained with methylene blue (Sigma-Aldrich) and counted. All experiments were performed in triplicates.

Luciferase assays

In studies of NanoLuc protein transduction, HeLa cells were seeded in a 96-well plate (10^4 /well) 1 day before the transduction of the cells with equal volumes of LP-NanoLuc produced with or without Vesicular Stomatitis Virus envelope glycoprotein (VSV-G) pseudotyping. Luminescence was analyzed 48 h posttransduction. For studies of transfer of the Firefly luciferase (Fluc) gene, HaCaT and ARPE-19 cells were seeded in a 96-well plate (1000 cells/well) 1 day before transduction with increasing amounts of IDLV-hyPBBase/Fluc-PGK-PBT or IDLV-hyPBmut/Fluc-PGK-PBT. Ten days after transduction, cells were analyzed for luminescence. HEK293 cells, primary keratinocytes and NHDFs were seeded in 24-well plates (5000 cells/well) on the day before transduction and then transduced with IDLV-hyPBBase/Fluc-PGK-PBT or IDLV-hyPBmut/Fluc-PGK-PBT (200 ng p24). Luminescence analysis was carried out 8 days after transduction, and cells were transferred to 96-well plates 6 h before analysis. NanoLuc luciferase and Firefly luciferase activity were analyzed by Nano-Glo™ and ONE-Glo™ luciferase assay system (Promega), respectively. Luminescence analysis was performed on a multi-sample plate-reading luminometer (Berthold).

Western blot analysis of protein-engineered LPs

To analyze the protein content of protein-engineered LPs, LP-containing supernatants were centrifuged through a 20% (w/v in PBS) sucrose cushion. LPs and LP-producing 293T cells were lysed in the presence of a protease inhibitor. 293T cells transfected with 2 μ g of pCMV-HAhyPBBase, or pCMV-SB100XCO, served as positive control and IDLVs without protein incorporated served as negative control. Either 30 μ g of cellular protein or 30 μ l of concentrated viral protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to the polyvinylidene difluoride (PVDF) membrane. Membranes were blocked by 5% fat-free milk dissolved in tris-buffered saline /0.05% Tween-20 for 1 h and incubated with a HIV-1 p24 polyclonal antibody (Thermo Scientific) or an HA monoclonal antibody (Covance) overnight at 4°C. The membranes were incubated with either peroxidase-conjugated anti-rabbit (Dako) or anti-mouse (Dako) secondary antibodies and visualized by enhanced chemiluminescence using a horseradish peroxidase substrate (Thermo Scientific). In studies of LP-HA-hyPBBase, the PVDF membrane was re-used for incubation with HA monoclonal antibody after washing away HIV-1 p24 polyclonal antibody with stripping buffer (Thermo Scientific). To detect SB100XCO, the PVDF membrane was incubated with primary polyclonal antibody against SB transposase (provided by Perry Hackett, University of Minnesota, Minnesota, USA).

Identification of hyPBBase-directed transposon insertions

To rescue integration sites from HeLa cells treated with lentivirally delivered transposase protein, individual puromycin-resistant HeLa colonies were isolated and separately expanded. Genomic DNA was extracted by saturated NaCl, digested with restriction enzyme NheI and XbaI and ligated by T4 ligase to generate DNA circles of random lengths harboring junction sites between the transposon and the neighboring genomic DNA. A two-step nested PCR (using first primers 5'-cctaaatgcacagcgacgga-3' and 5'-gtggttctaagtactgtgtttcc-3' and next the nested primers 5'-cgcgctatttagaagagagagc-3' and 5'-gaagaacgagatcagcagcctc-3') was performed to amplify the junctions between inserted transposons and genomic DNA. The resulting PCR fragments were purified and sequenced.

Copy number analyses by Southern blotting

HeLa cells transduced with IDLV-hyPBBase/puro-PGK-PBT (250 ng p24) or co-transfected with pCMV-hyPBBase (50 ng) and pLV/puro-PGK-PBT (250 ng) were treated with puromycin, and Southern blot analysis was performed on puromycin-resistant colonies as follows. Fifteen micrograms of genomic DNA from each clone was digested overnight with DraI before gel electrophoresis and vacuum blotting. Random labeling of the puromycin probe (using a PCR-amplified labeling template) was carried out by using the Prime-It random primer labeling kit (Agilent Technologies) according to the manufacturer's instructions.

Flow cytometry and confocal microscopy

HEK293 cells were seeded in 6-well plates (2×10^5 cells/well), and on the following day transduced with LP-GFP prepared with or without VSV-G pseudotyping. Twenty-four hours after transduction, cells were washed with PBS before being harvested and fixed with 4% paraformaldehyde. Data were collected on a FACSCalibur (Becton Dickinson) and analyzed with FlowJo (Tree Star). For confocal microscopy, HeLa cells were seeded at a concentration of 2×10^5 cells/well 1 day before transduction or transfection. Cells were then transduced with LP-HAhyPBBase (250 ng p24) or transfected with 2 μ g pCMV-HAhyPBBase. After 24 h, cells were washed three times with PBS and fixed with 4% paraformaldehyde. Slides were stored in 70% ethanol for at least 15 min. Fixed cells were then incubated with primary HA monoclonal antibody (Covance) and secondary Alexa Fluor® 488 Goat Anti-Mouse IgG (Invitrogen). Sequential imaging was done by using a 488-nm line of a multiline argon laser (detection of Alexa-488), and the 405-nm line of a 405–30 diode laser (detection of DAPI) on a confocal laser scanning microscope (LSM 710, Zeiss, Jene, Germany) using 63 oil-immersion objective with a numerical aperture of 1.4.

Quantitative real-time PCR analysis of viral RNA

RNA was purified from viral particles (15 ng p24) using the GeneJET Viral DNA and RNA Purification Kit

according to manufacturer's protocol (Thermo Scientific). Purified viral RNA was treated with RNase-free recombinant DNase (Thermo Scientific) and used for cDNA synthesis using Maxima First Strand cDNA Synthesis Kit for qPCR (Thermo Scientific) according to manufacturer's guidelines. qPCR reactions were performed using the TaqMan Universal Master Mix II (Applied Biosystems). For quantification of viral genomes, we used primers (5'-GGCACTGACAATTCCGTGGT-3' and 5'-AGGGACGTAGCAGAAGGACG-3') and a probe (5'-ACGTCCTTCCATGGCTGCTCGC-3'; FAM-BHQ) located in the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) of the lentiviral vector genome. Primers and probe were purchased from TAG Copenhagen. qPCR reactions were performed in four technical replicates on a LightCycler 480 (Roche). PCR conditions were 10 min at 95°C, 45 cycles of 15 s at 95°C and 60 s at 60°C. Total viral RNA levels were determined using the standard curve method. Briefly, a standard curve was made from serial dilutions of the lentiviral transfer plasmid pCCL-PGK-puro-H1-MCS harboring the WPRE sequence (39). The standard curve was used to calculate the total levels of viral RNA, and the data were normalized to the amount of p24 used for qPCR.

Statistical analysis

Data are presented as mean \pm SD in all experiments ($n \geq 3$). Student's *t*-tests were performed as indicated. (* indicates statistical significance, $P < 0.05$).

RESULTS

Incorporation, processing and transduction of foreign protein in LPs

As a main strategy for achieving LP-directed protein delivery to cells, proteins of interest (POI) including GFP, nano luciferase (NanoLuc) and the hypBase transposase were incorporated into the Gag polypeptide at a position between a heterologous myristoylation signal derived from the Lyn kinase and the HIV-1 matrix protein (Figure 1A). To release the POI from Gag and GagPol precursors, the HIV-1 protease cleavage site SQNY/PIVQ was inserted at the border of the POI and the matrix protein (Figure 1A). LPs were generated by co-transfecting HEK293T cells with pPOI-gagpol-D64V and plasmid DNA encoding the viral surface protein VSV-G (Figure 1B). Negative controls included LPs produced in the absence of VSV-G. Efficient VSV-G-dependent protein transfer to cells was detected with LPs containing GagPol-fused GFP and NanoLuc. In both HEK293 and HeLa cells (for delivery of GFP and NanoLuc, respectively), the protein was delivered by VSV-G-coated LPs, facilitating an uptake of cellular protein that was significantly higher than that obtained with VSV-G-negative controls (Figure 1C and D).

To explore LP-directed delivery of hypBase to target cells, we first confirmed the incorporation of hypBase in LPs. Owing to the lack of an anti-hypBase antibody, we inserted an HA-tagged version of the protein in the GagPol

precursor and confirmed, by western blot analysis, incorporation of either HA-hypBase or a mutated catalytically inactive version of the protein (HA-hypBmut) in the LPs (Figure 1E). Viral lysates of integrase-deficient lentiviral vector (IDLV) served as a negative control, and a cellular lysate of 293T cells transfected with pCMV-HAhypBase served as positive control. Notably, HA-hypBase released from the GagPol polypeptide included an N-terminal heterologous myristoylation signal as well as residual amino acids from the protease cleavage site in the C-terminus, and therefore was slightly larger than the protein encoded by pCMV-HAhypBase. In LPs produced with a packaging construct, in which the transposase was not flanked by a protease cleavage site (HA-hypBase Δ PC), we detected a protein that was larger than LP-delivered HA-hypBase (Figure 1E), indicating that the HA-hypBase even in the absence of a protease cleavage site was released by cleavage at an alternative site closer to the C-terminus of Gag. These findings indicated that HA-hypBase flanked by the SQNY/PIVQ sequence was released from the GagPol polypeptide by proteolytic cleavage. To confirm this, we treated LP-producing cells with 2 μ M saquinavir (SQV), an HIV-1 protease inhibitor, and verified reduced GagPol processing both in lysates from LP producer cells and in purified LPs using an anti-p24 antibody (Figure 1F, two left panels). In the presence of saquinavir, we were not able to detect released HA-hypBase in the LPs, indicating that processing by the protease was responsible for release of protein and exposure of the HA tag for detection by the antibody (Figure 1F, right). This conclusion was supported by the fact that we in repeated western blot analyses using the anti-HA antibody were not able to detect HA-tagged Gag or GagPol in LP- or vector-producing cells (Supplementary Figure S1A and B, left), whereas the proteolytically released HA-hypBase was detectable in particles derived from the LP- and lentiviral vector-producing cells (Supplementary Figure S1A and B, right).

To demonstrate the activity of LP-delivered hypBase, HeLa cells were transfected with pPBT/PGK-puro, carrying a transposon expressing the puromycin *N*-acetyl-transferase gene (puro), and on the following day treated with LP-hypBase, LP-hypBmut or VSV-G-deficient LP-hypBase. Notably, only among cells treated with VSV-G-pseudotyped LP-hypBase, we detected a high number of puromycin-resistant colonies (Figure 1G). For comparison, we performed in parallel an optimized standard plasmid-based transposition experiment based on co-transfection of pPBT/PGK-puro and pCMV-hypBase and found that this treatment did not create as many puromycin-resistant colonies as with VSV-G-pseudotyped LP-hypBase (Figure 1G). In summary, our findings document efficient incorporation and release of hypBase in LPs as well as robust *PB* transposition in cells treated by hypBase protein transduction.

Efficient *PB* transposition by co-delivery of hypBase protein and transposon donor by IDLVs

As hypBase could be successfully delivered by LPs, we then asked whether DNA transposition could be achieved by co-incorporating the transposase protein

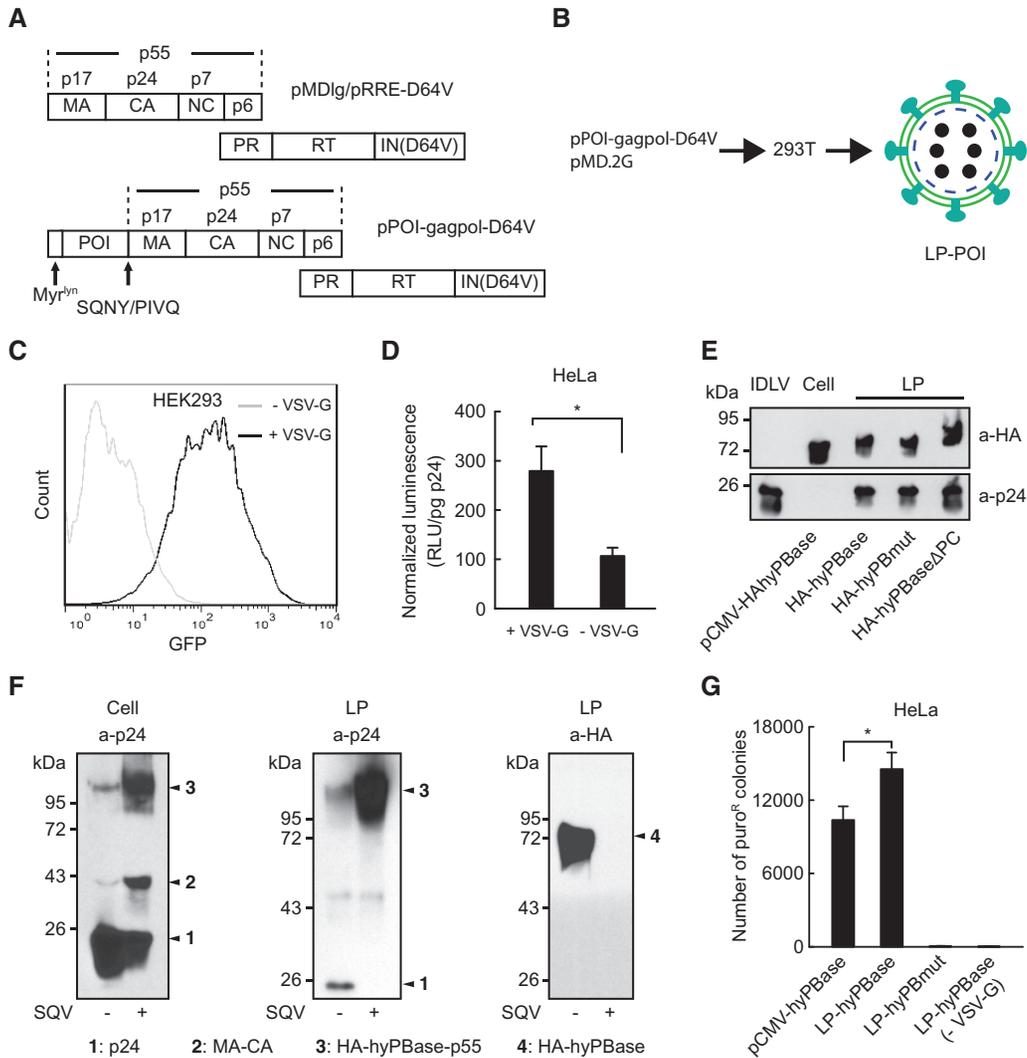


Figure 1. Incorporation of *PB* transposase in LPs leads to efficient DNA transposition by lentiviral transduction of target cells. (A) Schematic representations of third-generation lentiviral packaging constructs showing the individual entities of the GagPol polyprotein. In the standard packaging construct (upper), Gag is composed of matrix (MA), capsid (CA), nucleocapsid (NC) and p6, whereas Pol consists of protease (PR), reverse transcriptase (RT) and integrase (IN; here, a defective integrase containing the D64V mutation). The protein reference based on molecular mass is provided above the corresponding entity. For production of protein-transducing viruses, the protein of interest (POI) is inserted between a Lyn-derived myristoylation signal (Myr^{lyn}) and a codon-optimized GagPol-coding sequence (lower). A HIV-1 protease cleavage site SQNY/PIVQ is inserted between POI and GagPol. In this study, POIs include GFP, NanoLuc and hyPBBase transposase protein. (B) Schematic representation of a LP containing a specific protein of interest (LP-POI). LPs were generated by co-transfecting 293T cells with pPOI-gagpol-D64V and pMD.2G encoding the viral surface protein VSV-G. Black dots indicate units of hyPBBase protein. (C) Lentiviral transduction of GFP protein to HEK293 cells. Flow cytometric analysis was performed 24 h post-transduction. Histogram shows the relative level of GFP fluorescence. The black curve represents cells that were transduced with LP-GFP pseudotyped with VSV-G (152 ng p24), whereas the gray curve is indicative of cells that were treated with LP-GFP without VSV-G (174 ng p24). (D) Lentiviral transduction of NanoLuc to HeLa cells. Luminescence was measured 48 h post-transduction and normalized to the p24 content of LP-NanoLuc particles with and without VSV-G. (E) Evidence of incorporation of hyPBBase into LPs. Western blot analysis was performed on LP lysates (three right lanes) using antibodies for the HA tag (top blot) and p24 (bottom blot). IDLV that did not incorporate hyPBBase was used as negative control, and lysate from 293T cells transfected with pCMV-HAhyPBBase served as positive control. (F) Protease-directed cleavage of the GagPol-fused hyPBBase after virus release and maturation. To restrict viral protein processing, LP-producing 293T cells were treated with the protease-inhibitor saquinavir (SQV), or DMSO as a control. Blotting membrane of LP was initially incubated with anti-p24 antibody (middle blot) and reused for anti-HA antibody after stripping (right blot). (G) DNA transposition catalyzed by lentivirally delivered hyPBBase protein. HeLa cells were transfected with 1.95- μ g pPBT-PGK-puro (or co-transfected with 50-ng pCMV-hyPBBase as a positive control, left column) 1 day before transduction with LP-hyPBBase, LP-hyPBmut or LP-hyPBBase(-VSV-G) (in all cases 250 ng p24) and selected for puromycin resistance.

and vector RNA carrying the transposon element into LPs. A schematic representation of the generation, transduction and integration of such hybrid viruses is provided in Figure 2A. We first delivered the transposase and transposon separately as LP-contained hyPBBase protein and

transposon RNA carried by IDLVs, respectively, to validate that each component was functional (Figure 2B). We have previously shown that *SB* transposon elements can be delivered as RNA by IDLVs and efficiently transposed—after reverse transcription—into the

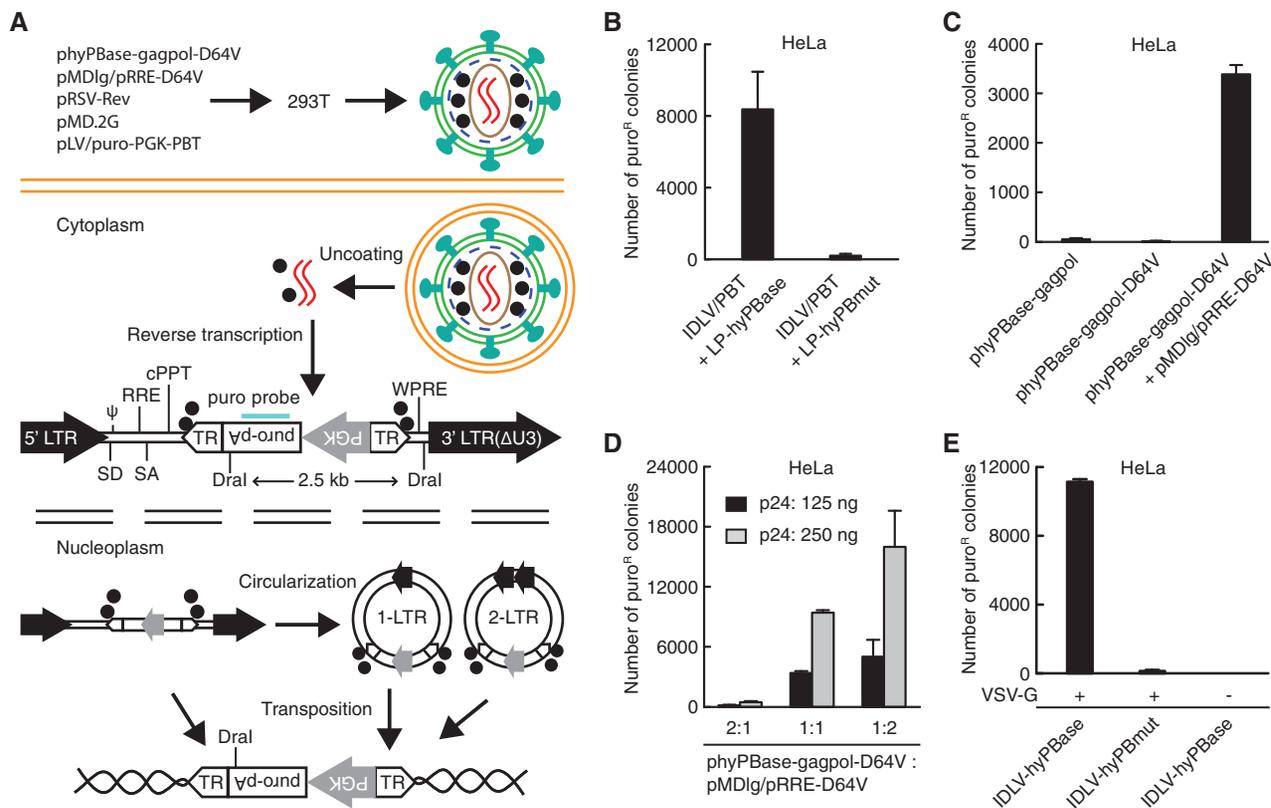


Figure 2. The hypBase protein incorporated into lentiviral vectors catalyzes mobilization of co-delivered and reverse-transcribed transposon vector. (A) Schematic representation of the production, transduction and integration of an IDLV containing (i) hypBase protein and (ii) the PBT/PGK-puro transposon carried by the lentiviral vector. This 'hybrid' vector, IDLV-hyPBBase/puro-PGK-PBT, was generated by co-transfecting 293T cells with phyPBBase-gagpol-D64V, pMDIlg/pRRE-D64V, pRSV-Rev, pMD.2G and pLV/puro-PGK-PBT. On viral entry, this vector is taken up by endocytosis and released to the cytoplasm ('uncoating'). Linear double-stranded DNA is formed by reverse transcription of vector RNA (indicated by red lines). Circular DNA can be formed either by homologous recombination (HR, leading to 1-LTR circles) or non-homologous end joining (NHEJ, leading to 2-LTR circles). The third-generation SIN vector pLV/puro-PGK-PBT contains the inverted *PB* transposon cassette flanked by terminal regions (TR). The puromycin resistance gene (*puro*) is driven by the PGK promoter. SD, ψ , RRE, SA, cPPT, WPRE indicate the location of the splice donor, packaging signal, Rev-responsive element, splice acceptor, central polyurine tract and the woodchuck hepatitis virus post-transcriptional regulatory element, respectively. Units of transduced hypBase protein, indicated throughout as black dots, eventually facilitate DNA transposition from linear and circular transposon substrates. (B) Evidence of transposition from an IDLV-delivered DNA transposon (125 ng p24) catalyzed by LP-delivered hypBase transposase (125 ng p24). LP-directed delivery of the inactive hypBmut transposase did not lead to colony formation. (C) Vector transfer is restricted in IDLVs packaged with hypBase-GagPol only, but rescued by co-incorporation of unfused GagPol polypeptide into the virus particles. LVs carrying the normal active IN or IN-D64V were not able to transfer the vector (left and middle column), but vector transfer and active transposition was re-established in IDLVs generated by using a 1:1 ratio (w/w) of phyPBBase-gagpol-D64V and pMDIlg/pRRE-D64V (right column). In all cases, 125 ng p24 was used. (D) Increased transposition efficiency with a reduced ratio (w/w) of the packaging constructs phyPBBase-gagpol-D64V and pMDIlg/pRRE-D64V. (E) Gene insertion by DNA transposition catalyzed by IDLV-directed delivery of hypBase transposase and transposon is VSV-G-dependent. Only VSV-G-coated IDLVs harboring hypBase (along with standard, unfused GagPol polypeptide) facilitated efficient DNA transposition. In all cases, 250 ng p24 was used.

genome (22). In accordance, solid *PB* transposition was achieved from DNA generated by reverse transcription of lentivirally delivered vector RNA containing the PBT/PGK-puro transposon (inserted into the vector in an orientation that was opposite to the orientation for production of full-length vector RNA). Notably, LP-delivered hypBase was able to get access to the *PB* transposon and facilitate efficient transposition, resulting in >8000 puromycin-resistant colonies in LP/IDLV-treated cells (Figure 2B). In contrast, treatment of the cells with LPs carrying the inactive hypBmut resulted only in few colonies.

We subsequently sought to package the transposon-carrying lentiviral vector in viral particles consisting of hypBase-fused GagPol polypeptides. However, when

such viruses were produced with either phyPBBase-gagpol or phyPBBase-gagpol-D64V (harboring normal and D64V-mutated integrase, respectively) as the only source of GagPol, neither vector transfer (in the case of phyPBBase-gagpol) nor transposition (in the case of phyPBBase-gagpol-D64V) was observed (Figure 2C). These findings demonstrated that hypBase-containing LVs were not able to transfer the vector. However, by supplementing pMDIlg/pRRE-D64V, the standard packaging construct containing the D64V-mutated integrase, in the mixture of plasmids used for vector production, we were able to rescue DNA transposition. Hence, by co-transfecting phyPBBase-gagpol-D64V and pMDIlg/pRRE-D64V in a 1:1 ratio (w/w), we registered a marked increase in *PB* transposition (Figure 2C, right column). As shown in

Figure 2D, the efficiency of transposition was increased at a 1:2 ratio of phyPBBase-gagpol-D64V and pMDlg/pRRE-D64V, whereas more extreme ratios of the two packaging constructs, 1:4 and 4:1, resulted in lower levels of transposition (Supplementary Figure S2). Finally, we verified that the production of puromycin-resistant colonies was both hyPBBase- and VSV-G-dependent (Figure 2E), demonstrating that gene insertion was catalyzed by LV-delivered hyPBBase protein. In accordance, we confirmed that insertion of the *PB* transposon was directed by the transposase. We sequenced 15 transposon junction sites derived from puromycin-resistant HeLa clones and found that all integrations were flanked by the TTAA sequence as a hallmark of *PB* transposition (Supplementary Table S2).

In a parallel series of experiments, we attempted to establish a similar approach for transduction of a codon-optimized version of the hyperactive SB100X transposase (SB100XCO) (Supplementary Figure S3A and B). However, despite the fact that SB100XCO was incorporated into viral particles and proteolytically cleaved from Gag (Supplementary Figure S3C), we never achieved DNA transposition by this approach. In control experiments, we tested the transposition activity of plasmid-encoded SB100XCO harboring a C-terminal extension consisting of five amino acid residues. This short tag rendered the transposase completely inactive compared with appropriate controls (Supplementary Figure S3D), suggesting that the *SB* transposase delivered in IDLVs was inactive and incapable of catalyzing mobilization of the transposon vector due to the presence of the C-terminal tag created after cleavage by the lentiviral protease.

***PB* transposition facilitated by transduced hyPBBase transposase in cell lines and primary cells**

To examine the versatility of a transposase delivery approach based on transduction from lentiviral Gag precursors, we transduced a series of cell lines including F9 murine embryonic carcinoma cells, HaCaT human keratinocytes, HEK293 embryonic kidney cells, HT1080 fibrosarcoma cells and CHANG and HepG2 human liver cells with IDLV-hyPBBase/puro-PGK-PBT (250 ng p24). For all cell lines, we observed efficient DNA transposition, as reflected by the number of puromycin-resistant colonies obtained with IDLV-hyPBBase/puro-PGK-PBT relative to the background level observed for IDLV-hyPBmut/puro-PGK-PBT carrying the inactive hyPBmut transposase (Figure 3A).

To carry out transposition assays that did not rely on selection, we also examined gene insertion by viral particles (designated IDLV-hyPBBase/Fluc-PGK-PBT) carrying the transposase protein and a transposon encoding the Fluc reporter gene. With this transposase/transposon carrier, we observed robust transposition in HaCaT cells (Figure 3B) providing 16-fold higher levels of persistent gene expression in hyPBBase- relative to hyPBmut-treated cells. Similar levels of DNA transposition were found in ARPE-19 human retina pigment epithelium cells treated with IDLV-hyPBBase/Fluc-PGK-PBT (Figure 3C). For both cell lines, the transposition rate increased with the amount of virus

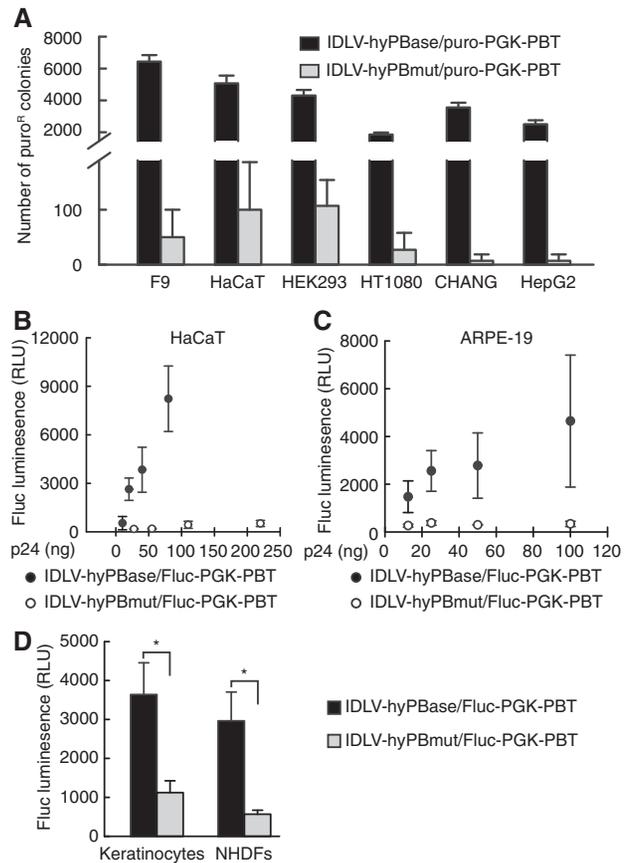


Figure 3. Potent DNA transposition by IDLVs carrying hyPBBase and transposon vector in cell lines and primary cells. (A) Efficient transposition by IDLV-hyPBBase/puro-PGK-PBT in a variety of cell lines. Cell lines were transduced with IDLV-hyPBBase/puro-PGK-PBT or IDLV-hyPBmut/puro-PGK-PBT (250-ng p24) and selected for puromycin-resistant colonies. (B and C) Increasing transposition in HaCaT and ARPE-19 cells with increased amounts of IDLV-hyPBBase/Fluc-PGK-PBT relative to the negative control, IDLV-hyPBmut/Fluc-PGK-PBT. Dose-response assays were carried out with increasing amounts of p24, as indicated. Luminescence was measured 10 days post-transduction. (D) DNA transposition facilitated by lentivirally delivered hyPBBase in primary cells. Primary human keratinocytes and NHDFs were transduced with IDLV-hyPBBase/Fluc-PGK-PBT or IDLV-hyPBmut/Fluc-PGK-PBT (200 ng p24) and measured for luminescence 8 days after transduction.

used, whereas the vector carrying the mutated transposase did not induce levels of Fluc expression that were over background. Finally, we exposed primary human keratinocytes and NHDFs to IDLV-hyPBBase/Fluc-PGK-PBT and the counterpart containing hyPBmut (Figure 3D). Increased luminescence, measured 8 days after transduction, verified that solid DNA transposition could be achieved also in primary cells, although episomal persistence of the lentiviral vector in these cells increased background expression. Taken together, these findings show the versatility of an integration system based on lentiviral transposase transduction.

Uptake of hyPBBase on lentiviral protein transduction is limited in each cell but wide-spread in a population and leads to high-efficiency production of single-copy clones

To characterize in further detail the fate of virus-delivered transposase protein in transduced cells, we performed

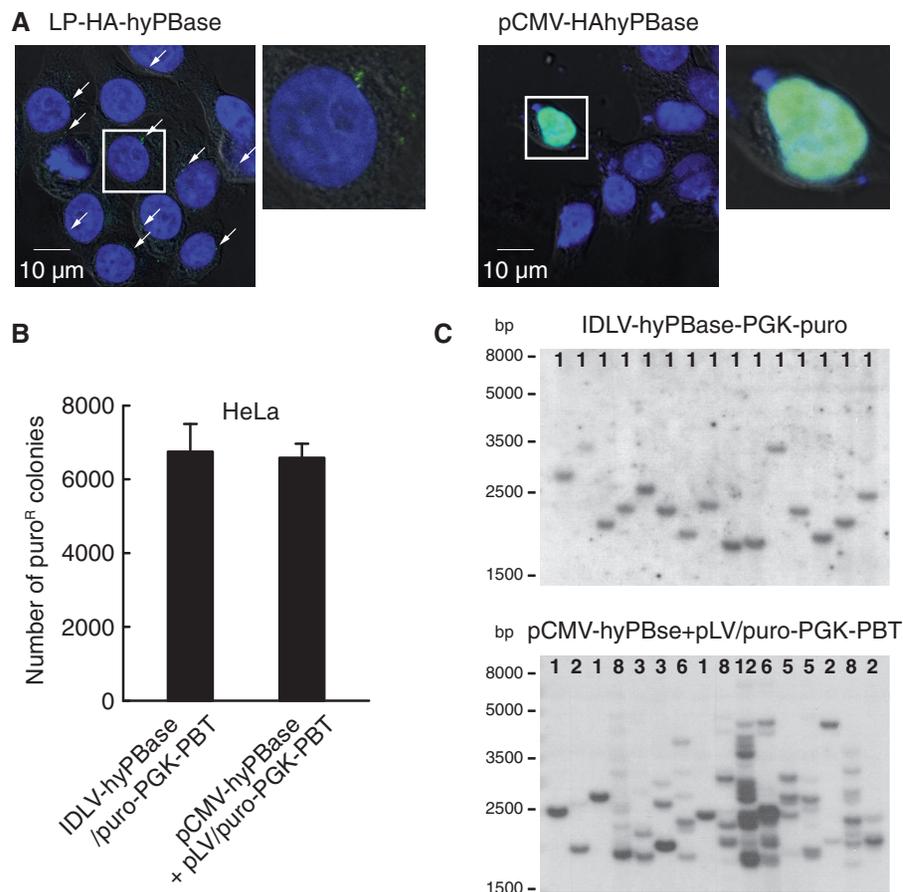


Figure 4. Robust DNA transposition, facilitated by a lentivirally restricted uptake of hyPBase, leads to generation of single-copy clones only. (A) Cellular prevalence of hyPBase on lentiviral protein delivery and plasmid-based expression in HeLa cells. Confocal laser scanning microscopy analysis was carried out to compare hyPBase distribution patterns in cells treated with LP-HA-hyPBase (left) and pCMV-HAhyPBase (right). By lentiviral protein delivery, few distinct dots, indicative of aggregates of hyPBase, could be registered in almost all cells. White arrows point to intracellular assemblies of hyPBase protein. In sharp contrast, the minority of the HeLa cells that had taken up plasmid DNA after transfection demonstrated accumulation of hyPBase in the nucleus. (B) Comparable levels of DNA transposition in HeLa cells obtained with IDLV-hyPBase/puro-PGK-PBT (250-ng p24) and by co-transfection of pCMV-hyPBase (50 ng) and pLV/puro-PGK-PBT (250 ng). (C) Lentiviral hyPBase transduction facilitates the production of single-copy clones. Copy numbers in IDLV-hyPBase/puro-PGK-PBT-treated cells and cells co-transfected with pCMV-hyPBase and pLV/puro-PGK-PBT were determined by Southern blot analysis of genomic DNA from puromycin-resistant colonies. HyPBase transduction led to production of single-copy clones only (15 single-copy clones out of 15 analyzed clones), whereas a plasmid transfection approach generated high copy number variability (3 single-copy clones out of 16 analyzed clones) and colonies that were mosaic. Estimated copy number of each clone is listed on top of each lane.

immunohistochemistry on LP-HA-hyPBase-treated HeLa cells with anti-HA antibody and examined the cells by confocal microscopy (Figure 4A). We reproducibly detected a limited number of fluorescent foci in each cell that had been transduced with the LPs. However, this pattern was evident for a large proportion of the cells (as indicated by white arrows in Figure 4A, left), suggesting that many cells in the population carried relatively few but locally highly concentrated transposase subunits. As a control, we transfected HeLa cells with pCMV-HAhyPBase and observed a different pattern; in this case, a relatively low percentage of the cells showed evidence of the protein (in accordance with the low transfection efficiency in HeLa cells), but cells that had been transfected showed substantial nuclear accumulation of the HA-hyPBase protein (Figure 4A, right).

We reasoned that such differences possibly could result in distinct patterns of integration. To directly compare

integration patterns of plasmid- and virus-based systems, we carried out a series of transposition experiments in HeLa cells with the two systems (transduction of IDLV-hyPBase/puro-PGK-PBT relative to co-transfection of pCMV-HyPBase and pLV/puro-PGK-PBT). Based on experimental settings that created high and comparable numbers of puromycin-resistant colonies (Figure 4B), we picked individual colonies and analyzed the number of insertions in each clone by Southern blot analysis (Figure 4C; location of probe and restriction sites shown in Figure 2A). Intriguingly, among 15 colonies that were randomly picked from cells transduced with IDLV-hyPBase/puro-PGK-PBT, all clones carried only a single transposon integrant (Figure 4C, upper) inserted by DNA transposition (as indicated by the size of *Dra*I cleavage products, which varied among the clones and differed from the 2.5-kb fragment that would be expected for an insertion of the entire lentiviral vector [Figure 2A]).

In contrast, only 3 of 16 clones that were isolated after plasmid transfection contained a single insertion, whereas the majority of the clones contained numerous insertions (Figure 4C, lower). As a typical consequence of prolonged transposase expression after transfection, some of these colonies were mosaic, as indicated by the different intensities of bands detected for individual colonies. In summary, based on these findings we conclude that a limited uptake of hyPBase protein, facilitated by virus-delivered protein transduction, leads to potent transposition and provides the unique capacity to deliver only a single copy of the transposon to the treated cells. This feature offers an attractive alternative to current plasmid-based transposon vector systems that are prone to form heterogeneous and multicopy clones due to difficulties of controlling the level and time frame of gene insertion on plasmid transfection. We conclude that transduction of the hyPBase transposase from lentiviral Gag precursors is applicable for uses that require both high efficiency and low-copy transgene insertion.

DISCUSSION

The use of DNA transposases as catalysts of gene insertion has impact in many areas of biomedicine including stem cell research (14,15) and gene transfer with therapeutic objectives (16,40). The engineering of hyperactive transposases, such as hyPBase (19) and SB100X (18), has improved the applicability of transposases, in particular for *in vivo* applications and in cell types, in which efficiency is compromised by low uptake of transposase-encoding nucleic acids and/or low levels of transposon donor. Common for all current uses of DNA transposon-based vector systems is that gene insertion by transposition relies on intracellular production of the transposase. Typically, the transposase is encoded by plasmid DNA (41,42) but may be expressed also by viral vectors carrying a transposase expression cassette (20,22). In an alternative approach, retroviral vectors carrying RNA incapable of undergoing reverse transcription have been adapted for delivery of mRNA as a source of *SB* transposase (26), just as transfection of *in vitro*-transcribed RNA facilitates short-term transposase expression (25,43). Reported attempts to produce recombinant *SB* and *PB* transposase protein are rare, reflecting the general challenges related to restoring activity of purified transposases. Purified *PB* and *SB* transposases fused to or incubated with cell-penetrating peptides induced only low levels of transposition (29,30). Still, delivery of transposase protein remains highly attractive, as this approach allows an immediate and short boost of transposase activity followed by intracellular decay of the protein. This reduces the risk of long-term transposase expression and limits any cytotoxicity related to prolonged production and potential insertional mutagenesis.

The present work establishes efficient DNA transposition in mammalian cells induced by delivery of transposase protein. We describe a new strategy to deliver the hyperactive hyPBase transposase in the context of engineered viral particles. By taking advantage

of the organized assembly of Gag and GagPol polypeptides during virus production, the transposase was incorporated in LPs as part of Gag/GagPol and subsequently liberated from the viral proteins by processing directed by the viral protease during maturation of the virus particle. We demonstrate that virally delivered transposase subunits bind transposon donors in transduced cells and are capable of catalyzing robust DNA transposition. These findings establish engineered lentivirus-derived nanoparticles, which do not carry any vector information, as safe and potent carriers of transposase protein and open up for the use of such carriers in many applications of transposon-based vector systems. Notably, by using an approach based on the release of transposase by proteolytic cleavage of the Gag polypeptide, we managed to deliver active hyPBase transposase, but not SB100X transposase. Evidently, the *SB* transposase was vulnerable to the short C-terminal extension originating from the protease cleavage site and therefore did not facilitate transposition in this context.

In an ideal scenario, transposase protein and transposon donors may be co-delivered in lentiviral complexes that facilitate efficient DNA transposition. This strategy is complicated by the fact that the transposon is incorporated in the particle in the context of vector RNA and is accessible for DNA transposition only after formation of double-stranded DNA by reverse transcription in the target cells. However, we and others have previously shown that such lentivirally delivered transposons may serve as substrates for the SB100X transposase (22,23). Therefore, we sought to combine transposase transduction from lentiviral Gag precursors with the delivery of vector-encoded transposon substrates. In the course of doing so, we found that IDLVs carrying only the hyPBase-Gag and hyPBase-GagPol fusion polypeptides were not capable of transferring the vector. Quantitative real-time PCR on particle RNA indicated that packaging of vector RNA was not affected by fusing hyPBase to Gag (Supplementary Figure S4). We assume that vector transduction is inhibited at a later stage, e.g. due to limitations during reverse transcription, but this is still unclear. To rescue vector transfer, we produced IDLV particles by co-expressing hyPBase-fused and unfused GagPol (both carrying the D64V mutation), facilitating most likely the generation of 'hybrid' particles carrying both wild-type and hyPBase-containing polypeptides. Notably, such IDLV preparations were able to co-deliver the hyPBase protein and the transposon vector, and the IDLV-hyPBase configuration facilitated stable gene expression by potent DNA transposition in a variety of cell lines as well as in primary cells.

Transposition facilitated by lentiviral transposase-transposon complexes varies in several ways from conventional plasmid- or RNA-based systems. Notably, with LPs as carriers of transposase, the cellular uptake of transposase in each cell was restricted, as documented by confocal microscopy, but evident in almost all cells in an LP-treated cell population. In contrast, plasmid-based delivery by transfection resulted in transposase expression only in relatively few cells, but these cells showed strong nuclear accumulation of the transposase. In accordance

with these differences, we identified the unique capacity of IDLV-hyPBase to create clones harboring only a single insertion of the DNA transposon, whereas plasmid co-transfection resulted in clones that were heterogeneous and contained multiple insertions. These findings support the notion that a high overall transposition rate by lentivirally delivered transposases is achieved through a modest uptake of both transposase and transposon in many cells, leading to a single event of transposition in only part of the cells carrying the transposase. In plasmid-based approaches, in contrast, several events of transposition occur in the relatively few cells expressing the transposase, leading to clones with multiple insertions. Our results establish transposase-carrying IDLVs as a new tool for inserting a single copy of a transgene expression cassette by transposition. This feature of protein transduction may be useful especially in gene therapy and transgenesis in which a single insertion is often favored.

DNA transposition facilitated by lentivirally delivered transposases and substrates may benefit from virus-based internalization and the option of targeting specific cell types. However, the method also has inherent limitations including the limited packaging capacity of lentiviral vectors, which does not allow incorporation of giant transposons (as large as 100 kb) that have been mobilized by the *PB* transposase (44). Also, the efficiency of protein delivery may vary among different cargo proteins depending on the size and structure, just like the efficiency and function may potentially be compromised by the existence of internal HIV-1 protease cleavage sites. Yet with the establishment of lentiviral transduction of transposases, our findings are paving the way for novel protein delivery approaches that may prove to be generally applicable in the field of genetic engineering with likely relevance for the delivery of programmable nucleases.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Ammar, I., Izsvak, Z. and Ivics, Z. (2012) The sleeping beauty transposon toolbox. *Methods Mol. Biol.*, **859**, 229–240.
2. Di Matteo, M., Matrai, J., Belay, E., Firdissa, T., Vandendriessche, T. and Chuah, M.K. (2012) PiggyBac toolbox. *Methods Mol. Biol.*, **859**, 241–254.
3. Ivics, Z., Hackett, P.B., Plasterk, R.H. and Izsvak, Z. (1997) Molecular reconstruction of sleeping beauty, a Tc1-like transposon from fish, and its transposition in human cells. *Cell*, **91**, 501–510.
4. Fraser, M.J., Ciszczon, T., Elick, T. and Bauser, C. (1996) Precise excision of TTAA-specific lepidopteran transposons piggyBac (IFP2) and tagalong (TFP3) from the baculovirus genome in cell lines from two species of Lepidoptera. *Insect Mol. Biol.*, **5**, 141–151.
5. Ding, S., Wu, X., Li, G., Han, M., Zhuang, Y. and Xu, T. (2005) Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice. *Cell*, **122**, 473–483.
6. Woodard, L.E., Li, X., Malani, N., Kaja, A., Hice, R.H., Atkinson, P.W., Bushman, F.D., Craig, N.L. and Wilson, M.H. (2012) Comparative analysis of the recently discovered hAT transposon TcBuster in human cells. *PLoS One*, **7**, e42666.
7. Li, X., Ewis, H., Hice, R.H., Malani, N., Parker, N., Zhou, L., Feschotte, C., Bushman, F.D., Atkinson, P.W. and Craig, N.L. (2013) A resurrected mammalian hAT transposable element and a closely related insect element are highly active in human cell culture. *Proc. Natl Acad. Sci. USA*, **110**, E478–E487.
8. Mitra, R., Li, X., Kapusta, A., Mayhew, D., Mitra, R.D., Feschotte, C. and Craig, N.L. (2013) Functional characterization of piggyBat from the bat *Myotis lucifugus* unveils an active mammalian DNA transposon. *Proc. Natl Acad. Sci. USA*, **110**, 234–239.
9. Rad, R., Rad, L., Wang, W., Cadinanos, J., Vassiliou, G., Rice, S., Campos, L.S., Yusa, K., Banerjee, R., Li, M.A. *et al.* (2010) PiggyBac transposon mutagenesis: a tool for cancer gene discovery in mice. *Science*, **330**, 1104–1107.
10. Collier, L.S., Carlson, C.M., Ravimohan, S., Dupuy, A.J. and Largaespada, D.A. (2005) Cancer gene discovery in solid tumours using transposon-based somatic mutagenesis in the mouse. *Nature*, **436**, 272–276.
11. Dupuy, A.J., Akagi, K., Largaespada, D.A., Copeland, N.G. and Jenkins, N.A. (2005) Mammalian mutagenesis using a highly mobile somatic sleeping beauty transposon system. *Nature*, **436**, 221–226.
12. Marh, J., Stoytcheva, Z., Urschitz, J., Sugawara, A., Yamashiro, H., Owens, J.B., Stoytchev, I., Pelczar, P., Yanagimachi, R. and Moisyadi, S. (2012) Hyperactive self-inactivating piggyBac for transposase-enhanced pronuclear microinjection transgenesis. *Proc. Natl. Acad. Sci. USA*, **109**, 19184–19189.
13. Al-Mashhadi, R.H., Sorensen, C.B., Kragh, P.M., Christoffersen, C., Mortensen, M.B., Tolbod, L.P., Thim, T., Du, Y., Li, J., Liu, Y. *et al.* (2013) Familial hypercholesterolemia and atherosclerosis in cloned minipigs created by DNA transposition of a human PCSK9 gain-of-function mutant. *Sci. Transl. Med.*, **5**, 166ra161.

14. Yusa, K., Rad, R., Takeda, J. and Bradley, A. (2009) Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. *Nat. Methods*, **6**, 363–369.
15. Grabundzija, I., Wang, J., Sebe, A., Erdei, Z., Kajdi, R., Devaraj, A., Steinemann, D., Szuhai, K., Stein, U., Cantz, T. *et al.* (2013) Sleeping beauty transposon-based system for cellular reprogramming and targeted gene insertion in induced pluripotent stem cells. *Nucleic Acids Res.*, **41**, 1829–1847.
16. Filareto, A., Parker, S., Darabi, R., Borges, L., Iacovino, M., SchAAF, T., Mayerhofer, T., Chamberlain, J.S., Ervasti, J.M., McIvor, R.S. *et al.* (2013) An *ex vivo* gene therapy approach to treat muscular dystrophy using inducible pluripotent stem cells. *Nat. Commun.*, **4**, 1549.
17. Nakazawa, Y., Huye, L.E., Salsman, V.S., Leen, A.M., Ahmed, N., Rollins, L., Dotti, G., Gottschalk, S.M., Wilson, M.H. and Rooney, C.M. (2011) PiggyBac-mediated cancer immunotherapy using EBV-specific cytotoxic T-cells expressing HER2-specific chimeric antigen receptor. *Mol. Ther.*, **19**, 2133–2143.
18. Mates, L., Chuah, M.K., Belay, E., Jerchow, B., Manoj, N., Acosta-Sanchez, A., Grzela, D.P., Schmitt, A., Becker, K., Matrai, J. *et al.* (2009) Molecular evolution of a novel hyperactive sleeping beauty transposase enables robust stable gene transfer in vertebrates. *Nat. Genet.*, **41**, 753–761.
19. Yusa, K., Zhou, L., Li, M.A., Bradley, A. and Craig, N.L. (2011) A hyperactive piggyBac transposase for mammalian applications. *Proc. Natl. Acad. Sci. USA*, **108**, 1531–1536.
20. Yant, S.R., Ehrhardt, A., Mikkelsen, J.G., Meuse, L., Pham, T. and Kay, M.A. (2002) Transposition from a gutless adeno-transposon vector stabilizes transgene expression *in vivo*. *Nat. Biotechnol.*, **20**, 999–1005.
21. Bowers, W.J., Mastrangelo, M.A., Howard, D.F., Southerland, H.A., Maguire-Zeiss, K.A. and Federoff, H.J. (2006) Neuronal precursor-restricted transduction via *in utero* CNS gene delivery of a novel bipartite HSV amplicon/transposase hybrid vector. *Mol. Ther.*, **13**, 580–588.
22. Staunstrup, N.H., Moldt, B., Mates, L., Villesen, P., Jakobsen, M., Ivics, Z., Izsvak, Z. and Mikkelsen, J.G. (2009) Hybrid lentivirus-transposon vectors with a random integration profile in human cells. *Mol. Ther.*, **17**, 1205–1214.
23. Vink, C.A., Gaspar, H.B., Gabriel, R., Schmidt, M., McIvor, R.S., Thrasher, A.J. and Qasim, W. (2009) Sleeping beauty transposition from nonintegrating lentivirus. *Mol. Ther.*, **17**, 1197–1204.
24. Huang, X., Haley, K., Wong, M., Guo, H., Lu, C., Wilber, A. and Zhou, X. (2010) Unexpectedly high copy number of random integration but low frequency of persistent expression of the sleeping beauty transposase after trans delivery in primary human T cells. *Hum. Gene Ther.*, **21**, 1577–1590.
25. Wilber, A., Frandsen, J.L., Geurts, J.L., Largaespada, D.A., Hackett, P.B. and McIvor, R.S. (2006) RNA as a source of transposase for sleeping beauty-mediated gene insertion and expression in somatic cells and tissues. *Mol. Ther.*, **13**, 625–630.
26. Galla, M., Schambach, A., Falk, C.S., Maetzig, T., Kuehle, J., Lange, K., Zychlinski, D., Heinz, N., Brugman, M.H., Gohring, G. *et al.* (2011) Avoiding cytotoxicity of transposases by dose-controlled mRNA delivery. *Nucleic Acids Res.*, **39**, 7147–7160.
27. Mellert, K., Lamla, M., Scheffzek, K., Wittig, R. and Kaufmann, D. (2012) Enhancing endosomal escape of transduced proteins by photochemical internalisation. *PLoS One*, **7**, e52473.
28. Gaj, T., Guo, J., Kato, Y., Sirk, S.J. and Barbas, C.F. III (2012) Targeted gene knockout by direct delivery of zinc-finger nuclease proteins. *Nat. Methods*, **9**, 805–807.
29. Lee, C.Y., Li, J.F., Liou, J.S., Charng, Y.C., Huang, Y.W. and Lee, H.J. (2011) A gene delivery system for human cells mediated by both a cell-penetrating peptide and a piggyBac transposase. *Biomaterials*, **32**, 6264–6276.
30. Järver, P., Fernaeus, S., El-Andaloussi, S., Tjörnhammar, M.-L. and Langel, Ü. (2008) Co-transduction of sleeping beauty transposase and donor plasmid via a cell-penetrating peptide: a simple one step method. *Int. J. Peptide. Res. Therap.*, **14**, 58–63.
31. Aoki, T., Miyauchi, K., Urano, E., Ichikawa, R. and Komano, J. (2011) Protein transduction by pseudotyped lentivirus-like nanoparticles. *Gene Ther.*, **18**, 936–941.
32. Miyauchi, K., Urano, E., Takizawa, M., Ichikawa, R. and Komano, J. (2012) Therapeutic potential of HIV protease-activable CASP3. *Sci. Rep.*, **2**, 359.
33. Voelkel, C., Galla, M., Maetzig, T., Warlich, E., Kuehle, J., Zychlinski, D., Bode, J., Cantz, T., Schambach, A. and Baum, C. (2010) Protein transduction from retroviral Gag precursors. *Proc. Natl. Acad. Sci. USA*, **107**, 7805–7810.
34. Kaczmarczyk, S.J., Sitaraman, K., Young, H.A., Hughes, S.H. and Chatterjee, D.K. (2011) Protein delivery using engineered virus-like particles. *Proc. Natl. Acad. Sci. USA*, **108**, 16998–17003.
35. Izmiryan, A., Basmaciogullari, S., Henry, A., Paques, F. and Danos, O. (2011) Efficient gene targeting mediated by a lentiviral vector-associated meganuclease. *Nucleic Acids Res.*, **39**, 7610–7619.
36. Sharma, N., Cai, Y., Bak, R.O., Jakobsen, M.R., Schroder, L.D. and Mikkelsen, J.G. (2013) Efficient sleeping beauty DNA transposition from DNA minicircles. *Mol. Ther. Nucleic Acids*, **2**, e74.
37. Moldt, B., Miskey, C., Staunstrup, N.H., Gogol-Doring, A., Bak, R.O., Sharma, N., Mates, L., Izsvak, Z., Chen, W., Ivics, Z. *et al.* (2011) Comparative genomic integration profiling of sleeping beauty transposons mobilized with high efficacy from integrase-defective lentiviral vectors in primary human cells. *Mol. Ther.*, **19**, 1499–1510.
38. Sharma, N., Moldt, B., Dalsgaard, T., Jensen, T.G. and Mikkelsen, J.G. (2008) Regulated gene insertion by steroid-induced PhiC31 integrase. *Nucleic Acids Res.*, **36**, e67.
39. Bak, R.O., Stenderup, K., Rosada, C., Petersen, L.B., Moldt, B., Dagnaes-Hansen, F., Jakobsen, M., Kamp, S., Jensen, T.G., Dam, T.N. *et al.* (2011) Targeting of human interleukin-12B by small hairpin RNAs in xenografted psoriatic skin. *BMC Dermatol.*, **11**, 5.
40. Doherty, J.E., Huye, L.E., Yusa, K., Zhou, L., Craig, N.L. and Wilson, M.H. (2012) Hyperactive piggyBac gene transfer in human cells and *in vivo*. *Hum. Gene Ther.*, **23**, 311–320.
41. Mikkelsen, J.G., Yant, S.R., Meuse, L., Huang, Z., Xu, H. and Kay, M.A. (2003) Helper-independent sleeping beauty transposon-transposase vectors for efficient nonviral gene delivery and persistent gene expression *in vivo*. *Mol. Ther.*, **8**, 654–665.
42. Yant, S.R., Meuse, L., Chiu, W., Ivics, Z., Izsvak, Z. and Kay, M.A. (2000) Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system. *Nat. Genet.*, **25**, 35–41.
43. Bire, S., Gosset, D., Jegot, G., Midoux, P., Pichon, C. and Rouleux-Bonnin, F. (2013) Exogenous mRNA delivery and bioavailability in gene transfer mediated by piggyBac transposition. *BMC Biotechnol.*, **13**, 75.
44. Li, M.A., Turner, D.J., Ning, Z., Yusa, K., Liang, Q., Eckert, S., Rad, L., Fitzgerald, T.W., Craig, N.L. and Bradley, A. (2011) Mobilization of giant piggyBac transposons in the mouse genome. *Nucleic Acids Res.*, **39**, e148.