HIV-1 assembly, release and maturation

Eric O. Freed

Virus-Cell Interaction Section, HIV Dynamics and Replication Program, Center for Cancer Research, National Cancer Institute, Bg. 535, Room 110, 1050 Boyles St., Frederick, Maryland 21702-1201, USA.

Abstract

Major advances have occurred in recent years in our understanding of HIV-1 assembly, release and maturation, as work in this field has been propelled forwards by developments in imaging technology, structural biology, and cell and molecular biology. This increase in basic knowledge is being applied to the development of novel inhibitors designed to target various aspects of virus assembly and maturation. This Review highlights recent progress in elucidating the late stages of the HIV-1 replication cycle and the related interplay between virology, cell and molecular biology, and drug discovery.

HIV-1 is the causative agent of AIDS, one of the most devastating viral pandemics in history. Although effective treatments are available to suppress HIV-1 replication in infected patients, current drugs do not eradicate the virus, making life-long treatment necessary. Prolonged treatment is associated with drug toxicities and a risk of viral resistance. Thus, the HIV research community must continue to explore novel therapeutic strategies, including those that target steps in the viral replication cycle that are not disrupted by currently available drugs.

The HIV-1 replication cycle can be divided into an early and a late phase. The early phase encompasses the events that occur from virus binding to the surface of the host cell until the integration of the viral DNA into the host cell genome. These early events include virus binding to cell surface receptors; cell entry; reverse transcription of the viral RNA to DNA; uncoating of the viral capsid; nuclear import of viral DNA; and DNA integration. The late phase refers to the events that occur from gene expression to the release and maturation of new virions, and includes the transcription of viral genes; export of the viral RNAs from the nucleus to the cytoplasm; translation of viral RNAs to produce the Gag polyprotein precursor (also known as Pr55^{Gag}), the GagPol polyprotein precursor, the viral envelope glycoproteins (Env glycoproteins), and the regulatory and accessory viral proteins; trafficking of the Gag and GagPol precursors and of the Env glycoproteins to the plasma membrane; assembly of the Gag and GagPol polyproteins at the plasma membrane; encapsidation of the viral RNA genome by the assembling Gag lattice; incorporation of the

efreed@nih.gov.

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viral Env glycoproteins; budding off of the new virions from the infected cell; and particle maturation (FIG. 1).

This Review provides an overview of what is currently known about the late stages of the HIV-1 replication cycle and how these steps are orchestrated by the Gag precursor protein, which assembles to form the virus particle at the plasma membrane. This information may, in the future, be applied to the development of novel inhibitors that target these events. Given the similarities in the replication strategies of different viruses, this information has also provided fundamental insights into the basic biology of virus replication.

Gag protein: structure and function

During the late phase of the HIV-1 replication cycle, viral genes are transcribed and viral RNAs are exported from the nucleus to the cytoplasm, where viral proteins are synthesized. Gag is produced as a 55 kDa precursor protein that forms the virus particle. At the same time, the 160 kDa GagPol polyprotein precursor — which contains the viral enzymes protease, reverse transcriptase and integrase — is also expressed at ~5% of the level of Gag, owing to a programmed ribosomal frameshifting event during Gag translation.

The Gag precursor contains matrix (MA), capsid (CA), nucleocapsid (NC) and p6 domains, as well as two spacer peptides, SP1 and SP2 (FIG. 2). As part of the uncleaved Gag precursor, the MA domain targets Gag to the plasma membrane and promotes incorporation of the viral Env glycoproteins into the forming virions; CA drives Gag multimerization during assembly; NC recruits the viral RNA genome into virions and facilitates the assembly process; and the p6 domain recruits the endosomal sorting complex required for transport (ESCRT) apparatus, which catalyses the membrane fission step to complete the budding process (BOX 1; FIG. 1). After virion release, the viral protease — which is encoded within the GagPol polyprotein precursor — cleaves the Gag precursor to the mature Gag proteins MA, CA, NC and p6. Cleavage of the Gag polyprotein triggers the morphological transformation in virion structure known as maturation (see below).

Although the structure of the intact, full-length Gag precursor has been elusive owing to its large size and the presence of flexible interdomain connections, atomic-level structures are available for the individual Gag proteins (FIG. 2). MA folds into a highly globular structure comprising five α-helices, a 3_{10} helix and a three-stranded mixed (β-sheet. A carboxy-terminal α-helix projects away from the globular core, serving to connect MA with the adjacent CA domain[1]. A number of basic residues cluster at the top of MA, allowing the protein to interact electrostatically with the negatively charged lipids in the inner leaflet of the plasma membrane. The amino terminus of the MA domain is co-translationally modified by the covalent attachment of a myristic acid moiety; this 14-carbon fatty acid, which can adopt a sequestered or an exposed conformation[2], plays an essential part in Gag-membrane binding (see below).

The CA domain folds into two independent, largely helical domains — the N-terminal domain (CA_{NTD}) and C-terminal domain (CA_{CTD}) — connected by a flexible linker[3–5]. In the mature protein, the N terminus of CA_{NTD} folds back into a β-hairpin structure (FIG. 2).

A proline-rich loop serves as the binding site for the host proline isomerase cyclophilin A (CYPA; also known as PPIA)[4], which is incorporated into virions and may help to shield the viral capsid from antiviral innate immune defences after virus entry into the target cell. The CA_{ctd} contains a dimer interface, which plays an important part in Gag multimerization[5], and the major homology region (MHR), which is highly conserved among orthoretroviruses and participates in virus assembly. Nuclear magnetic resonance (NMR) data obtained with a Gag fragment containing MA and the CA_{NTD} have provided insights into how the structure of CA is affected by cleavage between MA and CA[6]. In this pre-cleavage structure, the N terminus of the CA_{NTD} is unfolded, suggesting that the N-terminal p-hairpin of the mature CA protein (FIG. 2) forms after MA-CA cleavage.

NC contains two zinc-finger-like domains (often referred to as zinc knuckles) that are important for genome recognition and the recruitment of viral RNA to the assembling Gag complex. In the mature protein, the zinc fingers form independently folded domains analogous to beads on a string[7]. The C-terminal p6 domain is largely unstructured[8], consistent with its primary role as an adaptor for the host ESCRT machinery (BOX 1).

HIV-1 assembly

The path to virus assembly begins with synthesis of the Gag polyprotein in the cytosol and its translocation to the site of assembly. During its trafficking to the plasma membrane, or after its arrival there, Gag interacts with dimeric viral RNA. Virus assembly proceeds at the plasma membrane, and viral Env glycoproteins accumulate at the site. Gag then recruits the cellular ESCRT machinery, which drives the membrane scission reaction required for particle release (BOX 1; FIG. 1).

Gag trafficking to the site of assembly.

Although at times this has been the subject of controversy, it is now well established that the inner leaflet of the plasma membrane is the primary site at which HIV-1 particles assemble. However, the mechanism by which Gag is specifically targeted to the plasma membrane has been a topic of active investigation for many years. An important role for the MA domain in Gag targeting was demonstrated by the observation that mutations in MA, particularly those involving the highly basic region, caused Gag to be mistargeted to a late endosome or multivesicular body (MVB) compartment[9]. The phosphoinositide phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P$_2$), a phospholipid that is highly enriched in the inner leaflet of the plasma membrane, plays a crucial part in Gag localization[10]. Depletion of plasma membrane PtdIns(4,5)P$_2$ largely recapitulates the phenotype of MA basic-domain mutants, leading to retargeting of Gag to late endosomes or MVBs. Subsequent structural studies demonstrated that MA interacts directly with the negatively charged inositol headgroup of PtdIns(4,5)P$_2$ (REFS 11,12). Notably, NMR data revealed that binding of a soluble derivative of PtdIns(4,5)P$_2$ to MA triggers exposure of the myristic acid moiety covalently linked to the N terminus of MA[11]. Myristate exposure probably facilitates its insertion into the inner leaflet of the plasma membrane, thereby stabilizing the Gag-membrane association. An additional level of regulation of Gag targeting to the plasma membrane was suggested by the observation, in liposome studies, that RNA — probably tRNA[13] —inhibits Gag-

membrane interactions by binding to the highly basic region of MA. However, PtdIns(4,5)P$_2$, but not other acidic phospholipids, is able to outcompete RNA for MA binding to liposomes[14,15]. Collectively, these findings show that PtdIns(4,5)P$_2$ regulates Gag targeting to the plasma membrane by mediating direct electrostatic interactions with MA, by displacing RNA bound to MA and by inducing the exposure of the N-terminal myristate, allowing it to insert into the target membrane. A number of cellular proteins have been implicated in Gag targeting to the plasma membrane and in subsequent assembly[16]; however, the mechanism of action of these host factors remains to be elucidated (BOX 1).

On reaching the plasma membrane, Gag induces the recruitment and coalescence of cholesterol- and sphingolipid-enriched membrane microdomains, often referred to as lipid rafts[17–19] (FIG. 1). The targeting of Gag to lipid rafts in the plasma membrane and the importance of PtdIns(4,5)P$_2$ in this plasma membrane targeting are reflected by the high levels of cholesterol, sphingolipid and PtdIns(4,5)P$_2$ that are found on the viral membrane[20,21]. Lipid rafts probably serve as platforms for virus assembly, and the targeting of Gag to these microdomains may facilitate the incorporation of Env glycoproteins into virions (see below). Given that areas of cell-cell contact — referred to by virologists as 'virological synapses' — are also rich in lipid rafts[22], the preference exhibited by Gag for lipid raft-like membrane microdomains may enhance assembly at the virological synapse, thereby promoting cell-cell viral transfer.

RNA encapsidation.

After synthesis in the nucleus, HIV-1 RNAs are transported to the cytoplasm, where they undergo two main processes: translation into viral proteins and packaging into newly assembled virus particles. A large number of viral mRNA species are synthesized, some of which are multiply spliced before nuclear export; others largely escape splicing and are exported in a partially spliced or unspliced form. Export of intron-containing viral RNAs is mediated by a highly structured *cis*-acting RNA element known as the Rev-responsive element (RRE), which is bound by the virus-encoded *trans*-acting protein Rev[23]. Rev, which shuttles between the nucleus and the cytoplasm, promotes nuclear export by forming a complex with the nuclear export factor CRM1 (also known as EXP1) and the GTPase RAN[24].

As is the case for other retroviruses, two copies of full-length viral RNA are packaged into HIV-1 virions[25]. The presence of two copies of genomic RNA in each particle provides the opportunity for recombination during reverse transcription and may also allow reverse transcription to proceed if one RNA copy is damaged[26,27]. The bulk of recent evidence suggests that RNA dimerization occurs in the cytosol or at the plasma membrane[28] and that an RNA dimer is the recognition unit for packaging into assembling virions[29].

The NC domain of Gag is the primary viral determinant that drives RNA packaging. NC contains two Cys-Cys-His-Cys zinc-finger-like (or 'zinc-knuckle') domains (FIG. 2), which are flanked by basic residues that increase the affinity of NC for RNA. NC functions as a nucleic acid chaperone, an activity that contributes to RNA packaging, to the incorporation and placement of the tRNA primer and to reverse transcription[30]. Interactions between NC and RNA also help to drive Gag multimerization (see below).

Retroviral NC domains direct the packaging of viral genomic RNA by binding to a packaging signal, often referred to as the ψ-element, that is located near the 5' end of the genomic RNA in the 5' untranslated region (UTR). In some retroviral systems, the ψ-element is fairly well defined. By contrast, a small, well-defined ψ-element has not been identified for HIV-1. The 5' UTR of the HIV-1 genomic RNA is a highly structured region that contains a number of folded RNA elements[31], including the transactivation response element (TAR), which serves as the binding site for the viral Tat protein and the positive transcription elongation factor b (P-TEFb) complex; a polyadenylation site; the primer-binding site (PBS), which binds a molecule of tRNA^{Lys} so that it is packaged into virions and can prime the initiation of reverse transcription; the dimer initiation signal (DIS), which contributes to RNA dimerization; the major splice donor (SD); and potentially other stem-loop structures. A variety of deletions and mutations in the 5' UTR reduce packaging efficiency, and a large number of alternative structures have been proposed for this region of the viral RNA (reviewed in REF 31). Therefore, it is likely that multiple folding conformations exist for the 5' UTR of the HIV-1 genomic RNA, and different conformations may serve distinct functions. For example, the 5' end of the viral RNA has been proposed to undergo a conformational switch from a structure that favours translation to one that promotes packaging[32]. Structures are available for NC bound to a number of different segments of the 5' UTR, although it is not clear which of these is most biologically relevant for packaging.

To follow the movement of HIV-1 RNA together with Gag in living cells, total internal reflection fluorescence (TIRF) microscopy (BOX 2) was used to visualize RNA and Gag localization near the cell surface[33]. In the absence of Gag, the HIV-1 RNA moved dynamically to and away from the plasma membrane. In the presence of Gag, the RNA docked stably at the plasma membrane and then became immobile as Gag co-assembled around the RNA. RNA docking in the presence of Gag occurred before Gag-RNA colocalization could be detected, suggesting that initial contacts are made between the RNA and a small (subdetectable) number of Gag molecules. These observations, which are consistent with the hypothesis that Gag and RNA first interact in the cytosol[34], indicate that a few molecules of Gag recruit the viral RNA to the plasma membrane, where the RNA then nucleates particle assembly (FIG. 1). A separate study used live-cell imaging to track single molecules of HIV-1 RNA in the cytosol and observed that the overwhelming majority of RNA molecules moved by passive diffusion, whether or not Gag was also expressed[35]. To better understand the nature of HIV-1 Gag-RNA interactions, the technique of crosslinking immunoprecipitation (CLIP) sequencing was used (BOX 2). This analysis revealed striking changes in Gag-RNA interactions during HIV-1 assembly and maturation; Gag in the cytosol bound primarily to several distinct sites in the 5' UTR and the RRE, whereas Gag at the plasma membrane and in the assembling virion was crosslinked to a large number of sites throughout the HIV-1 genome[13]. In mature virions, in which NC is thought to coat the viral genomic RNA, NC binding was observed at specific sites that again included the 5' UTR and the RRE. These results provide a window into the dynamic nature of the interactions between HIV-1 Gag and viral RNA.

Gag assembly.

Following their arrival at the plasma membrane, the Gag protein and the full-length genomic RNA assemble into the nascent, immature virus particle together with the GagPol precursor. As mentioned above, Gag-RNA contacts help to nucleate assembly, but the main viral determinant that drives Gag multimerization is the CA domain. In the immature particle, Gag molecules are aligned and packed radially, with the MA domain bound to the inner leaflet of the viral membrane and the C terminus of Gag oriented towards the centre of the particle (FIG. 1). Recent biochemical results suggest that the MA domain is trimeric in virions[36], consistent with data indicating that HIV-1 MA crystallizes as a trimer[37] and forms hexamers of trimers on a model membrane monolayer[38]. To accommodate the curvature necessary to form a particle with a diameter of ~120 nm — the size of the HIV-1 virion — the immature Gag lattice is continuous but contains gaps that appear to be devoid of Gag protein[39–41] (FIG. 1).

An extensive amount of high-resolution structural information is available for the hexameric lattice formed by CA in the mature, conical capsid (see below). However, largely because of its curved and flexible nature, the structure of the immature Gag lattice has been technically more challenging to define. Cryoelectron tomography (cryo-ET) (BOX 2) data based on subtomogram averaging provided a low-resolution view of the immature Gag lattice in virus-like particles (VLPs); these studies showed a hexameric arrangement for the immature Gag lattice, with a hole at the centre of the hexamer[39,41]. More recently, cryoelectron microscopy (cryo-EM) and cryo-ET techniques were combined to solve the structure of the immature Gag lattice in in vitro-assembled tubes formed by a truncated version of the Gag protein from the betaretrovirus Mason-Pfizer monkey virus (MPMV)[42]; the atomic-resolution HIV-1 CA crystal structures were then modelled into the electron densities of the MPMV cryo-EM reconstruction. One of the most significant features of this analysis was to reveal that the CA-CA contacts in the immature Gag lattice differ from those in the mature CA lattice. This MPMV-based model has recently been revised with the realization that, despite conservation of the tertiary structures of HIV-1 and MPMV CA proteins, the quaternary arrangements of the CA proteins from these two retroviruses differ substantially[43]. Even in this most recent, subnanometre-resolution structure of the immature Gag lattice, the precise structure of SP1 is not resolved[43], although the observed electron densities are consistent with SP1 forming a six-helix bundle as proposed earlier[41]. Further studies will be required to obtain a structure of the CA-SP1 boundary region of Gag at atomic-level resolution. This is an important objective, as this region not only promotes virus assembly but is also the likely binding site for HIV-1 maturation inhibitors (see below).

Env glycoprotein incorporation.

The mechanism by which the Env glycoprotein complex is incorporated into virus particles remains incompletely understood. Lentiviruses, such as HIV-1, are unique among retroviruses in bearing Env glycoproteins with very long cytoplasmic tails; these long tails must in some way be accommodated by the underlying Gag lattice during Env incorporation[44,45]. Env incorporation could, in theory, take place by several non-mutually exclusive mechanisms: a passive process; co-targeting of Gag and Env to a common site on the plasma membrane (for example, a lipid raft-type microdomain); direct recruitment of

Env by Gag; and indirect recruitment of Env by Gag via a host cell bridging protein[44] (FIG. 3).

Genetic data support a central role for the MA domain of Gag and the gp41 cytoplasmic tail of Env in Env glycoprotein incorporation, and suggest direct or indirect interactions between Env and MA[44]. Mutations in MA and the gp41 cytoplasmic tail can block Env incorporation, and an MA mutant can rescue an Env-incorporation-deficient Env mutant[46]. The ability of MA mutations to block Env incorporation is relieved by truncation of the gp41 cytoplasmic tail, suggesting that these MA mutants block Env incorporation by imposing a steric clash between MA and the long gp41 cytoplasmic tail. The notion that the gp41 cytoplasmic tail and Gag interact is supported by the observation that the Env complex in immature virions (in which Gag is uncleaved) is non-fusogenic; activation of fusion activity is triggered by cleavage of Gag by the viral protease[47,48]. Fusion activity can also be activated by truncation of the gp41 cytoplasmic tail, suggesting that an interaction between uncleaved Gag and the gp41 cytoplasmic tail locks Env in a non-fusogenic conformation. Consistent with this hypothesis, Env was able to cluster on the surface of mature but not immature virions, and this clustering was found to depend on the gp41 cytoplasmic tail[49]. Furthermore, Env was retained on immature particles from which the lipid envelope had been removed with detergent, and deletion of the gp41 cytoplasmic tail resulted in a loss of Env from such detergent-stripped particles, suggesting that direct or indirect contacts take place between the immature Gag lattice and the gp41 cytoplasmic tail[50].

One of the major impediments to understanding HIV-1 Env incorporation is the lack of structural information about either MA in the context of an infectious virus particle, or the unusually long cytoplasmic tail of gp41. Early studies showed that HIV-1 MA crystallizes as a trimer[37], a finding supported by a more recent analysis of crystals assembled on 2D membrane monolayers[38]. However, direct analysis of HIV-1 virions by cryo-ET has not revealed any long-range order in the MA shell[43]. Even less is known about the structure of the gp41 cytoplasmic tail. This region of gp41, which is approximately 150 amino acids long, could be folded in several different conformations. Peptides derived from strongly helical motifs in the cytoplasmic tail, often referred to as lentiviral lytic peptides (LLPs), bind lipid bilayers, consistent with the idea that the cytoplasmic tail interacts with the membrane. Alternatively, the gp41 cytoplasmic tail could adopt a more rod-like, helical-bundle conformation in which it projects inwards, perhaps passing through the large gap in the underlying Gag lattice[38]. It is plausible that the gp41 cytoplasmic tail could adopt a different conformation in immature versus mature virions and at different stages of the fusion process. Although the higher-order arrangement of MA in the context of the infected cell membrane or the virion is still unknown, as mentioned above, a recent study supports the hypothesis that MA trimers form in virions and are important for Env incorporation[36]. One analysis used subtomogram averaging of the putative MA lattice present in membrane-enclosed structures containing multiple CA cores, and the resultant data provided evidence for the formation of hexamers of trimers[51], consistent with the arrangement of MA on PtdIns(4,5)P$_2$-containing model membrane monolayers[38].

HIV-1 release and maturation

After assembly of the immature Gag lattice at the plasma membrane, the nascent particle must undergo a membrane fission event to be released from the cell surface. This release step is mediated by the cellular ESCRT machinery (BOX 1), which is hijacked by Gag. During or shortly after budding off of the particle from the cell surface, the viral protease cleaves the Gag polyprotein precursor to trigger HIV-1 maturation.

Virion release.

The path to our current understanding of virus release began with the observation that deletion of the C-terminal p6 domain of Gag resulted in a release block and the accumulation of particles that were attached to the cell by a thin membranous stalk[52]. Subsequently, a detailed mutational analysis mapped the virus release function of p6 to a highly conserved PTAP motif (in which the second residue can be threonine or serine) near the N terminus of p6 (REF 53). Studies performed in other retroviral systems observed similar defects in virus release following mutation of short peptide motifs located at various positions within the Gag polyprotein. These motifs, or late domains, are composed of three distinct sequences: the above-mentioned PTAP, PPXY and YPXL[54]. The basis for the ability of these viral late domains to drive virus release remained a mystery until several groups independently and almost simultaneously reported[55–58] that the PTAP motif of HIV-1 interacts directly with a cellular protein known as tumour susceptibility gene 101 (TSG101), which is part of ESCRT-I. The ESCRT machinery, which is made up of several multiprotein complexes and accessory factors, has a key role in a variety of membrane budding and scission processes in the cell (BOX 1).

Many retroviruses, including HIV-1, encode more than one late domain; in addition to the above-mentioned TSG101-binding PTAP motif, the p6 domain of HIV-1 Gag also bears a YPXL-type binding site for ALG2-interacting protein X (ALIX; also known as PDCD6IP) (BOX 1). Although the TSG101-binding PTAP motif plays the dominant part in HIV-1 release, the ALIX-binding site is also required for optimal virus replication[59]. In mammals, the ESCRT machinery is greatly expanded relative to that in other, more primitive organisms and comprises ~20 proteins[54]. However, HIV-1 (and, in all likelihood, other enveloped viruses) requires only a subset of these factors; most notably, ESCRT-II and several subunits of ESCRT-III seem to be dispensable for HIV-1 budding[60]. Ubiquitylation of cargo proteins often serves as a signal for recruitment of the ESCRT machinery, and several ESCRT components contain ubiquitin-binding domains[54]. Although it is well established that many retroviral Gag proteins are likewise ubiquitylated, a role for Gag ubiquitylation in ESCRT recruitment and virus release has not yet been definitively confirmed. However, evidence supports the hypothesis that ubiquitin, together with the viral late domains, can promote Gag-mediated ESCRT recruitment[61,62]. Recent work has demonstrated that the host protein angiomotin promotes HIV-1 budding by forming a bridge between HIV-1 Gag and the E3 ubiquitin ligase NEDD4L. Intriguingly, the virus release defect induced by angiomotin depletion seems to be imposed at an earlier stage in the budding process than that induced by disruption of the Gag-TSG101 interaction[63].

For more than a decade, a wealth of data has accumulated about the structure and function of the ESCRT machinery and its role in vesicle budding, cytokinesis and virus release. Although the precise mechanism by which the ESCRT machinery drives membrane scission remains to be fully defined, it is clear that ESCRT-III plays a central part in this process. ESCRT-III has been observed to assemble into circular arrays or spirals[64,65] that could help to constrict the membrane at the neck of the bud to drive scission (FIG. 4). Indeed, in an *in vitro* giant unilamellar system, ESCRT-III components were sufficient to drive the budding and detachment of intraluminal vesicles[66]. ESCRT-III also recruits the AAA ATPase vacuolar protein sorting 4 (VPS4), which may provide energy for the membrane scission event and is also required for the recycling of ESCRT components after budding has been completed.

Real-time imaging has been used to address the kinetics of HIV-1 assembly and release, and the timing of ESCRT recruitment to the site of assembly. Using fluorescently tagged Gag and a combination of TIRF microscopy, fluorescence resonance energy transfer (FRET) (BOX 2) and fluorescence recovery after photobleaching (FRAP), the kinetics of assembly — that is, the time from initial Gag detection to the completion of Gag accumulation — were estimated to be in the range of 5–9 minutes, with some particles taking ~20 minutes to assemble[67,68]. ESCRT-III recruitment was observed late in the assembly process, consistent with its role in mediating the final stage of particle release[69]. To delve more deeply into the mechanism of ESCRT-mediated membrane scission, advanced microscopy techniques were applied to examine the topology of ESCRT components with respect to the budding HIV-1 particle. The application of 3D super-resolution microscopy and correlative electron microscopy led to the conclusion that the ESCRT machinery localizes within the head of the budding virion, driving membrane scission from within the interior of the bud[70] (FIG. 4a). By contrast, another study using super-resolution microscopy observed the sequential accumulation of ESCRT components at the neck of the assembling virion, with the ESCRT-III component charged MVB protein 4B (CHMP4B) recruited ~10 seconds before VPS4 (REF 71) (FIG. 4b). Although these studies are all consistent with their being a central role for the assembled ESCRT-III components in driving membrane scission, details relating to the topology of the machinery and the precise role of VPS4 in the process await further enquiry.

Virus maturation.

When expressed alone, Gag is competent to drive the assembly and release of non-infectious, immature VLPs. Particle infectivity requires the proteolytic activity of the viral protease, which is expressed and brought into virions as part of the GagPol precursor. Concomitant with virus release, the viral protease cleaves a number of sites in both the Gag and GagPol polyproteins to trigger virus maturation. The HIV-1 protease is an aspartyl protease that functions as a dimer, in which the active site is located in a cleft at the dimer interface[72]. The efficiency with which the viral protease cleaves each of its target sites varies considerably, giving rise to a highly ordered, stepwise processing cascade[73]. Mutations that either increase or decrease the efficiency with which the protease cleaves a particular site or sites can be highly detrimental to maturation and particle infectivity, leading to the formation of aberrant, non-functional cores[74].

Protease-mediated Gag and GagPol processing is accompanied by a major change in virion morphology (FIG. 5a-c). In the immature particle, Gag molecules are packed in a radial manner. Following liberation of the individual Gag domains by the viral protease, the CA protein reassembles to form the conical capsid core (note that the term 'capsid' is used for both the CA protein or domain and the conical structure formed by the CA protein; here, I use the term 'CA' when referring to the protein or domain and 'capsid' when referring to the conical capsid core). It remains an open question whether and to what extent the immature CA lattice disassembles and reassembles to form the mature CA lattice, or whether it undergoes a transition to the mature lattice without complete disassembly[51,75].

The organization of CA in the mature HIV-1 capsid core is well defined. An early study correctly proposed that the capsid core assembles with fullerene-like geometry, such that CA forms predominantly hexameric rings to generate a lattice that is closed off at both ends by the inclusion of pentamers: seven at the wide end and five at the narrow end[76] (FIG. 5c). The formation of a hexameric CA lattice with 12 pentamers is a widespread feature among retroviruses, and the final shape of the capsid core is determined by the placement of the pentamers (in this sense, the structure of the retroviral capsid core is analogous to that of a soccer ball, the outer shell of which is composed of a combination of hexamers and 12 pentamers). Many studies over the years have contributed to our understanding of the core structure[77]. For example, a combination of cryo-EM and molecular dynamics simulation generated an atomic model for the HIV-1 capsid core[78]. The inter-hexamer and intra-hexamer contacts have been defined; as mentioned above, although both immature Gag and mature CA assemble into hexameric lattices, the intermolecular interfaces in the two structures differ considerably[43] (FIG. 5a,b). Notably, several studies used artificially crosslinked CA hexamers to overcome aggregation issues during the process of protein purification. However, this problem was recently solved by crystallizing unmodified CA at low protein concentrations[79]. The resulting structure provided a detailed view of the native CA-CA interfaces and revealed that water molecules play an important part in stabilizing inter-hexamer interactions[79].

In the next round of infection, the viral capsid core provides a degree of protection for the viral RNA genome as it undergoes reverse transcription. Details of how rapidly and to what extent the capsid core disassembles — a process known as uncoating — after infection are currently being investigated[80]. Sensitivity to several post-entry restriction factors maps to CA, indicating that some CA protein remains associated with the reverse transcription complex after entry[81]. An emerging view is that at least a remnant of the hexagonal CA lattice stays intact as the reverse transcription complex traffics to the nucleus; this lattice serves as the recognition signal for host cell restriction factors and is also recognized by nuclear pore components that play an active part in the nuclear import of viral DNA. The observation that both increased and decreased core stability inhibit HIV-1 infection[82] suggests that uncoating kinetics have been precisely optimized to promote infection (reviewed in REF 81).

Gag as a target for antivirals

Having recognized for many years that the Gag polyprotein precursor and the mature Gag proteins have vital roles at multiple steps in the virus replication cycle, the research community has directed considerable effort into developing inhibitors of Gag function[83]. These efforts have unfortunately not resulted in the highly efficacious drugs that have come from parallel efforts to inhibit the viral enzymes reverse transcriptase, protease and, most recently, integrase. However, recent progress has been noteworthy.

The capsid domain and protein as a target.

The central role of CA in several steps of the virus replication cycle makes it an attractive target for the development of anti-HIV-1 inhibitors. Disruption of CA-CA contacts by small molecules could block the assembly of immature particles or of the mature capsid core. As core stability seems to be precisely balanced, compounds that either accelerate or delay uncoating could also block infection by acting in the target cell. One could also imagine an inhibitor that works by 'unmasking' the core and making it more vulnerable to post-entry restriction factors that target the capsid lattice.

Although no clinically viable inhibitors targeting CA have been reported, an increasing number of compounds have been described that demonstrate antiviral activity in cell culture. Early inhibitors include the peptide CAI (for CA inhibitor) that binds the CA_{CTD} and disrupts CA dimer formation *in vitro*[84]. Although CAI is cell impermeable, circularized derivatives were developed using the technique of hydrocarbon stapling; these derivatives penetrate cells and display antiviral activity[85]. *In silico* screening identified a small-molecule inhibitor, CAP-1, that binds a pocket near the base of the CA_{NTD} by an induced-fit mechanism[86,87]. This pocket is also the reported binding site for several other small molecules, including BD3 and BM4 (REF. 88). Although all of these compounds dock in the same pocket in the CA_{NTD}, their binding seems to have different outcomes, either disrupting the assembly of immature VLPs or blocking core condensation. A second pocket in the CA_{NTD} serves as the binding site for several other CA-based inhibitors, the best characterized being PF74 (also known as PF-3450074)[89,90]. These compounds act at a post-entry, pre-integration step. Interestingly, the binding site for PF74 overlaps with that of host cell factors that regulate nuclear import (for example, nuclear pore complex protein 153 (NUP153) and cleavage and polyadenylation specificity factor 6 (CPSF6)), suggesting that compounds that occupy this pocket block infection by preventing the viral nucleoprotein complex from entering the nucleus[91]. PF74, NUP153 and CPSF6 make contacts with the CA_{NTD}-CA_{CTD} interface and the adjacent monomer in the CA hexamer, and thus bind with higher affinity to the intact hexamer than to monomeric CA[92,93]. Notably, the recently reported crystal structure of the native CA suggests that PF74 destabilizes the capsid by inducing subtle changes at inter-hexamer interfaces[79]. As anticipated from the many parts that CA plays during replication, compounds that bind CA can disrupt HIV-1 replication at several distinct steps, making them worthy of further development.

The nucleocapsid, matrix and p6 domains and proteins as targets.

Like CA, NC functions throughout the virus replication cycle; as a result, research aimed at blocking NC function has been underway for many years. Much of this effort has been aimed at developing compounds that eject zinc from the NC zinc fingers (see REFS 83,94). A limitation to this approach has been toxicity arising from a lack of specificity for retroviral NC versus host protein zinc-fingers. Although in theory MA and p6 could also be targeted by antivirals that disrupt their functions, progress in this area is at an early stage.

Maturation inhibitors.

The importance of highly ordered and complete Gag processing for HIV-1 maturation and infectivity indicates that Gag processing could be a target for novel antiretrovirals that block specific steps in the proteolytic cascade. Such inhibitors are fundamentally distinct from protease inhibitors, which act by targeting the protease enzyme rather than the substrate (Gag). The first-in-class 'maturation inhibitor' with this mode of action (that is, targeting Gag) was a dimethyl succinyl betulinic acid derivative known as PA-457 or bevirimat (FIG. 5d). This compound was shown to inhibit HIV-1 maturation by blocking the protease-mediated cleavage of the CA-SP1 processing intermediate to mature CA[95,96]. Selection studies demonstrated that resistance to bevirimat could be conferred by changes in the sequences surrounding the CA-SP1 cleavage site[95,97], and several lines of evidence supported the hypothesis that bevirimat acts by binding the CA-SP1 region of Gag in the immature particle, thereby blocking protease-mediated cleavage at this site[98,99]. Advances in defining the structure of the CA-SP1 boundary region in the context of assembled immature Gag will be required for the precise determination of the bevirimat binding site, although insights into the mechanism of maturation inhibitor binding were obtained from the study of the structurally distinct compound PF-46396. Based on the study of PF-46396-resistant mutants, it seems likely that bevirimat and PF-46396 bind the same pocket in the assembled Gag complex[100]. Phase II clinical trials with bevirimat demonstrated the proof of concept that maturation inhibitors can significantly reduce viral loads in infected patients[101]; however, naturally occurring polymorphisms in SP1 compromised the activity of bevirimat[102]. Recent progress has been made in developing bevirimat analogues with low-nanomolar potency even against strains of HIV-1 bearing a variety of SP1 polymorphisms (Urano, E., Ablan, S. and E.O.F., unpublished observations), and it is anticipated that these potent bevirimat analogues will be tested in future clinical trials.

Outlook

Recent years have witnessed remarkable advances in our understanding of the late phase of the HIV-1 replication cycle, including virus assembly, release and maturation. However, many questions remain to be fully answered. For example, how does Gag traffic to the plasma membrane, and what is the physical and chemical nature of the sites where Gag and Env make their first encounter at the membrane? What are the mechanisms of action for the various host cell proteins that have been reported to have roles in Gag trafficking, particle assembly and Env incorporation? How does the ESCRT machinery drive membrane scission, and how does virus budding bypass the need for ESCRT-II in the budding process? What is the structure of the MA lattice in the immature and mature virion? What is the

topology of the gp41 cytoplasmic tail with respect to the viral membrane, and in what manner does this tail interface with MA? What is the structure of the CA-SP1 boundary region in the assembled Gag lattice, and how do maturation inhibitors bind to this region? It is well established that the host restriction factor tetherin (also known as BST2) can block particle release from the cell surface after completion of the budding process[103,104], but to what extent do other host restriction factors target virus assembly and budding? Answers to these questions will continue to fundamentally advance our understanding of the HIV-1 replication cycle and will provide information that is key to exploiting the late stages of this cycle as targets for therapeutic intervention.

Acknowledgements

Glossary

Capsid
The electron-dense structure at the centre of the mature virus particle. The capsid is composed of an outer layer of capsid protein surrounding the viral RNA genome and the viral enzymes reverse transcriptase and integrase.

Maturation
The morphological transition from the immature virus particle, in which uncleaved Gag proteins are aligned in a radial manner inside the viral membrane, to the mature particle, which contains a condensed, conical core.

Envelope glycoproteins (Env glycoproteins).
The heterotrimeric complexes of surface glycoprotein (gp 1 20) and transmembrane glycoprotein (gp41) that are packaged into the viral membrane and mediate receptor-co-receptor binding and fusion in the next round of infection.

Assembly
The process by which viral proteins and nucleic acids come together in an infected cell to produce new virus particles.

Protease
The viral enzyme that cleaves multiple sites in Gag and GagPol during maturation.

Reverse transcriptase
The viral enzyme that converts the single-stranded viral RNA genome to double-stranded DNA after virus entry into the cell.

Integrase
The viral enzyme that is responsible for catalysing the insertion of the newly synthesized viral DNA into the host cell genome.

Endosomal sorting complex required for transport (ESCRT)
A multi-complex machinery that comprises ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III, and that promotes membrane scission reactions (for example, during vesicle budding, cytokinesis and enveloped-virus budding).

3_{10} helix
A structural element within a protein; it is composed of a right-handed helix with three residues per turn, in which the first and third residues hydrogen bond with each other. The 3_{10} helix is more tightly wound than the more common α-helix.

Cyclophilin A (CYPA)
A member of the family of peptidyl prolyl isomerases that facilitate protein folding.

Multivesicular body (MVB)
A late endosome containing intraluminal vesicles.

Phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2).
A phospholipid that plays an important part in the association of HIV-1 Gag with the inner leaflet of the plasma membrane.

Virus-like particles (VLPs)
Non-infectious particles formed by the expression of Gag alone, or newly budded particles before maturation.

Maturation inhibitors
Small molecules that block virus maturation by preventing a specific late step in the Gag processing cascade.

Late domains
Small peptide motifs in retroviral Gag proteins that recruit cellular machinery (that is, endosomal sorting complexes required for transport) to the site of budding to promote virus release.

Angiomotin
A cellular protein that is implicated in endothelial cell migration and in the formation of tight junctions.

Fullerene-like geometry
The structural arrangement of atoms as observed in certain elemental forms of carbon; in their spherical arrangement, fullerenes (such as buckmin-sterfullerene) are composed of hexagonal rings of carbon with 1 2 pentameric rings, allowing the structure to adopt a closed conformation. An analogous arrangement of hexameric and pentameric rings is observed in the soccer ball (football, for the non-American reader) and in the retroviral core.

Hydrocarbon stapling
A chemical method of circularizing peptides, thereby stabilizing their conformation and enhancing their cellular penetration.

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Cellular factors involved in virus assembly and release

Gag partners during assembly

The steps that Gag follows to reach the plasma membrane have not been clearly defined, and likewise, the host factors that promote Gag trafficking to the site of assembly remain only partially characterized. The phospholipid phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P$_2$) is now well established as a host cell determinant of Gag binding to the plasma membrane (see the main text), and a number of host proteins have also been implicated in various aspects of Gag localization, membrane binding and assembly[16]. Further studies will be required to elucidate the specific roles of these protein factors in HIV-1 replication.

gp41 partners during envelope glycoprotein incorporation

The function of the gp41 cytoplasmic tail in envelope (Env) incorporation and virus replication is strongly cell type dependent[105]; it is indispensable in most T cell lines and in physiologically relevant primary T cells and macrophages, but is not required in several adherent cell lines and in the MT-4 T cell line. This cell type dependence suggests a role for host factors in Env incorporation. Several host factors have been postulated to bind matrix (MA) and/or the gp41 cytoplasmic tail and thereby promote Env incorporation[44]. For example, Rab11 family-interacting protein 1 isoform C (Rab11-FIP1C) was reported to promote Env trafficking to the plasma membrane and Env incorporation into virions[106].

Endosomal sorting complex required for transport

Studies in yeast in the early 2000s revealed that vacuolar protein sorting 23 (Vps23), the yeast homologue of tumour susceptibility gene 101 (TSG101), is part of endosomal sorting complex required for transport I (ESCRT-I), which together with several other ESCRT complexes (ESCRT-0, ESCRT-II and ESCRT-III) and the AAA ATPase VPS4 complex, forms a highly conserved apparatus that plays a vital part in the sorting of ubiquitylated cargo proteins into vesicles. These vesicles bud into late endosomes to form multivesicular bodies (MVBs). The PTAP late domain of Gag (in which the second residue is threonine or serine) interacts with the ESCRT-I component TSG101. The YPXL late domain of Gag was found to bind ALG2-interacting protein X (ALIX), a protein that in mammals interacts directly with both ESCRT-I and ESCRT-III. The ESCRT machinery was also shown to be required for other membrane scission events in the cell, most notably the abscission step of cytokinesis[107][108]. Indeed, the use of primitive ESCRT-III and VPS4 homologues in cell division seems to be an ancient and primordial activity of the ESCRT machinery, as this function is conserved in the domain Archaea[109]. Thus, in a remarkable convergence of virology and cell biology, it became clear that retroviruses evolved to co-opt a cellular machinery that promotes membrane scission reactions topologically equivalent to virus budding (that is, those facing away from the cytoplasm) (FIG. 4). The use of the ESCRT machinery for particle release has been established not only for retroviruses but also for several other families of enveloped

viruses (for example, filoviruses, paramyxoviruses and arenaviruses) and even for a virus that infects the archaeal organism *Sulfolobus solfataricus* [110].

Techniques to analyse virus assembly and release

Super-resolution fluorescence microscopy

The diffraction limit sets the resolution of the light microscope to approximately half the wavelength of light being measured (~250 nm for blue light). As a result, a point source emitter, such as a blue dye, is observed as being ~250 nm in diameter, preventing the resolution of objects closer together than this distance. Super-resolution techniques allow considerably higher resolutions, in the range of tens of nanometres, to be achieved. There are several types of super-resolution (or super-resolved) microscopy, such as photoactivated localization microscopy (PALM), stimulated emission depletion (STED) microscopy and stochastic optical reconstruction microscopy (STORM) (reviewed in REF 111). The use of super-resolution techniques to study HIV-1 budding and release is described in the main text.

Cryoelectron microscopy and cryoelectron tomography

Rapid freezing of the sample followed by electron microscopy analysis performed at very low temperatures enables data to be gathered on native samples in the absence of a fixative. Combined with controlled exposure to the electron beam, this minimizes damage to the sample. Analysis of a large number of identical particles allows averaging to generate a high-resolution 3D volume. A limitation of cryoelectron microscopy (cryo-EM) is that it requires the particles being analysed to be identical or nearly identical. Cryo-EM has thus been a powerful technique for the analysis of viruses with identical morphologies, but it is less useful for highly pleomorphic viruses such as HIV-1. In such cases, cryoelectron tomography (cryo-ET) is used to obtain a number of 2D electron microscopy images of an individual particle over a range of tilt angles. These 2D images are then combined to obtain a medium-resolution 3D reconstruction. Subvolume or subtomogram averaging techniques greatly increase the resolution by averaging common features present in the pleomorphic specimens. When available, atomic-resolution data from X-ray crystallography or NMR spectroscopy can be fitted into the electron microscopy density maps to produce pseudo-atomic models of large and complex structures (reviewed in REF. 112).

Total internal reflection fluorescence microscopy

Using an evanescent wave of light, total internal reflection fluorescence (TIRF) microscopy illuminates a thin section of the sample within ~100 nm of the cover slip. This method, which is often applied to single-molecule experiments, permits high-resolution information to be obtained about events occurring at the cell surface, such as virus budding (reviewed in REF. 113).

Fluorescence resonance energy transfer

In fluorescence resonance energy transfer (FRET; also called Forster resonance energy transfer), a donor fluorophore is excited by a specific wavelength of light; the donor can then transfer energy through non-radiative coupling to an acceptor fluorophore located in close proximity (in the range of 1–10 nm) to the donor, provided that the fluorescence emission spectrum of the donor overlaps with the absorption spectrum of the acceptor.

Energy transfer between donor and acceptor is highly dependent on their close proximity and thus provides a sensitive measure of the distance between the two fluorophores. This method is often used to measure protein-protein and protein-nucleic acid interactions in cells or *in vitro* (reviewed in REF 114).
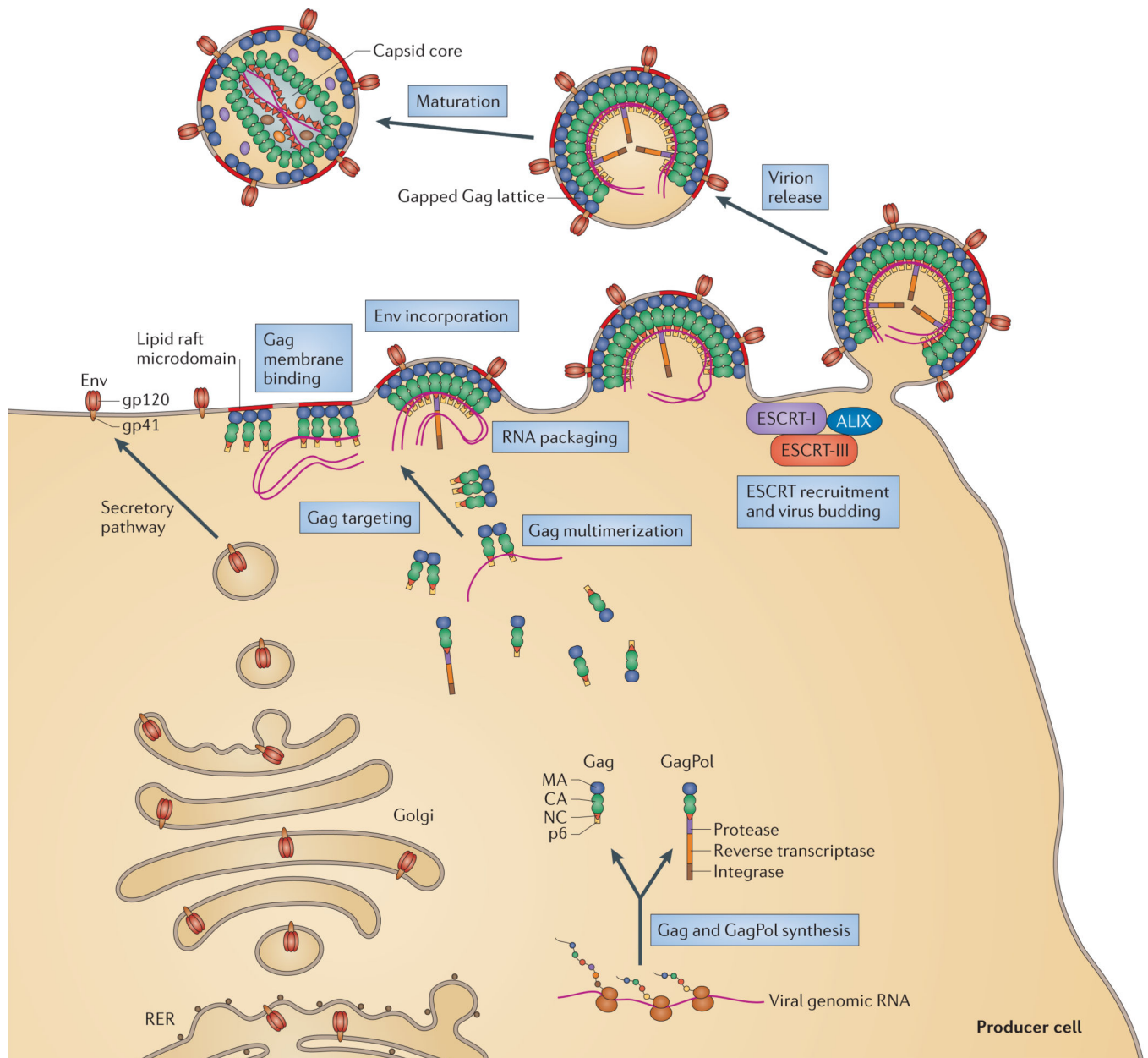
Figure 1 |. The late stages of the HIV-1 replication cycle.

The viral envelope (Env) glycoproteins traffic via the secretory pathway, from the rough endoplasmic reticulum (RER) to the Golgi and then in vesicles until they arrive at the plasma membrane. The Gag precursor polyprotein — which contains the matrix (MA), capsid (CA), nucleocapsid (NC) and p6 domains — is synthesized in the cytosol from full-length viral RNA. The GagPol precursor polyprotein — which contains MA, CA, NC, protease, reverse transcriptase and integrase domains — is synthesized as the result of a programmed frameshifting event during the translation of Gag-encoding viral RNA. Gag recruits the viral genomic RNA, begins to multimerize and reaches the plasma membrane by a still-undefined pathway. Gag is anchored to the plasma membrane in lipid raft microdomains via insertion of its amino-terminal myristate into the lipid bilayer and by

direct interactions with the phospholipid phosphatidylinositol-(4,5)-bisphosphate. The assembling particle incorporates Env and then recruits endosomal sorting complex required for transport I (ESCRT-I)via a direct association between the PTAP motif in p6 and the tumour susceptibility gene 101 (TSG101) subunit of ESCRT-I. Gag also engages in direct binding with the ESCRT-associated factor ALG2-interacting protein X(ALIX), primarily through the YPXL motif in p6. As the budding process proceeds, the ESCRT-III and vacuolar protein sorting 4 (VPS4) complexes are recruited and drive the membrane scission reaction that leads to particle release. Maturation, which leads to the formation of the conical capsid core, is triggered by proteolytic cleavage of the Gag and GagPol polyprotein precursors by the viral protease.
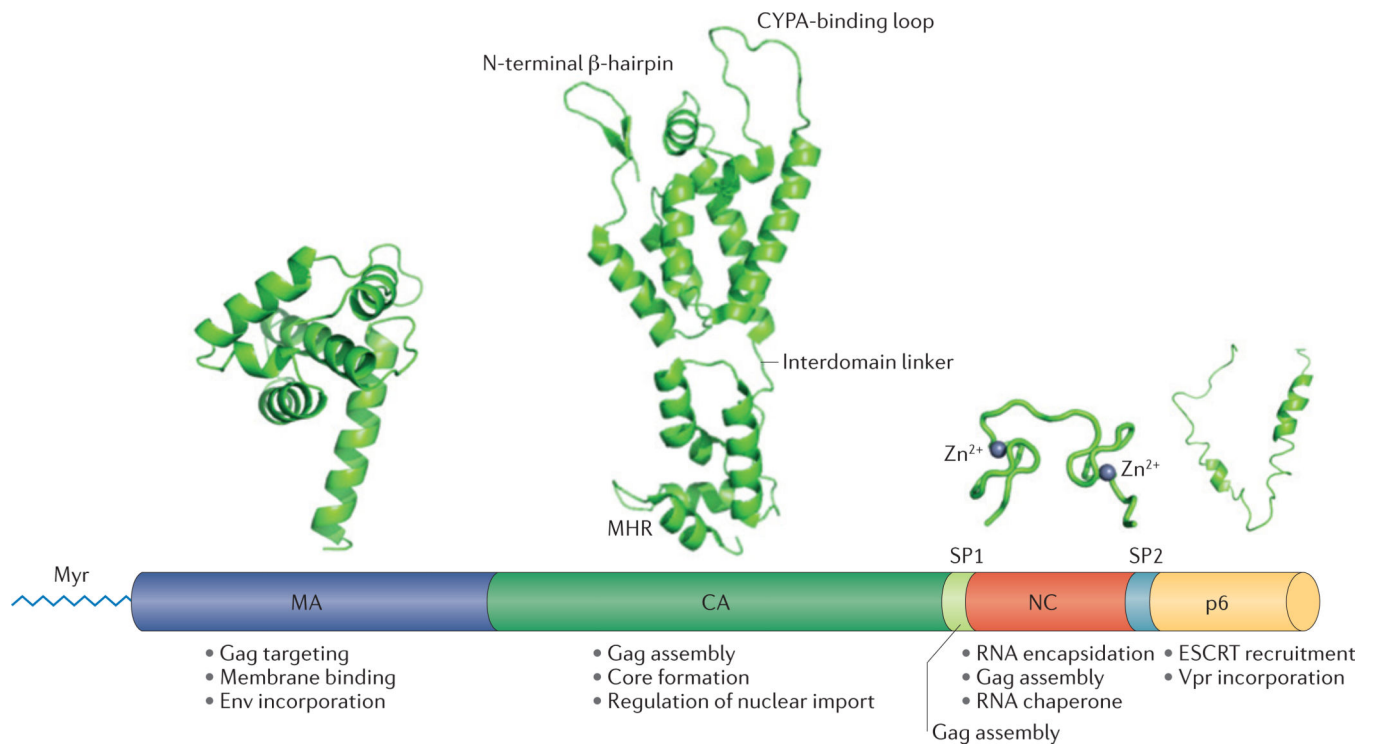
Figure 2 |. Gag structure and functions.

High-resolution structures of the major domains of Gag — matrix (MA), capsid (CA), nucleocapsid (NC) and p6 — are presented over a linear depiction of the Gag precursor polyprotein. The major functions of each Gag domain are indicated. MA (Protein Data Bank (PDB) accession 1HIW)[37] is involved in Gag targeting and binding to the plasma membrane, and in incorporation of the envelope (Env) glycoprotein. Membrane binding requires amino-terminal myristylation (Myr). CA (PDB accession 2M8N)[115] participates in Gag assembly, formation of the conical capsid core and regulation of the nuclear import of viral DNA. The CA N-terminal domain (CA_{NTD}) contains an N-terminal β-hairpin and a proline-rich loop that binds the host protein cyclophilin A (CYPA); the CA carboxy-terminal domain (CA_{CTD}) contains the major homology region (MHR). CA_{NTD} and CA_{ctd} are connected by a short, flexible interdomain linker. Spacer peptide 1 (SP1) is involved in Gag assembly. NC (PDB accession 1BJ6)[116] contains two zinc-finger domains; it participates in Gag assembly and RNA encapsidation, and serves as an RNA chaperone. p6 (PDB accession 2C55)[8] is involved in recruitment of the endosomal sorting complex required for transport (ESCRT) components and in Vpr incorporation.
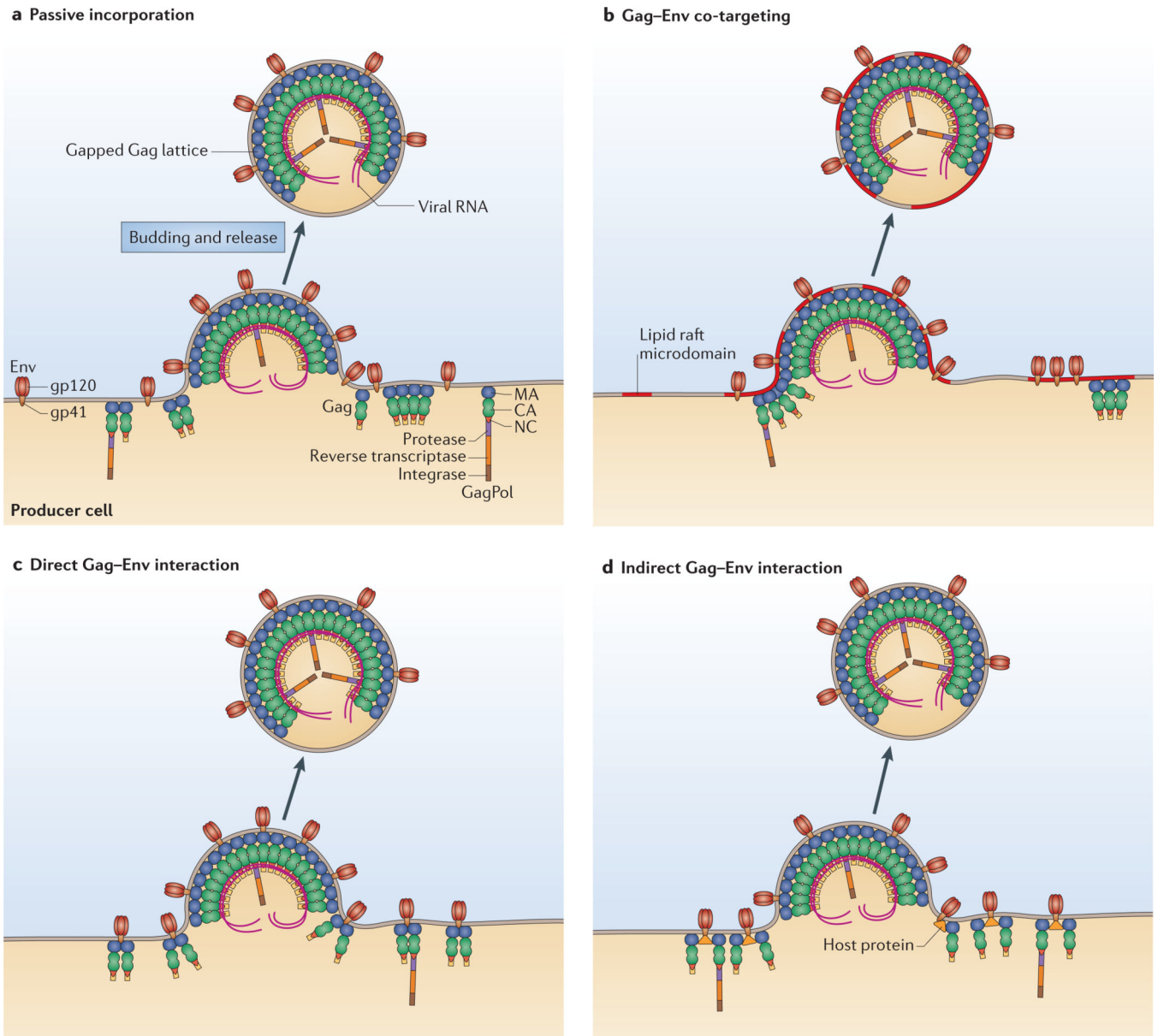
Figure 3 |. Models of viral envelope glycoprotein incorporation.
Several non-mutually exclusive mechanisms may participate in the incorporation of the viral envelope (Env) glycoproteins into new virions. **a** | Env expressed on the cell surface might be incorporated passively into virions as the particles acquire host-derived membrane during assembly and budding. **b** | In a putative Gag-Env co-targeting mechanism, both Gag and Env are targeted to a specific site on the plasma membrane (for example, a lipid raft microdomain), allowing Env to be concentrated at sites of assembly. **c** | Evidence suggests that the matrix (MA) domain of Gag and the cytoplasmic tail of the gp41 subunit of Env interact directly, resulting in the retention of Env complexes at sites of budding and their recruitment into virions. **d** | The MA domain of Gag and the cytoplasmic tail of gp41 could interact indirectly, with a host protein bridging the two viral proteins. CA, capsid; NC, nucleocapsid. Figure adapted from REF 44, Elsevier.
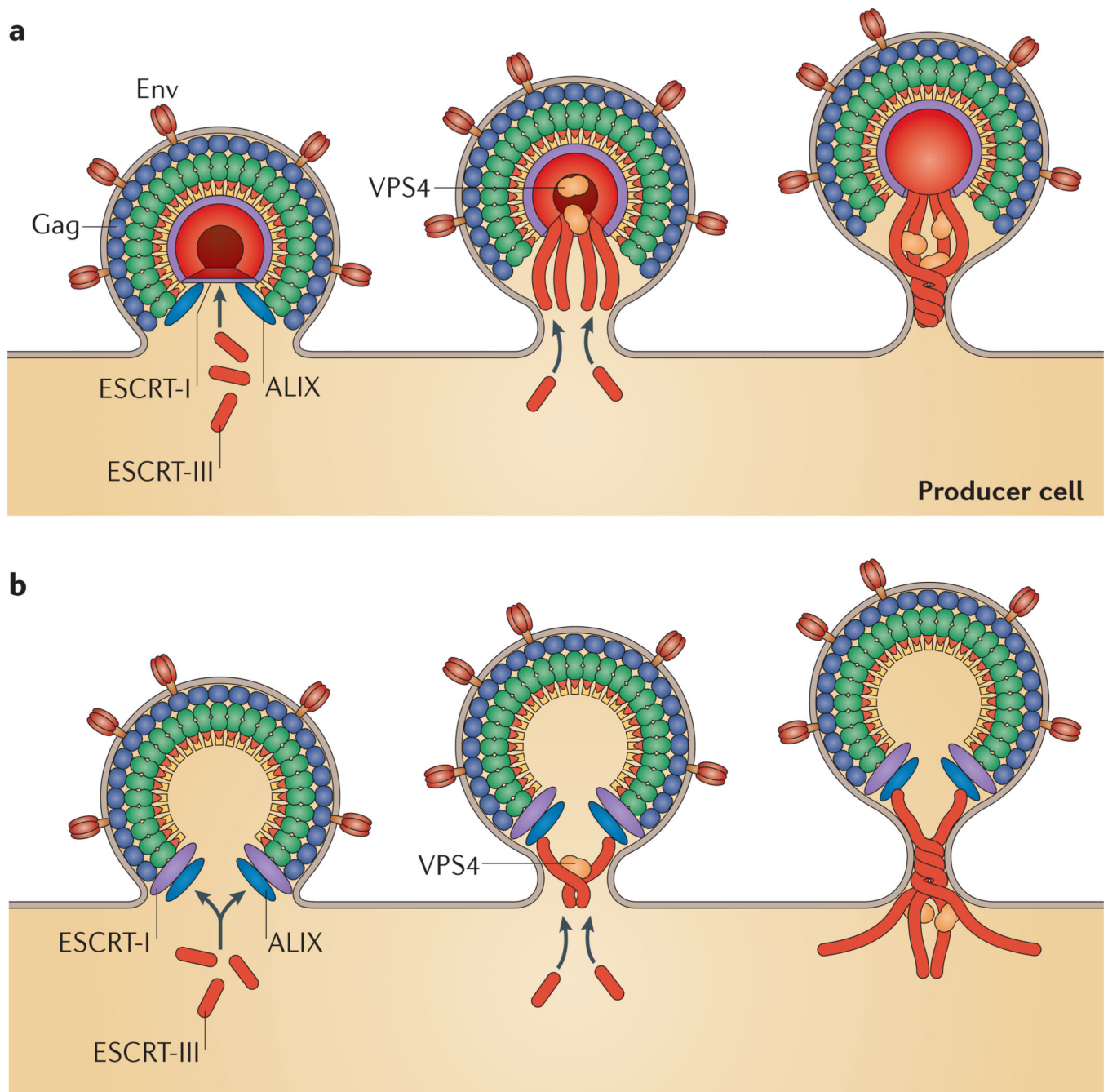
Figure 4 |. HIV-1 budding and release.

Models for membrane scission mediated by endosomal sorting complex required for transport III (ESCRT-III). In both models, the HIV-1 Gag protein interacts with ESCRT-I and ALG2-interacting protein X(ALIX), which recruit ESCRT-III. Membrane scission is driven by ESCRT-III polymerization in concert with the activity of the AAA ATPase vacuolar protein sorting 4 (VPS4). **a** | In the first model, ESCRT-III is localized within the budding particle[70]. **b** | In the second model, ESCRT-I and ALIX are located within the budding particle and recruit ESCRT-III and VPS4, which are mostly located outside the
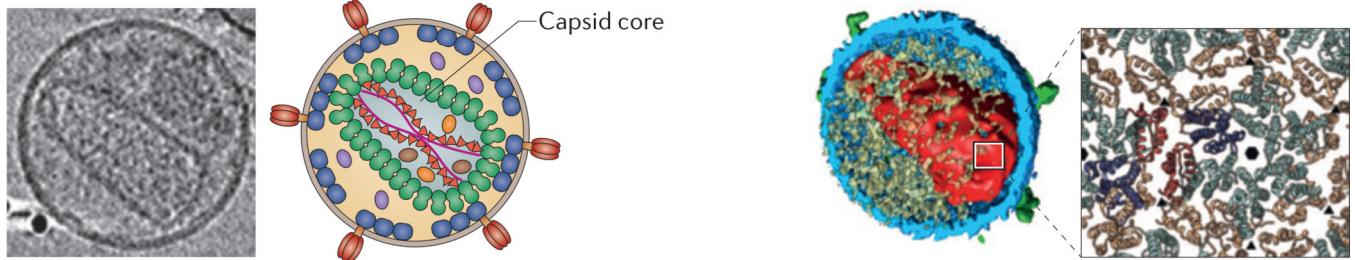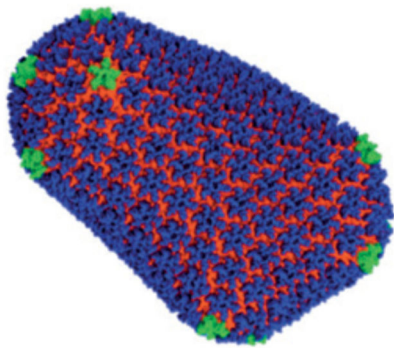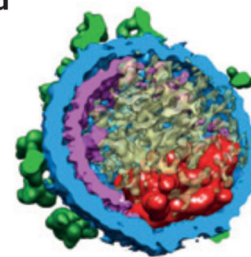
budding particle. Env, envelope. Part **a** adapted from Van Engelenburg, S. B. *et al.* Distribution of ESCRT machinery at HIV assembly sites reveals virus scaffolding of ESCRT subunits. *Science* **343**,653–656 (2014). Reprinted with permission from AAAS. Part **b** adapted from REF 117, © Cashikar *et al.*
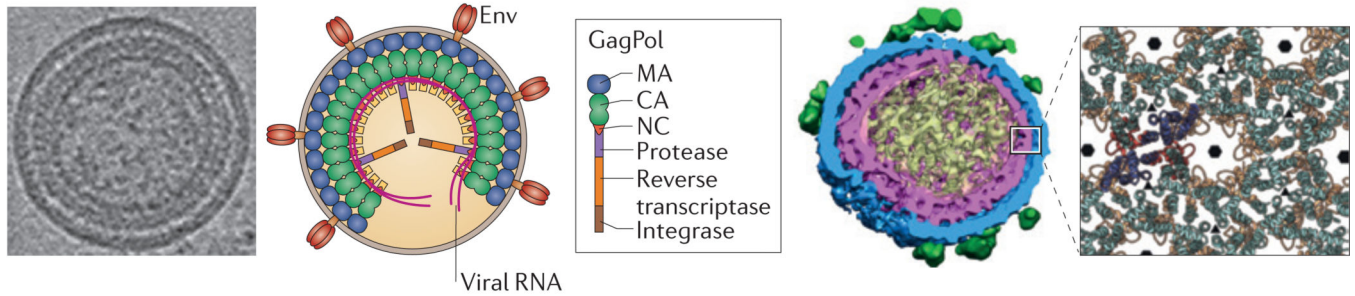
Figure 5 |. HIV-1 maturation.

During virus release, the viral protease cleaves a number of sites in both the Gag and GagPol polyproteins to trigger virus maturation, resulting in major changes in virion morphology, includin g the generation of the conical capsid core. **a** | A cryoelectron tomogram (left panel), an illustration (middle panel) and a cryoelectron tomography (cryo-ET) reconstruction (right panel) of the immature HIV-1 virion, together with a zoomed-in view of the immature Gag lattice (far-right inset). In the far-right panel, the capsid (CA) amino-terminal domain (CA_{NTD}) is depicted in blue and the CA carboxy-terminal domain (CA_{ctd}) is in orange. **b** | A cryoelectron tomogram (left panel), an illustration (middle panel) and a cryo-ET reconstruction (right panel) of the mature HIV-1 virion, together with a zoomed-in view of the mature CA lattice (far-right inset; the CA_{NTD} and CA_{CTD} are coloured as in part **a**). **c** | The all-atom structure of an HIV-1 capsid core, with CA pentamers (green) in the otherwise hexameric lattice. **d** | A cryo-ET reconstruction of a virion produced from cells treated with the maturation inhibitor bevirimat, showing disruption of capsid formation. Env, envelope; MA, matrix; NC, nucleocapsid. Cryoelectron tomograms in part **a** and **b** reproduced from REF. 118, Elsevier. Cryo-ET reconstructions in parts **a**, **b** and **d** republished with permission of the American Society for Microbiology, from HIV 1

maturation inhibitor bevirimat stabilizes the immature Gag lattice. Keller, P. W. *et al.* **85**, 4, 2015; permission conveyed through Copyright Clearance Center. Zoomed-in views in parts a and b reproduced from REF 43, Nature Publishing Group. Part **c** reproduced from REF 78, Nature Publishing Group.