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Rho family GTPases enhance HIV-1 infection

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Introduction

HIV-1, like other viruses, relies on host cell machinery to replicate. The proteins co-opted by HIV are collectively known as host dependency factors, and have been investigated through a variety of approaches including siRNA and CRISPR screens. Although these high-throughput screens have been informative in identifying novel host dependency factors, they rely on changes in HIV replication due to altered protein expression. However, it is well-recognized that protein function is controlled not only by expression levels but also by a variety of post-translational modifications (PTMs) that may be overlooked in conventional screening approaches.

In the context of HIV-1 replication, PTMs play important roles in several stages of the viral life cycle: (1) acetylation and methylation of histone residues on viral-associated nucleosomes impacts chromatin structure and accessibility of the viral promoter to cellular transcriptional machinery; (2) acetylation of HIV-1 integrase is necessary for viral integration into the host chromosome; (3) phosphorylation of the CDK9 T-loop residue Thr-175 regulates P-TEFb activity and proviral gene expression, and (4) acetylation of tubulin residues promotes stable microtubule networks that facilitate viral trafficking to the nucleus.

Many PTMs that regulate HIV infection are likely still undiscovered. Here, we screened a panel of small molecule inhibitors targeting enzymes in epigenetic and PTM pathways for effects on HIV-1 infection.

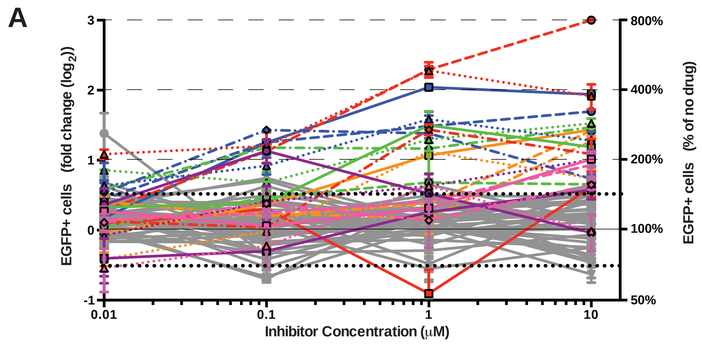
Present Study

A small-molecule library containing ~80 compounds regulating post-translational modification, epigenetic, and cytoskeletal pathways was screened for effects on HIV-1 replication in primary CD4+ T cells.
23 compounds enhanced HIV infection, including 17 HDAC inhibitors. An additional 16 compounds were found to reduce HIV-1 infection, including inhibitors of histone and DNA methylation and Rho GTPase signaling. We tested additional inhibitors of the Rho family GTPases Cdc42 and the Rho-associated protein kinase (ROCK) and observed similar reductions in HIV-1 infection. Finally, using phosphoproteomic and biochemical analysis of signaling pathways altered by HIV binding to CD4 and CCR5, be observed that HIV signaling activates the small GTPases Rac1 and Cdc42 and induces a dramatic restructuring of the cellular Rho GTPase landscape, suggesting that the activity of these pathways is directly modulated by the virus. Together, these data indicate that HIV signaling via its receptors at the time of attachment activates Rho family GTPases and that RhoA, Cdc42, and ROCK are host dependency factors for optimal HIV infection of primary CD4+ T cells.

Methods

Small molecule inhibitors. The 96-well "epigenetic screening library" was acquired from Cayman Chemical and stored at -20°C as 10 mM stocks in DMSO (Batch #0449323). Small molecule inhibitors were serially diluted in RPMI 1640/10% FBS/1% PenStrep for a final effective range of 10 nM - 10 µM immediately prior to experimental setup. Multiple freeze-thaw cycles were prevented to exclude potential variations in drug potency.
Infection experiments. 1 x 10^6 unstimulated primary CD4+ T cells were plated per well in a 96-well format and incubated for 4 hours at 37°C with media alone or media supplemented with 10 nM, 100 nM, 1 µM, or 10 µM of indicated small molecule inhibitor. Parallel plates were prepared for analysis of viral fusion and LTR-driven EGFP expression as previously described. Following incubation with small molecule inhibitors, cells were infected with HIV-1 reporter virus strain NL4-3-deltaE-EGFP (obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3-delta-E-EGFP (Cat #11100) from Drs. Haili Zhang, Yan Zhou, and Robert Siliciano) bearing CXCR4-tropic envelope JOTO.TA1.2247 [26] and a β-lactamase-Vpr fusion protein [27,28]. Plates were spinoculated for two hours at 1200 rpm and 25°C. Following centrifugation, cells were incubated for one hour at 37° C. Cells for viral fusion analysis were treated with CCF2-AM, washed, and incubated overnight at room temperature in the presence of probenecid. For EGFP expression, cells were incubated at 37°C for a total of 72 hours prior to staining and processing.
Flow cytometry. Cells were washed once with PBS containing 1% BSA and incubated with live/dead near-IR fixable viability dye (Invitrogen), CD3 Brilliant Violet 650 (BioLegend), and CD4 Allophycocyanin (eBioscience) at 4°C for 30 minutes. Cells were washed in PBS/BSA and fixed in PBS/BSA containing 1% paraformaldehyde prior to data acquisition. All samples were analyzed using a BD Fortessa (BD Biosciences) cell analyzer equipped with a High Throughput Sampler option. A minimum of 50,000 events were collected per sample at a flow rate of 2.5 µL/sec with 50 µL mixing and 3 x 200 µL washes. All experimental conditions were performed in triplicate. FlowJo version 9.7.6 (TreeStar, Inc) was used for analysis. For downstream validation of significant hits, the above experiments above were repeated in triplicate on a minimum of 3 healthy donor CD4+ T cell populations.
Phosphoproteomic analysis of HIV-exposed CD4+ T cells. Memory CD4+ T cells were purified by negative selection from a leukapheresis pack using custom RosetteSep kits (STEMCELL Technologies). Briefly, equal populations of 150 x 106 cells were exposed to 20 µg/ml p24 equivalent AT2-inactivated HIV-1 THRO or protein equivalent concentrations of non-viral microvesicles as control. HIV-1 THRO is a transmitted/founder virus isolated from a subject with early HIV infection that was found to be CCR5-tropic as evidenced by inhibition by TAK779 but not AMD3100 on TZM-bl cells and by its inability to replicate in PBMCs from a patient with the delta32-ccr5 mutation [29]. The AT2-inactivated viruses were derived from a cell clone of THRO (THRO CL.29) that was produced by co-culturing HEK293T cells transfected with the patient-derived THRO with A66-R5 cells and was identical in amino acid to the parental virus with the exception of Vif T68I, Vpu A8V, and Env R298K mutations. THRO CL.29 productively infected A66 cells expressing CCR5 but did not infect A66 cells that expressed CXCR4 (Julian Bess, personal communication). Following stimulation for 1, 15, and 60-minute periods, cells were incubated with cold PBS containing protease and phosphatase inhibitors. Cells were washed, lysed with 2% SDS, and digested enzymatically with trypsin. Phosphopeptides were enriched with titanium dioxide and purified by long gradient UPLC. Enriched samples were analyzed by LC-MS/MS and analyzed with Ingenuity Pathway Analysis (Ingenuity).
Small GTPase activation assay. Purified CD4+ T cells were resuspended at 5x106/ml in RPMI/10% FBS and rested overnight. Cells were then pelleted at 800xg for 5 minutes followed by resuspension in RPMI without FBS, rested at 37°C for 1 hour, and exposed to AT2-inactivated THRO or protein equivalent concentrations of non-viral microvesicles as detailed above. After a 1 minute incubation, ice-cold PBS containing protease and phosphatase inhibitors was immediately added, cells were pelleted at 800xg for 5 minutes and resuspended in ice-cold cell lysis buffer. Cells were transferred to a new tube, clarified by centriguation at 10,000xg for 1 min at 4°C and snap-frozen in liquid nitrogen. Following determination of protein concentration, cell lysates were thawed, normalized for protein concentration with lysis buffer, and analyzed using a RhoA, Rac1, and Cdc42 G-LISA activation assay (Cytoskeleton, Inc.) according to the manufacturers instructions.
Statistics. Data presented as mean values with standard error of the mean unless stated otherwise. All differences with a p value of <0.05 were considered statistically significant, correcting for multiple comparisons when appropriate. Statistical analyses were performed using student T-tests within GraphPad Prism v7.0.

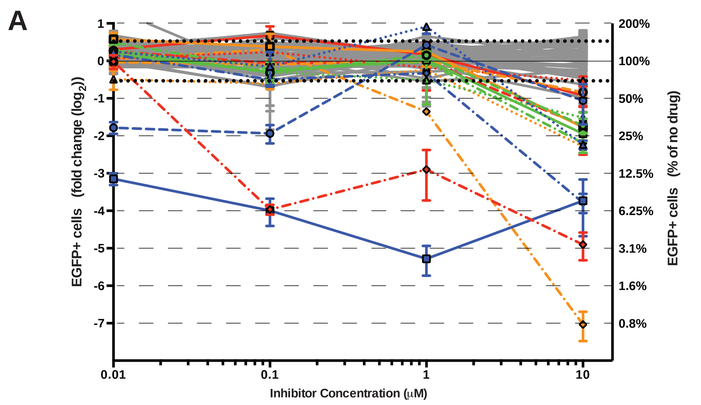
Results



HDAC inhibitor	Target	Infection (% of no drug)	Viability (% of no drug)
SAHA	Pan-HDAC	412 ± 19% ***	99.1%
Oxamflatin	Pan-HDAC	324 ± 16% ***	90.8%
Trichostatin A	Pan-HDAC	300 ± 19% ***	72.8%
SB939	Pan-HDAC	270 ± 14% ***	99.2%
M334	Pan-HDAC	283 ± 5% ***	93.5%
Chidamide	Pan-HDAC	160 ± 26 *	98.8%
CAY10603	Pan-HDAC	288 ± 24% ***	76.9%
(S)-HDAC-42	Pan-HDAC	226 ± 16% *	100.0%
Sodium Butyrate	Pan-HDAC	153 ± 25% ns	101.1%
CBHA	HDAC 1,3	490 ± 64% ***	97.7%
Apicidin	1,3,6	377 ± 80% ***	85.7%
4-iodo-SAHA	1,6	271 ± 24% **	93.8%
SBHA	1,4	271 ± 14% ***	88.3%
CAY10398	1	268 ± 23% ***	93.0%
Tubastatin A	6	218 ± 10% ***	96.6%
Salermide	Sirtuins	241 ± 9% ***	99.6%
Sirtinol	Sirtuins	202 ± 27% **	98.8%

Compound	Target	Infection (% of no drug)	Viability (% of no drug)
UNC1215	L3MBTL3 (inhibitor)	191 ± 36% **	99.0%
PFI-1	BRD2, BRD4 (inhibitor)	151 ± 8% ***	96.0%
N-Oxalyl-Glycine	hydroxylase (inhibitor)	159 ± 13% ns	97.1%
2,4,-Pyridine-dicarboxylic acid	hydroxylase (inhibitor)	150 ± 23% ns	96.8%
(+)-JQ1	BRD4 (inhibitor)	219 ± 44% ***	110.1%
DMOG	prolyl hydroxylase (inhibitor)	201 ± 20% **	99.8%

Figure 1: Small molecule inhibitors *enhancing* viral infection. A. LTR-driven EGFP expression in primary CD4+ T cells. **B.** 17 HDAC inhibitors increased susceptibility of CD4+ T cells to infection by HIV. (ns=not significant, *=p<0.05, **=p<0.01, ***=p<0.001). **C.** Six non-HDAC compounds also demonstrated greater than +0.5 log2 fold-change in EGFP. Infection and viability data correspon to the drug levels with the largest effect on infection with ≥70% viability compared to the no drug control.



Compound	Target	Infection (% of no drug)	Viability (% of no drug)
Decitabine	DNA MTase (inhibitor)	2.6 ± 1.2% **	94.3%
Lomeguatrib	O6-MeGu DNA MTase (inhibitor)	26.1 ± 7.6% *	107.5%
UNC0638	H3K9 methylation (inhibitor)	21.2 ± 2.9% *	49.5%
2,3,5 triacetyl-5-azacytidine	DNA MTase (inhibitor)	7.3 ± 2.2% ***	105.1%
trans-resveratrol	COX-1 (inhibitor)	26.0 ± 5.9% ***	105.2%
Tenovin-6	p53 (activator)	30.2 ± 7.0% ***	41.2%
CAY10591	Sirtuin 1 (activator)	35.1 ± 5.8% ***	101.3%
AG-014669	PARP1 (inhibitor)	21.8 ± 6.1% **	103.0%
GSK-J4	Histone DMase (inhibitor)	52.9 ± 16.6% ns	97.6%
C646	Histone ATase (inhibitor)	30.2 ± 21.7% *	78.1%
Garcinol	Histone ATase (inhibitor)	51.6 ± 7.4% *	100.2%
Chaetocin	Histone MTase (inhibitor)	6.5 ± 1.0% *	26.9%
CCG-100602	RhoA signaling (inhibitor)	21.6 ± 3.9% **	87.4%
Isoliquiritigenin	Guanylyl cyclase (activator)	56.1 ± 6.2% **	96.4%
Mi-2	Menin binding compound	20.5 ± 4.1% **	82.7%
5-azacytidine	Ribonucleoside analog	0.8 ± 0.4% ***	100.7%

Figure 2: Small molecule compounds *reducing* viral infection. A. LTR-driven EGFP expression was reduced by more than -0.5 log2 fold change by 16 compounds compared to no drug control (ns=not significant, *=p<0.05, **=p<0.01, ***=p<0.001). **B.** Table listing the 16 compounds reducing HIV infection with target proteins, percent infection, and viability data.

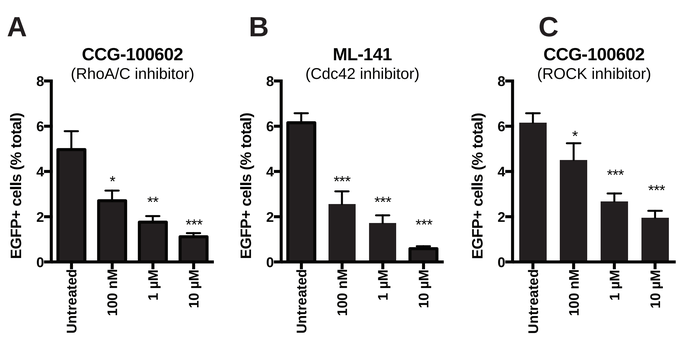


Figure 3: Inhibition of the Rho family GTPases RhoA and Cdc42 and the Rho-associated protein kinase (ROCK) reduce HIV infection. Primary CD4+ T cells were infected with CXCR4-tropic reporter viruses following treatment with small molecule inhibitors targeting A. Rho A/C signaling, B. Rho family member Cdc42, and C. Rho-kinase ROCK decrease HIV LTR-driven EGFP expression. *=p<0.05, **=p<0.01, ***=p<0.001. All experimental conditions performed in triplicate. Error bars represent SEM.

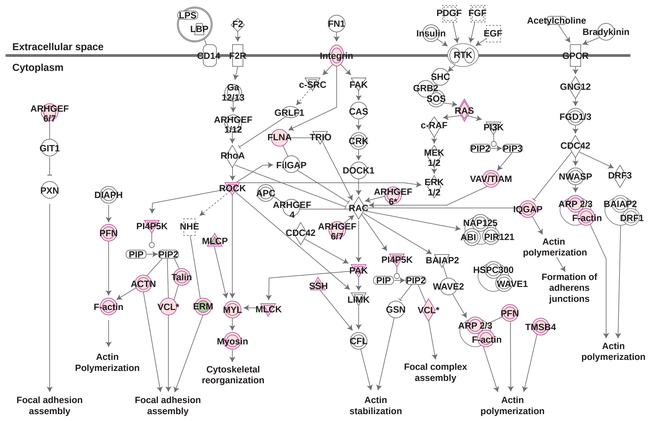


Figure 4: Canonical Rho signaling pathways were identified in a phosphoproteomics screen of HIV-1 signaling via CD4 and CCR5. Phosphoproteomic analysis reveals that numerous Rho family GTPase and ROCK signaling members are dysregulated (purple) in response to HIV exposure compared with a similarly prepared microvesicle control. Network analysis was performed using Ingenuity Pathway Analysis software.

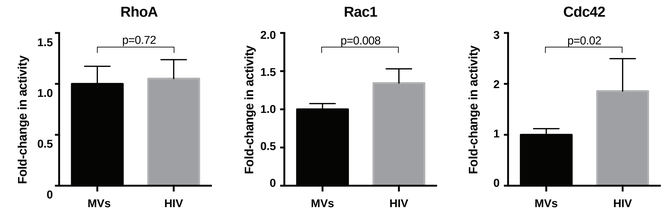


Figure 5: Biochemical GTPase activation assay reveals HIV significantly activates Rac1 and Cdc42. Primary CD4+ T cells were exposed to CCR5-tropic HIV-1 or microvesicles for 1 minute and then assessed for RhoA, Rac1, or Cdc42 activation using a small GTPase activation assay (G-LISA). Results were normalized to the microvesicle controls to determine fold-changes in activation.

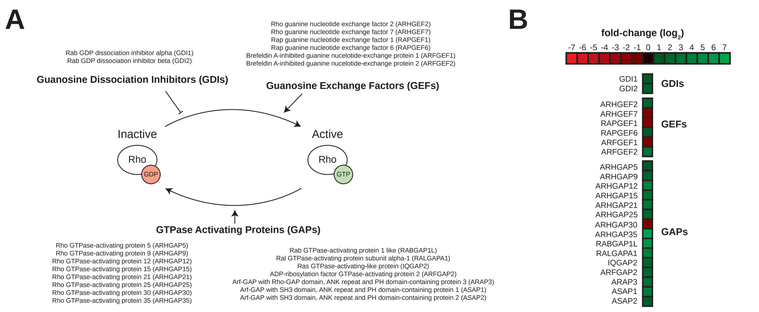


Figure 6: HIV signaling through CD4 and CCR5 alters the landscape of GTPase-regulating proteins. **A.** Rho family GTPases are regulated by guanine dissociation inhibitors (GDIs), guanosine exchange factors (GEFs), and GTPase activating proteins (GAPs). GTPase-regulating proteins with ±0.5 log2 fold changes in phosphopeptide expression levels following HIV-1 signaling via CD4 and CCR5 as compared to a microvesicle control are listed. **B.** Log2 fold changes in intensity of phoshopeptides from GTPase-regulating proteins 1 min after exposing cells to inactivated HIV-1 viruses.

Conclusions

This study has reinforced the importance of the cytoskeleton in optimal HIV-1 infection of host cells and implicated Rho family GTPases as host dependency factors for HIV-1. Additionally, we provide evidence that HIV-1 manipulates host GTPase activity via signaling upon binding to primary CD4+ T cells. Importantly, dependence on Rho family GTPase signaling for optimal infection has also been demonstrated for adenovirus, KSHV, and Influenza A viruses infections, suggesting that manipulation of the Rho GTPase signaling pathways to promote cytoskeletal reorganization may be a common feature among many viral infections.

Acknowledgements

This work was supported by NIH grant R01-HD077886 (JCT) and Case Western Reserve University CFAR NIH P30 AI036219.