Abstract

The establishment of a latent reservoir after infection by Human Immunodeficiency Virus type 1 (HIV-1) is a significant obstacle to an HIV cure. Antiretroviral therapies (ART) effectively target actively replicating virus, but are ineffective at targeting latently infected cells. Thus, an area of key interest lies in developing methods to induce expression of these latent proviruses. As such, an understanding of the molecular mechanisms which govern HIV transcription is of interest to identify new targets to mediate latency reversal. The aim of this research was to investigate the effect of transcriptional interference on HIV latency in cell models with characterized integration sites. In the model organism *Saccharomyces cerevisiae* (yeast), regulation of transcription involves a complex set of proteins that creates heterochromatin in regions after they have been transcribed to prevent cryptic transcription—transcription initiation in non-promoter regions. Evidence suggests this mechanism works in a similar capacity in humans, and we hypothesized this may contribute to HIV latency. Using CRISPR-Interference, we targeted a nuclease-deactivated Cas9 protein (dCas9) fused to the KRAB transcriptional repression domain to promoter regions of known genes of HIV integration to silence host gene transcription and examine the role of transcriptional interference in HIV latency. Cell lines expressing dCas9-KRAB and both single and multiple gRNAs targeted to host gene promoters showed moderate to high reduction of mRNA expression of the host gene of HIV integration but did not fully ablate transcription. In these systems, we did not observe an altered response in latency reversal. If transcriptional interference does indeed have an effect on latency, then it appears that merely diminishing transcription is not sufficient to alter reactivation, or that transcriptional interference plays a minor overall role in maintenance of latency.

Introduction

In the search for a cure for Human Immunodeficiency Virus (HIV), the rapid establishment of a latent reservoir of HIV-infected cells that cannot be targeted by traditional antiretroviral therapies (ART) remains a significant obstacle[1]. This latent pool is the source of plasma viral rebound upon cessation of ART. Numerous cellular mechanisms that contribute to latency have been characterized, including the sequestration of host transcription factors and the role of epigenetic modifications at the viral promoter[2]. Another proposed, but less characterized, mechanism is transcriptional interference. HIV preferentially integrates into intronic regions of actively transcribed genes[3]. It has been proposed that RNA Polymerase II (RNAPolII) initiation from the host gene promoter can collide with RNAPolII emanating from the provirus promoter (or LTR), resulting in transcriptional interference via steric hindrance that helps to maintain latency[4].

Interestingly, this mechanism may be more complex, as transcriptional regulation has been linked to chromatin regulation of gene bodies. Lysine methylation of histone H3 on lysine 36 (H3K36me) has been implicated in the regulation of cryptic transcription, a phenomenon where RNA Polymerase II initiates transcription at non-promoter regions[5]. Extensively studied in yeast, deposition of H3K36me by transcribing RNAPolII recruits protein complexes that deacetylate methyl-marked nucleosomes[5]. This mechanism prevents random initiation of RNAPolII within gene bodies after the creation of heterochromatin has occurred post-transcription. In yeast, this pathway uses the non-essential enzyme Set2 that can perform all 3 methylation reactions required to create H3K36 tri-methylation (me3). The ortholog present in humans, SETD2, interacts with RNAPolII, but it has been identified as an essential protein in humans[5]. In contrast to the system in yeast, SETD2 associates with heterogeneous nuclear ribonucleoprotein L (hnRNPL) for lysine

trimethylation; knockdown of hnRNPL expression results in a reduction of H3K36me[5]. The role of H3K36me in the maintenance of repressive chromatin during transcriptional elongation is similar in humans but may be independent of acetylation[6]. We are interested to determine if the H3K36me mechanism in yeast is similar in humans and if it may play a role in the maintenance of latency in HIV-infected cells. To examine this, guide RNAs targeted to the promoter of the host gene will be used to guide a defective Cas9 protein fused to the KRAB repression domain (dCas9-KRAB) to the host gene promoter in a technique termed CRISPR Interference (CRISPRi)[7]. Binding of dCas9-KRAB will induce repressive chromatin at guide RNA target sites and repress transcription of the host gene. We anticipate this system will allow further insight into the role of transcriptional interference in HIV latency reversal and may identify novel targets for latency reversal strategies for an HIV cure.

Methods

Cell Lines

2D10 and E4 cells were a gift from Dr. Jonathan Karn (Case Western Reserve). Cells were maintained in RPMI1640 (LifeTech) supplemented with 10% FBS (Millipore) and 100U/mL Pen/Strep (LifeTech) at 37°C/5%CO_2.

The 2D10 cell model is a Jurkat T-cell line that contains an integrated HIV reporter carrying LTR driven expression of regulatory proteins Tat and Rev, as well as a destabilized green fluorescent protein (d2EGFP). The integration site of this LTR-GFP construct in 2D10 cells has been characterized and identified to be the MSRB1 gene. This line also carries an attenuated H13L mutation in Tat which helps promote a cell's entrance into latency[8]. The E4 model was developed similarly to the 2D10 model, but E4 cells carry wild-type Tat and have an integration site in an

intron of the gene CD2AP[9]. In these models, HIV has an antisense orientation in 2D10 cells and a sense orientation in E4 cells relative to the host gene[10].

Guide RNA design

CRISPR gRNAs were targeted to the genes MSRB1 and CD2AP , the host gene of HIV integration for 2D10 and E4 cells, respectively. The non-targeted gene of one model served as a control gene for the other model (e.g., CD2AP was used as a control gene in 2D10 cells). All gRNAs were designed using the CRISPR Design tool (Table 1)(http://crispr.mit.edu). The gRNAs were selected based on predicted binding efficiency, low potential for off-target binding and location upstream and downstream of the host gene transcription start site (Figure 1). Multiple candidate gRNAs for each gene were selected; these sequences and their anti-sense strands (Sense – S, Antisense – AS) were designed with BsmBI or BbsI endonuclease sites for single- or multi-gRNA cloning, respectively. All gRNAs were created using the IDT Oligo Analyzer Tool (https://www.idtdna.com/calc/analyzer). The multi-gRNA cloning scheme was performed using the Golden Gate cloning method: the insertion of multiple DNA segments in one ligation reaction[12]. The vectors used for this cloning scheme—pLV hU6-sgRNA hUbC-dCas9-KRAB-T2a-Puro, phH1-gRNA, pmU6-gRNA, phU6-gRNA and ph7SK-gRNA—were purchased from Addgene and gifted by Dr. Charles Gersbach (Duke University)(Addgene plasmid # 71236, 53186, 53187, 53188, 531899)[11, 12].

Table 1 – *Guide RNAs designed with the CRISPR MIT tool* – sense strands shown below

Guide RNAs designed for Single- and Multi-gRNA targeting		
Multi	MSRB1	CD2AP
a	**GGGCGACGGCGCGAATCCGT**	**GATCCGTAAGTAACTTCGCA**
b	**ACGCGGGTACCGGCGGTCGT**	**AAAGGCGACACCGTAGACTA**
c	**ACATCACCCCCGGACGACGG**	**GACTGGTAGGAGAGCGCCGC**
d	**ATGCGATTGGACTGGCACCG**	**GAGAGAGCCCGAACGCGACG**
Single	MSRB1	CD2AP
1	**CACCATGCGATTGGACTGGCACCG**	**CACCGAGAGAGCCCGAACGCGACG**
2	**CACCGGGCGACGGCGCGAATCCGT**	**CACCTGCGGTGACGTCACGGCCG**

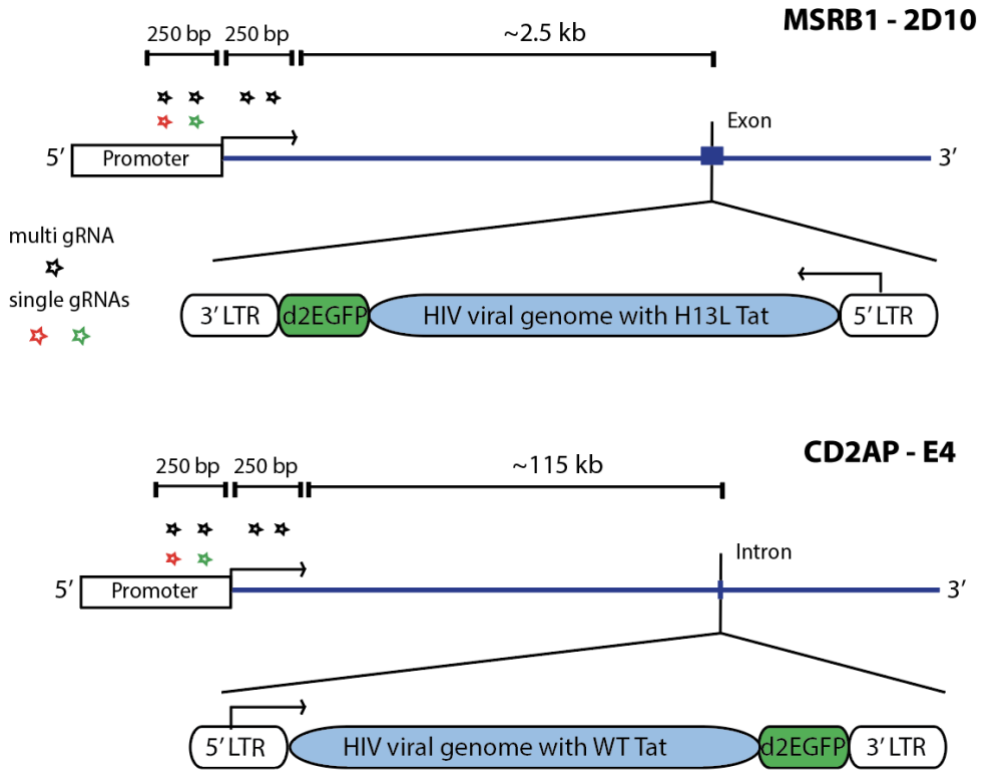

Figure 1 – *Schematic of proviral integration sites and gRNA targeting for MSRB1 and CD2AP* – Two gRNAs were targeted a short distance upstream of the transcriptional start site, and two gRNAs were targeted downstream. In cells with single-target systems, one gRNA was targeted to the promoter, and 2 different gRNAs were used.

Preparing gRNA oligonucleotides for Golden Gate[11] Cloning

In a 20µL reaction, stock of S/AS strands were added to a final concentration of 5 µM with T4 Polynucleotide Kinase (PNK) (NEB), 1x T4 PNK buffer (NEB), and 1mM ATP. The reaction was incubated at 37ºC for 1 hour, placed in a 95ºC heat block, and allowed to cool to 30ºC.

Plasmid design for single- and multi-gRNA constructs

Single–gRNA Constructs – The plasmid vector pLV hU6-sgRNA hUbC-dCas9-KRAB-T2a-Puro was digested with BsmBI (NEB) and dephosphorylated with rSAP (NEB) with 1x Fast-Digest buffer (ThermoFisher) and 0.1 mg/mL BSA (NEB) in a 30µL reaction. The digests were incubated at 37ºC for 2 hours. The digests were gel purified from a 1% agarose gel using a Molecular Biology GeneJET Gel Extraction Kit (ThermoFisher) using manufacturer specifications and eluted in 30µL 10mM Tris-HCl pH8.0. Guide RNA oligonucleotides were diluted 1:25 with water; 1µL diluted oligonucleotides, 30ng of gel purified plasmid, and T4 Ligase buffer (NEB) were added in a 10µL reaction with 0.1 mg/mL BSA and 1x T4 Ligase buffer. The reactions were incubated at room temperature for 30 minutes.

Each ligation reaction was transformed into 50µL Stbl2 competent E. coli (Invitrogen) by being heat shocked at 42ºC for 45 seconds. After heat shock, 250µL of SOC media was added to each reaction and cells were incubated in a 37ºC, 225 rpm shaker for 1 hour. Cells were plated on LB/Agar/Carbenicillin plates and incubated overnight at 37ºC. Colonies from each plate were picked and grown up in LB/Carbenicillin cultures, and plasmid DNA was isolated with a QIAprep Spin Miniprep Kit (Qiagen) to the manufacturer specifications and eluted in 30µL 10mM Tris-HCl pH8.0.

Multi–gRNA Constructs – To incorporate all 4 plasmids into 1 vector, gRNAs were cloned using a protocol modified from the Golden Gate assembly outlined in Kabadi et. al.[11]. For this

multiplexed system, a pre-designed vector containing dCas9-KRAB was not available. Therefore, the hU6 promoter of pLV hU6-sgRNA hUbC-dCas9-KRAB-T2a-Puro was removed such that BsmBI restriction sites could be added to make the vector suitable for Golden Gate cloning. To remove the U6 region, the plasmid was digested with of PacI (NEB) and rSAP using the Fast-Digest protocol previously outlined. The digested plasmid was gel purified from a 2% agarose gel with a Molecular Biology GeneJET Gel Extraction Kit (ThermoFisher) using manufacturer specifications and eluted in 30µL 10mM Tris-HCl pH8.0. The parent vector also needed BsmBI sites for the Golden Gate cloning scheme. Oligonucleotides containing BsmBI cut sites flanked with PacI cut sites were designed in the IDT analyzer tool (S5'-GATAAGAGACGTCTAGACGT CTCCACAAAT; AS5'-TTGTGGAGACGTCTAGACGTCTCTTATCAT). These annealed oligonucleotides were ligated into pLV hUbC-dCas9-KRAB-T2a-Puro with the same T4 ligation protocol previously outlined.

Guide RNA S and AS strands were designed with BbsI (ThermoFisher) endonuclease sites. Combined gRNA oligonucleotides were made using the same T4 PNK protocol previously outlined. Each gRNA for MSRB1 and CD2AP was cloned into 1 of 4 plasmid vectors: ph7SK-gRNA, pmU6-gRNA, phH1-gRNA, and phU6-gRNA. Each plasmid was digested with BbsI and rSAP using the Fast Digest protocol previously outlined. All digests were gel purified using a Qiaquick Gel Purification Kit (Qiagen) using manufacturer specifications and eluted in 30µL 10mM Tris-HCl pH8.0. Guide RNA oligonucleotides were diluted and used in the ligation reaction previously outlined. Each ligation reaction was transformed into 50µL Top10 competent E. coli (Invitrogen) with the same heat shock and 37ºC incubation protocol. Cells were plated on LB/Agar/Kanamycin plates and incubated overnight at 37ºC. Colonies from each plate were

picked and grown up in LB/Kanamycin cultures, and DNA was isolated with a QIAprep Spin Miniprep Kit.

Each of the gRNA plasmids were digested with BsmBI and run on a 1% agarose. The excised gRNA-containing segments were combined and gel purified into a one-pot mix of all excised segments using a Qiaquick Gel Purification Kit. A 2:1 ratio of one-pot gRNAs : pLV hUbC-dCas9-KRAB-T2a-Puro was used in the Golden Gate ligation protocol outlined in Kabadi et. al.[8]. Control gRNA vectors were made by adding an RNAPolIII polyA signal prior to the to the CRISPR gRNA sequence to prematurely terminate gRNA transcription while still expressing dCas9-KRAB within these lines.

Each ligation reaction was transformed into 50µL SURE competent E. coli (Agilent), heat-shocked, and incubated with the same procedure previously outlined. The cells were plated and grown up with LB/Carbenicillin, and plasmid DNA was isolated with the same protocol as previously outlined.

Lentivirus generation and transduction

293T cells were plated at 500,000 cells/well in a 6-well plate in RPMI (Gibco) supplemented with tetracycline-free (tet-free) FBS (Omega Scientific) and Pen/Strep (100U/mL:100ug/mL)(Gibco). 293T cells were transfected with the following to generate lentiviral particles: 0.5µg lentiviral plasmid vector, 4.6µL Mission Lentiviral Packaging Mix (Sigma-Aldrich), 2.7µL Fugene HD (Promega), and 30.3µL Opti-MEM (ThermoFisher). 48 hours after transfection, supernatant from each well was collected and filtered with a 0.45µm filter (Millipore) and 0.5mL aliquots of lentivirus were stored at -80ºC.

2D10 and E4 cells were plated at 30,000 cells/well in a 96-well plate in 50µL of tet-free RPMI. Cells were transduced with 50µL vector in the presence of polybrene at a final

concentration of 6µg/mL. The media was changed after 24 hours and replaced with tet-free RPMI supplemented with 500 ng/mL puromycin. The cells were maintained under puromycin to maintain dCas9-KRAB/gRNA lentiviral expression and cells were expanded for experiments.

RNA Isolation and quantitative Real-Time PCR

RNA was isolated from 2D10 cells using an RNeasy Mini Kit (Qiagen), including an on-column DNase digestion in the RNA isolation, using the manufacturer specifications. RNA was reverse-transcribed into cDNA using a Maxima cDNA synthesis kit. Gene expression was assayed by qRT-PCR using FastStart Universal SYBR Green Master (Roche) on the QuantStudio 5 (Applied Biosystems) with the primer sets in Table 2.

Table 2 – *RT-qPCR Primer sets* — Forward and reverse primers shown

Real Time qPCR-Primer Sets		
	Forward Primer	Reverse Primer
TBP	**GAGAGTTCTGGGATTGTACCG**	**ATCCTCATGATTACCGCAGC**
GFP	**TCAAGATCCGCCACAACATC**	**GTGCTCAGGTAGTGGTTGTC**
β-Actin	**AGGTCATCACCATTGGCAATGA**	**TCTTTGCGGATGTCCACGTCA**

10-fold serial dilutions of 2D10 cDNA was used to determine primer efficiency and relative expression of GFP to control genes TATA-binding protein (TBP) and β-actin using the Pfaffl method[13]. Two biological replicates of each cell population using a different targeting gRNA was used, and each biological replicate was plated in triplicate.

Latency Reversal and Flow Cytometry

Cells were plated in 96-well plates at 25,000 cells/well and treated with 250nM SAHA or 10ng/mL TNFα for 24 hours. Cells were stained with LIVE/DEAD Fixable Aqua Dead Cell Stain (ThermoFisher) for 30 minutes, followed by a DPBS wash and fixation in 1.5% paraformaldehyde/

DPBS. Cells were assayed by flow cytometry using the iQue Screener Plus (Intellicyt) and GFP expression with dead-cell exclusion was calculated using the ForeCyt analysis software (Figure 2)(Intellicyt).

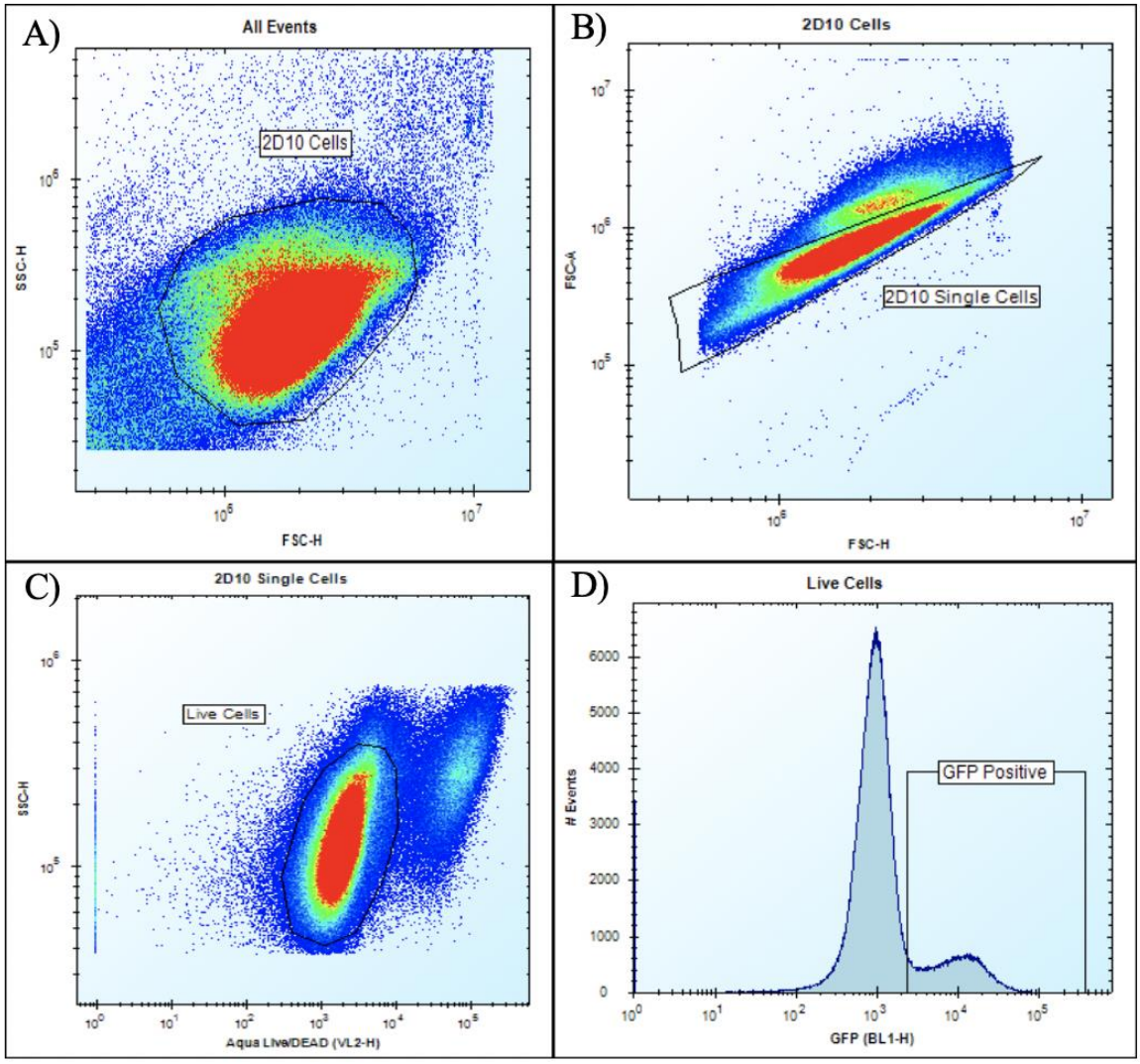

Figure 2 - *Flow cytometry gating scheme*– 2D10 cells were gated (A), doublets excluded (B), and dead cells excluded via live/dead stain (C) prior to gating for GFP expression (D). E4 cells were gated in the same manner.

Results

In the course of this project, gRNAs targeted to the host genes of HIV integration in 2D10 and E4 cells were cloned into lentiviral vectors co-expressing dCas9-KRAB—targeting the host gene for transcriptional repression. Both cell lines are Jurkat-derived models of HIV latency which contain an integrated proviral reporter which expresses GFP upon latency reactivation[9]. Each gene of integration was selected as a control gene because it was the integration site in another Jurkat model. It was hypothesized that altering host gene expression would alter the reactivation profiles in these models if transcriptional interference plays a role in maintenance of HIV latency. We first used an all-in-one CRISPRi system which expressed a single gRNA and dCas9-KRAB within the same construct and assed if this could effectively reduce target gene expression. We generated lentiviral vectors, transduced 2D10 and E4 cells, and puromycin selected to generate stable cell lines. After stable populations were established, we assayed MSRB1 and CD2AP gene expression via reverse-transcription/quantitative real-time PCR (RT-qPCR) to determine the level of CRISPRi-induced knockdown (Figure 3).

In the real-time PCR analysis of the single-gRNA system used in 2D10 cells, mRNA expression was reported relative to β-Actin and TBP reference genes. These values were then standardized to a 2D10 control that did not contain dCas9-KRAB. As shown in Figure 3, mRNA expression was reduced in genes that were targeted, and no significant change was seen in genes that were not targeted. We observed a 50% reduction of MSRB1 expression in the MSRB1-1 gRNA transduced cells, but saw a more sizable reduction, a 95% decrease, in cells harboring the MSRB1-2 gRNA. The reduction of CD2AP mRNA expression by both CD2AP-1 gRNA and CD2AP-2 gRNAs were comparable, demonstrating an approximate 65-75% reduction (Figure 3).

Encouraged by our RNA results demonstrating significant knockdown of targeted gene transcription, our established CRISPRi cell lines were then treated with two characterized latency reversing agents (LRAs) to examine the impact of targeting host gene expression on latency reversal. We treated our dCas9/KRAB/gRNA cell lines with various previously characterized LRAs including the HDAC inhibitor SAHA and TNFα, a cytokine which induces the NF-κB pathway. We then analyzed the level of latency reactivation to these LRAs via flow cytometry for GFP expression and excluded dead or dying cells via staining with a live/dead stain. Cell populations containing dCas9-KRAB single gRNAs targeted to either MSRB1 or CD2AP showed no significant difference in reactivation profiles when compared to normal 2D10 cells when reactivated with the HDAC inhibitor SAHA. When the same populations were reactivated with TNFα, cells with dCas9-KRAB+gRNA targeted to both MSRB1 and CD2AP showed a 9% increase in GFP expression when compared to the control 2D10 cells harboring no dCas9/gRNA expression cassette (Figure 4). However, there was no significant difference found when comparing GFP expression between MSRB1 or CD2AP gRNA populations, and as 2D10 cells do not harbor integrated virus in the CD2AP gene, this suggested this effect may be the result of puromycin selection of these cell populations and not specific to latency reversal.

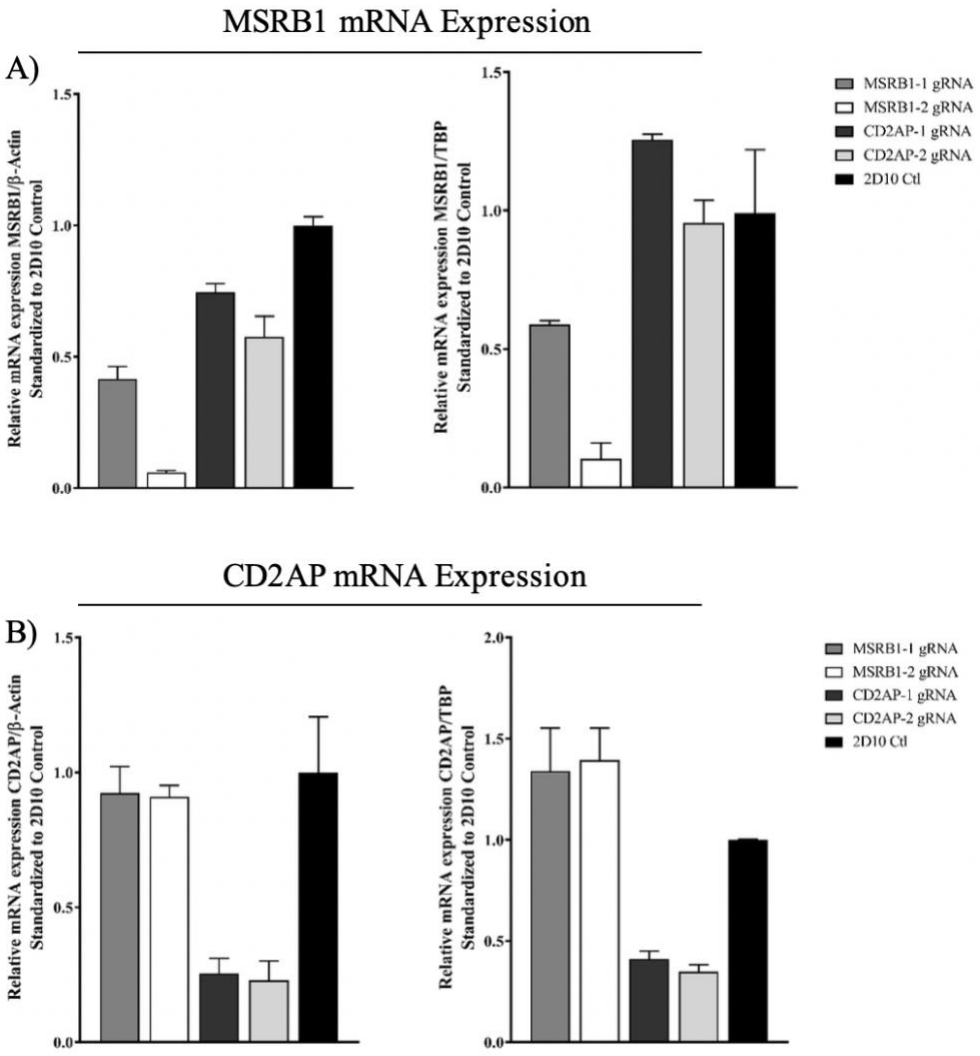

Figure 3– *Analysis of MSRB1 and CD2AP Gene Expression in 2D10 cells with Single gRNAs* – mRNA expression of MSRB1 and CD2AP was performed by RT-qPCR. (A) MSRB1 gene expression was reduced over 90% by MSRB1-2 gRNA, whereas the MSRB1-1 gRNA was less effective. (B) Both CD2AP-1 and -2 gRNAs show an similar reduction in CD2AP gene expression. Statistical analysis (T-Test with Dunn's Multiple Comparison Correction) confirmed no significant difference in mRNA expression of non-targeted genes of normal and transduced 2D10 cells. Error bars represent biological replicates.

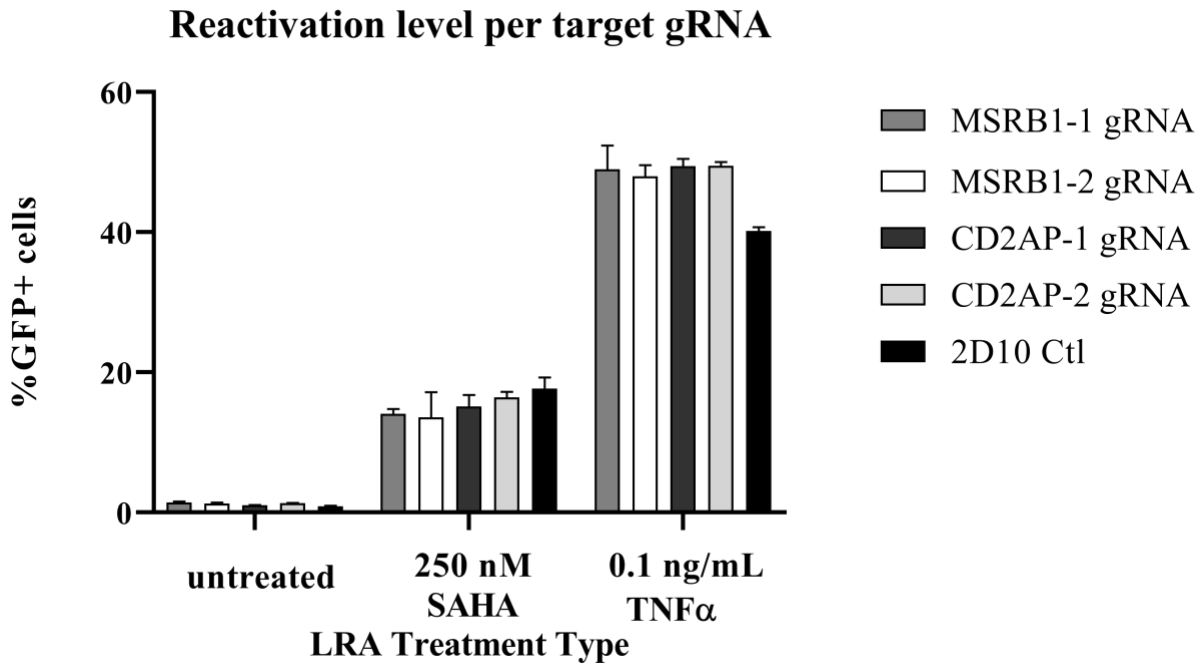

Figure 4 – *GFP Induction in Single-gRNA Constructs Upon Treatment with LRAs* – 2D10 cell lines containing one gRNA with dCas9/KRAB were treated with 250nM SAHA or 10ng/mL TNFα and assayed for GFP expression via flow cytometry. Statistical analysis was performed comparing the different populations and no significant difference was detected in GFP expression between populations. Error bars represent biological replicates.

While our single gRNA system achieved knockdown of host gene transcription, we looked to improve the level of targeting in an attempt to completely ablate gene transcription. To this end, we next used a more advanced CRISRPi system which expressed 4 independent gRNAs which could be used to increase overall targeting of the host gene[11]. Here we created multi-gRNA lentiviral vectors which were used to generate both 2D10 and E4 stable cell lines. In the analysis of the multi-gRNA system used in 2D10 and E4 cells, mRNA expression was reported relative to TBP expression, and these values were standardized to 2D10 and E4 populations containing dCas9-KRAB and terminating gRNA sequences (term 4gRNA). Those populations acted as more appropriate controls for the multi-gRNA-transduced cells because they contained vectors with dCas9/KRAB but could not target dCas9 to host genes due to lack of gRNA expression. 2D10

lines were generated first and thus expression of mRNA was assayed at multiple time points. E4 lines were developed later and as such are still in development with limited data. As shown in Figure 5, MSRB1 mRNA expression in both 2D10 and E4 cells with four gRNAs experienced approximately 66% reduction initially, which reduced to 50% reduction at a second time point. Conversely, CD2AP expression was reduced by 75% with high variation in biological replicates, which improved to approximately 83% with low variation at a second time point. Using T-tests with multiple comparisons, it was determined that there was no significant change in GFP expression in these lines upon host transcription knockdown. While there appears to be an increase in GFP mRNA expression in the E4 lines, it is not significant when compared to the MSRB1 gRNA containing E4 cells, suggesting an effect of gRNA/CRISPRi targeting (Figure 5). Further, we have observed that E4 reporter lines show increased GFP expression during initial puromycin selection which returns to basal levels after an extended amount of time under selection. Considering both of those points, it is likely that E4 GFP expression shown is a result of puromycin selection as opposed to gRNA targeting, and that GFP expression in E4 cells will decrease over time to a level similar to that shown in other figures.

Latency reversal in response to the LRAs SAHA and TNFα in reporters with multiple gRNAs proved to be similar to populations with single-gRNA constructs (Figure 6). E4 cells at the 25 day timepoint show what appears to be significant differences in latency reactivation compared to control populations, but when comparing treated versus untreated populations in that figure, one can see that populations are amplified at proportional levels. Between-population T-tests also showed no significant difference.

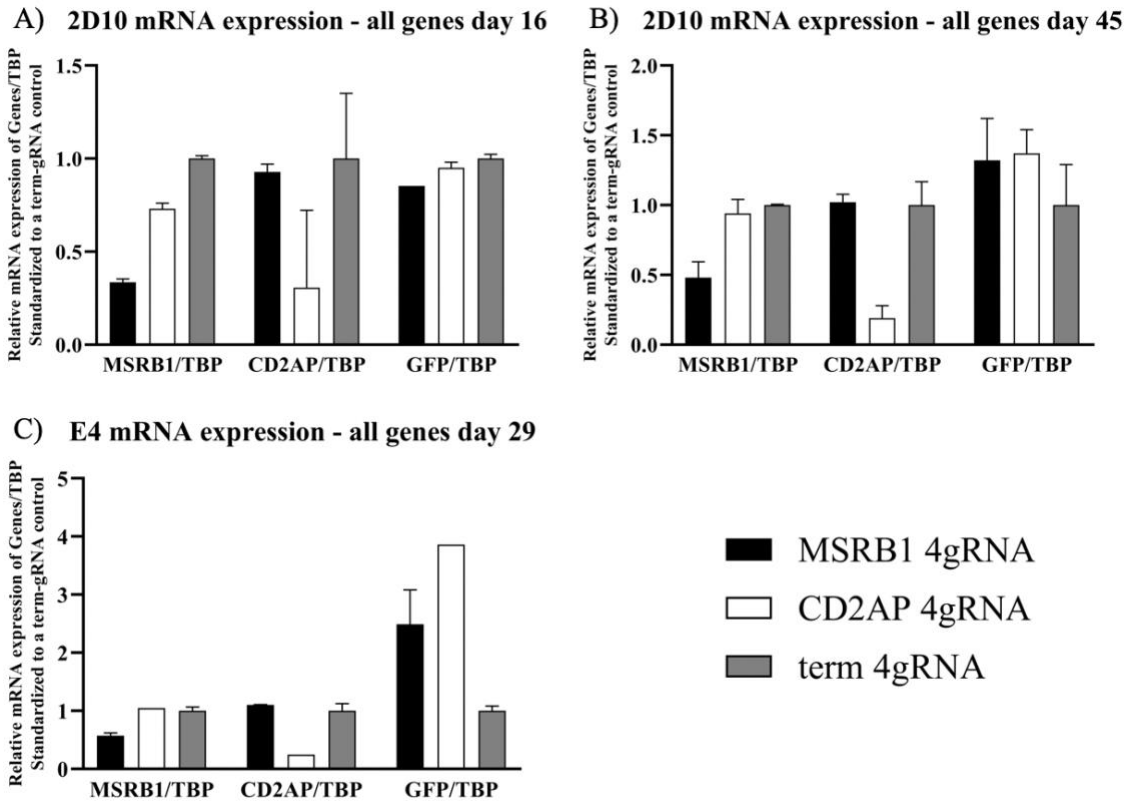

Figure 5 – *Analysis of MSRB1 and CD2AP Gene Expression in cells with multi-gRNA targeting* – mRNA expression of MSRB1 and CD2AP was performed by RT-qPCR. (A) MSRB1 gene expression was reduced by 66% at 16 days and 50% at 45 days in 2D10 cells. (B) CD2AP expression decreased with less variance in biological replicates from day 16 to 45. (C) One E4 biological replicate with a CD2AP 4gRNA construct was showing unusual expression levels compared to other replicates that was not representative of typical reduction levels observed in these experiments and was excluded from analysis. Statistical analysis confirmed no significant difference in mRNA expression of non-targeted genes compared to cell populations that did not express gRNAs. Error bars represent biological replicates.

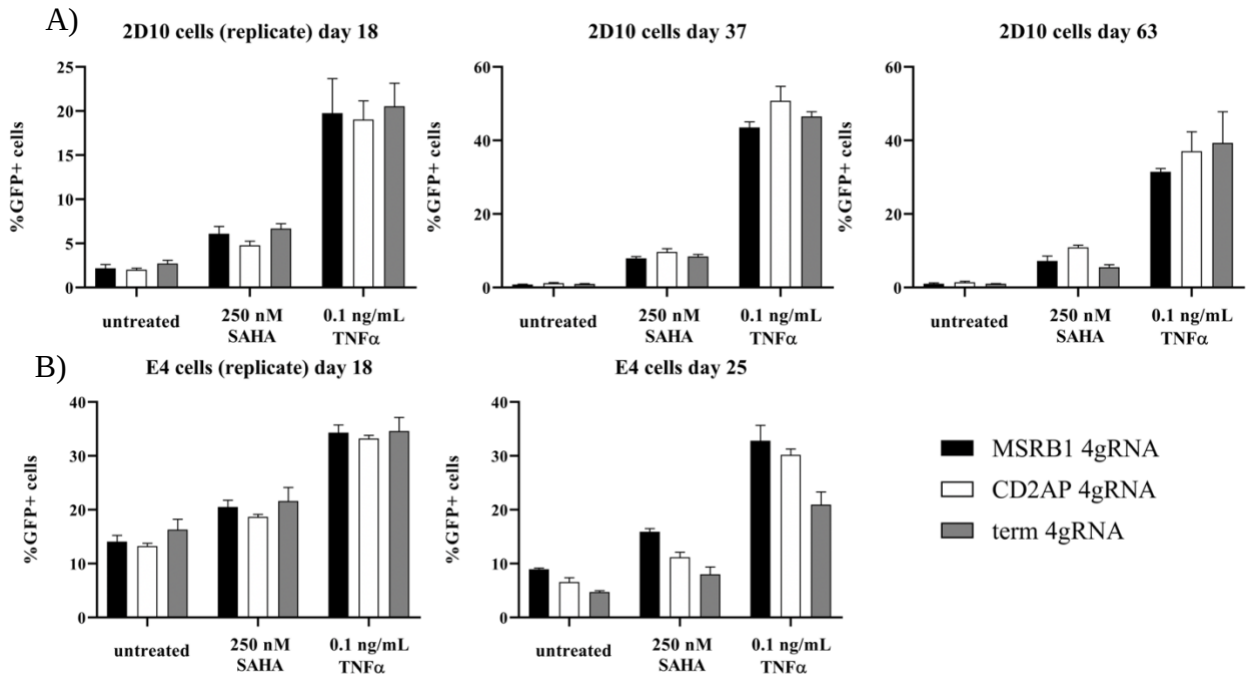

Figure 6 – *GFP Induction in Multi-gRNA Constructs Upon Treatment with LRAs* – (A) 2D10 and (B) E4 cells containing multi-gRNA constructs were treated with 250nM SAHA or 0.1 ng/mL TNFα. More-recent replicates of 2D10 and E4 cells with multi-gRNA constructs (2D10 day 18, E4 day 18) were created and assayed as well. Statistical analysis was performed comparing the different populations and no significant difference was detected in GFP expression between populations. Error bars represent biological replicates.

Discussion

The results demonstrate reducing the transcriptional activity of MSRB1 in 2D10 cells or CD2AP in E4 cells did not alter the level of latency reversal in either HIV latency model for both single- and multi-gRNA targeting. Real-time PCR data for single-gRNA targeting shows that the MSRB1-2 gRNA targeted to MSRB1 was very effective at reducing mRNA expression compared to moderate reduction by MSRB1-1 but increased targeting by the 4gRNA construct did not improve transcriptional knockdown. Inversely, the CD2AP 4gRNA construct was much more effective at reducing transcription compared single gRNAs. However, comparison of flow cytometry analysis between those populations showed no difference in GFP expression to suggest

an effect on maintenance of HIV latency. This may indicate that the transcriptional interference may play a significantly lesser role in the maintenance of HIV latency.

While we identified a highly effective MSRB1-targeted gRNA (MSRB1-2 gRNA) which initially reduced MSRB1 mRNA expression by approximately 95%, transcription was not completely ablated. To date, there is no literature on the level of transcription required to result in transcriptional interference of the HIV provirus. The use of a multiplex gRNA vector was employed in an attempt to entirely ablate transcription, as it was hypothesized that multiple targets of promoter regions would be more effective than a single target. However, while the CD2AP multiplex construct further decreased transcription of CD2AP, the MSRB1 construct decreased transcription at levels comparable to or worse than its single-gRNA counterpart for MSRB1. Additionally, neither construct completely ablated transcription for either gene. The utility of these genes in cell function was not assessed prior to the outset of this project. It is possible that these gene products are necessary for cell function, so there may be cellular mechanisms that are preventing complete ablation of transcription. The possibility remains that an impact on latency reversal will only be seen when transcription is entirely prevented—not merely reduced. Further work should clarify whether these genes are necessary for cell function and seek out other HIV latency models with known integration sites where such genes may not be vital for cell function.

Future work with our current models will involve the use of chromatin immunoprecipitation assays to confirm H3K9 methylation presence in targeted promoter regions, and to examine H3K9 methylation levels as a result of multiplex gRNA targeted relative to single gRNA targeting. Current populations will be kept under puromycin selection and assayed to evaluate whether mRNA expression or GFP induction changes over time. However, if future efforts manage to completely prevent transcription in these models or others and there is still no

change in the latency reversal response, then transcriptional interference mechanisms may indeed be insignificant in the maintenance of HIV latency. As the role of transcriptional interference in HIV latency has never been truly examined in this context, this may lead a change in the current thinking regarding the molecular mechanisms which govern HIV latency.

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