Role of I-BAR proteins and membrane curvature in HIV-1 assembly and release

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En Biologie Cellulaire
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ROLE OF I-BAR PROTEINS AND MEMBRANE CURVATURE
IN HIV-1 ASSEMBLY AND RELEASE

Présentée par Kaushik INAMDAR
Le 3 Décembre 2020

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“Your right is to perform your work, but never to the results. Never be motivated by the results of your actions, nor should you be attached to not performing your prescribed duties”
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List of Publications and Communications

PUBLICATIONS

Full assembly of HIV-1 particles requires assistance of the I-BAR IRSp53. Kaushik Inamdar, Feng-Ching Tsai, Aurore dePoret, Rayane Dibsy, Peggy Merida, Rémi Muller, Pekka Lappalainen, Johnson Mak, Philippe Roingeard, Cyril Favard, Patricia Bassereau and Delphine Muriaux*. (submitted)


COMMUNICATIONS IN SCIENTIFIC MEETINGS


Involvement of a Membrane Curving Protein in HIV-1 Gag Assembly. Kaushik Inamdar, Feng Tsai-Ching, Aurore de Poret, Peggy Merida, Johnson Mak, Cyril Favard, Patricia Bassereau and Delphine Muriaux*. ASCB-EMBO 2019, Washington DC, USA, Awarded Travel Grant from SBCF
Abstract

During the HIV assembly and budding, the plasma membrane undergoes a curvature driven by HIV-1 Gag self-assembly at the assembly site towards the exterior of the cell. However, the multimerization of Gag may not be sufficient and Gag may need to recruit cell factors for inducing local membrane curvature. We recently reported that the HIV-1 Gag particle release was dependent on the activation of the signalling pathway Rac1/IRSp53/Wave2/Arp3 in Jurkat T cells and primary blood lymphocytes. This cellular complex, when activated, is recruited to the cell plasma membrane and promotes the recruitment of actin branching, actin polymerisation and membrane remodelling in lamellipodia. In particular, the protein IRSp53 contains an I-BAR domain capable of inducing membrane curvature via the recognition of the plasma membrane phospholipide PI(4,5)P2. This phospholipid is also specifically recognized by the N-terminal Matrix domain of Gag and is a lipidic cofactor of Gag targeting to the plasma membrane and of HIV-1 assembly. This research project aimed at characterizing the cellular and molecular mechanisms of IRSp53 involvement in HIV-1 Gag assembly in CD4 T cells and HEK293T cells. Our results show that IRSp53 is associated with viral particles and that its knockdown by siRNA decreases HIV-1 release in T lymphocytes and HEK293 T cells. Electron microscopy of cells knocked down for IRSp53 revealed a striking phenotype of viral buds arrested at an early stage of assembly. Immunoprecipitation of IRSp53 showed a p6 independent pulldown of HIV-1 Gag, indicating intracellular complexing of Gag and IRSp53. Cellular fractionation and membrane flotation showed that IRSp53 recruitment to cellular membrane doubles upon expression of HIV-1 Gag. Dual colour single molecule PALM/STORM microscopy and subsequent analyses showed IRSp53 in close proximity to Gag clusters. We also found specific incorporation of IRSp53 in HIV-1 Gag particles as compare to other I-BAR proteins, a phenomenon dependent on its I-BAR domain. As the I-BAR domain is involved in membrane curvature, we then probed this aspect of IRSp53 involvement in HIV-1 assembly. Analysis of electron microscopy images revealed a curvature defect for buds from IRSp53 knocked out cells. Concomitant studies in cell free in vitro GUV systems also indicated a role for IRSp53 induced membrane curvature in Gag membrane binding. These results affirm the essential role of IRSp53 in the early stages of HIV-1 assembly. Finally as IRSp53 is a vital player in scaffolding actin signaling proteins, we established a role for activated Rac1 in IRSp53 membrane recruitment downstream of HIV-1 Gag, and test the involvement of the RacGEF Tiam1 in HIV-1 particle production. Super resolution microscopy also reveals the presence of actin nanostructures at HIV-1 assembly sites. All these results highlight the novel and essential role of the membrane curving I-BAR protein IRSp53, and the mobilization of cortical actin, in the late phases of HIV-1 replication.
Résumé

Lors de l'assemblage et du bourgeonnement du VIH, la membrane plasmique subit une courbure entraînée par l'auto-assemblage du VIH-1 Gag au site d'assemblage vers l'extérieur de la cellule. Cependant, la multimérisation de Gag peut ne pas être suffisante et Gag peut avoir besoin de recruter des facteurs cellulaires pour induire une courbure de membrane locale. Nous avons récemment rapporté que la libération de particules de VIH-1 Gag dépendait de l'activation de la voie de signalisation Rac1 / IRSp53 / Wave2 / Arp3 dans les cellules Jurkat T et les lymphocytes sanguins primaires. Ce complexe cellulaire, lorsqu'il est activé, est recruté dans la membrane plasmique cellulaire et favorise le recrutement de la ramification de l'actine, la polymérisation de l'actine et le remodelage de la membrane dans les lamellipodes. En particulier, la protéine IRSp53 contient un domaine I-BAR capable d'induire une courbure membranaire via la reconnaissance du phospholipide de membrane plasmique PI (4,5) P2. Ce phospholipide est également spécifiquement reconnu par le domaine Matrix N-terminal de Gag et est un cofacteur lipidique du ciblage de Gag vers la membrane plasmique et de l'assemblage du VIH-1. Ce projet de recherche visait à caractériser les mécanismes cellulaires et moléculaires de l'implication de l'IRSp53 dans l'assemblage du VIH-1 dans les cellules T CD4 et HEK293T. Nos résultats montrent que l'IRSp53 est associé à des particules virales et que son renversement par l'ARNsi diminue la libération du VIH-1 dans les lymphocytes T et les cellules T HEK293. La microscopie électronique des cellules renversées pour IRSp53 a révélé un phénotype frappant de bourgeons viraux arrêtés à un stade précoce de l'assemblage. L'immunoprécipitation de l'IRSp53 a montré une conversion indépendante de p6 de HIV-1 Gag, indiquant une complexation intracellulaire de Gag et de l'IRSp53. Le fractionnement cellulaire et la flottation de la membrane ont montré que le recrutement de l'IRSp53 vers la membrane cellulaire double lors de l'expression du VIH-1 Gag. La microscopie PALM / STORM à molécule unique bicolore et les analyses ultérieures ont montré l'IRSp53 à proximité des amas Gag. Nous avons également trouvé une incorporation spécifique de l'IRSp53 dans les particules de VIH-1 Gag par rapport à d'autres protéines I-BAR, un phénomène dépendant de son domaine I-BAR. Comme le domaine I-BAR est impliqué dans la courbure de la membrane, nous avons ensuite sondé cet aspect de l'implication de l'IRSp53 dans l'assemblage du VIH-1. L'analyse des images de microscopie électronique a révélé un défaut de courbure pour les bourgeons des cellules assemblées IRSp53. Des études concomitantes dans des systèmes GUV in vitro sans cellule ont également indiqué un rôle pour l'IRSp53 dans la liaison à la membrane Gag. Ces résultats confirment le rôle essentiel de l'IRSp53 dans les premiers stades de l'assemblage du VIH-1. Enfin, comme l'IRSp53 est un acteur essentiel dans l'échafaudage des protéines de signalisation de l'actine, nous avons établi un rôle pour le Rac1 activé dans le recrutement de la membrane IRSp53 en aval du VIH-1 Gag, et testé l'implication du RacGEF Tiam1 dans la production de particules du VIH-1. La microscopie à super résolution révèle également la présence de nanostructures d'actine sur les sites d'assemblage du VIH-1. Tous ces résultats mettent en évidence le
rôle nouveau et essentiel de la protéine I-BAR à courbure membranaire IRSp53, et la mobilisation de l'actine corticale, dans les phases tardives de la réplication du VIH-1.
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List of Abbreviations

aa: amino acid
ABBA: actin-bundling protein with BAIAP2 homology
BAR: Bin-Amphyphysin-Rvs
CA: capsid
CBC: coordinate based colocalization
CD+: Cluster of Differentiation 4
Cdc42: Cell division control protein 42 homolog
DBSCAN: density based spatial scan with added noise
dSTORM: direct stochastic optical reconstruction microscopy
ESCRT: Endosomal Sorting Complexes Required for Transport
GFP: green fluorescent protein
GTPase: guanosine triphosphatase
GUVs: giant unilamellar vesicles
HEK293T: human embryonic kidney 293T
HIV-1: Human Immuno-deficiency Virus 1
I-BAR: inverse BAR
IMD: IRSp53/MIM homology domain
IRSp53: Insulin Receptor tyrosine kinase Substrate protein 53
IRTKS: insulin receptor tyrosine kinase substrate
KD: knockdown
MA: matrix
MIM: missing-in-metastasis
mRNA: messenger RNA
NC: nucleocapsid
NEDD4L: NEDD4 Like E3 Ubiquitin Protein Ligase

PALM: Photo-activated localization microscopy

PinkBAR: planar intestinal and kidney specific BAR domain protein

PI(4,5)P₂: phosphatidylinositol (4,5) bisphosphate

Rac1: Ras-related C3 botulinum toxin substrate 1

RhoA: Ras homolog family member A

RNA: ribonucleic acid

siRNA: small interfering RNA

Tsg101: tumour susceptibility gene 101

VLPs: virus-like particles
INTRODUCTION
1. Chapter 1: HIV and its Replication

1.1 HIV/AIDS and its global disease burden

Human Immunodeficiency Virus (HIV), a retrovirus belonging to the genus *Lentivirus*, is the causative agent of AIDS (Acquired Immunodeficiency Syndrome), which occurs in the more advanced stages of HIV infection. First isolated in 1983 by Montagnier and Barre-Sinoussi at the Pasteur Institute in Paris, it was subsequently found to be the causative agent of AIDS by Gallo and his colleagues at the National Cancer Institute in Bethesda, Maryland (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984). As of 2018, this condition had claimed 32 million lives, while 37.9 million people were estimated to be living with HIV/AIDS over the world. Rapid diagnostic tests and antiretroviral therapy (ART) have made HIV/AIDS a manageable chronic condition for most of the infected population. However, infections continue in vulnerable populations, and an estimated 770,000 people died as a result of HIV-related conditions in 2018 alone, and 1.8 million were newly infected (WHO). While ART has proven efficient in managing the disease, there is no cure currently available for HIV/AIDS thus making it a large part of global disease burden. In addition, HIV drug resistance poses a long term threat to the ART based management of this disease (Clutter *et al.*, 2016), making our understanding of the fundamental processes underlying HIV infection a necessity for future therapies.

![Figure 1: Global prevalence of HIV by regions as defined by WHO (as of 2016).](image-url)
1.2 Health & Pathogenesis of HIV/AIDS

Sexual transmission is the most common mode of HIV transmission globally, while less frequent modes include injection via contaminated needles, exposure to contaminated blood or vertical transmission from mother to fetus. Following transmission in absence of any previous immune exposure, HIV disseminates rapidly by targeting the host CD4+ T lymphocytes causing acute HIV syndrome. This is characterized by flu-like symptoms and lymphadenopathy (Moir, Chun and Fauci, 2011). Due to lack of immune response, the viral load in the blood is extremely high in the first few weeks accompanied by a massive depletion of the target CD4+ T cells. Subsequently, the viral load decreases to a certain level and the virus has been shown to establish reservoirs in the lymph nodes and even in CD4+ T cells. This latency can last from months to years in the absence of ART. During this phase there is a persistent decrease in the CD4+ T cell count and a progressive increase in the viral RNA copy number. This chronic phase can last for over 10 years, before the individual develops AIDS, although this time period has been known to vary widely (Tough and McLaren, 2019).

![Disease progression of HIV/AIDS](image)

Figure 2: Disease progression of HIV/AIDS. Following acute infection, the viremia increases rapidly with a sharp decrease in CD4+ T cell count. This recovers briefly and then decreases progressively in the absence of ART to finally culminate in AIDS. (Tough et al Front Genet 2019)
The HIV genome is a double stranded positive sense RNA packaged with the nucleocapsid of the virus. This is reverse transcribed into the proviral DNA genome, which is integrated into the host cell genome in the nucleus. The genome codes for all the viral structural i.e. \textit{gag}, \textit{env}, replicating i.e. \textit{pol} and several accessory proteins. There are two known strains of HIV, HIV-1 and HIV-2. Both are zoonoses and cause AIDS. They also share similarities in genome and structure, however, HIV-2 infection is characterized by lower plasma viral loads and higher proportion of long term non-progressors. HIV-1, is thus the main causative agent of the global AIDS pandemic.

The \textit{gag} gene encodes the viral structural proteins (MA), the capsid protein (CA), the nucleocapsid (NC). The next \textit{pol} reading frame codes for all the viral proteins which function as enzymes such as the protease (PR), reverse transcriptase (RT), RNase H and integrase (IN). The \textit{env} reading frame codes for the envelope glycoproteins gp120 and gp41 (‘Human Immunodeficiency Virus (HIV)’, 2016). The remainder of the genome codes for the several accessory proteins which play a major role in promoting infection and interacting with cellular immune response. These include Tat, Rev, Nef, Vif, Vpr and Vpu. Vpu is replaced by Vpx in the case of HIV-2 (Nomaguchi \textit{et al}., 2012). There are reports of the anti-sense fragment of the \textit{env} gene which codes for the anti-sense protein (asp) in HIV-1. Reports indicate that this small hydrophobic protein plays a role in autophagy and HIV-1 replication, and cellular and humoral immune responses to this protein have been found in patients infected with HIV-1 (Bet \textit{et al}., 2015; Cynthia Torresilla and Jean-Michel Mesnard and Benoit Barbeau, 2015; Liu \textit{et al}., 2019).
1.4 Viral proteins and Structure

1.4.1 Gag

The viral structural protein Gag (pr55 Gag) is the main component of the viral particle, and is able to form such virus-like particles (VLPs) independently of other viral proteins upon expression. Structurally, it is a polyprotein composed of the matrix (p17, MA), capsid (p24, CA), nucleocapsid (p7, NC) and a P6 domain involved in viral bud release via interactions with the ESCRT-III complex.

![Figure 4: The different domains of the HIV Gag polyprotein from 5' to 3'. The myristoylated MA is membrane targeting followed by the self-assembling capsid CA domain. Separated by a spacer peptide SP1, the NC domain encapsidates the viral genome followed by another spacer SP2. The p6 domain is involved in ESCRT recruitment and scission of the viral bud.](image)

The matrix domain is composed of the N terminal 128 amino acids (aa). The MA domain, specifically the highly basic region (HBR) of the MA domain, interacts with the negatively charged phospholipids in the plasma membrane. The myristate moiety at the N terminal end helps anchor the protein to the plasma membrane (Resh, 2004). Gag, deleted for the MA but retaining the myristate was shown to still form infectious particles, indicating that the MA domain does not participate in the actual formation of the particle per se, but serves as a membrane binding surface and fine-tunes assembly by the capsid domain. The PI(4,5)P₂ specificity of HIV Gag assembly sites is dependent on the matrix domain (Chukkapalli and Ono, 2011). The MA domain has also been implicated in the intracellular transport of Gag by interacting with several cellular adaptor proteins (Ghanam et al., 2012).

The capsid CA domain, which is capable of oligomerizing, forms the structural core of the viral particle. CA has an N terminal domain (NTD) and a C terminal domain (CTD) separated by a flexible linker or
hinge region. Studies have shown that this hinge region is important for correct assembly, replication as well as infectivity (Melamed et al., 2004). The hexamerisation or pentamerisation of the NTD, accompanied by the dimerization of the CTD is necessary for forming the spherical viral core (Bocanegra et al., 2013). The NTD has been shown to be involved in incorporation of the prolyl peptidyl isomerase cyclophilin A into the budding particle. This interaction has been proposed to play a role in inhibiting the binding of the restriction factor TRIM5α to the CA-NTD, thus preventing its degradation at the proteasome (Kim et al., 2019). Since CA-CA oligomers form the core of the virion, this has been an attractive target for Gag-specific antiviral candidates (Carnes, Sheehan and Aiken, 2018).

Separating the CA and the NC domain is the 14 aa SP1 (or p2) spacer peptide. This spacer has been shown to play an important role in viral assembly. Mutational studies have revealed that any mutations within this stretch of residues affected correct particle formation and infectivity (Kräusslich et al., 1995). Cryo-electron tomography studies have shown that hexamers of Gag are, in fact, stabilized by six SP1 helices. The SP1-NC cleavage, required for immature core assembly, occurs first, followed by CA-SP1 cleavage late in the viral assembly, which is necessary for core maturation (Bell and Lever, 2013). Due to this vital CA-SP1 interaction and cleavage, this has also been an attractive antiviral target, with a novel inhibitor being already described targeting the CA-SP1 cleavage site. However, due to the naturally occurring mutations and genetic diversity in viruses, this drug faces a high level of resistance (Adamson et al., 2006; Nguyen et al., 2011).

The subsequent NC domain has three distinct roles in viral replication: nucleic acid recognition, role in virion structure formation and interactions with ESCRT components for scission along with the p6 domain. The NC has two zinc fingers separated by short basic domain. NC domain has the ability to renature nucleic acids and has a role in reverse transcription as well (Guo et al., 2000). Furthermore, as the viral RNA has an important role in coordinating Gag self-assembly (Floderer et al., 2018), the NC domain plays a vital role in optimizing the assembly process. Indeed, the NC domain has been shown to be important for Gag clustering in uropods, and for cell to cell transmission (Llewellyn et al., 2010). It has also been implicated in intracellular trafficking of Gag (El Meshri et al., 2015), and finally for ESCRT interactions as well. Deletion of the NC domain, or its zinc fingers reduced Gag-Tsg101 complexes in cells, suggesting that the NC domain is involved in the recognition of Tsg101 by Gag (El Meshri et al., 2018). NC also binds ALIX possibly in an RNA dependent manner (Popov et al., 2008), thus emphasising its multifunctional role in viral assembly and release.

The NC domain is followed by the second spacer peptide SP2, a short 16 aa stretch of residues containing two highly conserved prolines. Mutations in the C terminal or N terminal of these peptides have produced differential results; C terminal cleavage mutations affect proviral integration in studies of infections, while N terminal mutations do not, suggesting a role in proviral integration.
The p6 domain is the C terminal domain of Gag and has been well described to have a role in ESCRT recruitment via interactions with Tsg101. Deletion of this domain shows a drastically different phenotype of viral buds attached to the membrane via a stalk, indicating a clear deficiency in budding. The p6 domain is a 52 aa domain which has two helices and contains a PTAP motif which interacts with Tsg101. The second helix can also bind and recruit ALIX, thus reinforcing its prominent role in budding and scission via the ESCRT complex (Bell and Lever, 2013).

1.4.2 Pol

The *pol* gene codes for three proteins which fulfill necessary enzymatic functions in the viral replication cycle. These three proteins, protease (PR), reverse transcriptase (RT) and integrase (IN) are incorporated in the viral particle.

The protease is involved in the late steps of the viral replication, specifically in the maturation of the released viral particle by processing of the viral Gag polyprotein and the GagPol polyprotein. It is a 99 aa aspartic protease (having a catalytic aspartate at position 25/25\prime) which functions as a dimer of proteins (Louis *et al.*, 2007). It has also been implicated in cell death via proteolysis of vital cellular proteins such as cytoskeletal proteins and proteins involved in apoptotic pathways (Yang, Nkeze and Zhao, 2012). Due to its essential activity in viral maturation and its cytotoxicity, it has been an attractive antiviral target and protease inhibitors (PIs) are a part of the HAART cocktail of drugs. However, most PIs are competitive inhibitors of protease action, and thus are susceptible to development of drug resistant mutations in the virus.

The retroviral reverse transcriptase, a heterodimer of two subunits (66 kDa and 51 kDa) has two activities: A DNA polymerase activity transcribing double stranded DNA from single stranded viral RNA, and RNase activity, which degrades the viral RNA from the RNA-DNA hybrid. Since these polymerase do not have exonuclease proofreading activity, the rate of mutations induced with reverse transcribing is high as compared to cellular polymerases (Hu and Hughes, 2012). Despite its apparent self-sufficiency, it has been reported to interact with several cellular proteins in order or fulfil its role (Warren *et al.*, 2009). Several antiretroviral drugs such as NRTIs/NNRTIs target the polymerase of viral RT, however, no drugs are available as of yet for the RNAse activity of the RT. The RT has been shown to interact physically and biochemically with the viral integrase (IN). This has been implicated in the viral replication in cells (Tasara *et al.*, 2001).

The integrase protein functions to integrate the proviral reverse transcribed viral genome into the host cell genome. In infected cells, the IN functions as a part of the larger pre integration complex (PIC) which is composed of several viral and cellular proteins (Levy *et al.*, 2013). The viral DNA is processed at its 3’ ends and is then integrated into the host genome by the IN. It is usually present as a multimer
and its multimerization is stabilized by interactions with DNA (Kessl et al., 2009). Integrase inhibitors form the third part of the ART disease management, with several drugs currently approved for treatment of HIV/AIDS.

1.4.3 Env

The envelope glycoproteins gp120 and gp41 are present on the surface of the mature virion. They function in the recognition of the host cell receptors which enable binding and fusion of the viral particle to the host cell. The envelope protein gp120 is responsible for binding to the host cell via the transmembrane CD4 receptor and co-receptors CCR5 or CXCR4 on the surface of the cell. This binding then triggers a conformational change exposing the fusion peptide which then inserts into the cell plasma membrane, enabling fusion of the viral and cellular membranes. Env is a trimer, composed of three gp120 proteins and gp41 transmembrane proteins. It shows high heterogeneity among viruses, even in the number of envelope proteins present on the surface of the virion. Since it is the most prominent antigen, and its structure has been resolved by X-ray crystallography and cryo electron tomography, there are ongoing efforts to design vaccines against this protein (Arrildt, Joseph and Swanstrom, 2012; Ringel et al., 2018).
1.5 Viral Replication Cycle

The viral replication cycle starts with the binding of the infectious mature virion to the host cell surface receptors. The first step is the docking of the Env glycoprotein gp120 onto the cell surface receptor CD4. This induces a conformational change in gp120 which then enables it to recognize and bind the co-receptor (CCR5 or CXCR4). In receptor independent variants of HIV, which are capable of infecting receptor negative cells, the gp120 displays a “pre-triggered” or “receptor-primed” structure which is reminiscent of CD4 bound gp120. CD binding also induces a weakening or dissociation of gp120 from gp41, a process which is necessary for the subsequent membrane fusion. There is also evidence that receptor binding provokes conformational changes in the gp41 subunit. Fusion proceeds into two major steps: insertion of fusion peptides into the target membrane, and subsequent folding into hairpin structures to force the membranes into close proximity. The hydrophobic fusion peptide is in the N terminal of the HIV gp41, followed by a polar region and then two heptad repeats (HR1 and HR2 respectively). The long C terminal stretch has several domains, some of which have been reported to be involved in Env stability, association with lipid rafts and the fusion activity. The fusion of membranes proceeds as an initial hemifusion, i.e. lipid mixing of the distal leaflets of both membranes followed by the formation of the fusion pore. Formation of the fusion pore induces the final change in gp41 structure, the formation of the hairpins in HR1 and HR2 coils, which in turn stabilize the pore. The energy barrier required to enlarge the pore and facilitate release of the viral capsid could be provided by cellular machinery such as actin cytoskeletal signalling underneath the cell plasma membrane (Melikyan, 2011). Alternatively, HIV has also been shown to enter by conventional pathways of trafficking such as endocytosis (Miyauchi et al., 2009; van Wilgenburg et al., 2014; Chauhan and Khandkar, 2015).

The release and disassembly of viral capsids inside the host cell is known as uncoating. This process is concomitant with the reverse transcription of the viral genome into proviral DNA for integration. In fact, a study has shown that uncoating is triggered by the first strand transfer of the reverse transcription (Cosnefroy, Murray and Bishop, 2016) and inhibiting reverse transcription delays the uncoating process (Hulme, Perez and Hope, 2011; Yang, Nkeze and Zhao, 2012). Microtubular motors dynein and kinesin have been shown to be involved in uncoating (Lukic et al., 2014; Pawlica and Berthoux, 2014) via CA interactions with adaptor proteins FEZ1 and BICD2 (Malikov et al., 2015; Dharan et al., 2017). The eukaryotic translation elongation factor eF1α is also involved in uncoating and reverse transcription by interactions with the reverse transcriptase (Rawle et al., 2018). The dynamics of uncoating have been hotly debated, with previous studies saying that uncoating occurs directly after entry, while recent live cell single particle tracking studies indicate that capsid integrity loss takes at least 30 minutes post fusion (Mamede et al., 2017). However the as the nascent proviral DNA cannot be exposed to the cytoplasm, the capsid disassembly is a gradual process, with the final remaining core molecules docking at the nuclear pore (Francis and Melikyan, 2018). Proteins of the nuclear pore complex could be involved in disassembling the remaining CA molecules associated with the post
nuclear entry entry pre-integration complex (Chen et al., 2016; Dharan et al., 2016). A very recent study tracking single cores labelled with CA-GFP showed that nearly intact cores enter the nucleus, complete reverse transcription and uncoat near sites of integration about 1.5 hours before integration commences (Burdick et al., 2020). Thus the dynamics and location of HIV uncoating remain under intense study and speculation till date.

The integration of the proviral DNA into the host genome is mediated primarily by the viral integrase (IN) which is present in the viral core along with the other viral enzymes and the RNA genomes. Integration of the viral DNA in the host cell genome ensures that the viral genes are subject to the transcription along with the cellular genes. The proviral DNA thus serves as a template for transcription of viral genes. After reverse transcription occurs, the proviral DNA is initially stabilized with the IN protein in a large nucleoprotein complex known as the pre integration complex (PIC). Integrase tetramers associate with proviral DNA during integration in a complex then known as intasome. The integration process involves processing of the proviral DNA at its 3’ ends by the viral integrase followed by formation of phosphodiester bonds with the target DNA and subsequent integration. The nuclear
import, the associated cellular proteins such as RANBP2, TNPO3 and CFSP6 affect the choice of the site of integration (Lusic and Siliciano, 2017). In the three-dimensional nuclear space, proviral integration is preferable towards the periphery of the chromatin, in proximity to the nuclear pore (Marini et al., 2015). The site of integration also affects the transcription fate of the provirus, with permissive transcriptionally active sites allowing immediate transcription of viral genes, while less permissive silent sites delaying viral transcription and possibly contributing to formation of latent viral reservoirs. Studies have shown that active transcriptional sites are preferred including those genes which are activated post HIV infection (Schröder et al., 2002). Integration is also subject to physical constraints, with accessible and flexible chromatin regions of the nucleolus being favoured over condensed regions (Michieletto et al., 2019).

The transcription of viral genes is staggered, with the regulatory proteins Tat and Rev being transcribed first as short splices mRNA transcripts. Larger transcripts of Env, Vif, Vpr and Vpu are produced as the transcription increases sharply later in the infection. Viral genomes and the large GagPol transcripts are the last to be produced during the transcription of the proviral DNA. This regulation of transcription is largely controlled by the early Tat and Rev proteins. Tat (transactivating factor) activates transcription by binding to the LTRs and promoting elongation of transcripts, thus producing the longer transcripts of the viral proteins. Tat is able to recognize and bind a specific transactivator responsive region (TAR) RNA, located in the first 59 nucleotides of the LTR. Along with the cellular pTEFb complex, and its component the CD9 kinase, Tat induces hyperphosphorylation of the RNA polymerase at the TAR site, promoting elongation of the mRNA transcripts (Wu and Marsh, 2003; Karn and Stoltzfus, 2012). While necessary to produce the large number of different transcripts required for the different viral proteins, the HIV splicing mechanism is relatively inefficient as compared to cellular spliceosomes (Martin Stoltzfus, 2009). As unspliced or incompletely spliced mRNA transcripts are degraded in the nucleus, HIV and other retrovirus such as HTLV express regulatory factors which aid in nuclear export of these transcripts.

While early completely spliced transcripts are exported by cellular pathways, later larger transcripts (GagPol and Env) typically need the retroviral regulatory protein Rev for their export. Rev can oligomerize on specific elements in these transcripts with high affinity (Daugherty, D’Orso and Frankel, 2008) and then direct their export via nuclear pore complex proteins (Köhler and Hurt, 2007). Early transcripts of Tat, Rev and Nef exported via cellular pathway are translated first, but later on their translation is suppressed. These proteins are imported into the nucleus for their regulatory purposes. The other viral mRNAs interact with cellular helicases and initiation factors to promote initiation of their translation (Soto-Rifo et al., 2012; Soto-Rifo, Rubilar and Ohlmann, 2013). Furthermore, HIV-1 targets cellular translation factors to modulate native cellular translation and promote viral translation (Guerrero et al., 2015). Translation proceeds mainly by a cap-dependent, but also by IRES dependent mechanisms. HIV genome has two IRES, one in the 5’ UTR and another in the gag reading frame. The first IRES is supposed to drive the translation of the pr55 Gag and pr160 GagPol proteins while the
other one is involved in the translation of the structural proteins and the p40 isoform of Gag. The synthesized proteins are and the viral genomes are trafficked to the plasma membrane where Gag driven assembly of the virion takes place (Monette et al., 2013), followed by budding and scission of the viral particle. The immature viral particle undergoes maturation following cleavage of the polyprotein Gag into its constituent domains.

1.6 Structure of the Virion

The HIV virion is a spherical enveloped particle approximately 100-140nm in diameter. The lipid envelope is sourced from the host cell plasma membrane as the virus assembles and buds at the plasma membrane. The outer surface has the envelope glycoproteins as trimers. There are few Env molecules on the surface, and their numbers vary from 6-20 (on an average 14 Env trimers per virion) (Zhu et al., 2003; Liu et al., 2008; DeSantis et al., 2016). Env clustering is important for receptor binding and fusion with the host cell membrane (Roy et al., 2013). The density of Env molecules on the surface thus affects the transmission (Parrish et al., 2013) and also the infectivity of the virus (Bachrach et al., 2005; DeSantis et al., 2016). The Env gp120 is exposed on the surface while the gp41, linked to gp120, is embedded in the lipid membrane by a single transmembrane helix.

The immature capsid is composed of about ~2500 uncleaved pr55 Gag molecules. They are oriented as follows: the MA domain is towards the exterior of the capsid shell and binds the lipids in the membrane as well as the tails of the gp41 protein (Saad et al., 2006; Chojnacki et al., 2012). The capsid-capsid interactions along with the SP1 spacer form the structural base of the immature capsid shell. The NC domain is oriented towards the interior while binding the copies of the viral genome. The p6
domain does not have any apparent role in virion structure, but is involved in the late steps of viral budding (Garrus et al., 2001; Chamontin et al., 2015; El Meshri et al., 2018). It is also noted that the immature Gag shell is not continuous on the virion surface; it does not cover the entire particle, but only up to ~40% of the surface (Wright et al., 2007).

During maturation, the polyprotein Gag is cleaved by the viral protease into MAp17, CAp24 and NCp7. This triggers a rearrangement within the viral particle, with the MA remaining associated with the viral membrane. The mature CA assembles into the well-known capsid core, with the NC proteins condensing with the viral RNA and the viral enzymes PR, RT and IN. On rearranging the Env molecules on the surface, the particle is now primed for infection (Ganser-Pornillos, Yeager and Pornillos, 2012).
2. Chapter 2: Late stages of Viral Replication

2.1 Trafficking of Viral Proteins

HIV assembly in host T cells and other cell types has generally been accepted to be a process which takes place at the plasma membrane (Ono and Freed, 2004; Rudner et al., 2005; Jouvenet et al., 2006; Hübner et al., 2007; Welsch et al., 2007; Jouvenet, Bieniasz and Simon, 2008; Floderer et al., 2018). Viral proteins synthesized in the cytoplasm are thus trafficked to the plasma membrane. The retroviral Gag protein, synthesized on free cytosolic ribosomes, is the structural unit of the assembling virus. While the intracellular trafficking of Gag is still a topic of ongoing study, it has been shown to interact with several cellular proteins which could be implicated in its transport to the membrane. Gag-MA interacts with the cellular calcium binding CaM protein (Ghanam et al., 2010), which is involved in shuttling proteins to the plasma membrane. Some studies have also shown interactions of the Env gp160 with this CaM (Srinivas et al., 1993; Radding et al., 1996). Gag-MA (Dong et al., 2005) and Env (Batonick et al., 2005; Camus et al., 2007) also interact with adaptor proteins AP-2 and AP-3 respectively, which are also involved in intracellular transport. Gag also interacts with other proteins such as TIP47, a retrograde transport protein (Lopez-Vergès et al., 2006) and SOSC1, the depletion of which severely affects Gag trafficking (Ryo et al., 2008; Nishi et al., 2009). Interestingly, Gag-interactions with many of these proteins is mediated by the MA domain (Engeland et al., 2011), which is also involved in plasma membrane binding and lipid recognition.

The viral accessory proteins Nef and Vpu also manipulate the host cell trafficking apparatus, however, for the purpose of misdirecting cellular transport and mislocalizing cellular proteins. Both Nef and Vpu affect the cell surface distribution of the CD4 receptor, Nef by inducing the endocytosis of CD4 via the AP complexes (Greenberg et al., 1998; Fauré et al., 2004; Basmaciogullari and Pizzato, 2014) while Vpu induces ubiquitination and degradation of newly synthesized CD4 (Willey et al., 1992; Chen et al., 1993; Meusser and Sommer, 2004; Binette et al., 2007; Magadán et al., 2010). This CD4 downregulation and mislocalization lowers the probability of superinfection by released virions, and also facilitates release by reducing the CD4 molecules available at the surface and the possibility of post release Env-CD4 interactions. Finally, the Vpu mediated ERAD manipulation also ensures that Env is able to exit the ER, where the strong affinity of CD4 and Env could retain the Env in the ER (Buonocore and Rose, 1993).

Gag targeting to the plasma membrane also implicates the membrane lipids. Downregulation of the phospholipid PI(4,5)P_2 at the plasma membrane cause mislocalization of Gag at the MVBs (Ono et al., 2004) while cholesterol depletion causes loss in membrane binding and reduces virus assembly and production (Ono and Freed, 2001; Ono, Waheed and Freed, 2007). There is also evidence that HIV-1 may use the cholesterol trafficking pathway for Gag transport to the membrane (Tang et al., 2009). The Env protein gp41 also demonstrates some membrane lipid affinity (Hollingsworth et al., 2018) in addition to its interactions with the Gag protein.
The MA domain of Gag (along with its myristate) thus plays a major role in HIV-1 Gag trafficking by interacting with cellular adaptor proteins as well as securing the specificity of membrane lipids for optimal Gag membrane binding and assembly.

2.2 HIV-1 Assembly and Budding

2.2.1 Role of Viral Factors

HIV assembly, driven by the retroviral protein Gag, takes place predominantly at the plasma membrane of the host cell. It is a complex interplay between the self-assembly of Gag on one hand, and the membrane components on the other. At the base of the assembly process are about 1500-3000 molecules of Gag which self-assemble on the plasma membrane by binding phospholipids to form the shell of the immature virion (Vogt and Simon 1999). HIV Gag when expressed in cells is sufficient and capable of forming non-infectious virus like particles (Gheysen et al., 1989). The accessory proteins Vif, Vpr and Vpu have been implicated in the assembly of infectious virions. Vif has been reported at the membranes of infected cells and Vif mutants exhibit low viral titers (Goncalves et al., 1995, 1995; Volsky et al., 1995). This could be related to its role in increasing Env incorporation in assembling virions (Sakai et al., 1993; Borman et al., 1995). It also binds the pr55 Gag protein (Bouyac et al., 1997; Zhang et al., 2000) via the NC domain of Gag. The other accessory proteins also have been known to be incorporated in the budding virion for roles in early steps of infection, or they perform functions related to Env processing and incorporation.

The viral RNA has been proposed to play an important role in HIV assembly. A majority of viral particles produced from cells infected with the wild type virus contain genomic RNA (Chen et al., 2009), however in absence of viral RNA, in cells expressing Gag alone, viral particles have been found to contain cellular RNA (Rulli et al., 2007). The specificity of genomic RNA incorporation is dependent on the Ψ packaging signal, at the 5’ end of the viral RNA (Comas-Garcia et al., 2018). Even recombinant Gag while assembling in vitro could do so on any strand of nucleic acid, DNA or RNA (Campbell and Rein, 1999; Campbell, Crowe and Mak, 2001). Thus nucleic acids, and more specifically RNA, are not just passively incorporated into the assembling particle, but they certainly play a role in facilitating Gag assembly. A thermodynamic study involving recombinant Gag and RNA showed that Gag-RNA interactions, more specifically, the energy released from Gag-RNA interactions, play a role in overcoming the energy barrier necessary to form viral particles, and that these interactions are thus energetically favourable for assembly (Tanwar et al., 2017). In agreement with this, an elegant sptPalm study and trapping energy analysis in CD4+ T cells showed that approximately two-thirds of the energy required for Gag assembly came from Gag-RNA interactions, while only one third came from Gag CA-CA interactions (Floderer et al., 2018). Another recent study involving sptPalm tracking
of single Gag molecules showed that Gag deleted for the NC domain (thus unable to interact with RNA), was monomeric at the plasma membrane and did not form higher order clusters (Yang et al., 2018). Overexpression of Gag, however, has been shown to reduce this RNA-dependence of the assembly (Dilley et al., 2017), indicating the Gag-RNA interactions facilitate and enhance viral assembly while not being absolutely necessary for the process to take place.

The binding between Gag-MA and the plasma membrane is mediated predominantly by the membrane phospholipid PI(4,5)P$_2$ and cholesterol (Ono et al., 2004; Saad et al., 2006; Chukkapalli et al., 2008; Chukkapalli, Oh and Ono, 2010; Hamard-Peron and Muriaux, 2011; Charlier et al., 2014; Olety and Ono, 2014; Mercredi et al., 2016). In contrast to previous models, recent studies show that Gag is able to cluster these lipids in host cell membranes, essentially forming its own membrane nanodomains for assembly (Favard et al., 2019a). This was earlier shown on supporte lipid bilayers (SLBs) as well, where it was shown that Gag clusters PI(4,5)P$_2$ and cholesterol on these model membranes, but excludes sphingomyelin (Yandrapalli et al., 2016). This lipid composition of assembly sites has been also seen by others (Barklis et al., 2018; Chen et al., 2020). Interestingly, Barklis et al showed that PI(3,4,5)P$_3$ association with Gag remained unchanged irrespective of the presence of the membrane binding MA domain, suggesting that Gag could have lipid interactions apart from those required directly for assembly. PI(3,4,5)P$_3$ is an important intermediary in several pathways involving growth factors as well as cortical actin polymerization signalling, making this an intriguing proposition.

Figure 7. Representation of HIV-1 Gag assembly. Gag assembly showing the different domains of Gag and their interaction with membrane components and viral RNA (vRNA) to form the assembly platforms via Gag-Gag multimerization. (adapted from Mariani et al Front Microbiol 2014).
2.2.2 Structural aspects of HIV assembly

Structurally, the assembly of HIV-1 Gag corresponds to a lattice of self-assembling Gag molecules which incompletely cover a roughly spherical bud of about 100-150 nm (Wright et al., 2007). As mentioned before, multiple domains of Gag contribute to the assembly process; The MA and the myristate anchor the Gag to the membrane, while the CA-SP1-NC domains induce self-assembly through interactions with other Gag molecules and the RNA. The assembly process thus is a combined product of all these interactions, and is a co-operative process dependent on these individually weak interactions (Ganser-Pornillos, Yeager and Sundquist, 2008). The basic unit of assembling Gag is a hexameric unit of Gag rings formed primarily via CA-SP1 interactions with a spacing of about 8 nm (Nermut et al., 1998; Mayo et al., 2003; Briggs et al., 2004, 2006b) in neighbouring Gag molecules. Cryo-electron tomography of HIV virions (Wright et al., 2007), showed distinct hexameric lattice formation by the CA\textsuperscript{CTD}-SP1 domains, while no such arrangement was observed for the other domains. The CA\textsuperscript{CTD} makes intrahexamer as well as interhexamer contacts, contributing to overall lattice formation. Coarse grained simulations by Pak and colleagues found that the CA\textsuperscript{NTD} contributed to the membrane curvature generated due to assembly, thus coupling higher order multimerization of Gag to the necessary membrane curvature (Pak et al., 2017). Other studies (Grime et al., 2016) showed that formation of higher order structures was triggered by the increase in local CA concentration and density; at lower concentrations CA existed as several metastable “trimers of dimers”, a certain threshold needed to be overcome to form lattices corresponding to assembly site nuclei. Correspondingly, a recent study on modelling assembly kinetics via Gag-Gag interactions found that at least 50 Gag molecules were required to form stable nucleation sites for further assembly (Tomasini et al., 2018, 2018). This study further confirmed the notion of “trimers of dimers” as basic units of Gag assembly with monomers have a higher $K_{\text{off}}$ (dissociation constant) from modelled membranes.

![Figure 8. Representation of assembling Gag oligomers. a) Hexagonal lattice of Gag formed of three hexamers. The dimeric, trimeric and hexameric axes of symmetry are denoted by the ellipse, triangle and circle respectively. b) Monomers, dimers, trimers and the hexamers of Gag (adapted from Morger et al 2018 J Virol).](image-url)
The nucleating structure required to start Gag assembly is thus much smaller than the entire assembly site. A linear model of Gag assembly (Liu and Zou, 2019) predicted that free hexamers do not assemble into lattice structures until having reached a threshold value, thus emphasising the dynamic nature of Gag assembly.

2.2.3 Dynamics of HIV Assembly

HIV Gag assembly is a dynamic process which involves a gradual buildup and multimerization of Gag molecules from the cytosolic pool to form an assembly platform and the viral bud (Ivanchenko et al., 2009). A pioneering study using TIRF microscopy by Jouvenet and colleagues in 2008 showed that fluorescently tagged Gag appeared as punctate spots on the membrane and that the increase in fluorescence corresponded with assembling Gag took about five to six minutes (an average of 8.5 minutes) to complete its course (Jouvenet, Bieniasz and Simon, 2008). This study also noted a second rapid population of Gag punctae which were associated with tetraspanin markers, indicating pools of Gag in late endosomes. Using FRAP analysis, they were able to show that Gag assembly is a results of accumulation of Gag molecules in the punctae as evidenced by recovery of fluorescence, and also that this assembly process reaches an end, as evidenced by no recovery of fluorescence in static spots, indicating no further exchange of Gag molecules in these spots and hence completion of assembly. In the analysis of hundreds of assembling particles, they did not associate any organelle discharging pre-assembled VLPs at the plasma membrane indicating that, in their cell type, Gag assembly was exclusively at the plasma membrane. A subsequent study then demonstrated the associated of viral RNAs with the assembling Gag particles, and the synergistic relationship between Gag membrane binding and assembly on one hand, and RNA persistence at the membrane on the other (Jouvenet, Simon and Bieniasz, 2009). The fluorescent Gag puncta had already been shown as spherical virions bulging outward from the host cell membrane by correlative light and electron microscopy (Larson et al., 2005).

A more quantitative kinetics study of HIV-1 (labelled with GagGFP) assembly by coupled live cell time lapse TIRF/Wide field microscopy supported these results with an average time of 8-9 minutes to complete Gag assembly at the plasma membrane (Ivanchenko et al 2009 Plos Path). They fitted the fluorescence intensities of assembling particles to a saturating exponential function and obtained an average rate constant of Gag accumulation at 5x10^{-3}/second, corresponding to an assembly time of 8-10 minutes i.e. time needed to for exponential increase in fluorescence to reach a peak, indicating the end of assembly. Furthermore, three distinct phases of fluorescence dynamics were observed, viz. an exponential increase followed by a temporally variable plateau phase where the particle fluorescence fluctuated around a constant mean, and third phase of fluorescence decline. The first phase being assembly, the second phase was described as an assembled particle diffusing on the surface of the cell, following which ESCRT protein recruitment promotes viral bud scission thus inducing the third stage of
fluorescence decrease. The entire process took 1500±700 seconds to complete (~25±11 minutes), including assembly and release. The saturating exponential model also showed that Gag assembly was eventually retarded and stopped at a plateau, indicating the completion of the spherical shell of Gag, and prevention of further addition of Gag molecules to a complete particle. However, the Gag shell on viral particles is does not completely cover the surface of the particle (Wright et al., 2007; Carlson et al., 2008), indicating that factors other than Gag polymerization play a role in restricting the further addition of Gag in the budding particle. As ESCRT recruitment and scission of the bud occur in the late steps of viral assembly, membrane curvature of the nascent bud could play a major role in restricting further Gag multimerization of Gag at the assembly site. Mutants of Gag deleted for ESCRT interacting domains followed the same kinetics of assembly as wild type Gag (Jouvenet, Bieniasz and Simon, 2008; Ivanchenko et al., 2009). Expression of such a Gag mutant tagged with the pH-sensitive pHluorin showed that viral particles are distinct from the cytosol and yet remain membrane associated in the absence of ESCRT recruitment, indicating the well-known role of the ESCRT complex in scission and separation of the viral bud from the host cell plasma membrane. In accordance, budded particles (Ivanchenko et al., 2009) showed a dramatic increase in their recorded velocities, upon being released from the membrane.

These studies were performed in HeLa cells, which are not the host cell types permissive for HIV infection in a physiologically relevant context. Building on this, a quantitative single molecule sptPALM study of different mutants of HIV-1 Gag tagged with mEos2 was performed in host CD4+ T cells (Floderer et al., 2018). This single-molecule approach measuring localisation densities, diffusion and trapping energy of Gag molecules afforded a nanoscale level of precision to study this process. Localisation densities were analysed as indicators of Gag assembly (as compared to fluorescence intensities in earlier studies). A similar “three-phase” model of localisation density was observed, corresponding to a rapid increase, followed by plateau and then an abrupt decrease, mirroring, on a single molecule level, the aforementioned model by Ivanchenko and colleagues. Increased localisation density corresponded with decreased diffusion of Gag molecules, indicating that Gag assembly sites acted as “attraction traps” for additional Gag molecules in the vicinity. They showed that assembly kinetics in the relevant host T cells were similar to those observed before, with an average of 15 minutes required for assembly and release, and ~5 minutes were required for assembly to complete, as observed before by Jouvenet and colleagues in HeLa cells. Taken together, these results suggest that Gag assembly dynamics are independent of cell type and take place primarily at the plasma membrane of the respective cells.
2.2.4 Budding and scission of the viral particle

The process of scission of a vesicle from a membrane consists, in general, of three steps: the formation of a neck on the vesicle, constriction of the neck beyond a critical diameter and fission of the membrane and fusion at the neck, forming a complete particle (Rossman and Lamb, 2013). The second step of constriction is facilitated by mainly by protein-membrane interactions in cells, forcing the membrane to constrict beyond the critical threshold and thus catalysing scission of the membrane. While lipid-based membranes can spontaneously form vesicles by scission, cellular proteins are highly effective at this since their spatiotemporal concentration is tunable by the cell, thus making their action coordinated and specific. One such family of proteins are the endosomal sorting complex required for transport (ESCRT) proteins. They consist of four complexes, ESCRT-0, I, II, III and IV. ESCRTs 0 to III mediated targeting and membrane binding of the ESCRT complex, while ESCRT-III proteins are required for the final scission step (Hurley and Hanson, 2010; Hurley and Cada, 2018). THE ESCRT-III complex promotes scission of the membrane by polymerizing in spirals of decreasing diameter at the inner leaflet of the membrane bud, constricting the neck to induce scission and formation of the free bud (Fabrikant et al 2009, Wollert et al 2009).

The NC and p6 domains of HIV Gag are known to recruit members of the ESCRT-I family by direct interactions (Garrus et al., 2001; Chamontin et al., 2015; El Meshri et al., 2018). The ESCRT-0 and ESCRT-II functions of cargo clustering and ESCRT-I recruitment respectively are essentially taken over...
by HIV Gag in the context of viral budding, as it is capable of forming its own clusters as well as recruits Tsg101 and ALIX by itself (Hurley and Hanson, 2010). Mutations or deletions in the C terminal end of Gag affecting the p6 domain were found to produce defects in budding (Göttlinger et al., 1991), and the PTAP motif in this domain was found to recruit Tsg101 and Vps4 ATPase (Garrus et al., 2001). Deep etch electron microscopy by Cashikar and colleagues showed characteristic ESCRT-III spirals at the base of the viral bud (Cashikar et al., 2014).

ESCRT proteins are recruited prior to scission and also disassemble before scission. Study of membrane curvature and ESCRT recruitment showed that ESCRT recruitment, while not instrumental in generating this curvature, occurs after the membrane has been curved during Gag self-assembly (Johnson, Bleck and Simon, 2018). In fact, the negative curvature resulting from Gag assembly has been proposed to be a factor in the recruitment in of ESCRT-III complexes to the membrane (Lee et al., 2015). The pleiomorphy seen in immature shell assembly can be explained by the competition of the ongoing membrane curvature versus ESCRT recruitment. Mutants defective for budding show a more complete Gag shell than the wild type viruses, having more Gag molecules on an average (Carlson et al., 2008, 2010) on the viral bud. Thus, a combination of direct interactions, lipid clustering and recruitment by increasing membrane curvature serve to induce the neck constriction of the viral bud by the cellular ESCRT machinery. Post constriction, the neck fuses and the viral bud is release into the extra cellular space as a free viral particle. The immature virus is then processed by the viral protease into the mature infectious virus.

2.2.5 Involvement of cortical actin pathways in viral assembly and release

Membrane adjacent cortical actin and its cofactors play a vital role in directing the dynamics of plasma membrane. Formation of membrane ruffles, lamellipodia, filopodia and other membrane protrusions, as well as budding of vesicles and the spatial dynamics are directly influenced by the polymerization-depolymerization of the underlying cortical actin (Weed and Parsons, 2001). Maintenance of membrane tension and cell shape, and cellular migration downstream of extracellular stimuli, is also an indispensable function of the cortical actin (Chalut and Paluch, 2016). This is especially true for the small T lymphocytes which depend heavily on cortical actin for cell shape, migration and communication with other cells of the immune system (Kumari et al., 2014; Dupré et al., 2015; Fritzsche et al., 2017). Ubiquitous G proteins (also known as small GTPases) such as Rac1, Cdc42 and RhoA are essential players in this actin signalling at the plasma membrane.
Figure 10. a) The cyclic GTP-GDP bound state of small GTPases which also influences their membrane binding and targeting to sites of active actin signalling. Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) mediate the GTP-binding and GDP-binding respectively. b) GTP-binding of small GTPases, Cdc42, Rac and Rho enables their membrane translocation and binding to their effectors, of the WASP family, which in turn bind actin nucleating or capping factors such as Arp2/3 and formins to influence directly the organisation of cortical actin for cytoskeletal structures such as filopodia, lamellipodia or migration. (adapted from Bento et al 2013 JCS and Pixley 2012 IJCB).

Cortical actin polymerization also regulates spatiotemporal diffusion of receptors and membrane phospholipids (Andrade et al., 2015; Mattila, Batista and Treanor, 2016) has implications on membrane trafficking, targeting of proteins and signalling.

Since HIV buds from the plasma membrane, cortical actin dynamics can be instrumental for several processes such as protein targeting to membrane, lipid clustering and facilitating membrane scission and resealing. Early studies looking at protein content of HIV particles and virion associated cellular factors found that released virions contain F-actin and other cofactors of the cytoskeletal apparatus (Ott et al., 1996) and that Gag itself is sufficient to interact with F-actin (REY, CANON and KROGSTAD, 1996). The NC domain of Gag was initially implicated in direct interactions with cortical F-actin (Wilk, Gowen and Fuller, 1999), but later studies found this domain to be dispensable for actin incorporation into budding particles (Stauffer et al., 2014). Replacement of the NC domain with a dimerizing leucine zipper did not affect actin incorporation into the budding particle, however the intracellular actin concentration appeared to influence this association, which, taking into consideration the nanometric scale of HIV assembly, seems to suggest that actin is present in the close vicinity of budding HIV. Concentration of F-actin was found to be high at budding sites by cry-electron tomography (Carlson et al., 2010), with several buds occurring on actin structures corresponding to cellular protrusions such as filopodia and spikes. A recent study (Aggarwal et al., 2019) also showed the subversion of the filopodial
actin regulating Cdc42/IQGAP1 axis in budding of HIV in the context of cell-cell transmission in adherent cells. Other actin cofactors such as filamentin A (Cooper et al., 2011) and ROCK1 and LIM kinase, regulators of the actin turnover protein cofilin (Wen et al., 2014) were also found to be involved in HIV assembly and release, suggesting a global subversion of the cortical actin cytoskeleton for the retroviral assembly and release mechanisms, not restricted to specific proteins or mechanisms (Ospina Stella and Turville, 2018). Complete depolymerisation of F-actin by a potent toxin mycalolide B resulted in cytosolic aggregates of the viral capsid proteins, interpreted as a defect in transport of these proteins to the plasma membrane. The exact role of F actin remains unclear as some studies involving live cell TIRF imaging of HeLa cells (Rahman et al., 2014) found that perturbing the cortical actin network did not have a significant effect on HIV assembly dynamics, suggesting that actin dynamics could have other roles than directly influencing the self-assembly and release of HIV Gag in HeLa cells. Thomas and colleagues showed the involvement of a Rac1/IRSp53/Wave2/Arp3 pathway in HIV -1 particle production in the relevant host T cells (Thomas et al., 2015). This actin signalling pathway is involved in the formation of membrane ruffles and lamellipodia in adherent cells, downstream of growth receptor mediated extracellular signals. The activation of Rac1 (GTP binding), via GEFs, takes place downstream of phospholipid signalling and ends in Arp2/3 induced branching and polymerization for generation of an actin meshwork underneath the plasma membrane at leading edges of migrating cells (Bosco, Mulloy and Zheng, 2009; Sit and Manser, 2011; Campa et al., 2015). The cyclic GTP-GDP bound states of Rac1 are known to proceed in rapid succession at sites of lamellipodia formation (Mehidi et al., 2019) resulting in bursts of actin polymerization. A similar phenomenon was observed in the case of HIV-1 budding, where filamentous actin structures were observed at the initiation of budding which disappeared upon release of the particle (Gladnikoff et al., 2009), indicating a very transient recruitment of F-actin to facilitate and expedite the assembly and release. Since HIV-1 Gag binds to and clusters the membrane phospholipid PI(4,5)P₂ into membrane nanodomains, which play a large role in shaping the local membrane organization via phase separation of lipids as well as inducing local actin polymerization. Since all these pathways lead to deforming the plasma membrane for formation of the viral bud, membrane curvature during Gag multimerization becomes an important aspect of the assembly and release process, potentially implicating membrane lipids, proteins and also F-actin in this process. Lipid nanodomain signalling and actin polymerization have a synergistic interplay, with polymerized F-actin limiting diffusion of membrane bound signalling proteins, and clustered lipids inducing actin polymerization (Chichili and Rodgers, 2007, 2009; Gómez-Llobregat, Buceta and Reigada, 2013). Protein crowding, lipid clustering and actin polymerization form a trinity of events leading to localized membrane deformation or curvature, which is an essential part of the spherical HIV-1 particle formation.
3. Chapter 3: Mechanisms of Cellular Membrane Curvatures

Eukaryotic cell membranes are composed of lipid bilayers whose elastic properties make them resistant to spontaneous curvature and bending. However, plasma membranes are also sites of great activity, including endocytosis, exocytosis and other protrusions, which make curving these membranes of absolute necessity to maintain life. Thus, eukaryotic cells have evolved and conserved several mechanisms to actively bend and shape the membrane. Of these, lipid composition of the bilayer, membrane binding proteins and cytoskeletal forces are the primary mechanisms. More often than not, these mechanisms work in tandem to locally deform membranes.

3.1 Lipid composition and phases

Several cellular events such as vesicle formation and budding, membrane fusion, endocytosis, egress of enveloped viruses, blebbing during apoptosis and cell division during mitosis are influenced by membrane curvature and lipid composition of the implicated membranes (Yesylevskyy, Rivel and Ramseyer, 2017). Lipid composition in cellular membranes thus varies widely from organelle to organelle, for example, the ER contains very low amounts of negatively charged phospholipids and cholesterol, which is in complete contrast to the cell plasma membrane, which can contain up to 30% mol cholesterol and phospholipids (Vanni et al., 2014). Lipid bilayers in cell plasma membranes are asymmetric in terms of their lipid composition; phosphatidylcholine (PC) and sphingomyelin, for example, are generally located in the extracytosolic or outer leaflet while phosphatidylserine (PS), phosphatidylethanolamine (PE) and the phosphatidylinositols (PIs) are found in the cytosolic or inner leaflet of the plasma membrane (Ikeda, Kihara and Igarashi, 2006). Lipids have intrinsic shapes depending on their headgroups and chain length and saturation. Clustering of certain lipid types thus influences locally the shape of the lipid membrane. This, in addition to asymmetry in lipid composition of the inner and outer leaflets, constitutes the basic mechanism of membrane curvature. Cylindrical lipids such as PC and PS form flat monolayers, while lipids having smaller headgroups bend the membrane in such a way so as to move the headgroups closer. Conical lipids with larger headgroups such as the PIs bend the membrane away from the headgroups. Thus the energy needed to overcome the resistance of the membrane to bend depends on the deformability of the lipid bilayer, which in turn depends on its constituent lipids (Callan-Jones, Sorre and Bassereau, 2011) and the interactions between lipids. Studies involving dilute lipid systems to study asymmetry and lipid sorting were inconclusive at less drastic curvatures (larger vesicles >50nm, as compared to smaller <10nm vesicles, or tubes of higher diameter pulled from vesicles), indicating that lipid proximity and lipid-lipid interactions based on local concentrations play a role in sorting of lipids to areas of different curvature (Kamal et al., 2009; Tian and Baumgart, 2009). In addition, biological membranes are close to a demixing or phase change point (Sorre et al., 2009; Callan-Jones, Sorre and Bassereau, 2011), making them sensitive to changes in lipid compositions and concentrations, and in fact make membrane dynamics easier to regulate from a cellular point of view.
Figure 11. Influence of lipid shape geometry on lipid bilayer curvatures. Spontaneous intrinsic curvature induced by inverted conical, cylindrical and conical lipids in lipid layers. (adapted from Brown 2012 Biochemistry)

The degree of lipid saturation also affected the rigidity of the membrane; saturated lipids increase rigidity (thus reducing curvature), while unsaturated phospholipids decrease membrane rigidity making the membrane easier to deform (Rawicz et al., 2000; Morlot et al., 2012; Manni et al., 2018), Morlot et al 2012 Cell, Manni et al 2018 eLife). Also, phase separation of membranes i.e. transition from a liquid disordered gel state to a liquid ordered fluid state, has immense biological relevance since it influences the “fluidity” of the membrane as well as binding and clustering of proteins specific for those lipids. Cholesterol, for example, is known to reduce stiffness and decrease the gel-fluid transition temperature of phospholipid bilayers by intercalating between phospholipids and preventing their clustering. Lipid phases possessing high stiffness naturally do not favour curvature (Katz and Givli, 2017). This has implications for HIV assembly and budding, since assembly sites are enriched in phospholipids and cholesterol (Favard et al., 2019a), making the membrane more amenable for curvature. On the other hand, membrane shape, in turn, plays a role in lipid sorting (Callan-Jones, Sorre and Basseaou, 2011; McMahon and Boucrot, 2015; Katz and Givli, 2017); lipids prefer to sort to membranes with appropriate geometry to minimize the energy of the system.

However, biological plasma membranes are highly complex structures involving lipids, proteins and influenced by the underlying cytoskeleton. Protein binding to lipid membranes amplifies lipid sorting, by favouring concentration of similar lipid species (Römer et al., 2007; Ewers et al., 2010) which also influences protein sorting and membrane curvature. In addition, biological membranes do not exhibit macrodomains of similar lipid species, and do not have large scale mixing of lipids as seen in model membranes. A recent study in model systems and simulations showed that the underlying actin cortex
is responsible for restricting diffusion of lipids (Honigmann et al., 2014), thus influencing formation of lipid rafts or nanodomains, and finally playing a role in membrane deformation.

3.2 Membrane deformation by proteins

Lipid composition of the membrane and the spontaneous curvature of the membrane due to local changes in lipid compositions are a membrane intrinsic mechanism of curvature. While external factors like membrane binding proteins and actin polymerization can affect the lipid composition locally by binding and clustering, or restricting locally some lipid species, these membrane extrinsic factors can also directly curve the membrane by several mechanisms.

Proteins are essentially rigid molecules as compared to the soft and deformable membranes. Due to this, binding of rigid proteins can impart the curvature of the membrane binding region of the protein. Thus membrane curvature induced by proteins is specific to each protein and its membrane binding mechanism; each protein has a unique mechanism to deform membranes by its specific interactions with the membrane (Agrawal et al., 2016).

Proteins with intrinsic curvatures can directly affect membrane shape by binding the membrane, and single proteins can affect local membrane shape at nanometric scales. Such proteins can also homo- or hetero-dimerize forming clusters which can deform the membrane over larger micrometric areas. This clustering of intrinsically curved proteins is known as scaffolding since they impart their native shape to the membrane while stabilizing the generated curvature. Proteins having the Bin-Amphyphysin-Rvs (BAR) domain, belonging to the superfamily of the same name, for example, have an intrinsic curvature in the BAR domain. This curvature promotes their binding to suitable curved membranes (i.e. sorting

Figure 12. Different mechanisms of membrane curvature. Lipid asymmetry is a membrane intrinsic mechanism of spontaneous curvature. Membrane curvature by proteins can be mediated by scaffolding of curved proteins, by hydrophobic peptide insertion into the bilayer, by membrane binding and oligomerization of proteins or by crowding of proteins on the membrane surface. (adapted from Chabanon et al 2017 Wiley Interdiscip Rev Syst Biol Med).
based on their intrinsic curvature) and dimerization of these proteins then generates and stabilizes membrane curvature (i.e. scaffolding). Thus, the BAR superfamily proteins are involved in several processes involving vesiculation of the cellular membranes, such as endocytosis, exocytosis, formation of membrane invaginations and protrusions etc. Formation of ordered macromolecular structures by peripheral membrane proteins can also result in curvature and scaffolding of the membrane. Proteins of the dynamin family can bind the inositol lipids in the plasma membrane and oligomerize to form helical structures which scaffold the plasma membrane into membrane tubules (Hinshaw and Schmid, 1995; Marks et al., 2001). This enables these proteins to constrict the necks of budding vesicles and enable the fission of their membranes. As in the case of HIV budding, the ESCRT-III proteins also oligomerize in a well described spiral for bending the membranes at necks to enable scission. The self-assembly of the membrane binding retroviral protein Gag (see Section 2.2.2) has also been proposed to generate the requisite membrane curvature for its assembly into a spherical immature particle.

Amphipathic helices (AHs), as the name suggests, have both hydrophobic as well hydrophilic residues. The hydrophobic end of the helix is inserted into the membrane generating a local defect in lipid packing and thus potentially curving the membrane, while the hydrophilic residues bind the lipids in the membrane, stabilizing this curvature (Drin and Antonny, 2010; Roberts et al., 2013). Several BAR superfamily proteins also possess AHs in addition to their curved BAR domain for sensing and generating membrane curvature. AHs can also function as curvature sensing motifs, and many proteins having this domain localise to areas of high membrane curvature, and are notably involved in membrane scission (Jensen et al., 2011; Zhukovsky et al., 2019).

As opposed to true scaffolding, crowding of proteins is simply a result of macromolecular aggregation of proteins within a confined area on the membrane which generates steric constraints on the membrane, forcing it to deform to compensate for the geometrical shape of the crowded proteins (Jarsch, Daste and Gallop, 2016). This effect is thus dependent on the local concentration of the proteins, as well as their size, flexibility and density on the membrane (Derganc and Čopič, 2016). An adaptor protein of the endocytic pathway, epsin, has been shown to curve membranes by protein crowding (Snead et al., 2017). Stachowiak and colleagues also showed in model systems that protein crowding by epsin and the subsequent membrane curvature is a function of the lateral pressure generated by collisions between membrane-bound proteins (Stachowiak et al., 2012). This study concluded that membrane curving by crowding is a nonspecific mechanism based on surface coverage and local density of the protein, rather a result of specific motifs such as amphipathic helices.

3.3 Actin mediated membrane deformation

As stated before, actin polymerization can manipulate protein and lipid diffusion, and thus their local concentration, by creating cortical barriers underneath the plasma membrane. But, actin can also actively reshape the plasma membrane via the force generated by its polymerization. The actin cytoskeleton is involved in maintaining membrane tension by direct interactions with the plasma
membrane and provides a scaffold to generate and hold macroscopic changes in cell shape (Doherty and McMahon, 2008).

Figure 13. Different mechanisms of membrane deformation by actin polymerization. Cortical actin polymerization underneath the plasma membrane maintains membrane tension and thus is instrumental in maintaining as well as manipulating cell shape for micrometre scale deformations such as podosomes, lamellipodia, filopodia, microvilli and membrane ruffles.

Membrane deformation by actin polymerization is involved in cell migration, cell adhesion as well as inter-cellular communication. Several pathogens including viruses (Taylor, Koyuncu and Enquist, 2011) subvert the cytoskeleton for entry, intracellular transport as well as egress.

Polymerization of actin within lipid vesicles has been shown to induce changes in vesicle shape, i.e. dramatic protrusions from the lipid membrane (Cortese et al., 1989) as well as for tubulin (Hotani and Miyamoto, 1990). This indicates the basic mechanism of membrane deformation by cytoskeletal elements; the polymerization of actin against an essentially flat bilayer will overcome the energy barrier necessary to deform this membrane at its minimal energy state. This is seen very clearly in filopodial protrusions from the cellular membrane where parallel filaments of polymerized actin are observed within the ultra-structures of filopodia (Aramaki et al., 2014). In case of lamellipodia at the leading edge of cells, branching of actin filaments by the actin nucleator Arp2/3 provides a short and stiff network of polymerized actin underneath the membrane to form large micrometre scale membrane curvatures (Doherty and McMahon, 2008).
However, plasma membrane curvature \textit{in vivo} is an interplay of several cellular as well as extracellular factors. Actin polymerization itself is a part of a signalling cascade downstream of several intracellular or extracellular stimuli, and phospholipids $\text{PI}(4,5)\text{P}_2$ and $\text{PI}(3,4,5)\text{P}_3$ play important roles in recruiting actin polymerizing complexes to the membrane to trigger changes in cortical actin. Thus certain lipids can induce changes in cortical actin structures, while actin itself can cluster lipids and proteins into nanodomains. Many of the actin regulatory complexes are peripheral membrane proteins or scaffold proteins which can bind the membrane as well as cofactors of actin. Chief among these are proteins of the BAR superfamily, whose BAR domain can bind and cluster plasma membrane lipids, and also sense and induce plasma membrane curvature. In addition, they also have motifs which are involved in multi-protein interactions to bind and recruit small GTPases, their upstream and downstream modulators. They are thus uniquely positioned to manipulate membrane dynamics.

3.4 BAR Superfamily of proteins

BAR domain proteins are an evolutionarily conserved superfamily of ~750 proteins found in organisms ranging from single cell yeasts to complex mammals (Sharma and Caplan, 2016). The BAR (Bin Amphiphysin Rvs) domain is a stretch of about 200 residues that are able to influence membrane shape and curvature. These proteins generally form a dimer of their banana shaped BAR domains, which is the basic membrane binding unit of BAR proteins. \textit{In vitro} BAR domain proteins induce tabulation of lipid based liposomes indicating their membrane deforming function (Itoh \textit{et al.}, 2005). Each dimer has a differently curved membrane binding surface, ranging from almost flat (for the I-BAR protein PinkBAR) to highly curved BAR domains which have positively charged residues for electrostatic interactions with negatively charged phospholipids in the membrane (Rao and Haucke, 2011). The BAR superfamily includes subfamilies based on the subtype of their BAR domain and their phylogeny: the canonical BAR/N-BAR family, F-BAR (Fes-CIP4 homology) family and the inverted BAR or I-BAR family (IRSp53-MIM homology domain or IMD).
3.4.1 Protein–protein interactions and regulation of BAR superfamily proteins

Apart from the membrane curvature sensing/inducing BAR domains, these proteins commonly have the SH3 domain (Safari and Suetsugu, 2012). Src homology 3 (SH3) domains are stretches of ~60 residues which bind principally proline rich residues in target proteins. This assumes importance for membrane dynamics since all members including WASP and Wave of the actin modulating WASP family contain a large proline rich sequence (Mayer, 2001). Members of this family are capable of activating the actin branching complex Arp2/3, directly influencing cortical actin organisation and membrane shape. These proteins also interact with other cytoskeletal cofactors like dynamin and formins (Aspenström, 2014). Through this SH3 domain, hence, the BAR proteins are directly linked to downstream actin regulation (Takenawa and Suetsugu, 2007). In addition, several members of this family also interact with Rho GTPases and their GEFs or GAPs as a part of multiprotein complexes (Aspenström, 2014), and thus act as scaffolds for the signalling pathways regulating cortical actin dynamics. Due to their membrane curvature sensing and binding, the BAR proteins thus modulate the
precise spatiotemporal induction of membrane shape deformations in response to various stimuli (Rao and Haucke, 2011).

Taking into consideration their wide ranging interactions, BAR domain proteins are tightly regulated by a variety of mechanisms, including autoinhibition, phosphorylation/dephosphorylation and interaction with other proteins. Autoinhibition i.e. an inactive native conformation of the protein, and its release from this autoinhibited state has been proposed to be a mode of regulation for several proteins in this family, including syndapin, srGAP1 and SRGP1 (Rao and Haucke, 2011). This regulation is mediated by SH3 domain of the BAR proteins; binding of ligands to SH3 domains to this motif regulates the autoinhibition of BAR proteins (Stanishneva-Konovalova et al., 2016). Phosphoregulation by upstream kinases is another mechanism of BAR protein regulation; phosphorylation typically “inactivates” a protein, while dephosphorylation “reactivates” it. This has been shown for the F-BAR protein Cdc15, which is involved in membrane scission during cytokinesis (Roberts-Galbraith et al., 2009), wherein it was seen that multi-site phosphorylation of this protein induced a closed inactive conformation, promoting its dissociation from the membrane clusters and blocks interactions with its binding partners. On the other hand dephosphorylation releases the protein from this inactive state and promotes binding to its partner proteins.

![Figure 15. Regulatory mechanisms of BAR superfamily proteins. A) Different functions of BAR domain proteins at cellular membranes i.e. membrane protrusions, invaginations and vesicle budding. B) Regulation by a) autoinhibitory conformation, b) phosphorylation or c) inhibitory protein binding mediates inactivation of proteins. C) upon activation, the protein is released from its inhibited state and is then targeted to the membrane for its function. (adapted from Rao and Haucke 2011 Cell Mol Life Sci).](image)

Membrane targeting of BAR proteins is another regulatory mechanism to control their spatiotemporal action; they bind negatively charged phospholipids with high affinity and have the capacity of
clustering these phospholipids in the vicinity of their dimers (Zhao et al., 2013). This generation of stable lipid microdomains not only plays a role in membrane curvature, but is also instrumental in recruiting members of the actin signalling pathways. N-WASP is recruited to and activated by binding PI(4,5)P₂ via its basic domain downstream of the small GTPase Cdc42, following which it can then activate Arp2/3 for branching actin at the membrane (Rohatgi et al 1999 Cell). On the other hand, another ubiquitous Arp/3 modulator, Wave2, is activated by binding PI(3,4,5)P₃ downstream of Rac1 activation to form Arp2/3 mediated lamellipodia at the leading edge of migrating cells (Oikawa et al., 2004; Suetsugu et al., 2006). PI(4,5)P₂ is concentrated in the so-called lipid rafts and can thus control actin polymerization at these locations on the membrane via Wave complexes (Golub and Caroni, 2005). PI(3,4,5)P₃ is present in negligible concentrations at resting levels in the cell, however upon activation by several extracellular stimuli, it is synthesized by the PI-3-Kinases at the membrane, and is frequently found at the leading edges (Rameh and Cantley, 1999; Vanhaesebroeck et al., 2010). Thus lipid clustering by BAR domain proteins can link extracellular stimuli to actin dynamics via phosphoinositide signalling, as these lipids also serve to recruit cytoplasmic effectors, especially those with pleckstrin homology (PH) domains.

Despite having similar domains to regulate cytoskeletal effectors, and clustering similar negatively charged lipids, BAR domains proteins localize to different intracellular organelles based on their function and preferred curvature. They play roles in a variety of vital cellular processes such as cell division, cell migration, endocytosis, membrane protrusions, organelle biogenesis and secretion (Rao and Haucke, 2011). The primary function of BAR proteins relies not just on the curvature of each individual protein, but rather the ordered assembly/oligomerization of these proteins as scaffolds to impart large curvatures to the membrane. Depending on the geometry of the membrane binding surface, the curvature induced by these proteins can either be positive (BAR/F-BAR), negative (I-BAR) or flat stabilized domains (the I-BAR protein PinkBAR) (Frost et al., 2009; Pykäläinen et al., 2011; Suetsugu et al., 2010; Zhao et al., 2011). Thus BAR/F-BAR proteins are involved in generating positively curved invaginations at the membrane, and are hence involved in processes such as clathrin or caveolin mediated endocytosis as well as in membrane constriction and scission (Collins et al., 2011; Kukulski et al., 2012; Qualmann et al., 2011, Bhatia et al., 2009; Boucrot et al., 2012; Gallop et al., 2006; Masuda et al., 2006; Saarikangas et al., 2009). Assays using liposomes have been typically used to study the membrane curving functions of these proteins. Cryo-EM studies of the F-BAR protein CIP4 and the N-BAR protein endophilin N1 show that helical oligomerization and lateral contacts between adjacent dimers drive membrane tabulation and scaffolding (Frost et al., 2008; Mizuno et al., 2010). F-BAR proteins seem to prefer larger liposomes (>500nm) as compared to amphiphysin, a BAR protein, which prefers smaller liposomes due to it’s steeply curved BAR domain (Shimada et al., 2007). I-BAR proteins, on the other hand, having an inverted BAR domain, prefer and generate negative curvature to form membrane protrusions such as filopodia and lamellipodia (Figure 16). I-BAR proteins are as such candidates of choice for outward budding events such as vesicle formation and virus budding, which typically involve negative curvature towards the exterior of the cell.
Figure 16. Curvatures and cellular structures of BAR proteins. A) F-BAR proteins have a relatively shallow concave membrane binding surfaces as compared to the steeper BAR domain of amphiphysin, while I-BAR domains have a relatively shallow convex membrane binding surfaces as compared to the steeper BAR domain of amphiphysin, while I-BAR domains have a relatively shallow convex membrane binding surface. B) Thus F-BAR and BAR domain proteins scaffold membranes to produce invaginations while I-BAR proteins induce outward membrane protrusions. C) Differences in helical oligomerisation and scaffolding of membrane tubules by BAR/F-BAR and I-BAR proteins.
3.4.2 I-BAR (IMD) proteins

Membrane protrusions (Fig. 12) were previously thought to be dependent solely on actin polymerization via the Wave2-Arp2/3 pathway (Pollard and Borisy, 2003). Subsequent studies in cells and model systems, however, uncovered the role of I-BAR proteins in this process (Yamagishi et al., 2004; Bompard et al., 2005). However these studies still initially suggested actin bundling as a likely mechanism for filopodia formation, attributing this activity to the I-BAR domain. The inverse BAR (I-BAR) domain was initially identified at the N termini of IRSp53 (insulin receptor tyrosine kinase substrate protein 53) and MIM (missing-in-metastasis), and thus is also known as the IRSp53/MIM homology domain (IMD) (Zhao et al., 2013). A later study (Mattila et al., 2007), implicated the phospholipid PI(4,5)P₂ in the mechanism of membrane curvature induced by the I-BAR subfamily of proteins. This study also provided the first evidence that these proteins tubulate phospholipid rich liposomes in a direction opposite to that seen for BAR/F-BAR proteins, i.e. towards the interior of the liposomes, confirming their “inverse” mechanism of membrane deformation.

![Figure 17. Schematic representation of I-BAR proteins and their domains. All the five proteins contain the IMD/IBAR membrane binding domain at their N termini. The C termini have the protein-protein interaction motifs such as the WH2 domain and the SH3 domain. IRSp53 has an additional CRIB domain to bind Cdc42. (adapted from Safari and Suetsugu 2012 MDPI Membranes).](image)

There are five known I-BAR proteins: IRSp53, the founding member of the family, MIM (missing-in-metastasis), ABBA (actin-bundling protein with BAIAP2 homology), PinkBAR (planar intestinal and kidney specific BAR domain protein), and IRTKS (insulin receptor tyrosine kinase substrate) (Zhao et al., 2013). Structural studies of the N terminal 250 residues long I-BAR domain revealed it’s conformation as a zeppelin shaped dimer with antiparallel helix bundles, having homology with canonical BAR domains (E. Lee et al., 2002; Millard, Dawson and Machesky, 2007). IRSp53, IRTKS and PinkBAR also possess SH3 domains which are used to interact with Wave2, Mena, Eps8. IRSp53 contains an additional partial CRIB domain which binds the small GTPase Cdc42 while MIM has a proline rich
domain to bind the actin regulator cortactin. The C terminal end of all these proteins, however, contains the WH2 (WASP homology 2) domain (Zhao et al., 2013). This domain is the most abundant motif in most of the actin nucleators (Dominguez, 2016). The structure and domains of the I-BAR proteins thus clearly indicate their role in generating negative curvature and regulating local actin dynamics by recruiting and binding small GTPases as well as their effectors, putting these proteins firmly in the midst of membrane protrusion formation and cytoskeletal regulation.

Figure 18. Mechanisms of membrane deformation by I-BAR proteins and coupled actin polymerization. a) I-BAR proteins sense negatively regions of the membrane and localize there based on their preferred curvature; subsequent actin polymerization further deforms the membrane. B) De novo membrane curvature generated by I-BAR binding to membrane and subsequent actin polymerization increases this effect. (adapted from Zhao et al 2013)

The I-BAR/IMD domain has been shown to bind and cluster the phospholipid PI(4,5)P₂, more so than BAR domain proteins, in studies involving vesiculations of liposomes. Within the family, MIM and ABBA generated tubules of a larger diameter on an average, as compared to IRSp53 and IRTKS, and also seemed to interact with membranes in the absence of electrostatic charges, indicating additional mechanisms of membrane binding. This was revealed to be amphipathic helices which can be inserted into membrane to enable curvature, in contrast to IRSp53, IRTKS and PinkBAR, marking the difference within the family (Saarikangas et al., 2009). Subsequent studies also showed a difference in membrane transition densities and membrane curving induced by MIM and ABBA on one hand, and IRSp53 on the other (Chen, Shi and Baumgart, 2015), even proposing that the N terminal helices in MIM and ABBA inhibit induction of membrane curvature in their system, as higher densities of these proteins were required for the membrane shape transition. This suggests significant evolutionary divergence within the family.
Accordingly, phylogenetic analyses of the amino acid sequences of human and mouse I-BAR proteins along with *C. elegans* I-BAR, showed that MIM and ABBA (MIM-like) constitute a separate branch within the family while IRSp53, IRTKS and PinkBAR (IRSp53-like) are grouped together. IRSp53 and IRTKS are the closest relatives in this I-BAR family (Chen, Shi and Baumgart, 2015) (Figure 19).

Figure 19. Divergence within the I-BAR family. Comparison of amino acid sequences human and mouse (Hs and Mm) I-BAR proteins along with *C. elegans* (Ce) shows that there is clear divergence within the I-BAR family with MIM and ABBA constituting the MIM-like group while IRSp53, IRTKS and PinkBAR (denoted here as FLJ22582) constitute the IRSp53-like group. (adapted from Chen et al 2015 Biophys J).

Co-expression of fluorescently tagged constructs of these proteins, even the IMD domain alone, indicated that they localize to different compartments within filopodia, however overexpression of these proteins globally induces filopodia formation (Yamagishi *et al.*, 2004; Millard *et al.*, 2005; Saarikangas *et al.*, 2009; Chen, Shi and Baumgart, 2015). However, quantitative analysis of filopodia formation showed that IRSp53 is more efficient at sensing and inducing curvature, as seen by its efficiency in forming filopodia, than MIM (Breuer *et al.*, 2019). Characterisation of IRTKS revealed that overexpression of this protein forms microspikes at low levels of expression, and forms F-actin clusters at higher expression levels, indicating a different actin regulation function than IRSp53 which is well described to localize to filopodia (Millard, Dawson and Machesky, 2007). PinkBAR, though possessing an I-BAR domain, does not form protrusions in model systems or in cells, forming and stabilizing instead planar membrane sheets enriched in phosphoinositides. Resolution of the crystal structure of the protein showed that the I-BAR domain of PinkBAR is shorter than the other I-BAR proteins, and that it is also considerably flatter as well, forming planar oligomers rather than the helical oligomers commonly observed for BAR proteins, pointing to a possible explanation of its difference in morphological phenotype *in vitro* and *in vivo* (Pykäläinen *et al.*, 2011). MIM and IRTKS also exhibit antagonistic activities in the context of CXCR4 receptor endocytosis, via interactions with Rab7 and
Rab11 respectively, in an SH3 domain dependent manner (Li et al., 2019). MIM is also implicated in inhibiting endocytosis in migrating cells by competitive cortactin binding (Quinones, Jin and Oro, 2010).

Thus there is significant functional plasticity evident within the I-BAR family of proteins. A part of their functional plasticity can be attributed to their interactions with different actin regulators. IRSp53 is an intermediary between Rac and Wave2 for membrane ruffles and lamellipodia formation (Miki et al., 2000) and also binds Cdc42, Mena and Eps8 for filopodia formation (Krugmann et al., 2001; Lim et al., 2008). IRTKS, on the other hand, binds Rac rather than Cdc42 (Zhao et al., 2013) and also signals with another GTPase Rif and Wave2/Eps8. Rif also binds PinkBAR in some cell types (Sudhaharan et al., 2016). ABBA binds Rac1 via its IMD for promoting membrane ruffling (Saarikangas et al., 2008; Zheng et al., 2010) in glial cells. MIM also binds Rac1 (Bompard et al., 2005; Machesky and Johnston, 2007; Dawson et al., 2012) to promote actin polymerization. There is wide variation over different cell types as well for I-BAR protein function and signalling.

3.4.3 Physiological and pathological significance of I-BAR proteins

Considering overlaps in signalling pathways, there could have been redundancy in function for these proteins. There are some reports of IRSp53 and IRTKS having functional redundancy in embryonic cell function (Chou et al., 2017). But in a physiological context, these proteins show differential tissue expression in the body. IRSp53 is widely expressed in several cell types and tissues, but shows especially high expression in the neurons and plays a role in dendritic spine density. Accordingly, IRSp53 knockout mice show defects in learning, memory and synaptic plasticity. IRSp53 alleles are also implicated in ADHD in humans (Kim et al., 2009; Ribasés et al., 2009; Sawallisch et al., 2009). Dendritic development was also impaired in cells derived from IRSp53 knockout mice (Choi et al., 2005). IRTKS is expressed at low levels in the brain, being expressed more in the bladder, liver, testes, heart and lungs. Both IRSp53 and IRTKS are implicated in actin polymerization during enthemorrhagic E. coli infection (Vingadassalom et al., 2009). IRTKS is also elevated in centroblasts and centrocytes (Li et al., 2019). PinkBAR is expressed in kidney and intestinal epithelial cells (Pykäläinen et al., 2011). MIM was identified originally as being “missing” in some metastatic cell lines (hence the name), but has also been shown to be elevated in other metastatic cancers (Y.-G. Lee et al., 2002; Machesky and Johnston, 2007; Zhao et al., 2013). It is expressed in the heart, skeletal muscles and the central nervous system during development (Zhao et al., 2013). In addition, it is expressed in naïve and memory B cells (Li et al., 2019). ABBA, whose expression has been studied in adult mice, is present in the glial cells of the cerebral cortex (Saarikangas et al., 2008). IRSp53 remains the best described of this family, and its role in several cell types has been elucidated.

3.5 IRSp53
This protein was first discovered in a screen for tyrosine kinase substrates phosphorylated downstream of the insulin receptor activation by insulin (Yeh et al. 1996 JBC); hence the name. This study also proposed the presence of an SH3 domain in the C terminal, giving first clues to its actin regulatory function. In a subsequent study by the same group, they further elucidated that this putative SH3 domain, and also a possible WW domain, had no effect on the subcellular localization i.e. membrane binding of this protein, and neither did it affect its phosphorylation by tyrosine kinases (Yeh et al., 1996). Another study identified a human homologue of this protein, previously identified in hamster cells by Yeh and colleagues, as a cytoplasmic binding partner of brain-specific angiogenesis inhibitor 1 (BAI1), a transmembrane protein (Oda et al., 1999). This study confirmed the presence of the SH3 domain and its association with BAI1 through SH3 interaction with the proline rich region of BAI1. Importantly, it showed that this protein (named BAIAP2 for brain specific angiogenesis inhibitor associated protein 2), associated with BAI1 at the plasma membrane of cells. Confirmation of the SH3 domain led to further investigation into its role in cytoskeletal regulation.

3.5.1 Role in scaffolding actin regulatory proteins

Small GTPases and their effectors are primary regulators of the cortical actin network, and thus influence membrane dynamics and shape (Section 2.2.2, Section 3.3). IRSp53 acts as the link between Rac1 and Wave2 for formation of membrane ruffles (Miki et al., 2000). This study showed that IRSp53 bound Rac by its N terminal region, while it bound Wave by the SH3 domain. It also participates in filopodia formation by interacting with Cdc42 by a partial CRIB (Cdc42 and Rac binding domain) and the ENA/Vasp family member Mena by its SH3 domain (Govind et al., 2001; Krugmann et al., 2001) (Figure 21). Localization of IRSp53 to lamellipodia and filopodia however, was found to be independent of Mena, showing colocalization instead with another WASP family member Wave2, at the tips of lamellipodia (Nakagawa et al., 2003). This localization was shown to be dependent on its N terminal region (then referred to as the Rac binding domain), providing the first clues about the membrane binding activity of this region. The first hints about auto-inhibition were also obtained by studying its interactions with Rac1 and Wave2; The N terminal fragment bound Rac1 better than the full length protein, while Wave2 binding improved Rac1 interactions of the full length protein (Miki and Takenawa, 2002). This study also, in a first, proposed the role of the RacGEF Tiam1, in Rac1-IRSp53-Wave2 mediated morphological changes in spreading cells. Tiam1 (T-lymphocyte invasion and metastasis inducing protein 1), is a RacGEF which directs IRSp53 to Rac-induced lamellipodia instead of Cdc42 derived filopodia, binding IRSp53 and enhancing its binding to both Rac1 and Wave2; the Tiam1-IRSp53 complex directs localized Rac1 activation (Connolly et al., 2005; Rajagopal et al., 2010). Eps8 also forms a tri-molecular complex with Abi-1 and Sos-1 to activate Rac, along with IRSp53, downstream of active Ras. Similarly, the IRSp53-Eps8 complex was shown to direct Cdc42 induced filopodia formation (Disanza et al., 2006), as well as binding the formin mDia (Fujiwara et al., 2000), indicating that specificity of IRSp53 localization and action depends on upstream stimuli and binding of effectors via the SH3 domain (Robens et al., 2010) (Figure 20).
Consistent with its brain specific role, IRSp53 also interacts with post-synaptic density proteins PSD-95 and Shank via the SH3 domain and PDZ domains (Boeckers et al., 2002; Soltau et al., 2004; Sawallisch et al., 2009; Dosemeci et al., 2017) in neuronal cell types. As with other members of the BAR superfamily (Figure 15), IRSp53 is regulated by auto-inhibitory mechanisms by conformational constraints and phosphorylation by kinases. There is an intramolecular binding of the CRIB and the SH3 domain which is relieved by Cdc42 binding to the CRIB domain, or by effector binding to the SH3 domain (Kast et al., 2014). IRSp53 is also regulated by phosphorylation/dephosphorylation, and there are several phosphorylation sites between the CRIB and the SH3 domain (Kast and Dominguez, 2019). The multifunctional adaptor protein 14-3-3 is proposed to bind these phosphorylated sites and promote the dissociation of IRSp53 from the membrane and inhibit effector binding (Robens et al., 2010; Kast and Dominguez, 2019). AMPK and AMPK-related kinases are proposed to phosphorylate IRSp53 (Banko et al., 2011; Cohen et al., 2011; Schaffer et al., 2015), and this phosphorylation mediated 14-3-3 mediated inhibition as a counter to Cdc42 mediated activation of IRSp53 for filopodia formation (Kast and Dominguez, 2019).
Figure 21. Schematic representation of IRSp53 and its domains. IRSp53 possesses several protein-protein interacting domains. The N terminal IMD/I-BAR domain is primarily a membrane binding domain, however it also binds proteins such as the small GTPase Rac1. The partial CRIB binds to Cdc42. The SH3 domain has multiple binding partners depending on cell type and signalling pathway, notably the Wave2 complex for lamellipodia formation. The C terminal WW and ODZ domains are also protein-protein interacting sites. (adapted from Chen et al 2015 Comm Int Biol)

The phosphoinositide PI(3,4,5)P₃ plays an important role in this signalling cascade leading to Rac1 activation (Scheffzek et al 1998 Trends Biochem) and Wave2 mediated actin polymerization (Figure 20). IRSp53 optimized Wave2 activity in presence of PIP₃ and active Rac (Suetsugu et al., 2006). PIP₃ is also a membrane binding factor for the Wave complex, though it is not sufficient for its activation of Arp2/3 (Oikawa et al., 2004). In fact, this membrane targeting and PIP₃ binding is mediated by IRSp53 (Takahashi and Suzuki, 2010, 2011). It is also implicated in the membrane translocation and activation of Tiam1 through PI3K activity which locally synthesizes PIP3 from PIP2 (Ruch et al., 2016). Thus lipids, and the phosphoinositides PI(4,5)P₂ and PI(3,4,5)P₃ in particular, play important roles in IRSp53 mediated signalling at the plasma membrane.

3.5.2 IRSp53 isoforms

Alternate splicing of the 3’ exon of IRSp53 has produced four reported isoforms in humans (Okamura-Oho, Miyashita and Yamada, 2001) known as IRSp53-L, IRSp53-S, IRS-58 and IRSp53-T respectively. The N terminal 511 residues are identical in these proteins are identical, but the C terminal stretch of ~9 to 40 amino acids is different. The principal signalling domains of CRIB and SH3 are in the common identical part of the protein. Another study proposed a fifth isoform in rats, suggesting that it is involved in the auto-inhibition of the protein (Alvarez, Sutcliffe and Thomas, 2002). The different functions of these isoforms are as of yet unknown, but an early study implied that IRS-58 (so named for its molecular weight 58kDa), preferentially interacts with Cdc42 rather than Rac1 or RhoA (Govind et al., 2001).
3.5.3 The membrane binding I-BAR/IMD domain

In tandem with discovering the role of IRSp53 in actin mediated signalling via its scaffolding activity, structural studies revealed the presence of the I-BAR/IMD domain, which had some homology with the canonical known BAR domains (Yamagishi et al., 2004; Millard et al., 2005). The crystal structure of the N terminal I-BAR domain showed that it exists as a zeppelin shaped dimer of coiled coil domains. Subsequent studies then discovered the membrane deforming function of the I-BAR/IMD domain (then also known as the Rac binding domain), independent of actin filaments (Suetsugu et al., 2006; Mattila et al., 2007; Lim et al., 2008). Lim and colleagues also noted that IMD induced filopodia were morphologically different from those induced by full length IRSp53 (Lim et al 2008 JBC). IMD bound to several different phospholipids, PI(3,4,5)P$_3$, PI(3)P, PI(3,4)P$_2$, PI(4,5)P$_2$, in nonspecific electrostatic interactions (Suetsugu et al., 2006). Later studies however uncovered the principal role of PI(4,5)P$_2$ in IMD membrane binding. Increasing PI(4,5)P$_2$ concentration corresponded to increasing IMD binding to model membranes (Saarikangas et al., 2009; Futó et al., 2013). The PI(4,5)P$_2$ binding site was mapped to the positively charged ends of the dimer (Mattila et al., 2007), and this interaction was shown to be necessary for formation of membrane protrusion in cells, underscoring the importance of this phospholipid in membrane curvature. However other lipids such as PC and cholesterol are more abundant in membranes, hence clustering of phospholipids and nonspecific interactions with other lipids could very well regulate membrane binding via the IMD/I-BAR domain. In addition to generating curvature, the geometry of the shallow curved I-BAR domain also means that the protein is able to sense curvature and thus is able to phase-separate along areas of negative curvature, sorting maximally at a curvature of 18 nm (Prévost et al., 2015).

A space filling model of the 18 nm long dimer suggested that it would form membrane tubules with a diameter of ~95 nm, close to the one obtained in experimental studies (~80nm). Later studies however showed that IRSp53 preferably sorted at curvatures of 18nm diameter in model systems and cells (Breuer et al., 2019) and formed tubules of 40-60nm in diameter (Saarikangas et al., 2009). The IRSp53 I-BAR domain strongly and stably clustered PI(4,5)P$_2$ as compared to other I-BAR and BAR domains. Its interactions with the membrane are also based solely on electrostatic interactions, in contrast to MIM and ABBA whose amphipathic helix insertion aids in membrane binding (Saarikangas et al., 2009). IRSp53 also proved to be more efficient in inducing membrane shape transitions, requiring 50% less density to deform membranes as compared to other BAR and I-BAR proteins. This study also observed that membrane tension and protein density co-regulated membrane deformation. It also scaffolds and stabilizes existing curvature (Prévost et al., 2015). In light of efficient shape transition by IRSp53 at relatively low densities, and its wide ranging cortical actin manipulation, this model of co-regulation becomes important for membrane curvature by IRSp53 (Chen, Shi and Baumgart, 2015), and explains its expression and function in different tissues and cell types. Also, the binding and clustering of PI(4,5)P$_2$ at the ends of the membrane bound dimer aids in membrane curvature, as the I-BAR domain assumed a flatter structure in MD simulations (Takemura et al., 2017); this ties in with predicted
minimal energy localization of PI(4,5)P$_2$ molecules relative to negative membrane curvature (Tiberti, Antonny and Gautier, 2020) indicating that inducing packing defects and asymmetry in the plasma membrane bilayer is also involved in IRSp53-I-BAR mediated membrane curvature.

### 3.5.4 Involvement of IRSp53 in HIV-Gag assembly

A 2015 study (Thomas et al., 2015) discovered that the actin signalling pathway downstream of Rac1 and involving IRSp53-Wave2-Arp2/3 was involved in HIV-1 particle production in host CD4+ T lymphocytes. An overall increase in Rac1 activation levels and intracellular actin content was noted upon expression of HIV-1. SiRNA and drug mediated inhibition of the members of this pathway significantly decreased viral production in these cells. Notably, a knockdown of IRSp53 was more deleterious to viral production than other proteins, decreasing it by as much as 70%, revealing its essential role in this process. A double knockdown of IRSp53 and Wave2 perturbed actin content in the cortex while the HIV-1 protein Gag, which is plasma membrane bound for its assembly, was found to be mainly cytosolic in this condition. Taken together, these results highlight a role of IRSp53 mediated actin signalling in HIV-1 Gag assembly and particle production.

A recent study also implicated the filopodia generated downstream of another small GTPase, Cdc42, as being involved in cell-cell transmission of HIV-1 (Aggarwal et al., 2019). Interestingly, since IRSp53 is also an effector and scaffold for Cdc42 mediated signalling (see section 3.5.1), it is the common factor of these two independent studies.

As seen in Section 3.5.1, IRSp53 primarily senses and generates membrane curvature by scaffolding membrane, clustering lipids and generating local actin polymerization. The involvement of this protein in HIV-1 assembly indicates a possible role of its membrane curving function in addition to triggering actin polymerization. As HIV-1 bud formation requires a definite deformation of the plasma membrane to induce a negative curvature (see Section 2.2.2) for bud formation and release, IRSp53 could potentially play a vital role in this process.
RESEARCH QUESTION & OBJECTIVES

Assembly and budding of the enveloped retrovirus HIV-1, which takes place at the plasma membrane of host cells, necessitates an outward curvature of the membrane to form a complete bud which then is released from the cell. Previous studies have shown the involvement of cellular factors in this process, notably the ESCRT machinery which functions at the late steps of budding to perform scission of the assembled virus. However, the initiation of membrane curvature needed for the viral bud formation was thought to be mainly driven by the self-assembly of the viral structural protein Gag; the continuous multimerization of Gag provided the gradual induction of curvature needed to form the bud, discounting the possible role of any other factor in this process. We previously showed the involvement of IRSp53, an I-BAR protein, in HIV-1 particle production, as a part of a Rac1 dependent actin signaling pathway. I-BAR proteins are known to induce outward membrane curvature at the plasma membrane while acting as adaptor proteins for several cortical actin signaling pathways. We then decided to focus on the I-BAR protein IRSp53 and its possible role in membrane curvature at the HIV-1 assembly site. Furthermore, the recruitment of IRSp53 was examined in the context of Rac1 dependent signaling as well as actin proximity to the HIV-1 buds, in accordance with the involvement of IRSp53 in actin polymerization.

To study comprehensively the role of IRSp53 in HIV-1 assembly, a minimal system of HIV-1 Gag particle production was used to probe the interactions of IRSp53 at the assembly site at a molecular level. HIV-1 Gag is capable of forming non-infectious virus-like particles independent of any other viral proteins, retaining the assembly kinetics and structural characteristics of immature viral particles. As our hypothesis hinged on Gag interplay with cellular cofactors as a cornerstone of IRSp53 involvement in HIV-1 assembly, the minimal system was best suited to study this question. The research objectives were divided into a first major objective of probing the direct involvement of IRSp53 in HIV-1 assembly and the second objective examining the involvement of other cofactors as well as actin at the assembly site.

To answer these questions, we used a combination of biochemical and cell biological techniques as well as single molecule localisation microscopy to essentially probe the functional involvement, specificity and recruitment of IRSp53 in the context of HIV-1 Gag particles. In addition, cell free systems were used to examine the molecular interplay of Gag and the IRSp53 I-BAR membrane binding domain. After having established the functional role of IRSp53 as a membrane curving essential protein for HIV-1 assembly, we then probed its recruitment downstream of Rac1 and attempted to establish the identity of the RacGEF involved in this process, opening the doors for future studies to identify other cellular cofactors, and thus potential antiviral targets, upstream of this process.
RESULTS
The I-BAR proteins are a family of proteins known to sense and induce negative curvature at the cell plasma membrane. They also act as scaffold proteins for several actin signalling pathways at the cell membrane. Our lab has revealed that IRSp53 is an I-BAR domain protein involved in HIV-1 assembly via an actin-modulating small GTPase Rac1 signalling pathway using siRNA mediated strategy. Hence, the specific role of IRSp53 in HIV-1 assembly was investigated. During HIV-1 assembly and budding, the viral structural Gag proteins are targeted to the plasma membrane where they oligomerize on the viral genome, leading to the virus budding from the cell membrane. Here, we first studied the effect of siRNA mediated gene inhibition of IRSp53 on Gag VLP production in 293T cells and wild-type HIV-1 production in host CD4 T cells. Secondly, we analysed the possible interactions between Gag and IRSp53 by immunoprecipitation. Then we examined the incorporation of the IRSp53 protein in infectious wild-type HIV-1 as well as Gag VLPs produced in 293T cells. We further tested the specificity of this incorporation across other I-BAR family proteins. Our results show that inhibition of IRSp53 by siRNA leads to a significant reduction in VLP production while it is not the case for IRTKS. A direct or indirect interaction between Gag and IRSp53 was established, and we also reported an increased cell membrane localization of IRSp53 upon Gag expression in the cells. We performed studies of purified Gag and IRSp53 domain in a cell free system to analyse their molecular interplay. Our results indicate an enhancement of Gag binding to lipid membranes in presence of IRSp53 domain indicating that Gag binds better to membranes by interacting with IRSp53. Furthermore, imaging and gradients of purified VLPs also show that IRSp53 is specifically incorporated into Gag VLPs as compared to other I-BAR family proteins, and is likewise incorporated in HIV-1 particles. We further examined the presence of IRSp53 at thousands of virus budding sites by dual colour super resolution PALM/STORM microscopy. Imaging analysis indicated the presence of IRSp53 in close proximity within Gag assembly sites as compared to IRTKS. Taken together, our results point towards a specific new role for IRSp53 in HIV-1 particle assembly and it may aid in Gag binding to the cell membrane while possibly providing the initial curvature necessary for bud formation.
Full assembly of HIV-1 particles requires assistance of the I-BAR IRSp53.

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Abstract

During HIV-1 particle formation, the requisite plasma membrane curvature is thought to be driven solely by the retroviral Gag protein. Here, we reveal that the cellular I-BAR IRSp53 is required for the progression of HIV-1 membrane curvature to complete particle assembly. Partial siRNA knockdown of IRSp53 induces a decrease in viral particle production and a bud arrest at half completion. Using single molecule localization microscopy in cells, we show a preferential localization of IRSp53 around Gag assembly sites. In vitro, using giant unilamellar vesicles, we reveal that HIV-1 Gag protein localizes preferentially to IRSp53 I-BAR domain induced curved membranes, confirming a strong interplay between HIV-1 Gag and the I-BAR IRSp53. Finally, we observe the presence of IRSp53 in purified HIV-1 particles. Overall, our results highlight IRSp53 as a novel host factor in HIV-1 membrane curvature and its requirement for initiation of local membrane deformation and for full HIV-1 particle assembly.

MAIN TEXT

Introduction

The cell plasma membrane is a dynamic organelle, where crucial processes such as endocytosis and exocytosis take place through local membrane deformations. Several pathogens, such as bacteria and enveloped viruses interplay with the plasma membrane in the course of their replication cycle. Pathogens often enter the cells by endocytosis\(^1,2\) and exit by membrane vesiculation\(^3,4\), which are processes linked to generation of plasma membrane curvature; either inward or outward deformations. HIV-1 is an enveloped positive strand RNA virus belonging to the family *Retroviridae*, known to
assemble and bud outward from the host cell plasma membrane\textsuperscript{5}. The structural Gag polyprotein of HIV-1, by itself, is responsible for particle assembly\textsuperscript{6}: It can oligomerize at the inner leaflet of the plasma membrane forming virus-like particles (VLPs). The force required to bend the membrane to achieve VLP formation has been proposed to be provided by Gag self-assembly\textsuperscript{7}. The self-assembly of Gag has also been recently shown to segregate specific lipids \textsuperscript{8,9} and proteins\textsuperscript{10}, generating plasma membrane domains that could favor budding\textsuperscript{11,12}. However, only a small proportion of initiated clusters of Gag reaches the full assembly state leading to VLP release in living CD4 T cells\textsuperscript{13}, a majority being aborted events. Therefore, the mechanism by which the virus overcomes the energy barrier associated with the formation of the full viral bud remains an open question. Recently, coarse grained model of HIV assembly has shown that self-assembly of Gag might not be sufficient to overcome this energy barrier\textsuperscript{14} leaving the assembly in intermediate states supporting the fact that other factor may be necessary to assist Gag self-assembly during the generation of new VLPs.

Indeed, the plasma membrane curvature can be also generated by diverse host cell proteins. For example, I-BAR domain proteins sense and induce negative membrane curvature at a few tens to one hundred of nanometer-scale, i.e. in the HIV-1 size range, while generating outward micrometer-scale membrane protrusions such as membrane ruffles, lamellipodia, and filopodia. IRSp53 was first discovered as a substrate phosphorylated downstream of the insulin receptor\textsuperscript{15}. It is also the founding member of membrane curving I-BAR domain protein family, whose other mammalian members are MIM (missing-in-metastasis), ABBA (actin-bundling protein with BAIAP2 homology), PinkBAR (planar intestinal and kidney specific BAR domain protein), and IRTKS (insulin receptor tyrosine kinase substrate)\textsuperscript{16}. In addition to interactions with plasma membrane, IRSp53 binds both Rac\textsuperscript{17} and Cdc42 directly through its the unconventional CRIB domain\textsuperscript{18}, as well as downstream effectors of these GTPases such as WAVE2, Mena, Eps8 or mDia, through the SH3 domain. Thus, IRSp53 functions as a
scaffold protein for the Rac1/Cdc42 cascade\textsuperscript{19}. IRSp53 was reported to exhibit a closed inactive conformation that opens synergistically upon binding to Cdc42 and effector proteins\textsuperscript{10,11}. Regulation of IRSp53 activity was recently shown to occur through its phosphorylation and interaction with 14-3-3\textsuperscript{20}. Structurally, the I-BAR domain of IRSp53 is composed of \textit{crescent}-shaped rigid six alpha-helix bundle dimer. Due to its concave membrane binding surface and lipid interactions, IRSp53 is able to generate negative membrane curvature\textsuperscript{16}. While capable of forming homo-dimers, IRSp53 is also able to recruit and form hetero-dimers with other proteins to form clusters for initiation of membrane curvature\textsuperscript{21}.

Since the Rac1/IRSp53/Wave2/Arp2/3 signaling pathway is involved in the release of HIV-1 particles\textsuperscript{22}, we hypothesized that IRSp53 may be a prime candidate for membrane remodeling required during viral bud formation. Hence, we investigated the possible role of IRSp53 and its membrane curvature generating activity in HIV-1 Gag assembly and particle budding. Importantly, we discovered that IRSp53 is present in an intracellular complex with HIV-1 Gag at the cell membrane, incorporated in Gag-VLPs and associated with purified HIV-1 particles, and that IRSp53 functions in HIV-1 assembly as a facilitator of optimal HIV-1 particle formation through its membrane bending activity. Thus, we identify IRSp53 as an essential non-redundant novel factor in HIV-1 replication, and demonstrate that it is critical for efficient HIV-1 membrane curvature and full assembly at the cell plasma membrane.
Results

1. IRSp53 knockdown decreases HIV-1 Gag particle release by arresting its assembly at the cell plasma membrane.

We report here that partial knockdown of IRSp53 reduces HIV-1 particle release in host Jurkat T cells and in model cell line HEK293T (Figure 1a,b), as we also reported previously in primary T lymphocytes\(^{22}\). Cells were treated with siRNA targeting IRSp53 or IRTKS (validated by extinction of the transfected ectopic IRSp53-GFP or IRTKS-GFP proteins – Figure S1b and S1c respectively). In the context of Jurkat T cells, when expressing the viral Gag proteins in the context of HIV-1(\(\square\)Env) in order to only monitor the late steps of the viral life cycle, the partial knockdown of IRSp53 reduced particle release by 2 to 3 fold as compared to the control siRNA (Figure 1a, Left). To compare the role of different I-BAR domain proteins from the same family, we also measured the effect of siRNA targeting IRSp53 and IRTKS (Fig 1b) on HIV-1 Gag virus-like particle (VLP) production in 293THEK cells (see graph Fig 1b, and immunoblots Fig S1d,e). IRTKS shares similar protein domain organization and high sequence homology with IRSp53 (40% amino acid sequence identity and 59% sequence similarity, Fig. S2), and displays some functional redundancy with IRSp53\(^{23}\). IRTKS can also induce plasma membrane curvature\(^{24}\). Partial knockdown of IRSp53 (~50% gene inhibition), resulted in a 2-fold decrease in HIV-1 Gag particle production (Fig. 1b, Right, S1d). In contrast, knockdown of IRTKS (Fig.1b, S1e) did not have any significant effect on HIV Gag particle release, thus precluding the possibility of redundant functions between IRSp53 and IRTKS in the context of HIV-1 Gag particle formation.

Electron microscopy imaging of siRNA IRSp53 treated 293THEK cells expressing HIV-1 Gag revealed particle budding arrests at the cell plasma membrane (Fig 1c, Lower Panel), as compared to the siRNA-control cells (Fig 1c, Upper Panel). While the control cells exhibit
the normal phenotype of Gag-VLP budding from the cell plasma membrane, the IRSp53 knockdown cells display a series of viral buds arrested in assembly decorating the cell plasma membrane (Fig 1c, Fig S3). These results revealed arrested Gag assembly at the membrane and thus the involvement of IRSp53 in the assembly process. Since IRSp53 is an I-BAR protein involved in cell membrane curvature, we measured the curvature exhibited by HIV-1 buds in IRSp53 knockdown cells. While control cells displayed a range of HIV-1 Gag particles at different stages of assembly and budding, cells knocked down for IRSp53 displayed arrested buds at an early assembly stage (Fig 1d). Once measuring the dimensions of these arrested buds, we found that buds from cells knocked down for IRSp53 displayed a narrower range of curvature height (48 ± 22nm) as compared to the control (85 ± 53 nm), while the bud widths presented no difference between siIRSp53 (135 ± 64nm) and the control (140 ± 87 nm) (Fig 1e). The control cells thus exhibit a range of height and widths consistent with the range of buds seen at the membrane of these cells. The result indicates that in the absence of IRSp53, the viral buds are unable to progress beyond a certain curvature.

2. HIV-1 Gag and IRSp53 are associated preferentially at the cell membrane.

Since both Gag and IRSp53 target the cell plasma membrane upon interaction with PI(4,5)P₂ (6, 15, 16), we then tested if Gag and IRSp53 could associate directly or indirectly using immuno-precipitation assays (Fig 2). Our results show that immuno-precipitation of endogenous IRSp53 resulted in co-precipitation of Gag (Fig 2a, lane 1) as compared to controls (lanes 2 to 4). Thus, Gag and IRSp53 are components of the same intracellular complex. We then compared the percentage of IRSp53 attached to the cell membranes in the presence or in the absence of Gag using membrane flotation assays (Fig 2b). The lysosomal membrane protein
Lamp2 was used to validate the proper separation of the membrane fraction from the cytosol. In the absence of Gag (“HEK 293T control cells”), one can observe the presence of IRSp53 both in the cytosol (fractions 6-8) and at the cell membranes (fractions 1-2). Thus, at equilibrium, 20 to 30% of IRSp53 is bound to cell membranes (Fig. 2c). The same experiment was repeated with cells expressing HIV-1 Gag, where ~70% of Gag was bound to the cell membranes (Graph, Fig. 2c). Notably, we observed that expression of HIV-1 Gag led to at least a two-fold increase of IRSp53 in the cell membrane fractions (Fig 2c), as compared to the control cells where Gag is absent. This effect is similar with the one of Tsg101, a protein of the ESCRT-I complex known to interact mainly with the p6 domain of Gag\textsuperscript{26-28}, which also shows more than a 2-fold increase in cell membrane binding upon Gag expression (Fig 2c).

Furthermore, we examined if the Gag/IRSp53 interaction is dependent on the p6 domain of Gag to reveal if this interaction occurs before ESCRT complexing with Gag. We thus used a C-terminal mutant of Gag, Gag\textsuperscript{p6}, which is deficient in ESCRT-Tsg101 recruitment\textsuperscript{28}, still capable of binding the plasma membrane and assembling particles, but that buds poorly. Thus, Gag\textsuperscript{p6} viral particles are tethered and stay attached to the plasma membrane (see\textsuperscript{13} for the characterization of Gag(i)mEos2Δp6). Our experiments revealed that Gag, Gag(i)mEos2 and Gag\textsuperscript{p6(i)mEos2} were all pulled down with IRSp53 (Fig S4), showing that addition of the internal mEos2 protein did not affect the complexing of Gag with IRSp53. Moreover, the p6 domain, and thus the ESCRT-1 recruitment and budding, is not required for Gag/IRSp53 molecular interplay.

Together, these results suggest that HIV-1 Gag and IRSp53 can associate, directly or indirectly, in the same intracellular complex, reinforcing the idea of a strong molecular interplay between these two proteins. Moreover, these results provide evidence that
association between IRSp53 and HIV-1 Gag complexes occur at the cell plasma membrane before particle budding.


Our finding that IRSp53 and HIV-1 Gag are present in the same molecular complex at the cell membrane motivated us to assess whether IRSp53 was present specifically at the Gag assembly sites. Because HIV-1 assembly are ~100 nm in diameter\(^{13,29}\), we used PALM (Photo-Activated Localization Microscopy) coupled to dSTORM (direct Stochastic Optical Reconstruction Microscopy) with TIRF illumination, to investigate with high resolution the localization of I-BAR proteins in Gag(i)mEos2 assembly sites at the plasma membrane. Using density based spatial scan (DBSCAN) of Gag localizations, we quantified the size distribution of the Gag clusters, at a localization precision of ~16 nm (Fig.S5), and we found a diameter of 80 to 100 nm, which is within the size-range of HIV-1 Gag assembly sites\(^{13,29}\) (Fig 3a,b). Reconstructed dual color PALM/STORM images revealed that Gag(i)mEos2 assembly sites follow IRSp53 immuno-labeling along the cell plasma membrane, and that several Gag clusters overlap with IRSp53 (Fig 3a, S6). In contrast, Gag clusters did not overlap with IRTKS (Fig 3b, S6), consistent with the results of siRNA presented in Fig. 1b. We quantified these observations by performing coordinate-based colocalization\(^{30}\) (CBC) analysis of HIV-1 Gag and IRSp53 (or IRTKS) (Fig S7 for the process workflow). The CBC coefficient ranges from -1 to +1, where -1 corresponds to anti-correlation, 0 indicating non-correlation and +1 corresponds to perfect correlation between the two molecules. Since CBC values are calculated for each localization, we plotted the CBC values as cumulative frequency distributions for all localizations (n>10\(^5\)) of IRSp53/Gag and IRTKS/Gag. As shown in Fig. 3c, the CBC distribution for IRSp53/Gag has a higher proportion of values
exhibiting strong colocalization (27% of CBC>0.5) in comparison with IRTKS/Gag values (14% of CBC>0.5) (Fig 3d). This comparison directly shows that IRSp53 displays stronger single molecule colocalization with Gag than IRTKS in assembling clusters.

However, the average positions of IRTKS or IRSp53 molecules with respect to Gag molecules within the assembling clusters were unclear. Thus, we performed simulations to generate different patterns of PALM/STORM localizations (Fig S8). Comparison of the CBC values of simulated data with our experimental values indicated that IRSp53 localization, on average, displays a restricted pattern around and in the assembly sites. This corresponds to a circular band surrounding the assembly site at 80 nm from the center of the Gag budding sites with a width of 80 nm (Fig 3e, Fig S8e). On the other hand, IRTKS was present as a large diffuse pattern centered at 140 nm from the Gag assembly site center with a width of 200 nm, explaining why fewer IRTKS molecules were detected in the assembly sites (Fig 3f, Fig S8f). Our results thus show that IRSp53 indeed specifically localizes at HIV-1 Gag assembly sites at the cell plasma membrane, whereas IRTKS does not.

Finally, the same imaging (Fig. S9) and CBC analyses (Fig. S10) were applied to the HIV-1 host CD4 T cells, i.e. Jurkat T cells expressing Gag(i)mEos2 and immuno-labelled for IRSp53 (Fig S10a) or for IRTKS (Fig S10b). The results showed that the CBC cumulative distributions for IRSp53 (Fig S10c,e), as well as for IRTKS (Fig S10d,f), are similar to those obtained in HEK293T cells (Fig 3c,d). These analyses confirm the preferred IRSp53 localization to Gag assembly site in the host CD4 T cells. The involvement of IRSp53 around Gag assembly sites seems to be conserved regardless of the cell type, reinforcing the idea of a specific role for IRSp53 in HIV-1 Gag particle assembly.
4. IRSp53 is incorporated in HIV-1 particles.

To assess IRSp53 incorporation into HIV-1 Gag particles, we purified Gag virus-like particles (VLP) from cells transfected with Gag-mCherry and several GFP-tagged I-BAR domain proteins (Fig 4a). IRTKS displays functional redundancy with IRSp53, being able to curve membranes. IRSp53-I-BAR-GFP construct only contains the membrane curving I-BAR domain of IRSp53. PH-PLCδ-GFP, a PI(4,5)P₂ binding protein, was used as a control, because it binds PI(4,5)P₂ but does not generate membrane curvature. Fluorescent VLPs purified from these transfected cells were then visualized for two colors (green: GFP and red: mCherry), and Mander’s coefficient was calculated as an indicator of incorporation of the ectopic (green) GFP-tagged proteins within the (red) Gag-mCherry VLPs (see Materials and methods) (Fig 4a). We found that correlation for IRSp53-GFP and Gag-mCherry was 0.95-1 (Fig 3b, Left Graph, Red Column), indicating that almost all Gag-mCherry VLPs contained IRSp53-GFP. When using the IRSp53-I-BAR domain alone, we also obtained a high Mander’s coefficient, ~0.8 (Fig 4b). In contrast, for IRTKS-GFP, the Mander’s coefficient was 0.4-0.5, indicating no significant correlation between IRTKS-GFP and Gag-mCherry. Taken together, these results show the preferential incorporation of IRSp53-GFP, or its I-BAR-GFP domain, into released HIV-1 Gag particles.

To study the incorporation of endogenous IRSp53 in HIV-1 particles, cells were transfected with plasmids expressing either wild-type infectious HIV-1 or codon optimized immature HIV-1 Gag protein (without genomic RNA). The virus particles were purified through a 20%-sucrose cushion or further through continuous iodixanol density gradient (as in 32,33). IRSp53 was found to be associated with the viral particles in both conditions, i.e. in infectious HIV-1 and in Gag VLPs, indicating that Gag alone is sufficient to recruit IRSp53 in the viral particles (Fig 4c,d, “IRSp53”). Tsg101 also showed an association with viral particles in both conditions (Fig 4c, d, “Tsg101”), as reported previously26,27,34. In contrast,
IRTKS was not associated neither with Gag-VLP nor HIV-1 particles (Fig 4c, d, “IRTKS”).
Upon further purification (Fig. 4e), IRSp53, and the ESCRT proteins, Tsg101 and ALIX, were found associated within the same fractions containing the HIV-1 Gag viral particles, together with other well-known viral particle cofactors such as CD81, CD63 tetraspanins. Thus, endogenous IRSp53 is most probably incorporated in HIV-1 particles in a Gag dependent manner.

5. HIV-1 Gag is enriched at membrane tube tips generated by IRSp53 I-BAR domain.

The results above demonstrate that not only IRSp53 is incorporated in Gag-VLPs, but it is present at the budding sites and its deletion strongly reduces HIV-1 particle release in a Gag-dependent manner by arresting its assembly at the cell plasma membrane (Fig 1). Thus, we assessed the IRSp53 I-BAR/Gag interplay on model membranes using giant unilamellar vesicles (GUVs). We found that Gag binding to GUV membranes is increased ~7 fold when IRSp53-I-BAR domain was introduced first on GUVs before adding Gag (median value 6.7), compared to the condition of Gag only (median value 0.9) ($p < 0.0001$, $t$-test) (Fig 5a, “Gag only” vs. “I-BAR+Gag”). However, in the condition where Gag was introduced before adding the I-BAR domain, Gag intensity on GUV membranes increased only ~3 fold as compared to the Gag only condition ($p < 0.0001$, $t$-test) (Fig 5a, “Gag only” vs. “Gag+I-BAR”). Notably, by comparing I-BAR+Gag and Gag+I-BAR conditions, we observed a 2-fold higher Gag intensity on GUV membranes in the first condition ($p < 0.0001$, $t$-test) (Fig 5a, “I-BAR+Gag” vs. “Gag+I-BAR”). Taken together, these results show that IRSp53 facilitates the recruitment of Gag to PI(4,5)P$_2$-containing membranes.

Given these results, and that IRSp53 is a membrane curving protein involved in the early stage of generation of cell protrusions, we asked whether the local membrane deformation induced by IRSp53 could be a preferred location for HIV-1 Gag assembly. Previous in vitro
studies showed that when placing IRSp53 I-BAR domain outside PIP$_2$-containing GUVs, even at a relatively low bulk concentration (about 0.02-0.06 µM), I-BAR domain can deform GUV membranes, generating tubes towards the interior of the vesicles$^{24,36,37}$. We thus incubated GUVs with I-BAR domain at 0.05µM, which allows for the generation of inward membrane tubes, followed by the addition of Gag (Fig 6b, see Materials and Methods). This experiment revealed that Gag was sorted preferentially to the tips of the tubes generated by the I-BAR domain (Fig 5b,c, S11). Furthermore, we observed that addition of HIV-1 Gag resulted in the formation of shorter I-BAR tubules as compared to GUVs incubated with the IRSp53-I-BAR domain alone (Fig. S11), indicating an interference in I-BAR tubule elongation when Gag sorted to the tubule tips.

Together, these results demonstrate that HIV-1 Gag binding to membranes is enhanced by the presence of IRSp53 I-BAR domain, and that Gag preferentially binds to highly curved membranes generated by the I-BAR domain of IRSp53.

Discussion

The findings of this study uncovered the role of the host cellular I-BAR factor IRSp53 in HIV-1 Gag assembly and membrane curvature upon bud formation. In vitro, we showed that the IRSp53 I-BAR domain enhances Gag binding to PI(4,5)P$_2$-containing membranes (Fig 5). In cells, we showed that IRSp53 is retained to the cell membranes upon Gag expression (Fig 2). IRSp53 is found at, or in the close vicinity of, Gag assembly platforms at the cell membrane (Fig 3), and is incorporated into Gag virus-like particles and in HIV-1 virions (Fig 4). Importantly, we revealed that IRSp53 partial knockdown arrests Gag assembly at the mid-bud formation stage (Fig 1) and that Gag preferentially locates at the tube tips induced by IRSp53 I-BAR domain, interfering with its long tubule formation in vitro (Fig 5). Altogether, IRSp53 appears instrumental in membrane curvature upon HIV-1
budding and is locally subverted as an essential factor needed for full HIV-1 Gag particle assembly.

Using GUVs, we observed that Gag not only colocalizes with the IRSp53 I-BAR domain on the vesicles, but that the IRSp53-I-BAR domain increases Gag binding to the membrane (Fig 6a). Indeed, BAR domain proteins, in general, and IRSp53, in particular, are known to induce strong PI(4,5)P\textsubscript{2} clusters\textsuperscript{24,38}, and PI(4,5)P\textsubscript{2} was shown to play a role in Gag binding to cell plasma membrane\textsuperscript{39}, as well as to be strongly clustered during virus assembly\textsuperscript{9,10,40}. Thus, these results suggest that membrane binding of Gag on IRSp53-enriched membrane domains could promote the plasma membrane binding of both proteins (Fig 3, S6). This is in agreement with super resolution imaging in the cell, where Gag/IRSp53 interactions may take place at the Gag assembly sites, since, here, IRSp53 was localized in close proximity to Gag assembly sites in HEK293T cells (Fig 3) and in host CD4 T cells (Fig S9, S10). Our experiments suggest that Gag and IRSp53 are associated in a common complex at the cell plasma membrane (Fig 2). The fact that Gag retains IRSp53 to the cell membrane may suggest that Gag activates IRSp53, perhaps by releasing its auto-inhibition\textsuperscript{20}. This remains to be tested.

HIV-1 particles are known to incorporate a large number of cellular proteins, many of which are directly involved in virus budding\textsuperscript{34}. Here, we showed that IRSp53 is incorporated in Gag-VLPs, as well as in purified HIV-1 virions (Fig 4), which most likely depends on the I-BAR domain of IRSp53 (Fig 4). Using the IRSp53-I-BAR domain on GUVs, we induced membrane protrusions that have a negative mean curvature similar to a viral bud; Gag was found enriched in these tubular structures but particularly at the tips that have a half-sphere geometry similar to a viral bud (Fig 5). This indicates that Gag binds preferentially to IRSp53 I-BAR-curved membranes \textit{in vitro} in contrast to other almost-flat areas of the GUVs. Similarly, in cells, single molecule localisation images reveal some Gag clusters enriched at
IRSp53 labelled protrusions at the plasma membrane (see Fig S6a). IRSp53 clusters have already been reported prior to filopodia formation and in negatively curved area at the onset of endocytic buds. Moreover, it was shown that, inducing local membrane curvature, helps to initiate Gag lattice formation. We thus propose that IRSp53 induces local membrane curvature, upon activation through Rac1/Cdc42 and effectors, which promotes local Gag recruitment and initiation of the viral assembly (knowing that expression of Gag can activate Rac1).

We furthermore observed that siRNA knockdown of IRSp53 induces a decrease in viral particle production and arrests the assembly at half completion (Fig 1). Coarse grained simulations of HIV-1 Gag assembly recently showed that Gag self-assembly by itself is unable to overcome the free energy penalty required to curve the membrane above a certain threshold. Although the presence of RNA can facilitate the growth of the Gag network, this favours membrane bending due to the intrinsic curvature of assembling Gag hexamers. Since IRSp53 stabilizes curvature by scaffolding, another role of IRSp53 could be to lower this free energy barrier involved in the progression of the budding process beyond the half-sphere geometry, by stabilizing the bud curvature. This, either directly by organizing linearly around the assembly site and mechanically constricting the nascent bud, or indirectly with the help of actin polymerization.

Finally, ESCRT recruitment occurs at the end of virus assembly, after the membrane has been curved, forming a vesicle ready to bud. Overexpression of a mutant of the ESCRT protein Tsg101 was previously shown to block HIV-1 budding at a late stage, arresting the budding with a characteristic bulb shaped phenotype indicative of a defect in the late stage of the bud scission, in contrast with our observations with the IRSp53 siRNA phenotype (Fig 1, S3). Consequently, this suggests that Gag-IRSp53 association is ESCRT independent (as shown in Fig.S4) and occurs at an earlier stage of virus assembly.
Another study\(^{45}\) showed that angiomotin, which acts as an adaptor protein for HIV-1 Gag and the ubiquitin ligase NEDD4L, functions in HIV-1 assembly prior to ESCRT-I recruitment. Interestingly, angiomotin also contains a BAR domain\(^{46}\), but it is canonically involved in inducing positive curvature, as opposed to the negative curvature induced by I-BAR IRSp53. Thus, it is possible that angiomotin functions in another way, for example, at the viral bud neck which has both positive and negative curvatures by facilitating ESCRT recruitment.

IRSp53 itself is a scaffold protein for cofactors of cortical actin signalling\(^{16}\) and we have previously shown that a Rac1 signalling pathway, including IRSp53, is involved in HIV-1 particle production\(^{22}\). Thus, it is possible that IRSp53 could also play a role in generating local cortical actin density in the vicinity of the viral bud. The role of cortical actin associated with IRSp53 scaffolding in that context remains to be elucidated.

Our work thus illustrates a novel role for the host cellular I-BAR factor IRSp53, which is subverted by the retroviral Gag protein, in HIV-1 induced membrane curvature and in favoring the formation of fully assembled particle.

Materials and Methods

Antibodies.

Rabbit polyclonal anti human IRSp53 (07-786 – Merck Millipore), mouse monoclonal anti CA (24.2 - NIH AIDS Reagent Program – FisherBioServices), mouse monoclonal anti GFP (B-2 – Santa Cruz biotechnology sc-9996). Rabbit polyclonal anti-human IRTKS (Bethyl), mouse monoclonal anti human CD63 (MX-49.129.5 – Santa Cruz biotechnology sc-5275), mouse monoclonal anti human CD81 (5A6 – Santa Cruz biotechnology sc-23962), rabbit
polyclonal anti human IRSp53 (07-786 – Merck Millipore), mouse anti CA (183H125C – NIH3537), rabbit monoclonal anti human TSG101 (Abcam – ab125011), rabbit polyclonal anti human ALIX (Covalab – pab 0204).

**Plasmids.**

The plasmid expressing HIV-1 codon optimized Gag alone (pCMVGag [named pGag]), the plasmid expressing Pol and Env-deleted HIV-1 (named pNL4.3ΔPolΔEnv) encoding Gag alone with its packageable viral RNA and the plasmid expressing full wild-type HIV-1 (named pNL4.3) were described previously. Plasmids IRSp53-GFP, IRTKS-GFP, PinkBAR-GFP and IRSp53-I-BAR-GFP were obtained from University of Helsinki (Finland). Plasmids expressing PH-PLCδ-GFP was a gift of B. Beaumelle (IRIM), Gag(i)mCherry (named Gag-mCherry), Gag tagged with internal photo-activable mEos2 (named Gag(i)mEos2), p6-deleted Gag tagged with mEos2 (named pGagΔp6-mEos2) were described in.

**siRNA.**

Stealth siRNA (Invitrogen) targeting IRSp53 (BAIAP2) and IRTKS (BAIAP2L2), and Smartpools (Dharmacon) targeting IRSp53 (BAIAP2) or random sequence for siRNA controls were used in this study.

**Cell culture and transfection.**

A human embryonic kidney cell line (HEK-293T) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, GIBCO) and Jurkat T-lymphocytes were maintained in RPMI (GIBCO). Media were supplemented with 10% fetal bovine serum (FBS, Dominique Dutscher)), complemented with sodium pyruvate and antibiotics (penicillin-streptomycin) at 37°C with 5% CO2 atmosphere. HEK-293T cells were transfected as in . Based on different plasmids conditions, cells were transfected as follows (2*10^6 cells/transfection): PLASMID,
the amount of transfected plasmid was normalized by adding pcDNA3.1 empty plasmid. The cell medium was replaced 6 hours post-transfection and experiments were performed 24-48h post transfection. SiRNA transfections in HEK293T cells were performed with either RNAiMax (Invitrogen) or with JetPRIME (Polyplus) or by electroporation for Jurkat T cells. One day prior to transfection, 2x10^5 cells/well were seeded in 2mL of growth medium without antibiotics, in a 6-well plate. Transfection was performed using the manufacturer’s protocol. 24 hours after siRNA transfection, the cells/well were again transfected using CaCl_2/HBS. These cells were incubated at 37°C with 5% CO_2 atmosphere, for 24/48 hours.

**Immunoprecipitation assay.**

HEK-293T cells were transfected following the calcium-phosphate technique. Based on different conditions plasmids were transfected as follows (2*10^6 cells/transfection): Gag/pNLdPoldEnv (8ug each) and the amount of transfected plasmid was normalized by adding pcdna3.1 empty plasmid. The cell medium was replaced 6 hours post-transfection. 24 hours post-transfection, the cells were washed with cold 1X-PBS prior to collection with 800µL of chilled lysis buffer (50mM TRIS-HCl [pH=7.4]; 150mM NaCl; 1mM EDTA; 1mM CaCl_2; 1mM MgCl_2; 1% Triton, 0.5% sodium deoxycholate; protease inhibitor cocktail [Roche] 1 tablet/10mL lysis buffer). The cells were incubated on ice for 30 minutes and then centrifuged at 13,000 rpm/ 15minutes/ 4°C. The supernatant was collected in a new tube and the pellet was discarded. For each condition, 1000µg of protein (the collected supernatant) was incubated with 1µg of anti-IRSp53 antibody for overnight on a tube rotator at 4°C. 25µL of beads (Dynabeads Protein A, Life Technologies) was added to each tube of protein-antibody complex and incubated for 2 hours on the tube rotator at 4°C. The samples were then washed 5 times with the lysis buffer, followed by addition of 20µL 2X Laemmli’s buffer.
to the beads. The samples were denatured at 95°C for 10 minutes and then processed for Western blot.

**Western blot and analysis.**

50µg of each protein (intracellular) samples or 20µL of purified VLP samples, added with SDS loading dye, were resolved on a 10% SDS-PAGE gel. The gels were then transferred on to PVDF membranes. Immunoblotting was performed by incubating the membranes overnight with primary antibody at 4°C, and 2 hours with HRP conjugated secondary antibody at room temperature. The blot signals were detected using ECL Prime/ECL Select substrate (Amersham) and images were taken using ChemiDoc (BioRad).

**VLP purification and quantification.**

24 or 48 hours post-transfection, culture supernatants containing Gag-VLPs were collected, filtered through a 0.45uM filter, and centrifuged first at 2000rpm/5mins/4°C and then at 5000rpm/5mins/4°C. The supernatant was then purified by loading it on a cushion of 25% sucrose (in TNE buffer) and ultracentrifuged at 100000g for 100 minutes (SW41Ti, Beckman Coulter) at 4°C. The pellets were resuspended in TNE buffer at 4°C overnight. Gag-VLP release was estimated by performing anti-CAp24 immunoblot and by quantifying Gag signal in the blots using ImageJ software as described in . The calculation for Gag-VLP release is:

\[
\% \text{ of Gag in VLP} = \frac{\text{Gag}^{\text{released}}}{(\text{Gag}^{\text{released}} + \text{Gag}^{\text{intracellular normalized to GAPDH}})}.
\]

**Membrane flotation assay.**

For each condition, 4x10^6 cells were transfected and viral supernatants harvested 48h post-transfection, as described above. Cells were washed with ice-cold PBS and resuspended in Tris-HCl containing 4mM EDTA and 1X Complete protease inhibitor cocktail (Roche). Every step was then performed at 4°C. Cell suspensions were lysed using a dounce homogenizer, then centrifuged at 600g for 3min to obtain Post-Nuclear Supernatants (PNS). A cushion of 820µL of 75% (wt/vol) sucrose in TNE buffer (25mM Tris-HCl, 4mM EDTA,
150mM NaCl) was loaded at the bottom of an ultracentrifuge tube and mixed with 180 μL of PNS adjusted to 150mM NaCl. Two milliliters and 300 μL of 50% (wt/ml) sucrose cushion followed by 0.9 mL of 10% (wt/ml) sucrose cushion were then layered to obtain the gradient that was then centrifuged in a Beckmann SW60Ti rotor at 35 000rpm, 4°C, overnight. Eight 500μL fractions were collected from the top to the bottom of the centrifuge tube and analyzed by western blotting.

**Sample preparation for electron microscopy.**

SiRNA treated HEK-293T cells were fixed in 4% paraformaldehyde and 1% glutaraldehyde in 0.1M phosphate buffer (pH 7.2) for 48h, washed with PBS, post-fixed in 1% osmium tetroxide for 1h and dehydrated in a graded series of ethanol solutions. Cell pellets were embedded in EPON™ resin (Sigma) that was allowed to polymerize at 60°C for 48h. Ultrathin sections were cut, stained with 5% uranyl acetate and 5% lead citrate and deposited on colloidon-coated EM grids for examination using a JEOL 1230 transmission electron microscope.

**Sample preparation for super resolution PALM/STORM microscopy.**

HEK293T cells expressing HIV-1 Gag/Gag(i)mEos2 cultured on poly-l-lysine (Sigma) coated 25 mm round #1.5 coverslips (VWR) were fixed using 4%PFA + 4% sucrose in PBS for 15 min at RT. Samples were subsequently quenched in 50mM NH₄Cl for 5 min. Samples were then washed in dPBS and then blocked for 15 min in room temperature using 1%BSA in PBS and subsequently in 0.05% Saponin in 1%BSA in PBS. Samples were stained using a 1:100 dilution of the primary antibodies (rabbit polyclonal anti-human IRSp53, Sigma and rabbit polyclonal anti-human IRTKS antibody, Bethyl) for 60 min in room temperature. Samples were washed 3x5 min using 1% BSA in PBS followed by 60 min staining using a 1:2000 dilution of the anti rabbit Atto647N antibody (Sigma). Samples were washed 3x5 min
with PBS and stored in light protected container in +4°C until imaged. Samples were mounted on a StarFrost slide with a silicon joint with the STORM buffer (Abbelight). Cells were imaged within 60 minutes after application of buffer.

**PALM/STORM Imaging.**

Single-molecule localization microscopy was performed on a Nikon inverted microscope equipped with 405-, 488-, 561- and 642-nm lasers, an EMCCD Evolve 512 Photometrics camera (512*512, 16µm pixel size) with an oil immersion objective 100X NA1.49 Plan Apochromat. PALM imaging of Gag mEos2, activation was performed with lasers irradiance set to 0.3 kW/cm² for 405 nm conversation and ~ 2.2 kW/cm² for 561 nm excitation. Illumination was performed over a 25x25 µm area in the sample (1/e² spatial irradiance distance) in TIRF-mode. 20-50,000 images were acquired for each cell with 50 ms integration time. The mean precision localisation in PALM measurements was found to be 20±5 nm (Fig S3). 2D-STORM imaging of Alexa647, was performed using a ~5 kW/cm² irradiance with the 642 nm excitation. 25000 images were acquired for each condition. Image reconstruction was performed using the ThunderStorm plugin of ImageJ using Tetraspeck 100 nm multicolour beads (Life Technologies) as fiducial markers to correct for drift.

**Super Resolution Microscopy Analysis.**

The module DBSCAN of the super resolution quantification software SR Tesseler⁴⁸ was used to analyse the PALM localizations for quantification of Gag cluster sizes. In order to monitor the localisation of I-BAR proteins in the vicinity of Gag assembling particles, a binary mask was introduced into the PALM images. The centre of each Gag assembling cluster was determined and a custom MATLAB (Mathworks) code was used to extract localizations in a radius of 80nm around each Gag cluster center and to extract IBAR proteins localisations.
belonging to a disk of 150nm radius around the centre of each Gag clusters. These subsets of coordinates were then used to calculate coordinate-based colocalization (CBC), developed by Malkusch et al.\textsuperscript{30}, and implemented in the ThunderSTORM plugin of ImageJ (Fig. S4). The coordinate-based colocalization (CBC) values are calculated from single-molecule localization data of two species (Gag and IBAR proteins (IRSp53 or IRTKS)). A CBC value is assigned to each single localization of each species. We analysed the distributions of these CBC values by plotting and comparing the cumulative frequency distributions of the CBCs obtained in the two conditions (IRSp53 vs IRTKS). Finally, we performed a set of numerical simulations of images in ImageJ (see supplemental methods) to estimate the average localisation pattern of the immuno-labelled I-BAR proteins with respect to the mEos2 tagged Gag in PALM (Fig. S5).

**Preparation and Imaging of Fluorescent VLPs.**

24h after seeding $2.10^6$ HEK293T cells were transfected with 8µg of pI-BAR-GFP proteins with or without 8µg of pGag/pGag(i)mCherry (2/3 1/3 respectively). 24h after transfection cells media (9mL) were filtered before performing VLPs purification by ultracentrifugation (SW41Ti rotor (Beckman) 29 000rpm, 1h30) on TNE 20% sucrose cushion. Pellets were resuspended with 110µL of TNE and allowed to sediment on round 25mm coverslips during 45 minutes in a chamber. VLPs were imaged with a Nikon Ti Eclipse 2 TIRF microscope. Images were taken with an Evolve EMCCD camera – 512 photometrics, using 100X objective and 488 and 561nm lasers.

**Image Analysis for Colocalization.**

Images were acquired with Zeiss LSM780 (for fixed cells) or Nikon Eclipse Ti-2 in TIRF mode (for fluorescent viral particles). Colocalization analysis based on Mander’s coefficients was performed using JaCOP (Just another Colocalization Plugin)\textsuperscript{49}. Mander’s coefficient are
defined as \( M1 = \frac{\sum_i a_{loc}}{\sum_i a_i} \) and \( M2 = \frac{\sum_i b_{loc}}{\sum_i b_i} \), \( A \) and \( B \) being the two respective channels (mCherry and GFP). \( 0 < M < 1 \), with 1 full colocalization and 0.5 random colocalization. The \( M1 \) and \( M2 \) coefficients were calculated for several images and then represented as column graphs with red columns representing the degree of overlap of mCherry images with GFP images, and green columns representing the inverse.

**Iodixanol gradient.**

Cell culture medium of HEK293T (\( 2.5 \times 10^6 \) cells plated) transfected with \( 8 \mu g \) of pNL4.3\( \Delta \)pol\( \Delta \)env were collected 48h after transfection and filtered by 0.45\( \mu m \) filter. The medium was then ultracentrifuged on a TNE 20% sucrose cushion using a SW41Ti rotor (Beckman) at 40,000rpm during 1h30. A solution with 0.25M sucrose, 1mM EDTA, 10mM tris HCL pH 7.4 was used to diluted the 60% iodixanol stock solution (OptiPrep from Sigma) and to prepare 40% and 20% iodixanol solution. 1,5mL of each dilution (60%, 40% and 20% iodixanol) were successively layered in a SW55Ti tube (Beckman) and the pellet of VLPs obtained after ultracentrifugation on sucrose cushion was loaded on the top. The tube was ultracentrifuged at 50 000rpm with a SW55Ti rotor (Beckman) during 3h. Then 20 fractions of 200\( \mu l \) were collected from top of the tube. 20\( \mu l \) or 18.75\( \mu l \) of each fraction were loaded for western blotting.

**GUV reagents.**

Brain total lipid extract (131101P) and brain L-\( \alpha \)-phosphatidylinositol-4,5-bisphosphate (PIP\(_2\), 840046P) were purchased from Avanti Polar Lipids/Interchim. BODIPY-TR-C5-ceramide, (BODIPY TR ceramide, D7540) and Alexa Fluor 488 C5-Maleimide (AX 488) were purchased from Invitrogen. \( \beta \)-casein from bovine milk (>98% pure, C6905) and other reagents were purchased from Sigma-Aldrich. Culture-Inserts 2 Well for self-insertion were purchased from Ibidi (Silicon open chambers, 80209).
Protein purification and fluorescent labelling.

Recombinant mouse IRSp53 I-BAR domain was purified and labeled with AX488 dyes as previously described\textsuperscript{24,36}. Recombinant HIV-1 immature Gag protein was purified by J. Mak as described in Yandrapalli et al, 2016\textsuperscript{8} and labelled with Alexa488 maleimide dyes (Invitrogen). Briefly, a 200µM solution of the maleimide dye was incubated overnight at 4°C with a 20µM solution of the Gag purified protein in a buffer of pH 8.0 with 1M NaCl and 50mM Tris. Post incubation, the labelled mixture was subjected to dialysis with the Slide-A-Lyzer Mini Dialysis Device (Thermo Scientific), following the manufacturer’s instructions to remove the excess unbound dye from the solution.

GUV preparation and observation

Lipid and buffer compositions. Lipid compositions for GUVs were brain total lipid extract \textsuperscript{50} supplemented with 5 mole% brain PIP\textsubscript{2}. If needed, 0.5 mole% BODIPY TR ceramide was present in the lipid mixture as membrane reporter. The salt buffer inside GUVs, named I-buffer, was 50 mM NaCl, 20 mM sucrose and 20 mM Tris pH 7.5. The salt buffer outside GUVs, named O-buffer, was 60 mM NaCl and 20 mM Tris pH 7.5.

GUV preparation. GUVs was prepared by using the polyvinyl alcohol (PVA) gel-assisted method\textsuperscript{44}. Briefly, a PVA solution (5% (w/w) of PVA in a 280 mM sucrose solution) was warmed up to 50°C before spreading on a coverslip that was cleaned in advance by being bath sonicated with 2% Hellmanex for at least 30 min, rinsed with MilliQ water, sonicated with 1M KOH, and finally sonicated with MilliQ water for 20 min. The PVA-coated coverslip was dried in an oven at 50°C for 30 min. 5-10 µl of the lipid mixture (1 mg/mL in chloroform) was spread on the PVA-coated coverslip, followed by drying under vacuum for 30 min at room temperature. The PVA-lipid-coated coverslip was then placed in a 10 cm cell culture dish and
0.5 mL -1 mL of the inner buffer was added on the coverslip, followed by keeping it stable for 45 min at room temperature to allow GUV to grow.

Sample preparation and observation. GUVs were first incubated with either Gag or I-BAR domain at bulk concentrations depending on the designed experiments for at least 15 min at room temperature before adding either I-BAR domain or Gag, respectively, into the GUV-protein mixture. In experiments where there was only Gag but no I-BAR domain, the stock solution of I-BAR domain was used in order to obtain a comparable salt strength outside GUVs as those where I-BAR domain was present. The GUV-protein mixture was then incubated at least 15 min at room temperature before observation. For Gag/I-BAR membrane recruitment assay, samples were observed by a Nikon C1 confocal microscope equipped with a X60 water immersion objective (Nikon, CFI Plan Apo IR 60X WI ON 1,27 DT 0,17). For Gag/I-BAR tubulation assay, samples were observed with an inverted spinning disk confocal microscope, Nikon eclipse Ti-E equipped with Yokogawa CSU-X1 confocal head, 100X CFI Plan Apo VC objective (Nikon) and a CMOS camera, Prime 95B (Photometrics).

For all experiments, coverslips were passivated with a □-casein solution at a concentration of 5 g.L⁻¹ for at least 5 min at room temperature. Experimental chambers were assembled by placing a silicon open chamber on a coverslip.

GUV Image analysis.

Image analysis was performed by using Fiji². 

Quantification of AX488 Gag binding on GUV membranes. Florescence images were taken at the equatorial planes of GUVs using identical confocal microscopy settings. The background intensity of the AX488 channel was obtained by manually drawing a line with a width of 10 pixels perpendicularly across the membrane of a GUV. We then obtained the background
intensity profile of the line where the x-axis of the profile is the length of the line and the y-axis is the averaged pixel intensity along the width of the line. The background intensity was obtained by calculating the mean value of the sum of the first 10 intensity values and the last 10 intensity values of the background intensity profile. To obtain Gag fluorescence intensity on the membrane of the GUV, we used membrane fluorescence signals to find the contour of the GUV. Then, a 10 pixel wide band centered on the contour of the GUV was used to obtain the Gag intensity profile of the band where the x-axis of the profile is the length of the band and the y-axis is the averaged pixel intensity along the width of the band. Gag fluorescence intensity was then obtained by calculating the mean value of the intensity values of the Gag intensity profile, following by subtracting the background intensity.

Gag sorting map. Florescence images of GUVs were taken using identical confocal microscopy settings. For every GUV, we first calculated the fluorescence intensity ratio for every pixel of the Gag and membrane images of a GUV using \( \frac{I_{\text{Gag}} - I_{\text{background}}^{\text{Gag}}}{I_{\text{membrane}}} \), where \( I_{\text{Gag}} \) is the Gag intensity, \( I_{\text{background}}^{\text{Gag}} \) is the background intensity in the Gag channel, and \( I_{\text{membrane}} \) is the membrane intensity. The sorting map was then obtained by converting the resulting image from the previous step to a pseudo-colored image via the “Look Up Table, Phase”. The background intensity value in the Gag channel was the mean intensity value of a 50 pixel wide square in the background outside GUVs. The sorting map of I-BAR domain was obtained by using the same procedure as those for Gag.

Statistics.

All notched boxes show the median (central line), the 25th and 75th percentiles (the bottom and top edges of the box), the most extreme data points the algorithm considers to be not outliers (the whiskers), and the outliers (crosses).
References and Notes


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Author contributions:

K.I., FT, PB and D.M. designed the study and planned the experiments. KI, AdP, RD, FT, and PM carried out the experiments. KI, FT and RM performed the microscopy experiments. PL furnished the GFP tagged I-BAR expression plasmids and expertise on I-BAR proteins. KI, AdP, RD and PM carried out the biological assays. JM produced and purified the Gag protein in vitro. FT carried out the GUV assays with Gag. FT and PB analysed GUV results. RM and KI performed PALM/STORM imaging. CF resolved STORM analysis and performed simulations. KI, AdP, FT, CF, PB and D.M. contributed to interpretation of the results. KI and D.M. took the lead in writing the manuscript. Mainly K.I. and D.M., but also F.T., C.F. and P.L., edited the manuscript. P.B. and D.M. provided funding. All authors provided critical feedback and helped shape the manuscript.
Competing interests: No conflicts of interest to disclose.
Figure 1: Partial knockdown of IRSp53 decreases HIV-1 Gag particle release by arresting assembly at the cell plasma membrane. a) siRNA knockdown of IRSp53 in Jurkat T lymphocytes leads to a decrease in pNL4-3ΔEnv particle release (left graphs, immunoblots for IRSp53 and loading controls beneath the graphs). Similarly,
knockdown of IRSp53 in HEK293T cells led to a decrease in HIV-1 pGag particle release. Knockdown of a closely related I-BAR protein IRTKS did not have a significant effect on particle release (right graphs, immunoblots for IRSp53, IRTKS and loading controls beneath the graphs). c) Transmission electron microscopy images of HEK293T cells expressing HIV-1 Gag with control siRNA (upper panel) and siRNA IRSp53 (lower panel). Cells knocked down for IRSp53 show arrested buds at the plasma membrane as compared to the control cells which display a normal range of buds in different stages of assembly and budding (scale bar 100 nm). d) Measurement of the dimensions of the buds (height and width) in the control siRNA and siRNA IRSp53 conditions. The knocked down cells exhibit a narrow range of heights corresponding to the arrested buds visible in the images while the control cells display a wider range of heights corresponding to assembly progression (left graph, “Height of bud”). The widths of the buds in both conditions did not display large differences in distributions.
Figure 2: HIV-1 Gag associates with IRSp53 and promotes retention of IRSp53 at the cell membranes. a) Immunoprecipitation of HIV-1 Gag with an anti-IRSp53 antibody. HIV-1 Gag is enriched in anti-IRSp53 pulldown, as compared to the controls. b) Membrane flotation assay protocol. 1) 293T cells were dounced and 2) the post-nuclear supernatant was loaded onto a discontinuous sucrose gradient. Following ultracentrifugation, cell membranes were separated from the cytosolic fraction. c) Immunoblots for the indicated proteins (upper panel) and quantification and graph (lower panel) showing that upon Gag expression in cells, IRSp53 is enriched in the cell membrane fraction. A similar increase is seen for Tsg-101, a known interactor of the Gag polyprotein.
Figure 3: Super resolution microscopy imaging and simulations reveal preferential IRSp53 localization at HIV-1 Gag budding sites. a) Super-resolved dual color images of HEK293T cells expressing Gag(i)mEos2 immuno-labelled for IRSp53 (scale bar 10 µm) with magnified view (scale bar 500 nm) and Gag cluster size distribution. HIV-1 Gag assembly sites at the plasma membrane coincide strongly with cellular structures marked by IRSp53 (left panel, and magnified views) using coupled PALM/STORM microscopy in TIRF mode. Density based scan (DBScan) of HIV-1 Gag clusters of super resolution microscopy data showing size distribution of the clusters reveal distributions falling within the range of HIV-1 particles (80-150nm). b, Super-resolved dual color images of cells expressing Gag(i)mEos2 immunolabelled for IRTKS (scale bar 10 µm) with magnified view (scale bar 500 nm) and Gag cluster size distribution. c, Quantification of coordinate based colocalisation (as in 20) at Gag assembly sites: CBC values for IRSp53 (c) and IRTKS (d) were plotted as relative frequencies. c) IRSp53 CBC values show a peak highly correlated (>0.5) with 27% of IRSp53 localizations highly correlated with Gag (>0.5). d) IRTKS CBC values show a peak anti-correlated/non correlated (-0.5 to 0). d, Comparison of cumulative frequency distributions of IRSp53/Gag and IRTKS/Gag. Only 14% of IRTKS localisations are highly correlated, with a peak of negatively correlated values. e) Comparison of experimental IRSp53/Gag CBC cumulative distribution with the simulated values. (See Fig S5 for details). IRSp53 shows an experimental cumulative CBC distribution corresponding to a belt of 80nm width (waist=40nm) centered at a distance of 80 nm from the center of Gag assembly sites (left graph, bold grey line corresponds to experimental data for IRSp53, bold blue line corresponds to simulated values closest to experimental data). IRSp53 thus corresponds to restricted pattern in and around a Gag assembly site (panel 1 schematic of simulated data, panel 2
simulated data and panel 3 experimental data). f) IRTKS experimental CBC distribution (bold grey line in graph) correspond to simulated ones using a belt of 200 nm width (waist=100 nm) centered at a distance of 140 nm from the center of Gag assembly site (bold red line). IRTKS belt surrounding assembly sites is more diffuse and spreads out (panel 1 schematic of simulated data, panel 2 simulated data and panel 3 experimental data). Scale bars in the panels are 100 nm.
Figure 4: IRSp53 is incorporated into HIV-1 particles in a Gag dependent manner. a) Schematic for the protocol followed for imaging and analysis. HIV-1 Gag VLPs were purified from HEK293T cells expressing HIV-1 Gag/Gag-mCherry and IRSp53-GFP or other GFP tagged proteins (IRSp53-IBAR-GFP, IRTKS-GFP and PH-PLCδ-GFP). Purified Gag VLPs were then spotted over a poly-lysine treated glass slide and imaged by TIRF-Microscopy (particles were imaged in the red, and IRSp53 in the green channel). For each condition, 3000 particles (~300 particles/image, 10 images) were counted. Fluorescence overlapping fraction (Mander’s coefficient, see Materials and Methods for details) were determined for Gag-mCherry and for IRSp53-GFP, IRTKS-GFP and PH-PLCδ-GFP and reported in the graphs. b) The 0,5 value indicates the random incorporation level (indicated by black line across the graph). IRSp53-GFP and IRSp53-IBAR show high correlation values (0.95-1 and 0.8, respectively). The other I-BAR domain proteins were not significantly correlated with Gag-mCherry particles (0.4-0.5). PH-PLCδ-GFP, a known marker of the phospholipid PI(4,5)P$_2$, shows a slightly higher correlation (0.6), since HIV-1 Gag is known to associate with this phospholipid. c) Incorporation of IRSp53 into wild type pNL4-3 HIV-1 or d) Gag VLPs revealed by immunoblots against Gag(p24), IRSp53, IRTKS, Tsg101 or actin, as indicated. Following a 25% sucrose cushion purification, IRSp53 was found to be associated with released wild type HIV-1 (left panel) and Gag VLPs (right panel). Tsg101, known to be incorporated into released particles, was also found associated with viral particles. IRTKS, a closely related I-BAR protein to IRSp53, was not incorporated in purified HIV-1 viral particles or Gag-VLPs. e) Protocol of VLPs purification using sucrose cushions and iodixanol gradient. Briefly, pellets obtained after ultracentrifugation of cell culture medium of HEK293T transfected with pNL4.3HIV-1 or pGag were deposed on iodixanol gradient (20, 30
and 60%). 20 fractions of 200 µL were collected from top of the tube. Fractions collected following iodixanol gradient purification of NL4-3ΔPolΔEnv Gag VLPs were analyzed using western blots for IRSp53 and Gag, TSG101 and ALIX, CD81 and CD63 revealed respectively on the same membrane (blot 1 and blot 2) revealing IRSp53 association with Gag viral particles and known cofactors.
Figure 5: IRSp53 I-BAR domain enhances Gag recruitment to PIP$_2$-membranes and at the tip of I-BAR domain-induced tubes. a) (Left) AX488 Gag fluorescence intensity on membranes in the absence of I-BAR domain (named “Gag only”), in the presence of I-BAR domain where GUVs were first incubated with I-BAR domain and then Gag (named “I-BAR + Gag”) and GUVs were first incubated with Gag and then I-BAR domain (named “Gag + I-BAR”). Each circle presents one GUV analysis. N = 82 GUVs, n = 4 sample preparations for “Gag only”, N = 67 GUVs, n = 4 sample preparations for “I-BAR + Gag”, and N = 104 GUVs, n = 4 sample preparations for “Gag + I-BAR”. To pool all data points from the 4 sample preparations, in each preparation for all three conditions, Gag intensities were normalized by the mean Gag intensity in the “Gag only” condition. Protein bulk concentrations: 0.3 µM for AX488 Gag and 0.5 µM for I-BAR domain (not fluorescently labelled). (Right) Representative confocal images of AX488 Gag on GUV membranes in “I-BAR + Gag” condition. b) Representative confocal images of AX594 Gag in I-BAR domain-induced tubules. Inverted grayscale images are shown for I-BAR domain and Gag. Protein bulk concentrations: 0.3 µM for AX594 Gag and 0.05 µM for I-BAR domain (70% unlabeled and 30% AX488 labeled I-BAR domain). Cyan arrowhead points out a I-BAR domain-induced tubule and white arrow indicates the co-localization of Gag and I-BAR domain at the tip of the tubule. c) Representative confocal images of AX488 Gag in I-BAR domain-induced tubules. Inverted grayscale images were shown for membranes and Gag. Protein bulk concentrations: 0.3 µM for AX488 Gag and 0.05 µM for I-BAR domain (not fluorescently labelled). Cyan arrowheads point out I-BAR domain-induced tubules and white arrows indicate the co-localization of Gag and I-BAR domain at the tip of the tubules. Sorting map was obtained by
calculating the fluorescence intensity ratio of Gag and membranes (see Material and Methods for more details). Scale bars, 5 µm.
Figure S1: Effect of siRNA-based IRSp53 and IRTKS knockdown on HIV-1 Ga particle release. a) Expression of HIV-1 Gag in HEK293T cells (from
molecular clones pNL43ΔPolΔEnv or pGag(myc)) and a schematic representation of the viral Gag structure. Gag is known to have four main domains, the matrix MA interacting with cell membrane, the capsid CA for Gag oligomerization, the nucleocapsid NC interacting with the viral genomic (+)RNA and p6 recruiting Tsg101 for particle budding. b) Validation of siRNA targeting IRSp53. siRNA targeting IRSp53 diminishes expression of IRSp53-GFP as seen by immunoblot (Left, Lane 1 siCTRL, Lane 2 siIRSp53) and fluorescence imaging of 293THEK cells expressing IRSp53-GFP with corresponding siRNA (right). c) Validation of siRNA targeting IRTKS. siRNA targeting IRTKS diminishes expression of IRTKS-GFP as seen by immunoblot (Left), Left, Lane 1 siCTRL, Lane 3 siIRTKS and fluorescence imaging of 293THEK cells expressing IRTKS-GFP with siRNA, as indicated (right). d) Reduced viral release are seen in cells knocked down for IRSp53 (representative immunoblots, Lane 1 siCTRL, Lane 2 siIRSp53). e) IRTKS knockdown has no significant effect on HIV-1 Gag particle release (representative immunoblots, Lane 1 siCTRL, Lane 3 siIRTKS).
Figure S2: Protein sequence comparison of IRSp53 and IRTKS. a) IRSp53 and IRTKS are membrane curving I-BAR proteins. b) IRSp53 (Query, Accession Q9QB8) shares a 40% sequence homology with IRTKS (Subject, Accession Q9UHR4) Sequence alignment performed with NCBI Protein BLAST. Most of this homology is centered in the I-BAR/IMD domain of the three proteins and the C terminal SRC Homology 3 (SH3) domain common to the two proteins.
Figure S3: Transmission electron microscopy of siRNA treated HEK293T cells expressing HIV-1 Gag. SiRNA control (a) or siRNA IRSp53 (b) treated cells reveal that IRSp53 KD cells display HIV-1 Gag arrested assembly at the cell plasma membrane as shown by transmission electron microscopy.
Figure S4: Complexing of IRSp53 with Gag is independent of Gag-p6 domain.

Addition of an internal mEos2 tag within the Gag protein does not affect its complexing with IRSp53 (Compare lane 1 and Lane 2). Gag-mEos2Δp6, a mutant of Gag deficient in p6 dependent-ESCRT recruitment, is also immuno-precipitated (IP) with IRSp53 antibody (lane 3), indicating that loss of the p6 domain and Tsg101 recruitment is not a limiting factor for Gag-IRSp53 complexing. Lane 4 as an IP control.
Figure S5: Super resolution PALM-STORM microscopy and localization precision

a) Single HIV-1 assembly sites detected using PALM (yellow) as compared to TIRF-M (green) b) Nanoscale structures detected in STORM (white) as compared to TIRF-M c) Localization precision in PALM and d) STORM measurements.
Fig. S6 | Super resolution PALM/STORM images of HEK293T cells expressing HIV-1 Gag(i)mEos2 (green) and immunolabelled for a) IRSp53 (Atto647N, red) and b) IRTKS (Atto647N, red), with magnified images adjacent to the main image. Scale bar is 10µm for the large images and 500 nm for the magnified images.

Figure S7: Workflow of image analysis for PALM/STORM images. 1) Reconstructed PALM images 2) The PALM images were thresholded in ImageJ to obtain a mask of the assembly sites of Gag. 3) The coordinates of centres of these
assembly sites were then obtained using the Analyze Particles feature of ImageJ. 4) These coordinates were then used to analyze the original PALM and STORM single molecule localizations, and extract PALM coordinates from an area corresponding to size of assembly sites (r<80nm from center), and STORM coordinates in a larger area around the assembly site (r<150nm from center). 5) Using these filters, a new set of PALM and STORM localizations were generated, which corresponded to Gag assembly sites and the area around these sites. 6) These localizations were then used to calculate the coordinate based colocalization (CBC) for PALM (Gag) and STORM (IRSp53/IRTKS). The CBC values were then plotted as cumulative probability distributions.
Figure S8: Cumulative probability distributions of CBCs for simulated PALM/STORM data. Simulations corresponding to different patterns of STORM localizations around PALM clusters were performed using ImageJ. Each molecule
position was randomly distributed within a fixed size disk of 100nm diameter for the PALM localisations (which represent 70% of all the experimental Gag cluster diameter found) or within a belt surrounding this disk for the STORM localization. The STORM belt size ranged from a waist of 20 nm (width 40nm) to a waist of 100 nm (width 200nm), with distances from centre of the simulated viral bud (PALM disk) to the centre of the surrounding belt ranging from 0 nm to 160 nm for each waist size. Each simulated localization was then convoluted by a 2D spatial Gaussian function with a waist of 200nm in x and y direction to simulate the point spread function of the microscope. The number of photons emitted by each simulated position was randomly distributed according a Gaussian distribution centered to a value equivalent to the one experimentally observed, this in order to obtain a localization precision equivalent to the experimental ones (see Fig S3 for the experimental localization precision). Each set of simulated images was then analyzed using the Thunderstorm plugin of ImageJ with the same parameter as the one used for experimental data. Single molecule localizations obtained from this analysis were then used for CBC analysis using the CBC plugin of Thunderstorm as it was done for experimental data sets.
Fig.S9| Super resolution PALM/STORM images of CD4+ Jurkat T cells expressing HIV-1 Gag(i)mEos2 (green) and immunolabelled for a) IRSp53 (Atto647N, red) and b) IRTKS (Atto647N, red), with magnified images adjacent to the main image. Scale bar 10µm for the large images and 500 nm for the magnified images.
Figure S10: IRSp53, but not IRTKS, is localized at HIV-1 Gag budding sites in host CD4+ Jurkat T cells as revealed by super resolution microscopy. a) Super resolved dual color PALM/STORM images of Jurkat T cells immuno-labelled with IRSp53. Density based scan (DBScan) of HIV-1 Gag clusters of super resolution data showing size distribution of the clusters that reveal distributions falling within the
range of HIV-1 particles (80-150nm). Gag clusters overlap with IRSp53 in some areas (indicated). b) Jurkat cells immuno-labelled with IRTKS show a Gag cluster size distribution within the acceptable range. Gag clusters do not seem to overlap with IRTKS. CBC values for IRSp53 (c) and IRTKS (d) were plotted as relative frequencies. c) IRSp53 CBC values show a peak of IRSp53 localizations (26%) highly correlated with Gag (>0.5). d) IRTKS CBC values show a peak anti-correlated/non correlated (-0.5 to 0) with a peak of negatively correlated values. e) Cumulative probability distribution of CBCs for IRSp53/Gag. CBC values for IRSp53 and IRTKS were plotted as cumulative frequency distributions and compared with distributions of simulated values corresponding with different distances and structures (See Supplementary Fig.3). IRSp53 shows a cumulative CBC distribution corresponding to a simulation of waist at 40nm (width 80 nm) and a distance of 80nm (left graph, bold grey line corresponds to experimental data for IRSp53, bold blue line corresponds to simulated values closest to experimental data). IRSp53 thus corresponds to restricted pattern in and around a Gag assembly site (panel 1 schematic of simulated data, panel 2 simulated data and panel 3 experimental data). d, IRTKS CBC distribution (bold grey line in graph) is close to simulations of waist 100nm (width 200nm) and a distance of 140nm (bold red line). IRTKS is more diffuse and spread out (panel 1 schematic of simulated data, panel 2 simulated data and panel 3 experimental data). Scale bar in the panels= 100nm.
**Figure S11: In vitro GUV experiments** showing incubation of low concentration of the IRSp53-I-BAR domain (green) with GUVS (red) inducing long membrane tubulation towards the interior of the vesicle (top panel-a) while addition of HIV-1 Gag after incubation of I-BAR results in the formation of shorter tubules with HIV-1 Gag sorting to the tip of the short tubules (bottom panel-b).
Part B: Complementary Results of Part A: Research Article
B1. Complementary Results on IRSp53 involvement in HIV-1 Gag assembly and particle production

IRSp53 is a membrane binding protein involved in HIV-1 assembly. We previously established complexing of endogenous IRSp53 and HIV-1 Gag (Results, Part A). In this section, this complexing was further studies using different solubilisation buffers as well as different mutants of HIV-1 Gag. These results revealed a Gag membrane binding and multimerization dependent complexing of these proteins. In order to visualise IRSp53 localisation with respect to assembling Gag at the plasma membrane we attempted to label endogenous IRSp53 in resin-embedded samples for transmission electron microscopy of cells expressing HIV-1 Gag. We observed labelling of HIV-1 Gag buds at the plasma membrane of cells, however labelling of endogenous IRSp53 did not appear to be successful. We then proceeded to immunolabel endogenous IRSp53 in fixed cells and perform immunofluorescence to test the efficacy of this technique. Successful immunolabelling of IRSp53 was performed in fixed HEK293T cells, revealing characteristic membrane protrusions at the cell plasma membrane, validating this technique for further studies. This technique was later used for single molecule localisation microscopy and analyses to examine IRSp53 in relation to HIV-1 assembly sites (Results, Part A). As both IRSp53 and Gag bind and cluster the same plasma membrane phospholipids, we then turned to ectopic expression of GFP-tagged I-BAR proteins along with HIV-1 Gag followed by cellular fractionation and membrane flotation assays. Overexpression of IRSp53-GFP did not have any significant effect on HIV-1 Gag membrane binding. Taking advantage of this, we used ectopic expression of fluorescently tagged IRSp53 to study its recruitment at individual Gag assembly sites by live cell TIRF microscopy. Preliminary results revealed peaking of IRSp53 fluorescence in the initial period of Gag assembly, confirming its role in the assembly stage before budding and release of the virus. Together, these results, while validating optimisation of techniques for future studies, offer further insights into IRSp53 involvement in Gag dependent HIV-1 particle formation.
**B.1.1 The 53kDa IRSp53 isoform-S/T, and not IRS-58, is involved in HIV-1 Gag particle production.**

IRSp53 exhibits mRNA splice variants and four isoforms are currently known and described based on their relative molecular weights (Okamura-Oho et al 2001 BBRC). The isoforms are named as IRSp53-L, S, T and M (IRS-58). As the variation in these isoforms takes place in the C terminal end of the protein (Section 3.5.2), an antibody targeting the conserved common region (corresponding to amino acids 293-280, a region between the partial CRIB and SH3 domains, Figure 21) revealed that HEK293T expresses 3 detectable isoforms (L, IRS-58 and S/T), while Jurkat T cells express L and S/T only (Results, Part A, Figure 1a). SiRNA targeting IRSp53 knocked down IRSp53-S/T and IRS-58, or IRS-58 alone. (Figure 22) Interestingly, partial knockdown of IRSp53-S/T resulted in impaired HIV-1 Gag particle production, while a complete knockdown of IRS-58 had no effect on particle production, indicating that HIV-1 Gag particle production requires a specific isoform of IRSp53. Jurkat T cells do not seem to express detectable levels of the IRS-58 isoform, while the function of IRSp53-L, a 74kDa isoform, has no known function. Thus IRSp53 involvement in HIV-1 Gag particle production is isoform-specific and the 53 kDa isoform is involved in HIV-1 Gag particle production.

![Gene Knockdown](image1)

**Figure 22: Fig. Knockdown of IRSp53, and not IRS-58, diminishes HIV-1 Gag VLP release.** Partial knockdown of the 53 kDa isoform leads to a corresponding decrease in HIV-1 Gag VLP release (right panels, graph). Complete knockdown of the 58 kDa isoform, with no decrease in the IRSp53 isoform has no significant effect on VLP release (left panels, graph).
B.1.2 IRSp53 interaction with Gag persists with different buffers indicating a possible lipid independent interaction.

Pulldown of endogenous IRSp53 in cells expressing HIV-1 Gag results in precipitation of Gag suggesting a direct/indirect interaction between these proteins (Results, Part A, Figure 2a). Detergents are commonly used to permeabilize cells and solubilize proteins in order to identify proteins, and protein-protein interactions, particularly for membrane proteins. Detergents are classified as ionic, non-ionic and zwitterionic based on the charges on their headgroups. Ionic detergents have charged headgroups and are typically harsher, denaturing detergents with a higher capacity of disrupting protein-protein and protein-lipid interactions by actively binding proteins and altering their charges. Non-ionic detergents are less harsh and are considered non-denaturing, but are still capable of solubilizing membrane proteins while keeping protein structure intact. Zwitterionic detergents have both positive and negative charges, giving them a net neutral charge, but are typically harsher than non-ionic detergents. In this case, we tested the effect of a zwitterionic detergent (CHAPSO) on the IRSp53-Gag complex as compared to a combined non-ionic/ionic buffer (Triton X-100 + Sodium deoxycholate). CHAPSO solubilisation resulted in higher lipid recovery and less protein content as compared to Triton X-100, which showed an opposite effect of high protein recovery and low lipid content (Banerjee et al., 1995). CHAPSO solubilisation of cells expressing HIV-1 Gag and subsequent pulldown of the endogenous IRSp53 precipitated Gag (Figure 23 Buffer 1 Lane 1 “With Antibody”), but showed a high level of background (Lane 2 “Without Antibody”). This indicates a strong interaction as zwitterionic detergents are capable of disrupting protein-protein interactions, but the background could be due to unsolubilized lipid-bound Gag. On the other hand, the lipid disrupting action of Triton X-100 coupled with the relatively harsh bile salt detergent sodium deoxycholate reduced the background while preserving the precipitation of Gag by IRSp53 pulldown (Figure 23 Buffer 2 Lane 1 “with Antibody”, Lane 2 “Without Antibody”). This indicates that the interactions of IRSp53 and Gag could be independent of lipids, and are resistant to denaturation. Optimal conditions of immunoprecipitation of the IRSp53/Gag complex were thus determined, while possibly uncovering the nature of their interactions.
B1.3 Identification of the possible HIV-1 Gag domain involved in Gag association with IRSp53

HIV-1 Gag is a polyprotein composed of multiple domains (MA, CA, NC, p6), each having distinct functions in assembly and maturation of the HIV-1 virion (Section 1.4.1). As we established intracellular complexing of Gag and endogenous IRSp53 (Figure 2a, Section Results, Part A), we then attempted to ascertain the domain of HIV-1 Gag involved in this complexing. To this end, we expressed different mutants of Gag in HEK293T cells (characterised in (Floderer et al., 2018), Figure 24 A, B) and immunoprecipitated endogenous IRSp53 to compare the pulldown of each Gag mutant. The mutants used were tagged internally with fluorescent mEos2 to verify the expression and intracellular localisation of these proteins. We first verified that addition of this internal tag did not adversely affect its complexing with IRSp53 (Figure 24 C,D, lane 2 “Gag(i)mEos2”). Since the expression of the mutants varied between the conditions, and extensive washes failed to completely remove the non specific binding of Gag to the Protein A beads, we calculated the pulldown as the enrichment of the mutant pulled with the endogenous IRSp53 as compared to the non specific pulldown by the beads (Figure 24D). Upon comparing the enrichment for the mutants we found positive enrichments for the mutant ΔHBR (deficient in membrane binding), WM (deficient in multimerization) and Δp6 (deficient in Tsg101 interactions). However, the double mutant ΔHBR/WM, deficient in membrane binding as well as multimerization showed high negative enrichment. Correspondingly, ΔHBR/WM showed a cytosolic/nuclear localization in epifluorescence imaging (Figure 24B, “ΔHBR/WM”). The mutants MACA and MACA/WM, also cytosolic and deficient in membrane binding and multimerization also displayed negative enrichment. Thus, these results could indicate that HIV-1 Gag needs to bind the plasma membrane and multimerize in order to
complex with IRSp53. However, these results are strictly preliminary and further experiments are needed to optimize the washing conditions and verify the pulldowns obtained in this experiment.

![Figure 24](image)

**Figure 24**: Immunoprecipitation of HIV-1 Gag mutants by endogenous IRSp53. A) Different mutants of HIV-1 Gag, with an internal mEos2 tag, and different effects on assembly and budding. B) These were expressed in HEK293T cells and their intracellular localization was examined. C) Immunoprecipitation experiments were then performed by precipitating endogenous IRSp53 and the results elutes were probed for HIV-1 gag using the anti capsid antibody. D) Enrichment of each mutant was calculated as a fraction of the total pulled down with the antibody and subsequently subtracting the non specific enrichment in the control without antibody.

**B1.4 Immuno-electron microscopy studies of Gag-VLP budding at the cell plasma membrane immune-labelled for endogenous IRSp53 to analyse IRSp53 location at budding sites.**

In order to probe IRSp53 localization at single viral buds, it was necessary to adopt techniques affording visualisation of morphology at nanometric resolution. Electron microscopy has long been used to study budding HIV, and has been used successfully to visualize and characterize the retroviral buds and particles (Fuller et al., 1997; von Schwedler et al., 2003; Larson et al., 2005). HEK293T cells expressing HIV-1 Gag were fixed and labelled for the HIV-1 capsid (10 nm beads) and endogenous IRSp53 (20 nm beads) and then imaged using transmission electron microscopy. Electron micrographs showed the characteristic membrane curvature and electron dense retroviral buds at the plasma membrane for both HIV-1 Gag conditions (Figure 26 A,B), confirming the expression and production of viral particles in these cells. However, no labelling of endogenous IRSp53 was seen in both conditions. Previous reviews have touched upon the limitations of immuno-labelling of cells embedded in resin, a necessary step in electron microscopic observations.
of fixed cells (Griffiths and Lucocq, 2014). As immuno-labelling of endogenous IRSp53 had already confirmed the efficacy of the antibody (Section B1.3), the harsh fixation and resin embedding required for electron microscopy seemed to be the impeding factor in the labelling of this protein. Use of another antibody optimized for immuno-histochemistry (Materials and Methods) proved equally ineffective for labelling endogenous IRSp53 (Figure 26, B). IRSp53 visualisation by immunogold labelling has been performed before (Choi et al., 2005; Burette, Park and Weinberg, 2014; Dosemeci et al., 2017), however these studies used custom antibodies raised against specific peptides or full length IRSp53, indicating that commercially available antibodies could be ineffective for this technique. Since the immuno-labelling of IRSp53 was adequate for immunofluorescence (Figure 24), super resolution microscopy (TIRF-coupled PALM/dSTORM) by immunolabelling endogenous IRSp53 was chosen to overcome the diffraction limit and access single viral assembly sites.

Figure 25: Transmission immuno-electron microscopy of HEK293T expressing HIV-1 Gag. HEK293T expressing HIV-1 Gag alone were fixed and embedded in resin and immunolabelled for HIV-1 capsid (10nm beads) and endogenous IRSp53 (20 nm). A) Cells were immunolabelled with the Millipore 07-786 antibody against IRSp53, while B) were immunolabelled with the Sigma HPA023310 antibody. No labelling of endogenous IRSp53 is seen in any conditions.
B1.3 Immuno-labelling endogenous IRSp53 in HEK293T reveals IRSp53-labelled cellular protrusions at the cell membrane.

Since both the proteins IRSp53 and Gag cluster and bind PI(4,5)P₂ (Mattila et al., 2007; Saarikangas et al., 2009; Favard et al., 2019a; Podkalicka and Bassereau, 2019; Sengupta et al., 2019), they could definitely co-exist at the plasma membrane as Gag assembles at the plasma membrane. To verify the localization of endogenous IRSp53 with respect to Gag assembly sites, it was necessary to label endogenous IRSp53 in situ. Since the plasma membrane plays an important role in the localisation and interplay of these proteins, it was necessary to preserve plasma membrane structure while permeabilizing the cell to access and label endogenous IRSp53. Conventionally used permeabilizing detergent Triton-X 100 irreversibly perturbs plasma membrane lipids (Koley and Bard, 2010) thus potentially affecting the localisation of these proteins. An alternative detergent, saponin was used instead for immuno-fluorescence in HEK293T cells expressing Gag. Saponin is a plant based membrane permeabilizing agent which interacts with membrane cholesterol to make holes of ~100nm-1µm in the membrane; the permeabilization is reversible, thus minimally affecting the plasma membrane and membrane proteins (Wassler et al., 1987; Jamur and Oliver, 2010). Immuno-labelling of endogenous IRSp53 revealed characteristic labelling of lamellipodia and filopodia like protrusions at the cell membrane, as seen in previous studies (Nakagawa et al., 2003; Robens et al., 2010), confirming the efficacy of the antibody as well as the permeabilizing efficacy of saponin. Colocalization analyses of cells expressing Gag tagged with fluorescent tags did not offer insights into the relative localizations of these proteins, due to the diffraction limited lateral resolution afforded by the confocal microscope. However, successful labelling of endogenous IRSp53 provided optimal conditions for the necessary use of single molecule localisation microscopy to access HIV-1 Gag single assembly sites with nanometric resolution.

Figure 26: Immunolabelling of IRSp53 (in red, Atto647N) reveals characteristic cellular protrusions resembling filopodia and lamellipodia at the cell membrane. However, expression of fluorescently labelled Gag(i)mEos2 (in green) does not reveal spatial information about assembly sites due to the diffraction limit.
B1.5 Overexpression of IRSp53-I-BAR domain alone, and not full length IRSp53, affects Gag membrane localization

All the I-BAR proteins bind to the plasma membrane and are known to cluster the phospholipid PI(4,5)P$_2$ as well as show some dependence on cholesterol (Mattila et al., 2007; Saarikangas et al., 2009). HIV-1 Gag binding to the plasma membrane is also PI(4,5)P$_2$ dependent, and this lipid along with cholesterol are clustered at HIV-1 assembly sites (Yandrapalli, Muriaux and Favard, 2014; Yandrapalli et al., 2016; Favard et al., 2019a). Thus I-BAR proteins could potentially compete with HIV-1 Gag for membrane binding sites. To probe this aspect, different I-BAR proteins, IRSp53, IRSp53-IBAR domain alone, IRTKS and PinkBAR, tagged with GFP, were co-expressed in HEK293T cells expressing HIV-1 Gag, and 48h post transfection the cells were subjected to membrane flotation assay. Co-expression of IRSp53-GFP with Gag resulted in a slight mean decrease in Gag membrane bound fraction (43%) as compared to the control (47%, eGFP co-expressed with Gag) (Figure 27). On the other hand, co-expression of the IRSp53-IBAR domain led to sharp decrease in membrane bound Gag (23%). This indicates that this protein either compete with or displace Gag from membrane binding sites. The drastic difference between IRSp53-full length and the IRSp53-IBAR, however is notable. Previous studies have compared the effect of over-expression of these proteins on filopodia formation in living cells. They noted that expression of full length IRSp53 resulted in formation of dynamic filopodia with typical actin bundles, while expression of IRSp53-IBAR domain alone led to formation of filopodia-like protrusions which were stable, did not exhibit actin bundles and were resistant to actin-destabilizing drugs. This indicates that the absence of the C terminal signalling domains, notably the SH3 domain, alters the membrane binding properties of IRSp53, and also is expected to be dissociated from actin signalling. Either of these factors could be responsible for the sharp decrease seen in Gag membrane bound fraction upon expression of the IRSp53-IBAR domain. Actin signalling leading to cortical actin polymerization in particular has been noted to restrict diffusion of proteins and lipids on the plasma membrane, indicating that the actin signalling scaffold function of the SH3 and the WW domain of IRSp53 could play vital roles in retaining Gag at the plasma membrane.
Expression of the full length IBAR proteins does not seem to impact HIV-1 Gag membrane binding significantly however binding of the IRSp53 IBAR domain reduces membrane bound Gag (condition Gag + IRSp53-IBAR-GFP).

B1.6 Preliminary TIRF live cell imaging in HEK293T cells shows that ectopic IRSp53-mCherry recruitment peaks at the beginning of HIV-1 Gag assembly at the cell plasma membrane.

Since IRSp53 appears to be in proximity of assembling Gag, but not co-localising with all visible assembly sites, there is possibly a dynamic aspect to the localised IRSp53 presence at the assembly site. To probe this further for the temporal aspect of IRSp53 presence at the Gag assembly site, cells expressing HIV-1 Gag tagged with GFP and IRSp53-mCherry (Materials and Methods) were imaged in TIRF mode to capture Gag assembly events at the cell plasma membrane. The dynamics of Gag assembly have been extensively studied before (See Section 2.2.3, Page 30). Briefly, Gag assembly was interpreted from the increase in fluorescence intensity at identified clusters of Gag. Overexpression of ectopic IRSp53 did not seem to significantly affect HIV-1 Gag membrane binding (Section B1.5, Figure 25), validating the use of this method to observe the assembly process. The rise in intensity (assembly) followed by a plateau phase and a decrease in fluorescence (ESCRT recruitment and budding) took an average of 25 minutes, with the assembly phase lasting 5-15 minutes, in different cell types. The initial aim of this experiment was to obtain a similar profile for Gag assembly and then study the fluorescence intensity changes of IRSp53 with respect to this profile of HIV-1 Gag. As Ivanchenko et al had noted, there were a large fraction of cells which had produced early after transfection, and were saturated with virions at the plasma membrane, without any detectable new assembly event, while another fraction was observed which were fluorescent and expressing HIV-1 Gag but did not produce detectable virions. A minority of cells had detectable assembly events, and these were analysed for IRSp53 dynamics. To summarise, early imaging after transfection did not yield fluorescent cells in two channels, while imaging late after transfection resulted in observable cells being maximally saturated with assembled virions at the plasma membrane. HEK293T cells are frequently used for production of different virus stocks, being known to produce large quantities of virus, thus it is quite possible that the window of imaging available to visualise ongoing assembly events is very narrow for these cells. Notably, other studies involving live
cell imaging of HIV-1 assembly used HeLa cells (Jouvenet, Bieniasz and Simon, 2008; Ivanchenno et al., 2009). In the HEK293T system used in this study, only a few isolated assembly events were found among saturated cells and these were analysed for IRSp53-mCherry signal at assembly sites. Gag assembly events were identified and tracked over time and space and their fluorescence at each frame was recorded and analysed for mCherry and GFP channels. For assembly events corresponding to known profiles, IRSp53 signal was plotted and overlaid on Gag fluorescence intensity profile to assess its dynamic relative to the assembly event. IRSp53 fluorescence seemed to start increasing at the beginning of the assembly event and peak while the assembly event was still ongoing (Figure 28E, red), and decreased to the baseline before Gag fluorescence (green) reached a peak of intensity i.e. before the end of assembly. This suggests that IRSp53 plays a role in the initial period of HIV-1 Gag assembly, when the bud formation is in a nascent stage, and is possibly excluded from the assembly site as Gag assembly progresses. Further experiments are needed to have a statistically significant sample size and conclude with confidence.

Figure 28: Live cell imaging of cells transfected with Gag-GFP and IRSp53-mCherry. A) HEK293T cell expressing Gag(i)GFP and IRSp53-mCherry in TIRF showing HIV-1 Gag buds at the plasma membrane (in green). B) Assembling Gag bud was identified by measuring changes in GFP fluorescence intensity. C) Its trajectory was examined to verify its spatial restriction, a characteristic of assembling Gag. D) Relative fluorescence intensities measured for both channels were plotted as a function of time. E) Gaussian smoothing of the obtained intensity curves was performed to identify the global changes in intensity for comparison of the two channels. The rise in intensity of Gag(i)GFP till its peak was considered as assembly, and took approximately 8 minutes. The rapid loss in intensity following this peak, interpreted as budding and release, occurred approximately 25 minutes after the initial increase in intensity. IRSp53*mCherry fluorescence peaked and diminished in the first half of 8 minute assembly period.
Part B.2  Actin signalling pathways associated with IRSp53 in HIV-1 Assembly

IRSp53 involvement in HIV-1 Gag particle production was previously reported as a part of a Rac1 dependent signalling pathway. Here we studied the activation of Rac1 as a factor downstream of HIV-1 Gag expression in IRSp53 membrane binding. We find that activated Rac1, expressed with HIV-1 Gag, and not activated Rac1 alone increases IRSp53 binding to cell membranes. We identified the RacGEF Tiam1 as the possible activator of Rac1 involved in HIV-1 Gag particle production. Finally, as these pathways end in actin polymerization and brancing at the cell membrane, we examined the single molecule localisation of F-actin with respect to Gag assembly sites. Analyses of the data revealed a high correlation of actin molecules with HIV-1 Gag assembly sites, making way for studies probing the functional role of F-actin in HIV-1 assembly and release.
**B2.1 Activation of Rac1 affects IRSp53 membrane localization upon Gag expression.**

Small GTPases such as Rac, RhoA and Cdc42 are cornerstones of cortical actin signalling at the plasma membrane. IRSp53 is capable of interacting with both Rac1 and Cdc42 (Figure 21), and previous studies have shown the involvement of the Rac1-IRSp53 signalling pathway in HIV-1 cell free particle production (Thomas et al., 2015). Membrane binding of Rac1 is an important factor in the activation of this GTPase, along with the nucleotide exchange catalysed by GEFs (Das et al., 2015). Expression of dominant positive (V12Rac1) and dominant negative (N17Rac1) have been previously used for studies probing the role of this GTPase in cellular processes (Minden et al., 1995; Wheeler et al., 2006); the V12 substitution reduces endogenous GTPase and GAP activity of the protein, leading it to remain in a constitutively GTP-bound active state, while the N17 substitution increases its affinity for the GDI proteins, causing it to remain cytosolic and inactive. The dominant positive Rac1 induced lamellipodia generation while dominant negative Rac1 inhibited formation of lamellipodial extension, membrane ruffles and migration induced by growth factors and cytokines in several cell types (Ridley et al., 2003; Di Marzio et al., 2005). Since strong Rac1 activation was seen upon HIV-1 Gag expression previously and Rac1 itself is known to activate IRSp53 for actin cytoskeletal reorganisation, the role of Rac1 activation in IRSp53 membrane binding was explored using the Rac1 dominant mutants. HIV-1 Gag and the dominant mutants of Rac1 were co-expressed in HEK293T cells and the membrane binding of IRSp53 was assessed by membrane flotation of these cells. In addition, cells were transfected with just the Rac1 mutants to determine the role of Gag in this process. Control cells were transfected with empty vectors for assessing baseline levels of IRSp53 membrane binding. As compared to mock transfected cells, cells expressing Gag alone showed an increase in IRSp53 membrane localization, as seen before. However, co-expressing the dominant negative N17Rac1 with Gag led to an overall decrease in IRSp53 membrane binding (Figure 28), suggesting that Rac1 activation plays a vital role in IRSp53 membrane binding. Interestingly, expression of the dominant positive V12Rac1 alone had no significant effect on IRSp53 membrane binding (Figure 29). This suggests that Rac1 activation and HIV-1 Gag work in synergy to augment IRSp53 levels at cellular membranes. As HIV-1 Gag expression itself results in Rac1 activation (Thomas et al., 2015), this suggests that Gag is the dominant factor in IRSp53 activation and cell membrane binding. This could be via direct interactions or indirect interactions via other cofactors of this pathway. Rac1 activation is primarily mediated by nucleotide exchange catalysed by RacGEFs, opening up possibilities of these factors being involved in HIV-1 Gag directed Rac1 activation.
Figure 29: Fig. Co-expression of Rac1 dominant positive (Rac+) and dominant negative Rac1 (Rac-) along with HIV-1 Gag and its effect of membrane localization of endogenous IRSp53. As compared to mock transfected cells, the expression of HIV-1 Gag alone was sufficient to drive an increase in IRSp53 membrane binding. Co-expression of the Rac1 dominant negative mutant (Rac-) with Gag reduced IRSp53 binding to cellular membranes to near baseline levels. Expression of the dominant negative mutant (Rac-) and the dominant positive mutant (Rac+) alone did not affect IRSp53 membrane binding indicating that HIV-1 Gag plays an essential role in this process, possibly by interacting directly or indirectly with IRSp53.

B2.2 RacGEF Tiam1 is involved in HIV-1 Gag particle production and is possibly present in an intracellular complex with HIV-1 Gag.

There are about 20 guanine nucleotide exchange factors identified which act with membrane bound Rac1 to facilitate the GDP to GTP exchange for Rac1 activation (Marei and Malliri, 2017). There is evidence that GEFs direct the specificity of downstream effectors for the multi-functional small GTPases. As such, the GEF Tiam1 plays this role for the Rac1-IRSp53 pathway, directing IRSp53 to bind Rac1 rather than Cdc42 (Connolly et al., 2005). Tiam1 was first discovered as a factor inducing invasiveness in T-lymphoma cell lines, and was identified as having homology to RhoGEFs (Habets et al., 1994). Since it is implicated specifically in the Rac1-IRSp53 pathway, it is the prime candidate for Rac1 activation downstream of HIV-1 Gag expression in cells. To determine its functional role, if any, in HIV-1 particle production, siRNA targeting Tiam1 was co-transfected in HEK293T cells with HIV-1 Gag, and extracellular Gag was quantified as before (Materials and Methods). A partial knockdown of Tiam1 (~50%) resulted in a corresponding decrease in extracellular HIV-1 Gag (Figure 30), indicating its functional role in HIV-1 Gag particle production. In our previous study, a small molecule inhibitor of the Tiam1-Rac1 interaction similarly perturbed viral particle production in host CD4+ T cells (Thomas et al., 2015), confirming the involvement of this pathway of Rac1 activation in HIV-1 Gag dependent actin signalling. Since Tiam1 directly interacts with IRSp53 for its association with Rac1, and Gag-IRSp53 form an intracellular complex (Connolly et al., 2005), similar experiments were carried out to assess if Tiam1 is a part of this complex. HEK293T cells expressing HIV-1 Gag were lysed using the dual ionic/bile salt buffer of Triton X-100 and sodium deoxycholate as before (Materials and Methods). Following immuno-precipitation with the anti-Tiam1 antibody, the results were
assayed by immunoblot (Fig). As seen, the pulldown of Tiam1 induces a strong precipitation of HIV-1 Gag as compared to the controls (Fig.Lane.). This suggests that Tiam1 and HIV-1 Gag are present in an intracellular complex and may very well be the pathway by which Gag influences Rac1 activation and IRSp53 membrane recruitment. As the PI3K pathway and synthesis of PI(3,4,5)P$_3$ is involved in Rac1 activation by Tiam1, a pulldown was also performed with an antibody targeting this lipid. Pulling down cellular PI(3,4,5)P$_3$ also immunoprecipitated HIV-1 Gag. However, inspite of the high concentrations of the detergents as compared to their critical micellar concentrations, solubilisation of the phospholipid PI(3,4,5)P$_3$ is relatively unknown and thus impedes complete analysis of the result. In addition, the antibody may cross-react with the more ubiquitous PI(4,5)P$_2$, and as Gag is known to interact strongly with this lipid, the pulldown by PI(3,4,5)P$_3$ remains inconclusive.

Figure 30: Involvement of the RacGEF Tiam1 in HIV-1 Gag particle production and its interactions with HIV-1 Gag. A) siRNA mediated knockdown of the RacGEF Tiam1 impaired HIV-1 Gag particle release in HEK293T cells. B) Pulldown of endogenous Tiam1 resulted in immuno-precipitation of exogenous HIV-1 Gag (lane 1). Immunoprecipitation of endogenous PIP3 also pulled down HIV-1 Gag (lane 2).

B2.3 F-Actin nanostructures are present at HIV-1 Gag budding sites.

Previous results (Thomas et al., 2015) show a global increase in F-actin levels in host CD4+ T cells on expression of HIV-1. In order to assess the presence, if any, of F-actin in proximity to HIV-1 assembly sites, coupled TIRF-PALM/STORM imaging of HEK293T cells, transfected with Gag(i)mEos2, doped with Gag, as before, was performed. These cells were fixed 24h after transfection followed by labelling of F-actin by phalloidin tagged with Alexa-647. Following labelling, the samples were mounted in the dSTORM buffer and imaged. Localization uncertainty distributions were plotted as before (Materials and Methods) to assess the quality of acquisitions. Subsequently, to verify that Gag clusters observed upon reconstruction correspond to assembling/assembled particles, the PALM localizations were
analysed by the DBSCAN module of the SMLM tessellation software SR-Tesseler (Levet et al., 2015). Cluster size distributions (Figure 31, B) indicate that observed Gag clusters fall within the acceptable range of HIV-1 particle sizes, as before (Results, Part A, Figure 3a,b). Having verified the quality of the acquired images, coordinate based colocalization (CBC) analyses (Malkusch et al., 2012) were calculated for the two channels. Briefly, CBC values were calculated for each STORM localization with respect to PALM localizations within a diameter of 100nm (corresponding to average cluster size obtained), and cumulative frequency distributions were plotted. Coordinate based colocalization (CBC) studies as previously established for IRSp53, show an even higher proportion of actin single molecule localizations which are highly correlated with Gag localizations (45%) as compared to IRSp53 (27%) (Results, Part A, Figure 3c). Since we interpreted this correlation of IRSp53 to be an indicator of its transient presence at Gag assembly sites, it is clear that actin is present longer at individual assembly sites as compared to IRSp53. Overlap analyses of these images further showed that ~76% of Gag assembly sites are positive for actin, indicating an important role of F-actin in Gag assembly. Previous super resolution studies of host cell factors (the ESCRT complex subunits) and HIV-1 assembly sites also measured the fraction of Gag assembly sites coinciding with these factors (Prescher et al., 2015). They found that a very low fraction (1.5-3.4%) of assembly sites coincided with ESCRT components, indicating that HIV-1 Gag interactions with these proteins were transient. Our results indicate that the presence of F-actin at HIV-1 assembly sites is not a transient phenomenon, as the Gag clusters observed present a cross section of assembling, assembled and budded particles. Furthermore, observation of reconstructed images (Figure 31, A) reveals Gag clusters decorating sites of F-actin labelling, including cellular plasma membrane protrusions, indicating that HIV-1 Gag possibly prefers assembling at sites of pre-existing actin polymerization activity suggesting an important role of actin signalling cofactors in this process.
Figure 31: Super resolution PALM/STORM microscopy for actin nanostructures at HIV-1 Gag assembly sites. A) HEK293T expressing HIV-1 Gag(i)mEos2 doped with HIV-1 Gag and stained with Phalloidin-AlexaFluor-647 to mark F-actin were imaged in TIRF mode and subjected to successive PALM/STORM microscopy to obtain super resolved images of assembly sites and cellular actin (scale bar 10µm for large images and 100nm for the magnified “zoom” images). B) Gag cluster detection and cluster size obtained in SR-Tesseler C) 45% of F-actin single molecule localizations are highly correlated with HIV-1 Gag localizations as compared with 27% IRSp53 localizations, indicating that, on an average, actin is present more consistently at assembly sites.
DISCUSSION
The aim of our study was to decipher the functional role and recruitment of I-BAR proteins in the process of HIV-1 assembly and particle production, and therefore also study the involvement of cellular pathways associated with these proteins in the late steps of viral replication. HIV-1 assembles and buds primarily from the host cell plasma membrane, and the assembly of a particle requires coordination of membrane curvature and multimerization of the retroviral protein Gag at the inner leaflet of the plasma membrane. As I-BAR proteins act as scaffolds for small GTPase signaling pathways and are notably able to sense and generate curvature, their role in viral assembly was investigated.

Previous studies (Thomas et al., 2015) have revealed the involvement of a Rac1 mediated cortical actin polymerization pathway in HIV-1 particle production, along with its effectors Wave2, Arp2/3 and the scaffold protein IRSp53, an I-BAR protein. A siRNA based approach showed the requirement of an intact Rac1/IRSp53/Wave2/Arp3 pathway for HIV-1 particle production in T cells. Furthermore, a double knockdown of IRSp53 and Wave2 resulted in cytoplasmic localization of HIV-1 Gag and a disruption of the cortical F actin network, as well as a strong decrease in particle production, indicating a role of these proteins in global Gag membrane binding. Gag membrane binding, mediated by its myristate hook and the matrix (MA) domain, relies primarily on the phospholipid PI(4,5)P₂ at the inner leaflet of the plasma membrane and Gag is known to cluster this lipid and create nanodomains for its assembly (Yandrapalli et al., 2016; Favard et al., 2019a; Sengupta et al., 2019). Since IRSp53, being an I-BAR protein, is involved in modulating local lipid composition of the membrane, while also acting as a bridge between Rac1 and Wave2, it is a prime candidate for the key regulator of HIV-1 Gag assembly.

In this study, we confirmed the involvement of IRSp53 in HIV-1 Gag particle production, and also established its isoform preference as well as specificity and non-redundancy as compared to other IRSp53-like I-BAR proteins, IRTKS and PinkBAR. IRSp53 has multiple isoforms as a result of alternate splicing, and these isoforms differ mainly in their C-termini, while retaining 100% sequence identity in the first 511 residues. Distinctive phosphorylation has been attributed to the different isoforms, as well as sensitivity to different upstream stimuli (Okamura-Oho, Miyashita and Yamada, 2001). In our study, we found that partial knockdown of the isoform with a molecular weight of 53kDa (IRSp53-S) (Section B1.1) affected Gag particle production, while a complete knockdown of the 58 kDa isoform (IRS-58) had no significant effect. An early study had reported that the 58 kDa isoform interacted preferentially with Cdc42 rather than Rac (Govind et al., 2001). This acquires significance in our study, as previous results had observed Rac1 activation downstream of HIV-1 Gag expression, indicating that Gag preference for this Rho GTPase drives isoform selectivity for IRSp53.

While Rho GTPase specificity may direct isoform selection, other proteins within the I-BAR family interact with known partners of IRSp53. IRSp53 shares a 42% sequence identity with IRTKS, and these proteins share the eponymous I-BAR domain as well as the C terminal
SH3 domain with IRSp53. IRTKS also interacts with IRSp53 related actin regulators such as Rac1, Eps8 and Shank and the Rho GTPase Rif (Millard, Dawson and Machesky, 2007; Aitio et al., 2010; Sudhaharan et al., 2016; Postema et al., 2018), and some redundancy of function has been shown in embryonic mice for IRSp53 and IRTKS (Chou et al., 2017), indicating potential overlap in their signal scaffolding functions. Despite their similarities, however, a complete siRNA mediated knockdown of IRTKS failed to significantly affect Gag particle production, whereas only a partial knockdown of IRSp53 led to a corresponding defect in particle production (Results, Part A, Figure 1b). This preference for IRSp53 could be partially due to the differential expression of these proteins; IRTKS is expressed mainly in the gastrointestinal tract, liver and kidneys, with negligible expression in blood cells, in contrast to IRSp53 which is enriched in the brain and is expressed to some extent in blood cells (Human Protein Atlas: BAIAP2/BAIAP2L1). Accordingly, IRSp53 KO mice show defects in brain development (Chou et al., 2017), whereas KO of IRTKS confers insulin resistance, with diabetic mice showing low levels of IRTKS expression (Huang et al., 2013). While, IRSp53 and IRTKS are both phosphorylated downstream of the insulin receptor by tyrosine kinases, IRTKS seems to be physiologically involved in insulin signaling. Thus, differential tissue expression could be a possible explanation of the preferential involvement of IRSp53 in a physiological context. In our system, however, the chosen cell type (HEK293T) expresses both proteins, making this an ideal system to verify the specificity and non-redundancy of IRSp53 at a cellular level.

Since all these proteins interact with Rho GTPases and their downstream effectors, notably Wave2, the specific involvement and non-redundancy of IRSp53 alone strongly suggests a direct preference for this protein by the assembling retroviral Gag protein. There could be several intrinsic factors at play in I-BAR protein selectivity for this process. In spite of high similarity in their membrane binding I-BAR domain, several key differences have been noted in their interactions with PI(4,5)P2 enriched membranes. While studies have clearly elucidated the significant differences between IRSp53-like and MIM-like I-BAR proteins, differences within the subgroups remain unclear. IRSp53 clustered PI(4,5)P2 more efficiently than the other I-BAR proteins, a difference especially pronounced at high protein concentrations. A slight difference in the average size of tubules generated by IRSp53 (43 nm) was noted as compared to IRTKS (40 nm) (Saarikangas et al., 2009). IRSp53 also induced membrane shape transition at 50% lower concentrations as compared to MIM-like proteins (Chen, Shi and Baumgart, 2015). IRTKS induced formation of short actin microspikes upon expression in COS-7 cells, as compared to the filopodia generated by IRSp53, while retaining the conserved Rac1 binding region in the IMD domain (Millard, Dawson and Machesky, 2007), indicating an effect dependent on the C terminal region as well. Having some differences in their membrane binding, and also in generation of membrane protrusions, the specificity of IRSp53 could be a combined effect of its IMD and the C terminal scaffold motifs. Finally, IRSp53 could very well interact directly with HIV-1
Gag. In our study, IRSp53 pulldown immunoprecipitated HIV-1 Gag in different buffer conditions (Figure 23, Page 117), strongly indicating close interactions with each other.

The third IRSp53-like protein, PinkBAR, is less extensively studied as compared to IRSp53 and IRTKS. However, characterization of this protein has revealed that it is responsible for generation of flat, planar membrane domains as opposed to negative curvature generation by the other I-BAR proteins. This attributed primarily to significant differences in the morphology of the PinkBAR I-BAR domain, which is smaller and flatter as compared to the IRSp53 I-BAR domain. The P(4,5)P$_2$ binding sites are also distributed along the membrane binding interface of the I-BAR domain with low phosphoinositide specificity (Pykäläinen et al., 2011). The IRSp53 I-BAR domain, on the other hand displays high specificity for PI(4,5)P$_2$ and has specific pockets of phospholipid binding residues, explaining its efficiency in PI(4,5)P$_2$ clustering and thus inducing membrane shape transition (Takemura et al., 2017). Taking into consideration the morphological differences in the I-BAR domain and the similarities in C terminal signaling domain, this protein was used in our study as a control for testing the possible role of membrane curvature in HIV-1 assembly.

Ectopic expression of I-BAR proteins along with HIV-1 Gag and subsequent membrane flotation experiments revealed the effects of high concentrations of I-BAR proteins on Gag membrane binding (Figure 26, Page 122). Expression of IRSp53 induced a slight decrease in Gag levels in the membrane fraction as compared to control, IRTKS and PinkBAR expression, on the other hand, also did not drastically affect Gag membrane binding (Lane). As PinkBAR has relatively low specificity for PI(4,5)P$_2$, and generates flat, stable membrane domains, Gag multimers could possibly be excluded from PinkBAR bound membrane domains.

However, HIV-1 Gag assembles and buds from the plasma membrane and is known to incorporate proteins present at the assembly site and which are involved in the assembly and budding process. Thus, evaluation of the presence of these I-BAR proteins in released Gag particles would be a test of their involvement and proximity to assembling Gag and budding particles at the plasma membrane. Accordingly, studies of particles tagged with Gag-mCherry showed that IRSp53 is associated with ~95-100% of Gag particles, while this association falls to 40-50% for IRTKS positive particles (Results, Part A, Figure 4) indicating spatiotemporal exclusion of these proteins at the Gag budding site. Since IRSp53 and IRTKS have several similarities in their I-BAR domain, this test confirmed that it is the specificity of IRSp53 and its related membrane curvature that could play a role in Gag assembly, and not just PI(4,5)P$_2$ clustering (also induced by IRTKS). While PI(4,5)P$_2$ binding weakly influenced particle association, as exhibited by the PI(4,5)P$_2$ binding PH-PLCd (Results, Part A, Figure 4), it is not the deciding factor for this association. This indicates that the preference for IRSp53, by Gag, while probably influenced to some extent by its lipid clustering activity, depends on other intrinsic factors as well (Figure 23, Page 117).
Interestingly, a seemingly paradoxical result was observed for the IRSp53 I-BAR domain alone. Ectopic expression of this I-BAR domain drastically reduced Gag membrane attachment (Figure 26, Page 122), while it showed high association with released Gag particles (Results, Part A, Figure 4b). This indicates that membrane binding of the I-BAR domain alone could displace Gag from the membrane, however, there is an intrinsic property which ensures its incorporation into released particles. Previous studies comparing the effect of ectopic expression of the full length IRSp53 protein and its I-BAR domain alone (Lim et al., 2008; Yang et al., 2009; Sudhaharan et al., 2019) noted that the I-BAR domain alone generates stable protrusions which are devoid of actin bundles, as compared to the full length protein. In addition, a greater proportion of the I-BAR domain alone remained associated to the membrane. These studies point to a distinct difference in plasma membrane binding dynamics and actin polymerization between the full length protein and the I-BAR domain. This could explain the drastic reduction in Gag membrane binding upon I-BAR expression in cells; the I-BAR domain remains associated to the membrane longer and more stably than the full length protein, this could prevent Gag association to the plasma membrane. In addition, cortical actin polymerization, which is directed by the full length IRSp53 protein, could play a vital role in maintaining Gag at the membrane by generating a meshwork of local actin in the vicinity of assembling Gag. However, a high proportion of I-BAR domain alone remains associated with Gag particles, indicating a similarity of functional involvement in viral bud formation. As membrane protrusions formed the I-BAR domain are more stable, the curvature induced by the I-BAR domain could be the deciding factor for their association with Gag particles.

Membrane curvature is an essential phenomenon for virus assembly. As discussed previously (Chapter 3), membrane curvature could be a function of lipid composition, membrane-protein interactions, cortical actin dynamics or a combination of these phenomena. In the case of HIV-1 Gag, it is known to cluster lipids and its polymerization has been proposed to drive the main assembly process and expression of HIV-1 Gag alone can produce particles which bud from the plasma membrane. The requisite membrane curvature has also thus been proposed to be driven exclusively by the Gag polyprotein, via its membrane binding and multimerization (Grover et al., 2013). This aspect of Gag-membrane interactions has been studied exhaustively in cells and on model membranes and has been shown to induce clustering of specific lipids dependent on Gag self-assembly (Dick et al., 2012; Keller, Kräusslich and Schwille, 2013; Barros et al., 2016; Yandrapalli et al., 2016; Favard et al., 2019a; Sengupta et al., 2019). Gag binding was shown to be dependent on its myristoylation, multimerization and on the presence of the phospholipid PI(4,5)P2, however, multimerized Gag was binding to liquid disordered domains rather than liquid ordered “raft like” domains (Keller, Kräusslich and Schwille, 2013). This indicates that membrane binding and creation of de novo lipid nanodomains by clustering is an intrinsic property of the Gag polyprotein. While this lipid clustering action has been amply demonstrated in cells and model membranes, its proposed membrane curving action has so
far proven elusive to prove in these systems. A minimal study system involving giant unilamellar vesicles with HIV-1 Gag and RNA showed membrane deformation by aggregates of labelled Gag, however the diffraction limited imaging of these vesicles prevented molecular analysis of these data (Gui et al., 2015). In addition, the Gag aggregates seen on the vesicular membranes, while inducing deformation, did not seem to have a characteristic size, introducing the possibility of membrane deformation by simple crowding of proteins on the membrane surface (Chapter 3, Section 3.2). HIV-1 particles produced from cellular membranes have a well described average size of about 100-150 nm (Garnier et al., 1998; Faivre-Moskalenko et al., 2014), even the virus-like particles produced from cells expressing the Gag polyprotein alone. This apparent homogeneity in HIV-1 particle size distribution, even though influenced by the viral RNA, is not reproduced in minimal systems, indicating that the controlled membrane curvature needed to produce particles of a narrow range of diameters is possibly not an intrinsic property of multimerizing HIV-1 Gag alone. Other studies involving reconstitution of the Gag assembly used cytosolic extracts along with membrane vesicles in a cell free system, thus proving to be inconclusive about the role played by Gag alone in viral assembly (Lingappa et al., 1997). Even in these reconstituted systems, Gag assembly proceeded by way of assembly intermediates rather than a continuous process dependent on multimerizing Gag. This was also evident in cells, where the Gag assembly process exhibited pauses during formation of HIV-1 particles (Ku et al., 2013). These pauses were interpreted as an indication of a single rate-limiting event needed to promote extended assembly of Gag. Coarsed grained simulations of Gag constructs on lipid membranes revealed that spontaneous curvature of the membrane promotes extended assembly of Gag; Gag assembly cannot pass a certain point in its assembly without pre-existing membrane curvature, notwithstanding the intrinsic curvature of the hexamer (Pak et al., 2017). Combining these data with HIV-1 assembly dynamics (Ivanchenko et al., 2009), this barrier seemingly is present at the very beginning of Gag assembly (>300 Gags). Thus, early in the assembly, Gag-Gag and Gag-membrane interactions are not sufficient to overcome the energy barrier i.e. the membrane tension, which is necessary for curving the membrane. Taken together, these data point towards the distinct possibility that Gag assembly may require another factor for initiating and controlling optimal membrane curvature at the plasma membrane.

In contrast to HIV-1 Gag, ordered assemblies of the I-BAR proteins form well described and consistent invaginations on model membranes, with a narrow range of characteristic diameters of the induced tubules and sorting curvatures (Saarikangas et al., 2009; Prévost et al., 2015), and also in cells (Breuer et al., 2019). This clearly indicates that inducing and stabilizing specific curvatures is an intrinsic property of the I-BAR proteins. As discussed previously (Section 3.5.3), the I-BAR domain of IRSp53 is more efficient at clustering lipids as well as inducing membrane shape transitions at low protein density. Taking into consideration our results and the association of IRSp53 full length and its I-BAR domain with cell free Gag particles (Results, Part A, Figure 4), IRSp53 could play a role in
membrane curvature in the process of HIV-1 assembly. Hence, it was necessary to pinpoint the localization of IRSp53 with respect to Gag assembly sites. As Gag assembly sites are not visible by diffraction limited microscopy, we turned to single molecule localization microscopy; we coupled mEos2 tagged Gag in PALM and immunolabelled IRSp53 in dSTORM, to resolve single molecule localizations of these two proteins with nanometer resolution.

There have been previous studies using PALM and STORM for analyzing localization for host cell factors with respect to HIV-1 Gag assembly sites (Prescher et al., 2015). In this study, endogenous components of the ESCRT assembly were immuno-labelled and their localization was studied in the context of Gag assembly sites. ESCRT proteins were revealed as clusters of an average size of 45-60 nm, smaller than the average size of Gag clusters (116 nm). It has been amply demonstrated that ESCRT proteins are recruited to Gag assembly sites in the later stages of HIV-1 assembly (Bleck et al., 2014; Johnson, Bleck and Simon, 2018), indicating that Gag clusters are already formed before ESCRT recruitment. Live cell studies (Jouvenet, Bieniasz and Simon, 2008; Ivanchenko et al., 2009; Floderer et al., 2018) have also demonstrated that Gag assembly (as a function of observed fluorescence intensity) reaches a plateau phase before the release (indicated by a rapid loss in fluorescence intensity). This shows that Gag incorporation into the bud is complete before ESCRT recruitment, and thus ESCRT proteins are recruited to assembled buds. Thus, only about 1.5-3.4% of Gag assembly sites colocalised with Gag assembly sites in the super resolution study by Prescher and colleagues, interpreted as a consequence of the transient nature of Gag-Tsg101 interactions.

In our images reconstructed from coupled TIRF-PALM/STORM studies on cells expressing Gag(i)mEos2 and immuno-labelled for IRSp53, actually reveal Gag assembly sites decorating protrusions marked with IRSp53 (Results, Part A, Figure 3a) as opposed to IRTKS (Results, Part A, Figure 3b). Thus, it seems that Gag assembly sites are preferentially present at sites of IRSp53 activity (membrane protrusions, cell periphery), rather than the inverse which is seen in the case of ESCRT proteins, where these proteins are recruited to Gag assembly sites. In this case we decided to use a different approach to analyse the proximity of IRSp53 (or IRTKS), to individual Gag assembly sites.

Single molecule coordinate based colocalization (CBC) studies (Malkusch et al., 2012), of Gag single molecules (obtained from PALM acquisitions) and IRSp53/IRTKS localizations (obtained from STORM acquisitions), showed that 88% of all IRSp53 localizations lie within a 100nm of Gag localizations, as compared to only 30% of IRTKS localizations. Notably, on comparison with simulated PALM/STORM data (Results, Part A, Figure 3 e, f), IRSp53 is revealed to be in close proximity to Gag assembly sites (<80nm), again in stark contrast to IRTKS (~300nm), showing that IRSp53 is selectively present at Gag assembly sites. This is in agreement with aforementioned results of IRSp53 specificity with respect to functional involvement, and association with released Gag particles.
Proximity of IRSp53 to Gag assembly sites also points to its role in membrane curvature, as these assembly sites are indeed sites of plasma membrane curvature. We also measured the sizes of the assembly sites reconstructed from the PALM acquisitions, and verified that the Gag clusters seen fall within acceptable ranges known for Gag particles (Results, Part A, Figure 3a,b). Having obtained a distribution with a mean of 100 nm, the visible clusters included assembling, assembled as well as released particles trapped between the cell membrane and the coverslip. Assuming that the smaller assembly sites would be predominantly sites of ongoing assembly, it would have been insightful to analyse the differential, if any, colocalization of IRSp53 with different cluster sizes, as this would have shed some light on the temporal aspect of local IRSp53 presence with respect to assembling Gag. However, we could not correlate different cluster sizes to the CBC values obtained from single molecular colocalization analyses, thus precluding any attempts at extracting potential temporal data from our images. In addition, we find about 27% of IRSp53 localisations highly correlated with Gag assembly sites, and an equal fraction anti-correlated with these sites (Results, Part A, Figure 3c). Considering these results, we can then deduce that IRSp53 is not constitutively present throughout the assembly process, and there seems to be a temporal aspect to its presence at Gag assembly sites. It could be, thus, present at some point in the assembly process (highly correlated) and then is gradually excluded from assembly sites (anti-correlated). Considering the intrinsic membrane curving nature of I-BAR proteins, and the topology of viral buds, we can theorize that IRSp53 could be involved in membrane curvature at a point early in the membrane curvature associated with viral assembly.

As various studies have studied membrane curvature and lipid clustering of I-BAR domain proteins on giant unilamellar vesicles (GUVs) enriched with PI(4,5)P₂, we turned to this cell-free minimal system to observe the interplay between Gag-IRSp53 I-BAR-lipid membranes. Strikingly, pre-incubation of the IRSp53 I-BAR domain with GUVs increased Gag binding to these membranes by up to 7 fold as compared to Gag alone, an effect which was not observed upon reversing the order of incubation, i.e. Gag before IRSp53 I-BAR (Results, Part A, Figure 6a). Thus membranes previously enriched in the IRSp53 I-BAR domain promote Gag binding to such membranes. We can infer a certain temporal order of membrane binding of these two proteins from these results, i.e. IRSp53 is present at the membrane before HIV-1 Gag assembly, or when assembly is still in the membrane curving stage. As discussed previously, IRSp53 is efficient at inducing PI(4,5)P₂ clusters, and this mechanism could be a part of its role in promoting Gag binding to lipid membranes. However, considering its intrinsic role in curvature, we then looked for Gag binding in the context of IRSp53 induced membrane invaginations. Using a lower density of the IRSp53 I-BAR domain as described before (Prévost et al., 2015), inward invaginations or tubules were induced on the GUVs. Upon observing and analyzing the location of Gag clusters with respect to these tubules, we found that Gag bound preferentially to the tips of these tubules, with a mean Gaussian curvature similar to viral buds, rather than the cylindrical
walls of the tubule or the relatively flatter GUV membrane (Results, Part A, Figure 6b,c). Thus IRSp53 induced membrane curvature plays a vital role in Gag binding to lipid membranes.

As all the I-BAR proteins can, however, cluster lipids and induce curvature, notably IRTKS which is closely related to IRSp53. Thus the specificity of IRSp53 could also be related to direct protein-protein interactions between HIV-1 Gag and IRSp53. Immuno-precipitation of IRSp53 in a lipid disrupting anionic buffer also precipitated HIV-1 Gag (Figure 23, Page 117), indicating that Gag-IRSp53 interactions could be possibly independent of lipids, and thus could be due to close proximity of these proteins. This is not without precedent for IRSp53, since it has been shown capable of forming hetero-dimers with its regulatory proteins (Kast and Dominguez, 2019). In addition, its role in inducing initial curvature before recruitment of other factors is also described; IRSp53 is recruited about 1.4 seconds before membrane binding of the actin regulatory factor VASP at the leading edge of cells (Disanza et al., 2013). Another elegant study showed that ezrin, a protein which links membrane proteins to actin filaments, interacts with IRSp53 in order to sense negative curvature at the plasma membrane, a function which it is not capable of performing by itself (Tsai et al., 2018). This has interesting parallels with Gag, which is also incapable of sensing membrane curvature, and thus could associate specifically with IRSp53 for optimal membrane curvature in the initial steps of the viral assembly.

As discussed previously, Gag assembly proceeds by way of assembly intermediates in hexamer units (Section 2.2.2, Pages 30-31). In the CG study undertaken by Pak et al, the curvature necessary for assembling Gag hexamers was optimally set at 15 nm, considering the topology acquired by multimerized Gag due to its CA-CA interactions. Interestingly, IRSp53 has been shown to sort maximally at curvatures of 18 nm, in model systems (Prévost et al., 2015) and cells (Breuer et al., 2019). Thus the ideal curvature for IRSp53 is close to the optimal curvature required by assembling Gag, furnishing an additional reason as to why assembling Gag could prefer bind IRSp53 enriched sites. IRSp53 intrinsically induces and stabilizes curvature by scaffolding, while clustering PI(4,5)P₂; this creates an ideal nucleating point for assembling Gag hexamers, which then continue extended assembly at this location by further multimerization. In absence of IRSp53, Gag hexamers, while still capable of clustering lipids, fail to overcome the membrane tension and would thus lead to aborted assembly.

Electron microscopy images of cells knocked down for IRSp53 display a striking phenotype of arrays of buds arrested at an early stage of assembly (Results, Part A, Figure 1c). Quantification of the dimensions of these buds revealed that buds from IRSp53 knocked down cells had a narrow range of height as compared to control cells which displayed a wide range of heights which corresponded to buds in different stages of assembly. (Results, Part A, Figure 1d). This clearly indicates that the absence of IRSp53 leads to a curvature defect at HIV-1 Gag assembly sites. Interestingly, the width distribution for both the
conditions was not significantly different, indicating that Gag self-assembly continues irrespective of the presence or absence of IRSp53. However, self-assembly of Gag by itself is not sufficient to drive the requisite membrane curvature to form a complete bud. This would substantiate the aforementioned hypothesis that Gag self assembly, the absence of IRSp53, fails to overcome the energy barrier needed to form a spherical bud and thus remains arrested at an early stage of assembly.

Interestingly, our cell-free GUV studies involving membrane tubulation by the IRSp53 I-BAR domain show that, in presence of HIV-1 Gag, the tubules induced by IRSp53 are shorter and HIV-1 Gag sorts to the tips of these tubules (Results, Part A, Figure S12). This suggests that while HIV-1 Gag is sorted to the tips due to optimal curvature, this sorting impedes the elongation of the I-BAR tubules. This provides an insight into I-BAR tubule formation, which has been proposed to proceed by way of helical oligomers on the walls of these tubes. This has previously been shown for F-BAR domain proteins (Frost et al., 2008), while the mechanism remains unproven for I-BAR proteins (Nepal, Sepehri and Lazaridis, 2020). However, the I-BAR domain of IRSp53 is known to bind inner leaflets of these tubules (Saarikangas et al., 2009) and it scaffolds these tubules and stabilizes them (Prévost et al., 2015) at non saturating densities. In the case of HIV-1 Gag assembly, then, the IRSp53 could then oligomerise at the viral bud and stabilize the nascent bud curvature, allowing Gag self-assembly to pass the energy barrier required to bend the membrane. The assembled Gag on the curved membrane also blocks IRSp53 from oligomerising further, restricting its relative abundance at the assembly site.

Taking into consideration these factors, we can then hypothesize that HIV-1 Gag, globally, prefers to assemble at sites of high IRSp53 activity in cells, but as the assembly process continues, IRSp53 could be gradually excluded from the budding sites. Indeed, preliminary results from live cell TIRF microscopy with fluorescent Gag and IRSp53 (Figure 26, Page 124) shows that IRSp53 fluorescence intensity peaks at sites of assembling Gag early in the assembly, and then experiences a sharp drop before Gag intensity reaches its peak, i.e. before the end of assembly, indicating that a majority of IRSp53 is excluded from assembly sites before budding. However, we still find high correlation of exogenous IRSp53 with released viral particles (Results, Part A, Figure 4b), as discussed before, as well as association of endogenous IRSp53 with purified infectious particles as well as Gag VLPs (Results, Part A, Figure 5b,c). On comparing with endogenous Tsg101, which interacts with HIV-1 Gag for the penultimate steps involved in virus budding, we find that Tsg101 shows higher association with these particles. IRSp53, while present, does not exhibit a high amount associated with these purified particles, lending further credence to the hypothesis that it is possibly involved in the early steps of viral assembly. Its continued association with viral particle indicates some persistence at assembly sites throughout the process.

Electron microscopy analyses of released viral particles show that multimerized Gag is not present as a single contiguous layer covering the surface area of the released particle.
In fact, the Gag lattice covers only up to ~40% of the particle surface area, leaving a substantial area seemingly free, and thus available for possibly binding other proteins. A study examining the lipid composition of infectious HIV-1 as compared to producer membranes found significant enrichment of PI(4,5)P\(_2\) in viral membranes (3.4 fold increase) as compared to the producer cell membranes. While this is not unexpected considering the vital interactions of the viral Gag protein with this lipid, the study also noted a 3 fold molar excess of PI(4,5)P\(_2\) in the viral particle as compared to the average amount of HIV-1 Gag (Muksch et al 2019 Sci Rep). Considering that only 40% of the viral membrane is covered by Gag, the three-fold molar excess of this lipid could be bound by other proteins. As IRSp53 induces strong PI(4,5)P\(_2\) clustering, some IRSp53 thus could remain bound to free areas on the viral bud, and thus is released with the particle. Seeing its high correlation with released particles in our study, it is even possible that IRSp53 could be incorporated in low amounts in each particle potentially to stabilize the Gag-free areas of the viral bud.

Figure 32: Proposed scheme showing IRSp53 membrane clusters favouring Gag membrane binding and assembly. 1) IRSp53 and Gag have a stable equilibrium of binding/unbinding the cell plasma membrane and their turnover from the membrane to the cytoplasm. 2) IRSp53 is naturally induces some membrane curvature locally due to its multivalent assemblies, while polymerization of Gag as well induces local curvature, both inducing PIP\(_2\) clustering. 3) Gag preferentially initiates self assembly at sites curved by IRSp53, and this curvature, stabilized and promoted by IRSp53, is subverted by Gag to pass the energy barrier to bend the membrane. In the absence IRSp53, this barrier is not overcome and Gag remains self-assembled but blocked at the membrane curving stage. 4) Self-assembly of Gag continues the curvature of membrane generated by viral particle formation, with adjacent IRSp53 gradually being excluded from the budding site. 6) The viral particle buds out, with some IRSp53 molecules still trapped within the bud, while the majority resumes their equilibrium at the cell plasma membrane.
Other studies have also noted the role of BAR superfamily proteins in the HIV-1 particle production, confirming the intricacies of membrane curvature involved in this process. BAR domain proteins, as discussed before (Section 3.4), are involved in inducing positive Gaussian curvature, in the case of cellular membranes, towards the interior of the cells. A 2015 study showed that a cellular BAR domain protein, angiomotin, is involved in HIV-1 assembly and budding (Mercenne et al., 2015). Interestingly, in the absence of angiomotin, viral budding was arrested at an earlier stage before the budding, with viral buds having a larger arc than those seen in cells depleted for Tsg101. The viral bud, indeed, displays a combination of negative and positive Gaussian curvatures; negative curvature being at the tip of the bud, and positive curvature being at the neck of the bud. Angiomotin, with its BAR domain, could thus sense and induce positive curvature at the neck of the bud, promoting constriction of the neck and eventual scission by membrane fusion at the neck. Angiomotins are also negative regulators of Rac1 activation (Yi et al., 2011; Moleirinho, Guerrant and Kissil, 2014), potentially dissociating its role in assembly from our proposed model of curvature initiation by IRSp53 and our results tying Rac1 to this process. Another BAR domain protein, PACSIN2, was shown to be involved again in the late steps of viral assembly for cell-to-cell transmission of HIV-1, in a p6 dependent manner (Popov et al., 2008), as opposed to our results showing the p6 independence of IRSp53-Gag interactions. This reinforces the notion that positive curvature induced by these proteins could indeed play an important role in the late steps and budding of the virus. In addition, both of these studies implicated the ubiquitin ligase NEDD4L as an important factor in the interactions of Gag with these BAR domain proteins. Overexpression of this ligase has been known to rescue the well described budding defect in mutants of the Gag p6 domain (Usami et al., 2008), showing that these proteins could very well aid in constricting the bud neck for budding, even in the absence of ESCRT recruitment. Taken together with our results, these conclusions represent a significant paradigm shift regarding the overall role of HIV-1 Gag in optimal viral assembly. Gag, though being necessary as a structural component of the virus, is in fact dependent on several cellular factors for generating different types of curvature required for correct virus assembly and budding.

Involvement of the membrane manipulating IRSp53 and other BAR superfamily proteins also directly implicates the cortical actin cytoskeleton at HIV-1 assembly and budding sites. Previous studies from our lab (Thomas et al., 2015) have already established the role of a Rac1 induced actin signaling pathway in HIV-1 cell free particle production. In this study, a combined disruption of IRSp53 and its effector Wave2 has a drastic effect on the cortical actin phenotype, accompanied by delocalization of Gag from the plasma membrane. This is mirrored in over expression of the IRSp53-IBAR domain, incapable of scaffolding actin signaling proteins, which seemingly reduces Gag binding to cellular membranes (Figure 26, page 122). Cryo-electron tomography of HIV-1 buds revealed the presence of actin filaments in proximity to budding virus (Carlson et al., 2010). Live cell imaging analyses also showed HIV buds on filipodia-like structures in dendritic cells.
(Aggarwal et al., 2012), and a recent study implicated the Cdc42 mediated filopodial extensions to be instrumental in cell-cell spread of HIV-1 (Aggarwal et al., 2019). IRSp53 is the only I-BAR protein which can interact with both Rac1 and Cdc42 (Section 3.5), providing another possible explanation of its selective preference by Gag for the HIV-1 assembly process. Indeed, our super resolution images show Gag clusters decorating IRSp53 marked filopodia-like protrusions. Similar studies undertaken with cells expressing Gag tagged with mEos2, and marked for filamentous actin show a very high degree of correlation of Gag localizations with actin (Figure 30, Page 130), with Gag clusters marking areas of actin enrichment. In addition, ~75% of all Gag clusters overlapped with actin nanostructures. Thus, in our system, actin enrichment correlates with Gag clusters, most probably in an IRSp53 dependent manner.

The involvement of actin polymerization for cell-free virus assembly and release in the case of HIV has had conflicting reports, with high resolution microscopy consistently finding actin filaments in proximity to budding sites (Gladnikoff et al 2009 Biophys J, Carlson et al 2010 Plos Pathog), in agreement with our single molecule localization data. However, disruption of actin network by cytochalasin D in different studies proved inconclusive (Sasaki et al., 1995; Audoly, Popoff and Gluschankof, 2005; Jolly, Mitar and Sattentau, 2007; Graziano et al., 2011). Live cell imaging also appeared to show the apparent dispensability of actin in the kinetics of HIV-1 assembly (Rahman et al., 2014). HIV-1 Nef is also involved in modulating actin regulatory proteins (Lu et al., 1996, 2008), indicating that subverting the actin cytoskeleton at the plasma membrane by HIV-1 is a vital part of the infectious viral replication process. This raises pertinent questions about the actual role of actin polymerization in the context of viral particle production at the plasma membrane.

The actin cytoskeleton in the host CD4 T cells, the primary targets of HIV, is tightly regulated and performs a multitude of functions related to immune activation, maintaining membrane tension while being subjected to shear force and cell shape changes, especially during migration (Kumari et al., 2014; Rossy, Laufer and Legler, 2018). Thus its involvement in HIV-1 particle production which takes place at the plasma membrane of these cells, for both cell free and cell-associated particles, seems a very likely proposition. Local branched actin networks not only sustain membrane shape and micrometer scale membrane protrusions, but are also involved in regulating local lipid and protein movement (Section 3.3). Actin networks underneath the membrane have been shown to restrict diffusion of phospholipids, presumably compartmentalization of the plasma membrane, by single particle tracking (Fujiwara et al 2002 JCB, Clausen et al 2013 Nano Letters), and more recently by minimally invasive STED-FCS (Andrade et al., 2015). A similar mechanism has also been proposed and demonstrated for cell surface and membrane proteins (Edidin et al 1991 Science, Andrews et al 2008 Nat Cell Biol, Jaqaman et al 2011 Cell, Gowardshankar et al 2012 Cell). In fact, the “picket-fence” model proposes that cytoskeleton mediated restriction of membrane proteins is instrumental in creating membrane compartments, which is then largely responsible for creating the lipid microdomains, vital for several
processes at the membrane (reviewed in Trimble and Grinstein 2015 JCB). The trapping of lipids and proteins in such compartments has been hypothesized to create positive feedback loops for cytoskeletal signaling itself (Weisswange et al 2005 JCS). Phospholipids play an important role in cortical actin signaling, serving to recruit proteins and their effectors (see Section 3.1, Page 38). Trapping of these molecules thus creates optimal conditions for dynamic cytoskeletal reorganization at the plasma membrane. This could very well be a mechanism by which actin could influence viral assembly.

Actin polymerization has been implicated in the assembly and release of other enveloped viruses at the plasma membrane (Taylor, Koyuncu and Enquist, 2011; Spear and Wu, 2014). An interesting mechanism was proposed for assembly and budding of the influenza virus by Simpson-Holley and colleagues (Simpson-Holley et al., 2002). Influenza virions have two distinct morphologies; a spherical virion measuring about a 100nm in diameter, and large filamentous particles reaching several micrometres in length but of a similar diameter. In this study, disruption of the cortical actin cytoskeleton by the actin depolymerization inhibitor, jasplakinoside, specifically inhibited production of filamentous influenza virions, while not affecting production of spherical virions. Disruption of the actin network also disrupted lipid domains. They hence proposed a mechanism by which the actin cytoskeleton indeed forms lipid microdomains, in the absence of which there is insufficient material to form the larger filamentous virions, but still sufficient for production of the smaller spherical virions. This is borne out by another study implicating phosphorylation and inactivation of the actin depolymerizing factor ADF/cofilin in the infection by influenza virus (Liu et al., 2014). Cofilins are also involved in HIV-1 particle formation (Wen et al., 2014), downstream of the Rho GTPase ROCK and its effector LIM kinase.

Thus actin polymerization in the vicinity of the HIV bud could serve two purposes; the network could restrict the diffusion of phospholipids and serve as an additional inducer of phospholipid clustering apart from Gag itself and IRSp53, and furthermore could induce local clustering of Gag itself. By increasing local concentration of Gag with optimal membrane conditions, the system will thus tend to increase the probability of formation of viral particles. In the absence of these conditions, viral particle production will not be inhibited completely, and the dynamics of particle assembly will not change, however the efficiency of particle production could be affected. Indeed, electron microscopy studies of budding virions (Figure 25, Page 120) shows several buds in the close vicinity of each other, suggesting that optimal conditions for particle formation exist locally and thus promote formation of viral particles. Additional studies are required to confirm this hypothesis. A previous study from our lab measured the diffusion kinetics of different lipids at individual HIV assembly sites in CD4 T cells by STED-FCS (Favard et al., 2019a). In addition, sptPALM studies in these cells have also established kinetics for Gag itself (Floderer et al., 2018). The effect of disruption of cortical actin on the kinetics of lipid and Gag diffusion, by small molecule inhibitors of co-factors such as Arp2/3, or by depleting cells of these co-factors, could be then studied.
A previous study by Rahman and colleagues demonstrated that F-actin filaments appeared to be dispensable for HIV-1 assembly in HeLa by using alternatively Latrunculin B for actin depolymerisation and Jasplakinolide for actin treadmilling. Studies characterising actin binding kinetics of jasplakinolide have noted that saturation of actin binding sites by this molecule take 4h-24h at low concentrations, significantly longer time scales than the incubation times in the study. This leaves room for unbound actin filaments to continue dynamics at the nanoscale, which were inaccessible by the diffraction limit imposed by the TIRF microscopy used in this study. Floderer et al used single particle tracking PALM (sptPALM) to access localization density, diffusion and trapping energy of each single molecule in the vicinity of assembly sites. Disruption of actin dynamics and its effects on each assembling bud, if any, would be significantly more apparent using this super resolution technique. In addition to nanoscale dynamics, the cluster sizes obtained from the reconstructed images would be instrumental in determining the morphology of the assembling particle. Since this study analysed assembly kinetics only via intensity measurements of fluorescently tagged HIV-1 Gag at the cell plasma membrane, information regarding the final morphology attained by the assembling particle is missing. In the case of cells lacking IRSp53, electron microscopy images of cells expressing HIV-1 Gag exhibit buds arrested at an early stage of budding (Results, Part A, Figure 1c). Measurement of the dimensions of these arrested buds show significantly no difference in width as compared to particles budding from control treated cells (Results, Part A, Figure 1d,.). This indicates that IRSp53 deficiency and lack of its curvature inducing activity does not affect Gag-Gag self-assembly at the budding site. In terms of the number of Gag molecules present in each arrested bud, there may not be a significant difference as compared to control cells, however absence of IRSp53 causes a morphological defect which is visible, in this case, by electron microscopy. Similarly, the measurements of fluorescence intensity used in the 2014 study correlate only to the number of Gag molecules accumulating at each assembly site and does not reveal morphological features of these assembled particles. As they also note in the study, release of these particles was not examined, a factor linked to correct assembly and curvature of each bud. Thus the proper conclusion of the study would be that disruption of actin does not affect Gag self-assembly at the plasma membrane in terms of number of Gag molecules, however the conclusion that actin is dispensable for HIV-1 assembly, taken here to mean correct assembly leading to release, could possibly be erroneous. In addition, the study used fluorescently tagged LifeAct, an actin binding peptide, to monitor changes in actin dynamics vis-à-vis HIV-1 Gag assembly sites. Recent studies have demonstrated that binding of this peptide to cellular F actin alters actin organisation as well as cellular morphology and biophysical behaviour in the cell (Flores et al., 2019). Expression of Lifeact-GFP in the cells caused a dose dependent increase in cell spread area, total F actin amount as well as cellular viscosity, raising questions on the efficacy of this peptide as an actin probe for the rapid measurements needed for HIV-1 assembly sites. Another recent study showed that Lifeact binding sites on actin monomers overlap with sites bound by cofillin and myosin (Belyy et al., 2020). Cofilin plays a vital role in actin turnover while myosin
is an essential factor in regulating cytoskeletal forces and membrane tension. In addition, both of these factors have been shown to be involved in HIV-1 replication and release (Wen et al., 2014).

Taken together, these observations raise questions about the conclusions of this particular study, leaving the role of F-actin in HIV assembly and release a still open question.

The cortical actin network forms micrometre scale protrusions and membrane domains such as lamellipodia, membrane ruffles and filopodia in response to extracellular stimuli. Electron microscopy images of budding HIV-1 virions show groups of viruses budding at the membrane (Figure 25, Page 120), instead of individual viruses at isolated spots; this indicates the preference of assembly and budding at certain plasma membrane regions of the cell. Indeed in host CD4+ T cells, HIV has been shown to localize to structures known as uropods situated at the trailing edge of these cells ((Llewellyn et al., 2010, 2013; Ladinsky et al., 2019). Our PALM/STORM results, too, reveal HIV-1 Gag assembly sites decorating structures marked for actin (Figure 30, Page 130). This shows that HIV-1 may subvert existing global F-actin dynamics to aid in its assembly and release, leading also to nanoscale recruitment of F-actin at each viral bud. Our previous results (Thomas et al., 2015), show a global increase in Rac1 activation upon expression of HIV-1 Gag. This global activation in F-actin could indeed serve to augment cortical actin dynamics for an overall increase in the probability of successful assembly and release.

As the acto-myosin network is also a major factor in induction of force at the membrane, we cannot discount the role of actin in inducing local membrane curvature, or in budding, as actin regulatory BAR family proteins seem to be involved in both these steps of viral assembly and release. Three-dimensional single molecule localization studies (3D STORM) (Huang et al., 2008; Xu, Babcock and Zhuang, 2012) would provide insights into three-dimensional organization of actin near the viral bud.

Actin signaling at the plasma membrane takes places downstream of a variety of extracellular stimuli. As previous results from our lab implicate the Rac1 signaling pathway in the process of HIV-1 Gag particle release, we wanted to establish the possible RacGEFs involved in this process. Tiam1 (T-lymphoma invasion and metastasis inducing protein) is a very likely candidate for activating Rac1 in the context of HIV-1 release. First identified in T-lymphoma cells as a factor affecting invasiveness (Habets et al., 1994) and as an upstream activator of Rac1, its activity in different tumor derived cell lines was established (Habets et al., 1994; Michiels et al., 1995). Membrane localization of Tiam1, via its pleckstrin homology (PH) domain is needed for its activation of Rac1 to induce membrane ruffling (Michiels et al., 1997; Stam et al., 1997). Extracellular stimuli such as platelet derived growth factor (PGDF) were shown to have a role in this membrane translocation of Tiam1 to the plasma membrane (Buchanan et al., 2000). Another pathway involved in growth factor signaling, the PI(3,4,5)P3 synthesizing PI3K pathway, was also shown to be involved in Rac1 activation
via Tiam1, implicating the membrane phospholipids in this process (FLEMING, GRAY and DOWNES, 2000; Snyder et al., 2003; FLEMING et al., 2004).

Phospholipids are heavily implicated in plasma membrane signalling as well as in HIV-1 assembly. It is possible that creation of membrane micro domains during HIV-1 replication could create a positive feedback loop for cortical actin signalling at the plasma membrane (Page 125). Membrane proteins such as the tetraspanins are implicated in Rho GTPase manipulation by G proteins coupled receptor association, and these proteins are also found enriched in HIV-1 virions, as well as in our studies (Delaguillaumie et al., 2002; Hong et al., 2012; Termini and Gillette, 2017)(Results, Part A, Figure 5c). The clustering of these proteins could create membrane domains and activate GTPase signalling leading to the cascade ending in cortical actin manipulation which in turn could also restrict diffusion of certain lipids and proteins. This could create favorable conditions for HIV-1 assembly on a cellular scale. It remains to be seen how intercellular expression of Gag is capable of stimulating plasma membrane signalling. As this pathway is involved in cell migration, proliferation and notably dysregulated in several cancers, it will be interesting to focus future studies on this aspect of membrane domains and viral assembly.
Conclusion and Perspectives

We conducted this study with the aim of defining the functional role of the I-BAR protein IRSp53 and its cofactors in HIV-1 assembly and particle formation. We report the novel and essential role of IRSp53 in HIV-1 Gag induced membrane curvature and progression of the viral assembly. We show a membrane curvature defect in viral buds at the plasma membrane of cells knocked down for IRSp53. Our results also demonstrate the specific association of IRSp53 with viral particles, and we show the nanoscale localization of IRSp53 at HIV-1 assembly sites. Preliminary results also point towards an early presence of IRSp53 at sites of assembling Gag, emphasizing its role in membrane curvature. Cell-free studies reveal IRSp53 induced curvature and possible lipid clustering as a major factor in HIV-1 Gag membrane binding. In addition, these results point to a possible mechanistic explanation of IRSp53 oligomerization at the HIV-1 bud. Further results illustrate the membrane binding and multimerization dependent complexing of HIV-1 Gag and IRSp53. Over expression of the full length IRSp53 does not affect HIV-1 Gag cell membrane binding, while the I-BAR domain alone perturbs HIV-1 Gag binding to cellular membrane. Thus C terminal dependent regulation of IRSp53 is implicit in maintaining IRSp53 at the plasma membrane, and its presence at HIV-1 assembly sites is highly regulated.

Since we propose membrane curvature as a mechanism of IRSp53 involvement in HIV-1 assembly, and it is the I-BAR domain involved primarily in curvature, we can use tail-switch mutants of IRSp53 and IRTKS to ascertain the role of the C terminal domain in the specificity of this process. It will also be interesting to study IRSp53 recruitment in the context of the BAR proteins reported to be involved in HIV budding, to have a complete picture of membrane curvature at the budding site.

We also see a high correlation of F-actin with HIV-1 Gag assembly sites, thus raising questions about the role of F-actin in this process. As previous studies have touched upon lipid diffusion and HIV-1 Gag kinetics at assembly sites by sptPALM, it would be interesting to use small molecule inhibitors of different cofactors of Rac1 pathway to test the effect of perturbing this pathway on kinetics of assembly in live cells. 3D STORM studies could be undertaken at individual assembly sites, in these conditions to examine the nanoscale structures of F-actin at the viral bud, and concomitantly study the effect of the small molecules inhibitors of actin polymerization on these structures, if any.

On a cellular level, cortical actin forms different protrusions such as lamellipodia and filopodia at the cell surface, and HIV-1 has been reported to be present on these structures. These structures are highly dynamic and involve different actin structures, for example, lamellipodial actin consists of short branched actin networks below the plasma membrane, while filopodial actin is primarily composed of parallel actin bundles. The polymerisation and depolymerisation equilibrium of cortical actin combined with branching cofactors like Arp2/3 complex drive the formation of these structures. As the virus subverts this pathway
for its particle production, it will be interesting to look at changes induced by HIV localisation to these structures. In addition, the dynamics of these structures should be studied in live cells with respect to HIV assembly and release. Previous studies have shown viruses on actin enriched structures in fixed cells but live cell studies of cellular protrusions and virus budding are lacking. Finally, actin exists as a part of the larger actomyosin complex for membrane tension and generation of contractile forces, making it imperative to study the role of myosin, if any, in regulating HIV-1 particle production, especially in primary T lymphocytes which display a myosin dependent polarized phenotype.

We also established a role for activated Rac1 downstream of HIV-1 Gag in global IRSp53 membrane binding, while possibly identifying Tiam1 as the RacGEF involved in HIV-1 Gag article production. Further studies are required to identify the RacGEF involved in this process with a greater degree of confidence. Tiam1 (and other GEFs) is activated downstream of the PI3K pathway involving generation of PIP3. It is necessary to examine the activation of this pathway downstream of HIV-1 Gag expression, to ascertain whether it is this stimulus which leads to downstream activation of the Rac1 pathway. Using Gag mutants deficient in membrane binding, we can also verify the requirement of Gag membrane binding in this process.

Tiam1 is dysregulated in several cancers and is also considered as a marker of tumour cells. As such, there are efforts to target the PI3K pathway in cancer cells, and knockdown of Tiam1 has been shown to reduce invasiveness of tumour cells. If this pathway is indeed activated in the case of HIV-1 assembly, we can explore this as a therapeutic supplement to currently used antiretroviral therapy which targets only viral factors. This will help counter development of viral resistance to current therapies and open new possibilities of combating this global pandemic.
MATERIALS AND METHODS
Antibodies.

Plasmids.
The plasmid expressing HIV-1 codon optimized Gag alone (pCMVGag [named pGag]), the plasmid expressing Pol and Env-deleted HIV-1 (named pNL4.3ΔPolΔEnv) encoding Gag alone with its packageable viral RNA(Chen et al., 2014) and the plasmid expressing full wild-type HIV-1 (named pNL4.3) were described previously(Favard et al., 2019b). Plasmids IRSp53-GFP, IRTKS-GFP, PinkBAR-GFP and IRSp53-I-BAR-GFP were obtained from University of Helsinki (Finland)(Saarikangas et al., 2009). Plasmids expressing PH-PLCδ-GFP was a gift of B.Beaumelle (IRIM), Gag(i)mCherry (named Gag-mCherry), Gag tagged with internal photo-activable mEos2 (named Gag(i)mEos2), p6-deleted Gag tagged with mEos2 (named pGagΔp6-mEos2) were described in(Floderer et al., 2018).

siRNA.
Stealth siRNA (Invitrogen) targeting IRSp53 (BAIAP2) and IRTKS (BAIAP2L2), and Smartpools (Dharmacon) targeting IRSp53 (BAIAP2) or random sequence for siRNA controls were used in this study.

Cell culture and transfection.
A human embryonic kidney cell line (HEK-293T) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) and Jurkat T-lymphocytes were maintained in RPMI (Gibco). Media were supplemented with 10% fetal bovine serum (FBS, Dominique Dutscher)), complemented with sodium pyruvate and antibiotics (penicillin-streptomycin) at 37°C with 5% CO₂ atmosphere. Based on different plasmids conditions, cells were transfected as follows (2*10⁶ cells/transfection): PLASMID, 8µg; the amount of transfected plasmid was normalized by adding pcDNA3.1 empty plasmid. The cell medium was replaced 6 hours post-transfection and experiments were performed 24-48h post transfection. SiRNA transfections in HEK293T cells were performed with either Cacl₂/HBS, RNAiMax (Invitrogen) or with JetPRIME (Polyplus) or by electroporation for Jurkat T cells. One day prior to transfection, 2x10⁵ cells/well were seeded in 2mL of growth medium without antibiotics, in a 6-well plate. Transfection was performed using the manufacturer’s protocol. 24 hours after siRNA transfection, the cells/well were again transfected using CaCl₂/HBS. These cells were incubated at 37°C with 5% CO₂ atmosphere, for 24/48 hours.
**Immunoprecipitation assay.**

HEK-293T cells were transfected following the calcium-phosphate technique. Based on different conditions plasmids were transfected as follows (2*10^6 cells/transfection): Gag/pNLdPoldEnv (8ug each) and the amount of transfected plasmid was normalized by adding pcdna3.1 empty plasmid. The cell medium was replaced 6 hours post-transfection. 24 hours post-transfection, the cells were washed with cold 1X-PBS prior to collection with 800µL of chilled lysis buffer (50mM TRIS-HCl [pH=7.4]; 150mM NaCl; 1mM EDTA; 1mM CaCl_2; 1mM MgCl_2; 1% Triton, 0.5% sodium deoxycholate; OR 1% CHAPSO; protease inhibitor cocktail [Roche] 1 tablet/10mL lysis buffer). The cells were incubated on ice for 30 minutes and then centrifuged at 13,000 rpm/ 15minutes/ 4°C. The supernatant was collected in a new tube and the pellet was discarded. For each condition, 1000µg of protein (the collected supernatant) was incubated with 1µg of anti-IRSp53 antibody for overnight on a tube rotator at 4°C. 25µL of beads (Dynabeads Protein A, Life Technologies) was added to each tube of protein-antibody complex and incubated for 2 hours on the tube rotator at 4°C. The samples were then washed 5 times with the lysis buffer, followed by addition of 20µL 2X Laemmli’s buffer to the beads. The samples were denatured at 95°C for 10 minutes and then processed for Western blot.

**Western blot and analysis.**

50µg of each protein (intracellular) samples or 20µL of purified VLP samples, added with SDS loading dye, were resolved on a 10% SDS-PAGE gel. The gels were then transferred on to PVDF membranes. Immunoblotting was performed by incubating the membranes overnight with primary antibody at 4°C, and 2 hours with HRP conjugated secondary antibody at room temperature. The blot signals were detected using ECL Prime/ECL Select substrate (Amersham) and images were taken using ChemiDoc (BioRad).

**VLP purification and quantification.**

24 or 48 hours post-transfection, culture supernatants containing Gag-VLPs were collected, filtered through a 0.45µM filter, and centrifuged first at 2000rpm/5mins/4°C and then at 5000rpm/5mins/4°C. The supernatant was then purified by loading it on a cushion of 25% sucrose (in TNE buffer) and ultracentrifuged at 100000g for 100 minutes (SW41Ti, Beckman Coulter) at 4°C. The pellets were resuspended in TNE buffer at 4°C overnight. Gag-VLP release was estimated by performing anti-CAp24 immunoblot and by quantifying Gag signal in the blots using ImageJ software as described in . The calculation for Gag-VLP release is: % of Gag in VLP = Gag_{released} / (Gag_{released} + Gag_{intracellular normalized to GAPDH}).
Membrane flotation assay.

For each condition, 4x10^6 cells were transfected and viral supernatants harvested 48h post-transfection, as described above. Cells were washed with ice-cold PBS and resuspended in Tris-HCl containing 4mM EDTA and 1X Complete protease inhibitor cocktail (Roche). Every step was then performed at 4°C. Cell suspensions were lysed using a dounce homogenizer, then centrifuged at 600g for 3min to obtain Post-Nuclear Supernatants (PNS). A cushion of 820μL of 75% (wt/vol) sucrose in TNE buffer (25mM Tris-HCl, 4mM EDTA, 150mM NaCl) was loaded at the bottom of an ultracentrifuge tube and mixed with 180 μL of PNS adjusted to 150mM NaCl. Two milliliters and 300 μL of 50% (wt/ml) sucrose cushion followed by 0.9 mL of 10% (wt/ml) sucrose cushion were then layered to obtain the gradient that was then centrifuged in a Beckmann SW60Ti rotor at 35 000rpm, 4°C, overnight. Eight 500μL fractions were collected from the top to the bottom of the centrifuge tube and analyzed by western blotting.

Immuno-electron microscopy:

For immuno-EM, cells were fixed in 4% formaldehyde and 0.05% glutaraldehyde in PHEM buffer for 1 hour at 4 °C, then quenched with 0.05M NH4Cl for 30 minutes. After successive dehydration bathes, cells were infiltrated with mixes of resin (Lowicryl K4M) and ethanol with increasing concentrations of resin and then embedded and polymerised by UV at -20°C. Ultrathin sections were cut with a PTXL ultramicrotome (RMC, France) and collected on formvar/carbon-coated nickel grids. After blocking 20 minutes in Tris buffer (TB; 20mM Tris-HCl pH 8.2, 1% BSA, 1% goat serum), immunogold labelling was performed by co-incubating sections overnight at 4 °C with a rabbit anti-IRSP3 antibody diluted 1/50 in TBS and a mouse anti-capsid diluted 1/50 and one hour at room temperature with 20nm gold labelled goat anti-rabbit and 10nm gold labelled goat anti-mouse diluted 1/20 in TBS supplemented with 1% teleostean gelatine (Sigma). Finally, the grids were stained 20 minutes with 2% uranylacetate. Observations were made on a Tecnai G2 F20 (200 kV, FEG) TEM at the platform Plateau de microscopie electronique COMET, INM, Platform Montpellier RIO Imaging.

Single Molecule Localisation Microscopy:

While techniques like TIRF greatly improved access to events taking place at the plasma membrane of cells, the diffraction limit of conventional fluorescence microscopes still impeded access to nanoscale information contained in these events. HIV assembly sites and buds visualised by electron microscopy were revealed to be around 100-150 nm in diameter, well below the diffraction limit of fluorescence microscopes (~250-350 nm lateral resolution). A breakthrough study in 2006 (Betzig et al., 2006) found a way to circumvent this diffraction limit by using stochastic activation of fluorophores to pinpoint localisation of single fluorescent molecules by spatio-temporally separating their activation. This approach, now known as photo activated localisation microscopy (PALM), used photo activable
fluorescent proteins (PAFPs). A successive study, using the same basic principle of stochastic activation of fluorophores, demonstrated single molecule localisation using small molecules cyanine dyes for photoswitching and stochastic activation (Rust, Bates and Zhuang, 2006).

**Sample preparation for super resolution PALM/STORM microscopy.**

HEK293T cells expressing HIV-1 Gag/Gag(i)mEos2 cultured on poly-l-lysine (Sigma) coated 25 mm round #1.5 coverslips (VWR) were fixed using 4%PFA + 4% sucrose in PBS for 15 min at RT. Samples were subsequently quenched in 50mM NH₄Cl for 5 min. Samples were then washed in dPBS and then blocked for 15 min in room temperature using 1%BSA in PBS and subsequently in 0.05% Saponin in 1%BSA in PBS. Samples were stained using a 1:100 dilution of the primary antibodies (rabbit polyclonal anti-human IRSp53, Sigma and rabbit polyclonal anti-human IRTKS antibody, Bethyl) for 60 min in room temperature. Samples were washed 3x5 min using 1% BSA in PBS followed by 60 min staining using a 1:2000 dilution of the anti rabbit Atto647N antibody (Sigma). Samples were washed 3x5 min with PBS and stored in light protected container in +4°C until imaged. Samples were mounted on a StarFrost slide with a silicon joint with the STORM buffer (Abbelight). Cells were imaged within 60 minutes after application of buffer.

**PALM/STORM Imaging.**

Single-molecule localization microscopy was performed on a Nikon inverted microscope equipped with 405-, 488-, 561- and 642-nm lasers, an EMCCD Evolve 512 Photometrics camera (512*512, 16µm pixel size) with an oil immersion objective 100X NA1.49 Plan Apochromat. PALM imaging of Gag mEos2, activation was performed with lasers irradiance set to 0.3 kW/cm² for 405 nm conversation and ~ 2.2 kW/cm² for 561 nm excitation. Illumination was performed over a 25x25 μm area in the sample (1/e² spatial irradiance distance) in TIRF-mode. 20-50,000 images were acquired for each cell with 50 ms integration time. The mean precision localisation in PALM measurements was found to be 20±5 nm (Fig S3). 2D-STORM imaging of Alexa647, was performed using a ~5 kW/cm² irraniandce with the 642 nm excitation. 25000 images were acquired for each condition. Image reconstruction was performed using the ThunderStorm plugin of ImageJ using Tetraspeck 100 nm multicolour beads (Life Technologies) as fiducial markers to correct for drift.

**Super Resolution Microscopy Analysis.**

The module DBSCAN of the super resolution quantification software SR Tesseler(Levet et al., 2015) was used to analyse the PALM localizations for quantification of Gag cluster sizes. In order to monitor the localisation of I-BAR proteins in the vicinity of Gag assembling particles, a binary mask was introduced into the PALM images. The centre of each Gag assembling cluster was determined and a custom MATLAB (Mathworks) code was used to extract localizations in a radius of 80nm around each Gag cluster center and to extract IBAR
proteins localisations belonging to a disk of 150nm radius around the centre of each Gag clusters. These subsets of coordinates were then used to calculate coordinate-based colocalization (CBC), developed by Malkusch et al. (Malkusch et al., 2012), and implemented in the ThunderSTORM plugin of ImageJ (Fig. S4). The coordinate-based colocalization (CBC) values are calculated from single-molecule localization data of two species (Gag and I BAR proteins (IRSp53 or IRTKS)). A CBC value is assigned to each single localization of each species. We analysed the distributions of these CBC values by plotting and comparing the cumulative frequency distributions of the CBCs obtained in the two conditions (IRSp53 vs IRTKS). Finally, we performed a set of numerical simulations of images in ImageJ (see supplemental methods) to estimate the average localisation pattern of the immuno-labelled I-BAR proteins with respect to the mEos2 tagged Gag in PALM (Results, Part A, Fig. S9).

**Immunofluorescence and Confocal microscopy.**

For confocal microscopy, cells were grown as before and were fixed post treatment in 4% PFA+4% sucrose and permeabilized with 0.05% Saponin in 1% BSA in PBS (GIBCO). Cells were then incubated with the respective primary antibodies for 1 hour at room temperature and then washed with 1% BSA in PBS and then incubated with the secondary antibodies (Atto647 Sigma). Post incubation, coverslips were washed and mounted on a slide with Prolong Gold (Sigma) and left at room temperature in the dark to dry and cure. For transfected cells, cells were mounted post-fixation on the slides. Imaging was performed using a Zeiss LSM780 confocal microscope with an oil immersion objective of 63X with NA 1.4. Zeiss ZEN software was used to control the microscope and acquire images.

**Preparation and Imaging of Fluorescent VLPs.**

24h after seeding 2.10^6 HEK293T cells were transfected with 8µg of pI-BAR-GFP proteins with or without 8µg of pGag/pGag(i)mCherry (2/3 1/3 respectively). 24h after transfection cells media (9mL) were filtered before performing VLPs purification by ultracentrifugation (SW41Ti rotor (Beckman) 29 000rpm, 1h30) on TNE 20% sucrose cushion. Pellets were resuspended with 110µL of TNE and allowed to sediment on round 25mm coverslips during 45 minutes in a chamber. VLPs were imaged with a Nikon Ti Eclipse 2 TIRF microscope. Images were taken with an Evolve EMCCD camera – 512 photometrics, using 100X objective and 488 and 561nm lasers.

**Image Analysis for Colocalization.**

Images were acquired with Zeiss LSM780 (for fixed cells) or Nikon Eclipse Ti-2 in TIRF mode (for fluorescent viral particles). Colocalization analysis based on Mander’s coefficients was performed using JaCOP (Just another Colocalization Plugin)(BOLTE and CORDELIÈRES, 2006). Mander’s coefficient are defined as $M_1 = \frac{\sum A_{i,\text{color}}}{\sum A_i}$ and $M_2 = \frac{\sum B_{i,\text{color}}}{\sum B_i}$, A and B being the two respective channels (mCherry and GFP). 0<M<1, with 1 full colocalization and 0.5 random colocalization. The M1 and M2 coefficients were calculated for several images and
then represented as column graphs with red columns representing the degree of overlap of mCherry images with GFP images, and green columns representing the inverse.

**TIRF (Total Internal Reflection Microscopy) Imaging of HIV-1 Assembly:**

Since HIV assembly takes place primarily at the cell plasma membrane, a large body of work studying this process was achieved with the help of Total Internal Reflection Fluorescence Microscopy (TIRF-M). TIRF-M is an optical technique which uses incident light at an angle to the surface to generate an evanescent wave of light which illuminates a thin axial cross section of the sample close to the coverslip. In case of biological samples involving cells, this technique permits the excitation of fluorophores present close to the coverslip surface, i.e. present at the cell plasma membrane of adhered cells. Since a large population of fluorophores (at a distance >100 nm from the surface) remain dark, the signal-to-noise ratio for molecules at the plasma membrane is significantly better, allowing sensitive measurements of these membrane fluorophores. This technique has been successfully used to study trafficking of cell surface receptors. As discussed in Section 2.2.3 (Page 32), this was also used to study the assembly of HIV-1 at the plasma membrane by measuring the changes in intensity of fluorescent Gag at the plasma membrane. Further improvements in resolution were achieved by single molecule localisation microscopy in TIRF mode (See Annex, Page, Section, Page), HIV-1 Gag being one of the first proteins to be studied with this technique (REF).

![Figure 33 Principle of TIRF-M (adapted from Favier et al 2008 Methods)](image)

**TIRF Live cell Microscopy and analysis**

For visualizing cells in TIRF microscopy 293T cells were grown on poly-L-Lysine Fluorodishes (25 mm, WPI) in complete DMEM supplemented with 10% fetal bovine serum (Dominique Dutscher) sodium pyruvate (Invitrogen) and antibiotics (penicillin-streptomycin) for 18-20 hours. Cells were transfected with the necessary plasmids (Gag+Gag-mCherry+IRSp53-GFP 3:1:1). Before imaging, cells were washed with PBS and then medium was replaced with
Leibovitz L15 medium without phenol red for imaging. Cells were then imaged was performed using a Nikon widefield inverted microscope equipped with 488-nm, 561-nm activation and excitation lasers for epifluorescence and TIRF acquisition, controlled by MetaMorph software. The detector used for TIRF was the EMCCD Evolve 512 Photometrics camera (512*512, 16µm pixel size) with an oil immersion objective 100X Plan TIRF Apochromat with NA 1.49. Fluorescence was detected on a liquid cooled EMCCD camera (Andor Technology) in two channels (TIRF 488 and TIRF561) for a duration OF 30 min with a time interval of 3s. For live cell microscopy in TIRF, single particles of assembling HIV-1 Gag were detected with the Spot Detector plugin in ICY. The trajectory and the intensity profiles in two channels for each spot were obtained through the Intensity Tracker and Export Trajectory plugin. The intensity files thus obtained were analysed to extract spots which conformed to the kinetics of assembling Gag using custom MATLAB code (MathWorks). For spots conforming to known assembly kinetics, the intensities were plotted for the two channels and compared.
ANNEX: Review Article
Review

Monitoring HIV-1 Assembly in Living Cells: Insights from Dynamic and Single Molecule Microscopy

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Abstract: The HIV-1 assembly process is a multi-complex mechanism that takes place at the host cell plasma membrane. It requires a spatio-temporal coordination of events to end up with a full mature and infectious virus. The molecular mechanisms of HIV-1 assembly have been extensively studied during the past decades, in order to dissect the respective roles of the structural and non-structural viral proteins of the viral RNA genome and of some host cell factors. Nevertheless, the time course of HIV-1 assembly was observed in living cells only a decade ago. The very recent revolution of optical microscopy, combining high speed and high spatial resolution, in addition to improved fluorescent tags for proteins, now permits study of HIV-1 assembly at the single molecule level within living cells. In this review, after a short description of these new approaches, we will discuss how HIV-1 assembly at the cell plasma membrane has been revisited using advanced super resolution microscopy techniques and how it can bridge the study of viral assembly from the single molecule to the entire host cell.

Keywords: HIV assembly; SMLM; dynamics

1. Introduction

The human immunodeficiency virus (HIV) is an enveloped single stranded dimeric (+)RNA virus belonging to the family Lentiviridae. HIV is the causative agent of acquired immuno-deficiency syndrome (AIDS), estimated to have infected 70 million people since the first reported case, with 37 million people currently infected worldwide (source WHO 2017). However, since the introduction of protease inhibitors in HIV treatment, AIDS is considered a chronic disease. The HIV replication cycle has been widely studied. Briefly, HIV-1 infects mainly CD4+ T-lymphocytes by binding to the primary CD4+ receptor via its envelope (Env) protein and fuses by binding to the co-receptors, CCR5 or CXCR4, depending on the cell tropism. Post-fusion, uncoating of the virus takes place in the cytoplasm followed by reverse transcription and nuclear import of the viral DNA. The viral DNA integrates into specific sites in the host cell genome and then subverts the host cell machinery to transcribe and translate the viral genes into genomes and proteins, which are then trafficked to the membrane and assembled first into immature virions. The viral RNA genome is then packaged as a dimer into the forming particle. Concomitant to the particle release, post-cleavage of the structural Gag proteins by the viral protease occurs to give rise to its mature form, where a large structural rearrangement of the viral particle renders it infectious (for general reviews, see [1,2]).

The HIV-1 Gag structural protein is a 55 kDa polypeptide containing four domains; matrix (MA), capsid (CA), nucleocapsid (NC), and p6, in addition to two small spacer peptides, SP1 and SP2. Approximately 3000 Gag polyproteins assemble to form a single immature capsid shell [3]. Mutational analyses have revealed that only three domains of Gag (MA, CA, and NC) are required for immature particle assembly, whereas the fourth domain, p6, is required for budding and release.
by recruiting the endosomal sorting complexes required for transport (ESCRT) machinery host cell factors [4]. Membrane targeting of Gag requires the N-terminal myristate of MA, as well as residues in MA that form a basic patch (the highly basic region, HBR) which interacts with the acidic head groups of phospholipids at the inner leaflet of the cell plasma membrane (PM), especially including the phosphatidylinositol 4,5-bisphosphate \( \text{PI}(4,5)\text{P}_2 \) [5–13]. CA contains residues that form critical Gag–Gag interactions and NC is required for viral genomic RNA packaging, as well as non-specific interactions with RNA [14], and is essential for particle assembly [15]. Indeed, Gag is the only viral protein required for assembly and release of immature viral particles in cells, although production of infectious viruses requires other viral proteins, the Env proteins and the genomic RNA.

2. What Is Known about HIV-1 Assembly from Classical Microscopy?

The mature HIV-1 particle is 80–150 nm in diameter size [16–18] and thus the assembly site is below the resolution limit of conventional optical microscopy methods. Electron microscopy has long been a standard to study HIV-1 assembly and the particle structure [19]. Several studies have used electron microscopy to successfully demonstrate Gag multimerization at the plasma membrane, membrane curvature at the assembly site and viral budding. The role of cellular factors, such as the ESCRT machinery in HIV-1 particle release, has also been elucidated by this method [4,20]. Scanning and transmission electron microscopy has offered great insights into the formation and morphology of viral particles, providing evidence for electron-dense Gag layers underneath the plasma membrane and nascent particles connected to the cell surface by a thin stalk (for example see [21]). Further advances such as cryo-electron tomography have dropped the resolution to a few angstroms and allowed visualisation of viral protein complex structures close to the atomic level [22–24]. However, despite its stellar contribution to our understanding of HIV-1 particle structures, electron microscopy does not allow for study of the dynamics and the kinetics of nanoscale events taking place during virus assembly at the host cell plasma membrane.

The fluorescence microscopy advances, coupled with genetically encoded fluorescence proteins or external fluorophores added on live cells, has offered new insights into the dynamics underlying HIV-1 assembly. In the past decade, the introduction of a small Cys rich tag into the viral Gag protein enabled dynamic fluorescent imaging of Gag, using the membrane-permeable biosenal compounds, FlAsH and ReAsH, in model cell lines and in macrophages [25,26]. Since HIV-1 assembly occurs at the cell plasma membrane, most of the improvement in deciphering this process was achieved by total internal reflection fluorescence microscopy (TIRFM). The kinetics of HIV-1 Gag assembly and particle release could then be visualized thanks to fluorescent tagged Gag proteins, for the first time, in a transfected adherent model cell line [27]. Using live two color TIRF microscopy, the timing of the association of Gag with the genomic RNA at the cell membrane [28,29] could be observed for the first time, and thereafter with the ESCRTs for particle budding [30,31]. Using multicolor frame-by-frame alternating TIRF/wide-field imaging, Ivanchenko et al. pointed out a possible pre-association of several cytosolic Gag molecules with the genomic RNA [32]. It was suggested that there is a gradual formation of Gag multimers at the plasma membrane from the cytosolic pool [28,32]. Later on, advanced dynamic confocal microscopy techniques, such as raster image correlation spectroscopy (RICS) [33], confirmed the existence of the Gag-RNA oligomers in the cytosol that could be the precursors of assembly [34].

The recruitment of the ESCRT proteins, needed for bottle neck membrane scission of the viral particle, was also shown to be sequential [30,31]. Tsg101 is first recruited with Gag at the assembly site [30], interacting concomitantly with p6, and the NC domain of Gag [35], and persisting, like another ESCRT protein, ALIX [31,26], while CHMP and Vps4 proteins come later and are transient [30,31,37]. The entire process is no longer than 20 min [27,32] and seems to be cell type independent [38]. The scission and release process has been extensively reviewed in the past 5 years [36,39] but clarity still eludes us regarding the time interval between the 5 min of assembly/multimerisation of Gag proteins at the cell membrane and the late transient CHMP/Vps4 recruitment (Figure 1). Most of the above results are schematized in Figure 1.
Figure 1. Scheme of HIV-1 Gag protein dynamics in the cytosol and at the cell plasma membrane during the process of viral particle assembly. From the left to the right: (1) The immature HIV-1 Gag::eFP tagged protein is synthesized in the cytosol, where one can measure a diffusion coefficient of 3 μm²/s for cytosolic Gag, probably as a dimer and already in association with the genomic RNA (labelled as gRNA in the figure) [from (32,33)]. It is not known clearly if any cellular factors are involved at that stage or simple diffusion of Gag permits its targeting to the cell plasma membrane. (2) Once at the cell membrane, Gag interacts preferentially with acidic lipids, such as PE,PS, and anchors the cell membrane through its myristate domain. Then Gag can dynamically get back to the cytosol (measurable D=3 μm²/s) or pursue its multimerization on the viral RNA and at the inner leaflet of the cell PM (measurable D=1 μm²/s) [from (33)], thanks to concomitant NC-gRNA and the MA-lipid interactions. (3) After 5 min of assembly, i.e. Gag multimerization with incorporation of new Gag molecules, 10 more minutes are then required before the recruitment of the ESCRT machinery [4] [review in [34]], with a very low diffusion coefficient of Gag at the budding site (D=0.01 μm²/s) [from (35)]. During this phase (3), the cortical actin network could play a role in stabilizing Gag multimers at the cell membrane (from [4]). (4) Then after ESCRT recruitment, by the NC and p6 domains of Gag, (5) the immature viral like particle (VLP) is released.
Although conventional fluorescence microscopy methods such as wide-field or confocal microscopy have succeeded in elucidating many features of HIV-1 assembly in cells and continue to shed light on its dynamics, they are severely confined by the diffraction limit (d~250 nm), preventing access to the molecular details of the assembling virus (d < 150 nm).

3. Advanced Super Resolution Microscopy Shed a Light on HIV-1 Assembly

In these last two decades, far field optical microscopy has overcome the old resolution barrier, the diffraction limit, enabling super resolution visualization of previously invisible molecular details in biological systems. Since their conception, super-resolution imaging methods have continually evolved and one could expect that they will allow imaging cellular structures in living systems in three dimensions, with multiple colours, at the nanometer scale resolution, even though it remains very challenging. These techniques have been used to answer questions involving organization, interaction, stoichiometry, and dynamics of individual molecular building blocks and their integration into functional cellular machineries [41]. Super resolution microscopy (SRM) techniques can be divided into two categories. The first one is based on shaping the illumination light, such as (saturated) structured illumination microscopy (SIM) and stimulated emission depletion microscopy (STED). STED involves selective deactivation of fluorophores combined with this illumination shaping to narrow the emission spot down to 20–30 nm [42,43]. The second method is based on single-molecule detection and localization (often called single molecule localization microscopy, SMLM) taking advantage of the fluorophore ability to blink or to be photo-converted [44]. In this category, are found stochastic optical reconstruction microscopy (STORM) [45] and photo-activation localization microscopy (PALM) [46]. These approaches rely upon the acquisition of a set of several thousand images with each image having a random subset of fluorophores stochastically fluorescent at a given time point. By detecting each of single fluorophores and localizing precisely their center of mass, single molecule localizations from each of thousands of images can be obtained. These localizations are then used to reconstruct the final image with single molecule localisation precision (for a review see [47]). This localization precision is inversely proportional to the number of photons detected for each single molecule. Therefore, using very bright fluorophores, 10 to 20 nm precisions can be achieved.

Benefits from these nanoscale microscopy techniques have been rapidly applied to the study of immature HIV-1 Gag/i) mEOS2 assembly (see Figure 2 for an illustration). In fact, HIV-1 Gag was one of the first proteins to be visualized by PALM SRM [46] showing different cluster sizes of Gag, tagged with mEOS, at the cell plasma membrane of adherent model cell lines. Single molecule coordinate based analysis has shown the existence of three different types of Gag clusters in adherent cells: small random clusters (inferior to 50 nm diameter), clusters of defined size corresponding to assembling sites (between 50 to 130 nm) and large patchy aggregations of Gag corresponding to fully assembled structures (~140 nm diameter) [50]. Because the introduction of a protein tag into Gag could play a role, Gunzenhäuser et al. compared two photo-activatable tags, mEOS2 and tdEOS, with respect to Gag assembly [51]. The results show that Gag tagged with tdEOS forms unusually large clusters as compared to Gag-mEOS2, which in turn forms clusters well within the range for HIV-1 assembly sites. Their data suggests that the addition of protein tags may very well change the nature of Gag assembly. The larger tandem dimeric tdEOS possibly disrupts the regular hexameric Gag lattice structure in the VLPs, changing Gag organization. Introduction of fluorescent protein tags has previously been known to perturb Gag VLP structure. Indeed, electron microscopy studies have shown abnormalities in VLPs formed by Gag-GFP molecules [52,53] and in another study, HIV-1 tagged with an internal GFP in the C terminus of MA exhibited poor infectivity in the absence of helper untagged Gag molecules [54]. It is thus critical to add a certain quantity of untagged Gag along with the fluorescently tagged molecule. However, Hubner et al. shows that a Gag tagged internally with GFP, between the CA and the MA domains, flanked by protease cleavage sites, can facilitate native processing of the Gag polypeptide. This Gag(i)GFP was able to mimic native Gag in terms of localization and assembly dynamics [55].

Fluorescent protein tags appear as essential tools to study viral assembly in host living cells. However,
choosing the right fluorophore requires consideration of its intrinsic properties and its location into the targeted protein. Genetically encoded fluorescent tags and their properties have been reviewed in [56], and their applications and limitations for live cell imaging in [57,58]. Furthermore, recently, a comparative study of fluorescent tag properties for temporal or spatial fluorescence fluctuations was reported for quantifying protein oligomerization in living cells [59]. Several fluorescent proteins have been engineered from dimeric or tetrameric jellyfish or coral derived proteins and they may thus retain some residual tendency to aggregate, which may cause significant effects on the dynamics of protein–protein interaction studies. This is especially true when studying viral assembly: some FP proteins could thus reduce or increase HIV-1 Gag protein oligomerization properties and affect VLP biogenesis. In addition, fluorescence imaging in live cells carries with it an additional risk factor of phototoxicity, such as photobleaching, photodamaging, or the creation of photoreactive oxygen species due to the fluorophore and illuminations. This is especially evident in cases of photoactivable fluorescent proteins (PAFPs), such as EosFP, which require activation in the near-UV range of the spectrum. Strategies to reduce phototoxicity, by limiting the illumination to the focal plane and controlling phototoxicity effects have been reported [60]. In addition, recently, new labeling protocols based on fluorogenic systems [61] and biosensors in general have been developed for measuring acute spatiotemporal events in living cells (review in [62]). Thus, the choice of a suitable tag, the experimental conditions and the illumination plane are critical as it has clear implications on the results and interpretations.

Apart from the Gag protein self-assembly properties, the formation of an infectious HIV-1 particle involves the viral RNA genome (gRNA), other viral proteins, such as the envelope transmembrane glycoproteins (Env), and some host cellular proteins, such as ESCRT (reviewed in [15,20]). Most of the SRM studies on HIV-1 assembly have focused on HIV-1 Gag and Env clusters at the cell membrane. Dual color PALM/dSTORM and other dSTORM studies have illustrated the importance of the CT domain of Env in HIV-1 Gag assembly [63,64]. Mutations in the CT domain of Env resulted in loss of specificity of Env incorporation, as revealed by a coordinate based distance distribution analysis [63]. In addition, image-based morphological cluster analysis showed that Env-CT clusters are larger in cells expressing all HIV-1 proteins (except Nef) as compared to Env-CT only transfected cells, while it was not the case for EnvΔCT. This enhanced clustering of Env trimers in the presence of Gag was also observed by Roy and coworkers [64]. A few SRM studies have gone further and looked at cell-free virions to detect viral proteins and their positioning within the particle. A STED study in 2012 [65] revealed the distribution of Env on the virus surface which was dependent on the maturation process as well as on the Env CT domain.

Studies regarding host cell factors in HIV-1 assembly have mainly focused on the ESCRT machinery. Van Engelenburg et al. looked at relative distribution of ESCRT subunits within the budding particle by iPALM (interferometric PALM, a 3D SRM technique, see [66]) and correlative electron microscopy [67]. They showed the initial scaffolding of ESCRT subunits CHMP2A, CHMP4B and TSG101 within the viral bud followed by levels of CHMP2A decreasing significantly relative to Tsg101 and CHMP4B upon virus abscission. Apart from shedding light on the spatial distribution of ESCRT subunits within the particle, thanks to iPALM/correlative SEM, the differential incorporation of CHMP2A points to a distinct dynamic among the ESCRT subunits preceding viral abscission. TSG101 revealed a small cytosolic pool punctuated by localizations within the Gag lattice of assembling particles. Using two-color two dimensional super-resolution imaging, the ESCRT machinery (TSG101, ALIX, and Chmp4b/c proteins) was observed to be positioned at the periphery of the nascent virions, with TSG101 closer to the assembled Gag than ALIX, Chmp4b, or Chmp4c [30], certainly due to its direct interaction with NC and p6 [35]. Finally, Prescher and collaborators showed that the membrane scission process is driven from inside the HIV-1 budding neck by ESCRT-III protein assemblies including CHMP4B and CHMP2A as well as TSG101 and ALIX which were also located in clusters similar to the dimension of the neck [68].
Figure 2. A direct imaging comparison of wild-type HIV-1 Gag(i)mEos2 in Jurkat T cell using (a) classical TIRF microscopy and (b) photo activated localisation microscopy (PALM). Insets are showing assembling (part b, right inset) or assembled structures (part a, and b left inset). (c) Direct comparison of fluorescence intensity profile (green, TIRF image), or number of localizations (purple, PALM image) along the x direction (white line) crossing an assembled viral like particle (VLP) shows enhanced resolution in the case of PALM allowing for direct measurement of VLP sizes. (d) Purified HIV-1 immature Gag VLPs as seen by PALM (left). With the help of PALM microscopy, it is possible to measure the diameter of each single VLP and establish a distribution of it (graph, right). The average diameter found here is ~140 nm. Scale bars are 0.5 μm for a and b, 0.2 μm for b inserts and 0.1 μm for d (adapted from [48,49]).

All these studies show that SRM can now help in deciphering the respective positions of the different molecules involved in viral particle assembly in cells. Moreover, correlative iPALM and SEM studies by Van Engelenburg et al. [67] and Pedersen et al. [69] highlight the possibilities of SRM shedding light on a more resolved HIV-1 particle structure.

One of the main advantages of SRM over electron microscopy is its ability to observe, in real time and in living cells, ongoing processes at the nanoscale level, making it a technique of choice for studying virus assembly. However, most of the SRM studies cited above, barring a few [64], were
limited to fixed cell imaging. In the last five years, many efforts have now been put into deciphering the real-time molecular events occurring in living cells during the HIV-1 assembly and budding process.

4. Towards a Real Time Molecular Description of HIV Assembly in Living Cells

The first attempt to study the spatiotemporal aspect of Gag assembly in living cells at the single molecule level was conducted with single particle tracking PALM (sptPALM) [70]. By extracting the apparent diffusion coefficients (D) of each single Gag molecule from their mean square displacement (MSD), Manley et al. observed a strong decrease in Gag mobility that was correlated with the presence of Gag clusters at the cell plasma membrane (see Figure 3). Later on, a comparison of apparent diffusion coefficient distribution highlighted the role of the NC-RNA binding domain of Gag in HIV-1 assembly in adherent cells [71] or in CD4 T-cells [49]. Recently, studies by Floderer et al. [38,48] in T cells, and Yang et al. [72] in adherent cells, used sptPALM to track Gag molecules and to confirm the important role of the gRNA and the NC domain of Gag in the generation of Gag clusters at the plasma membrane of the host cell for efficient HIV-1 assembly. Floderer et al. [38,48] showed that gRNA in the context of a provirus acts as a spatiotemporal coordinator of the membrane assembly process, rather than only serving as an attractor for Gag molecules to the assembly site. By monitoring the trajectories of single HIV-1 Gag molecules in the vicinity of assembly sites, Floderer and collaborators [38,48] have shown the existence of directed motions towards these assembly sites. It is currently not clear or elucidated what these Gag directed motions towards the assembly site are; it could reflect Gag multimerization on the viral RNA, Gag–Gag interactions, Gag–host cell factor interactions or others. Using different Gag mutants and live SRM, several studies have revealed a major role of the NC–RNA interaction in coordinating HIV-1 Gag assembly at the cell plasma membrane [48,71,72]. Indeed, it was observed that, contrary to the hypothesis that gRNA only functions to drive formation of low-order Gag multimers, there was a crucial dependency of the assembly process at the cell membrane on the NC–RNA interactions.

Live SRM requires big data analyses that can be monitored in different ways. Yang et al. [58] classified the Gag trajectories using their mean square displacement and the deviation from the purely diffusive (random) motion. They used a $\alpha > 1.3$ exponent to consider directed motions from the generalized description of the MSD (see Figure 3b–d for detailed explanation). In the Floderer et al. approach, a Langevin description of Gag motions was used to rigorously disentangle the attractive (directed) and the random (Brownian) part of the overall Gag motion. Coupling this with Bayesian inference and Voronoi tessellation [73], Floderer et al. established Gag diffusion and attraction energy maps and monitored their fluctuations during the entire assembly and budding process (30 min) at the nanoscale level (see Figure 3e). Interestingly, their results show that the attraction strength sensed by single membrane Gag proteins at the vicinity of the assembly sites was not dependent on the nature of the RNA and was equivalent to an attractive energy of 3 to 4 kT (2–3 kcal/mol). Furthermore, it was revealed that the Gag–Gag interaction mediated by CA–CA dimerization represented only a third of Gag attractive energy, while the NC–RNA platform represented two thirds [48].

Measurement of apparent binding energies during viral assembly in living cells is opening new perspectives and allows comparison of live cell measurements with nicely tuned and controlled in vitro systems or in silico molecular simulations. For example, coarse grained modeling recently predicted that the presence of gRNA lowered the necessary SP1-CA hexameric interaction energy down to 3 kT to achieve correct HIV-1 assembly [74]. This energy value is indeed close to the Gag attractive energy found in Floderer et al. [48]. For the sake of comparison with another virus, an in vitro experimental approach found that the binding energy of icosahedral viral capsid subunit to its RNA genome was ~7 kT [75]. The capacity to directly measure the energy sensed by Gag during HIV-1 assembly using single molecule microscopy opens an incredibly large field of research that can now link coarse grained modeling and in vitro quantitative measurements and experiments in living cells.
Figure 3. Single Gag molecule dynamics during HIV-1 assembly. (a) Trajectories of numerous single Gag observed by TIRF-sptPALM Jurkat T-cells. (b) 3 classes of trajectories can be isolated, corresponding either to pure Brownian motion (1, green track), directed motion (2, red track) confined or trapped motion (3, yellow track). By plotting the mean square displacement (MSD) of the single molecule as a function of time, each type of motion could be theoretically distinguished. MSD(t) curves as a function of the motion type (Brownian, green, Directed, red, Confined, yellow) could be non-rigorously approximated by the following relation MSD(t) = D t^α where D is an apparent diffusion coefficient and α is either less than 1 (confined motion) or more than 1 (directed motion), with a special case where α = 1 (Brownian motion). (c) Due to noise in the MSD reconstruction it is often difficult to distinguish between confined, Brownian and directed motions, therefore a simple way to analyze the curves is to fit the MSD(t) with the simple and linear MSD(t) = Dt equation that usually leads to a broad distribution of apparent diffusion coefficients (distribution obtained from the trajectories observed in part (a)). These apparent diffusion coefficients could for example be arbitrarily divided in each class of motion (yellow, confined, low apparent diffusion coefficient, green Brownian, average apparent diffusion coefficient, red, directed, high apparent diffusion coefficient). (d) A less arbitrary method is to fit all the MSD(t) curve with the MSD(t) = D t^α approximation and take the benefit of the numerous noisy curves to establish a statistically relevant distribution of motion, allowing one to qualitatively classify the type of motion observed in the cell. This technique has been used to observe directed motion close to the assembly site in [72]. (e) A more rigorous analysis aims at disentangling the part of Brownian and directed motion in the trajectory using the Langevin equation of motion. This approach coupled to spatial tesselation, allows one to generate maps of directed motions containing the average direction as well as the strength of the attraction force generating these directed motions. By coupling this information to the changes in the density of single Gag localization over time, we monitored the attractive energy sensed by each single Gag molecule in the vicinity of the HIV-1 assembly site in host CD4 T cells [48].
Env incorporation into HIV-1 virions during particle assembly was recently studied by Buttler et al. [76]. Using iPALM, they showed that Env was incorporated in preformed Gag lattices to the neck of the assembling virions, as determined by the Env angular distribution on the surface of cell-associated virus. In parallel, a role of the C-terminal domain of Env in its trapping at the assembly site was elucidated, using sptPALM, by comparing the diffusion of the wt-Env with an Env deficient in the C-terminal tail (EnvΔCT). This nanoscale visualization of the budding site highlights a role of Gag in Env incorporation. Finally, Env mobility in the lipidic membrane of released virion was also studied using another SRM technique, i.e. scanning STED-FCS. This SRM technique uses the fluorescence fluctuations of several molecules to measure their motion in each STED voxel of the line scanned [77]. Chojnacki et al. reported that Env mobility was really low (D = 10^{-3} \text{ \mu m}^2/\text{s}) in the virions and constrained by its C-ter domain [78]. This Env mobility was doubled upon virus maturation. Interestingly, the Env diffusion coefficient found by Chojnacki et al. in HIV-1 particles [78] is a hundred times smaller than the one observed at the plasma membrane of virus producing cells [76,78], leaving an open window regarding the exact process and dynamics of Env incorporation during HIV-1 assembly.

In conclusion, advanced super resolution microscopies are nowadays opening incredible avenues in which to revisit some aspects of virology, looking at the extreme small (below 100 nm) with the possibility of coupling it to live cell imaging. This will enable one to decipher the virus life cycle in the host cell at the level of the single molecule and spatiotemporally offering a 3D + t view of the events, which was not possible 10 years ago.

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Figure 25: Transmission immuno-electron microscopy of HEK293T expressing HIV-1 Gag. HEK293T expressing HIV-1 Gag alone were fixed and embedded in resin and Immunolabelled for HIV-1 capsid (10nm beads) and endogenous IRSp53 (20 nm). A) Cells were immunolabelled with the Millipore 07-786 antibody against IRSp53, while B) were immunolabelled with the Sigma HPA023310 antibody. No labelling of endogenous IRSp53 is seen in any conditions. ........................................................... 122

Figure 26: Immunolabelling of IRSp53 (in red, Atto647N) reveals characteristic cellular protrusions resembling filopodia and lamellipodia at the cell membrane. However, expression of fluorescently labelled Gag(i)mEos2 (in green) does not reveal spatial information about assembly sites due to the diffraction limit. .................................................................................................................................. 123

Figure 27: Effect of over-expression of different I-BAR proteins on HIV-1 Gag membrane binding in HEK293T cells. Expression of the full length I-BAR proteins does not seem to impact HIV-1 Gag membrane binding significantly however binding of the IRSp53 I-BAR domain reduces membrane bound Gag (condition Gag + IRSp53-IBAR-GFP). ................................................................................ 125

Figure 28: Live cell imaging of cells transfected with Gag-GFP and IRSp53-mCherry. A) HEK293T cell expressing Gag(i)GFP and IRSp53-mCherry in TIRF showing HIV-1 Gag buds at the plasma membrane (in green). B) Assembling Gag bud was identified by measuring changes in GFP fluorescence intensity. C) Its trajectory was examined to verify its spatial restriction, a characteristic of assembling Gag. D) Relative fluorescence intensities measured for both channels were plotted as a function of time. E) Gaussian smoothing of the obtained intensity curves was performed to identify the global
changes in intensity for comparison of the two channels. The rise in intensity of Gag(i)GFP till its peak was considered as assembly, and took approximately 8 minutes. The rapid loss in intensity following this peak, interpreted as budding and release, occurred approximately 25 minutes after the initial increase in intensity. IRSp53*mCherry fluorescence peaked and diminished in the first half of 8 minute assembly period. ............................................................................................................. 126

Figure 29: Fig. Co-expression of Rac1 dominant positive (Rac+) and dominant negative Rac1 (Rac-) along with HIV-1 Gag and its effect of membrane localization of endogenous IRSp53. As compared to mock transfected cells, the expression of HIV-1 Gag alone was sufficient to drive an increase in IRSp53 membrane binding. Co-expression of the Rac1 dominant negative mutant (Rac-) with Gag reduced IRSp53 binding to cellular membranes to near baseline levels. Expression of the dominant negative mutant (Rac-) and the dominant positive mutant (Rac+) alone did not affect IRSp53 membrane binding indicating that HIV-1 Gag plays an essential role in this process, possibly by interacting directly or indirectly with IRSp53. .................................................................................... 129

Figure 30: Involvement of the RacGEF Tiam1 in HIV-1 Gag particle production and its interactions with HIV-1 Gag. A) siRNA mediated knockdown of the RacGEF Tiam1 impaired HIV-1 Gag particle release in HEK293T cells. B) Pulldown of endogenous Tiam1 resulted in immuno-precipitation of exogenous HIV-1 Gag (lane 1). Immunoprecipitation of endogenous PIP3 also pulled down HIV-1 Gag (lane 2) ................................................................................................................................................ 130

Figure 31: Super resolution PALM/STORM microscopy for actin nanostructures at HIV-1 Gag assembly sites. A) HEK293T expressing HIV-1 Gag(i)mEos2 doped with HIV-1 Gag and stained with Phalloidin-AlexaFLuor-647 to mark F-actin were imaged in TIRF mode and subjected to successive PALM/STORM microscopy to obtain super resolved images of assembly sites and cellular actin (scale bar 10µm for large images and 100nm for the magnified “zoom” images). B) Gag cluster detection and cluster size obtained in SR-Tesseler C) 45% of F-actin single molecule localizations are highly correlated with HIV-1 Gag localizations as compared with 27% IRSp53 localizations, indicating that, on an average, actin is present more consistently at assembly sites. ................................................ 132

Figure 32: Proposed scheme showing IRSp53 membrane clusters favouring Gag membrane binding and assembly.1) IRsp53 and Gag have a stable equilibrium of binding/unbinding the cell plasma membrane and their turnover from the membrane to the cytoplasm. 2) IRSp53 is naturally induces some membrane curvature locally due to its multivalent assemblies, while polymerization of Gag as well induces local curvature, both inducing PIP2 clustering. 3) Gag preferentially initiates self assembly at sites curved by IRSp53, and this curvature, stabilized and promoted by IRSp53, is subverted by Gag to pass the energy barrier to bend the membrane. In the absence IRSp53, this barrier is not overcome and Gag remains self-assembled but blocked at the membrane curving stage. 4) Self-assembly of Gag continues the curvature of membrane generated by viral particle formation, with adjacent IRSp53 gradually being excluded from the budding site. 6) The viral particle buds out, with some IRSp53 molecules still trapped within the bud, while the majority resumes their equilibrium at the cell plasma membrane................................................................. 143
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REFERENCES


