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Get in and get out: Remodeling of the cellular actin cytoskeleton upon HIV-1 infection

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Abstract

The Human Immunodeficiency Virus type 1 (HIV-1) is an intracellular pathogen whose replication cycle strictly depends on the host cell molecular machinery. HIV-1 crosses twice the plasma membrane, to get in and to get out of the cell. Therefore, the first and the last line of intracellular component encountered by the virus is the cortical actin network. Here, we review the role of actin and actin-related proteins in HIV-1 entry, assembly, budding and release. We first highlight the mechanisms controlling actin polymerization at the entry site that promote the clustering of HIV-1 receptors, a crucial step for the virus to fuse with the plasma membrane. Then, we describe how actin is transiently depolymerized locally to allow the capsid to cross the actin cortex, before migrating towards the nucleus. Finally, we review the role of several actin-binding proteins in actin remodeling events required for membrane deformation and curvature at the viral assembly site as well as for virus release. Strikingly, it appears that common actin-regulating pathways are involved in viral entry and exit. However, while the role of actin remodeling during entry is well understood, this is not the case during exit. We discuss remaining challenges regarding the actin-dependent mechanisms involved in HIV-1 entry and exit, and how they could be overcome.

Keywords: HIV-1 entry, HIV-1 assembly, HIV-1 budding, Actin cytoskeleton, ESCRT

I – Introduction

Actin is among the most abundant proteins in eukaryotic cells and is involved in many essential cellular processes, including the maintenance of the cellular architecture, intracellular transport, cell migration and contractility, phagocytosis and cell division to name a few. Globular actin (G-actin) assembles into filamentous polymers called actin filaments (F-actin), which are incorporated into complex networks. The dynamics of actin is regulated by many actin binding proteins (ABPs) which play essential roles in controlling F-actin nucleation, polymerization, disassembly, severing and formation of both linear and Arp2/3-dependent branched networks, either underneath the plasma membrane or within the cytoplasm (Gautreau, Fregoso, Simanov, & Dominguez, 2022; Pollard, 2016). Intracellular pathogens, including most human viruses, and many species of bacteria are therefore in constant interaction with the host cell's actin cytoskeleton, and use various strategies to manipulate the cellular actin networks to proliferate and propagate within the host.

HIV-1, responsible for AIDS (Acquired ImmunoDeficiency Syndrome), has been isolated at the Pasteur Institute in 1983 (Barre-Sinoussi et al., 1983). Its genome consists of two copies of a positive sense, single-stranded RNA, encoding structural and enzymatic proteins (Gag, Pol and Env), essential regulatory elements (Tat and Rev), and accessory regulatory proteins (Nef, Vpr, Vif, Vpu). The virus infects immune cells —mainly T lymphocytes and macrophages— that express the cellular receptor CD4 and the chemokine receptors CXCR4 or CCR5. Forty years of research to understand the life cycle of HIV-1 at the cellular and molecular levels have led to the detailed description of its replication cycle. In the course of its replication, the virus encounters the host actin cytoskeleton in many, if not all, stages of this cycle, including viral entry, reverse transcription, nuclear migration, transcription, nuclear export of genomic viral RNA, intracellular transport of viral components, assembly, budding and release (reviewed in (Lehmann, Nikolic, & Piguet, 2011; Y. Liu, Belkina, & Shaw, 2009; Ospina Stella & Turville, 2018; Rocha-Perugini, Gordon-Alonso, & Sanchez-Madrid, 2014; Spear, Guo, & Wu, 2012; Stolp & Fackler, 2011)). Both cell-free and cell-to-cell transmitted viruses participate in HIV-1 pathogenesis. Cell-to-cell transfer of viruses is strongly dependent on actin remodeling. This includes: (1) the formation of membrane protrusions involved in intercellular contacts between an infected donor-cell and an uninfected target-cell, (2) the formation and stabilization of the virological synapse between a donor-cell and a target-cell and (3) the

transfer of viruses from a presenting cell, that is not productively infected, to a target cell at the infectious synapse. The role of the actin cytoskeleton in cell-to-cell transmission of HIV-1 has been reviewed in (Bracq, Xie, Benichou, & Bouchet, 2018; Ospina Stella & Turville, 2018) and will not be discussed here.

At two crucial stages of its replication cycle, the virus meets the plasma membrane and the underlying actin cytoskeleton: for the entry in the target cells and during the assembly and budding of the newly-formed viral particles. This poses specific challenges since underneath the plasma membrane a dense and crosslinked actin filament meshwork —the actin cortex— is present. Here, we will review known strategies used by HIV-1 to dynamically remodel cortical actin to successfully enter into and exit from cells, and highlight the questions that remain unanswered regarding the molecular mechanisms involved.

II – The role of the actin cytoskeleton in HIV-1 entry

HIV-1 entry starts with the binding of the virus to the host cell and ends with the delivery of the viral capsid into the cytoplasm after fusion of the viral membrane with the host cell membrane. At the surface of the virus, the viral envelope protein (Env), composed of a trimer of gp120 and gp41 heterodimers, orchestrates these critical steps (reviewed in (Chen, 2019; Wilen, Tilton, & Doms, 2012a, 2012b)). Briefly, the binding of gp120 to the cellular receptor CD4 induces conformational changes in Env that allow coreceptor engagement, either CXCR4 or CCR5 depending on the viral tropism. Then, gp41 inserts into the host cell membrane to complete membrane fusion.

Viral fusion, whether at the plasma membrane and within endosomal compartments following endocytosis have been described in T cells and macrophages (reviewed in (Jakobsdottir et al., 2017)). However, in T cells, HIV-1 mainly fuses at the plasma membrane, and this represents the route for productive infection. Actin cytoskeleton dynamics is required for two critical steps of the HIV-1 entry at the plasma membrane in T cells: 1) clustering of the CD4 receptor and CXCR4/CCR5 coreceptors, and 2) delivery of the viral capsid into the cytoplasm. This will be detailed successively in the next two sections.

In macrophages, the productive route for virus entry —either fusion at the plasma membrane or at the endosomal membrane— is still under debate (reviewed in (Jakobsdottir et al., 2017; Melikyan, 2014)). Given the differences in the actin architectures at these two locations, in particular a thick actin cortex at the plasma membrane, it is likely that the actin cytoskeleton is differentially regulated at these two sites of entry when viruses enter macrophages. Additionally, the requirement of CXCR4 or CCR5 coreceptor for productive infection differs in macrophages and T lymphocytes (reviewed in (Yandrapally, Mohareer, Arekuti, Vadankula, & Banerjee, 2021)). Indeed, X4- and R5-tropic viruses lead to a productive infection in T lymphocytes whereas only R5-tropic virus does in macrophages. The evidence of actin remodeling in HIV-1 entry in macrophages will thus be described in a separate section.

1. Local actin polymerization at the viral entry site to cluster receptors and coreceptors and to promote fusion in T cells

The first paper reporting a role of the actin cytoskeleton in HIV-1 entry was published in 1998 (Iyengar, Hildreth, & Schwartz, 1998). At that time, important observations were made in T cells: 1) the viral envelope glycoprotein gp120 induces actin-rich polarized cocapping of the receptor CD4 and its coreceptor CXCR4 at the plasma membrane, 2) inhibition of actin polymerization by cytochalasin D (CytoD) fully inhibits HIV-1 entry and 3) actin depolymerization prevents the clustering of CD4 and CXCR4 and cell shape changes (pseudopod formation) upon gp120 addition. It was thus proposed that the interaction of Env with receptor and coreceptors induces actin-regulating signal transduction cascades, required for HIV-1 entry (Cicala et al., 2002; Iyengar et al., 1998). Since then, many studies have confirmed that the interaction of Env with its receptor and coreceptors induces actin polymerization through different actin-remodeling signaling cascades. This results in a burst of actin polymerization within 1 minute following virus incubation in primary CD4 T cells (Spear et al., 2014; Yoder et al., 2008), and within 5 minutes in Jurkat T cells (Gordon-Alonso et al., 2012; Gordon-Alonso et al., 2013). In this section, we will detail the signaling cascades and proteins that mediate the formation and maintenance of an actin-dependent cluster of HIV-1 receptors and coreceptors, facilitating entry of the virus in T cells.

Rac1-IRSp53-WAVE2-Arp2/3

The binding of HIV-1 Env to its receptor and coreceptors activates the three major Rho-family GTPases: RhoA, Rac1 and Cdc42. These small guanine nucleotide-binding proteins are well known to act as master molecular switches that orchestrate actin-dependent processes.

It was first reported that Env binding to its receptor and coreceptors induces Rac1 activation through a receptor-coupled G-protein $G\alpha_q$ and phospholipase C (PLC) signaling cascade (Harmon & Ratner, 2008; S. Pontow, Harmon, Campbell, & Ratner, 2007; S. E. Pontow, Heyden, Wei, & Ratner, 2004). Moreover, a specific inhibitor of a Rac1 activator, the guanine nucleotide exchange factors (GEF) Tiam-1, has an inhibitory effect on Env-mediated cell-cell fusion, virus-cell fusion and infection in a human glioblastoma U87 cell line expressing CD4 and CCR5 (S. Pontow et al., 2007). Later, depletion of Tiam-1 and of several other known actors of actin remodeling such as the tyrosine kinase Abl, IRSp53, WAVE2 or Arp3 were shown to impair Env-mediated cell-cell as well as virus-cell fusion and thus infection in the same cells (Harmon, Campbell, & Ratner, 2010). Furthermore, inhibition of the kinase activity of Abl blocks fusion at the hemifusion (lipid mixing) step, suggesting a role of actin and Abl in pore formation and expansion. The authors thus proposed the following model: first, Env-induced Rac1 activation is mediated by the Rac1 GEF Tiam-1, which associates with IRSp53 —an adaptor protein that binds Rac1 and the Arp2/3 activator WAVE2 (Abou-Kheir, Isaac, Yamaguchi, & Cox, 2008)— to link Rac1 to the WAVE2 complex. Then, activated Rac1 and Abl activate the WAVE2 complex and promote Arp2/3-dependent branched-actin polymerization. Altogether, it was concluded that the Rac1 signaling pathway leads to actin remodeling that facilitates Env-mediated HIV-1 fusion (Harmon et al., 2010).

More recent studies in primary T cells exposed to HIV-1 confirmed the Env-mediated activation of Rac1 at the time of entry (Lucera et al., 2017). Importantly, the role of WAVE2 and Arp2/3 complex in HIV-1 infection is also observed in resting CD4 T cells (Spear et al., 2014). Indeed, through gp120 binding to CXCR4, HIV-1 triggers WAVE2 activation. In addition, depletion of Arp3 reduces CD4 and CXCR4 surface levels and impairs HIV-1 infection in CD4 T cells. Based on these results, it was concluded that actin-nucleation and branching through the WAVE2-Arp2/3 pathway is important for the initial steps of HIV-1 infection (Spear et al., 2014). Of note, the assays used in this study could not address whether Arp2/3 plays a direct role in the entry step at the plasma membrane or in the subsequent genome integration.

Beside Arp2/3 activation by Rac1, it has been hypothesized that HIV-1 Env triggers a Cdc42-N-WASP-Arp2/3 pathway that facilitates viral entry (Swaine & Dittmar, 2015), but this needs to be experimentally confirmed. Nevertheless, a study shows that Env signaling through CD4 and CCR5 leads to the activation of Cdc42, and chemical inhibition of Cdc42 significantly reduces infection in primary CD4 T cells (Lucera et al., 2017). Whether the entry step or the later replication steps are affected remains to be investigated.

Altogether, these studies indicate that Env-mediated branched-actin polymerization is induced following the activation of Rac1 *via* the downstream nucleation-promoting factor WAVE2, which stimulates the Arp2/3 complex. This actin remodeling is important for the initial steps of viral entry.

Rac1-PAK-LIMK1-Cofilin and RhoA-ROCK-LIMK1-Cofilin together with Filamin-A

Beside activating actin polymerization, Env also induces RhoA and Rac1 signaling that inhibits the actin-disassembly factor Cofilin-1 (hereafter Cofilin). Cofilin is a key regulator of actin dynamics in cells, known to enhance actin filament turnover by severing and promoting dissociation of F-actin into G-actin (Wioland et al., 2017; Wioland, Jegou, & Romet-Lemonne, 2019). Cofilin is regulated by phosphorylation and dephosphorylation, the phosphorylated form of Cofilin being inhibited.

Phosphorylation-induced Cofilin inhibition is mediated by LIM kinase 1 (LIMK1). It was shown that HIV-1 Env triggers transient LIMK1 activation through a Rac1-PAK-LIMK1 pathway (Vorster et al., 2011). Moreover, depletion of LIMK1 in primary CD4 T cells induces a decrease in the levels of F-actin (which is mainly localized at the cell cortex in these cells) and an increase in the rate of CXCR4 internalization. It was thus suggested that Env-mediated actin polymerization through LIMK1 activation promotes CXCR4 coreceptor localization at the plasma membrane and thereby favors viral entry in CD4 T cells (Vorster et al., 2011). Consistent with a role of the LIMK1/Cofilin pathway, the secretory protein Slit2 inhibits HIV-1 entry in T cell lines and primary T cells, and this inhibition is mediated by the Slit2 transmembrane receptor Robo (Roundabout) (Anand, Zhao, Nagaraja, Robinson, & Ganju, 2013). Slit2 is known to regulate actin polymerization through Robo by preventing activation of the Rho family GTPases Rac and Cdc42 (Wong et al., 2001). Mechanistically, Slit2 has been proposed to prevent Env-induced actin polymerization through the Rac1-LIMK1-Cofilin pathway (Anand et al., 2013).

Upon gp120-binding to receptors, the transient inhibition of Cofilin by phosphorylation is also induced through RhoA-ROCK-LIMK1-dependent pathway (Jimenez-Baranda et al., 2007). This Cofilin inhibition is dependent on the interaction between CD4/CXCR4 and the actin-binding and crosslinking protein Filamin A (FLNa) (Jimenez-Baranda et al., 2007). In addition, it has been hypothesized that FLNa can link HIV-1 receptors to the actin cortex, enabling their mobility and anchorage after Env-binding (Jimenez-Baranda et al., 2007). Interestingly, FLNa is one of the most upregulated proteins in infected CD4 T cells (Chan et al., 2007).

Thus, upon Env binding to its receptor and coreceptors, both RhoA and Rac1 activation inhibits Cofilin *via* LIMK1 through RhoA-ROCK-LIMK1 and Rac1-PAK-LIMK1-dependent pathways, respectively. Noteworthily, the interaction between gp120 and CXCR4 induces chemokine-like signaling that mimics the natural binding of the stromal cell-derived factor 1 α (SDF-1 α) to CXCR4 (Balabanian et al., 2004; Nishita, Aizawa, & Mizuno, 2002). In non-infected cells, SDF-1 α induces the activation of LIMK1, thus negatively regulates Cofilin (Nishita et al., 2002). Interestingly, the use of inhibitors of the tyrosine kinase IKT, which is involved in SDF-1 α -mediated activation of Rac1 and Cdc42, blocks HIV-1 entry (Readinger et al., 2008).

In addition to virus/receptors interaction, other factors favor Cofilin phosphorylation and viral entry. It has been recently suggested that interferon- γ -inducible protein (IP)-10 has a role in HIV-1 entry in resting T cells (Wang et al., 2021). Indeed, IP-10 treatment triggers rapid LIMK1-dependent-phosphorylation/dephosphorylation cycle of Cofilin compared to untreated cells, remodels actin and increases viral entry. This study reinforces the idea that the LIMK1/Cofilin pathway is critical for the entry of HIV-1. Furthermore, in HIV-1-infected individuals, high-levels of plasma IP-10 are associated with rapid disease progression (Jiao et al., 2012; Ploquin et al., 2016). Moreover, the replicative capacity of the virus positively correlates with IP-10 (Muema et al., 2020) and the peak of plasma IP-10 level precedes that of HIV-1 viremia (McMichael, Borrow, Tomaras, Goonetilleke, & Haynes, 2010), making IP-10 a strong predictor for peak viral load (Gray et al., 2013; Lee et al., 2015).

ERMs and Gelsolin

Beside Cofilin, other actin-binding proteins have been identified as playing a role during HIV-1 entry: the ERMs (Ezrin, Radixin, Moesin) proteins and the actin-severing factor Gelsolin.

ERMs are proteins that reversibly crosslink cortical actin with the plasma membrane when phosphorylated (Fehon, McClatchey, & Bretscher, 2010). Activation of Ezrin and Moesin by phosphorylation is increased upon gp120/CD4 interaction (Barrero-Villar et al., 2009). Functionally, silencing Moesin or using a dominant-negative form of the protein impedes the reorganization of actin induced by Env, which impairs CD4-CXCR4 clustering and thus HIV-1 entry in T lymphocytes (Barrero-Villar et al., 2009). As a consequence, Moesin depletion reduces HIV-1 Env-mediated cell-cell fusion (Barrero-Villar et al., 2009). Consistently, HIV-1 infection is decreased in the presence of a non-phosphorylatable mutant and increased in the presence of a phosphomimetic mutant of Ezrin (Kamiyama et al., 2018). Altogether, it was thus proposed that activated ERMs are important for virus entry in permissive lymphocytes by promoting F-actin-dependent CD4-CXCR4 clustering and membrane fusion (Barrero-Villar et al., 2009). Of note, Moesin depletion is associated with increased microtubule cytoskeleton stability (Naghavi et al., 2007), indicating that ERMs could play multiple roles during infection.

The ABP Gelsolin, which participates in actin-remodeling by sequestering G-actin and by severing and capping F-actin, was also found to be involved in the regulation of cortical actin dynamics during HIV-1 entry. Both overexpression and silencing of Gelsolin inhibit Env-mediated F-actin reorganization, receptor clustering at one pole of the cell, and eventually membrane fusion in human CEM T cells and HeLa cells. Based on these observations, it was proposed that Gelsolin is a factor that acts at a step preceding membrane fusion to limit HIV-1 infection (Garcia-Exposito et al., 2013). Two other ABPs that localize at virus-cell contacts, Drebrin —which stabilizes F-actin (Mikati, Grintsevich, & Reisler, 2013)— and Syntenin-1 —which controls actin polymerization through Rac1 activation (Sala-Valdes et al., 2012)— also negatively regulate HIV-1 entry, since depletion of these proteins in T cell lines increases Env-mediated cell-cell fusion and HIV-1 infection (Gordon-Alonso et al., 2012; Gordon-Alonso et al., 2013).

In summary, the signaling induced by Env-binding to CD4 and CXCR4/CCR5 inhibits Cofilin and activates Arp2/3, thus mediating local branched-actin polymerization at the cell cortex. The increase in cortical actin polymerization leads to the clustering of the receptor and coreceptors which are stabilized and linked to the actin cortex *via* crosslinking proteins FLNa and Moesin. This results in the formation of an actin-capped cluster of HIV-1 receptor and coreceptor molecules at one pole of the cell. This is proposed to facilitate viral entry by

increasing the probability of Env/CD4-coreceptors interactions, and possibly to help enlarge the fusion pore and thereby the fusion of the viral membrane with the plasma membrane (Blumenthal, Durell, & Viard, 2012; Kozlov & Chernomordik, 2015) (**Figure 1**).

2. Local actin depolymerization to cross the actin cortex in T cells

As described above, the initial polymerization of cortical actin is necessary for the formation of the actin-cap and virus-cell fusion. However, following viral fusion, the actin cortex is believed to become a barrier for intracellular transport of the viral capsid. This is particularly the case in non-activated, resting T cells where the actin cortex is very dense, thus playing as an effective barrier for HIV-1 entry (Yoder et al., 2008). Of note, the restriction of viral infection by cortical actin is observed for different types of viruses (reviewed in (Delorme-Axford & Coyne, 2011; Swaine & Dittmar, 2015)). Consistently, in primary CD4 T cells, the burst of actin polymerization after HIV-1 incubation is rapidly followed by a decrease of F-actin to initial levels within 5 minutes and even below initial levels within 15 minutes in the overall cell population (Spear et al., 2014; Yoder et al., 2008). How the switch between actin polymerization and depolymerization occurs is not completely understood. It is thought that delayed Env/CXCR4 signaling via the receptor-coupled G α i leads to Cofilin activation, thus actin depolymerization after virus/membrane fusion. Remarkably, this dynamic remodeling of cortical actin during viral entry has been recently visualized in real time in resting T cells using live-cell imaging and tracking of fluorescent single viral particles labeled by quantum dots (QD) (Yin et al., 2020). When a single virus encounters the cell surface, rapid F-actin polymerization (visualized by a fluorescent LifeAct probe) can be observed at the virus/cell contact site within 1 minute. After 5 minutes, this is followed by a rapid, local actin depolymerization while the virus is crossing the actin cortex. Following capsid internalization into the cytoplasm, actin repolymerizes at the cortex (Yin et al., 2020). The mechanisms by which HIV-1 can actively overcome the cortical actin barrier by inducing actin depolymerization are described below (**Figure 2**).

The critical role of Cofilin when HIV-1 capsid crosses the cortical actin barrier was discovered in 2008 in resting T cells (Yoder et al., 2008). In these cells, Cofilin is largely inactive and the actin cortex is very dense, contrary to activated T cells. Treatment of resting T cells

with either viruses or Env rapidly triggers depolymerization of the actin cortex. This is due to the Env/CXCR4 signaling which mediates Cofilin activation (by an unknown phosphatase) through the receptor-coupled $G_{\alpha i}$. Indeed, Cofilin depletion leads to an increase of cortical actin and restricts viral entry, without affecting the fusion step. Conversely, Cofilin activation using a competitive peptide inhibitor of LIMK1 activity (S3 peptide) that prevents Cofilin phosphorylation, enhances viral replication. In conclusion, it was demonstrated that activation of Cofilin in resting T cells through Env/CXCR4 signaling is crucial to overcome the dense actin barrier and thus enable the post-entry step of viral infection (Yoder et al., 2008). Of note, Arp2/3-dependent branched-actin polymerization has been proposed to also promote HIV-1 capsid transport toward the nucleus after having crossed the cortex (Cameron et al., 2010; Spear et al., 2014; Vorster et al., 2011). However, while actin and ABPs mediate entry and early post-entry movement of HIV-1 at the cell periphery, it is now believed that long-range transport to the nucleus involves microtubules (Carnes, Zhou, & Aiken, 2018; Delaney, Malikov, Chai, Zhao, & Naghavi, 2017; Dharan et al., 2017; Malikov et al., 2015; Walsh & Naghavi, 2019).

Beside Cofilin, the F-actin cross-linker α -actinin may play a role in actin remodeling to facilitate viral progression through the actin cortex (Yin et al., 2020). Using fluorescent single viral particles labeled by QDs, the authors observed that α -actinin accumulates at the HIV-1 entry site and concomitantly cortical F-actin decreases at this location, while the capsid progresses through the actin cortex. Afterwards, α -actinin disperses and cortical F-actin locally returns to its original state (Yin et al., 2020).

3. Actin remodeling in HIV-1 entry in macrophages

As mentioned previously, the productive route for virus entry is not fully elucidated in macrophages, but there is evidence of HIV-1 fusion at the membrane of endosomes after endocytosis (reviewed in (Jakobsdottir et al., 2017)). As in CD4 T cells, the role of actin for HIV-1 entry in macrophages has been suggested by different studies. First, Env/CCR5-mediated activation of Rac1 is found in brain macrophages of HIV-1-infected patients (Woollard, Li, Singh, Yu, & Kanmogne, 2014). Second, HIV-1 entry in macrophages by endocytosis depends on dynamin, Rac1 and Pak1 (Carter, Bernstone, Baskaran, & James, 2011). Third, through gp120 binding to CCR5, HIV-1 triggers WAVE2 activation by phosphorylation in macrophages

(Spear et al., 2014). However, while the WAVE complex activates Arp2/3 downstream of Rac1, whether WAVE2 activation is required for viral entry remains to be investigated.

Consistent with these studies, live cell imaging of single QD-labeled viruses combined with actin-depolymerizing drugs showed that actin dynamics is critical for HIV-1 entry through clathrin-mediated endocytosis in infected primary macrophages (Q. Li et al., 2017). Single-virus tracking in infected human monocyte-derived macrophages further revealed that entry can also happen in specific actin-related structures, the podosomes, where the CCR5 coreceptor accumulates (W. Li et al., 2021). Stimulation of podosome formation (using scratch assay or treatment with transforming growth factor β , vascular endothelial growth factor or platelet-derived growth factor) promotes viral entry. In contrast, inhibition of podosome formation with drugs (SRC kinases inhibitor SU6656 or phosphoinositide 3-kinase inhibitor LY294002) or depletion of proteins affecting their formation (formin-like 1 or vinculin) significantly reduces entry. It was thus suggested that HIV-1 can use actin-rich podosomes to enter macrophages (W. Li et al., 2021). Nevertheless, it should be emphasized that these treatments may have other effects beyond podosome formation that could affect HIV-1 entry.

Actin remodeling at the HIV-1 entry site in macrophages has been recently proposed to be regulated by the purinergic receptor P2Y2 and the inflammasome NLRP3 (NACHT, LRR and PYD domains-containing protein 3) (Paoletti et al., 2019). Upon Env binding to receptor and coreceptors, ATP (adenosine triphosphate) is released from the infected cell and stimulates the purinergic receptor P2Y2. This facilitates actin polymerization at the entry site and viral fusion, through the activation of a proline-rich tyrosine kinase PYK2 by phosphorylation (Seror et al., 2011). The inflammasome NLRP3 inhibits PYK2 (proline-rich tyrosine kinase 2) phosphorylation in uninfected cells but was found to be degraded upon Env-mediated activation of P2Y2. Altogether, these results suggest that the inflammasome NLRP3 is an intrinsic restriction factor for HIV-1 infection in macrophages by repressing F-actin polymerization during entry and that the virus overcomes this restriction by inducing its degradation through P2Y2-dependent signaling pathway (Paoletti et al., 2019).

III – The role of actin in HIV-1 assembly, budding and release

The assembly, budding and release of HIV-1 particles involve a series of coordinated interactions between the virus and the plasma membrane of infected cells (reviewed in (Lerner, Weaver, Anokhin, & Spearman, 2022; Sundquist & Krausslich, 2012)). Gag —the central component of the late stages of the replication cycle— and Env reach the assembly sites via separate routes. Env traffics to the plasma membrane through the secretory pathway, then is endocytosed and recycles at the assembly site where it specifically incorporates into the forming viral particles (reviewed in (Anokhin & Spearman, 2022)). HIV-1 assembly occurs within specialized phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂)-enriched lipid microdomains of the plasma membrane through direct interaction between the matrix (MA) domain of Gag and these acidic lipids. Gag mediates all essential events of virion assembly, starting from the binding to the plasma membrane, to Gag multimerization necessary to generate spherical particles, genomic RNA packaging and concentration of Env proteins. Of note, the large RNA genome of HIV-1 complexed to Gag must cross the actin cortex which could represent a barrier that must be overcome before virus assembly (Isaac Angert, 2021). How Gag reaches the viral assembly sites, via membrane trafficking or cytosolic diffusion, remains unclear (Lerner et al., 2022). At the end of budding, Gag recruits the cellular ESCRT machinery (Endosomal Sorting Complexes Required for Transport) that mediates the release of the virion, through the polymerization of ESCRT-III filaments that pinch the plasma membrane. Finally, once the virion is formed, the protease of HIV-1 is activated (through an unknown mechanism) and cleaves the Gag precursor, resulting in structural rearrangement essential for the formation of a mature capsid. Molecular details regarding these late steps of the HIV-1 replication cycle can be found in these excellent reviews (reviewed in (Lingappa, Reed, Tanaka, Chutiraka, & Robinson, 2014; Meng & Lever, 2013; Rose, Hirsch, & Bouamr, 2020; Sundquist & Krausslich, 2012)).

While a relatively complete model of actin regulation for HIV-1 entry in T cells can be provided (see section II-), the exact role of actin in the assembly, budding and release of the viral particles remains debated. Mechanistically, physical interaction between the nucleocapsid (NC) domain of Gag and F-actin was observed (Ibarrondo et al., 2001; B. Liu et al., 1999; Rey, Canon, & Krogstad, 1996; Wilk, Gowen, & Fuller, 1999). Furthermore, deletion of the NC domain of Gag drastically reduces virus particle production in CD4 T cells upon infection (B. Liu et al., 1999; Rey et al., 1996). However, whether this results from disrupting

actin/Gag or other proteins/Gag interactions and reflects impaired assembly and budding of HIV-1 or other replication steps was not known.

In this section we will describe what is the current knowledge on the role of actin during the late stages of the HIV-1 replication cycle and emphasize the conflicting results that have been observed. We will then review the role of ABPs and actin-regulated proteins involved in assembly, budding and release of HIV-1.

1. Actin dynamics during assembly, budding and release of HIV-1 virus and conflicting results for a role of actin in these viral steps

The incorporation of actin and many ABPs in the viral particle was confirmed by several groups (Aggarwal, Stella, Henry, Narayan, & Turville, 2022; Chertova et al., 2006; Linde et al., 2013; Ott et al., 2000; Ott et al., 1996; Wilk et al., 1999). The presence of actin and ABPs can reflect a critical requirement of some of these proteins in the assembly, budding and release of the viral particles. Alternatively, it can result from passive incorporation and inform on the microenvironment at the time of budding.

First cues for a role of F-actin in late replication events came in 1995, when it was shown that treatment of infected T-lymphoblastoid H9 cells and monocytoid U-937 cells with the actin polymerization inhibitor Cytochalasin D (CytoD) partially inhibits virus release (Sasaki et al., 1995). Over the next few years, several studies using drugs that disrupt F-actin —such as CytoD, Latrunculin A (LatA), mycalolide B, or toxins inhibiting the Rho-family GTPases Rho, Rac and Cdc42— found similar results in various HIV-1-infected cell lines (Audoly, Popoff, & Gluschankof, 2005; Graziano, Elia, Laudanna, Poli, & Alfano, 2011; Jolly, Mitar, & Sattentau, 2007; Sasaki, Ozaki, Karaki, & Nonomura, 2004). Importantly, disruption of the actin cytoskeleton does not inhibit HIV-1 protein expression, but late viral steps in monocytic and T cell lines or differentiated macrophage-like cells, likely via RhoA overactivation in the latter cells (Audoly et al., 2005; Graziano et al., 2011; Sasaki et al., 1995). In addition, polarization of Env and Gag in capped, lipid raft-like assembly domains is impaired upon actin depolymerization (Jolly et al., 2007). Altogether, it was concluded that actin dynamics is essential for the last steps of the HIV-1 replication cycle. Interestingly, ADP-ribosylation of Rho by *Clostridium difficile* C3 exoenzyme does not alter virus release but reduces infectivity of HIV-1 particle, suggesting that the status of the actin cytoskeleton in the producer cells

impacts on the quality of released particles (Audoly et al., 2005). Moreover, treatment of HIV-1 infected cells with actin depolymerizing drugs (CytoD or Latrunculin) reduces Env incorporation into virions and thereby reduces infectivity (Jolly et al., 2007).

Actin is localized at virus budding regions in infected cells (Audoly et al., 2005; Jolly et al., 2007; Sasaki et al., 1995). Moreover, using atomic force microscopy (AFM), fluorescence microscopy and transmission electron microscopy (TEM), specific aster-shaped actin-related structures were described at the budding sites of virus particles in infected HeLa and 3T3 adherent cells (Gladnikoff, Shimoni, Gov, & Rousso, 2009). Interestingly, actin is actively remodeled during budding: viral buds emerge at the center of an actin aster and within 10 minutes aster arms elongate while virions reach their maximal height. After virus release, these structures gradually disappear. Mechanistically, virus-like particles (VLPs) made of Gag mutated in its nucleocapsid domain implicated in actin binding are unable to remodel actin, and bud slower than wild-type Gag by an order of magnitude (Gladnikoff et al., 2009). Thus, specific actin remodeling is induced by HIV-1 to promote viral assembly and release. Furthermore, it was modeled that protrusive forces exerted by actin polymerization at the budding site could promote local viral protein aggregation and thus accelerate virion assembly (Gladnikoff et al., 2009).

A study using cryo-electron tomography (cET) and human glioblastoma adherent cell lines transduced with adenoviral vectors expressing Gag and Gag-Pol proteins reveals that actin filaments are frequently observed in close proximity or at budding sites (Carlson et al., 2010). In addition, the existence of budding sites with different types of actin arrangements was reported. This includes budding sites with actin filaments parallel to the plasma membrane or directed towards the budding sites, and sometimes, budding sites not adjacent to F-actin. In some cases, the tips of actin filaments appear to be connected with the inner rims of the immature Gag lattice, consistent with direct interaction. Of note, half of the viral buds appears on the sides or at the tips of filopodia-like structures characterized by parallel actin organization (Carlson et al., 2010). This has been confirmed in different reports (Aggarwal et al., 2012; Aggarwal et al., 2022; Carlson et al., 2010; Ladinsky et al., 2014), and filopodia formation is induced upon infection in the human monocytic U-937 cell line (Aggarwal et al., 2022). However, it remains to be determined to what extent the high proportion of buds at filopodia might be the consequence of higher accessibility of cET to particular regions of the cell. Interestingly, the viral accessory protein Nef induces the

formation of filopodia (Aggarwal et al., 2012; Nobile et al., 2010). Consistently, Nef is a well-described regulator of the cellular actin cytoskeleton and it modulates the activity of actin regulatory proteins, such as Rac1 and Cofilin, to optimize immune evasion and HIV-1 replication (reviewed in (Ospina Stella & Turville, 2018; Stolp & Fackler, 2011)). Of note, electron tomography and 3D modeling of the bone marrow of HIV-1-infected humanized mice show that virions are associated with, and bud from, the plasma membranes of large cytoplasmic protrusions that are projected from T-cells and contact nearby cells (Ladinsky et al., 2019), confirming that these structures can be formed in a more physiological relevant *in vivo* model.

Based on these observations, it is tempting to speculate that different actin rearrangements occur during HIV-1 budding, with actin polymerization helping Gag-induced early membrane deformation and later actin remodeling allowing ESCRT-III recruitment. Indeed, it was shown that ESCRT-III polymerization is regulated by membrane tension (Booth, Marklew, Ciani, & Beales, 2019; Lafaurie-Janvore et al., 2013; Mercier et al., 2020), and it is well known that cortical actin is a key determinant of cell surface tension (Chugh & Paluch, 2018). Additional studies combining cET and manipulations of actin dynamics are necessary to understand how actin functions in HIV-1 budding and release. Extending this approach to infected T lymphocytes using cryo Electron Microscopy lamellae would also address this issue in a more physiological model.

Two studies from the same laboratory questioned the functional role of actin dynamics at the budding step (Rahman et al., 2014; Stauffer et al., 2014). In the first study, total internal reflection fluorescence microscopy (TIRFM) was used in HeLa cells co-transfected with a plasmid encoding Gag fused to GFP (Green Fluorescent Protein) and LifeAct-mCherry to analyze the viral assembly process and F-actin dynamics, respectively, in live cells (Rahman et al., 2014). The authors reported no difference in F-actin density at budding sites, compared to non-budding sites in the cell, and no transient changes in F-actin distribution over the course of Gag assembly. In addition, short term treatment with actin-depolymerizing or stabilizing drugs (Latrunculin B and Jasplakinolide, respectively) does not affect the rate of budding site initiation nor the kinetics of Gag assembly. Only longer treatment (30 min) with Jasplakinolide, which eventually induces a collapse of F-actin near the nucleus, inhibits the initiation of Gag assembly sites. Based on these results, it was concluded that actin dynamics is dispensable for

Gag transport and assembly at the plasma membrane. However, the authors do not exclude a role of actin in ESCRT recruitment after particles assembly and release of the virus (Rahman et al., 2014). In the second study, using similar cET experiments than the one described above, the authors compared the organization of F-actin at budding of VLPs in glioblastoma cell line expressing either wild type Gag or a Gag mutated in its nucleocapsid domain implicated in actin binding (Gag(LZ)) and previously reported to be important for actin remodeling (Stauffer et al., 2014). No differences regarding the actin association with budding sites—in contrast to (Gladnikoff et al., 2009) based on AFM—and no changes in actin incorporation in VLPs were found. This suggests that neither actin organization at the budding sites nor actin incorporation into VLPs depend on the interaction between NC and the actin filaments. Actin may thus be passively incorporated in virions during budding. Nevertheless, TIRFM at high temporal resolution in HeLa cells expressing GFP-tagged Gag(LZ) shows that this mutant Gag assembles 2-3 times slower than wild type Gag, which is interpreted by the authors as near wild type assembly kinetics (Rahman et al., 2014). In addition, Gag(LZ) budding sites tended to collapse in large patches at the plasma membrane, which were not seen in wild type Gag budding sites. Combined to the aforementioned results, it was concluded that actin and its interaction with Gag is not important for viral assembly.

Several factors might explain the conflicting results regarding the role of actin in the budding step of HIV-1. First, different cell lines have been used: adherent cells, such as HeLa or 3T3 cells are distinct from non-adherent monocytes or T lymphocytes regarding their morphologies, mechanical properties, and actin cortex. Second, while live cell imaging is a powerful approach that allows the analysis of individual budding events instead of mean values that can hide interesting individual behavior, it is carried out on cells that expressed individual tagged proteins and might not reflect infection conditions, as in release tests. Of note, LifeAct is now reported to detect only a subset of the cellular F-actin and it would be interesting to test other probes such as Utrophin-GFP (Belin, Goins, & Mullins, 2014). In addition, budding can be analyzed only in the ventral part of the cell in TIRFM, close to the substrate and cannot give information on budding events on the dorsal part of the cells with different mechanical properties. New live cell imaging techniques such as lattice-light sheet microscopy should help circumvent this issue. Third, TIRFM has limited resolution that cannot detect subtle changes in actin organization, beyond density, which could now be investigated by super-resolution microscopy. Finally, release tests were performed on bulk populations of

infected cells treated for at least 3 hours with actin drugs (used at different concentrations in the different studies), which could lead to indirect effects on virus assembly, budding and release. To avoid potential unspecific impact of actin-disrupting drugs, other studies have examined the role of specific actin binding proteins or actin regulators on HIV-1 assembly, budding and release, as described in the next section.

2. ABPs and actin-regulated proteins involved in assembly, budding and release of HIV-1.

Myosin II

A first ABP, the actin-based molecular motor Myosin II that acts as mechanoenzyme and slides actin filaments (reviewed in (Shutova & Svitkina, 2018)), was proposed to have a role in HIV-1 budding and release in 1995 (Sasaki et al., 1995). Using infected T-lymphoblastoid cells, two observations were made: (1) Myosin II was detected at the HIV-1 budding regions of the plasma membrane and (2) inhibition of myosin light chain kinase with Wortmannin reduced the release of HIV-1 in a concentration-dependent manner (Sasaki et al., 1995). Nevertheless, it should be pointed out that Wortmannin is not a specific inhibitor of this kinase.

ROCK1-LIMK1-Cofilin

Later on, a RNAi-based screen carried out in HeLa cells infected with HIV-1-pseudotyped with VSV-G revealed the involvement of several actin-related proteins in viral assembly or release (Wen, Ding, Hunter, & Spearman, 2014). In particular, depletion of either WAVE1, LIMK1, ROCK1, DIAPH1, or ARPC1B by siRNAs or shRNAs inhibits HIV-1 release. Of note, the role of DIAPH1 in HIV-1 release was not confirmed in Jurkat T cells and primary T lymphocytes (Thomas et al., 2015). The use of different cells —adherent vs in suspension— might explain this discrepancy. Focusing on three actin-related proteins highlighted in the aforementioned screen, an active role of the ROCK1-LIMK1-Cofilin pathway in HIV-1 release was reported (Wen, Ding, Wang, et al., 2014). Depletion of either LIMK1, Cofilin or ROCK1 in HeLa and COS-1 cells decreases HIV-1 release. Moreover, depletion of LIMK1 or ROCK1 induces Gag accumulation at the plasma membrane, indicating that these proteins play a critical role in the late steps of the replication cycle. Consistent with this finding, HIV-1 release

was also impaired when infected cells were treated with a competitive peptide inhibitor of LIMK1 activity (S3 peptide) that prevents Cofilin phosphorylation and thus overactivates Cofilin (Wen, Ding, Wang, et al., 2014). Interestingly, a more recent study identified a small-molecule inhibitor of LIMK1 that also inhibits the release step (in addition to viral DNA synthesis) in infected T cells (Yi et al., 2017). Finally, LIMK1 colocalizes with Gag VLPs and is incorporated in the virus (Wen, Ding, Wang, et al., 2014). Of note, Cofilin was also found incorporated into viruses (Aggarwal et al., 2022; Chertova et al., 2006; Linde et al., 2013; Ott et al., 1996). The fact that depletion of either Cofilin or LIMK1 —that inactivates Cofilin— reduces particle release, suggests that a proper balance between actin polymerization and depolymerization must be maintained during HIV-1 assembly and release. Live-cell imaging of individual budding events and EM would clarify the exact step that is inhibited (Gag polymerization at the membrane, membrane deformation, ESCRT-dependent scission) upon perturbation of Cofilin activity.

Rac1-IRSp53-WAVE2-Arp2/3

F-actin remodeling and membrane deformation required for virion assembly are likely linked. Because Rho GTPases are regulators of both actin dynamics and membrane remodeling, a comprehensive study aimed at understanding the role of each of the three main Rho family GTPases, RhoA, Rac1 and Cdc42, in Gag VLP release from T cells was carried out (Thomas et al., 2015). Only the depletion of Rac1 showed a major effect, with no effect after Cdc42 depletion and a mild effect upon RhoA depletion. Consistently, Rac1 (but not RhoA) specific inhibitors decrease VLPs release in Jurkat T cells. In addition, the expression of Gag alone in Jurkat T cells was found to activate Rac1 by 4 folds, but had no impact on RhoA activation. Furthermore, the authors found that depletion of IRSp53, WAVE2 or Arp3 but not the Rac1 effector Pak2, induces VLP release defects in Jurkat T cells and primary T lymphocytes. This was confirmed for the release of an HIV-1 Δ Env viral strain. It was thus concluded that the Rac1-IRSp53-WAVE2-Arp2/3 pathway is involved in HIV-1 release from T cells (Thomas et al., 2015). These results also suggest that Cofilin activity involved in HIV-1 release is not regulated through the Rac1-Pak2-LIMK1-Cofilin pathway. Mechanistically, depletion of either Rac1 or IRSp53 together with WAVE2 impairs Gag localization at the plasma membrane. Instead of being mainly punctate in control cells, Gag becomes essentially diffuse in the cytoplasm upon Rac1 depletion or upon IRSp53/WAVE2 depletion. Since F-actin levels

are increased in Gag-expressing cells, it was proposed that Rac1-IRSp53-WAVE2-Arp2/3 promotes actin polymerization at the assembly site to maintain Gag there and to prevent its return into the cytosol. Alternatively, protrusive forces exerted by branched actin could favor viral budding (Thomas et al., 2015). Of note, in contrast to IRSp53/WAVE2 co-depletion, a pool of Gag is still present at the budding sites when only IRSp53 is depleted (Thomas et al., 2015).

IRSp53 and AMOT

Later, it was found that IRSp53 is required for the progressive increase of membrane curvature necessary to complete HIV-1 particle assembly, as IRSp53 depletion induces viral bud arrest at half completion, in a “half-moon” shape (Inamdar et al., 2021). Furthermore, using super-resolution microscopy, IRSp53 was shown to surround HIV-1 Gag assembly sites, and was also incorporated into viral particles. It was also found that IRSp53 is in a complex with Gag. Thus, membrane curvature at the budding site is likely promoted locally at the plasma membrane by the Rac1-IRSp53 pathway. Importantly, IRSp53 possesses an inverted BAR (I-BAR) domain that can sense and induce negative membrane curvature (Zhao, Pykalainen, & Lappalainen, 2011) and is implicated in filopodia formation (Krugmann et al., 2001). It was shown *in vitro* that HIV-1 Gag protein preferentially localizes at the tip of curved membrane tubules induced by IRSp53 I-BAR domain on giant unilamellar vesicles (Inamdar et al., 2021). However, the role of actin in this context remains to be elucidated. These observations are nevertheless consistent with cET studies described above indicating a preferential association of virions to actin-rich filopodia. Intriguingly, another protein with a putative BAR domain, Angiomotin (AMOT), was implicated in HIV-1 assembly (Mercenne, Alam, Arii, Lalonde, & Sundquist, 2015). AMOT contains a candidate BAR-like domain that can remodel membranes *in vitro* (Heller et al., 2010), but it is not yet clear whether it functions as a classical or as an inverse BAR domain (Mim & Unger, 2012). Mechanistically, it was shown that the p130 isoform of AMOT (AMOT-p130) directly binds Gag, and that AMOT-p130 overexpression stimulates HIV-1 release whereas AMOT-p130 depletion reduces HIV-1 release (Mercenne et al., 2015). Importantly, AMOT-p130 depletion inhibits viral assembly, with viral buds blocked at “half-moon” shape, a phenotype reminiscent of IRSp53 depletion. It was thus hypothesized that AMOT-p130 is recruited at the half-moon stage of the HIV-1 assembly, through binding to Gag and/or to appropriate curved membranes. Then the BAR domain would promote further membrane curvature required for the recruitment of ESCRT-

III. Because Amot-p130 is an F-actin-binding protein, it was also proposed that F-actin recruitment and polymerization may help push the virion toward the final lollipop stage, when virions remain tethered to the host cell via thin membrane stalks (Mercenne et al., 2015).

Cdc42-IQGAP-Arp2/3 and DIAPH2

As mentioned above, budding particles have been observed at the tips and on the side of actin-rich filopodia structures in different studies (Aggarwal et al., 2012; Aggarwal et al., 2022; Carlson et al., 2010; Ladinsky et al., 2014). Using focused ion beam scanning electron microscopy (FIB-SEM), a recent study revealed that HIV-1 buds preferentially in Arp2/3-dependent actin structures of high positive membrane curvature, such as filopodia and ridges of lamellipodia, in the human monocytic U-937 cell line (Aggarwal et al., 2022). Depletion of either Rac1, WAVE2 or Cdc42 (leading to an absence of filopodia) inhibits virion release into the supernatant. Similar results were obtained when filopodia are shorter upon depletion of the actin-binding protein IQGAP1 —an effector of Rac1 and Cdc42. Altogether, these data suggest that HIV-1 hijacks a Cdc42-IQGAP1-Arp2/3 pathway to position budding sites at the tips of long filopodia and favors release.

The Cdc42-IQGAP1-Arp2/3 pathway is also critical for cell-to-cell transfer of the virus (Aggarwal et al., 2022). It was shown that IQGAP1 directly interacts with Gag, and its depletion increases virus release in adherent HEK293T cells as well as in Jurkat T cells (Sabo, de Los Santos, & Goff, 2020). In these cells, it was proposed that IQGAP1 negatively regulates Gag trafficking to the plasma membrane, and thus limits subsequent HIV-1 release (Sabo et al., 2020). However this was not observed in monocytic cells (Aggarwal et al., 2022). Thus, IQGAP1 could participate in virion release by regulating both filopodia formation and trafficking of Gag, to a degree depending on the cell type.

The budding of HIV-1 at the tip of filopodia and positively-curved membrane extensions is likely important for cell-to-cell spreading. In infected dendritic cells (DCs), it was found that the formation of filopodia bearing viruses on their tips, named HIV-1 filopodia, is dependent on the formin DIAPH2 and on Gag (Aggarwal et al., 2012). Importantly, the efficiency of viral spread from infected DCs to target CD4 T cells is correlated with high frequency of the HIV-1 filopodia. It was thus proposed that these filopodia allow the virus to engage a multitude of contacts with the target cells, thus creating multiple opportunities for cell-to-cell transmission of the virus (Aggarwal et al., 2012).

FLNa and Ezrin

It is noteworthy that the Rac1-IRSp53-WAVE2-Arp2/3 pathway is both involved in HIV-1 release and entry, as previously discussed in the section II-1. Two other proteins involved in HIV-1 entry, FLNa and Ezrin, have also been implicated in virus release (Cooper et al., 2011; Kamiyama et al., 2018).

FLNa was found in virions (Chertova et al., 2006), and directly interacts with Gag (Cooper et al., 2011; Ritchie, Cylinder, Platt, & Barklis, 2015). Moreover, FLNa colocalizes with Gag at the plasma membrane and is upregulated in infected cells (Chan et al., 2007; Cooper et al., 2011; Zhang et al., 2010). Importantly, FLNa depletion impairs HIV-1 release and conversely, FLNa overexpression increases virion release. Mechanistically, it was proposed that FLNa-Gag interaction is involved in Gag localization at the plasma membrane, since FLNa depletion leads to the accumulation of Gag in intracellular compartments (Cooper et al., 2011). More recently, a study revisited the role of FLNa in HIV-1 release. Indeed, it was demonstrated that FLNa associates with the restriction factor BST2/Tetherin (Dotson, Woodruff, Villalta, & Dong, 2016). BST2 tethers nascent viruses to the surface of the infected cells and is counteracted by the viral protein Vpu (Neil, Zang, & Bieniasz, 2008). It was shown that FLNa modulates BST2 turnover, since FLNa depletion enhances BST2 expression at the cell surface whereas FLNa overexpression reduces it (Dotson et al., 2016). EM experiments after FLNa depletion would clarify whether the observed decrease in virion release is due to a decrease in viral budding and/or an increase in virion tethering after the scission step.

The active, phosphorylated Ezrin (p-Ezrin) inhibits HIV-1 release in infected HeLa cells, without affecting Gag targeting at the plasma membrane (Kamiyama et al., 2018). Because p-Ezrin acts as a linker between actin cortex and plasma membrane, it has been proposed that p-Ezrin inhibits HIV-1 release by retaining particles attached to the cellular actin (Kamiyama et al., 2018). EM experiments would be important to confirm this hypothesis.

Taken together, the results presented in this section point toward a role for actin dynamics in HIV-1 assembly, budding and release in T cells (**Figure 3**). In infected macrophages, viral budding takes place mainly in the limiting membrane of a compartment that appears intracellular, named virus-containing compartment (VCC) or Intracellular plasma membrane-connected compartment (IPMC). The current view is that VCCs originate from preexisting tetraspanin-rich zones of the plasma membrane that have been internally

sequestered and are considered continuous with the plasma membrane (Berre et al., 2013; Gaudin et al., 2013; Mlcochova, Pelchen-Matthews, & Marsh, 2013). How virions are released from the VCCs and whether actin dynamics is involved in this mechanism remains to be determined. Nevertheless, it was shown that VCCs are surrounded by a meshwork of actin filaments, and actin depolymerization by Latrunculin-A enhanced the release of mature HIV-1 particles from infected macrophages (Mlcochova et al., 2013; Pelchen-Matthews, Giese, Mlcochova, Turner, & Marsh, 2012). Conversely, a recent study reported that stabilization of actin filaments with Jasplakinolide impairs HIV-1 release from macrophages, highlighting the importance of actin cytoskeleton dynamics in HIV-1 release from VCCs (Rodrigues, 2022). It was further suggested that the actin cytoskeleton is implicated in the movement of VCCs and in VCCs connection with the plasma membrane, which is essential for virion release. Moreover, inhibition of PYK2 —a protein kinase involved in regulating actin dynamics as described in the section II-3 and localized at the VCCs— induces virus accumulation in VCCs that are no longer connected to the plasma membrane. It was thus proposed that PYK2 regulates actin-dependent association of VCCs with the plasma membrane, allowing virus release from macrophages (Rodrigues, 2022).

V – Perspectives

Decades of research have started to highlight the complex interplay between cellular actin networks during entry, budding and release of HIV-1 viruses. While a relatively undisputed model can be proposed regarding the roles of the actin cytoskeleton in HIV-1 entry in T lymphocytes (**Figures 1 and 2**), controversy remains for the budding and release stages of the replication cycle (**Figure 3**). The role of the actin cytoskeleton in HIV-1 entry, budding and release in macrophages is, in comparison, less understood, and requires further investigations. Nevertheless, it is increasingly clear that actin dynamics is also essential for the virus to enter and exit these cells. It is important to note that all these findings arise from various cell models and experimental approaches. Although challenging, future work should focus on physiological relevant cells: primary CD4 T cells, DCs, monocytes and macrophages.

Strikingly, similar actin-regulating pathways and common actin-regulating proteins are involved in both viral entry and exit, as shown for the signaling complex Rac1-WAVE2-IRSp53-Arp2/3, Cofilin, LIMK1, FLNa and the Rho family GTPases Cdc42 and Rac1. We could thus

speculate that sequential actin polymerization and depolymerization is also important for HIV-1 budding and release, as it was demonstrated for entry. To better understand how each ABP controls HIV-1 entry and exit, it will be important to combine high resolution live-cell imaging and single virus tracking during these steps to reveal their precise temporal and spatial localization. Such state-of-the-art cell biology approaches (e.g. (Yin et al., 2020)), are required since virus entry and budding take place within only minutes. This crucial information is lacking in most studies. Furthermore, live cell imaging upon single ABP depletion should clarify at which exact step they act during entry (virus-cell fusion, capsid progression through the actin cortex and its intracellular trafficking) and exit (Gag targeting and assembly at the plasma membrane, ESCRT-dependent scission, release). Importantly, replacing long-term siRNA-mediated depletion —which can induce compensation pathways and indirect defects— by novel strategies of acute depletion (Nishimura, Fukagawa, Takisawa, Kakimoto, & Kanemaki, 2009) or delocalization (Farrants et al., 2020) of actin regulators should provide invaluable insights into the precise role of these proteins. Finally, different F-actin probes and FRET (Fluorescence Resonance Energy Transfer) probes for Rho GTPases should be used to precisely visualize actin cytoskeleton rearrangement and RhoA/Rac1/Cdc42 GTP-bound forms respectively, at different stages of the virus replication cycle.

Given the importance of actin regulation in the HIV-1 replication cycle, the development of drugs modulating the actin cytoskeleton or directly targeting the ABPs involved in viral entry or exit, could represent interesting new therapeutic strategies (Spear, Guo, & Wu, 2013; Yi et al., 2017). Many human viruses manipulate the cellular actin cytoskeleton to proliferate and propagate within the host (reviewed in (Spear & Wu, 2014; Taylor, Koyuncu, & Enquist, 2011)). This includes viral budding at the tips of filopodia-like structures that have been associated with the ability to mediate viral cell-to-cell transfer, or exploitation of Rho GTPases and Arp2/3 complex. Development of drugs that target the host actin cytoskeleton therefore has clinical potential beyond AIDS treatment and could be used to fight other viral diseases. Interestingly, a small-molecule inhibitor of LIMK1 inhibits HIV-1 release in infected T cells (Yi et al., 2017). This small molecule might represent a lead compound for broad spectrum anti-viral drug, since it inhibits the replication cycle of several viruses such as ebolavirus (EBOV), Rift Valley fever virus (RVFV), Venezuelan equine

encephalitis virus (VEEV) and herpes simplex virus 1 (HSV-1), in addition to HIV-1 (Yi et al., 2017).

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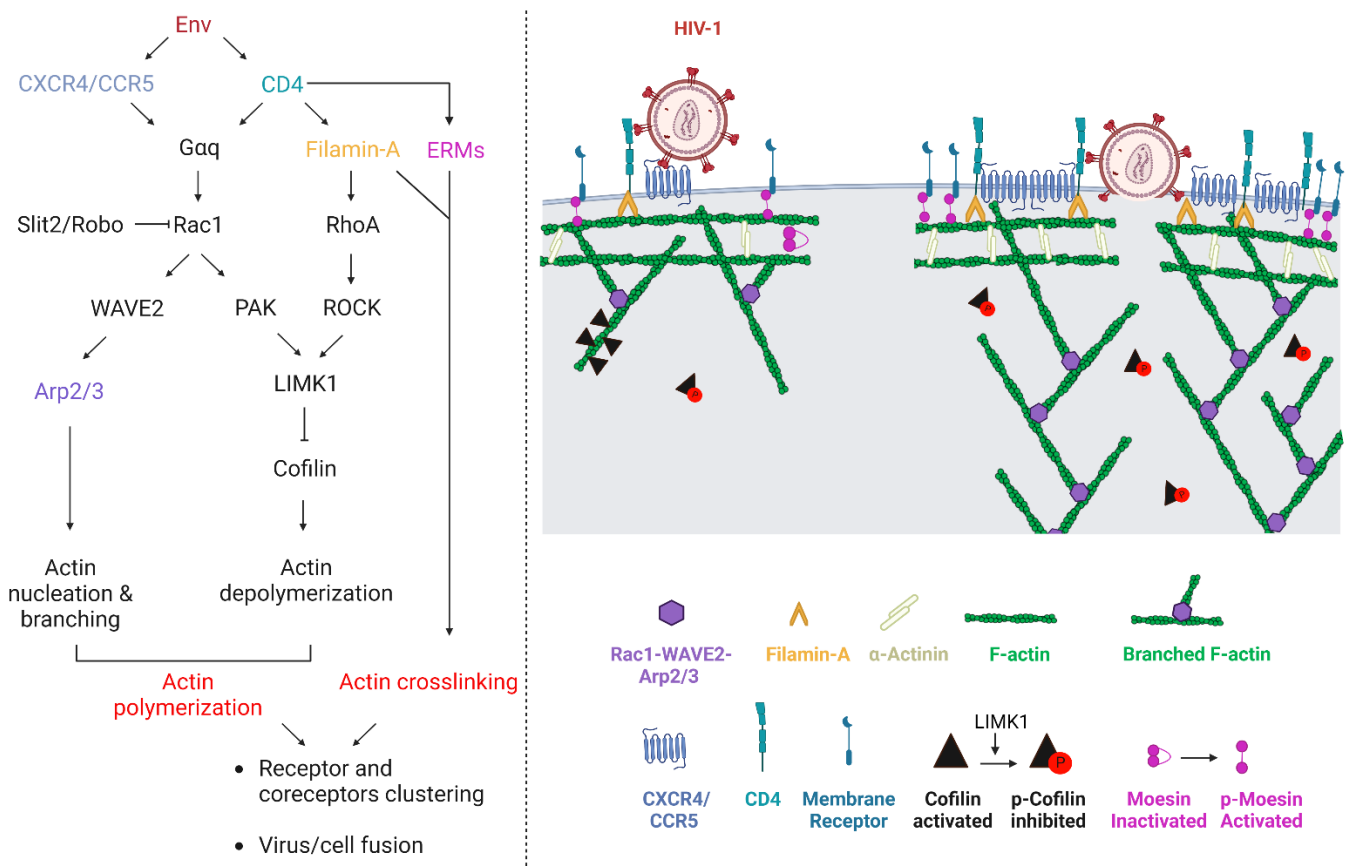


Figure 1

Role of the actin cytoskeleton in Env-mediated receptor and coreceptors clustering implicated in HIV-1 entry in T lymphocytes. The binding of Env to CD4 receptor and CXCR4/CCR5 coreceptors activates signaling through receptor-coupled Gαq and Filamin-A, which activates the Rac1 and RhoA GTPases, leading to Arp2/3-mediated branched-actin nucleation and Cofilin inactivation. This locally increases cortical actin polymerization and results in receptor clustering, which favors fusion pore expansion and viral fusion. Filamin-A and p-Moesin stabilize receptor and coreceptor clustering by crosslinking cortical F-actin to CD4 and to the membrane, respectively.

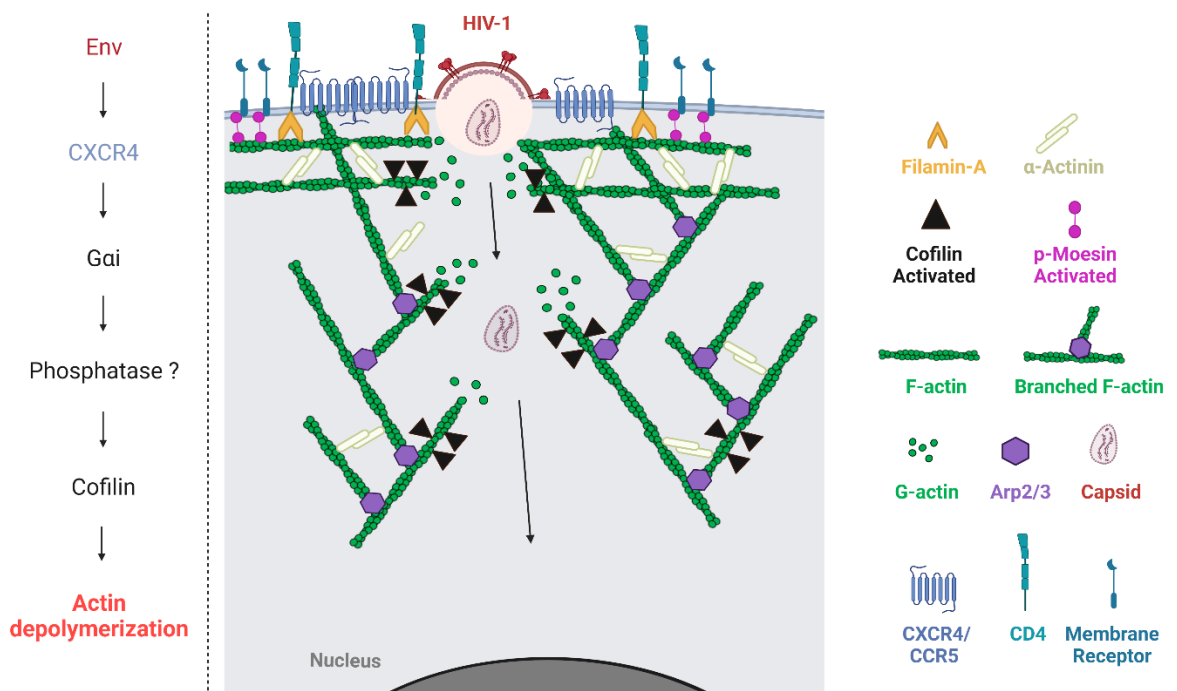


Figure 2

Env-mediated local actin depolymerization promotes the crossing of the capsid through the actin cortex in T lymphocytes. After virus-cell fusion, G α i-dependent Cofilin activation induces local F-actin depolymerization that enables the crossing of the viral capsid through the actin cortex. Moreover, the F-actin cross-linker α -actinin may play a role in actin remodeling to modulate viral progression through the actin cortex.

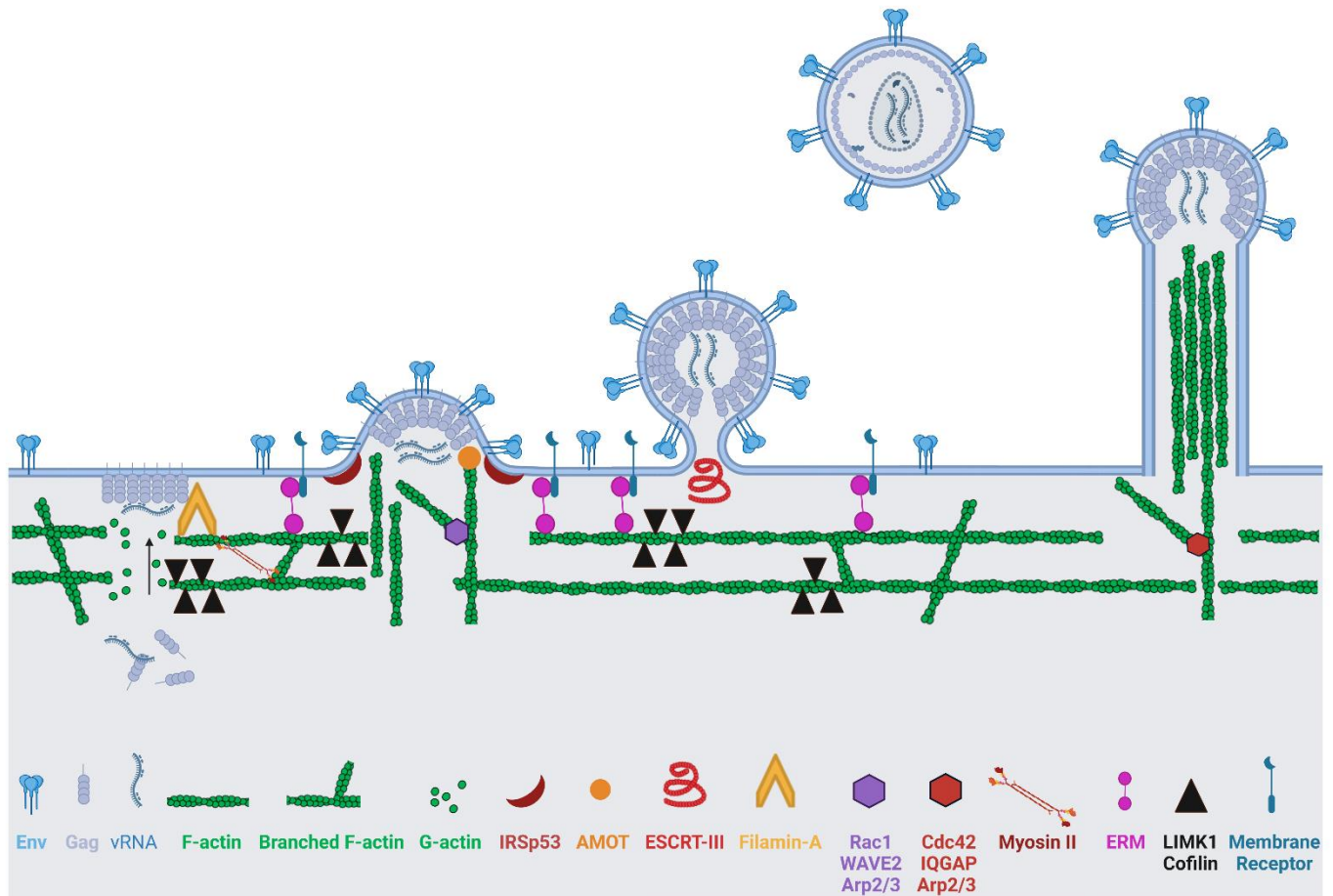


Figure 3

Hypothetical model for the role of the actin cytoskeleton in HIV-1 assembly, budding and release. Recruitment of Gag to assembly sites could be facilitated by a local depletion of cortical actin. Then, actin polymerization could support Gag stabilization at budding sites and produce protrusive forces helping membrane deformation for viral assembly. In addition, the BAR domain proteins IRSp53 and AMOT could further promote membrane curvature needed for membrane deformation during budding. ABPs involved in these steps are depicted in the figure, although the exact step at which they are involved is not fully understood. Budding at the tip of actin-rich filopodia-like structures depends on the Cdc42-IQGAP-Arp2/3 pathway.