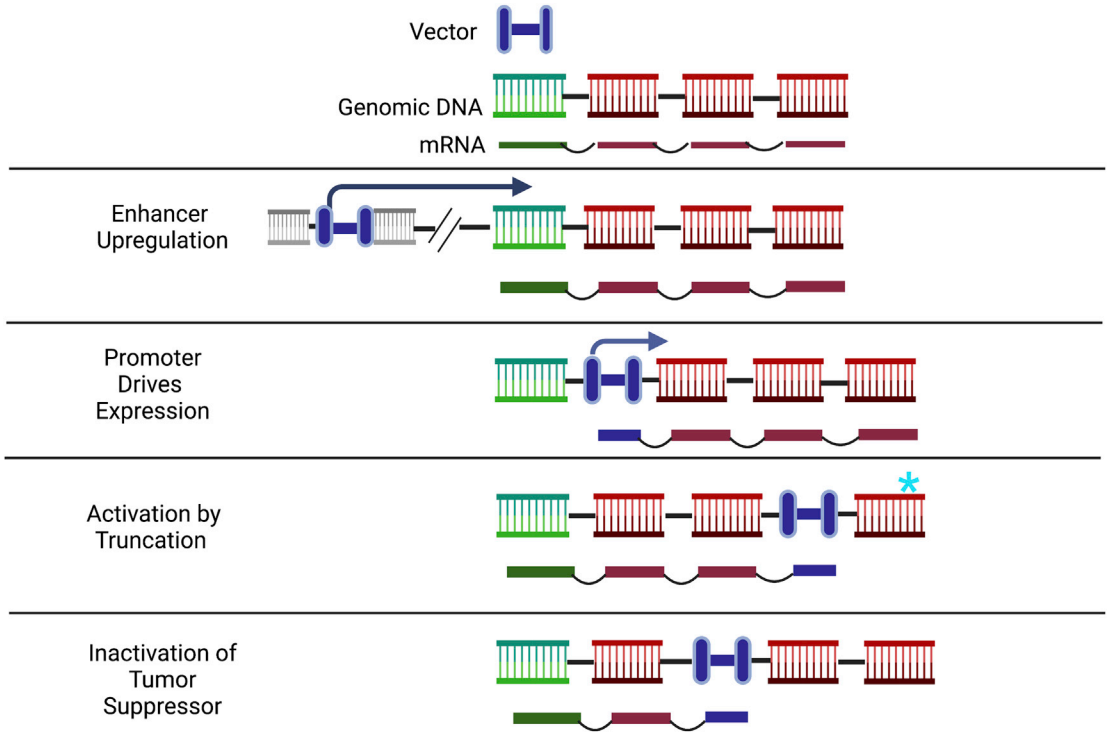
Gentoxicity

Retroviral Integration and Mutagenesis

Li, Z., Dullman, J., Schiedlmeier, B., Schmidt, M., von Kalle, C., Meyer, J., et al. (2002) Murine leukemia induced by retroviral gene marking. *Science* 296, 497.
«Integration has led to activation of the Evi-1 oncogene with subsequent development of myeloid leukemia in a murine bone marrow transplantation model»

To date, all of the clinical trials reporting secondary malignancies have utilized gammaretrovirus-based vectors, with the exception of one trial for the treatment of cerebral adrenoleukodystrophy that used a lentiviral vector.

Mechanisms for insertional alteration of gene expression



The most common mechanism for insertional oncogenesis has been oncogene promoter upregulation by the enhancer element within gammaretroviral vectors. While vector promoters driving oncogene transcription have been reported in murine cancers, this has not been noted in human cancers arising after vector insertion. Activation by truncation has been shown after insertion led to the truncation of mRNA, removing a let-7 site that normally regulated gene expression (noted by asterisk). This led to transient clonal expansion without cancer formation. Integration and disruption of gene expression has also inactivated a tumor-suppressor gene, which led to clonal expansion without malignancy in a patient with an inherited hypomorphic mutation in the non-transduced allele. Green, promotor region; red, exon.

Cornetta et al. 2023

Different Retroviruses Have Different Integration Preferences

HIV and Moloney murine leukemia virus (MLV) integration profile are the most extensively studied.

Integration studies revealed **different patterns of favored and disfavored target** sites for each retroviral family,

Integration properties of MLV, HIV, and ASLV retroviral vectors

MLV, murine lentivirus
HIV, human lentivirus
ASLV, avian sarcoma-leukosis virus

Retroviral integrases:

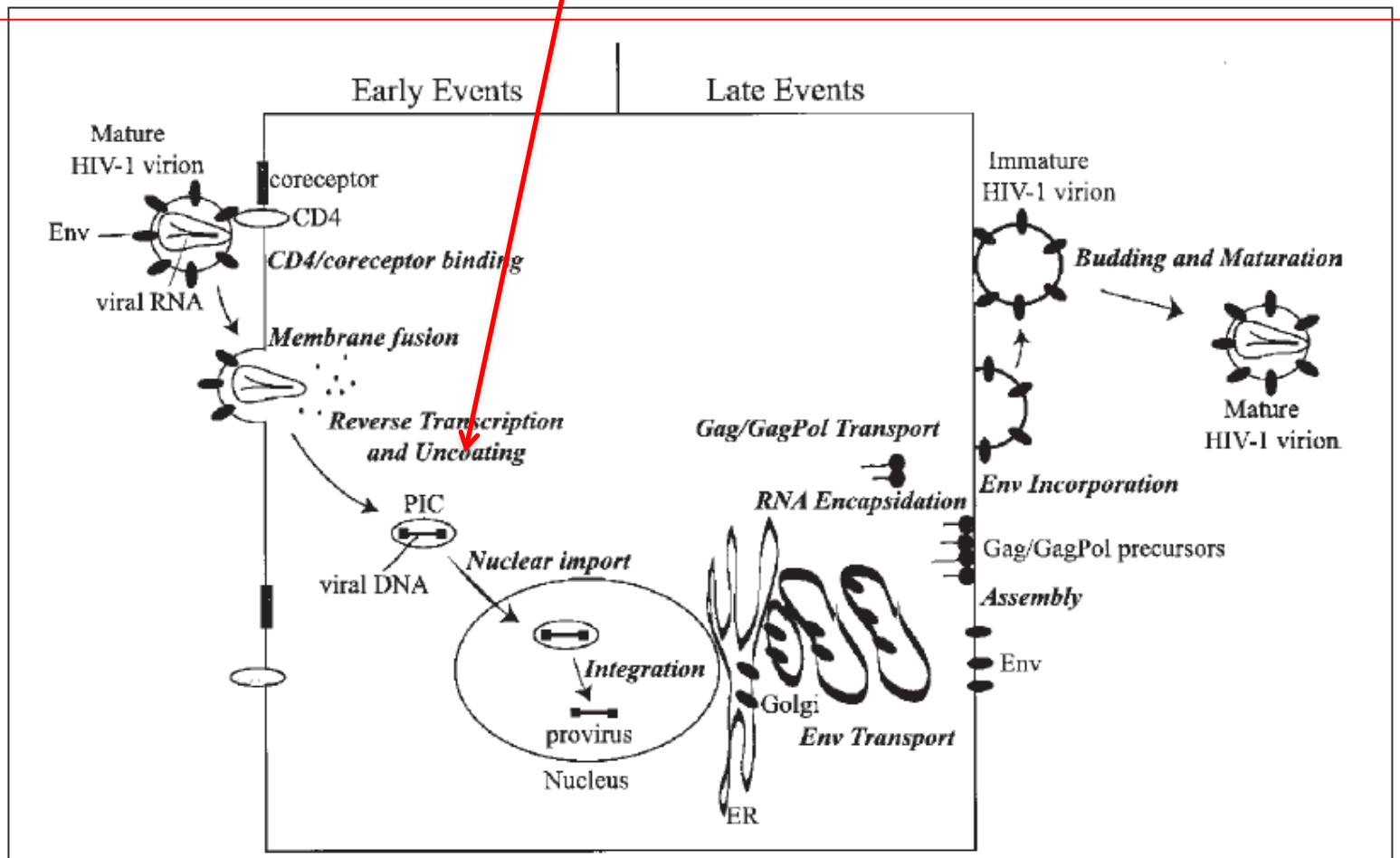
- strict sequence requirements for the viral DNA ends: the dinucleotide CA is invariably located 2 base pairs from both viral ends, and certain nucleotides may recur up to 15 base pairs away from the CA.
- target sites show some consensus sequences (statistically weak significance).

MLV vectors show a propensity to integrate around TSS and cis- acting regulatory regions of transcriptionally active genes, forming tight hotspots of clustered integrations.

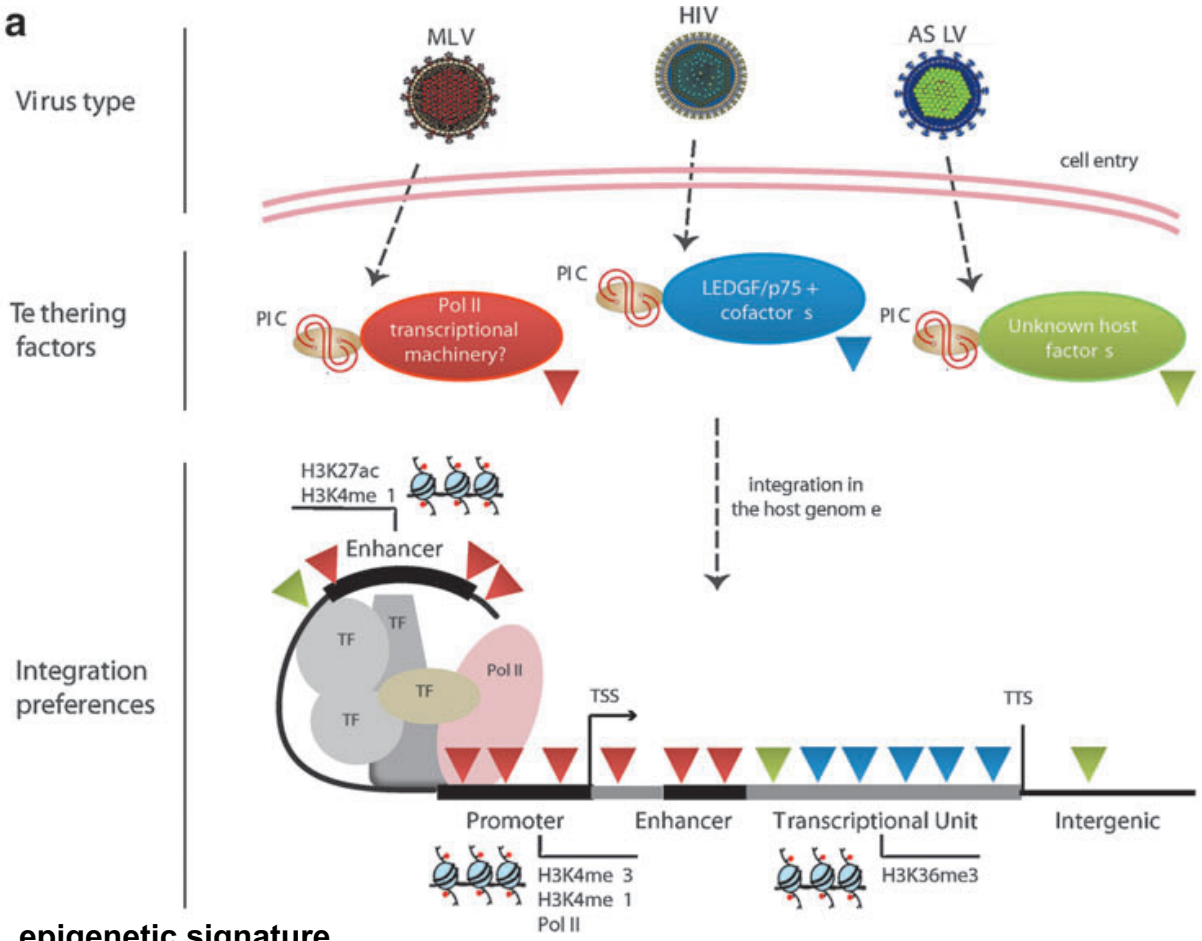
HIV vectors tend to form broader hotspots of integration, with a preference for the transcribed portions of genes.

ASLV integrations are evenly distributed along the genome with only a weak bias for gene-related genomic features.

retroviral PICs are tethered to specific genomic regions through association with different cellular factors.
LEDGF/p75, have been described to interact with HIV PICs and direct their integration
the cellular determinants of MLV and ASLV target site selection are still largely unknown.



integration properties of MLV (red), HIV (blue), and ASLV (green) retroviral vectors



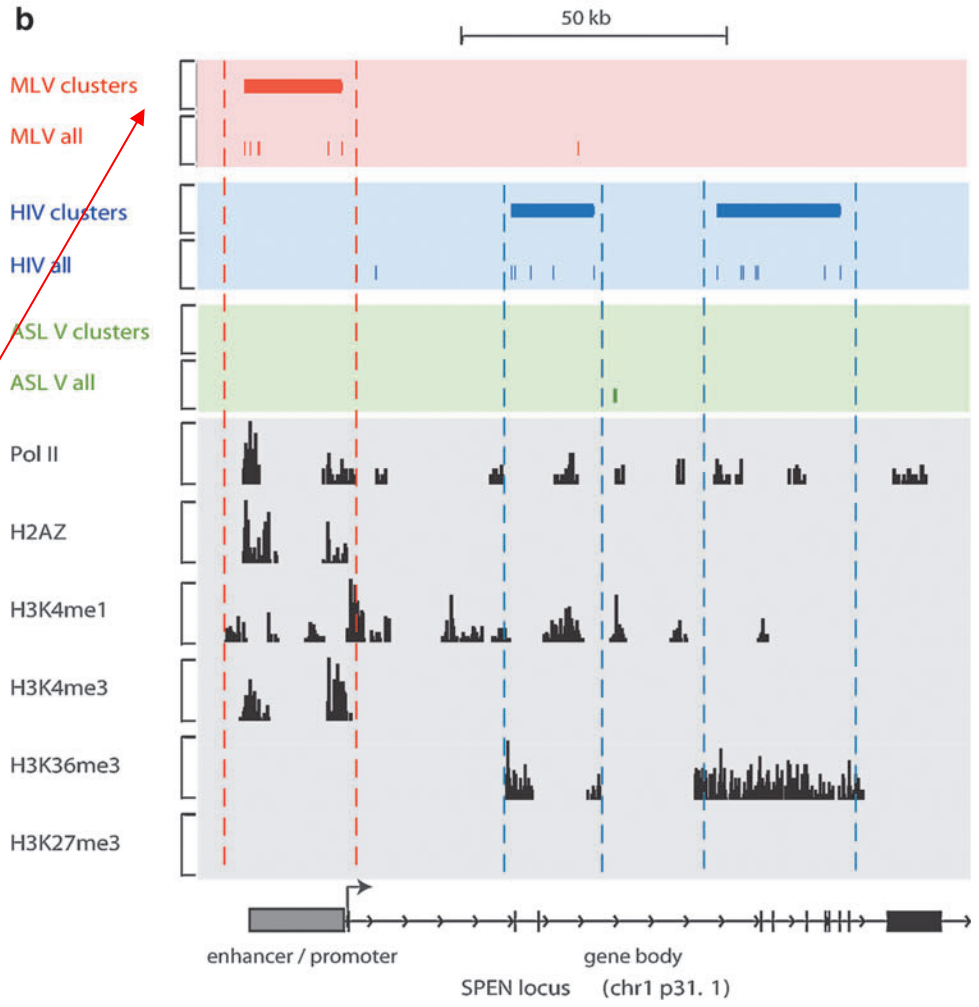
cell factors influencing provirus integration:
poor defined for MLV (PIC tethered to region bound by Pol II) and ASLV (random?)
integrase-LEDGF/p75 for HIV

ASLV, avian sarcoma-leukosis virus
TSS, transcription start site
LEDGF/p75, lens epithelium-derived growth factor (ubiquitously expressed nuclear protein associated with chromatin throughout the cell cycle)

epigenetic signature
MLV and HIV: region with transcriptionally active chromatin profile
MLV: histone acethylations and H3 methylations (H3K27me3, HeK0me3)
HIV: H3K4 mono-, di- and tri-methylated; H3-Ac, H4-Ac

Genomic browser screenshot of MLV, HIV, and ASLV vector integrations in the human SPEN gene locus gene locus.

The picture summarizes each vector's integration preferences and their association with epigenetic modifications. **MLV** integrations (red lines) form **clusters** (red bar) around the **TSS**, and in the **immediately upstream regulatory region** of the SPEN gene, perfectly co-mapping with histone modifications characteristic of active transcription (Pol II) and engaged regulatory regions (H3K4me1, H3K4me3, H2A.Z)

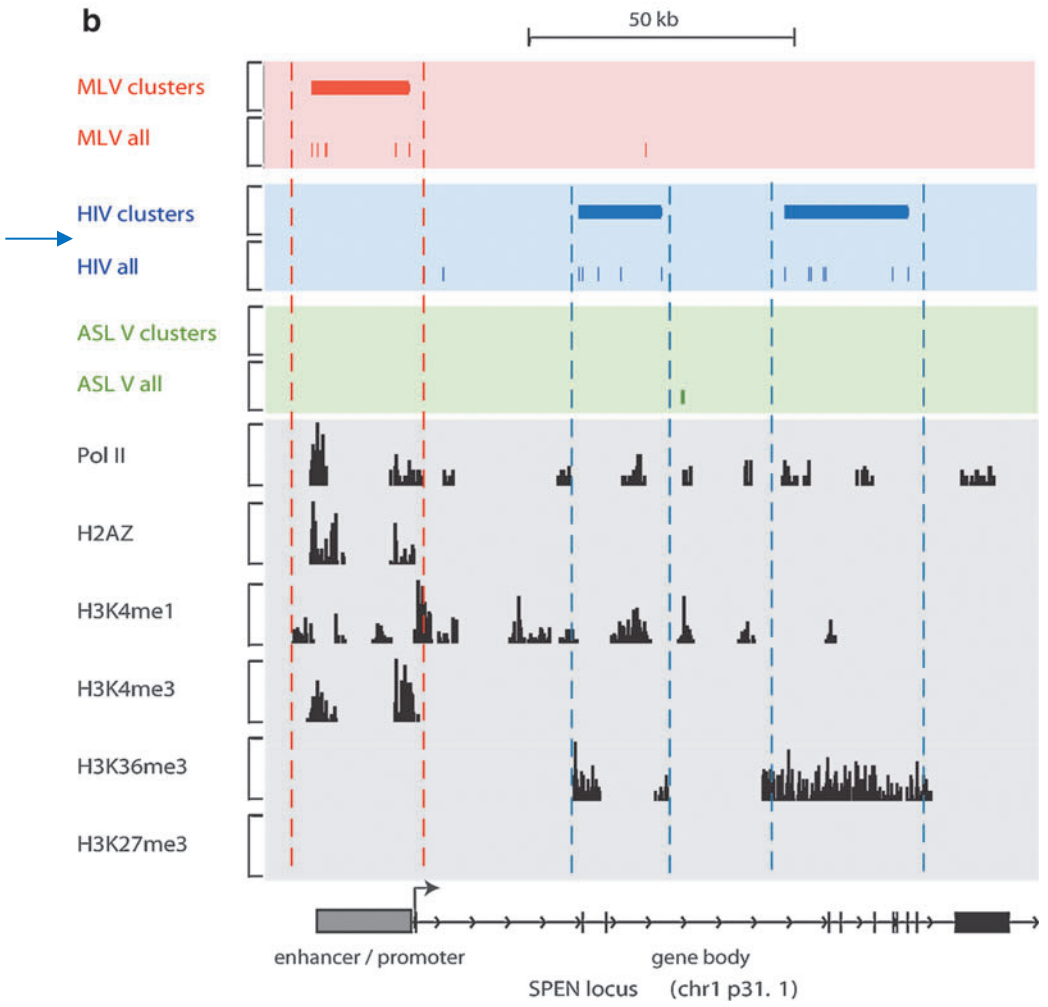


Integration strategy and viral evolution

MLV, by coupling target site selection to gene regulation, maximizes the chances of activating and maintaining proviral expression. Additionally, integration of the MLV provirus around promoters and regulatory elements of growth- and differentiation-controlling genes may increase the chances of inducing clonal expansion or transformation of the infected cells and ultimately favor viral propagation.

Genomic browser screenshot of MLV, HIV, and ASLV vector integrations in the human SPEN gene locus gene locus.

HIV integrations (blue lines) are **clustered in larger genomic hotspots** (blue bars), mainly localized **in the transcribed body** of SPEN, marked by H3K36me3, a histone modification associated to transcriptional elongation. ASLV integrations (green lines) are spread throughout the genome, rarely form clusters and do not appear to co-map with any specific chromatin mark. None of the vectors integrate in inactive and heterochromatic regions associated with the H3K27me3 histone modification



Integration strategy and viral evolution

HIV, by targeting open chromatin regions and avoiding control-regulatory elements minimize interference with host cell transcription and maximize virions production during a limited lifespan of the infected cells. Additionally, this strategy may be more permissive for a latent phase of the viral cycle.

retroviral integration and gene expression deregulation

the genotoxic consequences of retroviral gene transfer technology (from the clinic)

Insertional activation of proto-oncogenes by MLV-derived vectors:

- T-cell lymphoprolipherative disorders in patients undergoing gene therapy for X-linked severe combined immunodeficiency (SCID-X1) (Hacein- Bey-Abina et al., 2008; Howe et al., 2008) and Wiskott-Aldrich syndrome (WAS) (Avedillo Diez et al., 2011)
- pre- malignant expansion of myeloid progenitors in patients treated for chronic granulomatous disease (CGD) (Ott et al., 2006; Stein et al., 2010)

Insertion of a lentiviral vector in a proto-oncogene likewise caused clonal expansion in at least one patient undergoing gene therapy for beta-thalassemia (Cavazzana-Calvo et al., 2010).

Pre-malignant clonal expansion can predispose to subsequent accumulation of mutations or chromosomal aberrations, a classical model of neoplastic progression.

Secondary malignancy in clinical trials

Table 2. Reported events of clonal expansion or frank leukoproliferation in clinical gene therapy trials using hematopoietic stem cells

Condition	Vector	Cases/total	Gene(s)	Mechanism	Consequence	Reference
SCID-X1	RV: MLV-*IL2RG*	6/20	*LMO2*	LTR *trans*-activation	T ALL	PMID: 18688285
			CDKN2A			PMID: 18688286
XCGD	RV: SFFV-*CYBB*	3/3	*MECOM*	LTR *trans*-activation	MDS/AML	PMID: 20098431
WAS	RV: CMMP-*WASP*	7/10	*LMO2*	LTR *trans*-activation	ALL/AML	PMID: 24622513
			MECOM			
β-Thalassemia	LV: β-globin LCR-*HBB* (cHS4 insulator in LTR)	1/1	*HMGA2*	HMGA2 transcript truncation from splicing into insulator	transient clonal expansion	PMID: 20844535
ADA SCID	RV: GIADAl (MLV-*ADA*)	1/≥22	*LMO2*	LTR *trans*-activation	T ALL	https://ir.orchard-tx.com/news-releases/news-release-details/orchard-statement-strimvelisr-gammaretroviral-vector-based-gene
X-ALD	LV: lenti D (MND LTR-*ABCD1*)	3/67	*MECOM*	LTR *trans*-activation	MDS/AML	https://investor.bluebirdbio.com/news-releases/news-release-details/bluebird-bio-provides-update-severe-genetic-disease-programs
SCID-X1	LV: Cl20-i4-EF1α-hγcOPT (cHS4 insulator in LTR)	8/8	*HMGA2*	HMGA2 transcript truncation from splicing into insulator	transient clonal expansion	PMID: 35764638

A common factor in reported cases is the use of the MLV promoter/enhancer to drive gene expression. MLV LTR with its potent enhancer elements as a major factor in Inserional Oncogenesis.

In fact, a clinical trial of gene therapy for X-SCID safely used a gammaretroviral vector in which the LTR enhancer elements were self-inactivated (“SIN” vector); no clonal expansion or clinical leukoproliferation has occurred in more than 8 years of follow-up.

Non integrating lentiviral vectors

Integration-deficient Lentiviral Vectors: A Slow Coming of Age

Klaus Wanisch[1] and Rafael J Yáñez-Muñoz[1]

HIV- PIC complex is localized to genomic DNA through an interaction with lens epithelium–derived growth factor/p75 (LEDGF/p75) and the viral enzyme integrase subsequently mediates integration into host DNA.

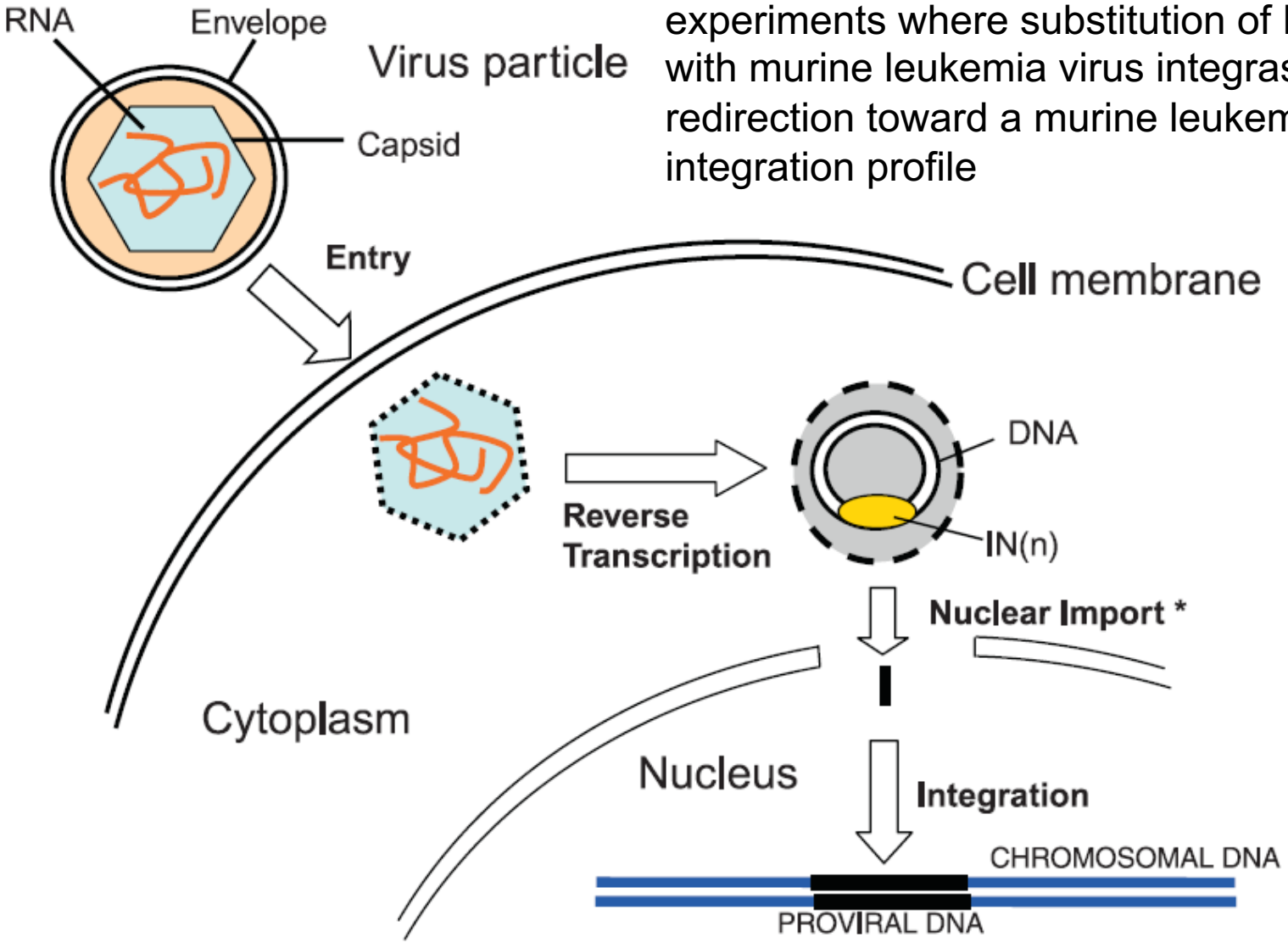
Site selection is influenced by a variety of factors and as for other retroviral vectors, cellular factors including higher order chromatin structures are likely to govern accessibility to target DNA.
Around **70% of HIV-1 integration sites occur in gene**s compared to a predicted level of around **30% if the process was purely random**

Viral integrase plays a key role in integration site selection, and this was demonstrated in experiments where substitution of HIV integrase with murine leukemia virus integrase resulted in redirection toward a murine leukemia virus– like integration profile

Risks associated with disrupted gene expression need to be carefully considered. How can the safety profile of lentiviral vectors be further improved?

Provirus integration is IN-dependent

Viral integrase plays a key role in integration site selection, and this was demonstrated in experiments where substitution of HIV integrase with murine leukemia virus integrase resulted in redirection toward a murine leukemia virus– like integration profile



avoid vector integration into genomic DNA entirely through the use of integration-deficient (nonintegrating) lentiviruses (NILV). Such an approach relies on **disabling conventional integration** pathways, for example through mutations in integrase.

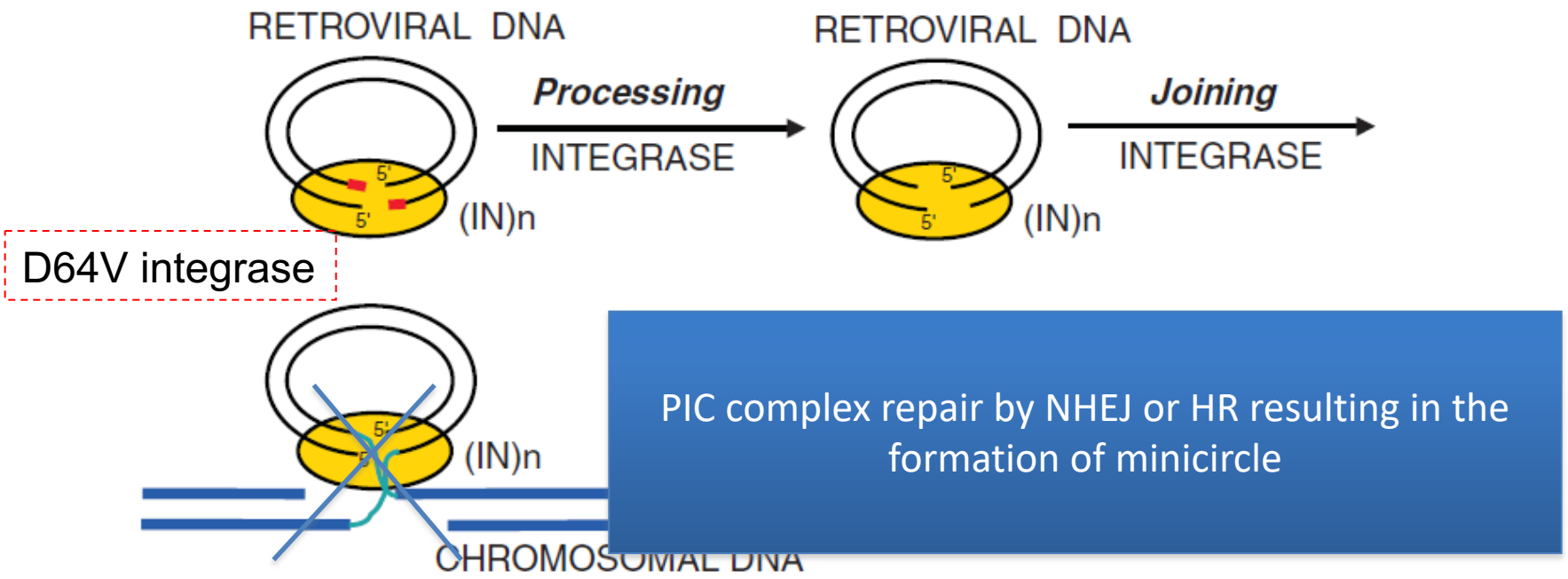


FIG. 2. Retroviral DNA integration. For details see text. IN(n), integrase multimer.

Mutations of integrase such as D64V are designed to disrupt its role in proviral integration, but not to compromise its other functions, including virus packaging and nuclear translocation of the preintegration complex. Thus**, following reverse transcription, lentiviral DNA fails to integrate into chromosomal DNA, and is predisposed to forming DNA circles as a consequence of host DNA repair proteins**

NILV - Generation of episome from lentiviral vectors

Circular provirus intermedia can be formed, when integration is blocked the amount of circular episome increases. It has been estimated that approximately one-third of linear lentiviral DNA forms either 1- or 2-LTR circles in target cells and these episomal forms remain transcription competent. This fraction increases in the case of integrase-deficient vectors

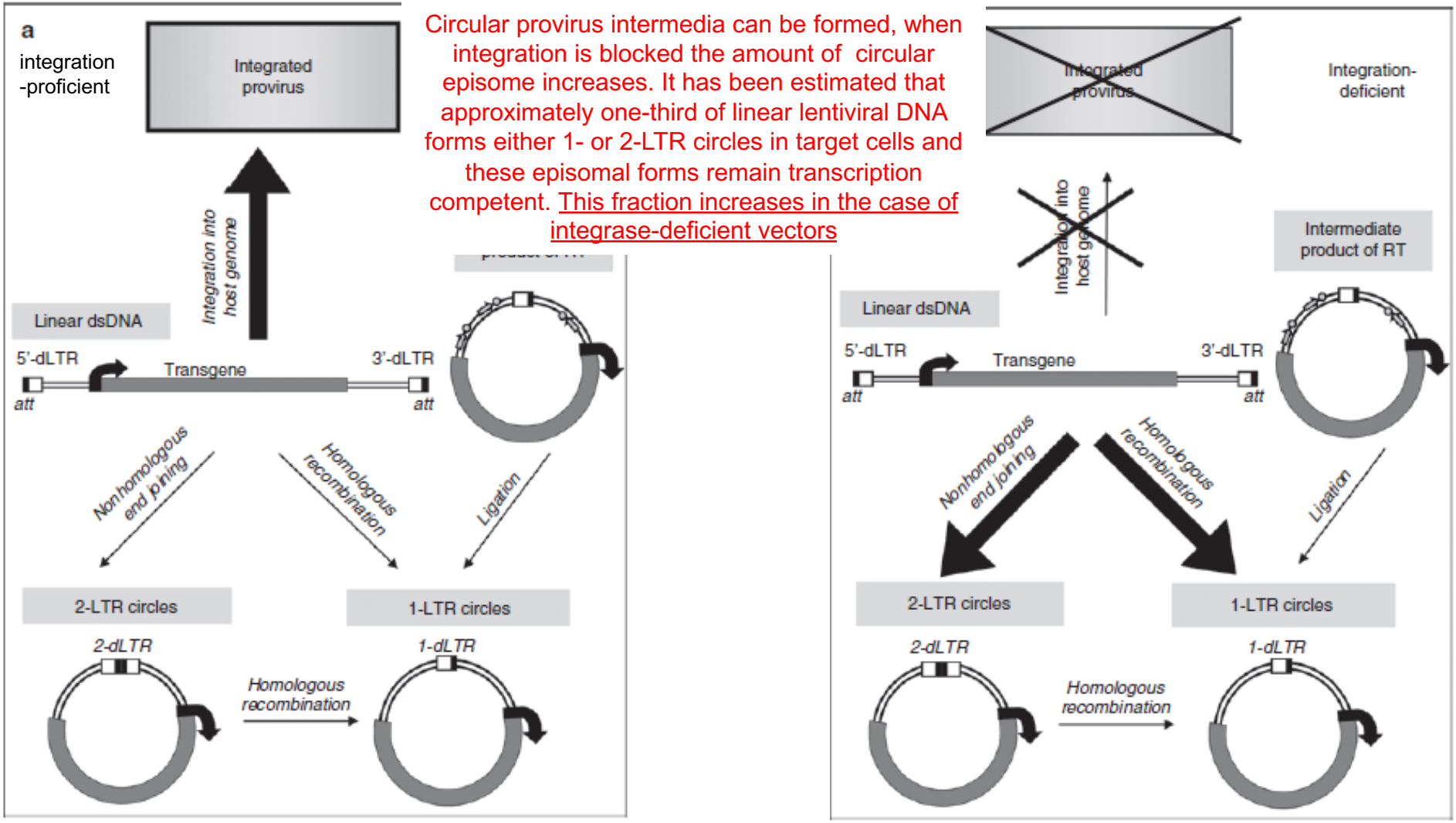


Figure 1 Generation of episomes from lentiviral vectors. The product of vector reverse transcription is a linear double-stranded DNA (dsDNA) with LTRs at both ends (deleted in the U3 region in self-inactivating vectors, here denoted as dLTR). This DNA is imported into the nucleus as part of the viral preintegration complex. (**a**) Conventional lentiviral vectors, harboring a functional IN, can be integrated into the host genome as proviruses. However, the linear DNA can also be circularized in several possible ways: nonhomologous end-joining produces 2-LTR circles, while intramolecular homologous recombination between the LTRs in linear DNA or 2-LTR episomes, or ligation of nicks (shown as arrow-bead structures) in intermediate products of reverse transcription, lead to 1-LTR circles. (**b**) When proviral integration is blocked through class I *IN* mutations, increased amounts of vector episomes are produced. *att*, IN attachment sites at the ends of viral DNA. LTR, long terminal repeat.

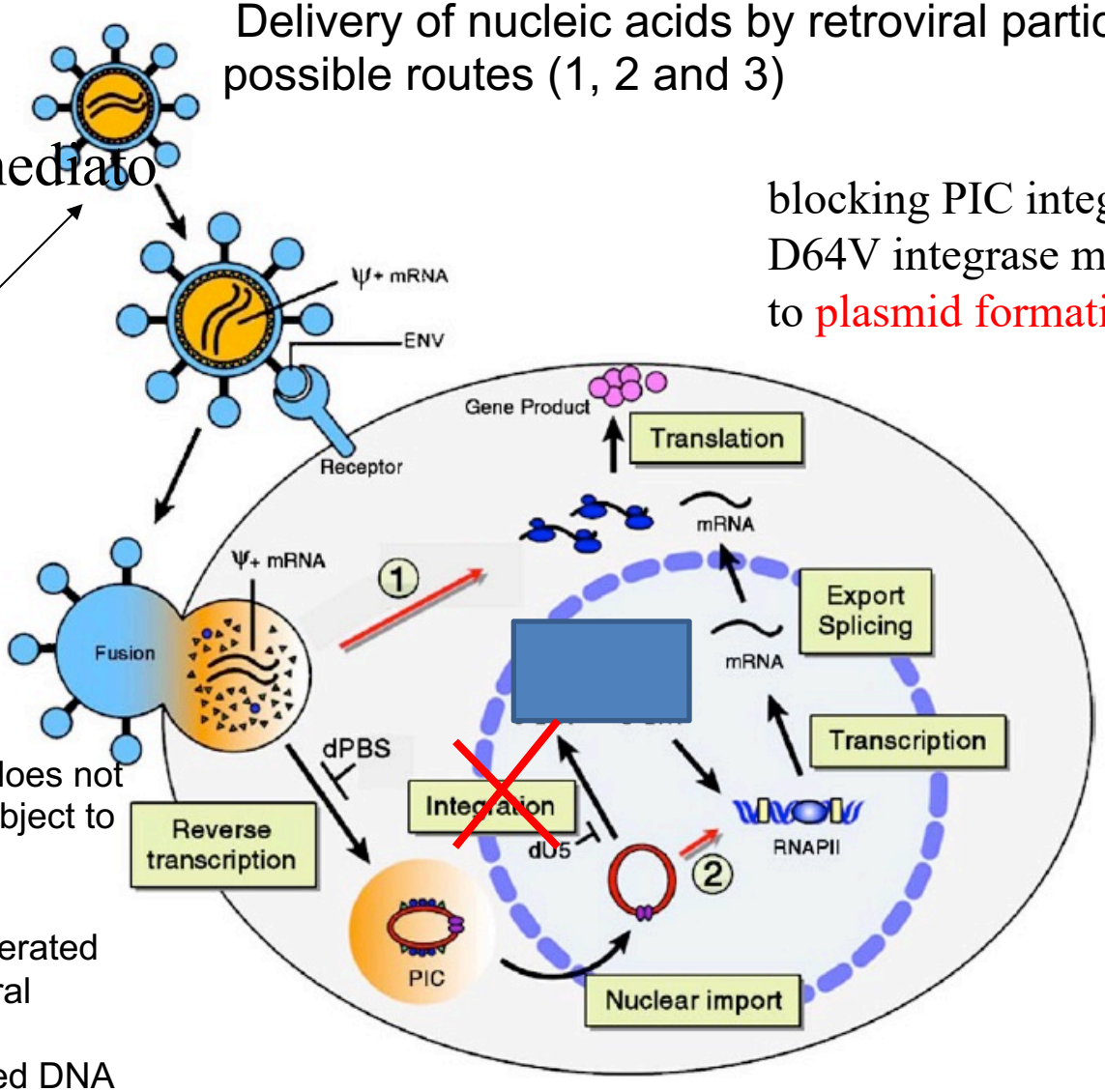
Delivery of nucleic acids by retroviral particles: three possible routes (1, 2 and 3)

Recettore mediato

entrata

fusione

blocking PIC integration by D64V integrase mutant, leads to plasmid formation (route 2)



1➔ if reverse transcription does not occur, the mRNA may be subject to immediate translation
2➔ if integration is blocked, episomal circles can be generated
3➔ if all steps of the retroviral transduction process are completed, a double-stranded DNA integrates

Following receptor-mediated uptake (by fusion or via endosomes, depending on the envelope protein), retroviral particles can deliver three forms of genetic information: (1) if reverse transcription does not occur, the mRNA may be subject to immediate translation; (2) if integration is blocked, episomal circles can be generated that may persist in non-dividing cells; (3) if all steps of the retroviral transduction process are completed, a double-stranded DNA integrates in cellular chromosomes. dPBS, deletion/mutation of the PBS (Primer Binding Site), datt, deletion/mutation of the att sites; RNAPII, RNA polymerase II.

NILV in mitotic and non-mitotic cells

In nonmitotic cells, gene expression from episomal lentiviral circular DNA (NILV vector) has been **sustained for many months**.

In dividing cell systems, such as the hematopoietic cells or skin tissue because episomal, nonreplicating, **lentiviral forms rapidly dilute** as cells turnover, the transgene effects is expected to be lost over a period of days or weeks.

Making NILV integrative

Nonintegrating lentiviral vectors can be integrated by alternative integrases such as meganucleases, zinc finger nucleases (ZFNs), transposone.integrase

Table 1 Integration-deficient lentiviral vectors (NILV) used to deliver alternative DNA-modifying systems and published efficiencies of gene transfer compared to integrating lentiviral vectors

Gene transfer vector	Integration mechanism	Target cell	Reporter gene	Maximum % stable transduction and/or integrants per NILV copy	Reference
ILV	Lentiviral integrase	HEK293T	eGFP	up to 100%	50
NILV	Flp recombinase	HEK293	Hygromycin	0.01% 2×10^{-5}/copy	25
NILV	I-*Sce*1 meganuclease	HEK293	eGFP gene repair	1%	27
NILV	Zinc finger nuclease	K562	eGFP/IL2Rγ chain locus/ CCR5 locus	35%	26
NILV	Sleeping Beauty (SB11)	HeLa	Neomycin	2.60% 4×10^{-4}/copy	42
NILV	Sleeping Beauty (SB100)	HEK293	Puromycin	% not reported 1×10^{-2}/copy	43

Abbreviations: eGFP, enhanced green fluorescent protein; ILV, integrating lentiviral; NILV, integration-deficient (nonintegrating) lentiviruses.

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integrating NILV

the system comprises:

A)NILV vector/s providing the integrase **– it provides the cutting system that we have selected**

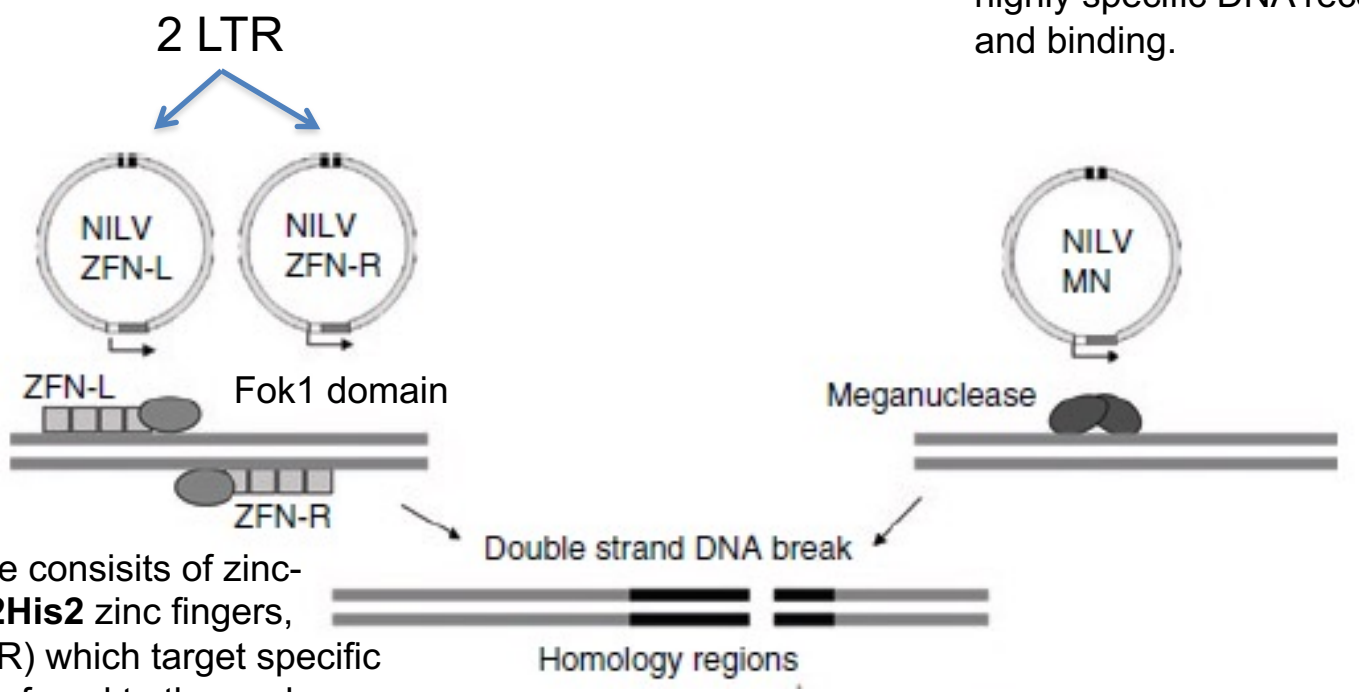
B)NILV-donor, carrying sequences homologous to the cleavage site, and the transgene. NILV donor provides a template for homologous recombination and repair of cleaved genomic DNA, and this can allow the insertion of the gene of interest **– it provides the genes that we want to integrate**

NILV vector Integration mediated site-specific recombinases

Schematic representation of nonintegrated lentiviral circles in which 2-LTRs have joined by nonhomologous end- joining in the absence of integrase activity. These episomal forms support transcription of nucleases capable of site-specific DNA modification.

In the case of zinc finger nucleases (ZFNs), the zinc finger motifs bind to opposite strands of target DNA sites and this allows dimerization of Fok1 endonuclease resulting in double-stranded cleavage of genomic DNA

Alternatively, meganucleases (MN) such as I-SceI, mediate precise DNA cleavage following highly specific DNA recognition and binding.



Here the integrase consisits of zinc-finger motif**, Cys2His2** zinc fingers, (ZFN-L and ZFN-R) which target specific DNA base triplets, fused to the nuclease domain of endonuclease Fok1. This has generated artificial ZFNs capable of inducing site-directed double-stranded DNA breaks.

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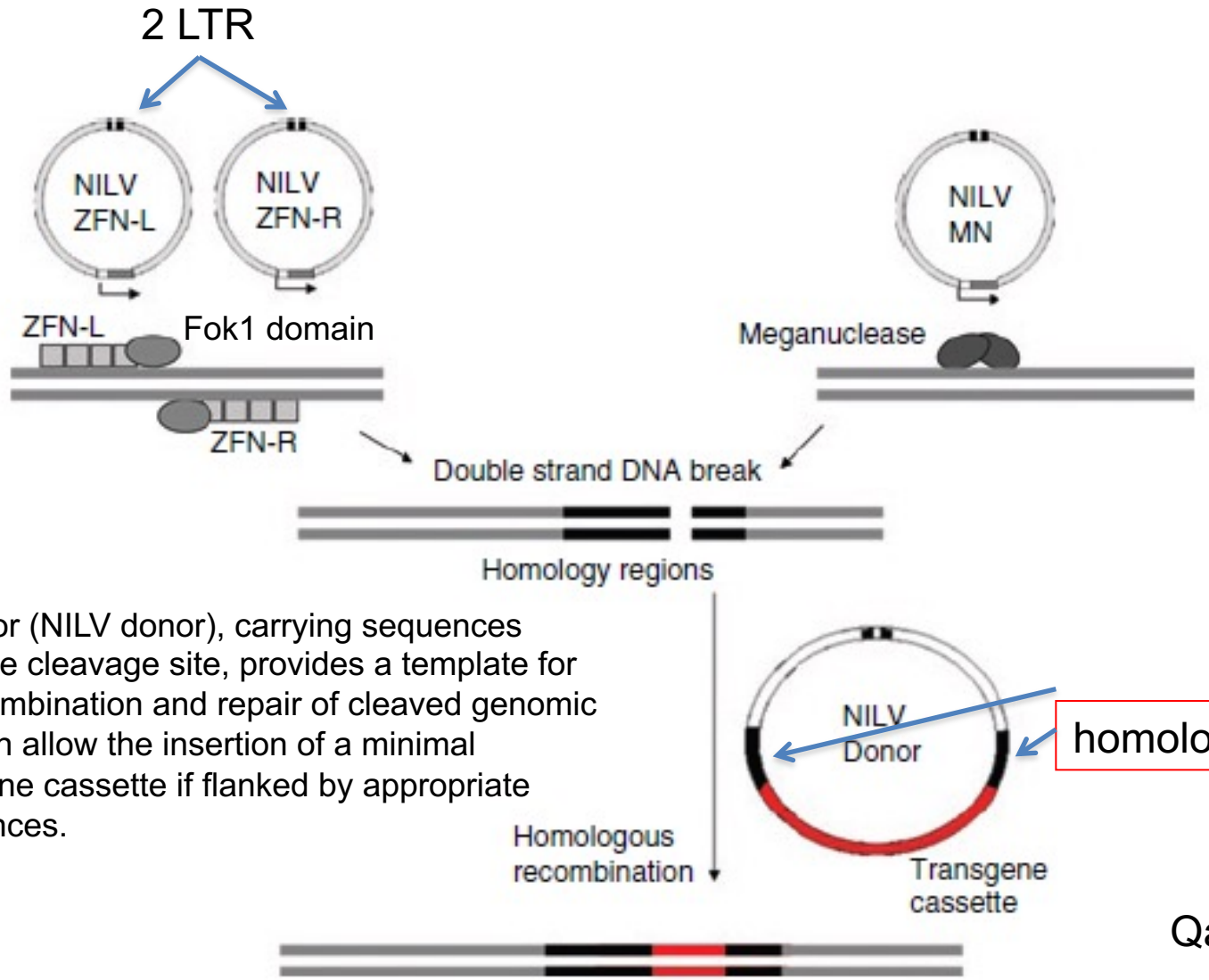
NILV vector Integration mediated site-specific recombinases

Schematic representation of nonintegrated lentiviral circles in which 2-LTRs have joined by nonhomologous end- joining in the absence of integrase activity. These episomal forms support transcription of nucleases capable of site-specific DNA modification.



A third NILV vector (NILV donor), carrying sequences homologous to the cleavage site, provides a template for homologous recombination and repair of cleaved genomic DNA, and this can allow the insertion of a minimal promoter/transgene cassette if flanked by appropriate homology sequences.

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NILV integration systems at work

Lombardo *et al.* generated transduced cells with three NILVs: two for expression of ZFN dimerizing pairs and one for delivery of the repair template encoding a transgene flanked by sequences homologous to the target site.
Gene conversion was observed at the IL2RG locus in 16% of K562 cells infected with all three vectors.

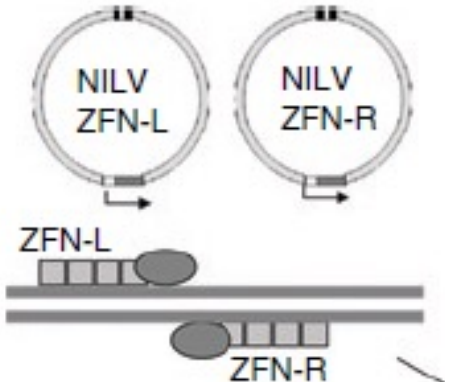
Similarly, ZFNs have been designed to target the *CCR5* locus. CCR5 is a coreceptor for HIV-1 entry and a homozygous Δ32 deletion in *CCR5* is linked to viral resistance in man. Perez *et al.* sought to permanently disrupt CCR5 expression and recreate the Δ32-CCR5 null phenotype in human CD4+ T cells. Using NILV vector addition at the *CCR5* locus of the Δ32-CCR5 occurred in 35% of K562 cells (0.005% background integration rate).

The CCR5 locus is being investigated as a “safe-harbor” site for integration, as disrupted CCR5 expression, present in around 1% of the population, does not appear to result in any significant reduction in immunity

nuclease-mediated genotoxicity

Finally the risk of nuclease-mediated toxicity will have to be considered for specific constructs, as it is likely to be dependent on a variety of factors including the specificity of DNA cleavage and the frequency of target-binding sites.

For example, engineering Fok1 endonuclease to require obligate heterodimerization for effective DNA cleavage can reduce “off-target” effects mediated by conventional Fok1 homodimers



La lezione è basata sulla seguente letteratura (in neretto le letture fortemente consigliate):

- **Qasim et al, 2010. Hybrid lentiviral vectors, Molecular Therapy 18:1263-1267**
- **Cavazza et al 2013, Mechanisms of retroviral integration and mutagenesis, Hum Gene Therapy 24, 119-131**
- **Cornetta et al 2023,** Meeting FDA Guidance recommendations

for replication-competent virus and insertional oncogenesis testing.