THE ELUCIDATION OF THE ROLE OF ARP2/3 IN HIV-1 REPLICATION IN CD4 T CELLS

by

Mark Spear
A Dissertation
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Doctor of Philosophy
Biosciences

Committee:

Dr. Yuntao Wu, Dissertation Director
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Date: 12-3-14

Fall Semester 2014
George Mason University
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Fall Semester 2014
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List of Abbreviations

HIV................................................................. Human Immunodeficiency Virus
UNAIDS........................................ Joint United Nations Programme on HIV and AIDS
AIDS................................................................. Acquired Immunodeficiency Syndrome
ART................................................................. Antiretroviral Therapy
Arp2/3............................................................. The Actin-related protein 2 and 3 complex
F-Actin ............................................................... Filamentous Actin
G-Actin............................................................... Globular Actin
NPF................................................................. Nucleation Promoting Factor
WASp............................................................... Wiskott-Aldrich Syndrome Protein
GEF ................................................................. Guanine Nucleotide Exchange Factor
GAP................................................................. GTPase Activating Protein
GDI................................................................. Guanine Nucleotide Dissociation Inhibitors
RTC................................................................. Reverse Transcriptase Complex
PIC................................................................. Pre-Integration Complex
TAR................................................................. Trans-Activation Region
RRE................................................................. Rev Response Element
Abstract

THE ELUCIDATION OF THE ROLE OF ARP2/3 IN HIV-1 REPLICATION IN CD4 T CELLS

Mark Spear, Ph.D.

George Mason University, 2014

Dissertation Director: Dr. Yuntao Wu

HIV-1-initiated receptor signaling and early actin dynamics are required for viral infection of resting CD4 T cells. WAVE2 is a component of a multiprotein complex linking receptor signaling to actin dynamics. WAVE2 directly activates Arp2/3, leading to actin nucleation and filament branching. Here we report that binding of HIV-1 to resting CD4 T cells and primary macrophages induces a rapid phosphorylation of WAVE2 at serine 351. This phosphorylation involves both Gαi-dependent and -independent pathways, and occurs in response to R5 and X4 viruses, suggesting that this signaling event is likely conserved in HIV infection. In addition, inhibition of WAVE2-mediated Arp2/3 activity through a specific Arp2/3 inhibitor, CK-548, inhibits both T cell chemotaxis and HIV-1 infection of CD4 T cells. Furthermore, inhibition of Arp2/3 through stable shRNA knockdown of Arp3 also inhibits HIV-1 infection of CD4 T cells. The inhibition is mainly at the level of viral nuclear migration. Our results suggest that
WAVE2 and Arp2/3 are actively engaged by HIV-1 following viral binding, and that
WAVE2-mediated Arp2/3 activity plays a critical role in mediating actin-based post-
entry nuclear migration.
Chapter One—An Introduction to the Human Immunodeficiency Virus

The HIV Pandemic

According to the Joint United Nations Program on HIV and AIDS (UNAIDS), 35.3 (32.2-38.8) million people are currently living with HIV, the etiological agent of Acquired Immunodeficiency Syndrome (AIDS). HIV infections and those living with HIV are also radically concentrated in sub-Saharan Africa, representing both a health and economic crisis. Broadly, infections and those living with infection concentrate in low and middle-income countries due, in large part, to the lack of resources (healthcare and prescription drug access, prophylactics, sanitation, etc.). Additionally, there were an estimated 2.3 million new HIV infections in 2012, which, though a significant decline from 3.4 million new infections in 2001, is a profound exemplification of the need for further intervention for the prevention of HIV transmission. The number of people living with HIV infection is also increasing, which both represents new infections and, importantly, the role of antiretroviral therapy (ART) in decreasing morbidity and mortality, and increasing longevity, in persons infected with HIV.

Clinical Immunobiology of HIV Infection

The canonical course of HIV infection, as observed in untreated patients, is exhibited in Figure 1. Initial infection is followed by an acute phase of viral replication, with flu-like or mononucleosis-like symptoms typically present, and this often emerges around 2-4
weeks post-infection\(^3\). During this time, a reduction in CD4 T cells may be observed. These CD4 T cells regulate post-exposure immune responses, and effectively guide the innate and adaptive immune response; as such, they are critical to normal immune function\(^3\). Following this phase, a combination of immunological and virological mechanisms leads to the establishment of clinical latency, a period that may last for years\(^3\). During this phase, low level viral replication occurs, and modest reductions in CD4 T cell counts may be observed\(^3\). After this phase, the immune system fails to contain ongoing replication, leading to chronically high levels of plasma viremia as well as a precipitous decline in CD4 T cell counts and T cell homeostasis, more broadly\(^4\). Once a patient’s CD4 T cell count drops below the threshold of 200 CD4 T cells/µl, this constitutes the primary clinical definition of AIDS\(^3\). At this juncture, the immune system is thoroughly dysregulated, exhibiting signs of CD4 T cell exhaustion, and opportunistic infections will occur\(^3\). Without treatment, AIDS-associated opportunistic infections typically lead to death of the patient. Notably, this clinical course is an “average,” and there are individual patients, termed Long-Term Non-Progressors (LTNP), who may never develop AIDS\(^5\).
Figure 1

Adapted From: Wu, Y. 2009. The co-receptor signaling model of HIV-1 pathogenesis in peripheral CD4 T cells. Retrovirology, 6:41.

**Figure 1**—The clinical course of HIV-1 infection. Shown are the viral titers and CD4 T cell counts canonically observed over time in untreated patients. The R5/X4 text denotes viruses of different tropism, either engaging CCR5 (R5) or CXCR4 (X4) coreceptors for entry. This particular figure was adapted from Wu (Retrovirology, 2009) indicating that the emergence of X4 virus variants contributes to the precipitous decline in CD4 T cells. The three phases of HIV replication (acute, clinical latency, and the collapse of the immune system leading to AIDS) are shown and separated by vertical dotted lines.
**HIV Biology**

HIV is a retrovirus, in which the free virus-associated nucleic acid is single-stranded RNA (ssRNA) ⁴. Additionally, the virus is enveloped, carrying host cell-derived lipids that once constituted a small portion of the host cell membrane (Figure 2) ⁴. The membranous envelope also bears the Envelope glycoprotein (referred to variably in the literature as Env, for the protein, env for the gene or mRNA species, gp160 for the unprocessed protein, and gp120:gp41, for the fully processed heterodimer). The viral Env protein directly contacts the cellular receptor, CD4 ⁶⁻¹¹, and one of two predominant co-receptors, CCR5 ¹²⁻¹⁷ or CXCR4 ¹⁸. This process also mediates cellular entry ³.

Following entry, virion-packaged reverse transcriptase (RT) catalyzes ssRNA->dsDNA conversion, and this occurs coincidently, as is currently thought, with viral core rearrangement and nuclear migration of the viral core ³. Prior to integration into the host genome, the core is generally referred to as the pre-integration complex (PIC), and is competent for integration ¹⁹, which is mediated by the viral Integrase (IN) protein ²⁰.

After integration, expression of the viral genome is necessary for completion of the viral life cycle. The Long Terminal Repeat (LTR), which is composed of the U3, R, and U5 regions, ultimately regulates the expression of the nine HIV-1 genes ³. In particular, the U3 region of the 5’ LTR acts as the promoter and enhancer, and the +1 R position as the transcription start site ³. However, expression from the 5’ LTR is limited in the absence of stimulation ³. Expression of the viral Tat ²¹,²² protein promotes transactivation of the HIV-1 LTR by binding the TAR (Trans-Activation Response
element) in the nascent mRNA \(^3,23\). More specifically, coordinated binding of Tat to the TAR along with CDK9 and Cyclin T1 drives RNA Polymerase II Carboxy Terminal Domain (CTD) hyperphosphorylation and transcriptional enhancement \(^{24}\). However, for expression of all viral gene products, both spliced and unspliced mRNA species must be exported from the nucleus, which requires the viral Rev protein. In the absence of Rev, only spliced early gene transcripts are exported, leading to expression of only Tat, Rev, and Nef protein. Expression of Rev \(^{23,25,26}\) promotes nuclear export of unspliced and partially spliced viral mRNA’s encoding the major structural proteins Gag, Gag-Pol, and Env, and the accessory proteins Vpr, Vpu, and Vif \(^3\). This process requires engagement of the Rev Response Element (RRE) by Rev along with Crm1 and Ran:GTP to promote the nuclear export of the intron-containing mRNA species \(^{27}\). Once the full suite of structural and accessory proteins, and the full-length genomic RNA (gRNA), are produced, assembly may occur.
Figure 2

**Figure 2**—HIV virion and Gag structure. Shown is the life cycle of HIV replication (a), as well as the structure of the HIV virion (d-g) and the major structural polyprotein Gag (b and c).

Gag, as shown in Figure 2, is the major structural protein of the viral particle. During viral budding, Gag and Gag-Pol polyproteins accumulate at assembly sites on the plasma membrane. The Gag-Pol polyprotein contains the major Gag components, as well as the three retroviral enzymes, RT (Reverse Transcriptase), IN (Integrase), and PR (Protease). Furthermore, this process requires recruitment of other viral proteins, cellular proteins, and, through the NC protein, the viral gRNA. PR ultimately cleaves Gag and Gag-Pol into its constituent components, MA, CA, NC, p6, PR, RT, and IN for the full Gag-Pol polyprotein. This occurs after budding of the viral particle through the plasma membrane, whereupon the virus acquires its membranous envelope. This proteolytic processing is associated with a dramatic change in virion core structure, as shown in Figure 2d-g. After this, the virus is competent for a new round of infection, thus completing the full viral life cycle.

Currently, ART drugs overwhelmingly target just two viral proteins: reverse transcriptase (RT) and protease (PR). IN has also become a clinically relevant target for ART and prophylaxis. For the sake of completeness, clinically useful antiretrovirals can also target the gp41 domain of Env, such as enfuvirtide; both coreceptors, with only CCR5 antagonists, like maraviroc, being used; and Tat, though no such drugs are currently approved by the FDA for use. For a variety of reasons, the latter targets, and the
relevant pharmaceuticals, are significantly less important for the consideration of ART, as is currently practiced clinically. Of the RT inhibitors, nucleoside/nucleotide analogue and non-nucleoside analogue reverse transcriptase inhibitors (NRTIs/NtRTIs and NNRTIs) are widely used as the frontline in ART, pre and post-exposure prophylaxis (PEP), and prevention of mother-to-fetus transmission. Most fixed-dose combinations sold by the major pharmaceutical companies contain NNRTIs/NRTIs by themselves, or mixed with PR inhibitors or IN inhibitors for regular ART as well as prophylaxis. The pharmaceuticals are added in combination to limit the resistance that is rapidly acquired during monotherapy (one drug therapy).

Importantly, even with ART, viral replication still occurs, and latently infected cells, those infected, but expressing little to no viral protein, are completely insusceptible to the effects of ART and immune surveillance mechanisms. Additionally, many patients receiving ART experience pharmacological failure to control viremia resultant from viral acquisition of resistance to at least one of the ART components. As a result of this clinical phenomenon, new targets and strategies are required to effectively curtail the ongoing HIV pandemic. Of note, cellular genes that act as essential viral HIV replication cofactors are likely ideal targets; particularly, if deletion of the cellular gene is tolerated. Unlike viral protein targets of antiretroviral therapy, cellular targets will not mutate, and lost cofactor activities may be indispensable, and irrecoverable by viral mutation.
**HIV and Actin**

When HIV gp120 binds to the primary viral receptor, CD4, and coreceptor, CXCR4 or CCR5, this engagement is not purely for fusion and entry. In particular, signal transduction induced by viral gp120 binding to coreceptor plays a pivotal role in early post-entry events. Most notably, fusion and entry, reverse transcription, and nuclear migration. Among one of the most critical cofactors for efficacious infection is the cellular actin cytoskeleton, whose dynamic structure and scaffolding properties appear to play a significant role during viral replication. My colleagues and I, along with other investigators, have amassed an enormous collection of works detailing the interdependencies between early post-entry events and actin dynamics: furthermore, not just that actin itself is important, but the interplay between specific actin regulatory proteins and viral processes are critical for the establishment of infection. For a general review on the topic of early post-entry events relating to HIV replication and the actin cytoskeleton, there are a number of reviews on the topic, including my own (Spear 2012). In brief, the mechanisms of HIV dependence on the actin cytoskeleton will be considered in this and the following sections.

As afore-mentioned, HIV gp120-coreceptor ligation introduces proximal signaling events that facilitate, in addition to subsequent steps, entry. For instance, Jiménez-Baranda *et al.* (2007) exhibited that Filamin-A, an actin-crosslinking protein, interacted with CD4 as well as both co-receptors and was required for receptor clustering and efficacious entry. This process appeared to require proximal signaling events...
leading to a RhoA-ROCK-LIMK-Cofilin pathway that led to inactivation/phosphorylation of the actin-depolymerizing factor, cofilin\textsuperscript{40}.

Ezrin/Radixin/Moesin (ERM) proteins, Moesin in particular, have also been implicated in the receptor clustering and actin rearrangements required for fusion and entry\textsuperscript{39}. This, process, subsequently confirmed and expounded upon by Yoder \textit{et al.} (2008)\textsuperscript{34}, has been postulated to create an actin-rich cap below the virus-receptor interface mediating stabilization of the entry/fusion complex, as reviewed in Liu \textit{et al.} (2012)\textsuperscript{44}. Furthermore, it has been shown that SDF-1, the natural ligand of CXCR4, HIV-1 X4 virions, and X4 viral gp120 all mediate actin dynamics at timespans that would typically include receptor engagement, fusion/entry, reverse transcription, and nuclear migration\textsuperscript{34}.

There is also evidence that reverse transcription may be an actin-dependent or actin-facilitated event\textsuperscript{42}. Following fusion, the viral core is deposited into the sub-membranous space along with the active signal transducers produced during receptor engagement, and it is here that the viral core must rearrange and undergo reverse transcription as the Reverse Transcriptase Complex (RTC). This space, the actin cortex, is densely packed with actin filaments, which likely influence core rearrangement and reverse transcription in as-yet unknown mechanisms. However, Bukrinskaya \textit{et al.} (1998) exhibited that Gag-derived matrix protein (MA), a component of the RTC, associates with actin\textsuperscript{42}. Furthermore, Hottiger \textit{et al.} (1995) showed that the RT protein, or the Gag-Pol polyprotein precursor, also associates with actin. Also, disruption of the actin cytoskeleton with cytochalasin D (CCD), which promotes filament disassembly,
decreased RTC association with actin and the production of late-phase RTC products when cells were pre-treated with CCD, but not when treated 2 hours after infection. Treatment with other cytochalasins exhibited similar effects on viral infection in indicator cells. As such, though the mechanism remains obscure, actin and actin dynamics appear to play a significant role in HIV reverse transcription as well.

**HIV Nuclear Migration**

Much of the focus of this research, as well as those of my colleagues, has been the effects of actin dynamics modulation on HIV-1 nuclear migration. As such, I will briefly discuss the role of actin dynamics and the associated signaling events and their role in this viral process. Yoder et al. (2008) exhibited that blockade of CXCR4-induced Gαi signaling, through Pertusis Toxin (PTX) treatment, dramatically reduced HIV-1 replication. The effect was found not to be the accumulation of cytosolic HIV-1 DNA, which was increased, but the nuclear pool of that DNA, which would be required for integration and productive infection. In effect, the PTX treatment appeared to stall the normal process of DNA migration to and localization within the nucleus.

Contrastingly, pre-treatment of cells with α-CD4/CXCR4-coated beads increased the accumulation of HIV-1 nuclear DNA, including the integrated form and a nucleus-specific HIV-1 DNA species, the 2-LTR circle. As the exact signaling mechanism was unknown at the time, further investigation indicated that actin dynamics were the crucial mediator of CXCR4/Gαi-induced HIV-1 nuclear migration facilitation. Specifically, that treatment with Jasplakinolide, which promotes polymerization and a
more static actin cytoskeleton, similarly prevents the accumulation of HIV-1 nuclear DNA\textsuperscript{34}. Also, that viral particles, or gp120 itself, induce the dephosphorylation and activation of the actin-depolymerizing factor, Cofilin\textsuperscript{34}. Knockdown of cofilin appeared to replicate the phenotype of PTX treatment—an increase in total DNA synthesis, and a decrease in nuclear DNA\textsuperscript{34}. From these data, the model developed to explain the phenotype was that Cofilin activation creates the actin dynamics required for the viral PIC to migrate to the nucleus. This is thought to occur in association with F-actin, as many PIC components can bind actin directly\textsuperscript{42,48–53}.

Similarly, Vorster \textit{et al.} (2011) exhibited that okadaic Acid (OA) enhanced viral replication in a dosage-dependent manner, and increased HIV-1 DNA synthesis and 2-LTR synthesis\textsuperscript{43}. Okadaic acid is a general phosphatase inhibitor, and this effect was likely mediated by the changes in activation and phosphorylation status of the various signal transduction pathways. For instance, the enhancement in HIV-1 replication was correlated with an increase in the cofilin kinase, LIMK-1, activation/phosphorylation, which was observed not only after OA treatment, but also after treatment of resting CD4 T cells with HIV-1 virions\textsuperscript{43}. Furthermore, the entire Rac-PAK1/2-LIMK1/2 pathway appeared to be activated, as ascertained by Western Blot\textsuperscript{43}. For further confirmation, shRNA knockdown of LIMK in a CD4 T cell line decreased HIV DNA synthesis and nuclear migration\textsuperscript{43}. Pseudotyping with VSV-G envelope glycoprotein, which forces the virus to bypass the cortical actin by endocytosis, largely abrogated these effects in knockdown cells\textsuperscript{43}. As such, the Rac-PAK1/2-LIMK1/2 pathway appears to be utilized
by the virus to induce cortical actin dynamics capable of efficaciously delivering the core
to the perinuclear area, which is required for subsequent viral replication events.

**On Cofilin and LIMK Activation**

Briefly, I will consider the implications of the superficial contradiction of the
activation of cofilin, by its dephosphorylation, and the activation of the cofilin kinase,
LIMK, by its phosphorylation. All else being equal, an increase in LIMK
activation/phosphorylation should result in an increase in phosphorylated/inactive cofilin
and net actin polymerization. However, Yoder *et al.* (2008) observed a net
depolymerization of actin in response to gp120 and HIV-1 viral particles, particularly
observable at 60 minutes post-treatment; furthermore, that cofilin dephosphorylation was
observable as early as 1-5 minutes with similar treatments, and was maximal at later
points (10 minutes and later among various donors) \(^{34}\). Vorster *et al.* (2011), conversely,
found oscillating patterns of LIMK phosphorylation with an early phase of high
phosphorylation (0.5-3 minutes) and another phase that was sometimes observed later
(20-60 minutes post-treatment) \(^{43}\). From this, the early LIMK activation and net cofilin
dephosphorylation are temporally coincident. As such, a phosphatase activity increase is
required, and ongoing research indicates that it may be slingshot, PP1\(\alpha\), and/or PP2A
(unpublished data). This early period most likely correlates with HIV virion binding,
since complete fusion is half-maximal around 30-60 minutes for HIV-1 X4 Env-mediated
fusion \(^{54}\). More recent kinetic analyses of HIV-1 binding and fusion indicate that
CD4/CXCR4 binding maximizes within minutes of virus-cell association \(^{55}\). As such, this
The process is likely analogous to “virus surfing,” in which the virus associates with the primary receptor and coreceptor and traffics with these receptors. Below the receptors, the actin cytoskeleton and associated factors, such as the ERM proteins, and Filamin A traffic the HIV fusion complex to a site competent for entry. Additionally, an actin-rich cap may also form below the fusion complex at the site of entry. Following entry, half-maximal at 30-60 minutes, a resurgence in LIMK phosphorylation may make a favorable environment for viral core rearrangements and RTC reverse transcription.

Indeed, the Rac-PAK-LIMK pathway, which should produce spatially localized polymerization due to Rac prenylation and membrane association, does extend to this period. The model that was proposed as a result of these findings and similar findings was that HIV binds to the cell, and perhaps traffics to a site favorable for entry, in an actin and receptor signal transduction-dependent manner. After this, the viral core then enters the cytosol in association with actin for reverse transcription, and then the viral PIC migrates to the nucleus also in association with actin. The contemporaneous activation of LIMK, Cofilin, and the other downstream effectors of Rac mediate actin treadmilling and HIV PIC nuclear localization.

**Arp2/3**

As a significant focus of this dissertation will be the role of Arp2/3 and the upstream regulators in HIV-1 infection, it would be germane to review the biological function of Arp2/3 in the introduction. Arp2/3 is a heptameric complex composed of the “actin-related protein complex” (ARPC) proteins ARPC1B, ARPC2, ARPC3, ARPC4,
and ARPC5, along with the actin-related proteins Arp2 and Arp3. As their names suggest, Arp2 and Arp3 are structurally related to actin, which derives its four-domain structure from its conserved ATPase domain and activity. The function of the complex is to nucleate an actin filament from the side of an existing actin filament, leading to dendritic branching. In this, the ARPCs predominantly mediate association with the preexisting filament, and Arp2 and Arp3 juxtapose to mimic a nascent F-actin oligomer, which induces filament nucleation and branching. ATP loading into Arp2 and 3 appears to be required for Arp2/3 function, while ATP hydrolysis appears to play a role in debranching, which must occur for normal cytoskeletal dynamics and actin filament turnover. The cellular purpose of Arp2/3 is quite pleiotropic. This is due to the consequence of Arp2/3 activation; force generation from processive, ATP-dependent filament nucleation, branching, and growth against a substrate—typically intercellular adhesions. As such, Arp2/3 plays a pivotal role in cell migration and lamellipodia formation, filopodia formation (a lesser role, albeit), endocytic vesicle separation and cleavage from the plasma membrane, intracellular vesicle trafficking (particularly in the intra and inter-trafficking in the ER and Golgi), and even aspects of cytokinesis. The complexity of regulation that is required to collectively organize these disparate activities has recently resulted in a burst of research on the upstream regulators of Arp2/3, and their very complex regulation, as discussed in the next paragraph and in later chapters.

In addition to this basic biology of the complex itself, Arp2/3 largely lacks activity without activation by nucleation-promoting factors (NPFs). The NPFs have, until very
recently in the literature, been divided into two prominent groups; the WASP family and
the WAVE family. The WASP family includes the Wiskott-Aldrich Syndrome Protein,
WASP, and neural-WASP, or N-WASP. WASP tends to be expressed in the
haematopoietic system, while N-WASP seems to be expressed, despite the name, more
broadly. The WAVE family, contrastingly, is composed of WAVE 1-3, with WAVE2
being ubiquitously expressed, and WAVEs 1 and 3 having more distinct tissue expression
patterns. In addition, new members of the WASP-homology domain-containing
family have recently been added, including JMY, WASH, and WHAMM; currently,
studies of these proteins is much more limited, and they will not be addressed
significantly in this dissertation. Both WASP and WAVE families, to the extent they
have been studied, are regulated as coincidence detectors, indicating that more than one
activating interaction must occur before the protein exhibits full activity, which would
result in maximal Arp2/3 activation. In both cases, association with Rho-family
monomeric GTPases, phosphoinositides, and certain protein interactions are required for
full activation; additionally, WAVE2 appears to require ERK2-mediated
phosphorylation for its full activation. In the case of N-WASP, the Rho-family
GTPase, Cdc42, SH3 domain-containing proteins binding the polyproline motifs, and
PIP2 are required for maximal activation. Similarly for WAVE2, when incorporated
into the WAVE regulatory complex (WRC), the Rho-family GTPase, Rac, ERK2
phosphorylation, associated with SH3 domain-containing proteins, and PIP3 are
required for its full activation. The results, in all these cases, is exposure of the VCA
domain, which stands for verprolin homology, cofilin homology (or, alternatively,
connector), and acidic region—also in the literature is “WCA” domain, to indicate the WASP homology 2 domain, which is the same as the verprolin homology domain \(^{64}\). The WCA directly activates Arp2/3 and recruits profilin and soluble G-actin, leading to Arp2/3 attachment to the mother filament and actin branching \(^{64}\). The combination of intracellular (and extracellular) concentration gradients of these regulators and signaling molecules creates remarkable specificity in the spatiotemporal dynamics of active Arp2/3 and, along with similar systems that are quasi-redundant, explains the emergent dynamic complexity of coordinated cellular activities (e.g. cellular migration).
Chapter Two—Initial Studies into the Knockdown Effects of Arp3

Introduction

As mentioned in the previous section, actin dynamics are critical to HIV-1 infection, particularly in primary cells, the blood, lymph, and tissue macrophages and CD4 T cells\(^{34,35,43}\). Furthermore, Arp2/3 is a critical regulator of these actin dynamics\(^{59,62}\). In addition, some research has come to light in the past decade indicating that Arp2/3 is important to HIV-1 infection\(^{36-38,70}\). Specifically, Komano \textit{et al.} (2004) found that expression of GFP-WCA, which dysregulates Arp2/3, led to a decrease in infection efficiency of HIV-1, among other viruses\(^{70}\). Furthermore, stable expression of GFP-VCA in H9 cells delayed HIV replication kinetics by a factor of roughly 2\(^{70}\). This effect was not due to a decrease in fusion\(^{70}\). However, the mechanism of viral inhibition was never fully explored beyond this\(^{70}\).

The results of Ratner’s group, which are not broadly inconsistent with the findings of this dissertation, found that a Rac GEF inhibitor blocked viral entry\(^{38}\), and that R5 HIV signals through a \textit{Gaq}-dependent pathway to mediate efficacious entry\(^{36}\). Also that Arp2/3 and upstream regulators were required, as determined by siRNA knockdown, for HIV fusion\(^{37}\). However, in all three of these publications, there was an extreme over-reliance on the glioblastoma cell line, U87, the reporter HeLa-cell derivative, TZM-bl, and recombinant viruses that do not replicate primary cell infection,
nor even transformed CD4 T cell infection. As such, there is no consensus on the role of Arp2/3 in HIV-1 infection.

However, to first create a system to study the mechanism by which Arp2/3 dysregulation inhibits HIV replication, we developed a CD4 T knockdown cell with reduced Arp3 expression. Characterization of the bulk culture was first performed. Then, through limiting dilution, a number of clones were isolated. Western blot analysis indicated a high degree of knockdown among all clones. Two of these clones, exhibiting a high degree of knockdown and HIV-1 infection reduction, were chosen for further analysis. These clones, shArp3-012 and shArp3-013, exhibited >90% knockdown of Arp3 protein expression. Furthermore, shArp-012 expressed reduced surface CD4, while shArp-013 expressed similar CD4 to the Non-Target Control (NTC). CXCR4 expression was reduced in both shArp-012 and shArp-013, albeit to different extents. Total cellular F-actin, in contrast, varied among the clones and over time, indicating complex compensation mechanisms.

Materials and Methods

Cells: CEM-SS cells are a CEM CD4 T lymphoblast derivative and were maintained at less than 1*10^6 cells per milliliter in RPMI 1640 (Life Technologies) supplemented with 10% FBS and, occasionally, penicillin/streptomycin (Life Technologies). Unless otherwise indicated, cells were cultured at 37°C and 5% CO₂. shArp3 clones were obtained by limiting dilution. Briefly, transduced cells were diluted to 0.5 cells/200 µl in 0.45 µm-filtered, CEM-SS-conditioned RPMI with 10% FBS and 1
µg/ml puromycin. Cells were then plated at 200 µl/well into a round-bottom 96-well plate, corresponding to 0.5 cells per well. Where colonies of cells were visible, cells were gradually transferred up to 24- and 6-well plates, and ultimately cultured in T-25 flasks for further analysis and for making stocks.

Viruses: NTC and Arp3 knockdown virus was created by Lipofectamine 2000 (Life Technologies) transfection of HEK293T cells in 10 cm Petri dishes using the following plasmids: 10 µg pLKO.1-Puro-Arp3 (Clone Number: TRCN0000029382, Sigma-Aldrich) or pLKO.1-Puro-NTC (Catalog Number: SHC016-1EA, Sigma-Aldrich); 7.5 µg pCMVΔ8.2, which expresses the packaging construct products, Gag, Gag-Pol, Tat, Rev, Nef, Vif, and Vpu, but lacking the HIV-1 Env protein; and 2.5 µg pHCMV-G, expressing the pan-tropic Vesicular Stomatitis Virus Envelope Glycoprotein G (VSV-G). Virus-containing supernatants were cleared by centrifugation at 1,200 rpm for five minutes to remove cell debris, and filtered through Whatman 0.45 µM filters. Virus-containing supernatants were then concentrated in Vivaspin 20 concentrator tubes (vivaproducts) to 20X.

Knockdown Lentiviral Transduction: $1 \times 10^6$ cells were resuspended into 200 µl of RPMI supplemented with 10% FBS. 200 ng of NTC and Arp3 shRNA-transducing lentivirus were added to the cells for 6 hours at 37°C and 5% CO$_2$. Cells were then plated into a 6-well plate, and puromycin was added to a concentration of 1 µg/ml at 48 hours post-infection.
Immunoblot Analysis of Arp3 and GAPDH: For SDS-PAGE samples, $1 \times 10^6$ cells were lysed into 100 $\mu$l 1X NuPAGE LDS Lysis Buffer, prepared by adding 250 $\mu$l NuPAGE 4X LDS lysis buffer, 100 $\mu$l NuPAGE 10X sample reducing agent, and 650 $\mu$l distilled water (Life Technologies). Samples were loaded onto NuPAGE Novex 4-12% Bis-Tris gels, with 50 $\mu$l NuPAGE antioxidant in the inner chamber (Life Technologies), and run at 150 V for approximately 1 hour. Gels were transferred to 8.5x13.5 cm 0.45 $\mu$m pore nitrocellulose membranes (Life Technologies) at 30 V for 2 hours in an ice bath. Membranes were washed in Tris-buffered saline (TBS) with 0.2% Tween-20 (Sigma-Aldrich) three times for five minutes. Membranes were then blocked in either 5% skim milk in TBS-T, or LiCor Blocking Buffer (LiCor). Staining was performed with a 1:1000 dilution of rabbit anti-Arp3 (Catalog Number: 4738, Cell Signaling Technology) or goat anti-GAPDH (Catalog Number: Ab9483, AbCam) into 2.5% skim milk TBS-T, and stained overnight at 4°C. Samples were then washed three times in TBS-T for fifteen minutes each. Samples were secondarily labeled, respectively, with a 1:5000 dilution of peroxidase-labeled goat anti-rabbit (KPL) or DyLight 800-labeled rabbit anti-goat (KPL), diluted into 2.5% skim milk in TBS-T, and stained for one hour at room temperature. Membranes were then washed three times for fifteen minutes each before imaging. For peroxidase-labeled secondary, 3 ml of SuperSignal West Femto Chemiluminescent Substrate (Pierce) was used for signal generation; whereas, IR dye-labeled stains were imaged on an Odyssey Infrared Imaging System (LiCor).
Surface Staining of CD4 and CXCR4: The following amounts and types of antibodies were used for staining: for general FcR blocking, 1 µl of mouse IgG (Cat. No. 015-000-003, Jackson ImmunoResearch Lab) was used; to stain for CD4, 5 µl FITC-labeled anti-CD4 IgG1κ (Catalog Number: 555346, BD Biosciences) was used, with 20 µl FITC-labeled IgG1κ as an isotype (Catalog Number: 530616, BD Biosciences); for CXCR4 staining, 20 µl PE-Cy5-labeled anti-CXCR4 IgG2aκ (Catalog Number: 306508, BioLegend) was used, with 20 µl PE-Cy5 IgG2aκ as an isotype (Catalog Number: 555575, BD Biosciences). To $5 \times 10^5$ cells, 3.5 ml of PBS with 0.1% BSA was added and cells were pelleted at 1,200 rpm for five minutes at 4°C. The supernatant was decanted, the residual cells were resuspended into residual buffer, and the cells were put on ice for the remainder of the stain. To the cell suspension, 1 µl of mouse IgG FcR-blocking antibody was added, followed by the indicated amount of isotype/stain antibody, followed by a 20-minute stain period carried out in the dark. Stained cells were then washed with 3 ml PBS with 0.1% BSA, and pelleted as before. After decanting the supernatant, cells were resuspended into 200 µl PBS with 0.1% BSA, and fixed with 200 µl 2% paraformaldehyde. Analysis was performed on a BD Biosciences FACSCalibur.

Actin Staining: To $5 \times 10^5$ cells, 3.5 ml of PBS with 0.1% BSA was added and cells were pelleted at 1,200 rpm for five minutes at 4°C. After decanting the supernatant and resuspending the cells, 400 µl of Cytoperm/Cytofix (BD Biosciences) was added, and cells were incubated at room temperature for 20 minutes. The remainder of the protocol was carried out on ice. To the fixed cells, 2 ml of BD Perm/Wash buffer (BD
Biosciences) was added, and cells were then pelleted at 1,200 rpm. After decanting the supernatant, cells were resuspended into residual buffer and 3-5 µl of 0.3 mM Phalloidin-FITC (Catalog Number: P5282-1MG, Sigma-Aldrich) was added before a 30 minute-stain in the dark. To wash off residual stain reagent, 2 washes with 2 ml BD Perm/Wash buffer were performed. After the final wash, cells were resuspended into residual buffer, and 100 µl of 2% paraformaldehyde was added prior to flow cytometric analysis. Viable cells were gated from FSC-SSC plots for FITC histogram plots of F-actin content.

Results

Infection of CEM-SS with shArp3 knockdown virus transiently reduces F-actin content

CEM-SS cells were transduced with 200 ng knockdown virus or NTC lentiviral vector for 6 hours for shRNA transduction. At 48 hours post-infection, 1 µg/ml puromycin was used to select for virally-transduced cells. Initial stains indicated that total F-actin content was reduced in bulk culture by FITC-Phalloidin stain (Figure 1). However, this effect was transient, and was predominantly lost by day 6 post-infection. As such, to achieve phenotypic stability, cells were cloned out by limiting dilution into 96-well plates. These clones were then individually characterized for their F-actin expression (Figure 2). Also, some cells were lost due to contamination issues; as such, clone numbers are not entirely continuous, representing lost clones and their corresponding numbers (e.g. clones shArp3-002, -003, and -004).
Figure 1—F-actin stain in bulk NTC and Arp3 knockdown cells. Cells were stained with FITC-phalloidin to stain F-actin. The above-indicated FSC-SSC gate (above, and corresponding to NTC Day 4 post-infection) was used to gate out cell debris and apoptotic cells from all samples, from which F-actin histograms (below) are based. The vertical line represents the approximate median F-actin stain for Day 8 NTC.
Figure 2: F-actin content in shArp3 Knockdown Clones. Cells were stained with Phalloidin-FITC to measure F-actin, and were gated as indicated above to exclude dead cells and debris. The vertical line approximates the median F-actin content of NTC for comparison between clones. Most clones exhibited less cellular F-actin compared with NTC; though some exhibited notably higher F-actin.
*shArp3 knockdown clones exhibit unique surface expression of CD4 and CXCR4*

To test whether Arp3 knockdown affected the viral receptor/coreceptor expression (i.e. CD4 and CXCR4 expression), which may impact later HIV studies, surface staining was performed on the resultant clones (Figure 3 and Figure 4). Most of the clones that were stained for CD4 expression exhibited similar, or reduced, expression (Figure 3). Whereas, all clones, save shArp3-004, exhibited reduced CXCR4 expression (Figure 4). This indicated that the knockdown of Arp3 had similar effects on surface protein processing, trafficking, surface expression, recycling, and degradation.

*Western Blot analysis of shArp3 knockdown clones*

To ascertain the relative expression of Arp3 in knockdown clones, whole cell lysates were produced from one million pelleted cells. After one-dimensional SDS-PAGE, samples were transferred to a 0.45 µm pore nitrocellulose membrane and probed for Arp3 expression (Figure 5A). Samples were subsequently probed for GAPDH, which is routinely used as a loading control (Figure 5B). Lastly, Arp3/GAPDH expression ratios, which better represents the Arp3 expression per cell and actual Arp3 knockdown, were calculated and plotted as a column graph (Figure 5C). All clones exhibited some degree of knockdown of Arp3 protein expression, with some exhibiting extreme protein expression reductions, having less than 10% original expression levels.
Figure 3

Figure 3—CD4 surface expression of shArp3 knockdown cells. Shown is the FITC-CD4 surface expression on NTC and knockdown clones. The same FSC-SSC gates as in figure 4. The vertical line represents the approximate median CD4 expression profile of NTC. As can be seen, CD4 expression was highly variable among the clones, but largely deviated by less than 50%.
Figure 4

Expression of surface CXCR4 in shArp3 knockdown clones. Cells were stained for surface CXCR4 or an isotype control. In the above panel is the FSC-SSC gate used for all samples, with some representative samples indicated. In the bottom panel, CXCR4 expression is shown in the histograms. The vertical dashed line represents the approximate NTC median CXCR4 expression. Most clones exhibited less CXCR4 surface expression than the NTC.
Figure 5

Expression of Arp3 and GAPDH in shArp3 knockdown clones. One million cells were lysed and stained for Arp3 expression (A). That same immunoblot was subsequently stripped, blocked, and reprobed for GAPDH (B). The relative ratio of Arp3-to-GAPDH was then calculated and plotted to more accurately reflect the percent knockdown in each cell clone (C), with NTC being normalized to 0% knockdown. As indicated, most clones exhibited 90% or greater Arp3 knockdown.
Discussion

Though Arp3 homozygous deletion is embryonic lethal in mice at the blastocyst stage, a stable knockdown platform for studying the role of Arp2/3 in HIV-1 infection was created $^{73}$. Additionally, this platform could be used to study any CD4 T cell actin-dependent processes more broadly. Furthermore, the actin, CD4 and CXCR4 surface expression, and Arp3 protein expression phenotypes were determined, allowing subsequent analysis of the effects of Arp3 knockdown on HIV-1 replication.

The bulk Arp3 knockdown cultures exhibited properties that indicated phenotypic instability. Most notably, total cellular F-actin rapidly returned to near-baseline within a week of culture, even in the presence of the knockdown vector selection antibiotic, puromycin. Additionally, they did not exhibit HIV-1 replication inhibition as a bulk culture (data not shown). There could be many reasons the bulk culture manifested these properties. As an essential component during cytokinesis, development, cell migration, and many other processes, Arp3 knockdown is likely to promote selection for cells with the least degree of functional Arp2/3 activity loss $^{59,63,73,74}$. Additionally, since Arp2/3 can function at nanomolar concentrations $^{75}$, even a small percent of residual expression of the functional complex can compensate for severe depletion $^{76}$. Additional compensatory mechanism may also exist: for example, the more static actin cytoskeleton that would likely be caused by Arp3 knockdown may be rendered more dynamic by hypophosphorylation of cofilin $^{77}$. Any of these compensatory effects could mask HIV-1
replication inhibition. As such, the bulk culture had to be cloned out to differentiate between Arp3 knockdown effects and compensatory effects.

To this end, several clones were isolated and characterized for their F-actin, CD4, and CXCR4 expression. Notably, most clones exhibited reduced F-actin content upon FITC-phalloidin staining, which is consistent with the role of Arp2/3 in \textit{de novo} filament nucleation \(^{59}\). However, F-actin content did vary considerably within the most studied clones, shArp3-012 and -013, between culture conditions, length of culture, and individual stains, indicating that bulk F-actin was not a stable phenotypic marker. Surface expression of CD4 was highly variable among the clones, which may indicate that CD4 surface expression regulation is not explicitly linked to Arp2/3 \textit{per se}, but may be regulated by actin dynamics more generally. In great contrast, all but one clone exhibited reduced CXCR4, which likely indicates that CXCR4 surface expression is more directly linked to Arp2/3 function. This is also consistent with Vorster \textit{et al.} (2009), wherein LIMK knockdown increased expression of surface CXCR4, and this was correlated with decreased internalization of the surface receptor \(^{43}\). Furthermore, clones exhibited considerable Arp3 knockdown by Western Blot, with individual clones exceeding 95%. This is consistent with a previous study, in which similar knockdowns were tolerated in mouse embryonic fibroblasts \(^{78}\).
Chapter Three—Initial Studies into the Effects of Arp3 Knockdown on HIV-1 Replication

Introduction

HIV-1 has been previously shown to require actin dynamics in multiple stages of viral replication, from entry, reverse transcription, nuclear migration, and perhaps even budding and cell-cell transmission, as reviewed in Spear et al. (2012). In particular, nuclear migration has been shown to be remarkably sensitive to perturbations in actin dynamics, including by chemokine stimulation. Among the many targets of these signaling pathways include the Rho GTPases and downstream effectors; germane to the focus of this dissertation are the WASp/WAVE family of Arp2/3 activators.

As afore-mentioned, Arp2/3 and, albeit to a lesser degree, WASp/WAVE have recently been identified as cofactors in HIV-1 replication; though, the mechanism is far from completely understood. In 2004, Komano et al. presented some of the first evidence that HIV-1, SIV, and intracellular mature Vaccinia Virus (IMV) require some level of Arp2/3 activity for productive infection. Specifically, that expression of GFP-VCA, which mimics the WASp/WAVE VCA domain and dysregulates Arp2/3, reduced infection using reporter virus. Using a proxy of virus-cell fusion, Komano et al. (2004) used the cell-cell reporter fusion system (described in Lin et al. (2005)) to explore
defects in HIV-1 Env-mediated fusion: in this system, cells expressing Env and T7 RNA polymerase are added to, and fuse with, cells expressing either GFP or GFP-VCA and a T7 RNA polymerase promoter-luciferase construct, resulting in luciferase activity after successful fusion. No fusion defects were observed when GFP-VCA was expressed, indicating that no defect in fusion occurred as a result of Arp2/3 dysregulation. To further corroborate their findings, stable expression of GFP or GFP-VCA in H9 cell clones infected with HIV-1 exhibited a defect in replication kinetics; furthermore, that cotransfection of HIV-1 proviral DNA vector and GFP-VCA expression vector in HEK293T cells resulted in reduced p24 release, indicating that late phases in the viral life cycle may be affected as well. These results would seem to indicate that Arp2/3 dysregulation prevents efficacious HIV-1 replication by affecting late phases and, perhaps, some early phases; fusion, however, was not affected in their study. In contrast Lee Ratner’s group found discrepant results in a series of studies from 2007, 2008, and 2010. Specifically, that inhibition and knockdown of Arp2/3 and upstream regulators inhibited HIV-1 fusion, but not later stages of viral replication (as they were not studied).

To resolve the apparent discrepancy, and to further probe the mechanism of HIV-1-Arp2/3 interactions, we created an Arp3 knockdown CD4 T cell line system using CEM-SS lymphoblasts. These cells, as previously described, expressed variable surface CD4, reduced CXCR4 surface expression, variable—though generally reduced—F-actin content, and markedly reduced Arp3 expression. Using these cell clones, we observed
reduced HIV-1 replication, with inhibitions exceeding 90%. Ultimately, shArp3 clones 012 and 013 were chosen for further characterization due to their more severe HIV-1 replication defects. These cells exhibited mildly reduced proliferation rates compared to the NTC control. Furthermore, pseudotyping with VSV-G envelope granted increased permissiveness to HIV-1 viral replication, but did not completely restore replicative capacity.

**Materials and Methods**

*Cells:* NTC and shArp3 cell clones were maintained at less than $1 \times 10^6$ cells per milliliter in RPMI 1640 (Life Technologies) supplemented with 10% FBS, 1 µg/ml puromycin (Life Technologies), and penicillin/streptomycin (Life Technologies). HEK293T cells were maintained in DMEM supplemented with penicillin/streptomycin (Life Technologies) and 10% FBS. Unless otherwise indicated, cells were cultured at 37°C and 5% CO$_2$.

*Viruses:* VSV-G-pseudotyped vNL4-3 was made by Lipofectamine 2000 (Life Technologies) cotransfection of 12 µg pNL4-3 (KFS), which lacks a functional HIV *env* gene, and 12 µg pHCMV-G, expressing the pan-tropic Vesicular Stomatitis Virus Envelope Glycoprotein G (VSV-G) $^{72}$. Wild-type vNL4-3 was made by transfection of 24 µg pNL4-3. Virus-containing supernatants were cleared by centrifugation at 1,200 rpm for five minutes to remove cell debris, and aliquoted into 2 ml cryotubes for -80°C storage.
**HIV viral infection:** $2 \times 10^5$ cells were resuspended into 100-200 µl of medium and infected for 2 hours at 37°C and 5% CO$_2$ with 100-400 ng of virus, which additionally corresponds to a TCID$_{50}$ of $10^{3.5} - 10^{4.5}$. Cells were then washed three times with serum-free RPMI 1640 to remove residual HIV-1 particles. Cells were then resuspended into 1 ml complete medium for the duration of p24 sample harvesting.

**Cell proliferation assay:** $5 \times 10^4$ cells/ml of NTC, shArp3-012, and shArp3-013 were cultured in 5 ml Falcon tubes for 8 days. Each 24 hours, 50 µl were used to measure the cell concentration, using three independent counts each time. Volumes were replaced after taking each sample, and the cultures were further diluted as needed to keep the cell concentration below $1 \times 10^6$. Final cell “densities” were adjusted for each dilution event.

**p24 analysis:** p24 samples were taken from 150 µl of resuspended culture, which was pelleted at 13,000 rpm for 30 seconds, and from which 135 µl was placed into a new microtube. Samples were then lysed with 15 µl of p24 lysis buffer (5% Triton-X in PBS). p24 was quantified using a modified p24 ELISA kit protocol. In brief, polyclonal p24 capture antibody (Catalog Number: 3537, NIH AIDS Reagent Program) was bound to ELISA plates overnight, washed, and blocked with 2.5% FBS in PBS. Subsequently, p24 lysates/samples were added, stained with biotinylated anti-HIV polyclonal antibody, stained with streptavidin-HRP, and then mixed with the chromogenic substrate, 3,3’,5,5’-tetramethylbenzidine, for plate $A_{630}$ reading.

**Statistical Analyses:** Unless otherwise indicated, all statistical analyses used two-sample, equal variance Student’s t-tests against the control, assuming a two-tailed distribution.
Results

HIV-1<sub>NL4-3</sub> replication phenotype in shArp3 clones

Having established a platform to determine the role of Arp2/3 in HIV-1 infection, shArp3 knockdown clones were infected with approximately 200 ng of wt HIV-1<sub>NL4-3</sub> (abbreviated as vNL4-3 throughout the manuscript) for two hours. After which, residual p24 was washed away repeatedly, and supernatant p24 samples were taken on day 0, 1, 3, 5, and 7 to determine the replicative capacity of vNL4-3 in these cells. This p24 was assayed quantitatively using a sandwich ELISA from p24 sample lysates. As shown in Figure 1, most clones reduced HIV-1 replicative capacity (90% of tested clones), with the vast majority of those (8 out of 9, or 89%) exhibiting greater than 70% reduction in HIV-1 p24. All tested clones showed significant reduction in vNL4-3 p24 release, with the exception of the one clone that enhanced, shArp3-008. Among those clones that inhibited replication, two clones exhibited reductions in HIV-1 p24 release of 87 and 95%; those being shArp3-012 and shArp3-013, respectively. Ultimately, it was these clones that were chosen for more detailed analyses.

Focusing further on shArp3-012 and shArp3-013, another vNL4-3 infection and p24 analysis was performed (Figure 2A). Notably, replication was much more robust in this experiment and, as such, comparative replicative diminishments were greater in the knockdown clones (Figure 2A). Percent inhibition of HIV-1 replication in shArp-012 was 97% on day 7 post-infection, and 98% for shArp-013 (Figure 2B). To determine if the diminishment in replicative capacity could not be overcome, a much longer timecourse
(25 days) of vNL4-3 infection was plotted (Figure 2C). As shown, the NTC p24 release peaked relatively early (day 15) before culture collapse, while that of the knockdown clones peaked around days 19 and 23 (for clones 012 and 013, respectively) before cytotoxic effects dominated the trends. Furthermore, percent inhibition increased over time up to days 9 and 11, as replication in shArp3-012 and shArp-013 was delayed as compared to the NTC control (Figure 2C).

Effects of Arp3 knockdown on cellular proliferation rate

To ensure that the relative reduction in p24 release from earlier experiments was not due to reduced cell growth, a cellular proliferation assay was performed (Figure 3). While shArp-012 exhibited cell growth rates similar to NTC, shArp3-013 grew at a lower rate. Specifically, day 8 cell numbers were around 60% of NTC; though, earlier samples had less of a difference in absolute cell number.

Comparison of VSV-G and wt vNL4-3 infection in Arp knockdown cells

As VSV-G envelope mediates an endocytic entry route, and appears to be much less reliant on the actin cytoskeleton, shArp-012 and shArp-013 were infected with both wild-type vNL4-3 and single-cycle vNL4-3 (VSV-G), which lacks the native env ORF. As shown in Figure 4, both shArp3-012 and shArp3-013 supported VSV-G envelope-mediated vNL4-3 infection, and subsequent p24 release, at a rate of approximately 33% of the NTC. Whereas, wt vNL4-3 p24 release was less than 6% of the NTC for shArp3-012, and less than 2% for shArp3-013. This would indicate that the inhibitory effect is
somewhat specific to wild-type Env-mediated infection, and that VSV-G pseudotyping mitigates the barrier to HIV-1 replication, albeit incompletely.
Figure 1

Figure 1—Replicative capacity of vNL4-3 in shArp3 clones. Top panel: Days 0-7 p24 release profile from infected NTC (black) and shArp3 (colored) cells. p24 was measured as ng per ml. Bottom panel: Day 7 p24 titer in ng/ml, with asterisks denoting statistical significance (p < 0.05) compared to NTC for all indicated clones, excluding shArp3-008, which enhanced viral replication.
Figure 2

Figure 2—vNL4-3 replicative capacities in shArp3-012 and shArp3-013 during short-term and long-term infections. Top panel: p24 release curve (ng/ml) over 7 days. Middle panel: A column graph of the % p24 release of the NTC control day 7 post-infection, at 3.1% for shArp3-012, and 1.9% for shArp3-013. Bottom panel: 25-day timecourse of p24 release (pg/ml) exhibiting the delayed replication kinetics of HIV-1 in these cells.
Figure 3—Effects of Arp3 knockdown on cell growth rates in shArp3-012 and shArp3-013. 5×10⁴ of each cell was resuspended into 1 ml of RPMI supplemented with 10% FBS and cultured over 8 days. Each day, 50 µl of sample was used to determine the cell concentration in triplicate, and cells were passed to ensure proper culture conditions. Cell number was adjusted from the combined passages and extrapolated from the initial 50,000 cells. As shown, shArp3-013 exhibited a more significant growth rate inhibition than shArp3-012 and NTC, the latter two being more similar in overall growth rates.
Figure 4—VSV-G versus Env-mediated infection in shArp3-012 and shArp3-013. Cells were infected with 200 µl of vNL4-3 wt or vNL4-3 (VSV-G) for two hours prior to washing and resuspension. In the top panel, VSV-G envelope infection-mediated p24 release is shown. In the bottom panel, wild-type p24 release is shown.
Discussion

In this chapter, having established a stable system for studying the role of Arp2/3 in HIV-1 replication, shArp3 clones were subjected to HIV-1 infection. 8 out of 9 clones, or 89%, exhibited statistically significant reductions in HIV-1 p24 release; furthermore, two of these clones, shArp3-012 and shArp3-013, reduced HIV-1 replication around 90% and were chosen for further study. This finding broadly recapitulated the findings of Komano et al. (2004) and Harmon et al. (2008 and 2010)\(^{36,37}\), indicating the importance of Arp2/3 in HIV-1 replication. shArp3-008, on the other hand, was an outlier, exhibiting relatively high levels of Arp3 knockdown with higher viral p24 release than NTC, indicating that this clone was unique. Perhaps other cellular factors, or a high level of residual Arp2/3 activity, compensated, allowing higher levels of viral replication.

Another p24 release experiment with more robust viral replication indicated that the replicative inhibition was quite severe, resulting in greater than 95% inhibition of p24 release. Moreover, that this reduction in p24 release could not be predominantly attributed to reductions in cellular proliferation rates, as shArp3-012 growth rates were similar to NTC, and shArp3-013 growth rates were only 40% less than NTC. This block in efficacious viral replication resulted in delayed viral replication kinetics, which broadly correlated with the severity of p24 release inhibition, and, further, with the level of Arp3 knockdown. Although, it should be noted that when p24 release inhibition was plotted against Arp3 expression levels across all clones (data not shown), no obvious trend was observed, and this might be partially related to the fact that 8 out of 9 clones
exhibited pronounced (>50%) reductions in Arp3 expression levels, with those 8 having >90% Arp3 knockdown; thus, the small differences in Arp3 expression levels (<10%) between most clones were not suitable for such an analysis. Furthermore, this may indicate that subtle differences in residual Arp2/3 function, or the function of other cellular factors, primarily determined the degree of severity in the HIV-1 p24 release phenotype. Additionally, the delay in replicative kinetics may indicate that HIV-1 overcame the diminishment in cellular Arp2/3 complex during later viral cycles; although, the mechanism by which this occurred is not known.

To determine if the inhibition was related to the viral mode of entry, VSV-G pseudotyping of vNL4-3 and subsequent infections with the wt vNL4-3 and vNL4-3 (VSV-G) viruses were performed. Pseudotyping with VSV-G reduced the viral replication defect to 40% of NTC, indicating that the mode of entry did affect the replicative capacity of HIV-1. Importantly, VSV-G pseudotyped HIV-1 enters cells through clathrin-mediated endocytosis \(^{87,88}\), whereas, HIV enters cells through plasma membrane fusion \(^{89}\) or a macropinocytosis-like route, and is likely cell-type dependent \(^{90,91}\). Furthermore, that VSV-G-mediated endocytosis of vNL4-3 allows it not only to enter the cells via a different route, but also bypass some of the post-entry requirements of the actin cytoskeleton \(^{45}\). Thus, the relative increase in the efficiency of VSV-G-mediated entry and infection is likely related to the reduced requirements for actin cytoskeletal dynamics observed in normal Env-mediate entry and post-entry events \(^{34,43,45}\). However, clathrin-mediated endocytosis, which is utilized by the VSV-G
envelope, does require Arp2/3 activity \(^92\): as such, it is tempting to speculate that most, and perhaps all, of the defect in VSV-G-pseudotyped HIV-1 infection is due to entry.
Chapter Four—exploring the mechanism of the reliance on the Arp2/3 complex for HIV-1 replication

Introduction

As afore-mentioned, HIV relies on the actin cytoskeleton at multiple stages in viral replication. These include receptor clustering and entry\textsuperscript{39–41,79}, reverse transcription\textsuperscript{42,53}, nuclear migration\textsuperscript{34,43,45,45}, nuclear export of viral mRNA\textsuperscript{93}, and assembly, release, and cell-cell transmission\textsuperscript{80,94–97}. Furthermore, the role of Arp2/3 in HIV-1 infection has been specifically addressed in prior studies, which generated discrepant results regarding the role of Arp2/3 as required for viral entry\textsuperscript{36–38} versus post-entry events\textsuperscript{70}.

In prior chapters, we created a panel of clones of stable Arp3 knockdown cells, derived from CD4 T cell lymphoblastic CEM-SS cells. Utilizing this, the HIV-1 replication defect upon Arp2/3 inhibition was broadly recapitulated in our cellular system\textsuperscript{36–38,70}. However, the mechanism by which Arp3 depletion results in reduced replication efficiency has not yet been addressed in our current study. In this chapter, the mechanism of viral replication inhibition is explored. Specifically, viral entry, reverse transcription, nuclear migration, and transcription of early viral transcripts are all explored.
From these experiments, we exhibited that Arp3 knockdown has no-to-modest effects of viral fusion, using two different entry assays. Furthermore, that Arp3 knockdown does not interfere with viral reverse transcription, but does block viral nuclear migration. Additionally, early nef transcripts were reduced in Arp3 knockdown cells, but only to the extent that nuclear migration was inhibited, corroborating the prior findings.

Materials and Methods

Virus Preparation and Infection: Virus stocks of HIV-1_{NL4-3} were prepared by transfection of HEK293T cells with cloned proviral DNA as described 98. Single-cycle virus HIV-1(VSV-G) and HIV-1(Env) were prepared as previously described 45. Levels of p24 in the viral supernatant were measured in triplicate on the same ELISA plates using an in-house ELISA Kit. Viral titer (TCID$_{50}$) was determined on the Rev-dependent GFP indicator cell Rev-CEM 99,100.

For viral infection, unless otherwise specified, $10^{3.5}$ to $10^{4.5}$ TCID$_{50}$ units of HIV-1 were used to infect 2×10$^5$ CEM-SS cells, either carrying shRNA knockdown or not. Infections were carried out for 2 hours at 37°C, washed twice, and then resuspended into fresh medium (2×10$^5$ cells per ml). Culture supernatant was taken for p24 ELISA.

Viral Entry Assays: The BlaM-Vpr-based viral entry assay was performed as previously described 34,101. In brief, 1×10$^6$ cells were infected with BlaM-Vpr virus for 2 hours at 37°C. Where indicated, cells were treated with 100 ng/ml T20 peptide to block entry.
After this, cells were washed and loaded with CCF2 substrate for 1 hour at room temperature. Cells were subsequently washed and resuspended in development solution, within which the fluorogenic reaction was allowed to develop for 16 hours in the dark before performing flow cytometry. A Nef-luciferase-based entry assay was also performed as previously described \(^{102}\). Briefly, cells (1×10\(^6\)) were infected with 130 ng of Nef-luciferase containing viruses at 37 °C for 2 h, and then washed three times with medium. Cells were resuspended in 0.1 ml of luciferase assay buffer (Promega), and luciferase activity was measured in live cells using a GloMax-Multi Detection System (Promega).

For measuring the entry of Nef-luciferase tagged HIV-1(VSV-G), 2×10\(^5\) cells were infected with 500 µl of virus for 2 h at 37°C. Where indicated, cells were also pretreated with 20 mM NH\(_4\)Cl for 1 h at 37°C, and infected in the presence of 20 mM NH\(_4\)Cl for 2 h. Afterward, cells were washed twice with 3 ml of ice-cold, serum-free RPMI, and once with 1 ml of PBS. Cells were resuspended in 0.1 ml of luciferase assay buffer (Promega), and luciferase activity was measured. Data were normalized to within-experiment NTC averages from three separate experiments, with five reads from each experiment.

**Quantitative Real-time PCR**: Viral DNA quantification was carried out using the Bio-Rad iQ5 real-time PCR detection system, utilizing the forward primer 5’LTR-U5, the reverse primer 3’ gag, and the probe FAM-U5/gag \(^{34}\). Pre-qualified, full-length proviral plasmid pNL4–3 was used as the DNA standard. Viral DNA and 2-LTR circles in shRNA lentiviral vector- transduced cells (shNTC, shArp-12, and shArp-13) were measured as
described previously. For measuring 2-LTR-circles, the DNA was amplified by real-time PCR with primers and probe MH536, MH535, and MH603. For real-time PCR quantification of the nef transcripts, total cellular RNA was extracted with SV Total RNA isolation system (Promega), and then reverse transcribed into cDNA using random decamers and M-MLV reverse transcriptase as previously described. Nef cDNA was further quantified by real time PCR using primers 5′ Nef (5′-GGCGGCGACTGGAAGAA-3′), 3′ Rev (5′-AGGTGGGTTGCTTTGATAGAGAAG-3′), and the probe Nef/Rev (5′-FAM-CGGAGACAGCGACGAAGGCTCATC-TAMRA-3′).

Statistical Analysis: All statistical analyses were performed by unpaired, two-sample t test assuming a two-tailed distribution.

Results

Entry assays exhibit differential effects of Arp3 knockdown on HIV-1 fusion

To determine the effects of Arp3 knockdown on the first step in viral replication, entry and fusion, two different fusion assays were performed. Firstly, a β-lactamase-Vpr (BlaM-Vpr) fusion assay was performed as described previously. In brief, virions co-package the BlaM-Vpr fusion protein, allowing for infection of target cells and delivery of the BlaM-Vpr protein; after this, cells are loaded with a fluorogenic BlaM substrate that is detectable fluorometrically by flow cytometry. In this assay, NTC displayed 0.97% cells bearing the BlaM-cleaved CCF2 fluorescent substrate; in contrast, only
0.01% of mock-treated cells exhibited fluorescence, and treatment with the fusion inhibitor, T20, reduced fluorescent events to 0.06%, nearly that of the control. These results broadly support the fidelity and specificity of the assay. shArp3-012 displayed enhanced entry, at 1.23% fusion events, and shArp3-013 exhibited a 75% reduction in viral entry, at 0.25% fusion events.

The other assay performed was a Nef-Luc assay, described in. The system is similar to the BlaM-Vpr assay, in that Nef-Luc fusion proteins are incorporated into virions, and cell-associated Luciferase activity, measured luminometrically, corresponds to fusion events. Using this assay, different results were obtained (Figure 2). shArp3-012 exhibited higher fusion activity, as before; however, shArp3-013 exhibited similar fusion activities to those observed in NTC. These results would imply that Env-mediated HIV fusion is not affected in this assay. In contrast, VSV-G pseudotyped virus entry was inhibited in shArp3-012 and -013 by approximately 40%; furthermore, the fusion activity could be completely blocked by treatment with ammonium chloride, which prevents the endosomal acidification necessary for VSV-G fusion.

HIV reverse transcription is not diminished in shArp3 knockdown cells

To determine the effects of Arp2/3 knockdown on HIV-1 reverse transcription, cells were infected over a 48-hour timecourse with single-cycle vNL4-3 (Env). Single-cycle vNL4-3(Env) lacks a functional env ORF, but is pseudotyped with wt Env, allowing only one productive viral infection cycle. Utilizing this virus, total cellular DNA was extracted, and total viral DNA was quantified by qRT-PCR (Figure 3). At all time
points, shArp3-012 and -013 exhibited modestly higher levels of DNA synthesis than the NTC control. This is in partial agreement with the Nef-Luc fusion data (Figure 2), but inconsistent with the BlaM-Vpr fusion data (Figure 1).
Figure 1—shArp3-012 and -013 exhibited divergent phenotypes in the BlaM-Vpr entry assay. 1×10^6 cells were infected with BlaM-Vpr virus for 2 hours at 37°C. Where indicated, cells were treated with 100 ng/ml T20 peptide to block entry. After this, cells were washed and loaded with CCF2 substrate for 1 hour at room temperature. Cells were subsequently washed and resuspended in development solution, within which the fluorogenic reaction was allowed to develop for 16 hours in the dark before performing flow cytometry.
Figure 2—shArp3-012 and -013 exhibit similar wt HIV entry levels in comparison with NTC in the Nef-Luc assay. 130 ng of vNL4-3(Nef-Luc) or 500 µl of vNL4-3(VSV-G, Nef-Luc) were incubated with $1 \times 10^6$ cells for 2 hours at 37°C prior to extensive washing. Where indicated, cells were pre-treated with 20 mM NH$_4$Cl for 1 hour, as well as during the 2-hour infection. Cells were subsequently resuspended in complete Luciferase Assay Buffer, and cell-associated luminescence was measured.
Figure 3: Arp3 knockdown does not reduce HIV-1 reverse transcription in shArp3-012 and -013 cells. At a ratio of approximately 200 µl vNL4-3(Env) to 10^6 cells, cells were infected at the indicated time points before sample lysis and DNA extraction. Total viral DNA was amplified from 100 ng input DNA, and total viral DNA is expressed as relative copies per 100 ng input DNA.
HIV-1 nuclear migration and resulting nef transcription are impaired in shArp3 knockdown cells

Following, or concomitant with, viral reverse transcription, the viral DNA in the PIC must migrate to and translocate within the nucleus. The movement of viral DNA to the nucleus can be measured by a correlative, the 2-LTR circle \(^\text{43,45}\): in brief, a portion of viral DNA enters the nucleus and is modified by host non-homologous end joining repair factors that join the two terminal repeats \(^\text{107}\). As this only occurs in the nucleus, these serve as a marker for nuclear localization of viral DNA, and, hence, of nuclear migration itself. Using qRT-PCR, as before, viral 2-LTR circles were quantified in single-cycle vNL4-3(Env)-infected cells (Figure 4). Nuclear migration was inhibited \(~70\%\) in shArp3-012 cells, and \(~90\%\) in shArp3-013 cells, as compared to the NTC control.

To further corroborate these results, and due to the low sensitivity of the 2-LTR assay, nef transcripts were also quantified by RT-PCR and qPCR. As above, cells were infected with single-cycle vNL4-3(Env). As shown in Figure 5, shArp3-012 and -013 exhibited reductions in viral nef transcripts, with respective diminishments of 78% and 88% at 48 hours post-infection. At 24 hours post-infection, shArp3-013 nef transcripts could not be quantified due to a lack of amplification, and shArp3-012 nef transcripts were reduced by 85% compare to the NTC control. These results are quantitatively similar to the 2-LTR circle data, indicating that this inhibition is not, per se, related to viral transcription, but the lack of viral DNA that migrates to the nucleus.
Figure 4: Arp3 knockdown blocks efficacious 2-LTR accumulation in the nucleus. At a ratio of approximately 200 µl vNL4-3(Env) to 10⁶ cells, cells were infected at the indicated time points before sample lysis and DNA extraction. 2-LTR circle DNA was amplified from 200 ng input DNA, and total viral DNA is expressed as relative copies per 200 ng input DNA.
Figure 5: Arp3 knockdown reduces the production of nef transcripts. At a ratio of approximately 200 µl vNL4-3(Env) to 10^6 cells, cells were infected at the indicated time points before sample lysis and DNA and RNA extraction. 50 ng of total RNA was subject to RT-PCR, followed by qRT-PCR from 10 µl of the resultant cDNA. nef transcript copy numbers are expressed in relative copies per amplification from 25 ng input RNA. ND is “not detectable,” as the signal did not cross the threshold for detection.
Discussion

In this chapter, the mechanism of Arp3 knockdown-mediated inhibition of HIV-1 viral infection was explored. Using two different entry assays, the BlaM-Vpr assay and the Nef-Luc assay, two divergent results were obtained. The BlaM-Vpr assay exhibited modest enhancement of viral entry for shArp3-012, and a 75% reduction in viral entry into shArp3-013 cells. Yet, no such inhibition was observed in the Nef-Luc entry assay, where shArp3-012 exhibited modest enhancement, and shArp3-013 was similar to the NTC. The two assays, although superficially similar, are different; specifically, the Nef-Luc entry assay was performed using 130 ng wt vNL4-3(Nef-Luc), a titer that is typically lower than the BlaM-Vpr assay, which can require 1-2 µg of input p24101,108. The resulting difference in titer may dramatically alter the requirements for viral receptor/coreceptor concentrations and clustering, and the associated signaling events necessary for entry. Furthermore, the total viral DNA data broadly supports the Nef-Luc entry data, showing enhancements in viral DNA in shArp3 knockdown cells at 2 hours post-infection. As such, these data suggest that at higher titers, or in cell-cell fusion experiments36–38, viral entry may require Arp2/3-mediated actin nucleation for receptor clustering, or some other process. Furthermore, the VSV-G-pseudotyped HIV-1 entry data suggested that the previously observed diminishment in VSV-G-mediated infection (Chapter 3) could largely (~66%) be attributed to a defect in entry. This indicates that in transformed cells, VSV-G pseudotyping does not completely block the need for actin effectors, particularly for endocytic entry.
Reverse transcription was positively affected by Arp3 knockdown, with modest (>1.5-fold) enhancements of viral DNA replication seen through most time points.

Though the reasons for this phenotype were not explicitly explored in this dissertation, the fact that actin and associated dynamics play a role in the efficiency of viral reverse transcription\textsuperscript{53,84} suggests that subtle divergences in the dynamics and structure of the cortical actin network may play a role.

The next step in the viral life cycle addressed was nuclear migration. Using two different correlatives of viral nuclear migration, 2-LTR circle accumulation and nef transcription, viral nuclear migration was found to be the predominant step affected by Arp3 knockdown. That the nef transcription inhibition was quantitatively similar to the 2-LTR circle diminishment indicates that transcription is not affected, \textit{per se}: rather, the viral nuclear templates that are required for transcription are reduced due to the lack of nuclear localization of these templates.

The resulting data seems to indicate that Arp2/3 is directly or indirectly involved in nuclear localization. The model that is currently being proposed in this dissertation is that Arp2/3 directly docks on the viral PIC, mediating nucleation, branching, and force generation necessary for intracellular motility and nuclear localization. Alternatively, HIV-1 may passively associate with the actin cytoskeleton, with Arp2/3 merely generating force and actin treadmilling: this would allow the PIC to migrate towards the nucleus. This model will be more specifically addressed in chapter 6.
As indicated earlier, the results obtained by Harmon et al., indicating a reliance on Arp2/3 and upstream regulators for entry, radically deviate from our own\textsuperscript{36–38}, whereas, our results are more consilient with the findings of Komano et al. (2004), indicating that WCA-mediated dysregulation of Arp2/3 affected viral replication, but not at the level of entry\textsuperscript{70}. Importantly, the Harmon et al. (2008 and 2010) studies used the astroglia cell line, U87.CD4.CCR5, in cell-cell fusion experiments with Env-expressing BSC40 cells, which are African green monkey kidney cells. As such, the effects therein observed may be attributed to the cell types in question, or to the requirements for cell-cell fusion, but not free-viral infection. Although these results were confirmed using free-viral infection of peripheral blood lymphocytes (PBLs)\textsuperscript{36,37}, only p24 release was measured. As such, they assumed that the reduction in p24 was due to entry, but may have been observing the same viral replication defect observed in our studies. From this, and later studies performed in this dissertation, it would appear the phenotypes observed by Harmon et al. (2008 and 2010) may be artifacts of the systems used for characterization.
Chapter Five—Inhibition of Arp2/3 with the Small Molecule inhibitors CK-548 and CK-636

Introduction

In the preceding chapters, the development of the stable Arp3 knockdown cells, shArp3-012 and shArp3-013, allowed the characterization of the role of Arp2/3 during HIV-1 infection. In brief, in corroboration with an earlier study, Arp2/3 was not required for entry or reverse transcription. However, Arp2/3 was critically required for HIV-1 nuclear migration, implying that Arp2/3 may directly associate with the PIC to mediate intracellular motility and nuclear localization, as seen with vaccinia virus and baculovirus.

Recently, Nolen et al. (2009) described two classes of small molecule inhibitors of the Arp2/3 complex. The two classes were based on the two molecules originally identified in a drug screen as Arp2/3 inhibitors; these were CK-548 and CK-636. The two compounds displayed high activity towards Arp2/3, with the CK-548 IC_{50} being 11 µM and CK-636 being 4 µM for Homo sapiens Arp2/3. Unlike CK-636, CK-548 did diminish the interaction with the NPF, N-WASP, implying different mechanisms of action. In line with these observations, CK-636 was observed in X-ray crystallography
to bind differently than CK-548, being bound at the Arp2 and Arp3 interface. It was believed that this interaction would prevent the juxtaposition of Arp2 and Arp3 that is required for Arp2/3 activation and filament nucleation/branching\(^{115}\). In contrast, CK-548 binds subdomain 1 of Arp3 and was postulated to have a less apparent mechanism, possibly related to preventing nucleotide binding by Arp3, or engagement of the pointed end of nucleated filaments by Arp3\(^{115}\).

Using these two inhibitors, we recapitulated their \textit{in vitro} activity in a pyrene-actin polymerization assay performed by Jeffrey Kuhn and colleagues at Virginia Polytechnic Institute and State University. \textit{In vivo}, we exhibited modest chemotaxis inhibition towards the chemokine, SDF-1, for CK-548, but not CK-636. Additionally, changes in cellular F-actin were observed flow cytometrically with FITC-phalloidin staining. In line with the chemotaxis data, CK-548, but not CK-636, exhibited potent anti-HIV activity utilizing a reporter cell line. This was recapitulated in CEM-SS cells, wherein p24 release was inhibited by 25 and 50 \(\mu\)M CK-548. These results were further confirmed in primary resting CD4 T cells, wherein 12.5 and 25 \(\mu\)M CK-548 reduced viral replication without affecting T cell activation. Using the same qRT-PCR techniques from the prior chapter, the block in viral replication was shown to predominantly occur at the level of nuclear migration, with some modest additional effects on viral budding.

\textbf{Materials and Methods}

\textit{Isolation of Resting CD4 T Cells and Monocytes from Peripheral Blood:} All protocols involving human subjects were reviewed and approved by the George Mason University...
Institutional Review Board. Resting CD4 T cells were purified from peripheral blood of HIV-1 negative donors by two rounds of negative selection as previously described. Briefly, for the first-round depletion, we used monoclonal antibodies against human CD14, CD56 and HLA-DR, DP, and DQ (BD Biosciences). For the second-round depletion, we used monoclonal antibodies against human CD8, CD11b, and CD19 (BD Biosciences). Antibody-bound cells were depleted by using Dynabeads Pan Mouse IgG (Invitrogen). For further negative selection of the memory and naïve CD4 T cell subsets, monoclonal antibody against either CD45RA (0.02 µl per million cells) or CD45RO (0.1 µl per million cells) (BD Biosciences) was added during the second round of depletion. Purified cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), penicillin (50 units/ml) (Invitrogen), and streptomycin (50 µg/ml) (Invitrogen). Cells were rested overnight before infection or treatment.

*Virus Preparation and Infection*: Virus stocks of HIV-1<sub>NL4-3</sub> were prepared by transfection of HeLa cells with cloned proviral DNA as described. Single-cycle virus HIV-1(VSV-G) and HIV-1(Env) were prepared as previously described. Levels of p24 in the viral supernatant were measured in triplicate on the same ELISA plates using an in-house ELISA Kit. Viral titer (TCID<sub>50</sub>) was determined on the Rev-dependent GFP indicator cell Rev-CEM.

For viral infection, unless otherwise specified, 10<sup>3.5</sup> to 10<sup>4.5</sup> TCID50 units of HIV-1 were used to infect 10<sup>6</sup> cells. For infection, resting CD4 T cells were incubated with the
virus for 2 h, washed once, and then resuspended into fresh medium (10⁶ cells per ml) and incubated for 5 days without stimulation. Cells were activated with anti-CD3/CD28 magnetic beads at 4 beads per cell. Culture supernatant (100 µl) was taken every 2 days or daily after stimulation. Cells were removed by centrifugation, and supernatant saved for p24 ELISA. Fresh medium was added when needed. CEM-SS cells, either carrying shRNA knockdown or not, were similarly infected for 2 h, washed twice, and then resuspended into fresh medium (2×10⁵ cells per ml). Culture supernatant was taken for p24 ELISA. Rev-CEM cells were also similarly infected, and viral infection was measured by flow cytometry (FACSCalibur, BD Biosciences) of GFP-positive cells. To exclude drug cytotoxicity, propidium iodide (PI) (2 µg/ml, Fluka) was added into the cell suspension prior to flow cytometry, and only viable cells (PI negative) were used for measuring GFP expression.

In Vitro Actin Bead Assay: Carboxylated polystyrene 4.5 µm diameter microspheres (Polysciences, Warrington, PA) were coated with 8.5 µM GST-tagged VCA by incubating for 1 h at room temperature. Particles were pelleted by low speed centrifugation and resuspended in storage buffer (10 mM HEPES pH 7.8, 0.1 mM KCl, 1 mM MgCl₂, 1 mM ATP, 0.1 mM CaCl₂, 0.01% NaN₃) containing 1 mg/ml bovine serum albumin (BSA, Sigma-Aldrich) to block subsequent nonspecific binding. Particles were stored at 4 °C for up to 1 week. For reconstitution of bead motility, glass slides and coverslips were cleaned and blocked overnight in 1% BSA at 4 °C and dried in air before use. We placed 16 µl of reaction mixture on a BSA coated slide, covered with a BSA
coated coverslip, and sealed the chamber with VALAP. Labeled and unlabeled Ca-ATP actin were diluted to the desired labeled fraction, mixed 9:1 with 10× magnesium exchange buffer (10× ME: 10 mM EGTA, 1 mM MgCl₂) and incubated on ice for 2 min to form 4× final concentrations of Mg-ATP actin. We placed 8 µl of Mg-ATP actin at the bottom of a 1.5 ml Eppendorf tube and added 7 µl of motility protein mixtures with or with no Arp2/3 complex inhibitor and 1 µl of coated nanofibers or beads on the side of the tube. We washed both drops together with 16 µl 2× TIRF buffer (2x: 100 mM KCl, 2 mM MgCl₂, 2 mM EGTA, 20 mM imidazole, pH 7.0, 200 mM DTT, 0.4 mM ATP, 30 mM glucose, 0.25% 1500 cP methylcellulose, 40 µg/ml catalase, 200 µg/ml glucose oxidase) and placed the reaction mixture in slide-coverslip as described above. For Image acquisition and processing, actin fluorescence was observed with a 60 × 1.49 NA TIRF objective on an Olym-EXi cooled CCD camera (QImaging) using SlideBook image acquisition software (Intelligent Imaging Innovations, Inc). All subsequent image-processing steps were performed in ImageJ, available at http://rsbweb.nih.gov/ij. Epi-fluorescence microscopy images were unprocessed. Images were cropped for publication.

**Chemotaxis Assay:** A half million Jurkat T cells were resuspended into 100 µl of RPMI 1640 medium and then added to the upper chamber of a 24-well transwell plate (Corning). The lower chamber was filled with 600 µl of medium premixed with SDF-1 (40 ng/ml). The plate was incubated at 37 °C for 2 h, and then the upper chamber was removed, and cells in the lower chamber were counted. Where indicated, CK-548 and
CK-636 (Sigma) were added to the culture supernatant for 2 h prior to the assay along with a DMSO control.

**Quantitative Real-time PCR:** Viral DNA quantification was carried out using the Bio-Rad iQ5 real-time PCR detection system, utilizing the forward primer 5’LTR-U5, the reverse primer 3’ gag, and the probe FAM-U5/gag. Pre-qualified, full-length proviral plasmid pNL4–3 was used as the DNA standard. Viral DNA and 2-LTR circles in shRNA lentiviral vector-transduced cells (shNTC, shArp-12, and shArp-13) were measured as described previously. For measuring 2-LTR-circles, the DNA was amplified by real-time PCR with primers and probe MH536, MH535, and MH603. For real-time PCR quantification of the nef transcripts, total cellular RNA was extracted with SV Total RNA isolation system (Promega), and then reverse transcribed into cDNA using random decamers and M-MLV reverse transcriptase as previously described.

**Conjugation of Antibodies to Magnetic Beads:** Monoclonal antibodies against human CD3 (clone UCHT1) and CD28 (clone CD28.2) were from BD Pharmingen (BD Biosciences). For conjugation, 10 µg of antibodies were conjugated with \(4 \times 10^8\) Dynabeads Pan Mouse IgG (Invitrogen) for 30 min at room temperature. Free antibodies were washed away with PBS + 0.5% BSA, and the magnetic beads were resuspended in 1 ml of PBS + 0.5% BSA.

**CD25 and CD69 surface staining:** Resting CD4 T cells were isolated and rested for 24 hours at 37°C and 5% CO₂. Cells were incubated for 2 hours in the presences of DMSO or 50, 25, or 12.5 µM CK-548. Cells were then activated using α-CD3/CD28 beads, at 4
beads per cell and incubated for an additional 24 hours. Beads were removed by pipetting the cells repeatedly, and then the tubes were placed on a magnetic particle concentrator. Cells were then transferred to flow cytometry tubes, and pelleted at 1,200 rpm for five minutes. After decanting the supernatant, surface IgG was blocked using 1 µl of ChromPure mouse IgG (Jackson ImmunoResearch Lab, Cat. No. 015-000-003). Cells were then stained for 30 minutes with mouse PE-CD25 (BD, Cat. No. 555432) or PE-CD69 (BD, Cat. No. 557050), or the isotypes, PE mouse IgG1κ (BD, Cat. No. 550617). After this, cells were washed twice with PBS + 0.1% BSA and resuspended into 400 µl 2% paraformaldehyde before flow cytometric analysis.

**Statistics:** All statistical analysis was performed using an unpaired Student’s t-test with an \( \alpha < 0.05 \). IC\textsubscript{50} calculations were based on sigmoidal curve fit of dosage versus inhibition using the equation \( S(x) = A + \frac{B}{1 + (x/C)^D} \), where A-D are solved coefficients for the minimized sum of squares, \( S \) is the % inhibition, and \( x \) is the dosage in \( \mu \text{M} \). In this equation, \( C \) corresponds the \( K_m/\text{IC}_{50} \).

**Results**

*CK-548 and CK-636 inhibit Arp2/3-dependent actin polymerization in vitro*

To corroborate the findings of Nolen *et al.* (2009), both CK-548 and CK-636 were tested *in vitro* using a pyrene-actin polymerization assay. In brief, and as illustrated in Figure 1, pyrene-labeled actin is mixed with recombinant profilin, capping protein, divalent cation-ATP, and Arp2/3 complex along with WCA-coated polystyrene beads:
inhibition of Arp2/3, the complex itself or its association with WCA, results in reduced polymerization-stimulated pyrene-actin fluorescence, which can be measured by fluorometry and observed by TIRF microscopy. As shown in Figure 1, in the DMSO control, beads developed significant polymerization, associated fluorescence, and acquired an actin comet tail, which promoted bead motility in the reaction. However, in the presence of the inhibitors, actin comet tail formation was abrogated, and bead-associated polymerization was reduced. This reduction in actin comet tail formation was quantified, indicating IC$_{50}$ values of ~15 µM, which is broadly consistent with Nolen et al. (2009).

*CK-548, but not CK-636, diminishes chemotaxis towards SDF-1 in Jurkat cells*

Having established and confirmed that CK-548 and CK-636 are *bona fide* Arp2/3 inhibitors, the *in vivo* effects of these two inhibitors were addressed. First, cells were treated with 50 µM CK-548 or CK-636 to determine whether these inhibitors promote actin depolymerization. As expected from their *in vitro* activities, modest actin depolymerization occurred in the presence of both inhibitors; however, the depolymerization observed in CK-548-treated cell was noticeably more pronounced at certain time points, further indicating that CK-548 may be a more potent inhibitor in this cell system (Figure 2). In broad agreement with this, chemotaxis in Jurkat cells towards the chemokine, SDF-1, was also reduced by ~33%, but only by CK-548. Although the mechanism of differential drug efficacy was not addressed in this work, it was assumed that likely factors would include (1) intracellular delivery of the drugs, (2) intracellular
stability of the drugs, and/or (3) differential mechanism of action creating notably divergent physiological responses.
Figure 1: CK-548 and CK-636 inhibit Arp2/3-mediated actin polymerization in vitro.

WCA-coated polystyrene beads were incubated with pyrene-labeled actin, profilin, capping protein, and Ca$^{2+}/$Mg$^{2+}$ ATP in the presence or absence of Arp2/3 inhibitors. Shown in the top panel is the actin comet tails (CT) observed in the DMSO control, and the lack of comet tails observed in the presence of Arp2/3 inhibitors, quantified in the lower right panel.
Figure 2: CK-548 and CK-636 depolymerize actin in treated CEM-SS cells. Cells were treated with 50 µM CK-548, CK-636, or DMSO for a 2-hour timecourse, and then stained for F-actin using phalloidin-FITC. DMSO treatment did not change cellular F-actin over the timecourse, and only the 2-hour data is shown.
Figure 3

Figure 3: CK-548, but not CK-636, diminished Jurkat cell chemotaxis towards SDF-1. Where indicated, cells were treated for 2 hours with CK-548, CK-636, or DMSO for 2 hours. Cells were then plated on the top of a transwell plate, with 40 ng/ml SDF-1 in RPM 1640 with 10% FBS lower chamber. After 2 hours, cells in the lower chamber, which had migrated to SDF-1, were counted in triplicate.
**CK-548 diminished HIV-1 replication in CD4 T cells**

To test the effects of CK-548 and CK-636 on HIV replication, a CD4 T reporter cell line, Rev-CEM, was used. In brief, this cell line expresses GFP in the presence of two viral accessory genes, Tat and Rev, which are necessary for transcription and export of the GFP-encoding mRNA, respectively\(^\text{100}\). As such, GFP expression should only occur in the presence of HIV infection. In this system, we pretreated cells for 2 hours using either DMSO, 12.5, 25, 50, or 100 \(\mu\)M of each drug, and analyzed the cells flow cytometrically 72 hours post-infection (Figure 4). Dose-dependent inhibition of HIV replication was observed for CK-548, but not CK-636. The IC\(_{50}\) of HIV-1 replication inhibition by CK-548, as calculated from a sigmoidal fit with the limited set of data points, was \(~22\ \mu\)M. Increased cytotoxicity was only observed at 100 \(\mu\)M of CK-548 by propidium iodide staining (Figure 5); as such, 25 and 50 \(\mu\)M were used for all future analysis. To corroborate these findings, HIV-1 p24 release was also monitored over a longer timecourse in CEM-SS cells (Figure 6). As shown, stronger inhibition of CK-548, at 25 and 50 \(\mu\)M, was observed in the longer timecourse, which likely relates to the additive effect of inhibition over multiple viral life cycles.
Figure 4: CK-548, but not CK-636, inhibits viral replication in Rev-CEM reporter cells. 2×10^5 Rev-CEM cells were pretreated with DMSO, CK-548, or CK-636 at the indicated concentrations for 2 hours prior to a 2-hour infection with wt vNL4-3 in the presence of drugs. Cells were subsequently washed, and incubated for 72 hours before performing a propidium iodide stain and flow cytometric analysis.
Figure 5: Only higher dosages of CK-548 are cytotoxic in CEM-SS-derived Rev-CEM reporter cells. Cells were treated and infected as described in Figure 4, and the propidium iodide-positive (dead) cell populations were plotted against CK-548 concentration.
Figure 6: CK-548 inhibits HIV p24 release in CEM-SS cells. $2 \times 10^5$ were pretreated for 2 hours with CK-548 or DMSO prior to a 2-hour infection with 500 µl vNL4-3 in the presence of drug. Cells were subsequently washed three times, and p24 samples from cleared supernatant were taken every 24 hours for an ELISA.
**CK-548 does not inhibit total viral DNA synthesis**

As with the knockdown cell lines, the mechanism of action of CK-548 was ascertained by following the viral life cycle, starting with total viral DNA synthesis. 1×10⁶ CEM-SS cells were pretreated with 12.5, 25, and 50 µM for one hour prior to infection. Cells were then infected with 1 ml vNL4-3 (Env) single-cycle virus, followed by extensive washing, and then samples were collected at 12 and 48 hours post-infection. After DNA extraction, qPCR was performed, exhibiting that total viral DNA synthesis was not inhibited at later time points (48 hours post-infection, Figure 7). However, the 12-hour time point showed reductions in total DNA synthesis, indicating that the kinetics of reverse transcription may have been delayed.

**CK-548 diminishes HIV 2-LTR circle accumulation**

The same DNA samples from the prior experiment were subjected to qPCR for 2-LTR circle DNA, which only appears in the nucleus. As shown in Figure 8, total 2-LTR circle DNA copies appeared to only decrease at higher dosages at 48 hours post-infection. However, at 12 hours post-infection, no detectable 2-LTR circles were observed. Furthermore, when normalized to total viral DNA, there was a significant effect at 25 and 50 µM CK-548. Furthermore, no 2-LTR circle DNA could be detected at 50 µM at any of the time points, indicating that the process of nuclear migration was almost completely abrogated.
Figure 7: Total viral DNA synthesis is not inhibited by CK-548 treatment. $1 \times 10^6$ cells per sample were pretreated for 1 hour, infected for 2 hours, washed, and then resuspended for 12 and 48 hours, whereupon samples were collected for DNA extraction. Shown are the resultant qPCR results as copies per 100 ng input DNA.
Figure 8: CK-548 reduces viral 2-LTR circle accumulation in the nucleus. The same DNA samples from figure 7 were probed for 2-LTR circle DNA by qPCR using 200 ng input DNA. Shown on the left is the total quantities of 2-LTR circle DNA; whereas, on the right, the ratio of 2-LTR:total viral DNA is shown, exhibiting the defect in nuclear accumulation. ND = Not detectable.
**CK-548 has a modest effect on HIV budding**

As actin has been implicated in HIV budding, the effects of CK-548 in viral budding were tested. $2 \times 10^5$ cells were infected with vNL4-3 (Env) single-cycle virus for 12 hours. After this, cells were washed extensively and 12.5, 25, 50 µM CK-548, or DMSO, was added and p24 samples were taken at 12-120 hours post-infection. As exhibited in Figure 9, modest effects on viral budding were observed at later time points for the higher dosages, with inhibition rates ~30% at 25 and 50 µM.

**CK-548 inhibits viral replication in latently infected CD4 T cells without affecting T cell activation**

To test the effects of CK-548 on primary, resting CD4 T cells from peripheral blood, $1 \times 10^6$ resting CD4 T cells were pretreated with DMSO, 12.5, 25, or 50 µM CK-548. After this, cells were infected for 2 hours, washed, and cultured for 5 days without T cell activation. During this time, p24 samples were harvested at days 0, 1, 3, and 5. Also, on day 5, each sample was activated by adding α-CD3/CD28 antibody-coated beads at a ratio of 4 beads per cell. After activation, p24 samples were taken every 24 hours through day 12 post-infection. As shown in Figure 10, all dosages of CK-548 blocked viral replication nearly completely. To determine if this effect was due to CK-548 interfering with the CD4 T cell activation, and thus diminishing the viral replication, surface staining of the T cell activation markers CD25 and CD69 were performed as described in the materials and methods (Figure 11). As indicated, the 12.5 and 25 µM CK-548 treatment
groups exhibited no defect in T cell activation marker expression; whereas, the 50 μM group exhibited a defect in CD25 expression, but not CD69 expression.
Figure 9: CK-548 modestly decreases HIV-1 budding. $2 \times 10^5$ cells were infected for 12 hours prior to washing and the addition of DMSO or 12.5, 25, or 50 μM CK-548. After this, p24 samples were taken from 12-120 hours post-infection and quantified by p24 ELISA. Samples were quantified in triplicate from the same experiment.
Figure 10: CK-548 also inhibits HIV-1 replication in primary resting CD4 T cells. $1\times 10^6$ resting CD4 T cells were pretreated with DMSO or CK-548 at the indicated dosages, and then infected with vNL4-3 wt for 2 hours. After this, cells were washed and then cultured for 5 days without cell activation, with p24 samples collected every 48 hours. Cells were
then stimulated with α-CD3/CD28 antibody-coated beads, and p24 samples harvested every 24 hours. Samples were quantified by p24 ELISA as before.
Figure 11: Neither 12.5 nor 25 µM CK-548 inhibit CD4 T cell activation marker expression. $1 \times 10^6$ resting CD4 T cells were treated with the indicated concentration of CK-548 for 2 hours prior to activation with α-CD3/CD28 beads. CD25 and CD69 surface staining was performed 24 hours post-activation.
Discussion

In this chapter, we described the *in vitro* and *in vivo* effects of the recently described Arp2/3 inhibitors, CK-548 and CK-636. Firstly, recapitulating the previously described *in vitro* function of these inhibitors, both inhibitors could inhibit *in vitro* Arp2/3-dependent actin polymerization in a pyrene-actin bead assay. Both inhibitors reduced the bead associated polymerization and prevented the development of stable actin comet tails that were observed in the DMSO control, and both these activities require active Arp2/3 in this particular assay. Furthermore, the IC$_{50}$’s for actin comet tail disruption were approximately 10-15 µM for both inhibitors, which were marginally higher than that reported by Nolen *et al.* (2009), with CK-548’s being 11 µM and CK-636’s being 4 µM. The first *in vivo* test, in our study, measured the effects of both inhibitors on cellular F-actin: notably, while both inhibitors clearly downmodulated cellular F-actin, CK-548 had more robust effects throughout most of the timecourse. The second *in vivo* test focused on the capacity to disrupt Jurkat CD4 T cell chemotaxis to SDF-1, a chemokine that binds the HIV-1 coreceptor, CXCR4. In this assay, CK-548 diminished Jurkat chemotaxis by 33%, but no such effect was observed for CK-636. It is important to note that though Arp2/3 is important for certain forms of motility, like extracellular matrix-associated chemotactic cues in fibroblasts, it may be partially dispensable in other contexts. As such, complete inhibition of Jurkat chemotaxis may not be possible with Arp2/3 inhibitors. Additionally, the fact that in both the actin staining and chemotaxis assays, CK-636 showed consistently lower activity, implies that
either the mechanism of CK-636 action, or some other feature, reduced its effectiveness 
in vivo. In line with this, Nolen et al. (2009) described two notable differences between CK-548 and CK-636 when initially describing their various in vitro and in vivo effects 
115. These differences were the effect of CK-548 on WCA affinity and the differing mechanisms of action due to different binding sites 115. More specifically, that CK-636, unlike CK-548, did not inhibit interaction with the WCA domain, and that CK-636 bound at the Arp2-Arp3 interface, which immediately indicated the likely mechanism of action—preventing the necessary juxtaposition and structural rearrangements necessary for actin nucleation by the Arp2-Arp3 dimer 115. The mechanism of action of CK-548 was far less clear, as it bound a pocket in Arp3, and the mechanism may be related to nucleotide binding or binding of the daughter filament 115,118.

Next, the effects of these two inhibitors in HIV infection were directly addressed using an HIV-1-dependent reporter cell line, Rev-CEM 100. As with Jurkat chemotaxis, CK-548, but not CK-636, inhibited HIV-1 infection in a dose-dependent manner, with an IC50 of 22 µM. Ultimately, CK-636 was abandoned for the rest of this study for its lack of activity, which, as discussed above, might relate to the different mechanisms of action of each drug. The results with CK-548 were recapitulated with CEM-SS p24 release data, which, due to the longer timecourse and the additive effects on multiple viral replication cycles, indicated that larger degrees of inhibition could be achieved.

This block in viral replication was not due to a decrease in net accumulation of total viral DNA, as at 48 hours post-infection, total viral DNA exceeded the DMSO
control. Conversely, at 12 hours post-infection, diminishment was observed for 25 and 50 µM CK-548, and this kinetic delay may reflect the need for particular configurations of cortical actin necessary for reverse transcription and, possibly, uncoating. Though DNA synthesis was not significantly inhibited over the entirety of the timecourse, nuclear accumulation of HIV-1 2-LTR DNA was inhibited. As above, delayed kinetics were observed, with all drug dosages tested reducing 2-LTR circles to below the limits of detection at 12 hours post-infection. Furthermore, 2-LTR circle accumulation relative to total viral DNA at 48 hours post-infection showed significant reductions (~60%) at 12.5 and 25 µM, and undetectable levels of 2-LTR circles at 50 µM. This, as with the Arp3 knockdown cells, exhibits that the predominant stage in viral replication inhibition occurs at the level of nuclear localization.

In order to quantify whether later stages in viral infection were involved, CK-548 was added 12 hours post-infection to negate most of the early impacts of CK-548 on nuclear migration. In this experiment, only modest reductions in viral release of p24 were observed. It is important to note that, even as late as 12 hours post-infection, some effect on nuclear localization may still have occurred, and it cannot be ruled out that the inhibition observed may relate to this antiviral activity of CK-548. Actin has been implicated in budding and release, but this has been recently disputed. Regardless, our results do not necessarily support or refute a small potential role for Arp2/3 in HIV budding.
Lastly in this chapter, we tested the effects of CK-548 on primary resting CD4 T cell latent infection. All dosages tested, including 12.5 µM, blocked viral replication throughout the timecourse. Given that 12.5 µM CK-548 inhibited replication to the same extent as 100 µM in Rev-CEM implies that resting CD4 T cells are more sensitive to subtle changes in the actin cytoskeleton as relates to the efficiency of HIV-1 infection. Congruently, this has been reported in other contexts, such as sensitivity to spinoculation-mediated HIV-1 infection enhancement, which is related to actin dynamics. This further implies that for primary CD4 T cells, the IC$_{50}$ of CK-548 may be quite low, perhaps sub-micromolar. Furthermore, this inhibition of HIV-1 replication did not reduce the markers of cellular activation, CD25 and CD69—with one exception—indicating that no cytotoxic or cytostatic effects were occurring. Regarding the exception, 50 µM CK-548 did reduce CD25 expression to 50% of the DMSO, but did not affect the expression of CD69. As such, this effect may itself be reliant on changes to CD25 trafficking, and may not necessarily reflect a defect in cellular activation, which might explain the discrepancy between the CD69 and CD25 data. Alternatively, 50 µM CK-548 may genuinely reduce cellular activation, and CD25 expression might be a more sensitive readout of this.
Chapter Six—Co-receptor-dependent signal transduction mediates Arp2/3 activation

Introduction

Arp2/3 is a critical regulator of actin dynamics, playing pivotal roles in endocytosis, lamellipodium formation and certain forms of chemotaxis, cytokinesis, and cadherin junctions. It is perhaps not surprising that HIV, which has previously been shown to require actin dynamics for infection, also requires Arp2/3. Dependence on Arp2/3 for motility and spread has also been observed for baculovirus, vaccinia virus, and the bacteria, *Listeria monocytogenes* and *Shigella flexneri*. Canonically, Arp2/3 is activated by the cellular Nucleation-Promoting Factors (NPFs), which including N-WASP and WASP, WAVE1-3, and the more recently described WASH, WHAMM, and JMY. Each of these NPFs contain, in addition to a variable N-terminus which defines the spatiotemporal regulation of each NPF, a C-terminal WCA domain that is directly responsible for Arp2/3 activation. For baculovirus and *Listeria monocytogenes*, Arp2/3 activation is directly encoded by WCA sequences in p78/83 and ActA, respectively. For *Shigella* and vaccinia, pathogen proteins indirectly activate Arp2/3 by coopting cellular NPFs and their associated signaling networks. In the case of HIV, no WCA-type sequences...
were observed in any of the HIV genes by protein BLAST, suggesting that if HIV actively engages Arp2/3, it likely does so indirectly by cooption of NPF-associated signaling networks. Alternatively, a WCA-type motif might be encoded discontinuously, but there are currently no reports that indicate this. As such, it would be germane to briefly consider the normal modes of Arp2/3 activation and their associated signaling networks.

Actin cytoskeletal reorganization is normally mediated by the Rho-family GTPases, which includes 23 members among 7 families\textsuperscript{130}. Like most other monomeric GTPases, the Rho GTPases cycle between an active GTP-bound form and an inactive GDP-bound form. The activation of the Rho GTPases is mediated by Guanine-nucleotide Exchange Factors (GEFs), and inactivation by GTP hydrolysis is greatly stimulated by GTPase-Activating Proteins (GAPs)\textsuperscript{130}. Additionally, Rho GDP Dissociation Inhibitors (Rho GDIs) sequester Rho GTPases in the cytosol, and provide an additional layer of negative regulation\textsuperscript{131}. There are currently 69 known Dbl-Homology (DH) GEFs in the human genome, along with 11 Dock-Homology Region (DHR) GEFs\textsuperscript{132}, and a predicted 59-70 GAPs\textsuperscript{133}, which implies the degree of specificity of Rho GTPase regulation during coordinated cellular processes.

Though most Rho-family members are not well studied, largely owing to tissue-specific expression and quasi-redundant functions, the canonical family representatives of the Rac, Cdc42, and RhoA GTPase families have been extensively studied. These proteins in turn regulate signaling motifs that directly impinge on the actin cytoskeleton
Among the most relevant Rho effectors for this dissertation are the NPFs. For instance, Rac1 primarily activates WAVE proteins by binding to Sra-1 in the WAVE Regulatory Complex (WRC); whereas, Cdc42 binds the GTPase-Binding Domain (GBD) of WASP/N-WASP. Although the regulation of the more recently described NPFs is quite poorly understood, RhoD may regulate WHAMM. However, N-WASP/WASP and WAVE1-3 regulation are not solely determined by Rho GTPase binding. For instance, WAVE activity can also be modulated through multiple phosphorylation events: the non-receptor tyrosine kinase, Abl, phosphorylates Y150 on WAVE2 (Y151 for WAVE1 and 3) to promote full activation, in addition to phosphorylating a number of residues in ABI1, a component of the WRC. WAVE proteins are also modulated by CK2, CDK1 and 5, and ERK-mediated phosphorylation. ERK, along with Abl, phosphorylation seem to be required for optimal WRC activation and function. Phosphoinositide-3,4,5-triphosphate (PIP₃) also promotes full WRC activation by binding the WAVE basic domain. WASP and N-WASP activity are also regulated by phosphorylation and PIP₂ binding, albeit with N-WASP being more reliant on PIP₂. Because WAVE and WASP proteins are so intricately regulated, it is often difficult to ascertain whether they are activated in a cell, without directly imaging WASP/WAVE-specific perturbations in the actin cytoskeleton. As such, we used the ERK phosphorylation site, S351, as a proxy for detecting WAVE activation. Additionally, it was previously reported that Rac1 and PI3K are activated downstream of HIV-1 gp120 binding to CXCR4, which broadly indicates that the Arp2/3 signaling axis is likely activated downstream of gp120.
In this chapter, we explore the upstream regulators, N-WASP/WASP and WAVE2. Furthermore, we show that the N-WASP/WASP-inhibitors 187-I and wiskostatin have no significant effect on HIV-1 infection in reporter cells. Furthermore, we show that WAVE2, contrarily, exhibits S351 phosphorylation, which is consistent with WAVE2 being activated downstream of receptor engagement. This phosphorylation event occurs following treatment with both R5 and X4 viruses, indicating that both coreceptors can induce WAVE2 S351 phosphorylation. Additionally, this activation is largely blocked by the CXCR4 antagonist, AMD3100 \(^\text{140}\). Treatment with PTX, which prevents G\(_i\)-dependent coreceptor signaling, also abrogates late, but not early, WAVE2 phosphorylation.
Figure 1: Signaling motifs of Rac1, Cdc42, and RhoA. The Rho GTPases are activated downstream of GPCR, integrin, and RTK pathways, among others, by GEFs. Each Rho-family GTPase then activates conserved signaling motifs like the Rac1-PAK-LIMK and Rac-WAVE-Arp2/3 pathways. These coordinated signaling modalities in turn drive specific actin rearrangements leading to lamellipodium, filopodium, uropod, or actin stress fiber formation. Adapted from Spear et al. (2014).
Materials and Methods

Isolation of resting CD4 T Cells and monocytes from peripheral blood: All protocols involving human subjects were reviewed and approved by the George Mason University Institutional Review Board. Resting CD4 T cells were purified from peripheral blood of HIV-1 negative donors by two rounds of negative selection as previously described

Briefly, for the first-round depletion, we used monoclonal antibodies against human CD14, CD56 and HLA-DR, DP, and DQ (BD Biosciences). For the second-round depletion, we used monoclonal antibodies against human CD8, CD11b, and CD19 (BD Biosciences). Antibody-bound cells were depleted by using Dynabeads Pan Mouse IgG (Invitrogen). For further negative selection of the memory and naïve CD4 T cell subsets, monoclonal antibody against either CD45RA (0.02 µl per million cells) or CD45RO (0.1 µl per million cells) (BD Biosciences) was added during the second round of depletion. Purified cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), penicillin (50 units/ml) (Invitrogen), and streptomycin (50 µg/ml) (Invitrogen). Cells were rested overnight before infection or treatment. Macrophages were differentiated from human monocytes from the peripheral blood of HIV-1 negative donors. Briefly, two million peripheral blood mononuclear cells were plated into each well of six-well plates in serum-free RPMI 1640 medium for 1 h. Adherent cells were cultured in RPMI 1640 plus 10% heat-inactivated fetal bovine serum
(FBS) with 10 ng/ml macrophage colony stimulating factor (M-CSF) (R&D System, Minneapolis, MN) for 2 weeks with medium change for every 2 days.

**Virus preparation and infection:** Virus stocks of HIV-1$_{NL4-3}$, HIV-1$_{NL4-3}$(AD8), and HIV-1(Yu2) were prepared by transfection of HEK293T cells with cloned proviral DNA. For viral infection, unless otherwise specified, $10^{3.5}$ to $10^{4.5}$ TCID50 units of HIV-1 were used to infect $10^6$ cells. Where indicated, cells were treated with 100 nM AMD3100$^{140}$, or 50 ng/ml of Pertussis Toxin (PTX)$^{141}$.

Rev-CEM cells were infected with 200 µl of vNL4-3 for two hours prior to washing and culturing for an additional 72 hours. Cells were pretreated with the WASP/N-WASP inhibitors, 187-1$^{142}$ or wiskostatin$^{143}$ (Tocris), or serum-free RPMI at the indicated concentration for 1 hour. Additionally, drugs were added during and after infection.

**Western blotting:** One million cells were lysed in 100 µl of NuPAGE LDS Sample Buffer (Invitrogen). Cell lysates were sonicated and separated in 4–12% Bis-Tris polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membranes (Invitrogen). The membranes were washed in TBS-T for 3 min and then blocked for 30 min at room temperature using either 5% skim milk in TBS plus 0.2% Tween 20 or LiCor Blocking Buffer (LiCor). Membranes were incubated with an anti-Phospho-S351 WAVE2 antibody (07-1514, Millipore) (1: 1000 dilution in LiCor Blocking Buffer) or an anti-GAPDH antibody (ab9483, Abcam) (1:1000 dilution in 2.5% skim milk) overnight at 4 °C. WAVE2 was similarly stained with a 1:1000 dilution of rabbit anti-WAVE2
(3659, Cell Signaling Technology) in Tris-buffered saline (TBS) with 0.2% Tween-20 (TBS-T) with 2.5% skim milk (w/v) overnight at 4 °C. After washing the membranes 3 times for 15 minutes each, secondary staining was performed using a 1:5000 dilution of Rabbit Anti-Goat IgG DyLight 680 (KPL, 072-06-13-06) or a 1:5000 dilution of goat anti-rabbit HRP conjugate in TBS-T with 2.5% skim milk for 1 h at room temperature. IR-dye conjugated stains were imaged on an ODYSSEY imaging system (Li-Cor Biosciences), and HRP-conjugated secondary antibodies were imaged using SuperSignal West Femto Chemiluminescent Substrate (34095, Thermo Scientific).

**Results**

*N-WASP/WASP inhibitors have modest effects on HIV-1 infection*

To ascertain whether the NPFs, WASP and N-WASP, contribute to HIV-1 infection—and are thus likely candidates for HIV-1-mediated Arp2/3 activation—the chemical inhibitors 187-1 and wiskostatin were used. Both drugs act by locking WASP/N-WASP in an autoinhibited conformation, preventing optimal NPF activation\(^\text{142,143}\). Rev-CEM cells were pretreated with both drugs, at various dosages above and below their reported IC\(_{50}\)’s, for 1 hour prior to infection. Both the infection and post-infection culturing were also performed in the presence of drug. As shown in Figure 2, neither inhibitor appreciably lowered HIV-1 infection in this cell system, indicating that WASP and N-WASP are unlikely mediators of Arp2/3 activation and regulation during HIV-1 infection. Though 5 µM wiskostatin had a modest effect on infection rates, higher
dosages were almost completely cytotoxic, and a number of off-target effects of wiskostatin have been observed\textsuperscript{144}.

\textit{HIV induces the phosphorylation of serine 351 of WAVE2, and late phase-phosphorylation is inhibited by PTX}

To test whether HIV-1 signals to WAVE2 from its receptor and coreceptor, resting CD4 T cells were stimulated with 200 µl vNL4-3. Cells were either untreated, or treated over a timecourse before pelleting and lysis. After sample processing, 10-15 µl of sample was loaded onto SDS-PAGE gels, transferred, and probed with for total WAVE 2, phospho-S351 WAVE2, and GAPDH. As shown, phosphorylation is observed as early as 1 minute, and is maintained throughout the timecourse with increased phosphorylation at 20 and 30 minutes post-infection (Figure 3).
**Figure 2:** WASP/N-WASP inhibitors have modes effects on vNL4-3 viral infection.

Cells were pretreated with the indicated concentration of WASP inhibitors for 1 hour before infection with 200 µl vNL4-3, which was also in the presence of inhibitors. After infection, cells were washed and cultured for 72 hours with inhibitors prior to propidium iodide staining and flow cytometric analysis. Although not shown, 10 and 20 µM wiskostatin treatments were tested and were fully cytotoxic at these concentrations.
To further probe the mechanism of WAV2 phosphorylation, the Gαi-inhibitor, Pertussis Toxin (PTX) \(^{141}\), was used. When viral gp120 binds to CD4 and CXCR4, multiple signaling pathways can be induced, and among them are the Gαi and Gaq-dependent CXCR4 signaling pathways. As shown in Figure 3, inhibition of Gαi blocks late-phase phosphorylation of WAVE2 S351, but not early phosphorylation, indicating that some Gαi-independent signaling may contribute to WAVE2 phosphorylation.

*WAVE2 S351 phosphorylation is CXCR4-dependent*

To further probe the role of CXCR4 signaling, the CXCR4 antagonist, AMD3100 \(^{140}\), was utilized. Resting CD4 T cells were either untreated or treated with 100 nM AMD3100 in a timecourse experiment, as above. As shown in Figure 4, AMD3100 prevented WAVE2 S351 phosphorylation. This indicates that most WAVE2 phosphorylation is dependent on CXCR4, and is likely to be independent of CD4.

*R5 envelopes also induce WAVE2 S351 phosphorylation in macrophages*

Up to this point, only CXCR4-dependent signaling was observed during HIV-1 infection; however, CCR5 is also an important coreceptor for R5 viral variants. To test whether CCR5-utilizing viruses can also induce S351 WAVE2 phosphorylation, macrophages were differentiated from PBMCs by culturing with 10 ng/ml GM-CSF for two weeks. Cells were then treated with vNL4-3 (AD8) and vYU2, both of which are R5 strains. As shown in Figure 5, both AD8 and YU2 viruses could induce S351
phosphorylation of WAVE, indicating that this signaling is conserved among R5 and X4 variants.
Figure 3: HIV-1 induces WAVE2 S351 phosphorylation in Gαi-dependent and independent pathways. After isolation and overnight culturing, 1×10⁶ resting CD4 T cells were either untreated, or treated with vNL4-3 for the indicated timecourse before sample lysis and analysis by SDS-PAGE and immunoblot. In the bottom panel, resting CD4 T cells were also pretreated for 1 hour with 50 ng/ml PTX to block Gαi-dependent signaling.
Figure 4: AMD3100 inhibits WAVE2 S351 phosphorylation. After isolation and overnight culturing, $1 \times 10^6$ resting memory CD4 T cells were either untreated, or treated with vNL4-3 with or without 100 nM AMD3100 for the indicated timecourse before sample lysis and analysis by SDS-PAGE and immunoblot.
Figure 5: vNL4-3(AD8) and vYU2 induce WAVE2 S351 phosphorylation downstream of CCR5 engagement. Adherent PBMCs were cultured with 10 ng/ml GM-CSF for two weeks, changing out medium every 2 days. After this, cells were left untreated, or treated with vNL4-3(AD8), pseudotyped with the R5 viral strain AD8 envelope, or vYU2, another R5 viral strain. Cells were then lysed and prepared for immunoblot as before.
Discussion

In this chapter, we explored the mechanisms of Arp2/3 activation during HIV-1 infection. Previously, we had shown that Arp2/3 inhibition or knockdown dramatically reduced HIV replicative capacity in CEM-SS and resting CD4 T cells. However, whether Arp2/3 was directly engaged by HIV-initiated signal transduction had not been exhibited. As such, the immediate upstream regulators of Arp2/3, the NPFs, were addressed in turn—focusing on the better described WASP, N-WASP, and WAVE2 proteins.

Treatment of a CD4 T indicator cell line with the WASP and N-WASP inhibitors, 187-1 and wiskostatin, had modest effects on HIV-1 infection percentages. While Rac1 has been shown to be activated and necessary for HIV-1 infection, Cdc42, which directly regulates WASP and N-WASP, has not. Furthermore, N-WASP plays a predominant role in endocytosis, and WASP facilitates T cell activation and development, neither of which are expected to play a role in early infection events. As such, N-WASP and WASP are unlikely regulators of Arp2/3 during early HIV infection events. In line with this, chemotaxis towards SDF-1, the natural ligand of CXCR4, from WASP mouse T cells is only mildly reduced in vitro. Although, knockout of Wiskott-Aldrich syndrome protein-interacting protein (WIP), which interacts with WASP and N-WASP, or a WASP/WIP double knockout did severely impair CXCR4 T cell chemotaxis, indicating that WASP and N-WASP may play some redundant role in regulating CXCR4 signaling and chemotaxis.
In contrast to N-WASP/WASP, WAVE-family proteins are activated by Rac1 and are much more likely to be engaged by the PI3K and Rac1 signaling axes activated by viral gp120. In support of this, WAVE2 becomes phosphorylated on S351 after treatment with HIV-1 particles. Furthermore, treatment with AMD3100, a CXCR4 antagonist, completely abrogated WAVE2 phosphorylation, indicating that CXCR4 signaling was critical for WAVE2 activation. Also, this indicates that CD4 plays only a minor or modulatory role in WAVE2 phosphorylation. In addition, treatment with PTX blocked late, but not early, WAVE2 phosphorylation. As PTX only inhibits Gαi, Gαo, and Gαt, and the associated βγ signaling, this would imply that Gαi-independent signaling might also promote WAVE2 activation. In line with this, Gαq signaling has been observed downstream of CXCR4 stimulation by its natural chemokine, SDF-1. CCR5 signaling from R5 viruses also induced the phosphorylation of WAVE2, exhibiting that this signaling event occurs and may be required for both R5 and X4 strains.

As aforementioned, the WAVE family proteins are coordinately regulated by tyrosine phosphorylation by c-Abl, serine/threonine phosphorylation by a number kinases, PIP, and Rac1-GTP. Additionally, the WRC components can also be phosphorylated, with Abi1 having a number of tyrosine and serine/threonine phosphosites targeted by Abl and ERK2. Given this, it is often difficult to directly assay the activities of the various WRCs, necessitating the use of proxies like membrane translocation, lamellipodium protrusion, Rac1 activation, actin
rearrangements, and phosphorylation. In this particular study, we showed that WAVE2 is phosphorylated on S351, which is required for optimal activation. This particular site is phosphorylated by ERK2, which has previously been shown to be activated during HIV infection downstream of CCR5 and CXCR4 engagement. Although not directly addressed in this study, Abi1 is likely to be phosphorylated as well.

Cumulatively, along with previous studies exhibiting the HIV-1 mediated activation of Rac1, PI3K, ERK1/2, and the HIV gp120-mediated induction of actin dynamics, there appears to be sufficient evidence to suggest that WAVE2 is directly targeted and activated during HIV infection. Furthermore, that this activation likely contributes to Arp2/3 activation, which facilitates HIV nuclear migration. This model is represented in Figure 6.
Figure 6: model of HIV-1-induced Arp2/3 activation and nuclear migration. Upon binding to the cell surface, HIV-1 gp120 engages both CD4 and CCR5 or CXCR4, inducing signal transduction. In particular, G\(\alpha\)i and G\(\alpha\)q pathways are activated, culminating in the activation of WAVE2, coflin, LIMK, and Arp2/3. The HIV PIC is shown undergoing two possible intracellular motility routes, either passively associating with actin during active treadmilling, or directly associating with Arp2/3 to propel the PIC in the cytoplasm as seen in vaccinia virus.
Chapter Seven—future studies

Introduction

In the previous chapters, a concerted effort was made to clarify the roles of Arp2/3 during HIV-1 infection in CD4 T cells and, to a lesser extent, macrophages. In this endeavor, stable CD4 T cell knockdown clones were created and characterized for F-actin, surface expression of CD4 and CXCR4, and HIV-1 replication phenotypes. During these experiments, the vast majority of clones exhibited significant defects in HIV-1 replication. Two of the clones, shArp3-012 and shArp3-013, were further probed for the mechanism of the HIV-1 replication defect. In brief, while fusion and reverse transcription were not inhibited, nuclear migration of the viral PIC appeared to be directly diminished in Arp3 knockdown cells.

These results were further corroborated with the use of the small molecule Arp2/3 inhibitor, CK-548 \(^\text{115}\). As described earlier, CK-548 inhibited HIV replication in reporter cells, and HIV p24 release in CEM-SS cells. This was not due to a reduction in reverse transcription, as later time points had similar or higher levels of total viral DNA. However, nuclear migration was impaired to a similar degree as in knockdown cells, with higher dosages reducing the quantity of nuclear 2-LTR circles below the limits of detection. To discern if there was any additional effect on HIV-1 budding and release,
CK-548 was added 12 hours post-infection, and the p24 release data indicated that there may be a modest affect on HIV-1 release.

Lastly, upstream regulators of the Arp2/3 complex were considered, with an emphasis on the WASP and WAVE family of NPFs. Treatment of indicator cells with the N-WASP/WASP inhibitors 187-1 and Wiskostatin did not reduce HIV-1 infection in reporter cells, indicating that these NPFs were unlikely to mediate Arp2/3 activation in infected cells. In the case of the WAVE family, where no inhibitor existed at the time of the writing of this manuscript, phosphorylation was used as a correlative of WAVE protein engagement. As such, the ubiquitously expressed WAVE2 was chosen as a target WAVE-family member, and the ERK2-mediated phosphorylation of serine 351 was utilized as a site indicating WAVE2 engagement during HIV-1 infection. WAVE2 was phosphorylated in response to HIV-1 treatment in resting CD4 T cells. Furthermore, this signaling could be completely abrogated by treatment with the CXCR4 antagonist, AMD3100, indicating that the signaling was downstream of CXCR4, and may be completely or partially independent of CD4. Treatment with PTX, which inhibits the Gαi signaling downstream of CXCR4, indicated that late-phase S351 phosphorylation was dependent on Gαi; however, earlier signaling may require another Gα protein, such as Gαq. Lastly, treatment of macrophages with R5 Env-bearing viruses also induced WAVE2 S351 phosphorylation, indicating that CCR5-dependent signals can also engage WAVE2.
Despite these findings, a number of residual questions remain regarding Arp2/3 and HIV-1 infection. For instance, further clarifying the roles of the NPFs and upstream signaling events during HIV-1 infection: there are a number of WAVE2 phosphorylation sites, two other WAVE-family members, three other NPFs, and the ultimate regulators of Rac1 activation that have not been sufficiently addressed here or in the literature. Additionally, in order to support the model of Arp2/3 activation of in HIV-1 infection, one would need to prove that HIV-1 proteins associate with either the Arp2/3 complex itself or one of the NPF complexes. As such, each of these will be considered in turn, focusing on the rationale and methodology of each issue. Additionally, these residual questions are outlined in Figure 1.
Figure 1: Outline of future studies regarding the role of Arp2/3 in HIV-1 infection.

Future directions include a more thorough analysis of the role of Gαi and Gαq, determining the identity of which GEF mediates Rac activation, further studies into phosphoregulation of the NPFs, knockdown analysis of which NPFs are responsible for HIV-1 nuclear migration, and IP and imaging studies to show an interaction between Arp2/3 and HIV-1 PIC proteins.
Signaling and the NPFs

As aforementioned, the WASP and WAVE family of proteins are dynamically regulated by Rho GTPase engagement, binding of SH3 domain-containing proteins, binding by phosphoinositide phosphates, and multiple phosphorylation events—each of which have subtle and unique effects relating to activation, subcellular localization, and colocalization with binding partners. It is important to note that this study did not clearly delineate (1) bona fide WAVE2 activation or (2) the necessity of WAVE2 for HIV-1 infection. Instead, this study exhibited that WAVE2 was engaged by signaling downstream of CXCR4, and that, along with previous studies exhibiting Rac1 activation and PI3K activation, WAVE2 activation was likely. As such, to further discern the roles of the NPFs during HIV infection, additional experiments are warranted.

Among the first experiments that can be performed is the analysis of other phosphosites in WAVE2, and their paralogous sites in WAVE1 and WAVE3. In addition to S351, ERK also phosphorylates S308, S343, and T346. Although, these sites are not conserved in WAVE1 and possibly WAVE3. Additionally, there may be an additional 8 ERK sites on the WRC protein, ABI1. The overall effect of all these sites is to promote WRC activation in the lamellipodium, and though observing all sites in impractical, showing additional phosphorylation of WAVE2 and ABI1 by ERK does bolster the argument that WAVE2 is being explicitly activated. Arguably more important is the determination of Y150 phosphorylation of WAVE1-3, which is mediated by Abl tyrosine kinase, as this site is predicted to also activate WAVE-family proteins via
VCA domain exposure. Src phosphorylation of Y125 also appears necessary for efficient WAVE2 activation. Similarly, CK2 inhibits WAVE2 activation by phosphorylation of the VCA acidic region. These sites are valid targets for immunoblots and cell imaging studies.

To more specifically address which NPFs are required for HIV-1 infection, knockdowns of each NPF would need to be performed. Notably, all the afore-mentioned NPFs are expressed in Jurkat cells, indicating that each potentially have a role during HIV-1 infection. Currently, an siRNA study is currently being conducted by a collaborator, focusing on the role of actin-binding proteins more broadly in HIV-1 infection; some of the early data seem to indicate WAVE1 and WASH1 may be more important during the infection process in reporter cells (data not shown). Although, it is important to note that reporter cells may not completely recapitulate the necessity of individual NPFs, as opposed to Arp2/3, due to some redundancy in function. As such, there may need to be significant clarification of which WAVE member, or other NPFs, is implicated in HIV infection.

Upstream of the NPFs

In the CXCR4 signaling cascade, above the NPFs and Rac1 activation, are the GEFs responsible for Rac1 activation. Previously, Pontow et al. (2007) had implicated Tiam1 or Trio using the Rac GEF inhibitor NSC23766. While these results are positively indicative of a role of Tiam1 or Trio in HIV-1 infection, the inhibitor in question may target a number of Rac1-directed GEFs, as it directly binds the
Rac1-GEF interface\(^{156}\). As such, the identity of the Rac1 GEF remains to be fully clarified during HIV-1 infection. Furthermore, although HIV-1 Nef protein has been previously shown to interact with the broad Rho-family GEF, Vav\(^{157-159}\), this inhibitor appears to be incapable of blocking Vav-mediated Rac-induced cell growth; accordingly, Vav may not play a role in Rac1 activation during early HIV-1 infection\(^{156}\).

Unfortunately, GEF regulation is quite varied\(^{132}\), necessitating the using of knockdowns targeting Rac1-directed GEFs, such as Tiam1/2, P-Rex1/2, TrioN, Duo, Sos1/2, and RasGRF1/2.

Further upstream, Harmon \textit{et al.} (2008) have explicitly implicated a Gaq-PLC-PKC-Pyk2-Ras-Tiam1-Rac pathway in R5 infection of cells\(^{36}\). However, our results indicate that Gai is also required for HIV-1 infection in resting CD4 T cells\(^{34}\), and at least partially responsible for WAVE2 S351 phosphorylation and possibly membrane localization. Furthermore, while Harmon \textit{et al.} (2008) indicated that cell fusion is independent of Gai, Yoder \textit{et al.} (2008) implicated PTX-sensitive signaling in HIV-1 nuclear migration later in infection\(^{34,36}\). As such, the relative contribution of Goq and Gai-dependent pathways needs to be ascertained.

**The Arp2/3 nuclear migration model**

As aforementioned, the current model for Arp2/3-facilitated nuclear migration of the HIV PIC predicts that HIV-1 either directly engages the Arp2/3 complex through interacting with it or some associated factor; alternatively, it is quite possible that HIV-1 associates with actin, and Arp2/3 complex activity merely pushes the viral PIC towards
the nucleus. As tempting as it may be to perform a direct co-IP of the viral PIC or Arp2/3 complex, this may not be an appropriate method to prove a direct interaction with Arp2/3. Since HIV-1 proteins directly interact with actin, including Nef\textsuperscript{49}, RT\textsuperscript{53}, Gag or NC\textsuperscript{51,52}, and possibly integrase\textsuperscript{160}, any IP of the PIC will necessarily recover actin-binding proteins. As such, it is quite possible that a co-IP may isolate Arp2/3 without truly indicating a direct interaction with it or an associated factor (e.g. WAVE2). Nonetheless, this remains the only viable mechanism to preclude the possibility of HIV not associating with the complex. Additionally, colocalization and imaging studies of Arp2/3 and the PIC may prove quite useful in showing meaningful associations in live cells.
References


Biography

Mary C. Smith graduated from Fairfax High School, Fairfax, Virginia, in 1983. She received her Bachelor of Arts from George Mason University in 1987. She was employed as a teacher in Fairfax County for two years and received her Master of Arts in English from George Mason University in 1987.