HIV-1 promoter is gradually silenced when integrated into BACH2

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Abstract

The persistence of the latent HIV-1 reservoir is a major obstacle to cure HIV-1 infection. HIV-1 integrates into the cellular genome and some targeted genomic loci are frequently detected in clonally expanded latently HIV-1 infected cells, for instance, the gene BTB domain and CNC homology 2 (BACH2). We investigated HIV-1 promoter activity after integration into specific sites in BACH2. The HIV-1-based vector LTatCL[M] contains two fluorophores: 1.) Cerulean, which reports the activity of the HIV-1 promoter, and 2.) mCherry driven by a constitutive promoter and flanked by genetic insulators. This vector was inserted into introns 2 and 5 of BACH2 of Jurkat T-cells via CRISPR/Cas9 technology in the same and convergent transcriptional orientation of BACH2, and into the genomic safe harbour AAVS1. Single cell clones representing active (Cerulean+/mCherry+) and inactive (Cerulean-/mCherry-) HIV-1 promoters were characterized. Upon targeted integration of the 5.3 kb vector LTatCL[M] into BACH2, active HIV-1 promoters were gradually silenced as reflected by decrease in Cerulean expression over a period of 162 days in culture. Silenced HIV-1 promoters could be reactivated by TNF-α and Romidepsin. This observation was independent of the targeted intron and the transcriptional orientation. BACH2 mRNA and protein expression was not impaired by mono-allelic integration of LTatCL[M]. Our results show that the HIV-1 promoter is silenced when integrated into BACH2 without impairing BACH2 mRNA and protein expression. This might contribute to HIV-1 persistence, enabling infected T-cells to complete differentiation into a memory phenotype, persist, and clonally expand over time.

Keywords

HIV-1 integration; recurrent integration gene (RIG); HIV-1 persistence; HIV-1-based, dual-fluorophore vector; BACH2; CRISPR/Cas9; genomic safe harbour AAVS1

Introduction

Antiretroviral therapy (ART) blocks efficiently virus replication, however, does not cure HIV-1 infection due to the presence of replication-competent but silenced proviruses preferentially integrated in long-lived resting CD4+ T-cells (Finzi et al. 1997, Wong et al. 1997). Various factors and molecular mechanisms that result in HIV-1 latency have been proposed (Ruelas and Greene 2013). One such factor might be the integration site of the provirus, which has been suggested to not only be responsible for silencing the provirus, but also supporting cell expansion, thus maintaining the size of the HIV-1 latent reservoir (Maldarelli et al. 2014, Wagner et al. 2014). Integration of HIV-1 into the human genome is not random. In vivo and ex vivo HIV-1 integration site analyses revealed that HIV-1 favours integration into introns of active transcription units in gene-dense regions, although a minority of integration events outside of these regions have consistently been observed (Ciuffi and Bushman 2006, Kok et al. 2016, Mitchell et al. 2004, Schroder et al. 2002, Stevens and Griffith 1996). Furthermore, HIV-1 appears to target active transcription units that are in close proximity to the nuclear pore (Marini et al. 2015). On the population level, intragenic HIV-1 does not have a preference for either transcriptional orientation of the targeted gene (Cohn et al. 2015, Schroder, et al. 2002, Stevens and Griffith 1996). HIV-1-infected cells can undergo clonal expansion and increase over time (Cesana et al. 2017, Cohn, et al. 2015, Maldarelli, et al. 2014, Rezaei and Cameron 2015, Satou et al. 2017, Wagner, et al. 2014), and the proviruses in these clonally expanded cells are often located in specific regions of the human genome. One recurrent integration gene (RIG) that has been observed across patients’cells in numerous independent studies is the gene BACH2, in which the provirus is
almost exclusively found in intron 5 and in the same transcriptional orientation as BACH2 (Cesana, et al. 2017, Ikeda et al. 2007, Imamichi et al. 2014, Mack et al. 2003, Maldarelli, et al. 2014, Wagner, et al. 2014). Since these BACH2 integration sites were identified in HIV-1-infected individuals who have been on ART for several years, it is conceivable that these proviruses are inactive, although it remains unknown whether this presumed inactivity is a result of integration site-dependent silencing of replication-competent proviruses or due to defective proviruses.

To address the question of whether HIV-1 would be silenced upon integration into intron 5 of BACH2 in the same transcriptional orientation, we employed a modified version of our dual-fluorophore HIV-1-based vector, LTatC[M], which reproduces features of active and latent HIV-1 infections (Kok et al. 2018). This vector comprises two fluorescent reporter genes: 1.) Cerulean, which reports the activity of the HIV-1 promoter, and 2.) mCherry, the expression of which is driven by a constitutive promoter and further protected from position-effect variegation by a pair of flanking genetic insulators to identify cells harbouring an integrated vector (Uchida et al. 2013, Villemure et al. 2001, Yahata et al. 2007). In this study, we investigate whether CRISPR/Cas9-mediated targeted HIV-1 integration in BACH2 would lead to inactivation of the HIV-1 promoter, and if so, whether it is locus and/or transcriptional orientation dependent.

**Material and Methods**

Generation of LTatCL[M] with target locus homologous arms and Cas9/guide RNA-encoding plasmids

In LTatC[M] the 3’LTR is located downstream of the second fluorophore mCherry to enable retrovirus production and subsequent infection of target cells (Kok, et al. 2018). LTatC[M] was modified to LTatCL[M], i.e., the 3’LTR (L) was inserted between Cerulean (C) and the insulator cHS4 (Figure 1A) to further enhance the transcriptional independence of the HIV-1 promoter controlled Cerulean. For targeted integration of our HIV-1-based, dual-fluorophore vector, retrovirus production is not required. Thus, the HIV-1 3’LTR was relocated immediately downstream of Cerulean. Additionally, a polyA signal was inserted between mCherry ([M]) and the second insulator sMAR8 (Figure 1A). The homologous regions on both sides of the targeted HIV-1 integration site in the human genome, were obtained from NCBI GenBank: BACH2 intron 5 (Accession No: NT_007299.13; 5’ arm nucleotides 93’502-94’355, 3’ arm nucleotides 94’356-95’206), BACH2 intron 2 (5’ arm nucleotides 339’363-340’186, 3’ arm nucleotides 340’187-341’034), and AAVS1 (Accession No: NC_000019.10; 5’ arm nucleotides 1’399-2’218, 3’ arm nucleotides 2’219-3’051). Targeted integration sites are depicted in Figure 1B.

Primers to amplify the respective target locus homologous arms are listed in Supplementary Table 1. Each PCR reaction contained 100 ng of human genomic DNA (Sup-T1 cell line; obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Dharam Ablashi), 1x Platinum Taq PCR buffer (ThermoFisher), 2 mM MgCl₂ (ThermoFisher), 0.2 mM dNTP (NEB), 0.4 µM of each forward and reverse primer, and 1 U Platinum Taq polymerase (ThermoFisher) in a total volume of 50 µL. The PCR cycling conditions were as follows: 94°C for 2 min; 35 cycles of (94°C for 30 s, 55°C for 30 s, 68°C for 1 min); 68°C for 5 min; 4°C hold.

The respective pair of target locus homologous arms were cloned into pGEM-T Easy vector (Promega). Subsequently, the dual-fluorophore vector LTatCL[M] was cloned into each plasmid in the same or convergent orientation via blunt-end cloning. The 6 plasmids pBACH2_i5-, pBACH2_i2-, and pAAVS1-LTatCL[M] were generated, containing the homologous arms of the targeted HIV-1 integration loci in BACH2 intron 5, BACH2 intron 2,
and in the safe harbour loci AAVS1, respectively, containing LTatCL[M] in both
transcriptional orientations (s and c) (Figure 1A).
Plasmids encoding Cas9 and guide RNAs were based on pX458 (Addgene plasmid 48138)
(Supplementary Table 1). Annealing of 100 μM 5’ phosphorylated primers for the guide
RNAs was performed using the following conditions: 80°C for 5 min, 65°C for 7 min, 60°C
for 7 min, 55°C for 7 min, 50°C for 7 min, 45°C for 7 min, 40°C for 7 min, 35°C for 7 min,
30°C for 7 min, 25°C for 7 min, and 4°C hold. The annealed guide RNA primers were
separately cloned into pX458.
All plasmid sequences were confirmed by next-generation sequencing. Briefly, plasmids were
gel purified, diluted to 0.2 ng/µl, and 1 ng of the plasmids were processed using the Illumina
Nextera XT DNA library Prep kit. The DNA libraries were subsequently paired-end or single-
end sequenced with an Illumina MiSeq using the MiSeq Reagent Kit v3 (150-cycle). Plasmid
maps are shown in Figure 1A.

Transfection of Jurkat T-cells and single cell sorting
Prior to transfection, the six LTatCL[M] vectors (pBACH2_i5-, pBACH2_i5c-, pBACH2_i2s-, pBACH2_i2c-, pAAVS1_s-, and pAAVS1_c- LTatCL[M]) were linearized using 100 units of
NsiI (NEB)/20 µg DNA. For transfection, 1 million Jurkat T-cells were resuspended in 100
µL Nucleofector solution (Cell Line Nucleofector™ Kits, Lonza) and combined with 2 µg of
the linearized dual-fluorophore vector and 2 µg of the corresponding gRNA/Cas9 plasmid:
pX458_gBACH2 (intr 5), pX458_gBACH2 (intron 2), and
pX458_gAAVS1 (AAVS1). pSpCas9(BB)-2A-GFP (pX458) was a gift from Feng
Zhang (Addgene plasmid # 48138) (Ran et al. 2013). Nuclofection was performed using the
program X-001 for Jurkat T-cells (Amaxa™ Nucleofector™ II, Lonza). Cells were cultured
in RPMI-1640 medium media supplemented with 10% fetal bovine serum (FBS) and 1%
Penicillin Streptomycin (10'000 units/ml Penicillin, 10 mg/ml Streptomycin).

At day 9 post transfection, cells were analysed using fluorescence-activated cell sorting
(FACS) and sorted at 20 cells per well in 96-well plates using a BD FACSARia™ III (BD
Biosciences). Two cell phenotypes were sorted: 1. Cerulean+/mCherry− and 2. Cerulean−
mCherry+. This was done for each nucleofected cell population: BACH2_i5s, BACH2_i5c,
BACH2_i2s, BACH2_i2c, AAVS1_s, and AAVS1_c. After 50 days, expanded cell cultures
were analysed by flow cytometry and subsequently single cell sorted. Longitudinal flow
cytometric analysis of the cells were done with the LSRFortessa II (BD Biosciences) and data
were analysed using the FlowJo Software v.10.0.8. (FLOWJO, LLC) (Figure 1C).

Activation of silenced HIV-1 promoter in cells with integrated LTatCL[M] in BACH2
Over time silenced HIV-1 promoter in sorted Cerulean+/mCherry+ monoclonal cell lines were
reactivated using 10 ng/µL Tumor Necrosis Factor Alpha (TNF-α) and 4 nM Romidepsin
(Ro) (Selleckchem). After 24 h the cells were analysed by flow cytometry.

Amplifying the junctions of LTatCL[M] integration into BACH2 and AAVS1
Genomic DNA was extracted from 5 million transfected Jurkat T-cells using the DNeasy
Blood and Tissue kit (Qiagen) and quantified by Quant-iTTM PicoGreen™ dsDNA Assay kit
(ThermoFisher Scientific). To verify the targeted integration of the dual-fluorophore vector
LTatCL[M] into BACH2 or AAVS1, the junctions of targeted integration were amplified by
(semi-)nested PCR using 300 ng genomic DNA containing 1x PCR buffer (Sigma Aldrich),
1.5 mM MgCl2, 0.2 mM dNTPs, 0.5 µM of each respective forward and reverse primer
(Supplementary Table 1), and 0.5 U JumpStart Taq DNA polymerase (Sigma-Aldrich) in a
total volume of 25 µL. The PCR cycling conditions were as follows: 94°C for 2 min; 35-40
cycles of (94°C for 30 s, 63°C for 30 s, 72°C for 2 min); 72°C for 5 min; 4°C hold.

Table 1, and 0.5 U JumpStart Taq DNA polymerase (Sigma-Aldrich) in a
total volume of 25 µL. The PCR cycling conditions were as follows: 94°C for 2 min; 35-40
cycles of (94°C for 30 s, 63°C for 30 s, 72°C for 2 min); 72°C for 5 min; 4°C hold.



Mono-allelic or bi-allelic integration of LTatCL[M] into BACH2 and AAVS1 was verified by PCR containing 100 ng genomic DNA, 1x PCR buffer, 1.5 mM MgCl$_2$, 0.2 mM dNTPs, 0.5 µM of each respective forward and reverse primers (Supplementary Table 1), and 0.5 U JumpStart Taq DNA polymerase in a total volume of 25 µL. The PCR cycling conditions were as follows: 94°C for 2 min; 35 cycles (94°C for 30 s, 63°C for 30 s, 72°C for 2 min); 72°C for 5 min; 4°C hold.

Amplicons were sequenced by Sanger sequencing. The sequencing reaction contains 1x Seqmix (Big Dye Termination v1.1, 1x Dilution Buffer (ThermoFisher)), and 100 ng DNA in a total volume of 40 µL. 0.2 µM primer was added (Supplementary Table 1). The PCR cycling condition were as follows: 40 cycles (96°C for 30 s, 50°C for 30 s, 60°C for 4 min); 4°C hold. Sequencing was performed following the manufacturer’s instruction on an 3130xl Genetic Analyzer (Applied Biosystems).

Near full-length amplification of integrated vector LTatCL[M]

Genomic DNA was extracted from 3 million transfected cells using the DNeasy Blood and Tissue kit and quantified by Quant-iT™ PicoGreen™ dsDNA Assay kit. To investigate the integrity of the integrated vector LTatCL[M], near full-length amplification of the vector was performed, using genomic 300 ng DNA, 1x Long Amp Taq Reaction Buffer (NEB), 0.4 mM dNTPs, 0.5 µM of each forward and reverse primer (Supplementary Table 1) and 2.5 U Long Amp Taq Polymerase (NEB) in a total volume of 25 µL. The PCR cycling conditions were as follows: 94°C for 30 min; 30 cycles of (94°C for 20 s, 58-61°C for 30 s, 72°C for 5 min); 72°C for 10 min; 4°C hold. 1 ng purified amplicons were processed with the Nextera XT DNA Library Preparation Kit (Illumina) and subsequently sequenced using the MiSeq reagent Kit v2 as described above.

Quantification of BACH2 mRNA expression

RNA was extracted from 3 million transfected cells using the All Prep DNA/RNA kit (Qiagen) and quantified by Nanodrop 1000. 800ng RNA was reverse transcribed with PrimeScript Reverse Transcriptase (Takara) according to manufacturer’s instructions. To exclude contamination of plasmid and genomic DNA contamination, reactions lacking reverse transcriptase were included for each RNA sample. To quantify the mRNA level of BACH2, qPCR reactions were performed in triplicates for each sample. cDNA was diluted 1:10 mixed with 1x PCR buffer (Sigma Aldrich), 1.5 mM MgCl$_2$, 0.2 µM dNTP (NEB), 0.5 µM of each Fw and Rv primers, 1x SYBR® Green, 50 nM Rox, 0.5 U JumpStart Taq Polymerase (Sigma-Aldrich) in a total volume of 25 µL. For each reaction, primer pair was chosen to amplify a specific region of the BACH2 mRNA, spanning exon 7-8. As a mRNA expression control glycerinaldehyde-3-phosphat-dehydrogenase (GAPDH) was amplified with GAPDH-specific primers (Supplementary Table 1). The qPCR cycling conditions were as follows: 94°C for 2 min; 40 cycles (94°C for 30 s, 55°C for 30 s, 63°C for 1 min (collect data)); 72°C for 2 min; 95°C for 15 s; 4°C hold. Melt curves were collected to analyze specificity of the amplification. The qPCR was performed in the ABI 7800 real-time PCR thermocycler then analysed using the 7500 Software v2.0.4, using the comparative C$_t$ method (Livak and Schmittgen 2001).

Quantification of BACH2 protein expression

Cells were lysed using assay lysis buffer (50 mM Tris-HCL pH8, 150 mM NaCl, 0.5% Na deoxycholate, 0.5% Triton X-100, 1x Protease inhibitor, ddH$_2$O) and protein was quantified using the Bicinchoninic acid assay (BCA) (Pierce™ BCA Protein Assay Kit). 40 µg protein were mixed with loading dye and loaded on a Bolt™ 4-12% Bis-Tris Plus Gels (ThermoFisher Scientific). Subsequently, proteins were transferred onto a nitrocellulose membrane (iBlot™ 2 Transfer Stacks, ThermoFisher Scientific) blocked for 1h at RT with
5% Top-Block wt/vol in PBS supplemented with Tween-20 (10x PBS pH7.4, 0.1% Tween 20, and ddH$_2$O). For protein detection, the membrane was incubated with primary antibody (1/400 diluted rabbit anti-BACH2 antibody (PA5-23642, ThermoFisher Scientific) and 1/5'000 diluted rabbit-anti-GAPDH antibody (ab9485, Abcam)) overnight at 4°C. After washing the membrane four times with 1x PBS-T, the secondary antibody, 1/10'000 diluted goat anti-rabbit IgG H&L (HRP) (ab97051, Abcam) was added and the membrane was incubated at RT for 1 h. The membrane was then washed four times with 1x PB-T. To visualize the protein, Immobilon Crescendo Western HRP substrate (Merck) was added to the membrane. Chemiluminescence was captured with the Stella system (model 3200, Matlab Group Companies).

Results

Targeted integration of the 5.3 kb HIV-1-based, dual-fluorophore vector LTatCL[M] into BACH2 and AAVS1 via CRISPR/Cas9-mediated homology directed repair

To investigate specific effects of HIV-1 integration into BACH2, four constructs were generated containing the vector LTatCL[M] flanked by BACH2 homologous arms (Figure 1A) and integrated in Jurkat T-cells by means of the CRISPR/Cas9 technology. The constructs were integrated into intron 5 of BACH2 in 1.) the in vivo observed same (s) and 2.) the convergent (c) transcriptional orientation as BACH2 to investigate the potential impact of transcriptional orientation on HIV-1 promoter activity. Furthermore, LTatCL[M] was integrated into intron 2 of BACH2 in 3.) the same and 4.) the convergent transcriptional orientation to investigate the position effect of HIV-1 integration into BACH2. Intron 2 was chosen as it has not yet been observed in HIV-1 infected patients, however, it was frequently observed as HIV-1 integration locus in human CD34$^+$ hematopoietic stem cells infected in vitro with HIV-1(Maldarelli, et al. 2014). In addition, LTatCL[M] was integrated into intron 1 of PPP1R12C (protein phosphatase 1 regulatory subunit 12C) in again both transcriptional orientations. This genomic locus was previously identified as preferred site for integration of adeno-associated virus (AAV) DNA and designated AAVS1 (AAV integration site)(Kotin et al. 1992). AAVS1 is widely used as genomic safe harbour (GSH) although not without some limitations particularly in the context of future use in gene therapy trials(Papapetrou and Schambach 2016, Sadelain et al. 2011). Nevertheless, for research purposes it is considered a GSH and we expect that the HIV-1 promoter will not be silenced when integrated in this GSH. These six vector constructs and subsequently generated Jurkat T-cell clones were named BACH2$_{i5s}$, BACH2$_{i5c}$, BACH2$_{i2s}$, BACH2$_{i2c}$, AAVS1$_{c}$, and AAVS1$_{s}$ (Figure 1A and B).

Jurkat T-cells were nucleofected with linearized plasmids of the six LTatCL[M] constructs. Nine days post transfection, the frequencies of stably transfected Cerulean$^+$/mCherry$^+$ and single mCherry$^+$ cells were between 0.1 and 1.8% (Figure 1C). Cells were sorted to enrich Cerulean$^+/mCherry^+$ (i.e., active HIV-1 promoter) and single mCherry$^+$ (i.e., inactive HIV-1 promoter) cells (Figure 1D).

To obtain monoclonal cell lines, a sequential flow cytometric sorting strategy was employed. For all targeted integration variants, Cerulean$^+/mCherry^+$ and single mCherry$^+$ cells were first sorted at 20 cells per well in 96-well plates and expanded in culture up to 50 days post transfection. We analysed by means of flow cytometry a total of 92 cell populations, and selected 29 for further expansion. These cells were single cell sorted to obtain monoclonal cell lines and cultured for another 25-30 days. In total, 43 monoclonal cell lines were generated. For each targeted integration variant (BACH2$_{i5s}$, BACH2$_{i5c}$, BACH2$_{i2s}$, BACH2$_{i2c}$, AAVS1$_{c}$, and AAVS1$_{s}$), at least one monoclonal cell line for each of the two phenotypes (Cerulean$^+/mCherry^+$ and single mCherry$^+$) were obtained with the exception of
AAVS1_c, single mCherry+ (Table 1). The monoclonal cell lines were characterized as follows.

First, targeted integration of the HIV-1-based, dual-fluorophore vector LTatCL[M] was verified by amplification of a fragment spanning the targeted gene up- or downstream of the 5' or 3' homologous arms, respectively, and LTatCL[M]. Targeted integration was confirmed in 39/43 monoclonal cell lines representing all three genomic loci in both orientations and for both fluorescence phenotypes, except for AAVS1_c single mCherry+ (Table 1).

Second, targeted integration of LTatCL[M] in those 39 monoclonal cell lines was verified to be mono-allelic by a PCR strategy that allows sufficient amplification only of the not targeted BACH2 or AAVS1 allele by using primers up- and downstream of the BACH2 or AAVS1 homologous arms (Figure 1A) and short PCR extension times. All monoclonal cell lines contained a not targeted allele of the respective targeted integration site (Table 1).

Third, BACH2 mRNA expression was quantified in 11 Cerulean+/mCherry+ monoclonal cell lines, representing each targeted integration variant. A decrease of BACH2 mRNA expression could not be observed in any Cerulean+/mCherry+ monoclonal cell line (Figure 2A, Table 1).

Forth, the potential impact on BACH2 protein expression after targeted integration of LTatCL[M] into BACH2 was investigated by Western blot analyses. In line with unaltered BACH2 mRNA expression, BACH2 protein expression was not impaired compared to non-transfected Jurkat T-cells in all 35 monoclonal cell lines tested (Figure 2B, Table 1).

In summary, we successfully performed targeted integration of the 5.3 kb HIV-1-based, dual-fluorophore vector LTatCL[M] into various loci of the human genome via CRISPR/Cas9-mediated homology-directed repair. We subsequently generated monoclonal cell lines modelling actively and latently HIV-1-infected cells with integrated LTatCL[M] in either transcriptional orientation in the targeted genomic loci.

Silencing of the HIV-1 promoter in cells with integrated LTatCL[M] in BACH2

To study the HIV-1 promoter activity over time in Cerulean+/mCherry+ monoclonal cell lines, i.e., active HIV-1 promoter, the fluorescence profile was frequently monitored for 162 days. In the majority of Cerulean+/mCherry+ monoclonal cell lines with integrated LTatCL[M] in BACH2, the HIV-1 promoter was gradually silenced as observed by the decline of the frequency of Cerulean+/mCherry+ cells to <50% in 16/18 monoclonal cell lines within 24-162 days and to <10% in 10/18 monoclonal cell lines within 58-162 days (Figure 3A and Supplementary Figure 2). This was observed independent of the BACH2 introns 2 or 5 chosen for targeted integration of LTatCL[M] and the transcriptional orientation of LTatCL[M] relative to BACH2. In contrast, in all 5 AAVS1_s and AAVS1_c Cerulean+/mCherry+ monoclonal cell lines the frequency of Cerulean+/mCherry+ cells remained relatively stable for 162 days (>80% in 4/5 cell clones) as observed for one BACH2_i5s clone (Figure 3A).

Overall, the HIV-1 promoter when integrated into the BACH2 gene is silenced over time in the majority of Cerulean+/mCherry+ monoclonal cell lines whereas it remains active when integrated into AAVS1.
Silenced HIV-1 promoters can be reactivated by TNF-α and Romidepsin

To evaluate the reactivation of silenced HIV-1 promoters in Cerulean+/mCherry+ monoclonal cell lines, which gradually lost Cerulean expression, we performed an activation assay using TNF-α and Romidepsin. TNF-α and Romidepsin in combination have been shown to activate the silenced HIV-1 promoter (Kok, et al. 2018, Sogaard et al. 2015). These compounds were used to evaluate the reactivatability of the silenced HIV-1 promoter in 8 Cerulean+/mCherry+ monoclonal cell lines with integrated LTatCL[M] in BACH2. After 99 days, 1.6-48.2% of cells were Cerulean+/mCherry+. Upon treatment with TNF-α and Romidepsin, the frequencies of Cerulean+/mCherry+ cells increased to 61.6-89.8% (Figure 3B), demonstrating that the silenced HIV-1 promoter was reactivatable in those monoclonal cell lines.

Monoclonal single mCherry+ cell lines harbour large internal deletions in LTatCL[M]

A total of 17 single mCherry+ monoclonal cell lines, presumably representing latently HIV-1-infected cells, silenced at an early time point upon targeted HIV-1 integration, were treated with TNF-α and Romidepsin to activate the HIV-1 promoter. Cerulean expression was not induced by TNF-α and Romidepsin in any of these 17 monoclonal cell lines. To further investigate this, the whole vector LTatCL[M] was amplified and sequenced in 8 of these 17 monoclonal cell lines. Surprisingly, the integrated vector LTatCL[M] in these monoclonal single mCherry+ cell lines harboured large internal deletions in the 5’HIV-1 LTR, tat, and/or Cerulean cassette (Figure 4). The monoclonal BACH2_i5s cell lines 3.1 and 3.2, which were derived from the same cell culture after the 1st sort, showed the same deletion in the IRES-Cerulean region (Figure 4A). A similar observation was made in the monoclonal BACH2_i2c cell lines 13.1 and 13.3, also derived from the same 1st sorted cell population: The same deletion spanning from the 5’LTR to the Cerulean cassette was observed in both monoclonal cell lines (Figure 4B). All independent single mCherry+ monoclonal cell lines contained large deletions in different regions of the vector suggesting that the plasmids were not the source of those deletions. This was confirmed by next-generation sequencing of the plasmids not showing any evidence for large deletions. Next, the integrated vector LTatCL[M] was sequenced in a cell population of single mCherry+ cells immediately after the 1st sort, 9 days post transfection (Supplementary Figure 1). Evidence for numerous different large deletions were observed in the 5’HIV-1 LTR, Tat, and/or Cerulean (data not shown), indicating that those deletions occurred early during targeted integration.

Discussion

We developed a novel HIV-1 latency model based on CRISPR/Cas9-mediated targeted integration of an 5’337 bp HIV-1-based, dual-fluorophore vector into selected sites in the human genome. In our previous study, we have shown that the HIV-1-based, dual-fluorophore vector LTatC[M] reproduces features of active and latent HIV-1 infections (Kok, et al. 2018). Here, we studied the fate of HIV-1 in specific HIV-1 integration loci/sites by targeted integration of the vector LTatCL[M] into BACH2 and AAVS1. In Jurkat T-cells, HIV-1 integration into BACH2 led initially to an active HIV-1 promoter as shown by the expression of HIV-1 LTR controlled Cerulean. Over time those monoclonal cell lines showed a gradual silencing of the HIV-1 promoter. HIV-1 integration into the two target loci of BACH2 intron 5 and intron 2 in both orientations showed similar silencing of the HIV-1 promoter over time. Differences were observed for the safe harbour locus AAVS1, in which no substantial silencing of the HIV-1 promoter was observed over a time period of 162 days. This raises the question, whether BACH2 is an exceptional HIV-1 integration site promoting HIV-1 latency or, vice versa, AAVS1 is an exceptional HIV-1 integration site preventing HIV-1 latency. In a future study, we will be expanding the repertoire of investigated HIV-1 integration sites.
In virally suppressed HIV-1-infected individuals, the provirus integrated into BACH2 has been found predominantly in intron 5 in the same orientation as the gene and has been linked with clonal expansion. However, in vitro in HIV-1 infected cell lines, integration has been found to occur randomly in the BACH2 gene (Maldarelli, et al. 2014, Wagner, et al. 2014). The preference of HIV-1 integration into BACH2 intron 5 in the same orientation in vivo could be caused by: 1.) Selection of BACH2 intron 5 integrants over other integrants over time (Hughes and Coffin 2016), 2.) BACH2 intron 5 is preferentially targeted by HIV-1 in primary CD4+ T-cells, presumably due to the spatial location of this locus in the nucleus of target cells (Marini, et al. 2015), or 3.) distinct alterations in BACH2 expression levels caused by different HIV-1 integration patterns. BACH2 expression levels vary between T-cells in distinct differentiation stages (Richer et al. 2016). An increase of BACH2 transcripts in regulatory and effector T-cells were observed when HIV-1 was integrated in BACH2 (Cesana, et al. 2017). Enhanced expression of the wild-type BACH2 in regulatory T-cells leads to increased proliferation capacity without affecting the cells’ phenotype (Cesana, et al. 2017).

Alteration of BACH2 expression might cause HIV-1 persistence in BACH2 intron 5 and expansion of the cell. In Jurkat T-cells, as compared to primary CD4+ T-cells, the BACH2 expression levels might be different (Shan et al. 2017). Nonetheless, we could not observe an inhibitory effect on BACH2 mRNA expression nor an effect on BACH2 protein expression when BACH2 introns 5 and 2 were targeted for integration with our HIV-1-based vector. This indicates that other factors might lead to the persistence of HIV-1 in this locus.

The expression of Cerulean decreased over time in almost all BACH2 monoclonal cell clones independent of the targeted intron and the orientation of HIV-1 integration. The slow decline and the presence of Cerulean positive cells for up to 162 days in some of those monoclonal cell clones shows that targeted integration of LTatCL[M] into BACH2 does not immediately and not completely lead to HIV-1 promoter inactivity in Jurkat T-cells. However, insulator-protected mCherry expression also declined in some of those cell clones, suggesting a gradual and strong silencing effect at the BACH2 loci. This silencing was reversible as shown by the treatment of cell clones harbouring silenced HIV-1 promoters with TNF-α and Romidepsin that led to HIV-1 promoter reactivation. These observations are partly in line with Lange et al. (2018): They observed a comparable reversible silencing of the HIV-1 promoter in BACH2 within 20-40 days targeting two sites within BACH2 intron 5 via CRISPR/Cas9 and inserting one reporter gene under the control of the HIV-1 LTR-promoter in the same transcriptional orientation as BACH2 (Lange et al. 2018). Here, we observed the same phenotypes in another intron of BACH2 and independent of the orientation of the HIV-1 integration. Furthermore, targeted integration of LTatCL[M] into the safe harbour site AAVS1 did not lead to silencing of the HIV-1 promoter.

Mono-allelic integration of 5.3 kb long LTatCL[M] into BACH2 and AAVS1 has been confirmed in monoclonal cell lines by amplifying and sequencing junctions of integration. So far, the longest fragment inserted in human lymphocytes was 1.5 kb long (Hung et al. 2018), hence, our findings show that insertion of a 3.5x larger fragment is possible in Jurkat T-cells. Fragments of similar length (5.5 kb and 7.4 kb) have been successfully integrated in other cell types (embryonic stem cells and zygotes)(Wang et al. 2015).

Monoclonal cell lines expressing initially only mCherry, i.e., modelling latently HIV-1 infected cells, were found to contain large deletions in the LTR-tat-Cerulean cassette. Similar deletions were already observed by us when we used a variant of our vector to generate retroviruses for infection of cells (Kok, et al. 2018). There, we speculated that the deletions were caused by error-prone HIV-1 reverse transcription (Bebenek et al. 1989, Patel and Preston 1994) or copy-choice recombination (Sanchez et al. 1997, Simon-Loriere and Holmes 2011), which is also observed in cells from HIV-1-infected individuals (Ho et al. 2013). However, our current system does not require reverse transcriptase prior to integration since we are using CRISPR/Cas9-mediated targeted integration. Therefore, the deletions might be
due to recombination events triggered by the LTRs flanking the Cerulean cassette (Mager and Goodchild 1989). Together with our previous observation (Kok, et al. 2018), our results underline the importance to further investigate single fluorescent cell populations (representing latently HIV-1 infected cells) in latency models using HIV-1-based vector systems. Beyond HIV-1, these findings might also have implications for lentiviral vectors used for gene therapy.

Conclusion

Our CRISPR/Cas9-mediated, targeted integration system based on the HIV-1 dual-fluorphore vector LTatCL[M] enables studies on effects of HIV-1 integration in selected genomic sites on viral and cellular phenotypes. Targeting the BACH2 gene revealed that loci within this gene are capable of supporting an active HIV-1 promoter upon integration but its activity diminishes over time. The temporal silencing of the HIV-1 promoter at these loci might be a component of a potentially complex mechanism by which HIV-1 persists.

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Author contributions

YLK an AI designed the study, performed the experiments, analysed the data, and wrote the manuscript. LJ supported the experiments and analysis of the data. KN, AK, and DH supported the experiments. TC was pioneering in the design of the study. KJM invented and designed the study, supported the analysis of the data, and edited the manuscript. All authors read and approved the manuscript.

Conflict of Interest Statement

Competing interests

KJM has received travel grants and honoraria from Gilead Sciences, Roche Diagnostics, Tibotec, Bristol-Myers Squibb, and Abbott; the University of Zurich has received research grants from Gilead, Roche, and Merck Sharp & Dohme for studies that KJM serves as principal investigator, and advisory board honoraria from Gilead Sciences. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Contribution to the Field Statement

Clonal expansion of latently HIV-1 infected cells and its association to certain HIV-1 integration hotspots is currently an important topic in HIV-1 reservoir research, since the stability of the HIV-1 reservoir is a major obstacle to cure HIV-1 infection. Our novel HIV-1 latency model using CRISPR/Cas9-mediated, targeted integration of the HIV-1-based, dual-fluorphore vector LTatCL[M] enables studies on effects of HIV-1 integration in selected genomic loci/sites on viral and cellular phenotypes. Using the HIV-1 integration hotspot
BACH2 as example, we could show that HIV-1 integration into BACH2, but not into AAVS1, leads to HIV-1 latency, which was reversible upon re-activation.

**List of Abbreviations**

ART: Antiretroviral therapy; BACH2: BTB domain and CNC homology 2; AAVS1: Adeno-associated virus integration site 1; LTR: Long terminal repeat; LTatCL[M]: 5’LTR –Tat-Cerulean-3’LTR-insulator-mCherry-insulator; TNF-α: Tumor Necrosis Factor Alpha; Ro:Romidepsin; RIG: recurrent integration gene;

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**Availability of Materials**

Not applicable.

**Availability of Data**

Not applicable.

**Mandatory data and required repositories**

| Data-type                              | Required Repositories                          | Metadata Standard |
|----------------------------------------|------------------------------------------------|-------------------|
| Genetic and genomic sequence (DNA)     | GenBank                                        | MiXS              |
|                                        | DNA Data Bank of Japan (DDBJ)                  |                   |
|                                        | European Nucleotide Archive (ENA)              |                   |

**Figures**

**Figure 1: Targeted integration of the dual-fluorophore vector LTatCL[M] into specific genomic loci in Jurkat T-cells.** (A) Schematic diagram of the six HIV-1 based, dual-fluorophore vectors LTatCL[M] (5’337 bp) flanked with the BACH2/AAVS1 homologous arms. LTatCL[M] contains two fluorophores (Cerulean and mCherry) to distinguish between inactive and active HIV-1 promoters, i.e., modelling latently and actively HIV-1 infected cells. The Cerulean cassette is under the control of the HIV-1 LTR whereas mCherry is under the control of an independent constitutive promoter (heIF4A1) and flanked by two insulators (cHS4 and sMAR8). LTR, long terminal repeat; tat, HIV-1 transactivator; IRES, internal ribosome entry site; cHS4, chicken hypersensitive site 4; tetO, tetracycline operator sequences; heIF4A1, human eukaryotic initiation factor 4A1, gene promoter; sMAR8, synthetic matrix attachment region 8. (B) Scheme of the targeted HIV-1 integration sites in BACH2 and AAVS1. Some described HIV-1 integration sites in vivo are marked by red arrows (Maldarelli, et al. 2014, Wagner, et al. 2014). (C) Percentage of Cerulean+/mCherry+ (white bars) and single mCherry+ (black bars) cells 9 days post transduction of Jurkat T-cells targeting the different loci in BACH2 and AAVS1. The means and standard deviations of 3 independent experiments are depicted. (D) Flow chart to generate monoclonal cell lines. Jurkat T-cells were separately transfected with the vectors shown in A and the corresponding
gRNA/Cas9 plasmid. Nine days post transfection, the six different targeted HIV-1 integration variants were each sorted by 20 cells per well for the phenotypes Cerulean+/mCherry+ and single mCherry+. Fifty days post transfection, the cells were single cell sorted for each targeted integration variant for the two phenotypes Cerulean+/mCherry+ and single mCherry+. After at least 25 days post 2nd sorting, cells were further characterized. (A-D). The in vivo observed preferential HIV-1 integration loci in BACH2, BACH2_i5s, is highlighted by red boxes.

**Figure 2:** The mono-allelic integration of LTatCL[M] into BACH2 did not impair BACH2 mRNA nor BACH2 protein expression. (A) BACH2 mRNA expression was quantified by amplification of a region in the mRNA downstream of BACH2_i5 and BACH2_i2. The bar blot consists of two independent qPCR experiments, consisting of two replicates each. The qPCR was carried out two times independently in duplicate. The means and standard deviations are depicted. (B) Western blot analysis of BACH2 protein expression, 110 kD BACH2 (upper panel) and 37 kD GAPDH (lower panel) are shown for an exemplary experiment. The in vivo observed preferential HIV-1 integration loci in BACH2, BACH2_i5s, is highlighted by red boxes.

**Figure 3:** Longitudinal analysis of Cerulean+/mCherry+ monoclonal cell lines and reactivation of silenced HIV-1 promoter. (A) Longitudinal frequency of Cerulean+/mCherry+ expression in 24 monoclonal cell lines for up to 162 days: 3x BACH2_i5s (depicted in red, first left panel), 4x BACH2_i5c, 7x BACH2_i2s, 4x BACH2_i2c, 3x AAVS1_s, and 3x AAVS1_c. Each symbol represents one Cerulean+/mCherry+ monoclonal cell line. (B) Reactivation of silenced HIV-1 promoter upon treatment of monoclonal cell lines with 10 ng/μL TNF-α and 4 nM Romidepsin for 24 hours followed by FACS analysis. The experiment was carried out three times independently. The in vivo observed HIV-1 integration loci in BACH2, BACH2_i5s, is highlighted by red boxes.

**Figure 4:** Mapping of large deletions within LTatCL[M] in TNF-α/Romidepsin non-responsive, single mCherry+ monoclonal cell lines. Deletions in the integrated vector LTatCL[M] are depicted for monoclonal BACH2_i5, BACH2_i2, and AAVS1 monoclonal cell lines with integrated LTatCL[M] in the same transcriptional orientation (A) and in the convergent transcriptional orientation (B). Black horizontal bars show the amplified and sequenced regions of LTatCL[M] and large deletions are shown in white horizontal bars. Light blue and light red vertical bars show the positions of the two fluorophores Cerulean and mCherry, respectively. Vector schemes are depicted on top. The in vivo observed HIV-1 integration region site in BACH2, BACH2_i5s, is highlighted by red boxes. Different starting points of amplicons were due to using different primer pairs for amplifications.

**Table 1:** Characteristics of monoclonal Jurkat T-cell lines after targeted integration of LTatCL[M] into BACH2 and AAVS1. Cerulean+/mCherry+ and single mCherry+ representing active and inactive HIV-1 promoters in LTatCL[M] transfected cells. The in vivo observed preferential HIV-1 integration loci in BACH2, BACH2_i5s, is highlighted by a red box.
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HIV-1 based, dual-fluorophore LTR-Tat/Cerulean-LTR-ins-heIF4A1/mCherry-ins (LTatCL[M]) vector

BACH2_15s

BACH2_15c

BACH2_12s

BACH2_12c

AAVS1_s

AAVS1_c

5'337bp

B

C

D

Characterization of single cell clone
- verification of LTatCL[M] integration
- verification of mono- or bi-allelic LTatCL[M] integration
- quantification of BACH2 mRNA expression
- quantification of BACH2 protein expression
- longitudinal analyses of Cerulean and mCherry expression
- HIV-1 promoter activation

9 days post transfection

Cerulean'+mCherry' (active HIV-1 promoter (modelling productive HIV-1 infection))
single mCherry' (inactive HIV-1 promoter (modelling latent HIV-1 infection))

sorted 20 cells/well

sorted single cells/well
50 days post transfection

single mCherry' (inactive HIV-1 promoter)
25-30 days post 2nd sort

Cerulean'+mCherry' (active HIV-1 promoter)
| LTatCL[M] integration site and orientation | HIV-1 promoter phenotype¹ | monoclonal cell lines (n) | targeted integration confirmed by PCR | mono-allelic targeted integration | unaltered BACH2 mRNA expression | unaltered BACH2 protein expression |
|------------------------------------------|---------------------------|--------------------------|--------------------------------------|----------------------------------|-------------------------------|----------------------------------|
| **BACH2_i5s**                           | active                    | 6                        | 5/6 (83.3%)                          | 5/5 (100%)                       | 2/2 (100%)                    | 5/5 (100%)                       |
|                                          | inactive                   | 7                        | 5/7 (71.4%)                          | 5/5 (100%)                       | n.a.                          | 4/4 (100%)                       |
| **BACH2_i5c**                           | active                    | 4                        | 4/4 (100%)                           | 4/4 (100%)                       | 2/2 (100%)                    | 4/4 (100%)                       |
|                                          | inactive                   | 1                        | 1/1 (100%)                           | 1/1 (100%)                       | n.a.                          | 1/1 (100%)                       |
| **BACH2_i2s**                           | active                    | 7                        | 7/7 (100%)                           | 7/7 (100%)                       | 2/2 (100%)                    | 7/7 (100%)                       |
|                                          | inactive                   | 4                        | 3/4 (75%)                            | 3/3 (100%)                       | n.a.                          | 3/3 (100%)                       |
| **BACH2_i2c**                           | active                    | 4                        | 4/4 (100%)                           | 4/4 (100%)                       | 2/2 (100%)                    | 4/4 (100%)                       |
|                                          | inactive                   | 2                        | 2/2 (100%)                           | 2/2 (100%)                       | n.a.                          | 2/2 (100%)                       |
| **AAVS1_s**                             | active                    | 2                        | 2/2 (100%)                           | 2/2 (100%)                       | 1/1 (100%)                    | 2/2 (100%)                       |
|                                          | inactive                   | 3                        | 3/3 (100%)                           | 3/3 (100%)                       | n.a.                          | n.a.                            |
| **AAVS1_c**                             | active                    | 3                        | 3/3 (100%)                           | 3/3 (100%)                       | 2/2 (100%)                    | 3/3 (100%)                       |
|                                          | inactive                   | n.a.                     | n.a.                                 | n.a.                            | n.a.                          | n.a.                            |
| **Total**                                |                           | 43                       | 39/43 (90.7%)                        | 39/39 (100%)                     | 11/11 (100%)                  | 35/35 (100%)                     |

¹active = Cerulean*/mCherry*, inactive = single mCherry*

n.a. = not available