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Perspective

Understanding latent HIV-1 reservoirs through host genomics approaches

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SUMMARY

Genetically intact HIV-1 proviruses are a major concern with regard to curing infection because they cause viral rebound after the cessation of antiretroviral therapy. However, intact proviruses are not prevalent in HIV-1 reservoirs. As such, it is essential to precisely determine the position of these proviruses before putting forward a better antiretroviral cure. Recently, a revised HIV-1 deeply latent reservoir concept has been proposed, stating that the progress of the establishment of HIV-1 reservoirs is influenced by immune-mediated selection during the course of infection. This selection force leads to the persistence of genetically intact proviruses as those with the best fit to avoid clearance. This hypothesis refreshes our understanding of HIV-1 latent reservoirs. For this reason, we reviewed current studies relevant to this theme and provide our perspectives to reinforce the overall understanding of HIV-1 latency in the context of the host genome.

INTRODUCTION: HIV-1 PROVIRUS GENOME INTEGRITY AND DEEPLY LATENT RESERVOIRS

Current antiretroviral therapy can block new infections of susceptible cells but cannot eliminate the virus production by cells with integrated latent proviruses. Latently infected cells forming latent reservoirs may cause viral rebound after antiretroviral therapy is interrupted, consequently impeding the efficacy of treatment. Determining the precise position of HIV-1 integration in latent reservoirs is thus crucial in order to develop a better functional cure to achieve a goal of sustained viral suppression without the need for life-long treatment. To achieve this task, it is necessary to develop methods that allow the characterization of proviral genomic sequences in reservoir cells.

To date, technical advances have made it possible to recapitulate a single proviral genome using clinical materials, which we will detail in this perspective. HIV-1 latent reservoirs were initially defined using a quantitative viral outgrowth assay[1–3] and PCR-based methods.[4,5] However, it has been known that the former provides only a minimal approximation of the frequency of latently infected cells, and the latter may overestimate the size of the reservoir. The limitations of these methods have been discussed in many articles; thus, we do not reiterate that part in this perspective. Following those advances, several methods were developed to characterize the integrity of the near-full-length HIV-1 genome, including single-proviral[6] and single-genome sequencing,[7] single-genome amplification, full-length individual proviral sequencing (FLIPS) assay[8] and FLIP-seq,[9] and intact proviral DNA assay (IPDA).[10] Some of the methods are analogous to each other; others are not. In this perspective, we rigorously selected research articles from the last 5 years, in which the authors applied techniques to assess HIV-1 proviral genome integrity (Subsection Current technologies used for characterizing the integrity of proviral genomic sequences). We systematically compare the methodologies used in each selected article in Table 1. Importantly, we also provide technical details, including the primer design and the usage of primers in different rounds of PCR amplification in different approaches (Table 2), thereby facilitating the readers to commence experiments. The limitations and shortcomings of the current methods are briefly discussed in Section Continued technical improvement for the study of HIV-1 latent reservoirs. Additionally, we also highlight the methods that allow viral genomic integrity to be associated with its corresponding integration site (Section Continued technical improvement for the study of HIV-1 latent reservoirs; Table 1). One of the reasons we wrote this perspective was that we are aware of the importance of the revised hypothesis regarding HIV-1 deep latency that was recently proposed.[11–16] The concept of HIV-1 deep latency (or silencing) originates from 1990s onward and was updated progressively. A deeper level of HIV-1 latency is most likely referred the latent proviruses that are refractory to be reactivated. These proviruses are often associated with either epigenetic features (e.g., DNA methylation) or the specific chromosomal conformation (e.g., heterochromatin). This presently renewed hypothesis of HIV-1 deep latency not only refreshes our current understanding of latency itself but also provides a window of opportunity to strengthen our knowledge about the role of the host genome coupled with immune response during HIV-1 infection.

The theoretical basis of this hypothesis is, in large part, supported by the results of experiments using methods to discriminate genetically intact proviruses from defective ones and assess their interactions with local chromosomal microenvironments. Therefore, in this perspective, we also highlight the success of these methods, which enables us to investigate dynamic alterations of HIV-1 deeply latent reservoirs (Section Reconfiguration of deeply latent HIV-1 reservoirs correlates with epigenetic- and 3D genomic features). One remarkable achievement of the

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Table 1. Systematical summary of the research articles using molecular methods to characterize the integrity of the viral genome

Title	Targeted individuals	Method type	Targeted cells	Longitudinal/ multiple time points study	HIV-1 IS included in the study	Reference
Virally suppressed people living with HIV who use opioids have diminished latency reversal	ART-treated individuals	ddPCR	$CD4^+$ T cells	+	–	Basukala et al. (2023)[18]
Phenotypic characterization of single $CD4^+$ T cells harboring genetically intact and inducible HIV genomes	ART-treated individuals	HTS	$CD4^+$ T cells	–	–	Dufour et al. (2023)[19]
HIV rapidly targets a diverse pool of $CD4^+$ T cells to establish productive and latent infections	Pretreatment HIV-1-infected individuals; ART-treated patients	HTS	$CD4^+$ T cells; lymph node mononuclear cells	+	–	Gantner et al. (2023)[20]
Unequal distribution of genetically intact HIV-1 proviruses in cells expressing the immune checkpoint markers PD-1 and/or CTLA-4	ART-treated individuals	HTS	$CD4^+$ T cells	–	–	Fisher et al. (2023)[21]
Varied patterns of decay of intact HIV-1 proviruses over two decades of art	ART-treated individuals	ddPCR	$CD4^+$ T cells	+	–	Gandhi et al. (2023)[22]
Host variation in type I interferon signaling genes (MX1), C-C chemokine receptor type 5 gene, and major histocompatibility complex class I alleles in treated HIV+ noncontrollers predict viral reservoir size	ART-treated individuals	ddPCR	$CD4^+$ T cells	–	–	Siegel et al. (2023)[23]
In-Depth Characterization of Full-Length Archived Viral Genomes after Nine Years of Posttreatment HIV Control	ART-treated individuals; posttreatment controllers; natural HIV infection controllers	HTS	PBMCs	+	–	Trémeaux et al. (2023)[24]
Phenotypic signatures of immune selection in HIV-1 reservoir cells	ART-treated individuals	HTS+ddPCR	$CD4^+$ T	+	+	Sun et al. (2023)[16]
Progressive transformation of the HIV-1 reservoir cell profile over two decades of antiviral therapy	ART-treated individuals	HTS	PBMCs	+	+	Lian et al. (2023)[15]
HIV-1 reservoir evolution in infants infected with clade C from Mozambique	Infants infected with HIV-1	HTS	PBMCs	+	+	Koofhethile et al. (2023)[25]

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Table 1. *Continued*

Title	Targeted individuals	Method type	Targeted cells	Longitudinal/ multiple time points study	HIV-1 IS included in the study	Reference
Intact provirus and integration sites analysis in acute HIV-1 infection and changes after one year of early antiviral therapy	Pretreatment and ART-treated individuals	ddPCR	PBMCs	+	+	Rozera et al. (2022)[26]
Stable HIV Reservoir Despite Prolonged Low-Dose Mycophenolate to Limit $CD4^+$ T cell Proliferation	ART-treated individuals	ddPCR	$CD4^+$ T cells; rectal biopsies	+	–	Schiffer et al. (2022)[27]
Early intervention with 3BNC117 and romidepsin at antiretroviral treatment initiation in people with HIV-1: a phase 1b/2a, randomized trial	ART-treated individuals	ddPCR	$CD4^+$ T cells	+	–	Gunst et al. (2022)[28]
Identification of CD98 as a Novel Biomarker for HIV-1 Permissiveness and Latent Infection	ART-treated individuals	ddPCR	$CD4^+$ T cells	–	+	Zhang et al. (2022)[29]
The effect of induction immunosuppression for kidney transplant on the latent HIV reservoir	ART-treated individuals	ddPCR	$CD4^+$ T cells	+	–	Benner et al. (2022)[30]
Measuring the latent reservoir for HIV-1: Quantification bias in near full-length genome sequencing methods	ART-treated individuals	HTS+ddPCR	$CD4^+$ T cells; J-Lat	–	–	White et al. (2022)[31]
Distinct gene expression by expanded clones of quiescent memory $CD4^+$ T cells harboring intact latent HIV-1 proviruses	ART-treated individuals	HTS+ddPCR	$CD4^+$ T cells	–	–	Weymar et al. (2022)[32]
SARS CoV-2 mRNA vaccination exposes latent HIV to Nef-specific CD8(+) T-cells	ART-treated individuals	ddPCR	$CD4^+$ T cells	+	–	Stevenson et al. (2022)[33]
HIV infected $CD4^+$ T cell clones are more stable than uninfected clones during long-term antiretroviral therapy	ART-treated individuals	HTS	$CD4^+$ T cells	+	+	Guo et al. (2022)[34]
Markers of immune activation and inflammation are associated with higher levels of genetically intact HIV in HIV-HBV co-infected individuals	Pretreatment and ART-treated individuals	HTS	$CD4^+$ T cells	+	–	Wang et al. (2022)[35]
Intact HIV proviruses persist in the brain despite viral suppression with ART	ART-treated individuals	ddPCR	Autopsy brain tissue; matched lymphoid tissues	–	–	Cochrane et al. (2022)[36]
Immune correlates of HIV-1 reservoir cell decline in early treated infants	Infants infected with HIV-1 before and during ART; ART-treated adults	HTS	PMBCs	+	–	Hartana et al. (2022)[37]

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Table 1. *Continued*

Title	Targeted individuals	Method type	Targeted cells	Longitudinal/ multiple time points study	HIV-1 IS included in the study	Reference
Deep sequencing analysis of individual HIV-1 proviruses reveals frequent asymmetric long terminal repeats	ART-treated individuals	HTS	PMBCs	–	+	Joseph et al. (2022)[38]
Altered T cell subset distribution in the viral reservoir in HIV-1-infected individuals with extremely low proviral DNA (LoViReTs)	ART-treated individuals	ddPCR	$CD4^+$ T cells	–	–	Gálvez et al. (2022)[39]
Plasma-derived HIV-1 virions contain considerable levels of defective genomes	ART-treated individuals	HTS	Plasma samples from viremic HIV-1-infected individuals	+	–	Fisher et al. (2022)[40]
The HIV-1 proviral landscape reveals that Nef contributes to HIV-1 persistence in effector memory $CD4^+$ T cells	ART-treated individuals	HTS	$CD4^+$ T cell subsets	–	–	Duette et al. (2022)[41]
ABX464 decreases the total human immunodeficiency virus (HIV) reservoir and HIV transcription initiation in $CD4^+$ T cells from antiretroviral-therapy-suppressed individuals living with HIV	ART-treated individuals	ddPCR	$CD4^+$ T cells	+	–	Moron-Lopez et al. (2022)[42]
Complex decay dynamics of HIV virions, intact and defective proviruses, and 2LTR circles following initiation of antiretroviral therapy	ART-treated individuals	ddPCR	$CD4^+$ T cells	+	–	White et al. (2022)[43]
HIV reservoir quantification using cross-subtype multiplex ddPCR	ART-treated individuals	ddPCR	PBMC; J-lat 5A8	+	–	Cassidy et al. (2022)[44]
Evaluation of HIV-1 reservoir size and broadly neutralizing antibody susceptibility in acute antiretroviral-therapy-treated individuals	ART-treated individuals	ddPCR	$CD4^+$ T cells	–	–	Moldt et al. (2022)[45]
Cellular activation, differentiation, and proliferation influence the dynamics of genetically intact proviruses over time	ART-treated individuals	HTS	$CD4^+$ T cell subsets	+	–	Horsburgh et al. (2022)[46]
Longitudinal clonal dynamics of HIV-1 latent reservoirs measured by combination quadruplex polymerase chain reaction and sequencing	ART-treated individuals	HTS+ddPCR	PBMCs	+	–	Cho et al. (2022)[47]

Table 2. Primers applied in methods for the characterization of the integrity of the HIV-1 genome

Method	HIV-1 genomic region[a]	Primer name (annealing position[b])	5′-3′ sequence	Reference
FLIPS	5′ U5 LTR	BLOuterF (623)	AAATCTCTAGCAGTGGCGCCCGAACAG	Simonetti et al. (2021)[53]
	3′ U5 LTR	BLOuterR (9686)	TGAGGGATCTCTAGTTACCAGAGTC	
	5′ U5 LTR	275F (646)	ACAGGGACCTGAAAGCGAAAG	
	3′ U5 LTR	280R (9676)	CTAGTTACCAGAGTCACACAACAGACG	
IPDA	psi	Psi-Forward (692)	CAGGACTCGGCTTGCTGAAG	Jiang et al. (2020)[12]; Simonetti et al. (2021)[53]; Rozera et al. (2022)[26]
	psi	Psi-Reverse (797)	GCACCCATCTCTCTCCTTCTAGC	
	env (Rev response element)	Env-Forward (7736)	AGTGGTGCAGAGAGAAAAAAGAGC	
	env (Rev response element)	Env-Reverse (7851)	GTCTGGCCTGTACCGTCAGC	
FLIP-seq/MIP- seq	5′ U5 LTR	U5-623F (623)	AAATCTCTAGCAGTGGCGCCCGAACAG	Einkauf et al. (2019)[11]; Jiang et al. (2020)[12]; Lian et al. (2021)[13]; Lian et al. (2023)[15]; Koofhethile et al. (2023)[25]
	3′ U5 LTR	U5-601R (9686)	TGAGGGATCTCTAGTTACCAGAGTC	
	5′ U5 LTR	U5-638F (638)	GCGCCCGAACAGGGACYTGAAARCGAAAG	
	3′ U5 LTR	U5-547R (9632)	GCACTCAAGGCAAGCTTTATTGAGGCTTA	
MIP-seq	*gag*	U5-623F (623)	AAATCTCTAGCAGTGGCGCCCGAACAG	Einkauf et al. (2019)[11]; Jiang et al. (2020)[12]; Lian et al. (2021)[13]; Simonetti et al. (2021)[53]; Lian et al. (2023)[15]; Koofhethile et al. (2023)[25]
	pol	NE1 (3333)	CCACTAACTTCTGTATGTCATTGACAGTCCAGCT	
	pol	ProA- (2735)	GGCAAATACTGGAGTATTGTATG	
	gag	U5-638F (638)	GCGCCCGAACAGGGACYTGAAARCGAAAG	
	pol	ProC- (2724)	GAGTATTGTATGGATTTTCAGGCCCAAT	
	gag	5CP1 (1981)	GAAGGGCACACAGCCAGAAATTGCAGGG	
	pol	RT3.1 (3859)	GCTCCTACTATGGGTTCTTTCTCTAACTGG	
	gag	2.5 (2011)	CCTAGGAAAAAGGGCTGTTGGAAATGTGG	
	pol	RT3798R (3798)	CAAACTCCCACTCAGGAATCCA	
	pol	RT3597mixF (3597)	AAAACAGGAAARTATGCAA	
	vpu	SC05R (6004)	AGCTCTTCGTCGCTGTCTCCGCTT	
	pol	RT3626F (3626)	TGCCCACACTAATGATGTAA	
	vpu	SC02R (5980)	CTTCCTGCCATAGGAGATGCCTA	
	vpr	VP5450F (5450)	CAGGACATAACAAGGTAGGATC	
	GP120	CO602 (7817)	GCCCATAGTGCTTCCTGCTGCTCCCAAGAACC	
	vpr	VP5549F (5549)	AGAGGATAGATGGAACAAGCCCCAG	
	GP120	V3CR (7760)	TGCTCTTTTTTCTCTCTSCACCACT	
	GP41	GP41Fo (7626)	TTCAGACCTGGAGGAGGAGATAT	
	nef	3LTRi (9628)	TCAAGGCAAGCTTTATTGAGGCTTAA	
	GP41	GP41Fi (7652)	GGACAATTGGAGAAGTGAATTAT	
	nef	3UTRi (9610)	AGGCTTAAGCAGTGGGTTCCCTAG	

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Table 2. *Continued*

Method	HIV-1 genomic region[a]	Primer name (annealing position[b])	5′-3′ sequence	Reference
Derivative	5′ U5 LTR[a]	Primary forward (623[b])	AAATCTCTAGCAGTGGCGCCCGAACAG	Brandt et al. (2011)[54]
	3′ U5 LTR[a]	Primary reverse (9686[b])	TGAGGGATCTCTAGTTACCAGAGTC	
	SL3: stem-loop of Psi element[a]	Secondary Forward (769[b])	GCGGAGGCTAGAAGGAGAGAGATGG	
	3′ LTR repeat[a]	Secondary reverse (9632[b])	GCACTCAAGGCAAGCTTTATTGAGGCTTA	
	5′ LTR U5[a]	NFLPAS_OuterFWD (611[b])	AGTCAGTGTGGAAAATCTCTAG	Joseph et al. (2022)[38]
	3′ LTR U5[a]	NFLPAS_OuterREV (9685[b])	GAGGGATCTCTAGTTACCAGAG	
	5′ LTR U5[a]	NFLPAS_InnerFWD (618[b])	GTGGAAAATCTCTAGCAGTGG	
	3′ LTR U5[a]	NFLPAS_InnerREV (9672[b])	TTACCAGAGTCACACAACAGAC	
	5′ LTR U5[a]	DNA F1 (623)	AAATCTCTAGCAGTGGCGCCCGAACAG	Matsuda et al. (2021)[55]
	3′ LTR U5[a]	DNA R1 (9676)	TGAGGGATCTCTAGTTACCAGAGTC	
	5′ LTR Lys tRNA primer binding site[a]	Nested F (638)	GCGCCCGAACAGGGACYTGAAARCGAAAG	
	3′ LTR repeat[a]	DNA R2 (9632)	GCACTCAAGGCAAGCTTTATTGAGGCTTA	
	5′ LTR Packaging loops[a]	DNA F2 (682)	TCTCTCGACGCAGGACTCGGCTTG	
	3′ LTR repeat[a]	DNA R2 (9632)	GCACTCAAGGCAAGCTTTATTGAGGCTTA	

[a]Viral genomic regions provided in this review due to the absence of information in the original articles.
[b]Primer annealing positions provided in this review due to the absence of information in the original articles.

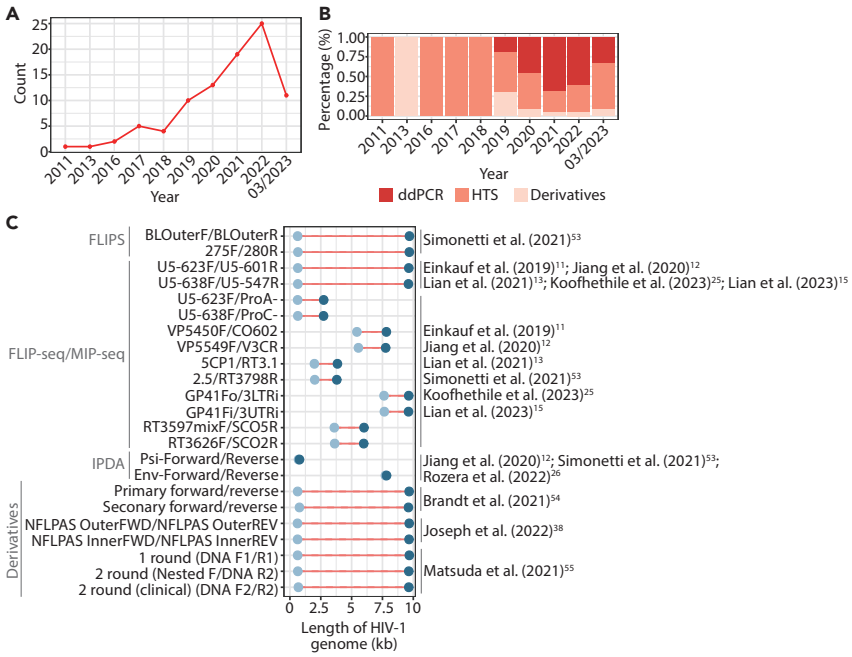

Figure 1. Overview of the technical progress in investigating HIV-1 reservoirs associated with the viral genome integrity

(A and B) Research articles using high-throughput-sequencing-based, ddPCR-based methods and analogous derivatives to study HIV-1 proviral genome integrity during the last 13 years. Increase of interest in research on HIV-1 proviral integrity (A). Proportion of techniques applied for assessing proviral genome integrity (B).

(C) Visualization of primers positions used to characterize HIV-1 proviral genome integrity. Sequences of primers are summarized in Table 2.

articles collected in this subsection is the precise identification of a distinct genomic region of intact proviruses at a deeper level of latency using methods that assess proviral genome integrity and track the evolution of such reservoirs coupled with immune-mediated selection. Essentially, we wish to introduce the concept that in addition to the 3D genome architecture associated with epigenetic modifications, functional genome properties may conjoin in the complicated process of the establishment of HIV-1 latency[17] (Subsection Task-evoked host genome functional network toward the evolution of HIV-1 reservoirs). We propose that the 3D genome could be seen as a space where various communities of gene sets share similar biological functions. Host genes in communities could be selectively targeted by HIV-1 at different stages of viral infection. At the same time, we also propose considerations for further technical improvement (Subsection Continued technical improvement for the study of HIV-1 latent reservoirs). Altogether, in this perspective, in addition to a summary of current technologies used to characterize the integrity of the proviral genomic sequence, there is an emphasis on the newly introduced "immune selection" hypothesis of HIV-1 deep latency and the involvement of the host genome in the evolution of HIV-1 latent reservoirs.

TECHNICAL BACKGROUND AND THE CURRENT STAGE OF THE TECHNOLOGIES USED FOR CHARACTERIZING THE INTEGRITY OF THE VIRAL GENOME

Search strategy and selection criteria

A rigorous literature search was performed on PubMed (accessed on 27 March 2023) with the keywords (((intact) OR (intact provirus) OR (intact proviruses)) AND ((HIV) OR (HIV-1))). Research articles were selected from the first 1,000 cited papers (from December 2005 to March 2023), limited to those published in the English language and excluding review articles, preprints, protocol papers, author corrections and errata, and news features. We then executed a text editing command (`awk '/intact HIV/ || /intact provirus/ || /full length/ || /nearly full length/ || /full-length/' PubMed_list_1000_articles`) on the title and abstract of each article to remove inappropriate articles and carefully examined all relevant articles. Eventually, 91 articles met the selection criteria. We observed a trend of an increasing number of studies focused on genetically intact HIV-1 (Figure 1A), especially after the FLIPS[8] and FLIP-seq[9] assays were published in 2017 (Figure 1A). This again emphasizes that characterizing the integrity of the HIV-1 proviral genome is necessary for further deep investigation of HIV-1 latent reservoirs. We also observed that IPDA[10] became one of the most popular methods for detecting the integrity of the HIV-1 proviral genome after it has been benchmarked (Figure 1B), because this ddPCR-based method is easy to manipulate and cost-effective compared with high-throughput sequencing. Due to the limitation of the references in this perspective article, we summarize studies published in the past 2 years (2022 and 2023) and list them in Table 1.

At present, the methods used to characterize the integrity of a proviral genomic sequence are mainly derived from FLIPS and IPDA assays, which are based on high-throughput sequencing (HTS) and ddPCR, respectively. Hence, the methods described in this perspective are mainly

categorized as either HTS-based or ddPCR-based (Figure 1B and Table 1). Methods that did not fit into either of these were assigned as "derivatives" (Table 1). Of note, while reviewing the articles, we realized that some methods were named differently even though they are highly analogous to either FLIPS or IPDA. To minimize confusion for readers, we only provide the method type in Table 1.

Current technologies used for characterizing the integrity of proviral genomic sequences

Technologies based on single genome amplification of proviral sequences followed by HTS have made remarkable progress since 2017. Two similar approaches, FLIPS[8] and FLIP-seq,[9] were developed to characterize proviral genomic sequences in reservoir cells isolated from HIV-1-infected individuals with resolution of single proviruses. The aim of both methods is to determine intact therefore potentially replication-competent proviruses in a defined cellular reservoir and overcome some of the shortcomings of previous methods based on Sanger sequencing. The principle is to sequence the near-full-length genome of a single provirus by amplifying its DNA using two rounds of nested PCR amplification, followed by high-throughput sequencing and *de novo* assembly of sequences *in silico*. The use of inner primer pairs for a second round of nested PCR amplification in both FLIPS[8] and FLIP-seq[9] differs from the previous methods[48–50]; the former covers the HXB2 genome from 646 to 9676 base pairs (bp) and the latter from 638 to 9632 bp. The outer primer pair covers the HXB2 genome from 623 to 9686 bp and is the same for FLIPS[8] and FLIP-seq.[9] In principle, a provirus is considered genetically intact when the retrieved sequence is over 8,900 bp without inversions, deletions larger than 100 bp, frameshift/out-of-frame indels, G-A hypermutations, premature/lethal stop codons, or defects in the *cis*-acting element of the 5′ long terminal repeat (5′ LTR).[8,9] Overall, both methods overcome the limitations associated with single-proviral sequencing (SPS) and provide a more accurate assessment of the integrity of proviral genomic sequences.

IPDA, developed by Bruner et al.,[10] is another commonly used method to characterize the integrity of proviral genomes due to its high precision and reproducibility. IPDA uses two sets of primers targeting the HIV-1 packaging signal sequence (692–797 bp) and the conserved part of the Rev-response element (RRE) in the HIV-1 *env* gene (7736–7851 bp) for PCR amplification. Primers are coupled with independent fluorescence-labeled probes, which makes proviral sequences detectable when PCR amplification is complete. A proviral sequence is considered to be genetically intact when both fluorescent signals are present. Given that primers and probes are designed for only two genomic targets throughout the HIV-1 genome, it has been reported that due to HIV-1 sequence diversity, IPDA could cause assay failure.[51] Regardless, possible solutions to address these issues have been provided in different studies.[51,52] Nonetheless, it is worth noting that none of these methods (both HTS- and ddPCR-based methods) available at present can purely distinguish intact proviruses from defective ones unless the entire viral genome can be sequenced. At present, it remains challenging to have an assay that allows specifically focusing on the extremely low levels of HIV-1 reservoir in resting $CD4^+$ T cells. In the future, apart from the specificity (intact versus defective proviruses), the sensitivity of the assay itself is another crucial layer to be taken into account for developing new technologies.

The genomic positions of primer pairs used in these methods are illustrated in Figure 1C. Of note, the primers in Figure 1C were selected from studies that used standard methods of FLIP-seq, MIP-seq (detailed in Section Continued technical improvement for the study of HIV-1 latent reservoirs), and IPDA and studies that used derivative techniques to characterize the viral genome integrity and map the integration site. Primer sequences and corresponding viral genes annealed by primers are summarized in Table 2.

MECHANISTIC INTERPLAY BETWEEN PROVIRUSES AND THE HOST GENOME

Reconfiguration of deeply latent HIV-1 reservoirs correlates with epigenetic- and 3D genomic features

During the last 5 years, an increasing number of studies using clinical samples from elite controllers[12,13] and patients on antiretroviral therapy (ART)[11,14–16,41,47,56,57] have shed light on latent reservoirs harboring proviruses that are refractory to be reactivated. These studies, focusing on reservoir cells harboring genetically intact proviruses, have paved the way for a hypothesis suggesting that immune-mediated selection might play an important role in reshaping the genomic locations of the proviruses during the progression of HIV-1 infection. From the selected 91 research articles, we listed the articles highlighting findings relevant to the immune-mediated selection on the establishment of HIV-1 latent reservoirs (Table 3).

Different selection pressures that may lie between intact and defective proviruses have been brought by previous studies[56,60] and a series of studies from the Lichterfeld laboratory, and others reinforce this concept. Einkauf and colleagues (2019)[11] first showed that relative to genetically defective proviruses, intact proviruses were preferentially present in nongenic chromosomal regions and distal to active transcriptional start sites as well as accessible chromatin regions. These observations imply that the force of immune-mediated selection toward intact proviruses may differ from the force affecting defective proviruses. Consistently, Lian and colleagues observed that intact proviruses in long-term ART-treated individuals were frequently present in heterochromatin regions.[15] Here, it is important to note that although *in vitro* experiments based on epigenetic studies and functional assays, such as ATAC-seq, have shown that features associated with heterochromatin impede HIV transcription and reactivation, the functional role of heterochromatin in the context of *in vivo* viral rebound requires further investigation. The different selection force between intact and defective proviruses may appear in the observation that reservoir cells harboring intact proviruses are more vulnerable to host immune responses, most likely resulting in a more pronounced decrease of cell numbers compared with that harboring defective proviruses.[26,37,47,57,58,61,62] Furthermore, using longitudinal samples from HIV-1-infected individuals, Einkauf et al.[14] demonstrated that relative to transcriptionally silent proviruses the decrease in transcriptionally active proviruses was more noticeable in ART-treated patients, indicating that transcriptionally active HIV-1 proviruses are actively selected against during prolonged ART. However, it is important to stress that in the case of large and transcriptionally active clonally expanded cells, transcriptionally active proviruses appear to be refractory to the host selection mechanism.[14] Indeed, numerous studies have reported that the persistent HIV-1 infection is at least partially due to the clonal expansion of infected cells. Although it is not completely clear why some proviral-integrated latently

Table 3. Summary of the research articles relevant to the immune-mediated selection on latent HIV-1 reservoirs

Title	Targeted individuals	Summary of the study	Reference
Phenotypic characterization of single CD4$^+$ T cells harboring genetically intact and inducible HIV genomes	ART-treated individuals	CD4$^+$ T cells harboring replication-competent HIV retrain VLA-4 expression irrespective of diversity of phenotypic signatures of HIV reservoir cells	Dufour et al. (2023)[19]
Phenotypic signatures of immune selection in HIV-1 reservoir cells	ART-treated individuals	Ensemble signatures of surface markers are frequently expressed in reservoir cells harboring intact proviruses and in large clones of virally infected cells	Sun et al. (2023)[16]
Progressive transformation of the HIV-1 reservoir cell profile over two decades of antiviral therapy	ART-treated individuals	Intact proviruses preferentially integrate toward heterochromatin. Such proviruses are less transcriptionally active and, possibly, less rebound competent	Lian et al. (2023)[15]
Longitudinal clonal dynamics of HIV-1 latent reservoirs measured by combination quadruplex polymerase chain reaction and sequencing	ART-treated individuals	The size of reservoir harboring defective proviruses is relatively stable with minimal decay during the 10 years compared with which harboring intact proviruses with an estimated half-life of 4.9 years	Cho et al. (2022)[47]
The HIV-1 proviral landscape reveals that Nef contributes to HIV-1 persistence in effector memory CD4$^+$ T cells	ART-treated individuals	The proportion of intact HIV-1 proviruses was higher and persisted over time in effector memory CD4$^+$ T cells, and CTL (cytotoxic T lymphocyte) escape mutations remain stable in intact proviruses present in effector memory CD4$^+$ T cells during antiretroviral therapy	Duette et al. (2022)[41]
Parallel analysis of transcription, integration, and sequence of single HIV-1 proviruses	ART-treated individuals	Epigenetic features in a linear- and 3D genome influence HIV-1 transcription. And transcriptionally active proviruses were actively selected against during prolonged antiretroviral therapy.	Einkauf et al. (2022)[14]
Immune correlates of HIV-1 reservoir cell decline in early treated infants	Infants infected with HIV-1 before and during ART; ART-treated adults	Longitudinal decline of intact HIV-1 proviruses during ART is associated with antiviral NK cell immune responses.	Haryana et al. (2022)[37]
Intact provirus and integration sites analysis in acute HIV-1 infection and changes after one year of early antiviral therapy	Pretreatment of HIV-1-infected individuals and ART-treated adults	The ratio between intact and total proviral HIV-1 DNA did not dramatically change between pretreatment HIV-1 individuals and patients subjected to 48 weeks of therapy.	Rozera et al. (2022)[26]
Selective decay of intact HIV-1 proviral DNA on antiretroviral therapy	ART-treated individuals	Intact provirus levels declined significantly over time (median half-life, 7.1 years) compared with defective provirus levels.	Gandhi et al. (2021)[58]
Signatures of immune selection in intact and defective proviruses distinguish HIV-1 elite controllers	Elite controllers	Different immune selection pressure on intact and defective proviruses observed in elite controllers: intact proviruses were frequently located in heterochromatin regions, whereas defective proviruses commonly integrate in permissive genic euchromatin regions.	Lian et al. (2021)[13]

(*Continued on next page*)

Table 3. *Continued*

Title	Targeted individuals	Summary of the study	Reference
Longitudinal study reveals HIV-1-infected $CD4^+$ T cell dynamics during long-term antiretroviral therapy	ART-treated individuals	Slow decay of intact proviruses but no changes in the proportions of various types of defective proviruses. The proportion of intact proviruses in expanded clones was similar to that of defective proviruses in clones.	Antar et al. (2020)[57]
Distinct viral reservoirs in individuals with spontaneous control of HIV-1	Elite controllers	Intact proviruses preferentially integrate toward heterochromatin (centromeric satellite DNA or in Krüppel-associated box domain-containing zinc finger genes on chromosome 19).	Jiang et al. (2020)[12]
Intact HIV-1 proviruses accumulate at distinct chromosomal positions during prolonged antiretroviral therapy	ART-treated individuals	Intact HIV-1 proviruses were enriched in non-genic chromosomal positions and more frequently showed an opposite orientation relative to host genes. In addition, such proviruses were preferentially integrated in either relative proximity or increased distance from active transcriptional start sites and to accessible chromatin regions.	Einkauf et al. (2019)[11]
Early antiretroviral therapy in neonates with HIV-1 infection restricts viral reservoir size and induces a distinct innate immune profile	Neonates with HIV-1 infection	Rapid antiretroviral initiation in neonates results in an extremely small reservoir of intact proviral sequences, a reduction in abnormal T cell immune activation, a more polyfunctional HIV-1-specific T cell response, and an innate immune profile that displays distinct features of improved antiviral activity and is associated with intact proviral reservoir size.	Garcia-Broncano et al. (2019)[59]
Longitudinal HIV sequencing reveals reservoir expression leading to decay, which is obscured by clonal expansion	ART-treated individuals	Intact and defective proviruses that contain genetic elements that favor protein expression are under negative selection pressure. And defective proviruses that lack these genetic elements but encode a strong donor splice sequence are under relative positive selective pressure.	Pinzone et al. (2019)[56]

infected cells can clonally expand and others cannot, one longitudinal study of infants infected with HIV-1 indicates that clonal proliferation of HIV-1-infected cells can occur early in life,[25] implying that the central determinant of whether reservoir cells clonally expand could be intrinsic and present early in life or inherent. Notably, studies by Einkauf et al.[14] and others point out that HIV-1-targeted genes are frequently involved in the regulation of cell proliferation and oncogenesis.[63–65] The mechanistic interplay between proviruses and cancer-related genes requires further investigation.

Elite controllers are a specific subgroup of HIV-1-infected individuals who spontaneously control viral replication without antiretroviral treatment. Although it is estimated that elite controllers represent less than 1% of the total population of HIV-1-infected individuals, integration sites that have been through long periods of elite control may offer us a unique opportunity to investigating HIV-1 deeply latent reservoirs and host factors involved in silencing proviral transcription. Jiang et al.[12] and Lian et al.[13] have shown that relative to defective proviruses, the majority of intact proviruses from elite controllers integrate into centromeric satellite DNA or chromosomal regions associated with heterochromatin features, resembling footprints left by the immune-mediated selection observed in ART-treated patients. It appears that the impact of immune selection pressure in elite controllers is more pronounced than in post-treatment controllers, rare individuals who maintain low levels of viremia after stopping ART. Here, it is important to note that some elite controllers do experience viral rebound during the period of elite control, and the reason is not yet clear. At present, many questions about the host genetic background/factors that contribute to the phenotype of elite control remain open for further research. Regardless, studying the fundamental mechanism of elite control enhances our understanding of HIV-1 deep latency. Altogether, precisely located HIV-1 deeply latent reservoirs can be seen as a subset of intact proviruses that are not susceptible to longitudinal immune-mediated selection processes and frequently integrate into chromosomal regions associated with heterochromatin features. Intact proviruses in elite controllers are transcriptionally "blocked and locked" and are at a deeper level of latency in ART-treated patients. In both cases, they are, as a result, less likely to reignite, thus representing a promising target as a functional cure for HIV-1 infections.

Task-evoked host genome functional network toward the evolution of HIV-1 reservoirs

In most cases, after HIV-1 DNA integration, the host genome is no longer able to distinguish the integrated proviral DNA from its own genes. It is thus not surprising that the host genome either directly or indirectly affects the fate of provirus in reservoir cells. Plenty of studies have contributed to our understanding of the influence of a linear genome on the selection of HIV integration and reactivation of the proviruses resident in latent reservoir cells and *in vitro* cellular models. In addition to the linear genome, spatial genomic features have also been documented to influence the selection of HIV integration using *in vitro* experiments. The 3D genome is usually seen to be organized in hierarchical layers. The topological structure of the 3D genome may correspond to gene expression[66] and epigenetic transcription regulation in the nucleus. Whether topological 3D genome organization influences the transcriptional state of proviruses becomes an obvious question, because persistent HIV-1 infection requires viral DNA to integrate into the host genome. HIV-1 DNA integration frequently occurs in the outer shell of the nucleus in close correspondence with the nuclear pore, preferentially into transcriptionally active regions of the chromatin,[67,68] once reversed-transcribed double-stranded linear viral DNA is transported in the nucleus. Studies from the Lusid laboratory have reported that HIV-1 DNA preferentially integrates in the topologically genomic regions associated with active histone marks in human $CD4^+$ T cells[69] and microglia cells.[70] Several indicative features of HIV-1 integration, including preferentially integrating in actively transcribed genes (denoted by H3K36me3), the analog of the frequently HIV-1-targeted genes, integrating in the vicinity to genic enhancers and super-enhancers, and integrating in the proximity to H3K36me3-dependent and CTCF (CCCTC-binding factor)-enriched topologically associated domain (TAD), are largely conserved between the microglia cellular model and $CD4^+$ T cells.[69,70] Remarkably, Rheinberger et al. have also shown that the dynamic nature of CTCF in defining 3D genome organization influences HIV-1 latency in the microglia cellular model.[70]

As we proposed in our recent publication,[17] the 3D genomic space can also be seen to consist of different functional communities embedded with a group of genes involved in similar biological functions and pathways. This concept is inspired by assortativity, which originates from graph theory and has been applied to measure the probability of connection between two individuals, frequently representing a social network.[71] Given that HIV-1 integration at a genetic level is not uniform, whether the genes in functional communities are selectively targeted by HIV-1 and respond to longitudinally viral infections is a question of interest. Reanalyzing HIV-1-targeted genes retrieved from longitudinal samples of ART-treated individuals[14] and elite controllers,[12] we found that different and unique so-called immunological gene set "signatures" are enriched alongside HIV-1 infections in ART-treated patients and elite controllers.[17] We assume that alterations of immunologic signatures appear significant and display a high degree of specificity in terms of which immune cell types and proinflammatory soluble factors are required at different stages of HIV-1 infection. Enriched signatures could then trigger downstream biological functions engaged in enriched signatures. In other words, HIV-1 integration frequency might be used as a surrogate for gene sets, in order to define specific immune cell types and proinflammatory soluble factors that satisfy the need for host immunity in the course of HIV-1 infection. Furthermore, different immunologic signatures were found to be enriched between ART-treated patients and elite controllers, suggesting that different selection pressure may exist between them.[17]

Remarkably, using the phenotypic and proviral sequencing strategy, Sun and colleagues observed that ensemble signatures of cell surface proteins linked with immune checkpoint receptors PD1, TIGIT, BTLA, 2B4, and KLRG1 were uniquely displayed in HIV-1-infected memory $CD4^+$ T cells harboring intact proviruses and large clones of HIV-1-infected cells.[16] Increases expressions in these immune checkpoint proteins confer increased resistance to immune-mediated killing and suppress proviral transcription. One possible explanation for this biological phenomenon may be that the activation of biological pathways is selective: different biological pathways are prone to be enriched, whereas others languish in reservoir cells. Still, it is important to note that in studies by Sun et al.[16] and Dufour et al.,[19] diverse phenotypic signatures

were noted, especially in clonally expanded reservoir cells. Whether or not this diversity of phenotypic signatures linked with immunity results from selective enrichment of functional genome properties that activate downstream biological pathways requires further investigation. Regardless, all these studies highlight that the topological domains and functional properties of the 3D genome constitute an additional layer of complexity that should be considered while we attempt to understand the establishment and configuration of HIV-1 deeply latent reservoirs.

CONTINUED TECHNICAL IMPROVEMENT FOR THE STUDY OF HIV-1 LATENT RESERVOIRS

At least two parameters, proviral genome integrity and integration site, are required to characterize the latent reservoir that is responsible for viral rebound. Matching the integration site to the integrity of a viral genomic sequence in a pool of cells thus becomes critical to accurately seek the location of intact proviruses in reservoir cells. Many current approaches combine different methods to overlay independent datasets containing proviral genome integrity and HIV-1 integration sites. However, it is not clear whether it is possible to recover all readouts from both datasets and whether overlapping pairs can fairly represent the status of each provirus in its physiological infection condition. Therefore, we believe that a method that allows concomitant characterization of the integrity of proviral genomic sequences and mapping of corresponding integration sites is required. Matched integration site and proviral sequencing (MIP-seq)[11] and multiple displacement amplification single genome sequencing (MDA-SGS)[72] are two examples of the technology that allows to sequence near-full HIV-1 genomic sequences and integration sites. The experimental procedure of MIP-seq is similar to that of FLIP-seq. First, input DNA materials are highly diluted to obtain roughly one proviral genome in each reaction. Then, single proviral genome dilution is followed by multiple displacement amplification to amplify DNA materials. Finally, the reaction is split in two: one proportion is subjected to amplification of the near-full-length HIV genome and another is subjected to sequencing of the genomic junction of viral and cellular DNA.

Given that our ultimate goal is to identify truly deeply latent reservoirs, knowing the transcriptional status of every provirus is thus required for further technical improvement. Certainly, it is feasible, as we split materials from single proviral genome dilution into more proportions, to characterize the proviral genome integrity, map the integration site, measure corresponding transcriptional levels, and then unite these independent datasets at the analytic step; however, these processes are time-consuming and labor-intensive. More importantly, during the use of such an approach, we may risk having noisy readouts or loss of readouts because the starting materials are highly diluted. For this reason, here we propose the use of a long-read sequencing platform such as PacBio or Oxford Nanopore for concomitant visualization of the integrated proviral transcriptional landscape associated with the integrity of proviral genomic sequences with the resolution of single proviruses. Molecular tools and approaches such as the barcoding strategy and the linear amplification method allow immediate amplification of single proviral genomic sequences in a pool of cell populations.

OUTSTANDING QUESTIONS

1. Does assortative structure of the host functional genome networks topologically overlay the 3D genomic structure?

It is well known that the topology of the 3D genome reflects its intrinsic and baseline functional architecture; however, whether host genome functional network also possesses topological attributes that exist along a spectrum, ranging from local individually enriched immunologic signatures to a global network reflecting the connection of the entire functional property network remains unknown. Further characterization of the principles by which a set of the genes reconfigures as a subject that performs demanding tasks (response to HIV-1 infection or not) is necessary to realize a new perspective on the network organization of the host functional genome properties alongside HIV-1 infection. In addition, although studies[16,19,73] have demonstrated that unique phenotypic signatures are present in reservoir cells harboring intact proviruses and large clones of HIV-1-infected cells, it is not clear whether the observed phenotypic signatures are apparent on cells upon primary HIV-1 infection or they are under the selective pressure of immune-mediated selection. Finally, whether such phenotypic signatures are specific to ART-treated patients or shared between ART-treated patients and elite controllers is another layer of the open questions required for further investigation.

2. Are all proviruses present in microenvironments of deep latency transcriptionally silent?

At present, whether viral blips can still occur in deeply latent reservoirs under the pressure of immune-mediated selection remains unclear. A careful examination of the transcriptional phenotype of individual proviruses that integrate in genomic microenvironments favorable for deep latency is required. It is important to stress that the results of *in vitro* experiments have shown that the transcriptional activity of proviruses retargeted by allosteric integrase inhibitors with regard to "block-and-lock" antiretroviral functional cues does not always correlate with epigenetic features surrounding proviral integration sites,[74] implying that the local genomic context associated with epigenetic features may not be sufficient to permanently "block and lock" proviruses in the state of transcriptional silence. In the case that viral blips are detectable in deeply latent reservoirs, it is critical to understand whether the cause of this phenomenon is intrinsic (e.g., stochastic HIV-1 gene expression) or extrinsic (e.g., insufficient effect of epigenetic modifications on chromosomal regions at the site of HIV-1 integration or caused by unknown cellular factors) before developing a better antiretroviral strategy.

A recent study by Clark and colleagues made remarkable progress by identifying host gene expression signatures in HIV-1-infected memory CD4 T cells.[73] They develop the microfluidic-based technology, termed focused interrogation of cells by nucleic acid detection and sequencing (FIND-seq) that allows to profile the transcriptomes of millions of HIV-1-infected single cells *ex vivo*, and conclude that host gene expression signatures of memory CD4 T cells isolated from HIV-1-infected individuals subjected to ART were not favorable for HIV

transcription.[73] Here, it is important to note that although RNA was separately isolated from single cells encapsulated in water-in-oil droplets, the whole transcriptomic analysis of cells infected by HIV-1 was performed based on a pool of transcriptomes, rendering it difficult to evaluate whether the identified signatures can reflect the transcriptional phenotype of individual memory CD4 T cell subsets or are overridden by a small fraction of single outliers within a pool of reservoir cells. In addition, although polyadenylated RNA-seq in HIV-1-infected memory CD4 T cells from patients subjected to ART did not reveal either full-length genomic HIV-1 transcripts or spliced HIV-1 messages, further characterization of transcriptomic signatures between cells harboring intact and defective proviruses at a single-virus level is nevertheless required.

Overall, due to the current technical limitations, at present, no compelling evidence showing the longitudinal transcriptomics profile of latent reservoir cells is available. In the first place, it is critical to verify whether HIV-1 integration frequency correlates with levels of endogenous gene expression of reservoir cells. Afterward, one can elucidate the longitudinal changes of the transcriptional profile of latent reservoir cells and compare them with that obtained from other reservoir cells, thereby facilitating our understanding of the mechanism of establishment of HIV-1 deep latency and the fate of proviruses.

CONCLUDING REMARKS

A proper technology that allows us to characterize the integrity of individual proviral genomes in a population of reservoir cells is required in order to better decipher the longitudinal evolution of HIV-1 reservoirs. Technical advances such as FLIPS, IPDA, and their derivatives address this challenge by revealing individual near-full-length proviral genomic sequences. Although these methods are not yet fully optimized, important observations have reshaped our knowledge about HIV-1 latent reservoirs that are critical for viral rebound. Moreover, HIV-1 reservoirs within individuals are not in a steady state; they change over time. The selection force may be driven by host immunity based on the findings that (1) a considerable proportion of HIV-1 reservoir cells remain transcriptionally active during ART and can be visible to immune recognition mechanisms[14,15] and (2) different and specific phenotypic signatures relevant to immune checkpoint markers are observed in reservoir cells harboring intact proviruses and large clones of HIV-1-infected cells.[16,19] Here, we further propose that a network of functional genome properties should be also taken into account.[17] In the future, a sufficient number of integration sites retrieved from clinical samples, and perhaps experimental models to recapitulate the progress of longitudinal HIV-1 infection, will be necessary to obtain a robust conclusion whether the evolution of latent HIV-1 reservoirs drives and accompanies a reconfiguration of the whole functional architecture of the host genome into an assortative network of functional genome properties.

Again, it is important to stress that according to the "immune selection" hypothesis, intact proviruses that persist under selection pressure are considered to be "blocked and locked" in a state of deep latency; however, large transcriptionally active proviral clones seem to be able to outcompete this selection force,[14] indicating that perhaps an additional unknown mechanism may be involved in this selection process, especially for clonally expanded reservoir cells. Further technical improvements that will allow us to combine proviral genome integrity, integration sites, and corresponding viral transcription are urgently needed in order to precisely decipher the fate of individual proviruses over time. Although current observations strongly suggest that the intrinsic properties of the host genome may participate in this selection process, it is not clear at present whether the host genome itself plays an active role in governing HIV-1 pathogenesis and whether it can be seen as part of the host immune system or is in cooperation with host immunity. Either way, the organization of the host genome and its functional properties should receive attention when we attempt to complete our understanding of HIV-1 deep latency.

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AUTHOR CONTRIBUTIONS

Conceptualization, H.-C.C.; Figure and tables preparation, K.W. and H.-C.C.; Writing— original draft preparation, K.W. and H.-C.C.; Writing—review and editing, K.W. and H.-C.C. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS

The author declares no conflict of interest.

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