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NEXT-GENERATION VECTORS

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Specifically integrating vectors for targeted gene delivery: progress and prospects

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Integrating vectors based on viruses or transposons are efficient gene delivery vehicles and promising tools for gene therapy. While different vector systems have different preferences and biases when it comes to target site selection, integration can always occur at vast numbers of potential sites throughout the human genome. This can result in unpredictable expression of the transgene (position effects), and can disrupt host genes or regulatory elements (genotoxicity), thereby potentially causing malignant transformations. Our knowledge about the natural target site selection properties of these gene insertion systems can be translated into artificial, experimental retargeting with the goal of introducing a bias into their insertion profiles. Here, we provide an overview of naturally occurring targeting mechanisms of viruses and transposons, and of the different molecular strategies that have been followed to manipulate their target site selection to derive stably integrating vectors with enhanced safety profiles.

Submitted for review: Jan 13 2017 ▶ **Published**: Mar 30 2017

INTEGRATING VECTOR SYSTEMS

The advent of gene therapy has significantly transformed the way we treat human genetic defects. For most of human history, treating genetic diseases meant attempting to ameliorate the symptoms. However, the ability to modify the human genome has made it possible to correct the underlying genetic defects. Apart from replacing defective versions of genes or regulatory elements, the same techniques can also be used to introduce novel functions to cells, for example for use in cellular therapies. Stable introduction of genes, whether to replace a

defective gene or to introduce a new function, is dependent on technologies that can integrate these pieces of DNA into the genome. Without genomic integration, the therapeutic gene is only transiently expressed and a treatment would have to be repeated on a regular basis. So-called integrating vectors are often based on viruses, which possess the natural ability to transfer DNA across the cell membrane and integrate it into the host genome. Non-viral integrating systems, for example transposon-based vectors, are also able to stably integrate DNA into target genomes, but need to be first introduced into the cell.

After initial enthusiasm, it became clear that gene therapy using integrating vectors is associated with considerable risks. Probably the most prominent cases were the studies attempting to treat X-linked severe combined immunodeficiency (X-SCID) using autologous hematopoietic stem cells, which were modified *ex vivo* using first-generation γ-retroviral vectors based on the murine leukemia virus (MLV) [1]. While the treatment successfully corrected the defect in almost all patients, five out of 20 developed leukemia within approximately 5 years [2–4]. These adverse events were shown to be related to insertion of the transgene near the *LMO2* gene and subsequent overexpression of LMO2 induced by the retroviral long terminal repeats (LTRs) [5], although other, insertion-unrelated events were also involved [6]. The development of leukemia after γ-retroviral gene therapy was also reported from trials treating other diseases [7,8].

Despite insertion-related complications, gene therapy was more efficient at treating X-SCID than hematopoietic stem cell transfer [9] and the survival rates of both therapies are similar [10]. Newer γ-retroviral vectors have been modified to be less genotoxic [11]. Clinical trials using integrating vectors have shown promise for the treatment of several diseases, including adrenoleukodystrophies [12,13], human immunodeficiency virus (HIV) infection [14], β-thalassemia [15], Wiskott–Aldrich syndrome [16,17] and B-cell malignancies [18–21]. In 2012, the first gene therapy product, marketed under the name Glybera, was approved in Europe; Glybera is an adeno-associated virus (AAV)-based vector for treatment of lipoprotein lipase deficiency. The first retrovirus-based treatment based on stable gene transfer into hematopoietic stem cells – called Strimvelis – was approved in 2016 to treat severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID).

The development of leukemias highlights an intrinsic problem of many integrating vectors: their integration can occur at large numbers of sites scattered around the genome (which is already potentially mutagenic) with vector-specific integration biases towards actively transcribed genes and their regulatory elements (which makes some vector systems even more mutagenic). For example, lentiviral vectors and γ-retroviral vectors actively target transcription units or transcriptional regulatory elements of genes, respectively [22–24]. Some vectors based on transposons, such as the *piggyBac* (PB) element, have integration profiles similar to viruses [25]. The *Sleeping Beauty* (SB) system, on the other hand, has been found to integrate in a close-to-random manner with only a small

DOI: 10.18609/cgti.2017.013

bias towards genes [25–29]. Indeed, when compared directly to MLV-based γ-retroviral vectors, HIV-based lentiviral vectors and the PB transposon in human $CD4^+$ T cells, the SB transposon was found to display the least deviation from random with respect to genome-wide distribution: no apparent bias was seen for either heterochromatin marks or euchromatin marks and only a weak correlation with transcriptional status of targeted genes was detected [25]. However, even vectors with a completely random integration profile can insert into or near genes by chance.

Random integration of transgenes into target genomes can have two consequences, both of which are highly problematic for gene therapy applications (Figure 1). The first of these are position effects. The expression of the transgene can be influenced by its position in the genome, which may lead to unpredictable therapy results and side effects. For example, if the transgene integrates into heterochromatin, it might be expressed at a very low level or not at all, making it therapeutically ineffective. Some transgenes may also have a deleterious effect on the cell if they are overexpressed. The second problematic effect of random integration is insertional mutagenesis. Insertion of a transgene can disrupt host genes and regulatory elements, including those responsible for cellular homeostasis. If a tumor suppressor gene is disrupted (loss-of-function mutation), or a proto-oncogene is overexpressed (gain-of-function mutation) as a result of vector integration, it can result in malignant transformation of the target cell, as has been observed in the X-SCID clinical trials mentioned above.

Assuming a completely random integration profile, the chance of insertional activation of a proto-oncogene has been estimated to be less than one in 10 million [30]. However, due to integration bias of many vectors, these events occur much more often, the exact frequency depending on both the vector system and the target cell (organism, cell type and individual genetic background) [24]. It should also be noted that insertion of an integrating vector near a proto-oncogene does not automatically result in malignant transformation [24], and clonally expanded cells retrieved from patients generally contained genetic alterations unrelated to vector insertion [6,31].

NATURALLY OCCURRING TARGETED INSERTION

When attempting to retarget vectors, it is possible to mimic nature, as some naturally occurring viruses and transposons have site- or region-specific insertion preferences (Table 1).

Lentiviruses like HIV preferentially integrate into active transcription units [22]. This targeting effect is based on interaction between the viral integrase (IN) and the host chromatin reader lens epithelium-derived growth factor (LEDGF) (Figure 2A) [32–35]. LEDGF binds near actively transcribed genes [36], marked by the histone modification H3K36me3 via its PWWP domain [37–39], and its chromatin-binding profile mirrors the HIV integration profile [36,40]. The interaction between lentiviral IN and LEDGF tethers the integration complex to these sites [41].

Insertions of MLV and γ-retroviral vectors are targeted towards

transcription start sites (TSSs), CpG islands and DNAse I-hypersensitive sites [23,42,43]. This characteristic bias has been shown to be the result of an interaction between host factors of the bromodomain and extraterminal domain (BET) family and the viral IN (Figure 2B) [44–46]. BET proteins are chromatin readers and recognize di- and triacetylated H4 and diacetylated H3 [47]. Disruption of the BET interaction domain of the MLV IN randomizes MLV integration distribution, thereby improving the safety profile of γ-retroviral vectors by 'de-targeting' [48]. Although this approach cannot be used to derive an integration profile that would be safer than completely random, it has the appeal that no overexpression of targeting proteins (see below) is required to achieve a change in chromosomal integration patterns [47].

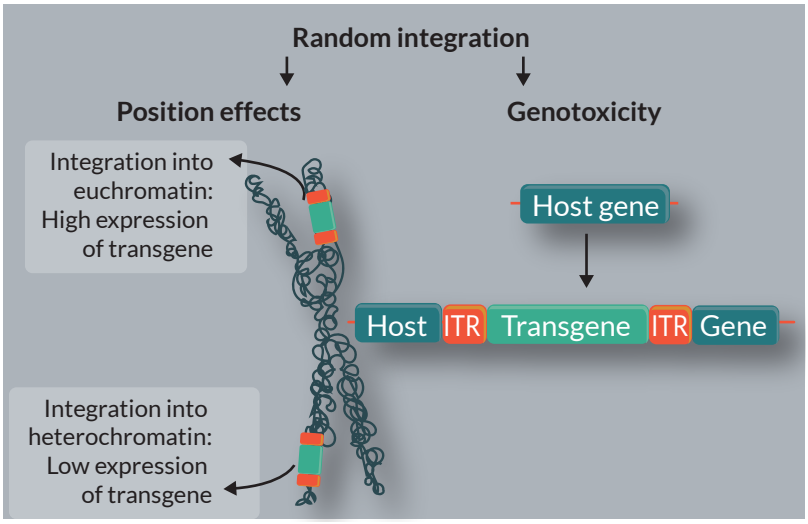
While lentiviruses and γ-retroviruses are targeted to elements that occur in the human genome many times, integration of wild-type AAV is site-specific. AAV integrates into the *AAVS1* locus located on human chromosome 19 [49]. The viral Rep protein simultaneously binds Rep recognition sequences (RRSs) in the viral inverted terminal repeats (ITRs) as well as in the human genome, bringing the integration complex in close vicinity to the target site (Figure 2C) [50–52]. Such target site specificity, combined with a lack of pathogenicity of AAV, would be highly advantageous for gene therapy applications [53]. However, in recombinant AAV vectors the Rep gene is replaced by a genetic cargo, and thus the resulting vectors, unfortunately, lack targeted genomic integration [54]. Additionally, AAV proteins have been shown to cause immune complications [55].

The preference of several viruses to insert near actively transcribed genes might be related to the fact that viral genomes need to be transcribed after integration for their propagation. Thus, viruses that preferentially integrate in genomic regions accessible to the transcription machinery likely gain an evolutionary advantage [24]. Transposons, on the other hand, lack an extracellular phase in their life cycle [56]. This means that integrations that disrupt the host cell's function are deleterious for the survival of the transposon itself. Consequently, many transposons have a lower preference for genes and transcription units than viruses [57].

The *Saccharomyces cerevisiae* retrotransposons Ty1 and Ty3 preferentially integrate into genomic regions upstream of RNA polymerase III (Pol III) TSSs [58,59]. While this might seem to contradict the general rule that transposons have a lower preference for transcriptionally

FIGURE 1

Position effects (effects of the genomic context on expression of the transgene) and genotoxicity (effects of the transgene on genes or their regulation) are the two major problems with randomly integrating vectors.



The figure uses a transposon-based vector as an example. ITR: inverted terminal repeat.

DOI: 10.18609/cgti.2017.013

TABLE 1

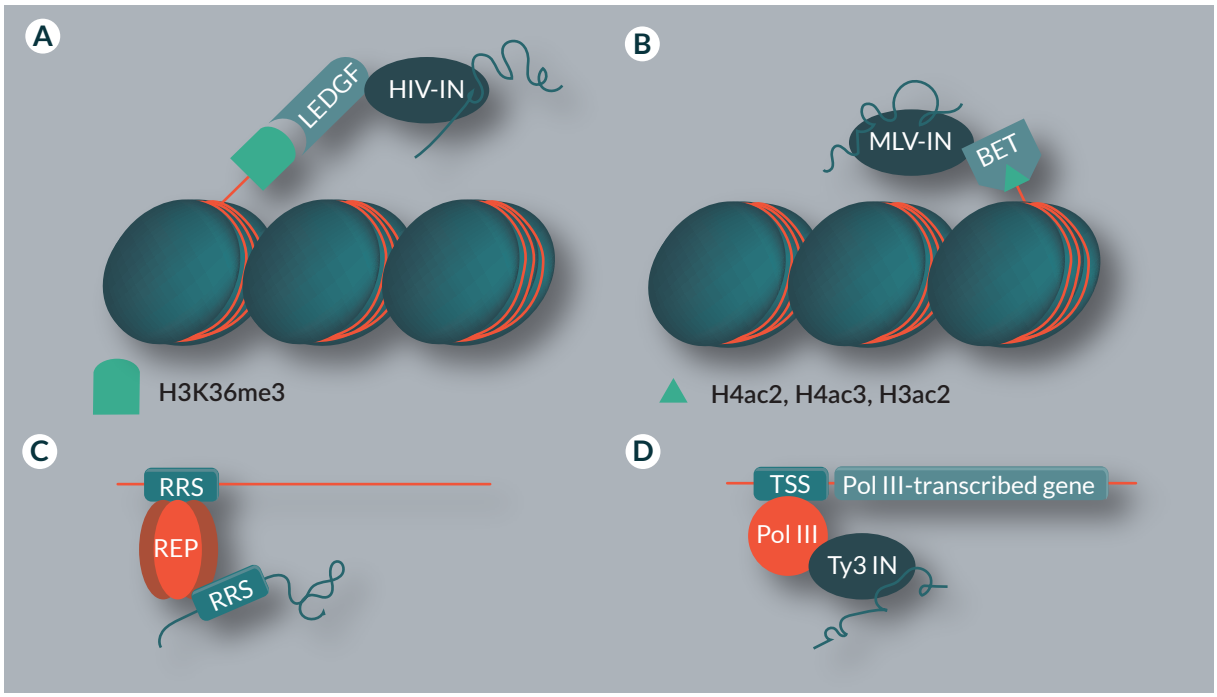
Naturally occurring targeted insertion systems.

Recombinase	Origin	Target	Cofactors	Ref.
Lentiviral IN	Lentiviruses	Transcription units	LEDGF	[32–35]
γ-retroviral IN	γ-retroviruses	TSSs, CpG islands	BET proteins	[44–46]
AAV Rep	AAV	*AAVS1* site	None	[50–52]
Ty1 IN	*S. cerevisiae*	Upstream of Pol III-transcribed genes	TFIIIB of Pol III	[61–65]
Ty3 IN	*S. cerevisiae*	Upstream of Pol III-transcribed genes	TFIIIB, TFIIIC of Pol III	[66–68]
Ty5 IN	*S. cerevisiae*	Heterochromatin	Sir4p	[76–81]
Tf1 IN	*S. pombe*	Promoters of Pol II-transcribed genes, arrested replication forks	Sap1	[72,73,75]
TRE ORF1	*Dictyostelium*	tRNA genes	Pol III	[69–71]
Tn7 transposase	Bacterial	Replicating DNA, *attTn7*	β clamp, TnsD, TnsE	[84,86–88]
PB transposase	Insects	TSSs	BET proteins	[25]
SB transposase	Fish	DNA sequences that resemble the transposase binding sites	The SB transposase itself	[25]
ϕC31 IN	ϕC31 phage	Pseudo *attP* sites in the human genome	None	[92]

active loci, it should be noted that the regions upstream of Pol III-transcribed genes are often gene-poor [60]. Ty1 has a particularly strong preference for tRNA genes and the 5S RNA gene, usually integrating in a window that extends several hundred base pairs upstream of the TSS [61–63]. An interaction between the Ty1 IN and the TFIIIB component of Pol III is responsible for this effect [64,65]. Ty3 also targets Pol III start sites, but integrations are found in a narrow window of one or two base pairs upstream of the TSS [57], and the TFIIIB and TFIIIC components of Pol III complexes are involved in the recruitment of Ty3 (Figure 2D) [66–68]. Other retrotransposons that specifically integrate near tRNA genes include the TRE (tRNA gene-targeting retrotransposable elements) elements from *Dictyostelium discoideum*, which are targeted via interaction with Pol III transcription factors [69–71]. The Tf1 retrotransposon in *Schizosaccharomyces pombe* preferentially integrates into promoters of Pol II-transcribed genes [72,73], and a major determinant of this target site selection is the DNA-binding protein Sap1, which binds to clusters of a 5-bp sequence motif [74]. Interaction of Sap1 with the Tf1 IN has been suggested to play an important role in tethering the preintegration complex – which consists of transposon DNA, IN and cofactors – to target sites [75]. In addition, it was recently shown that Sap1 guides Tf1 insertions into arrested replication forks [75]. In contrast to Ty1 and Ty3, the Ty5 yeast retrotransposon mostly integrates into heterochromatin [76–78]. This insertion preference is mediated via an interaction between a C-terminal domain of Ty5 IN and the host factor Sir4p [79–81].

FIGURE 2

Several viruses and retrotransposons have preferences in their target site selection.



(A) Lentiviruses like HIV integrate into active transcription units; this is mediated by an interaction between the host factor lens epithelium-derived growth factor (LEDGF) and HIV integrase (IN). LEDGF interacts with chromatin marked by the histone modification H3K36me3. **(B)** Similarly, γ-retroviruses like the mouse leukemia virus (MLV) are targeted to transcription start sites by an interaction between proteins of the bromodomain and extraterminal domain (BET) family and MLV IN. BET proteins bind to acetylated H3 and H4 tails. **(C)** AAV targets a specific locus called *AAVS1* in the human genome. The viral Rep protein achieves this by binding its genomic target site (Rep recognition sequence (RRS)) and an RRS in the viral genome. **(D)** The yeast retrotransposon Ty3 integrates upstream of genes transcribed by Pol III, mediated by interactions between the IN and components of the Pol III complex (TFIIIB and TFIIIC). TSS: transcription start site.

Sir4p is a chromatin component predominantly found in telomeric heterochromatin [82,83]; thus, the interaction between Sir4p and the IN increases the likelihood of insertions into these regions.

Not only retrotransposons, but also DNA transposons can be targeted to certain sites and genomic regions in their hosts. For example, the Tn7 bacterial DNA transposon is capable of both DNA sequence- and structure-specific targeting, i.e., targeting specific nucleotide sequences or DNA structures independently of their nucleotide sequence. Tn7 transposition is targeted into actively replicating DNA by a mechanism involving the transposon-encoded protein TnsE [84], which interacts with the β clamp processivity factor of the DNA replication machinery [85]. Alternatively, another transposon-encoded factor, TnsD, binds to specific nucleotide sequences called *attTn7* sites [86,87]. Targeting of Tn7 depends on which cofactor is used during integration [88]. Apart from the genome of the natural bacterial host, a small number of *attTn7*-like sites can be found in the human genome as well, but Tn7 transposition into these sites has not been established [89]. Finally, the eukaryotic DNA transposon PB, originally isolated from the cabbage looper moth, was recently shown to be targeted to TSSs through an interaction of the PB transposase with BET domain proteins, similar to the mechanism shown to be responsible for the enrichment of MLV integrations into TSSs [25].

DOI: 10.18609/cgti.2017.013

A unifying theme in the targeting mechanisms described above is that a DNA- or chromatin-associated factor recruits preintegration complexes to certain genomic sites by physically interacting with a virus- or transposon-encoded protein. Another mechanism of targeted gene insertion exists that is based on direct recognition and interaction of the recombinase with a given DNA sequence in the genome. For example, the ϕC31 IN from a *Streptomyces* phage [90] mediates unidirectional recombination between the *attP* site of the phage genome and the *attB* site in the bacterial host genome, but it is also active in human cells [91]. Since the recognition sequences are relatively short (<40 bp), a number of sites with high similarity are expected to occur in the human genome. Indeed, a number of pseudo *attP* sites were found, and it was shown that ϕC31 IN can integrate DNA into these sites in a directed manner [92]. This makes the ϕC31 system an interesting tool for gene therapy, and it has been tested in preclinical models both *ex vivo* and *in vivo* [93–98]. However, it has been shown that expression of the ϕC31 IN can result in a DNA damage response and chromosomal aberrations, limiting its utility for therapeutic applications [99,100].

Targeting based on pseudo sequences in the human genome has also been observed for the SB transposon system, originally isolated from fish genomes [101]. Namely, it has been shown that SB integrations are enriched near genomic sequences that resemble the transposase binding sites that are normally found in the ITRs of the SB transposon [25]. Because SB transposase molecules likely interact with one another during the transposition process, binding of transposase molecules to these pseudo SB sites might tether transpositionally active transposase molecules bound to the transposon ITRs to these sites, thereby resulting in a fraction of insertions in their vicinity [25]. This naturally occurring tethering mechanism resembles targeted integration of wild-type AAV into the *AAVS1* locus (simultaneous binding of Rep to the viral ITRs and to the genomic target site (Figure 2C)), and provides the molecular basis of artificial retargeting with the N-terminal N57 domain of the SB transposase (described below).

ARTIFICIAL RETARGETING

In order to avoid position effects and insertional mutagenesis, it is of great interest to establish technologies that artificially retarget otherwise semi-randomly integrating vector systems to a precisely defined genomic region or specific sequence. Artificially retargeted vectors are not only useful for gene therapy. For example, fusions with DNA-binding domains (DBDs) of unknown specificity can be used to determine the binding sites of these domains by analyzing the integration profile of the retargeted vector [102–104].

Instead of relying on chance occurrence of pseudo sites in the human genome that are recognized by a recombinase enzyme (described above for the ϕC31 IN), custom DBDs that can be engineered to specifically interact with practically any sequence in the human genome are of great utility. In previous decades, two major classes of engineered DNA-binding proteins have been used for site-specific

genome engineering: zinc finger proteins (ZFPs) [105] and transcription activator-like effectors (TALEs) [106]. In their most widespread applications, both ZFPs and TALEs serve as DBDs directly fused to an effector

TABLE 2

Retargeting via direct recombinase-DBD fusions.

Hybrid protein	Targeting effect	Activity	System	Ref.
Viral vectors				
HIV-IN/LexA	Enrichment near LexA binding site	Like wild-type	*In vitro*	[115]
HIV-IN/λR	Enrichment near λR binding site	Like wild-type	*In vitro*	[116]
HIV-IN/Zif268	Hotspots near Zif268 binding site	Integration like wild-type, abolished infectivity	*In vitro*	[117]
HIV-IN/E2C	32% (six-fold increase) within 30 bp of target	Like wild-type	*In vitro*	[118]
HIV-IN/E2C	1.45% (ten-fold increase) near *erbB-2*	<24% of wild-type	Cell culture (genomic)	[119]
ASV-IN/LexA	Hotspots <23 bp from LexA binding site	Similar to wild-type	*In vitro*	[120]
MLV-IN/Sp1	Ca. 13% near Sp1 binding sites	Like wild-type	Cell culture (genomic)	[121]
Transposon vectors				
SB/Gal4	25% (11-fold increase) in 443 bp window	26% of wild-type activity	Cell culture (plasmid)	[111]
SB/E2C	17.8% (8-fold increase) in 443 bp window	20% of wild-type activity	Cell culture (plasmid)	[111]
SB/E2C	Up to 2% of clones with integration near endogenous target site	<5% of wild-type activity	Cell culture (genomic)	[113]
SB/ZF-B	Up to 44.8% of insertions into L1 elements	<10% of wild-type activity	Cell culture (genomic)	[113]
SB/Rep	Up to 2-fold enrichment within 5 kb from consensus RRSs	20–80% of wild-type activity	Cell culture (genomic)	[26]
PB/CHK2-ZFP	50% enrichment in 500 bp window around target site	Like wild-type	Cell culture (plasmid)	[126]
PB/Gal4	4.5-fold increase (24%) within 800 bp of endogenous target sites	Like wild-type	Cell culture (genomic)	[110]
PB/TALE	<1% within 250 kb of endogenous target site	Like wild-type	Cell culture (genomic)	[109]
IS30/cI	Ten-fold increase in target plasmid	Similar to wild-type	*E. coli* (plasmid)	[127]
IS30/Gli1	Several insertions near target site, but mostly illegitimate	Significantly reduced	Zebrafish (plasmid)	[127]
Mos1/Gal4	96% within 1 kb of binding site	Transposition increased >ten-fold	Mosquito embryos (plasmid)	[128]
ISY100/Zif268	Hotspot 7-17 bp from binding site	Up to nine-fold lower than wild-type	*E. coli* (plasmid)	[129]
Other recombinases				
Tn3 resolvase/Zif268	Up to 100% recombination with appropriate target site	Like wild-type	*E. coli* (plasmid)	[131]
Gin recombinase/dCas9	Up to 32% recombination on plasmid target, <1% for genomic deletion	Depends on target sequence	Cell culture (plasmid, genomic)	[135]

DOI: 10.18609/cgti.2017.013

endonuclease domain derived from the restriction enzyme *Fok*I. A revolutionary, programmable new tool is the CRISPR/Cas nuclease system [107], which relies on an RNA molecule and DNA:RNA base pairing for providing specificity for the cleavage reaction.

An overview of experimental approaches to retargeting is provided in Table 2 (targeting via direct recombinase fusions) and Table 3 (targeting via adapter proteins). Concerning the experimental systems, *in vitro* refers to cell-free assays. For cell culture assays, it is indicated whether integration was analyzed on a target plasmid or in the genome.

Direct recombinase-DBD fusions

The most direct approach to retargeting of viral or transposon vectors is to directly fuse a DBD to the recombinase enzyme (IN or transposase for virus- or transposon-based vector systems, respectively) (Figure 3A & Table 2). The main drawback of this method is that some recombinases may suffer from a reduction of catalytic activity in the context of a fusion protein. For example, while the PB transposase can be fused to many DBDs without significant loss of activity [108–110], the SB transposase only tolerates protein domain additions to its N-terminus. Even N-terminal

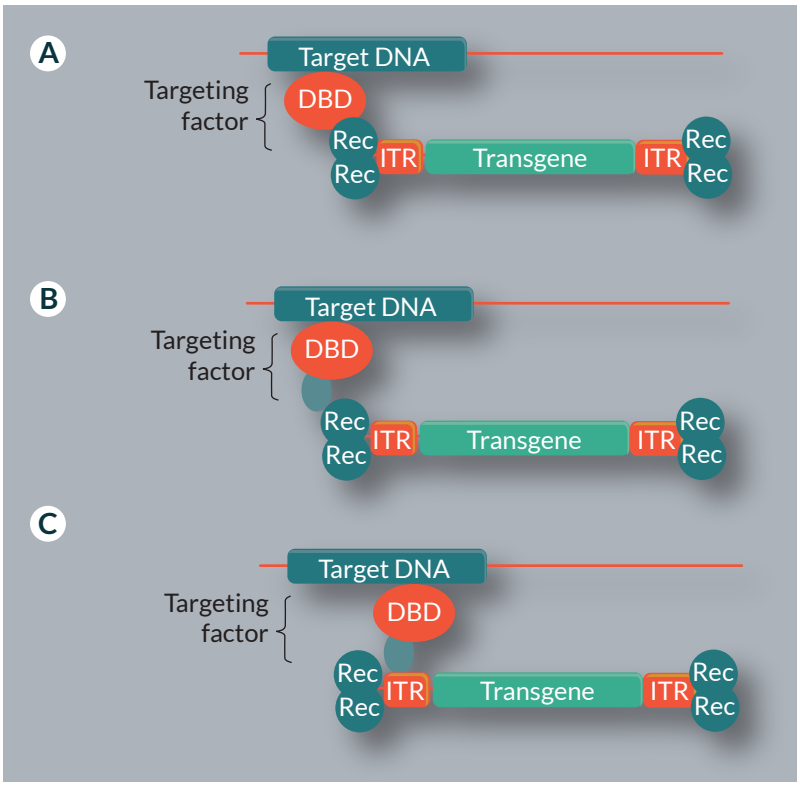
TABLE 3

Retargeting via adapter proteins

Adapter protein	Binding activities	Targeting effect	System	Ref.
Protein-chromatin adapters				
LEDGF/λR	HIV-IN, λR site (genomic)	Increased integration near λR site	*In vitro*	[136]
LEDGF/CBX1	HIV-IN, H3K9me3	50% decrease (from 67.2% to 32.6%) of insertions into genes	Cell culture (genomic)	[138]
LEDGF/ING2	HIV-IN, H3K4me3	13-fold enrichment (from 3.8% to 50.3%) within 2.5 kb of TSSs	Cell culture (genomic)	[140]
LEDGF/HP1α	HIV-IN, H3K9me2,3	Ca. 1.5-fold enrichment in intergenic regions	Cell culture (genomic)	[141]
Sir4p/LexA	Ty5-IN, LexA site (genomic)	<200-fold enrichment of integration into target plasmid (from <0.1% to 15%)	Yeast (plasmid)	[81]
N57/TetR	SB transposase, TRE	>10% of all cells contained insertion near TRE	Cell culture (genomic)	[112]
N57/ZF-B	SB transposase, L1 elements	4-fold enrichment within 400 bp of ZF-B sites	Cell culture (genomic)	[113]
N57/E2C	SB transposase, *erbB-2* locus	<1% insertion near *erbB-2* locus	Cell culture (genomic)	[113]
N57/Rep	SB transposase, RRSs	Ca. 2.5-fold enrichment near consensus RRSs	Cell culture (genomic)	[26]
DNA-chromatin adapters				
LexA/TetR	LexA site (SB transposon), TRE	<1% insertion near TRE	Cell culture (genomic)	[112]
LexA/SAF	LexA site (SB transposon), MARs	Ca. 4-fold enrichment in MARs	Cell culture (genomic)	[112]
TALE/Gal4	*CCR5* locus, UAS (PB transposon)	0.014% near *CCR5* locus	Cell culture (genomic)	[109]

FIGURE 3

Strategies to tether vector systems to target sites.



A targeting factor can consist of a DNA-binding domain (DBD) fused to the recombinase (Rec) enzyme **(A)**. For simplicity, only one subunit of Rec is shown as a fusion protein. Alternatively, a targeting protein may consist of a DBD and a second domain, which is capable of binding the Rec **(B)** or the DNA containing the transgene **(C)**. The figure uses a transposon-based vector as an example. ITR: inverted terminal repeat.

fusions generally have reduced transpositional activity [111–114] in the range of 10–80% of the wild-type, unfused transposase, depending on the fusion partner. For viral INs, *in vitro* enzymatic activity is often retained after the addition of a foreign domain [115–118]. However, this modification can negatively affect capsid packaging and result in reduced infectivity [117] and reduced *in vivo* activity [119].

Several attempts have been made to retarget the HIV system by fusing DBDs to HIV IN. Fusions of HIV IN to LexA [115], lambda repressor (λR) [116] as well as the ZFPs Zif268 [117] and E2C [118] have been shown to bias HIV integration in cell-free *in vitro* assays using artificial target DNA fragments. The IN-E2C fusion was also shown to retarget HIV integration in cell culture assays [119]. *In vitro*, this construct increased the number of integrations in a 30-bp region around the target site six-fold [118], while *in vivo*, the percentage of insertions that occurred near the E2C binding site increased ten-fold [119]. All of these hybrid proteins retained catalytic activities similar to wild-type IN, but some resulted in reduced infectivity when assembled into virions [117]. Other IN fusions that have been successfully used to retarget retroviral systems include avian sarcoma virus (ASV) IN with LexA [120], and MLV IN with Sp1 [121].

Retargeting of the SB system via direct transposase fusions was first demonstrated by fusing the DBD of the yeast transcription factor Gal4 and the synthetic ZFP E2C to the N-terminus of the SB transposase [111]. While UAS, the target sequence of Gal4, is absent from the human genome, E2C targets a unique site near the *erbB-2* gene [122,123]. These hybrid vectors were able to target integration near their respective target sites in inter-plasmid assays; integration near the target sites was increased up to 11-fold to 25% [111]. However, no targeting into the genome could be demonstrated. This was later achieved with the generation of new transposase hybrids: a fusion of E2C to a hyperactive SB mutant was shown to direct up to 2% of transposition events towards the endogenous target site in the promoter region of the *erbB-2* gene [113], as demonstrated by a locus-specific semi-nested PCR assay. However, when the integration profile was analyzed on a whole-genome level by linear amplification mediated

DOI: 10.18609/cgti.2017.013

PCR, no bias toward integration near E2C recognition sites could be shown, suggesting that detection of these rare events was dependent on the method applied to recover the insertions. Additionally, a synthetic ZFP targeting the 3′-end of L1 elements was used to retarget the SB system [113]. L1 elements are good targets for SB transposition because they are A/T-rich (SB transposase integrates into TA dinucleotides) and abundant, making up 17% of the human genome [124]. The presence of multiple binding sites in the genome is expected to improve the ratio of targeted to untargeted events. In fact, around 45% of all transpositions catalyzed by the hybrid transposase ended up in L1 elements. A direct fusion of the DBD of the Rep protein from AAV to the SB transposase has also been shown to direct transposition towards endogenous RRSs [26].

Hybrid transposases based on the PB system have also been generated. The CHK2-ZFP, which targets the *CHK2* gene with high specificity [125], can be fused to the PB transposase without significant loss of transpositional activity [126]. The hybrid vectors target a CHK2-ZFP binding site in an inter-plasmid transposition assay, but not the endogenous target site in the human genome [126]. However, a fusion of PB transposase and the Gal4 DBD was later shown to direct transposition to artificially introduced UAS sites as well as to endogenous UAS-like sites in the human genome (4.5-fold increase in an 800 bp window around endogenous sites) [110], and fusion with a synthetic TALE domain allowed targeting to the endogenous *CCR5* gene (with 0.014% of cells containing targeted events) [109].

Other transposon systems that were retargeted using direct transposase-DBD fusions include the bacterial IS30 transposon (with cI repressor and Gli1) [127], the *Drosophila* Mos1 transposon (with Gal4) [128] and the bacterial ISY100 element (with the Zif268 ZFP) [129]. However, for all of these systems, targeted transposition was only demonstrated for integration into plasmids or bacterial genomes.

Transposases and viral INs are not the only recombinases that have been retargeted by fusing DBDs to them. While in tyrosine recombinases like Cre and FLP the catalytic domains and the DBDs are structurally intertwined [130], this is not true for serine recombinases. This family of enzymes, which contains the abovementioned ϕC31 IN, has physically separable DNA-binding and catalytic domains, making it possible to replace the DBD to alter the enzyme's specificity [131]. Replacement of the original DBD with the Zif268 DBD has been shown to retarget Tn3 resolvase in bacterial cells [131]. Other serine recombinases, for example the Gin invertase, have also been fused to foreign DBDs and retargeting into the human genome has been demonstrated [132–134]. One such fusion protein consisted of the Gin catalytic domain and a catalytically inactivated Cas9 (dCas9) as a DBD, resulting in a recombinase that is active in human cells and can be targeted to extrachromosomal plasmids by supplying the proper gRNAs [135].

Targeting via adapter proteins

An alternative approach to vector retargeting, a mechanism found in most naturally targeted systems,

is the use of adapter proteins that simultaneously bind to the target DNA and to a component of the vector system. This may be the integrating enzyme, the viral or transposon DNA, or both (Figure 3B–C & Table 3). This method avoids problems with activity loss associated with direct recombinase fusions.

Some retroviruses use this mechanism to direct their integration, and experimental manipulation of their targeting systems can alter their integration profile. LEDGF, a factor responsible for targeting of lentiviral integration, recognizes particular chromatin marks while simultaneously binding to HIV IN, thereby tethering the integration complexes to target sites in the genome. It is possible to retarget HIV integration by replacing the chromatin reading-domain of LEDGF with other DBDs. Fusing λR to the LEDGF IN-binding domain (IBD) targets HIV integration towards binding sites of λR *in vitro* [136]. Replacing the chromatin-binding domain of LEDGF with CBX1 (HP1β), which recognizes H3K9me3 chromatin marks [137], successfully retargeted HIV integration to intergenic regions *in vivo* [138]. While wild-type IN inserted into genes 67.2% of the time, this value dropped to 32.6% with the CBX1/LEDGF fusion [138]. This construct was even validated in an X-linked chronic granulomatous disease (X-CGD) model, demonstrating stable integration and expression [139]. Similar constructs with DBDs from ING2 and HP1α also altered the HIV integration profile in cell culture-based assays [140,141]. Deletion of the LEDGF PWWP domain or replacement with unspecific chromatin binding domains was also demonstrated to increase the percentage of 'safe' integrations [142].

An adapter-based approach has also been used to alter the target specificity of the Ty5 retrotransposon. A fusion of Sir4p, which interacts with Ty5 integrase during its natural targeting process, with LexA was used to direct insertions to a LexA binding site on a target plasmid [81]. Additionally, it was shown that target specificity could be altered by replacing the Sir4p interaction domain of the Ty5 IN [81].

Several adapter proteins have been developed for the SB system. For example, fusion proteins consisting of the LexA DBD and a second DBD with a genomic target were able to direct transposition of a transposon containing a LexA binding site [112]. Using the tetracycline repressor (TetR) as the second DBD allowed targeting of more than 10% of insertions towards an artificial tetracycline response element (TRE) containing binding sites for TetR, whereas a SAF-box fusion directed insertions to endogenous matrix attachment regions (MARs) [112].

Instead of using a DBD that binds to the transposon DNA, adapter proteins can also bind to the transposase. For this purpose, an N-terminal fragment of the SB transposase (N57), which is a dual DNA-binding and protein dimerization domain [143], can be used. Fusions of N57 with TetR, E2C, ZF-B and the Rep DBDs were able to direct transposition catalyzed by wild-type SB transposase to TRE, *erbB-2* gene, L1 elements and RRSs, respectively [26,112,113]. Additionally, it has been shown that targeting efficiencies can be improved by utilizing fusion proteins that bind both the transposon DNA and the transposase [26].

DOI: 10.18609/cgti.2017.013

So far, no equivalent to the N57 fragment is available for the PB system. Without a domain interacting with PB transposase, it is not possible to design a protein that would tether the transposase to the target site. However, it is possible to retarget PB transposition using adapter proteins consisting of two DBDs that bind to the target site and to a site in the transposon. This has been demonstrated by a fusion of a TALE domain recognizing the *CCR5* locus in the human genome and a Gal4 domain in combination with a PB transposon containing a UAS site [109].

TRANSLATIONAL INSIGHT

The results of the studies mentioned above show that both viral and transposon vector systems can be retargeted using either direct recombinase fusions or adapter proteins. The efficiency of this targeting effect varies depending on the vector system used, the exact targeting mechanism and choice of target sites. However, for all of these approaches, the number of untargeted integrations is much higher than the number of targeted integrations. This is due to the fact that binding of the artificially introduced DBD is generally not required for the system to integrate. The number of potential integration sites is generally vastly greater than the number of desired integration sites, meaning that most integration events will occur bypassing the desired targeting effect. This problem could be addressed by modifying the vector system in a manner that makes integration dependent on binding of the foreign DBD. However, this has not been achieved yet.

Recent advances in the development of novel DBDs like ZFPs [144], TALEs [145] and the CRISPR/Cas system [146,147] have allowed the application of highly specific designer endonucleases. Introduction of a double-strand break (DSB) at a precisely defined genomic location allows disruption of endogenous genes or – when coupled with a homology template – any desired modification of the target sequence [148], including gene repair and gene addition. In light of these developments, particularly the CRISPR/Cas system, it might seem that the development of targetable recombinases has become obsolete.

There are several important aspects to consider when comparing targeted viral/non-viral gene integration systems and designer nucleases. The first is the efficiency at which a desired genetic modification can be introduced into a cell population. Designer nucleases are specialized in introducing a DSB into the DNA, and are therefore highly efficient in mutagenizing a target site [149,150]. However, gene addition at the cut site is a process executed by DSB repair mechanisms of the cells; the efficiency of which is considerably lower than introducing the DSB in the first place [151]. In other words, knocking out a gene by designer nucleases is far more efficient than knocking in a gene into a specific site. On the other hand, integrating viruses and transposable elements have evolved machineries for gene integration, because genomic insertion is a fundamental step of the life cycle of these genetic elements. That means that the efficiency of gene insertion by vector systems that are based on such genetic elements is robust, which is a key requirement for medically

relevant applications. An additional benefit of integrating vectors over nuclease-based approaches is that some integrating vectors, particularly those based on transposons, can deliver their cargo into the genomes of non-dividing cells [152,153]. Nuclease-based approaches, on the other hand, rely on DSB repair, as outlined above. In eukaryotic cells, DSBs can be repaired by at least two pathways, homology-directed repair (HDR) and non-homologous end joining (NHEJ). The two pathways act complementarily, but at different stages of the cell-cycle: NHEJ is preferentially active in the G1 and early S phases [154], whereas HDR is the preferentially used DSB repair pathway in the late S and G2 phases when homology templates are available [155], and is strongly downregulated in most post-mitotic cells [156]. Consequently, gene addition and gene repair require dividing target cells.

The second important aspect is the safety profiles of the diverse gene insertion technologies. Although gene insertion by designer nucleases is targeted to specific sites in the genome, cellular responses to DSBs can result in cytotoxic effects and off-target cleavage can lead to genotoxicity [157–162]. Due to its RNA-guided targeting mechanism, the CRISPR/Cas system is especially prone to off-target effects, which can be hard to predict. The extent of off-target effects varies with the actual gRNA sequence, but several mismatches can be tolerated, even if they occur consecutively [163]. *In vitro*, off-target sites with seven mismatches were observed [164] and modification of off-target sites can be as efficient or even more efficient than modification of the on-target site [163]. Genome-wide methods for off-target detection like GUIDE-seq [165] confirmed that the number of off-target sites and the efficiency at which they are modified strongly depends on the individual gRNAs. The number of off-target sites can range from none [165] to thousands [166], and bioinformatics tools like the MIT CRISPR Design Tool [167] or E-CRISP [168] might fail to predict experimentally determined off-target sites [165]. However, high-fidelity variants of Cas9 with greatly reduced off-target effects have been developed [169].

While the mechanism of HDR results in highly specific gene correction, any DSB can also be repaired by NHEJ. This means that, although cutting the DNA catalyzed by recombinases is highly specific, the actual outcome of the reaction on a genome-wide scale can be diverse. This has profound implications for the detection of off-target effects. It is relatively easy to determine the numbers and genomic locations of virus or transposon integrations in a target cell. Thus, picking cells with a single integration event mapped onto a genetic locus results in a high degree of certainty that: i) no other genomic modifications have been introduced; and ii) the insertion event has no negative impact on the cell as a risk factor. This is not possible when using endonuclease-based approaches because some off-target mutations – for example the deletion of a single nucleotide – are very difficult to detect. Thus, gene additions by integrating genetic vector systems (including the targeted recombinase approaches outlined in this article) could very well be safer than an endonuclease, in case clonal analysis of genetically engineered cell products is possible. In

DOI: 10.18609/cgti.2017.013

bulk cell populations, where prior annotation of every single insertion and off-target cleavage event is not feasible, potential risks associated with genome engineering can only be inferred on the basis of the type of vector used (insertional preferences of viral and non-viral vectors), the nuclease and the genomic target site (these will be the major determinants of off-target cleavage), the type of cell (stem cells with a high proliferative potential are more prone to oncogenic transformation) and the disease condition (which largely determines what cell types are to be engineered, and specifies a certain genetic background that might affect the risks associated with genetic engineering).

FINANCIAL & COMPETING INTERESTS DISCLOSURE

The authors have no relevant financial involvement with an organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock options or ownership, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

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