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# Applications of CRISPR/Cas9 tools in deciphering the mechanisms of HIV-1 persistence

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HIV-1 infection can be controlled but not cured by combination antiretroviral therapy. Indeed, the virus persists in treated individuals in viral reservoirs, the best described of which consisting in latently infected central memory CD4$^+$ T cells. However, other cell types in other body compartments than in the peripheral blood contribute to HIV-1 persistence. Addressing the molecular mechanisms of HIV-1 persistence and their cell-specific and tissue-specific variations is thus crucial to develop HIV-1 curative strategies. CRISPR/Cas9 editing technologies have revolutionized genetic engineering by their high specificity and their versatility. Multiple applications now allow to investigate the molecular mechanisms of HIV-1 persistence. Here, we review recent advances in CRISPR-based technologies in deciphering HIV-1 gene expression regulation during persistence.

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## Introduction

More than twenty years after its introduction, combination antiretroviral therapy (cART) is still the only available treatment for individuals infected with Human Immunodeficiency Virus type 1 (HIV-1). The current antiretroviral regimens are potent in suppressing viral replication and decreasing transmission rates and have substantially improved HIV$^+$ individuals life expectancy [1,2]. Unfortunately, cART interruption invariably leads to the rebound of plasmatic viremia in treated individuals, to similar levels than before treatment initiation [3]. Because the therapy is not curative, HIV$^+$ individuals must thus adhere to a lifelong daily antiretroviral drug regimen, which has led to a new set of complications of sustained chronic inflammation, premature ageing and higher risks of non-AIDS comorbidities [4]. Therefore, addressing the sources of HIV-1 persistence in cART-treated individuals is crucial to eradicate the virus and to allow a cART-free remission.

HIV-1 persists in cART-treated individuals under the form of viral reservoirs [5•,6]. The best-characterized cellular reservoir corresponds to latently infected long-lived resting memory CD4$^+$ T cells, especially central memory CD4$^+$ T cells [7,8], although other T-cell subsets significantly contribute to the HIV-1 reservoirs, such a naive and effector memory CD4$^+$ T cells [9,10]. Latent infections are a rare form of infection, with an estimated frequency as small as $10^{-7}$ per resting CD4$^+$ T cells, during which the integrated HIV-1 provirus is maintained silent in a continuum of epigenetic, transcriptional and post-transcriptional mechanisms [7]. Because latency is a non-productive state of infection, latently infected cells escape immune-mediated clearance, while the lack of viral replication limits the efficiency of antiretroviral drugs [11]. However, HIV-1 latency can be reversed and even one single reactivated cell can rekindle the infection [12•]. Further impediments to HIV-1 eradication arise from the intrinsic heterogeneous nature of HIV-1 reservoirs, that are not only established in other cell types than CD4$^+$ T cells but are also distributed in virtually every tissue of the HIV-1 infected individuals [13,14•]. Several studies have also reported the heterogeneity of silencing mechanisms among HIV-1 infected individuals [15,16]. In this context, the molecular understanding of HIV-1 persistence plays a crucial role in the development of novel curative strategies.

Genome editing technologies, especially the clustered regulatory interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas)9 system, have proven to be a formidable tool to interrogate biological processes. The CRISPR/Cas9 system originates from bacteria where it participates in adaptative immune protection against bacteriophage DNA [17••], and was subsequently adapted for genome engineering in mammalian cells [18,19]. This two-component system is composed of Cas9, a DNA endonuclease that is targeted to specific loci by CRISPR-associated guide RNAs. The double-strand break (DSB) induced by Cas9 will be

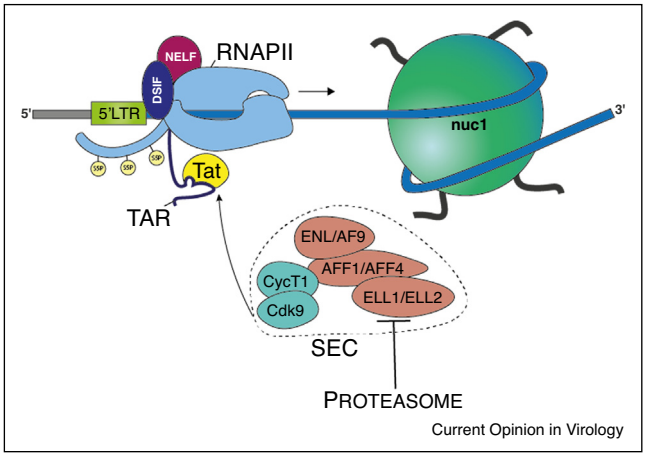
repaired by the cellular machinery, resulting in insertions or deletions (indels), that, if targeted to coding exons, will permanently abolish protein expression [17]. Multiple derivatives of CRISPR–Cas9 editing have been developed for a wide variety of applications, from functional screens to transcriptional and epigenetic modulations, and rely on different catalytic forms of Cas9 and on different DNA repair mechanisms [20••,21]. Here, we review three regulation modes of HIV-1 gene expression and highlight how the versatile CRISPR/Cas9 system can be used to address the molecular mechanisms of HIV-1 persistence.

## The proteasome as a key contributor to HIV-1 latency

HIV-1 proviruses are under the control of the cellular machinery for their transcription. As for many cellular genes [22], HIV-1 transcription elongation is a key limiting step [23]. Indeed, following the initiation of transcription, the RNA polymerase II (RNAPII) synthesizes only a short stem-loop transactivation response (TAR) element before pausing. RNAPII pausing at the HIV-1 proximal promoter region is due to the presence of a repressive nucleosome [24], and to the recruitment of the negative elongation factor (NELF) and of the DRB sensitivity-inducing factor (DSIF) [25]. To overcome RNAPII pausing, a multifunctional cellular complex is assembled by the virally encoded Tat protein and loaded to the TAR element [26,27]. This complex, referred to as a super elongation complex (SEC), contains the positive transcription elongation factor b (P-TEFb) [28], composed of Cdk9 and cyclin T1, a member of the ELL family of elongation stimulatory factors and several members of the AF4/FMR2 family of transcription factors (Figure 1) [26,27]. The different components of the SEC coordinately convert the paused RNAPII to an active elongating form capable of productive HIV-1 transcription [29]. Part of the functional characterization of SEC members in controlling transcriptional elongation at the HIV-1 promoter has been performed using a CRISPR/Cas9-mediated knockout in a $CD4^+$ T-cell model of latency [30]. In a recent publication, the same team conducted a loss-of-function CRISPR screen to search for additional molecular actors in HIV-1 transcriptional repression [31••].

Functional genetic screens aim at identifying genes that play an important role in a phenotype of interest, and have largely benefited from the efficiency of CRISPR/Cas9 genome editing [20]. The variant used by the team of Qiang Zhou relies on a catalytically inactive Cas9 (or dead Cas9, dCas9) fused with the chromatin modifier domain KRAB (Krüppel-associated box) that together mediate locus-specific transcriptional silencing, a process termed CRISPR interference (CRISPRi) [32]. In their study, Li *et al.* first generated a CRISPRi reporter cell line for HIV-1 latency by stably transducing a doxycycline-inducible red fluorescent reporter dCas9-KRAB construct

**Figure 1**

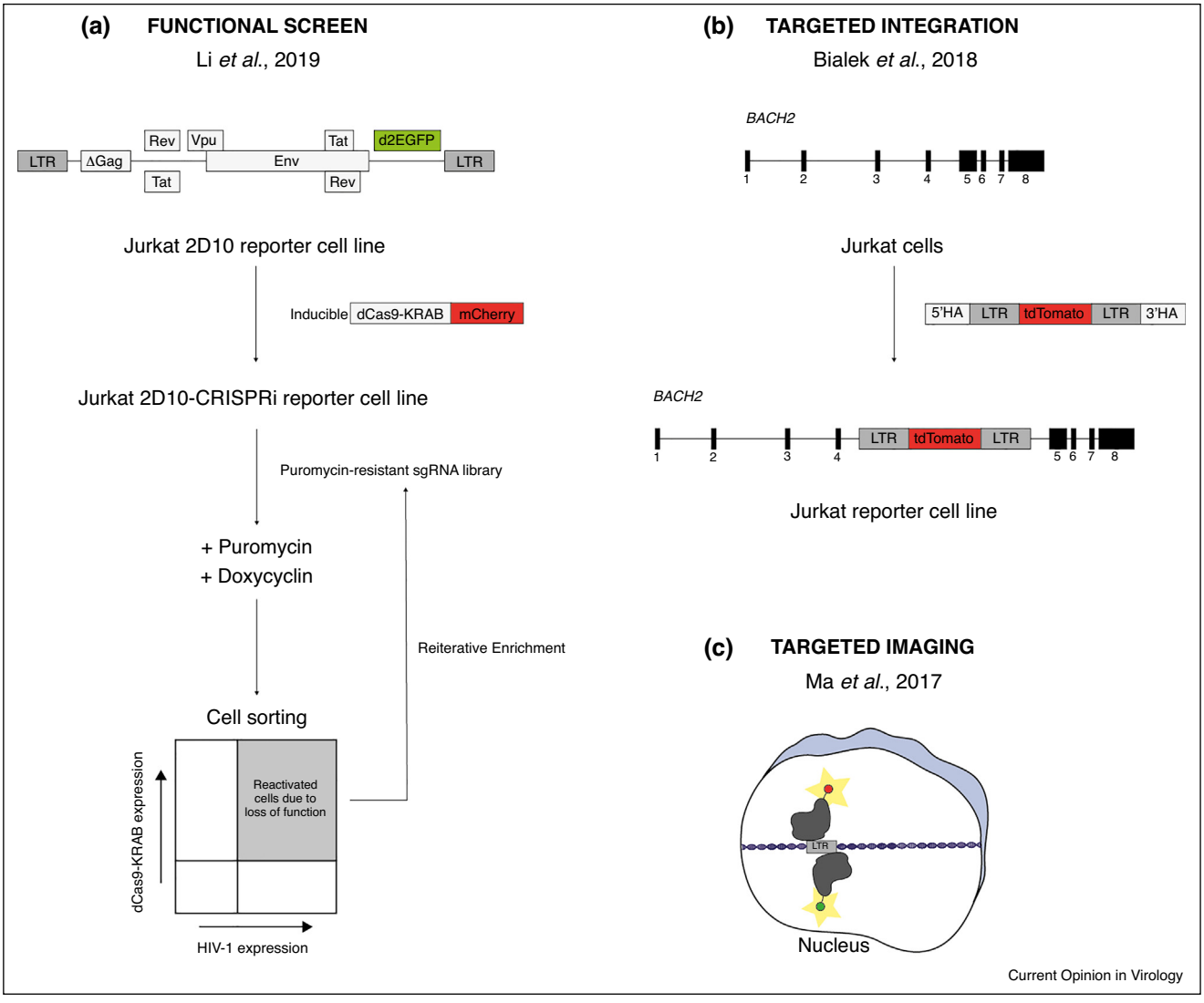



Pausing of the RNA Polymerase II at the proximal region of HIV-1 promoter is countered by a multicomponent complex.
Pausing of RNA Polymerase II (RNAPII) at the proximal region of HIV-1 promoter, located within the 5′LTR, is due to the recruitment of NELF and DSIF and to the presence of the repressive nucleosome nuc1. A multicomponent complex assembles under the control of the virally encoded Tat to overcome RNAPII pausing. This super elongation complex (SEC) contains the positive transcription elongation factor B (P-TEFb), composed of Cdk9 and Cyclin T1 (CycT1), ELL1 or ELL2, that are members of the ELL family of elongation stimulatory factors and several members of the AF4/FMR2 family of transcription factors.

in the Jurkat-based 2D10 minivirus reporter cell line, where the HIV-1 promoter controls the expression of the green fluorescent protein (Figure 2a). This cell line, named 2D10-CRISPRi, was subsequently used for the screen itself by transducing a lentiviral library containing about 200 000 single guide RNAs (sgRNAs) that redundantly target human genes [31] (Figure 2a). Flow cytometry was then used to sort cells, in which the targeted CRISPRi resulted in the reactivation of HIV-1 transcription from the latent state. This loss-of-function screen thus allowed to identify genes essential in the maintenance of HIV-1 latency in the Jurkat 2D10 cells. Because the first round of library transduction and reactivation yielded limited results, essentially due to spontaneous viral activation, the team reiterated the procedure four times, a technique they coined as Reiterative Enrichment and Authentication of CRISPRi Targets or REACT [31].

Sequencing of the library subset present in the reactivated cells identified six hit genes that were enriched in a statistically significant manner: *PSMD1*, *NFKBIA*, *CYLD*, *GON4L*, *PSMD3*, and *PSMD8*. These include factors suppressing the NF-κB pathway (*NFKBIA*), an important regulator of HIV-1 transcription, and an interactant of the histone deacetylase HDAC1 (*GON4L*), that maintains a strong heterochromatin environment at the HIV-1 promoter during latency. However, the three other genes (*PSMD1*, *PSMD3*, and *PSMD8*) were unexpected, and

**Figure 2**




CRISPR/Cas9 editing is a versatile tool to interrogate molecular mechanisms of HIV-1 latency.
**(a)** Experimental scheme for loss-of-function CRISPRi screen, as performed by Li *et al.* The Jurkat 2D10 reporter model for HIV-1 latency was stably transduced with a doxycycline-induced dCas9-KRAB construction to generate a CRISPRi reporter cell line termed '2D10 CRISPRi'. These cells were then transduced with a sgRNA library, where sgRNAs targeting human genes are co-expressed with puromycin (and a third fluorescent marker, not represented here). In the puromycin-resistant cell populations (i.e. cells co-expressing sgRNAs), the expression of dCas9 is then induced. The double positive red fluorescent (E2-CRIMSON$^{+}$) and green fluorescent (GFP$^{+}$) population corresponds to reactivated cells due to loss-of-function of HIV-1 latency.
**(b)** Targeted integration of an HIV-1 reporter construct, containing the red fluorescence marker tdTomato under the control of HIV-1 5′LTR, is achieved in the fourth intron of the cellular *BACH2* gene using homologous recombination. The guide RNAs are designed to be homologous to the chosen genomic integration site and will guide the Cas9 so as to generate a DSB at specific cellular coordinates. Repair using the HIV-1 reporter construct, flanked by homologous arms in 5′ and 3′ (5′HA and 3′HA, respectively), leads to the targeted integration.
**(c)** Dual targeting of 5′LTR by sgRNAs provokes the recruitment of the dCas9 labelled with two different quantum dots (TZ1-QD625 and SSA-QD525, respectively), allowing for locus-specific visualization of integrated HIV-1 provirus within the nuclear topography.

encode elements of the 19S regulatory particle of the 26S proteasome. The proteasome had already been linked with the maintenance of HIV-1 latency in a previous study [33]. However, the present report highlighted how mechanistically the proteasome machinery inhibits HIV-1 transcription in a Tat-dependent manner. Indeed, targeted CRISPRi against *PSMD1*, *PSMD3*, and *PSMD8* was associated with elevated protein levels of ELL2, and in a lesser extent, ELL1, but not of the other cellular components of the SEC machinery. Altogether, this CRISPRi functional screen thus allowed the identification of the proteasome as a key factor in the regulation of

HIV-1 latency, notably by decreasing the level of the SEC ELL2 component (Figure 1). However, increased proteasomal activity during latency is likely not to be affecting only transcription elongation of HIV-1 genes and further experiments will be needed to determine the targets of the ubiquitination-proteasome proteolytic pathway during HIV-1 latency. In this regard, it has been shown that HIV-1 also hijacks the ubiquitination machinery to degrade host restrictive factors [34], suggesting that the virus exploits similar mechanisms both during productive and latent infections.

## Role of the integration site in HIV-1 reservoir persistence and expansion

HIV-1 preferentially integrates into genomic regions that are transcriptionally active, a replication step that is controlled by cooperation between viral and cellular determinants [35•,36]. Intriguingly, several studies have pointed out specific hotspots of HIV-1 integration in $CD4^+$ T cells [37], associated with clonal expansion [38,39]. Indeed, Maldarelli *et al.* analyzed the integration sites distribution in $CD4^+$ T cells isolated from the blood of $HIV^+$ individuals and identified fifteen independent integrations events in the fourth and fifth intron of the BTB domain and CNC homolog 2 (*BACH2*) gene, an important regulator of T-cell differentiation [38]. Additional preferential multiple HIV-1 integrations were also shown in the MKL/myocardin-like 2 (*MKL2*) gene and in the signal transducer and activator of transcription 5B (*STAT5B*) gene [38,39]. The recurrent identification in $HIV^+$ individuals of identical integration sites, associated with clonal expansion, suggests that HIV-1 could exploit insertional mutagenesis to deregulate the target genes of integration contributing to long-term survival and division of infected cells, a mechanism reminiscent of the ones mediated by some onco-retroviruses [40]. Accordingly, chimeric mRNA transcripts containing viral HIV-1 sequences fused to coding exons of *BACH2* and *STAT5B* were found in one-third of the analyzed $HIV^+$ individual samples in a recent study [41]. Furthermore, the overexpression of chimeric HIV/*BACH2* and HIV/*STAT5* transcripts conferred increased proliferation in primary $CD4^+$ T cells from uninfected donors [41]. Altogether, these data prove the existence of HIV-1 infected $CD4^+$ T-cell clones that contain replication-competent proviruses, which can give rise to infectious viruses following HIV-1 transcriptional reactivation [42]. The implication of clonal expansion to HIV-1 persistence is undeniable; however, how the integration site contributes to HIV-1 silencing remains somewhat a subject of debate, with studies indicating that the integration site is a determinant of latency [43,44] and other disputing any contribution [45] or its relative mechanisms [46]. Indeed, heterogeneous populations of latent proviruses may be maintained in a variety of chromatin environments [44,47], which may or may not contribute to clonal expansion. Hence, characterizing the molecular mechanisms responsible for HIV-1 integration sites in the control of HIV-1 persistence and clonal expansion is needed.

Because of the low frequency of latently infected $CD4^+$ T cells *in vivo*, the majority of the molecular mechanisms of HIV-1 latency have been resolved in transformed cell lines, which are characterized by clonal HIV-1 provirus integration sites [48]. These cell lines are thus not representative of HIV-1 integration hotspots associated with clonal expansion *in vivo*. Site-specific transgene integration can be typically achieved by CRISPR/Cas9 targeted editing [20]. The most common strategy of 'knock-in' relies on exploiting homologous recombination to repair the induced DSBs using a transgene template, hence resulting in targeted integration [49]. This strategy of targeted integration has recently been proposed by the team of Ulrike Lange to generate reporter models for HIV-1 infection with selected proviral integration sites in *BACH2* (Figure 2b) [50••]. So far, the proposed protocol is time-consuming; however, it paves the way for the use of CRISPR/Cas9 targeted integration in mechanistic studies on position effects of HIV-1 integration in latency and clonal expansion. Furthermore, recent advances in knock-in strategies, using homology-independent DNA repair, will allow to study HIV-1 integration effects *ex vivo*, in primary cells [49].

## Nuclear topography and HIV-1 persistence

An important feature associated with HIV-1 integration sites is the local nuclear environment. Indeed, eukaryotic nuclei are highly dynamic and cellular genes are known to occupy preferred nuclear positions [35]. HIV-1 proviruses have been found to localize to the outer shell of the nucleus, at the proximity of nuclear pores [51], while transcriptionally silent HIV-1 proviruses can be localized in inner regions of the nucleus, in subcompartments such as promyelocytic leukemia (PML) nuclear bodies [52]. Still, it remains unclear how subnuclear positioning of HIV-1 proviruses relates to the state of transcription, and if dynamic transitions between these states and localizations occur.

Indeed, much of our understanding of the impact of nuclear topography to HIV-1 gene regulation relies on immuno-three-dimensional fluorescent *in situ* hybridization (immune-FISH). This technique uses multiple tagged probes to target a nucleotidic sequence of interest resulting in visualizable discrete bright spot indicative of a single locus [53]. Because the cells need to be fixed, FISH provides high spatial but static information [53]. On the contrary, CRISPR/dCas9-based techniques allow for noninvasive live imaging of genomic loci [53]. A wide variety of CRISPR-based imaging methodologies have been developed in the recent years, using dCas9 fused with classical fluorochromes, more stable and smaller organic dyes or specific nanoparticles [53]. This latter approach was recently reported by the team of Xian-En

Zhang to live visualizing single-copy of integrated proviral HIV-1 DNA in latently infected cells [54••]. Indeed, the team used a dCas9 fused with luminescent nanoparticles termed quantum dots, that are brighter and have a higher photostability than organic dyes and fluorochromes, and that allow single-molecule imaging [53]. More specifically, a pair of dCas9 fused to two differently colored quantum dots was co-targeted to the HIV-1 promoter region with optimized sgRNA and allowed to successfully image HIV-1 proviral DNA in latently infected cells (Figure 2c) [54]. At this prospective stage, the study of Ma *et al.* thus reveals the potential of using CRISPR/dCas9-based techniques to image single integrated HIV-1 provirus. Ultimately, CRISPR imaging will uncover the mechanisms of dynamical nuclear architecture into HIV-1 gene expression regulation.

## Conclusion

With the advent of the highly versatile CRISPR/Cas9 editing system, multiple techniques have been developed that can potentially interrogate the molecular mechanisms of HIV-1 persistence. In addition to CRISPR-based approaches with direct therapeutic purposes ([55•,56] and Atze Das' review in the current issue [62]), the new molecular actors identified in the control of HIV-1 gene expression during persistence constitute many potential therapeutic targets for anti-HIV strategies.

Several features of CRISPR/Cas9 editing render this system attractive in HIV-1 mechanistic studies. First, as in functional screens, HIV-1 molecular mechanisms of latency can be teased out without bias, with the potential of revealing unexpected novel regulatory mechanisms. Second, CRISPR/Cas9 editing relies mainly on ubiquitous cellular processes, such as base complementarity and DNA repair [57]. Thus, CRISPR-based techniques have the potential to address molecular mechanisms of HIV-1 persistence irrelevantly of the nature of the infected cells. In particular, genetic engineering advances have rendered editing possible in *ex vivo* primary cells [58]. This is crucial considering an increasing body of evidence that reservoirs are not only present in $CD4^+$ T cells, and not only in peripheral blood [14,59], yet, the cellular-specific and tissue-specific molecular mechanisms of HIV-1 latency are still poorly understood. However, the limitations of CRISPR-based technologies should be carefully considered in designing mechanistic studies. Off-target binding of Cas9 and dCas9 has been reported [60], which could be problematic considering the abundance and homology of endogenous retroviruses to HIV-1 proviral sequences. Optimization of sgRNA can prevent off-target effects; however, the binding of Cas9 and dCas9 remains dependent on the chromatin structure of the targeted locus [60]. Furthermore, some studies have reported that HIV-1 can escape genome editing, by accumulating mutations that prevent recognition by sgRNAs [61]. The consequences of these limitations are less dire when using CRISPR/Cas9 editing for molecular studies rather than for therapeutic approaches but should still been considered in experimental design to prevent false positives and false negatives.

Altogether, we have focused, in the present review, on three modes of HIV-1 gene expression regulation and we have shown how CRISPR/Cas9-based applications have already provided or will provide insights to deepen our molecular understanding of HIV-1 persistence.

## Conflict of interest statement

Nothing declared.

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## Further reading

62. "Elimination of infectious HIV DNA by CRISPR-Cas9" is to be published in the 38th Volume of COVIRO.