# Broadly active zinc finger protein-guided transcriptional activation of HIV

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**Research**

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## Abstract

# Background

Human Immunodeficiency Virus type 1 (HIV-1) is a lentivirus that causes a persistent viral infection and results in the demise of immune regulatory cells. Clearance of HIV-1 infection by the immune system is inefficient, and integration of proviral DNA into the genome of host cells provides a means for evasion and long-term persistence. A therapeutic compound that specifically targets and sustainably activates a latent HIV provirus could be transformative and is an overarching goal for the “shock-and-kill” approach to a functional cure for HIV.

# Results

Substantial progress has been made towards the development of recombinant proteins that can target specific genomic loci for gene activation, repression or inactivation by directed mutations. However, most of these modalities are too large, or too complex, for efficient therapeutic application. We describe here the development and testing of a novel recombinant zinc finger protein transactivator, ZFPb-362-VPR, which specifically and potently enhances proviral HIV transcription both in established latency models and across different viral clades. Additionally, ZFP-362-VPR activated HIV reporter gene expression in a well-established primary human $CD4^+$ T-cell latency model and was specific in targeting the HIV LTR as determined from off-target transcriptome analyses.

# Conclusions:

This study provides clear proof of concept for the application of a novel, and therapeutically relevant, protein transactivator to purge cellular reservoirs of HIV-1.


## Introduction

Human Immunodeficiency Virus type 1 (HIV-1) establishes a latent long-term infection that remains hidden from the immune system. Clearance of latent HIV-1 DNA from infected host cells was suggested to take over 73 years [1]. Activation and purging of provirus from macrophages, monocytes, and T-cells, with ~ 1 infected cell per million cells, has been an ongoing experimental struggle, the goal being to find latency reversing agents (LRAs) that can be used along with antiretroviral therapy (ART). This “shock-and-kill” approach aims to eliminate the reservoir by inducing HIV-1 activation resulting in death of the infected cell [2]. Several LRAs are known to activate latent provirus in patients [3], but LRAs are broad effectors of cellular processes and, therefore, inherent off-target effects are expected. The ideal LRA should also activate genetically distinct clades of HIV-1. A method that could specifically activate latent virus in quiescent $CD4^+$ T-cells [4] and in macrophages in those patients on ART, and across multiple

clades of virus, could prove useful to purge cellular viral reservoirs, expose infected cells to T-cell killing, and potentially lead to a functional cure.

We and others have reported the potent and broad activation of latent HIV-1 using defective CRISPR fused to transcriptional activating domains [5–7]. Using this system, the most effective locus in the HIV-1 LTR for modulating transcription was shown to be the LTR-362 site [5–7], as a single guide RNA directed to this site (sgF2-362) with a defective CRISPR activator (dCas9-VPR), potently activates latent HIV [7]. Notably, the LTR-362 site contains a NF-kB sequence doublet that is unique to HIV-1 and not found in the human genome [8]. This site is susceptible to non-coding RNA directed transcriptional control [9, 10], and represents an HIV-specific site for targeted control of proviral transcriptional activation. While dCas9-VPR can be targeted to the LTR-362 site and potently activate HIV at levels greater than available LRAs, including suberoylanilide hydroxamic acid (SAHA), tumor necrosis factor-α (TNFα), or prostratin [7], there are limitations, such as the requirement of a guide RNA administered with the dCas9-VPR protein. An alternative approach requiring a single smaller protein delivered systemically could prove useful in the activation of latent HIV. One such approach is outlined here and encompasses generating Zinc Finger Protein (ZFP) conjugates targeted to the promoter of HIV. ZFPs are small, versatile and modular, and can target virtually any gene of interest. Unlike CRISPR/Cas9, ZFPs require only a single component, which is much smaller compared with Cas9 (~ 0.6 kb versus ~ 4 kb), making them more compatible with delivery vectors that have size limitations.

Over the last decade much progress has been made towards the development of a bona fide therapeutic protein based on Zinc Finger (ZF) technology. ZF nucleases (ZFN) have been developed to induce indels at specific genomic loci, such as CCR5 [11], to generate HIV-resistant $CD34^{+}$ hematopoietic stem cells (HSCs) in bone-marrow transplants, and is currently being developed as a therapeutic strategy [12]. Importantly, ZFPs can be bioinformatically identified and tailored to target distinct sites using well-established algorithms [13].

ZFPs can be fused to other proteins, such as repressive epigenetic regulatory proteins that direct gene silencing at the targeted locus, to provide locus-specific functionality [14–16]. ZFNs have been used to targeted HIV's LTR [17], activate the LTR and latent virus transcription [18, 19], excise provirus [20], and repress HIV transcription [21]. However, no ZFP has been developed to target and activate all clades of HIV and latent virus. We describe here a new ZFP that can potently activate all clades of HIV with minimal off-targeting. This new ZFP may prove useful in "shock-and-kill" strategies to activate latent virus in the presence of ART or facilitating HIV targeted chimeric antigen T cell killing of those cells infected with virus.

## Results

# ZFP-362-VPR specifically binds and transcriptionally activates the LTR of HIV

Recently, we and others demonstrated that a defective CRISPR conjugated to the VP64 or VPR activation domains (dCas-VP64 and dCas-VPR, respectively) was capable of activating latent HIV-1 provirus [5–7, 22–24]. This work demonstrated that these constructs are the most potent activators found to date for HIV, exhibiting far greater activation of HIV than any LRAs currently employed to activate provirus, and that one locus appeared to be the most susceptible target site for CRISPR-directed modulation of HIV transcription. This locus, the LTR-362 site [5–7], contains an NF-kB doublet that is unique to HIV and not found elsewhere in the human genome [8], and is one of the most effective sites for small antisense RNA directed transcriptional modulation of HIV-1 [9, 10]. Because the LTR-362 site is the "Achilles heel" of HIV-1 transcription, we used ZF Tools Ver 3.0 to generate and screen three unique ZFP conjugates targeted to this site (Fig. 1A). The new ZFPs is fused to a Tat domain used previously in a ZFP targeting *UBE3A* as a potential therapy for Angelman Syndrome [14] and the VPR domain used previously with defective CRISPR to HIV[7]. One of these three ZFPs, ZFP-362b (referred to as ZFP-362), was found to potently activate HIV expression, at levels comparable to dCas-VP64 + sgF2-362 (Figs. 1B-C). Notably, this targeted activation was lost in pMoΔ362 cells, which lack the ZFP-362 target site (Fig. 1D). Next, we tested the breadth of ZFP-362 transcriptional activation. The ability of ZFP-362 to activate Clades A-G of HIV-1 was assessed in co-transfected HEK293 cells (Fig. 1E). ZFP-362 demonstrated the most potent activation of subtypes A, B, D and F, and less robust activation in subtypes C, E and G. A noteworthy observation is that the target site is conserved in subtypes A, B, D and F, while subtypes E and G contain point mutations and deletions in the LTR-362 site (**Figure S1A**). Subtype C contains a triple NF-kB site and the ZFP-362 binding locus aligns better between the second and third NF-kB motifs with a single mismatch deletion (**Figure S1B**). Overall, these data support the notion that ZFP-362-VPR is specifically active on the HIV LTR for a wide range of HIV subtypes.

To more clearly determine the targeting of ZFP-362-VPR, a chromatin immunoprecipitation assay (ChIP) [25] was performed on both LTR targeted dCas9-VPR + sgF2-362 and ZFP-362-VPR treated pMo-HEK cells, a HEK293 cell line that has been transduced with lentivirus that has an LTR-driven GFP. Both the dCas9-VPR and ZFP-362-VPR were found to localize specifically to the LTR (Fig. 1F) and ZFP-362 enrichment was lost when targeting a pMo vector lacking the 362 site (**Figure S2)**. To verify the presence of the ZFP-362-VPR, protein expression was confirmed by western blot (Fig. 3S). Collectively, these data suggest that the ZFP-362-VPR fusion construct can target HIV-1 at the LTR-362 site and potently activate viral transcription.

We have previously demonstrated that a dCas9-VPR and dCas9 + sgF2-362 can activate a variety of latent HIV models, whereas variable activation was observed with other LRAs [7]. We similarly tested the ZFP-362-VPR vector on well-established reporter cell lines for HIV latency and observed activation of J-Lat cell lines "6.3", "10.6" and "15.4", with similar potency to that of the dCas9 + sgF2-362 **(**Fig. 2**)**. Furthermore, the ZFP-362 reliably activated the LTR, with fold changes comparable to the most commonly used LRAs (Fig. 3). These data demonstrate that ZFP-362-VPR can consistently and potently activate HIV in different models of HIV latency, and that this activation is independent of activating NF-κB signaling pathways.

# ZFP-VPR-362 activates HIV in a primary CD4$^+$ T cell model of latency

It was determined if the ZFP-362-VPR can activate HIV in a primary HIV latency model. CD4$^+$ T-cells were either uninfected cells or HIV latently infected *in vitro* infected 17 days prior with a pNL4.3-Δenv-nluc replication incompetent vector expressing nano-luciferase pseudotyped with VSVG, and the latent population was separated from productively infected cells. The ZFP-362-VPR vector was transfected into the latently infected CD4$^+$ and 72 hours later, the level of luciferase activity was measured as a read-out of LTR activation. There was a significant increase in luciferase levels with the ZFP-362-VPR compared to a GFP or ZFP control (Fig. 4). Likewise, known activators of the LTR, PMA or anti-CD3/CD28 stimulation, resulted in high levels of LTR activation as measured by luciferase activity. Although high-levels of activity were observed in one donor, the levels of activity with the ZFP-362-VPR in two other donors was less pronounced (**Figure S4**) and may reflect differences in transfection efficiency. Nonetheless, these data demonstrate that the ZFP-362-VPR can activate the HIV LTR in primary CD4$^+$ T-cells.

# Assessment of ZFP-VPR-362 off-target effects

A major concern of using LRAs is the broad off-target cellular effects that can result in cellular toxicityor oncogenic mutations. As the ZFP-362 targets the same site as the sgF2-362, we compared the effects of ZFP-362-VPR on endogenous RNA expression. The ZFP-362-VPR, dCas9 + sgF2-362 or pcDNA3.1 was transfected into pMo cells and gene expression was assessed by bulk RNA sequencing analysis. Principal component analysis showed tight clustering of the sample groups, indicating experimental treatment is the major source of variation (**Figure S5A**). Expression plots show that ZFP-362-VPR and dCas9 + sgF2-362 potently activate the on-target GFP reporter, and CRISPR components were only detected in dCas9-VPR + sgF2-362 cells (**Figure S5B-C**). There were 191 differentially expressed genes observed in the dCas9-VPR-sgF2-362 (**Table 1**) and 219 observed in the ZFP-362-VPR treated samples (**Table 2**) compared to the control pcDNA3.1 vector ($|\log2FC| \geq 2$, FDR = 0.05). Importantly, the ZFN-B-VPR had a comparable gene expression profile to the dCas9-VPR + sgF2-362 (Fig. 5A**)**: 358 differentially expressed genes were common to both treatments, 12 genes unique to the dCas9-VPR + sgF2-362-treated samples, and 40 genes unique to the ZFP-362-VPR-treated samples (Fig. 5B, **Tables 3 & 4**). We looked for enriched GO terms and KEGG pathways associated with differentially expressed genes in ZFP-362-VPR treated samples with respect to controls and enrichment of terms associated with neurotransmitter transport and pathways specific for cardiomyocytes was observed (Fig. 5C). Similarly, enrichment for these GO terms and KEGG pathways was observed for dCas9-VPR + sgF2-362-treated samples, suggesting similar target profiles (Fig. 5D). Importantly, none of the differentially expressed genes or pathways is a known oncogene or is associated with cell proliferation.

# Discussion

ZFPs are versatile and modular. Several ZFPs have been developed to target and repress HIV, including one designed against the SP1 site in the LTR [26] while others have developed ZFPs targeted to three regions in the LTR and found that they were potent stable repressors of viral expression [27]. However, none of these previous studies developed a ZFP to the highly conserved NF-κB site in the LTR. Moreover, these first generation ZFPs, while functional, lacked several modifications that enhance nuclear function and trafficking. One recent and exciting example utilized a ZFP conjugated to a KRAB silencing domain and a Tat domain for targeting the brain and directed to the promoter of an antisense long non-coding RNA (lncRNA) involved in regulating genes active in Angelman Syndrome [14]. In that study it was conclusively shown that brain-directed targeting of a ZFP is feasible *in vivo* and contingent on a Tat localization peptide conjugated with the ZFP [14]. We capitalized on these recent developments and developed ZFP-362-VPR to contain the same Tat domain used previously in the ZFP targeting in Angelman Syndrome [14]. The use of this Tat domain may allow for the ZFP-362 to function as a soluble protein as well as to enhance nuclear targeting and retention of the ZFP-362 activator, though such a notion was not experimentally assessed in this study.

While ZFPs, transcription activator-like effector nucleases (TALEN), and several CRISPR and defective CRISPR enzyme systems containing various conjugates have been shown to target HIV for applications ranging from silencing [28], to excision [24, 29–33], to activation [5, 7], the problem of achieving target specificity while maintaining activity across the various HIV-1 clades has remained enigmatic. We report here a ZFP activator that specifically activates HIV transcription by targeting the LTR-362 site. The ZFP activator affects a limited subset of genes that appear transcriptionally activated in an off-target manner. Notably, ZFP-362 is capable of broadly activating all clades of HIV to varying levels of expression. To date, LRAs that function specifically and broadly activate viral transcription across all clades have not been reported.

The ZFP-362-VPR has a very similar pattern of differential gene expression compared to dCas9-VPR + sgF2-362 suggesting a comparable off-target profile for these vectors, and previously found that differentially expressed genes with the dCas9-VPR + sgF2-362 were significantly lower in number and magnitude compared with TNF-α [7] suggesting these vectors have a better on-target profile than LRAs with broad-acting cellular pathways. Nevertheless, ZFP-362-VPR affected genes enriched in pathways relevant to cardiomyocyte signaling and contraction (Fig. 5). These data suggest that even though the NF-κB doublet is not present in the human genome, the dCas9-VPR + sgF2-362 and ZFP-362-VPR may interact with some NF-κB binding sites leading to off-target effects. Clearly not all NF-κB sites are bound and activated as known NF-κB pathways, such as cytokine signaling or proliferation [34] were not identified by enrichment analysis, and 362 site-targeting in our previous work showed no effect on cell cycle progression and viability [7]. However, we cannot rule out non-specific ZF binding and activation; further work is required to determine the range of motifs bound by ZFP-362-VPR, enabling us to better identify the potential physiological effects of ZFP-362-VPR-mediated dysregulation.

Dysregulation of NF-κB activation can result in cardiac remodeling, hypertrophy, and contribute to heart failure [35], and it remains to be determined if ZFP-362-VPR is able activate downstream NF-κB target

sites involved in these processes. Although we did not assess ZFP-362-VPR *in vivo*, our data suggest that restricting delivery of ZFP-VPR-362 to cardiac tissue may be required, or that this approach should be used transiently to activate latent virus for a defined therapeutic window. Using delivery vectors that do not accumulate in cardiac tissue could be helpful in avoiding off-target effects. Alternatively, inclusion of tissue-specific miRNA target sites in the transgene has been used to prevent undesirable expression in specific tissues [36], and incorporating cardiac-specific miRNA target sites into ZFP-362-VPR may restrict expression in this organ [37].

ZFPs are endogenous regulators of gene transcription that can regulate transposable elements and viral elements [38], including human ZNF10, which inhibits HIV LTR function [39]. Interestingly, endogenous ZNF10 was found to block SP1/NF-kB interaction in the HIV LTR and inhibit viral transcription. It is notable that the cell has evolved a ZFP regulator to this same region that ZFP-362 is directed against, suggesting that over time perhaps HIV will be silenced by the action of endogenous KRAB containing ZFPs [40].

The ZFP-362 activator of HIV described here may prove useful in the development of a functional cure for HIV infection. For instance, the ZFP-362 activator could be used in a “shock-and-kill” strategy to activate latent virus while maintaining ART, leading to the death of the latently infected cells [2]. Alternatively, ZFP-362 could be used to induce sustained expression of HIV to allow for or enhance chimeric antigen T cell (CAR) targeting of infected cells. Notably, the lack of HIV antigen has proven problematic for developing robust anti-HIV CARs [41, 42]. However, for either of these approaches to prove viable, the issue of delivery remains to be solved. Indeed, while ZFPs, TALENs and CRISPR based gene therapies are all proving exceptionally valuable in modulating HIV expression and may in the next decade emerge as a bona fide means of eliminating HIV infection, a means of delivery to infected target cells remains enigmatic (reviewed in [43, 44]). ZFPs or TALENs could be delivered using lipid nanoparticles [45] or cellular exosomes [46]. Such an approach would be amendable to CAR T cell and “shock-and-kill” based strategies, however, more work in developing these systems and determining the ability to deliver ZFP-362 remains to be carried out as do many of the technical challenges inherent in cell delivery.

# Conclusion

This work describes a novel, potent, and target-specific activator for HIV for use in “shock-and-kill” strategies and represents a simple, compact modality that could be used in conjunction with ART, or targeted immune therapies, to eliminate latent HIV from infected patients.

# Materials And Methods

**Vectors**: The H1sgRNA (Control), dCas9-VPR and sgf2-362 have been previously described [7]. The amino acids sequence of ZFP-362 and ZFP-VEGF that bind their respective target sites were identified using the ZF Tools Ver 3.0 [47] and fused to VPR activation domain and cloned into pcDNA3.1 mammalian expression vector by Genscript. The ZFP-362 was ordered as a gBLOCK (IDT, IA, USA) and was clone into

a dCas9-VPR vector (addgene #63798) to replace the dCas9 in this vector using NEBuilder® HiFi DNA assembly Master mix (NEB, MA, USA). The LTR-driven luciferase from different subtypes were obtained from the NIH AIDS Reagent Program (Cat. No. 4787, 4788, 4789, 4790, 4791, 4792, 4793, NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pBlue3'LTR-luc-A from Dr. Reink Jeeninga and Dr. Ben Berkhout [48, 49].

**Cell Lines**: pMo-HEK and pΔ362 lentiviral vectors were generated as described in Shrivastava *et al.* [50]. HEK293T cells (ATCC) were transduced at a MOI of 0.1 to ensure a single integrated copy. 7 days post transduction, GFP positive cells were FACS sorted (FACSCalibur II, Becton, Dickinson and Company, East Rutherford, NJ at the Scripps Flow Cytometry core facility) to generate a cell line with a stably integrated HIV LTR or with a Δ362LTR that contains mutations at the 362 site. The J-Lat cell lines were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: J-Lat Full Length Clones 6.3, 10.6, and 15.4, which are collectivity referred to as J-Lat cells, which were deposited by Dr. Eric Verdin [51]. The LChIT reporter line, a CEM T-cell–based reporter system, comprising of the single-copy LTR integrant driving the expression of mCherry-IRES-tat has been described previously [52].

## Cell Culture

HEK293T cells (ATCC) and the derived pMo-HEK and pΔ362 reporter lines were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, M, USA) and 50 µg/ml Pen/Strep (Thermo Fisher Scientific, Waltham, MA) at 37 °C and 5% $CO_2$. J-Lat and LChIT cell lines were both maintained in RPMI 1640 (Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum, 2 mmol/l l-glutamine (Thermo Fisher Scientific, MA, USA), and 50 µg/ml Pen/Strep (Thermo Fisher Scientific, MA, USA) at 37 °C and 5% $CO_2$.

**CLADE-specific activation assays.** HEK293 cells were transfected in triplicate using Lipofectamine 3000® (Thermo fisher scientific, MA, USA) with pcDNA-ZFP-362-VPR or pcDNA-VEGF-VPR with vectors expressing Firefly luciferase off the LTRs from different subtypes of HIV. A vector expressing *Renilla* luciferase was included as a background control (pRL-CMV, Promega, WI, USA). At 48 hrs post-transfection, a Dual-luciferase® Reporter Assay was performed according to manufacturer instructions and luciferase activity detected on the Glomax® Explorer system (Promega, WI, USA). The levels of Firefly luciferase were normalized to *Renilla* luciferase, and made relative to the pcDNA-VEGF-VPR control.

**Activation of the HIV LTR**: The protocol to assess the ability of the ZFP-362-VPR construct to activate latent HIV-1 has been described in Saayman *et al.* [52]. Briefly, pcDNA-ZFP-362-VPR, pcDNA-ZFP-VEGF-VPR (control), or pcDNA3.1 (Mock) plasmids were transfected into the CEM LChIT cells and the J-Lat cells using the Neon Transfection System (Thermo Fisher Scientific, MA, USA) at a cell density of $2 \times 10^7$ cells/ml with the following electroporation parameters: 3 pulses, 1,350 V and 10 ms. The dCas9-VPR + sgF2-362 vectors, which have been previously shown to potently activate HIV, were included as a comparison [52]. The cells were cultured without antibiotics following electroporation. To determine the activation with LRAs, J-Lat cells were treated with TNF-alpha (10 ng/ml, Gibco, Cat#PHC3015),

CD3/CD28 beads (3:1 ratio beads:cells, Dynabeads™ Human T-Expander CD3/CD28 beads, Cat#11141D), prostratin (3 µmol/l, Sigma Cat#P0077-1 mg), PMA (50 ng/ml, Fisher Scientific, Cat#BP685-1) with/without Ionomycin (500 ng/ml, Tocris Biosciences, Cat#1704/1). A DMSO control was included with 0.5% v/v DMSO dissolved in RPMI media with 10% FBS. Seventy-two hours post-treatment, the ability of these constructs to induce latent activation was assessed by FACS with 10000 single events collected, and the data was analyzed using FlowJo vX3.05470 software.

**NF-κB site specificity of ZFP-362-VPR**: To test the target site specificity of the pcDNA-ZFP-362-VPR to activate the LTR of HIV, 100,000 pMo-HEK and pΔ362 reporter cells were seeded per well in a 24 well plate at 24 hours prior to transfection. Cells were transfected in triplicate using Lipofectamine 2000 (Thermo Fisher Scientific, MA, USA) and 72 hours post transfection, total cellular RNA was isolated from cells using the Maxwell RSC simplyRNA Cells Kit (Promega, WI, USA) according to manufacturer's instructions. The concentrations of the isolated DNase-treated RNA samples were standardized and then reverse transcribed using the Mu-MLV Reverse Transcriptase (Thermo Fisher Scientific, MA, USA) with a n-oligo-dT/random nonamer primer mix (IDT, IA, USA).Quantitative real-time PCR (qRT-PCR) was carried out using Kapa Sybr Fast universal qPCR mix (Kapa Biosystems, MA, USA) on a Roche LightCycler® 96 System (Roche Diagnostics Corporation, IN, USA) according to provided instructions. The following PCR primer sets were used - GFP Fwd Primer: 5'-GACAACCACTACCTGAGCAC-3, GFP Rev Primer:5'-CAGGACCATGTGATCGCG-3, RPLP0 Fwd Primer: 5'-CGCAGCCAATAGACAGGAG-3, RPLP0 Rev Primer: 5'-GCGCGTGCCTTTTATAATGC-3 [53], and the GFP expression was analyzed using quantitative reverse transcription PCR (qRT-PCR). pcDNA-ZFP-VEGF-VPR and pcDNA3.1 were included as negative controls. The qPCR data was analyzed using LightCycler® software version 1.1.1320.

### Activation of HIV LTR in $CD4^+$ T-cell latency model

Naïve cells were isolated and activated by anti-IL12 (Peprotech, Cat#500-P154G), anti-IL4 (Peprotech, Cat#500-P24), TGFbeta (Peprotech 100 – 21) and anti-CD3/CD28 beads (Invitrogen, Cat#11131D). Activated cells were infected by pseudotyped pNL4.3-deltaEnv-nLuc-VSVG viruses. At Day 17, $CD4^+$ cells were isolated using Dynabeads™ CD4 positive isolation kit (Invitrogen, Cat#11331D). At Day 18, $CD4^+$ cells were treated with 10 ng/ml PMA (Sigma, Cat#P1585) or anti-CD3CD28 beads or transfected by plasmids (GFP, ZFP-362-VPR, or ZFP-VEGF-VPR) using by Neon electroporation (Invitrogen, Cat#mpk1096). After 72 hrs, part of the cells was stained with fixable viability dye efluor™ 450 (eBioscience, Cat#65-0863-14), anti-CD4 (Invitrogen, Cat#MHCD0405) and anti-P24 (BD, Cat#556027) antibodies, and measured by flow cytometry. The cells were counted and samples containing 2000 live cells were lysed and measured using the Nano-Glo® Luciferase Assay System (Promega, Cat#N1120).

### ChIP analysis of ZFP-362-VPR

The ZFP-362-VPR, and dCas9-VPR + sgF2-362 plasmids were transfected in triplicate into 3 million pMo-HEK cells using calcium phosphate transfection. ChIP analysis was carried out on the transfected pMo-HEK cells for the myc-tagged activating complexes using anti-myc antibodies (Cell Signaling Technology,

Danvers, MA). The ChIP protocol was performed 72 hours post-transfection using previously described protocols [10, 54, 55]. The ChIP elutes were purified using the MinElute PCR purification kit (Qiagen, Hidlen, Germany) and analyzed by qPCR. Relative enrichment at the LTR was determined using LTR specific primers that were described in Saayman *et al*.[54].

### ZFP-362-VPR and dCas9-VPR + F2gRNA effects on global RNA expression

To determine the extent to which ZFP-362-VPR and dCas9-VPR + sgF2-362 disrupted RNA expression, we compared pMo-HEK cells transfected with either construct to those transfected with a pcDNA3.1 control. The pMo-HEK were seeded at 100000 cells per well in a 24-well plate and transfected with the ZFP-362-VPR, and dCas9-VPR + sgF2-362 plasmids in quadruplicate using calcium phosphate transfection. Total cellular RNA was isolated 48 hrs post-transfection from cells using the Maxwell RC simplyRNA Cells Kit (Promega, WI, USA). Sequencing was performed by Genewiz® on an Illumina platform with paired end 150 bp reads. Sequence data are available via Gene Expression Omnibus accession number GSE150382. Raw sequencing reads were processed with the nf-core [56] RNASeq pipeline version 1.4. Briefly, trimmed reads were mapped using Spliced Transcripts Alignment to a Reference (STAR) [57] to the GRCh37 reference using the ENSEMBL (release 75) annotation. Each library was subjected to extensive quality control, including estimation of library complexity, gene body coverage, and duplication rates, among other metrics detailed in the pipeline repository. Reads were counted across genomic features using Subread featureCounts [58] and merged into a matrix of counts per gene for each sample. Differential expression analysis was performed with DESeq2 [59] as implemented in the SARTools package [60]. Only genes with at least 1 read count in 1 sample were retained for the analysis. Multiple testing was corrected using the Benjamini-Hochberg method, where p-values are adjusted to control for false discovery. An adjusted p-value threshold of 0.05 was used to select differentially expressed genes. Gene enrichment analyses were performed with ClusterProfiler [61] using the full set of genes with at least 1 count in any sample as background and differentially expressed genes as the input list. Enriched GO terms or enriched KEGG pathways were selected using an FDR threshold of 0.05 with the enrichGO function of ClusterProfiler.

## Abbreviations

**HIV**
Human Immunodeficiency Virus
**ZFP**
Zinc Finger Protein
**ZFN**
Zinc Finger Nuclease
**TALEN**
Transcription activator-like effector nucleases
**CRISPR/Cas**
clustered regularly interspaced short palindromic repeats with CRISPR-associated proteins

**LTR**
Long Terminal Repeat
**TNF-alpha**
Tumor Necrosis Factor alpha
**IL**
Interleukin
**CAR**
Chimeric Antigen Receptor
**VPR**
VP64-p65-Rta
**ART**
Antiretroviral Therapy
**NF-κB**
Nuclear factor kappa-light-chain-enhancer of activated B cells
**SP1**
Specificity Protein 1
**GO**
Gene Ontology
**KEGG**
Kyoto Encyclopedia of Genes and Genomes
**DMEM**
Dulbecco's Modified Eagle Medium
**HEK293 cells**
Human Embryonic Kidney cells
**qRT-PCR**
Quantitative Real-Time Reverse Transcription PCR
**CD**
Cluster of Differentiation
**CCR5**
C-C Chemokine Receptor 5
**sgRNA**
Small Guide RNA
**LRA**
Latency Reversing agents

## Declarations

# COMPETING INTERESTS

K.V.M, M.S.W, T.A.S, and D.A.L have been issued a patent on this technology, W02019079819.

# Ethics approval

Ethics approval was obtained from the University of Utah to use primary tissues in the described assays (IRB 00067637).

### Consent for publication

Not applicable.

### Availability of data and materials

The data sets analyzed and materials used in this study are available from the corresponding author on reasonable request. RNA sequence data are available via Gene Expression Omnibus (accession number GSE150382).

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# AUTHOR CONTRIBUTIONS

K.V.M, M.S.W, T.A.S, Y.Z, and V.P designed the experiments; T.A.S., Y.Z., D.C.L, C.S, N.A.G, performed the experimental assays; T.A.S, D.C.L, N.A.G, Y.Z, and D.O.M performed data analysis; T.A.S, K.V.M., D.O.M, M.S.W, Y.Z. and V.P. revised the manuscript. All authors read and approved the final manuscript.

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Not applicable.

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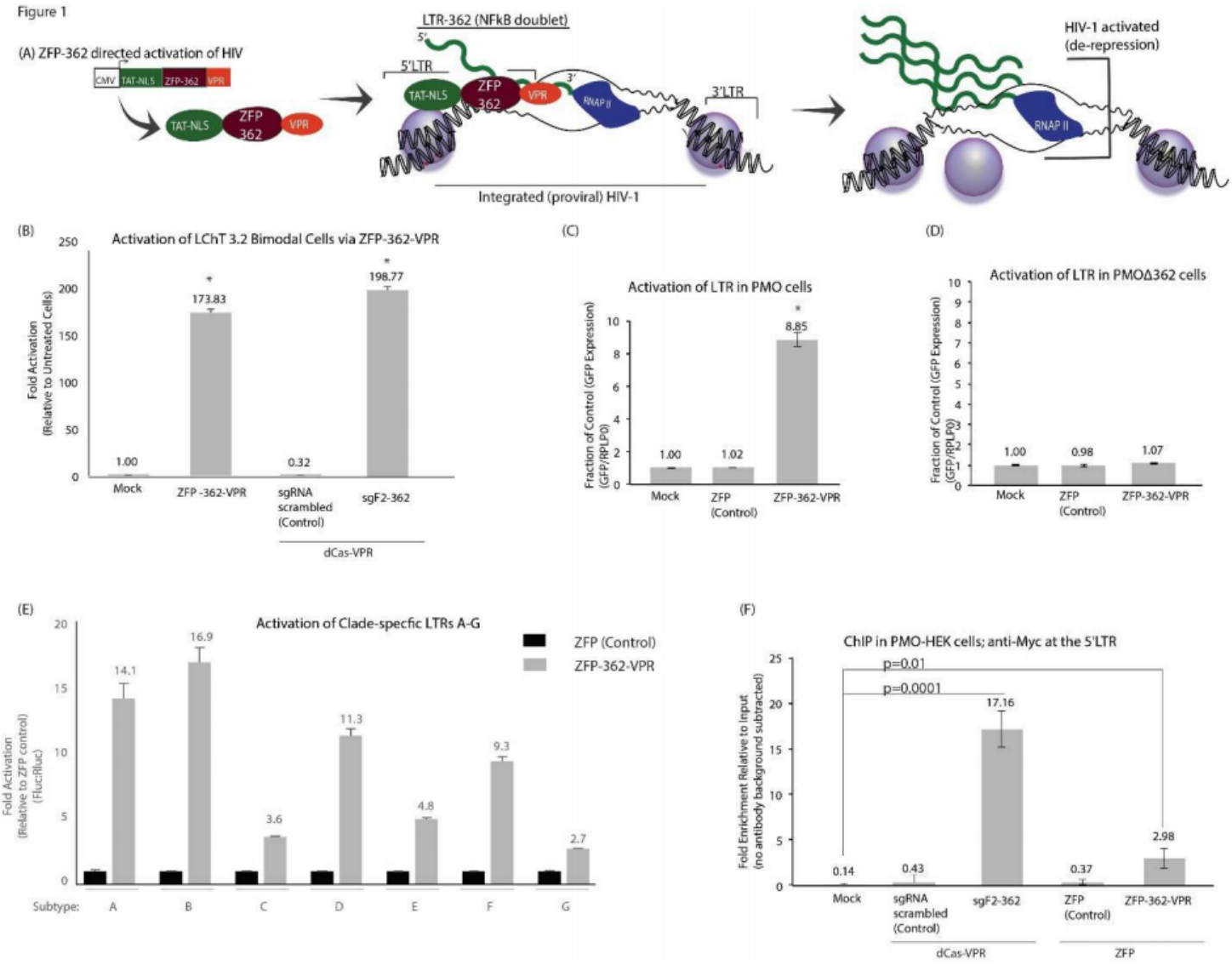
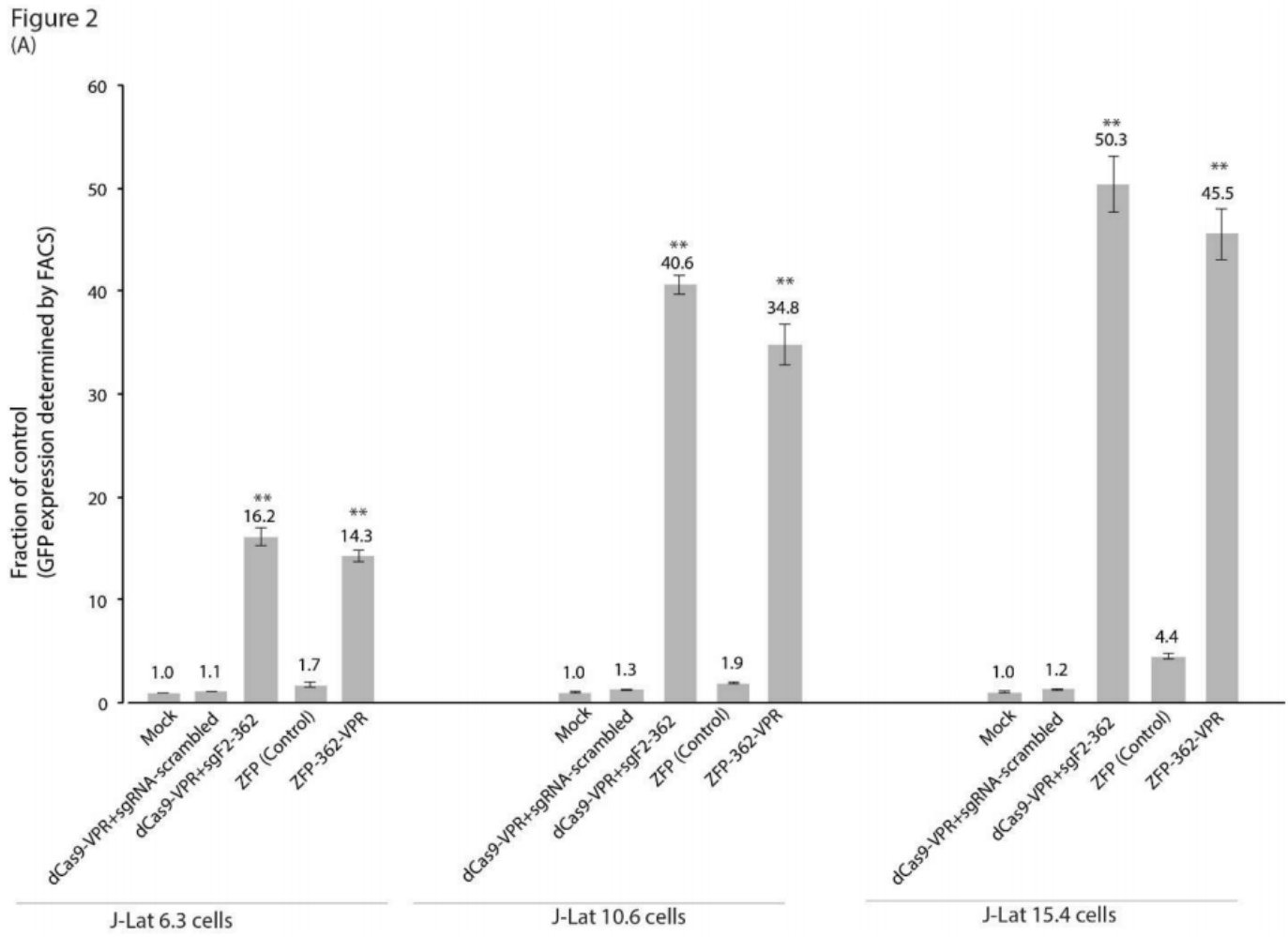
# Figures




## Figure 1

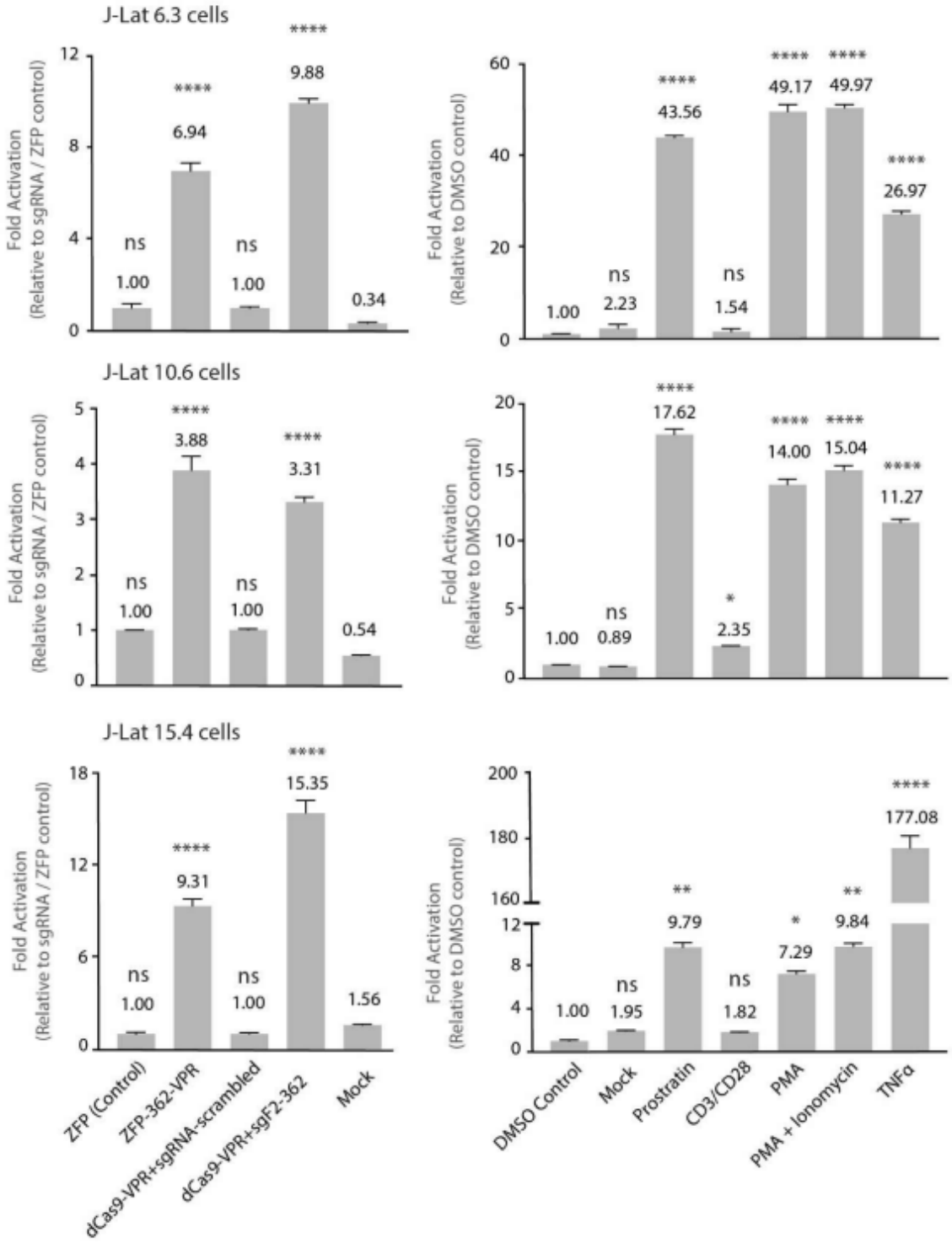
ZFP-362-VPR-mediated transcriptional activation of HIV. (A) a schematic is shown depicting ZFP-362-VPR, a zinc finger fused to the VPR activator domain with a Tat peptide as a cell penetrating motif and NLS peptide sequences for nuclear targeting, was developed to transcriptionally activate the NF-kB site in the HIV-1 LTR [10]. (B) ZFP-362-VPR activates LTR expression in LChiT 3.2 bimodal latent HIV reporter cells at levels that are comparable to dCas-VPR [52]. (C) ZFP-362-VPR activates LTR expression of GFP in pMO-HEK cells but (D) has no effect on pMOΔ362 cells containing a deletion in the LTR-362 site targeted by the ZFP-362. (E) The ability of ZFP-362-VPR to activate various subtypes of HIV was determined by co-transfection of ZFP with subtype variable LTR expressing luciferase clones. (F) ChIP analysis of ZFP-362-VPR and dCas-VPR binding to the HIV LTR. The pMO-HEK cells were transfected with the dCas-VPR+sgF2-362 or control sgRNA, or ZFP-362-VPR and ChIP assay was performed 72 hrs post-transfection to determine binding to the LTR-362 site [8]. The experiments were performed in triplicate treated cells and errors bars are calculated as standard deviations. (*) represents $p<0.001$ from a unpaired student's t-test.




## Figure 2

Activation effects of ZFP-362-VPR on different J-Lat latent infected cell models. Three different J-Lat cell lines (J-lat 6.3, 10.6 and 15.4) were subjected to neon transfection with dCas-VPR+sgF2-362, ZFP-VEGF-VPR (control), or ZFP-362-VPR. The expression of GFP was determined by FACS at 72 hrs post-treatment. The experiments were performed in triplicate treated cells and errors bars are calculated as standard deviations. Statistical significance was determined using an unpaired student's t-test (**) P<0.005.

Figure 3




## Figure 3

Comparison of ZFP-362-VPR and LRAs on in vitro latency models. Three different J-Lat cell lines (J-Lat 6.3, J-Lat 10.6, and J-Lat 15.4) were subjected to either electroporation with dCas-VPR+sgF2-362 or ZFP-362-VPR or treated with various known LRAs: TNF-alpha, CD3/CD28 beads, prostratin, PMA or PMA+Ionomycin. DMSO and untreated (mock) cells were included as negative controls. Activation of HIV was determined 72 hrs after treatment by FACS for the levels of GFP. Fold activation was the GFP

percentage made relative to a control vector set at 1. The results of triplicate treated cultures are shown with the SEM. Statistically significant differences, as determined from a one-way Anova and Dunnett's test are also shown (*) P<0.05, (**) P<0.005, and (****) p<0.0001 and were analyzed compare to the mock or DMSO control for the vectors and LRAs, respectively.

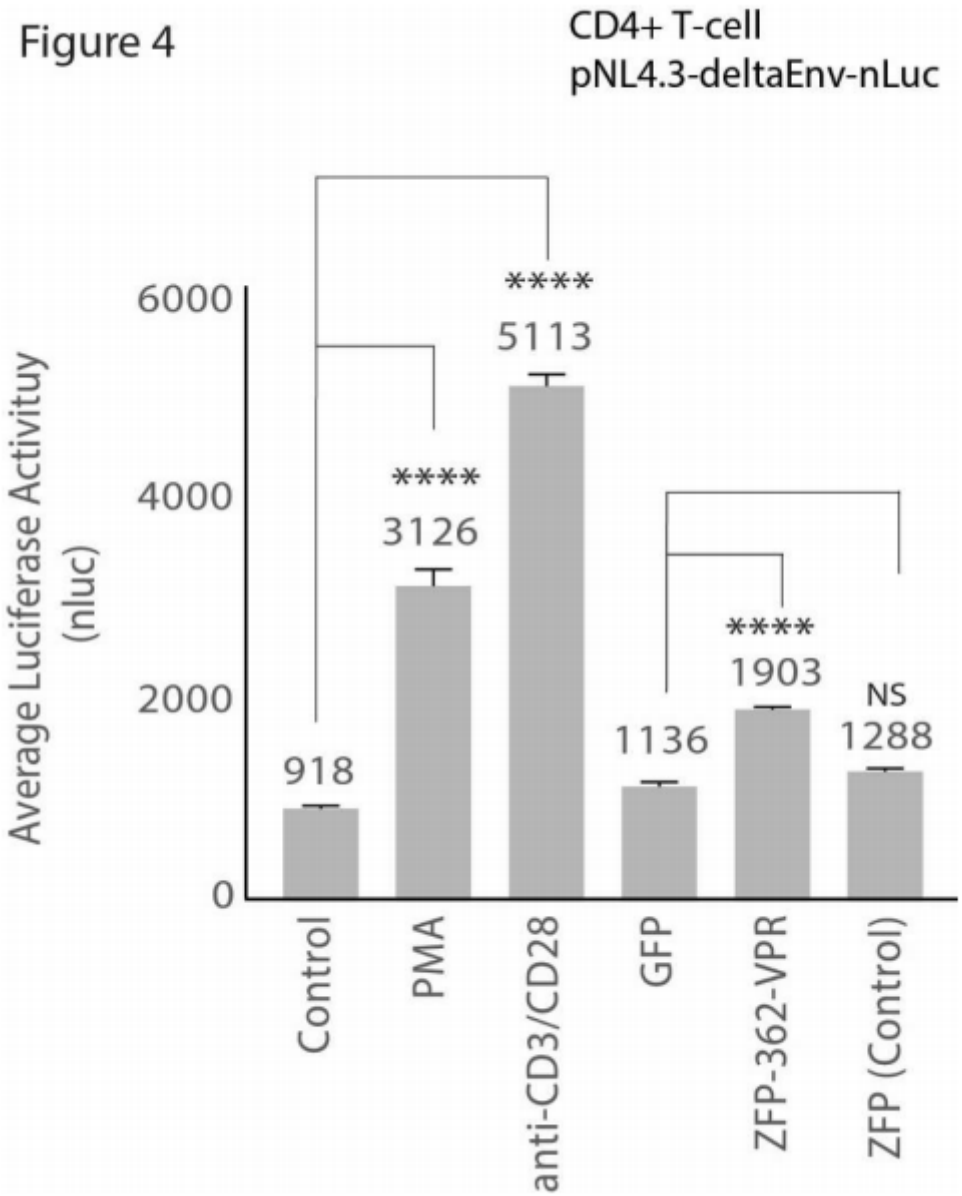
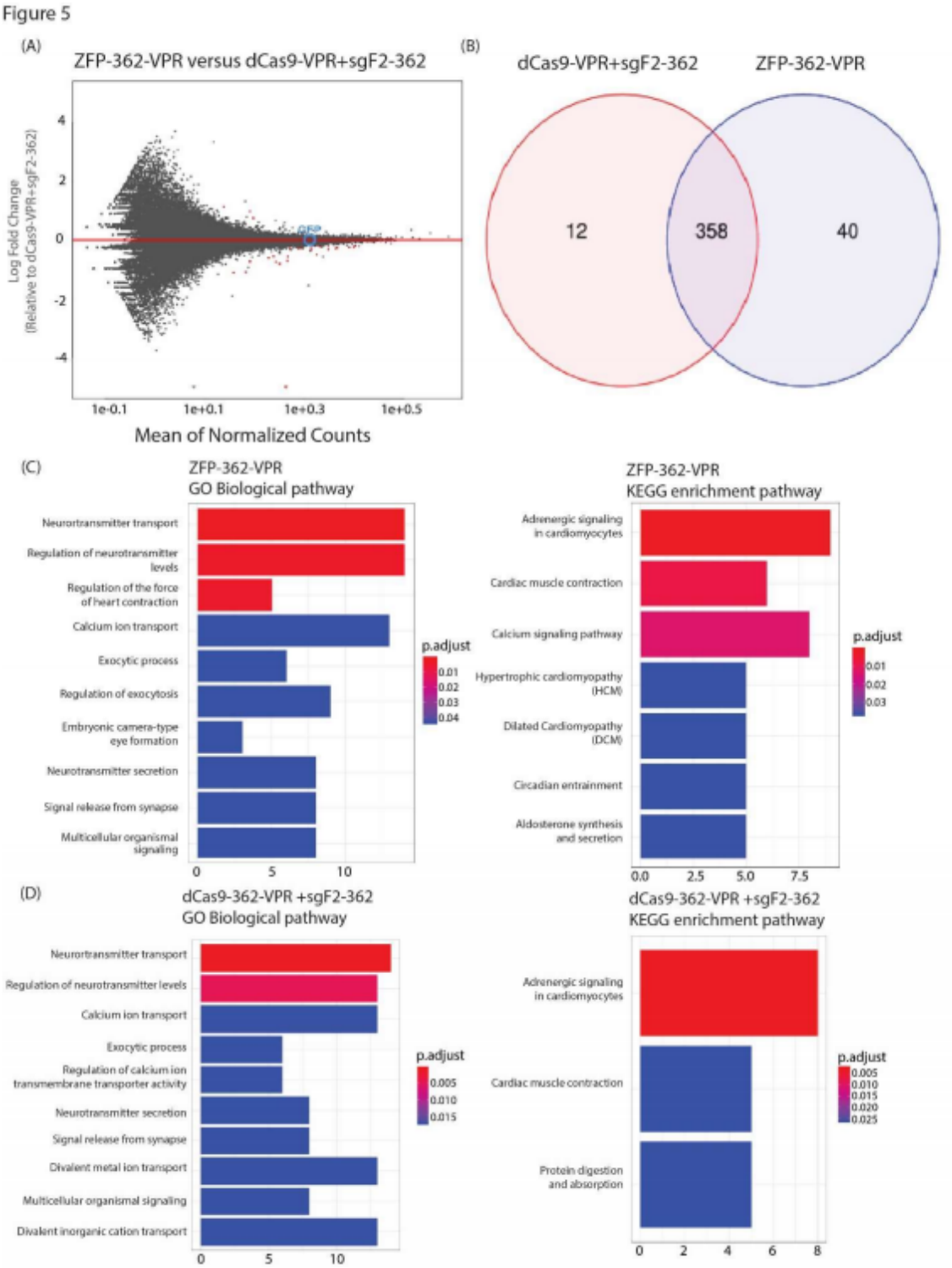



**Figure 4**

ZFP-362-VPR activity in primary CD4+ T-cell latency model. CD4+ T-cells (pseudotyped viruses pNL4.3-Env-nLuc-VSVG uninfected cells and latently infected cells) were transfected with ZFP-362-VPR and control vectors (GFP and ZFP-control), and 72 hrs later the levels of luciferase were measured. CD4+ T-cells were also treated with PMA (10 ng/ml) and anti-CD3/CD28 beads as positive controls. The average of triplicate treated cells are shown with the standard deviations, and p-values were determined using a one-way Anova and Dunnett's test. ****) p<0.0001. NS=not significant.




## Figure 5

ZFP-362-VPR induced differential gene expression. pMo-HEK cells were transfected with the pcDNA3.1, dCas9-VPR+sgF2-362 or ZFP-362-VPR expression vectors and total RNA was extract 72 hrs post-transfection and subjected to RNA sequencing analysis. (A) Comparison of the log fold change of ZFP-362-VPR treated samples with dCas9-VPR+sgF2-VPR mean expression. Differentially expressed genes (|logFC > 1, FDR 0.05) are colored red; GFP expression is equally and potently activated in both

treatments. (B) A Venn Diagram of the number of differentially expressed genes unique to ZFP-362-VPR and dCas9-VPR+sgF2-362 treated samples. Bar plot of enriched KEGG pathways indicated by differentially expressed genes in the (C) ZFP-362-VPR and (D) dCas9-VPR+sgF2-362 treated samples with respect to controls.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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