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Intracellular Destinies: Degradation, Targeting, Assembly, and Endocytosis of HIV Gag

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Abstract

The HIV-1 Gag protein assembles into immature capsids when expressed in human cells. Although self-assembly of Gag was once thought to be sufficient to explain capsid formation, in the past decade it has become increasingly apparent that in cells, the pathway from Gag synthesis to assembled capsids is coordinated and facilitated by host factors. These cellular factors likely direct the trafficking, membrane targeting, and multimerization of Gag, and could also assist with encapsidation of viral RNA. While some of these factors have been identified, much remains to be learned about the mechanisms by which they act to promote capsid formation. Moreover, studies suggest that the amount of intracellular Gag undergoing assembly per se at any given time may be quite low, with the majority of Gag in some cell types undergoing degradation or representing Gag that remains cell-associated after assembly. If this model holds true, then defining the Gag subpopulations on which individual cellular factors act will be important for understanding the role of host factors. Towards this end, it will be important to find markers and features that can distinguish subpopulations of Gag destined for different outcomes so that these populations can be quantified and tracked separately both at the biochemical and microscopic level. Thus, the challenge for the future will be to understand which cellular factors act during the pathway from Gag synthesis to assembly, and exactly where and how they act in this pathway. **(AIDS Rev. 2007;9:150-61)**

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Key words

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Gag and its structural components: different functions at distinct points in the viral lifecycle

It is well established that Gag is the only HIV-encoded protein that is needed to make noninfectious virus-like particles (VLP) that lack viral RNA. Despite requiring only one viral protein, the process of assembly is quite complex because many events that are critical for virus production need to be coordinated temporally and spatially within the host cell during VLP formation. These events include synthesis of the structural proteins Gag and GagPol, encapsidation of cellular RNA, proper targeting of viral particle components to the site of assembly, multimerization of Gag to form a spherical immature capsid (which will be referred to here as assembly), budding of the capsid into a host-derived lipid bilayer, and release of virus from the cell (Fig. 1A). The task is even more complicated during assembly of infectious virus, which requires all the events described above as well as specific encapsidation of two copies of the full-length HIV genome and packaging of additional viral proteins within the viral capsid. For all of these reasons, the events that lead to HIV assembly in cells are likely to be highly regulated.

The HIV-1 Gag polyprotein consists of four structural domains, matrix (MA), capsid (CA), nucleocapsid (NC), and p6, as well as two small spacer sequences. In addition to these structural subdomains, Gag also contains three functional domains defined by their spe-

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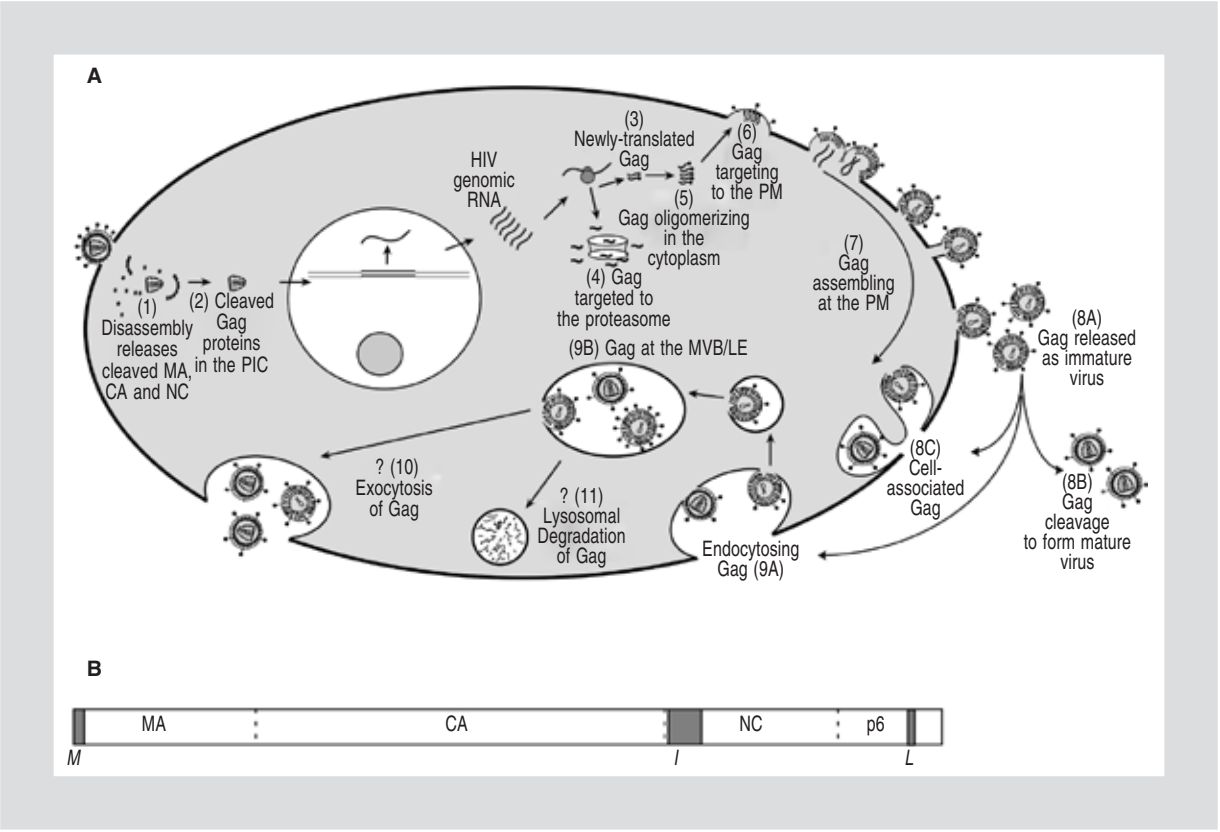

Figure 1. ***A.*** *The many subpopulations of HIV Gag in human cells. This diagram illustrates the many different fates and functions of the Gag cleavage products (early in the lifecycle) and of full-length Gag (late in the viral lifecycle). When the mature virus enters the cell, the cleaved Gag proteins are released into the cytoplasm upon disassembly of the viral core (1). Matrix (MA) becomes a component of the pre-integration complex (PIC), which transports the newly reverse-transcribed HIV genome to the nucleus (2). Following HIV integration and nuclear export of subgenomic transcripts, genomic RNA is exported to the cytoplasm where it is used as a template for translation of full-length Gag (3). A large fraction of newly synthesized Gag is degraded by the proteasome (4). During immature capsid (CA) assembly, only full-length Gag polypeptides, and not the cleaved products of Gag, undergo assembly. Gag polypeptides likely undergo oligomerization in the cytoplasm (5) and then target to the plasma membrane (6) where they undergo higher-order multimerization into completely assembled immature capsids (7). The completely assembled immature capsids bud out of the plasma membrane (PM) resulting in release of immature virus (8A), which undergoes maturation at the time of release, leading to cleavage of the Gag polyprotein into the MA, CA, nucleocapsid (NC) and p6 proteins (8B). Note that in some cell-types, such as macrophages, completed virus can be held in complex cell-associated compartments (8C), or can be endocytosed (9A) and transported via vesicles to multivesicular bodies (MVB) and/or late endosomes (LE) (9B). It remains to be determined whether infectious virus within intracellular compartments can be released from the cell via exocytosis (10) or can undergo endolysosomal fusion resulting in lysosomal degradation (11). As described in the text, some subpopulations and trafficking pathways outlined in this model are still controversial and will need further validation; furthermore, other populations not shown in this diagram may also exist.* ***B.*** *Structural and functional domains of HIV Gag. Full-length Gag is composed of structural domains, matrix (MA), capsid (CA), nucleocapsid (NC), and p6, that are cleaved during maturation, as well as functional domains (*M, I, *and* L*) indicated by shading.*

cific contributions to late events in the viral life cycle. These functional domains (shown in Fig. 1B) include the M domain in the N-terminus of MA (responsible for membrane binding); the I domain in NC (which mediates Gag-Gag interactions); and the L domain located in p6 (which governs budding and release). It should be noted that the first three domains of Gag (MA, CA, and NC) are sufficient for targeting and assembly. In contrast, p6 recruits a network of cellular factors (the ESCRT proteins, reviewed[61]) that promote budding and release, which are distinct steps in the HIV lifecycle that follow immature capsid assembly.

In an infected cell, full-length 55 kDa Gag polypeptides undergo assembly to form immature capsids. Approximately 2000 to 5000 Gag polypeptides are required to form a single spherical immature HIV-1 capsid[9], which has a diameter of ~100 nm[36] and a sedimentation value of ~750S[56]. Upon assembly into an immature capsid, full-length Gag is cleaved during virion maturation into its four structural domains by the HIV-1 protease. Gag cleavage induces a restructuring event, yielding a conical capsid that resides within the envelope of the mature infectious virus. Notably, each of the four structural domains of Gag has a specific

role within full-length Gag during assembly and a different role following cleavage, when each acts as an independent protein. Within full-length Gag, the MA domain promotes membrane targeting, CA and NC are critical for multimerization, and the p6 domain is required for budding; in contrast, the cleaved Gag domain proteins act after virus infection of a target cell during the post-entry events, which include viral uncoating and disassembly, interaction with host restriction factors, formation of the pre-integration complex (PIC), and transport of the PIC from the cytoplasm to the nucleus (reviewed[35,39,94]; partially diagrammed in Fig. 1A). The dramatically different behavior of full-length Gag polypeptides during assembly compared to cleaved Gag domains post-entry most likely reflects intrinsic differences in the structure of the intact versus cleaved proteins, disparities in their cytoplasmic concentrations, and the interaction of full-length Gag and the cleaved proteins with different cellular factors. Because the cleaved Gag domain proteins do not initiate immature capsid assembly *in vivo,* studies that rely exclusively on one or more cleaved Gag domains to address questions of targeting and assembly, either recombinantly or in cells, should be interpreted with caution. While such studies can lead to useful insights, they also have significant limitations, since only full-length Gag has all the properties required for targeting to membranes, assembly into proper immature capsids in cells, and release.

Shifting paradigms: from self-assembly to assembly facilitated by cellular factors

Two decades ago HIV-1 Gag assembly was viewed as resulting largely from Gag-Gag interactions, but evidence increasingly suggests that assembly is instead a product of complex viral-host interactions. This shift has resulted in part because of data obtained from new systems for studying assembly. In the mid 1990s, studies of recombinant Gag *in vitro* advanced our understanding of assembly by demonstrating that purified Gag polypeptides at high concentrations in isolation with RNA can assemble into small spherical structures[14,15]. Although these studies provided critical information about the intrinsic properties of Gag, they did not address how Gag assembles in an intracellular environment because this system does not contain other proteins besides Gag. In the late 1990s investigators began to ask how viral-host interactions contribute to Gag targeting and assembly in cell extracts and in cells. Cell-free HIV capsid assembly systems[56,96], which use cellular extracts to faithfully reconstitute synthesis and assembly of Gag into immature capsids, were devised at that time, allowing the viral-host interactions that occur during these events to be studied *in vitro.* In 1997 our group used such a system to provide evidence that one or more cellular factors present in a eukaryotic cellular extract are critical for immature HIV-1 capsid assembly[56] and from this system isolated ABCE1[109], a cellular factor that promotes HIV-1 capsid assembly. Since the identification of ABCE1, newer techniques like siRNA knockdowns, in combination with more classical techniques for overexpression of wild-type and dominant-negative proteins, have led to identification of a number of additional cellular factors that appear to be involved in Gag trafficking and/or assembly, including phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$)[67], the δ subunit of the adaptor protein 3 complex (AP-3δ)[30], Staufen1[18,63], and others. While selected factors implicated in the pathway from Gag synthesis to immature capsid assembly will be discussed in more detail below, a comprehensive discussion of cellular factors involved in the entire retroviral lifecycle has recently been published elsewhere[39].

Because of studies such as those referenced above, it is now generally accepted that cellular factors facilitate HIV-1 assembly when Gag is expressed in mammalian cells. However, consensus has not been achieved on exactly which of these factors are required for HIV capsid assembly, as well as when, where, and how they act. Disrupting events such as encapsidation of RNA or other virus components, targeting Gag to membranes, Gag multimerization, and virion budding will lead to effects on virus release from cells, an easily assayed endpoint. Thus, pinpointing the exact mechanism of action of individual cellular factors implicated in late events of the virus life cycle will require a rigorous assessment of how knockdowns affect each step in the pathway to virion formation.

Studies in murine cells have highlighted the importance of host factors acting at one particular step in the pathway from Gag synthesis to assembly, namely membrane binding of Gag. While membrane targeting of wild-type HIV-1 Gag occurs in most eukaryotic cell lines, targeting and the subsequent events of assembly fail to occur in some rodent cell lines, even when these cells are engineered to overcome restrictions in HIV entry and transactivation[6,58]. Although Gag targeting and assembly are clearly concentration-dependent and cooperative in human[76] and murine[43] cell lines, levels of Gag expression do not appear to explain the lack of wild-type Gag targeting and assembly in murine NIH3T3 cells[22,23,57,99]. In addition, myristoylation, which is required for proper membrane targeting of Gag, is not defective in murine cells[43,50,99]. Moreover, studies of murine-human heterokaryons have demonstrated that murine cells are missing a factor or factors required for HIV assembly[6,57]. Consistent with this, mouse-human somatic cell hybrids containing human chromosome 2 allowed virion production in murine

cells, further supporting a requirement for a specific cellular factor(s) in Gag targeting[23].

HIV genomic RNA: at the intersection of synthesis, packaging, and assembly

Studies of assembly in murine cells have also demonstrated that the pathway used by HIV genomic RNA during nucleocytoplasmic transport influences later events of Gag targeting and assembly[99]. These data suggest that understanding host factors involved in HIV genomic RNA trafficking will likely lead to important insights into the role of cellular factors in assembly. For this reason, we will discuss selected aspects of RNA trafficking that pertain to assembly.

Because Gag is translated in the cytoplasm from unspliced full-length (genomic) HIV-RNA, the virus requires a means of bypassing the mechanisms that eukaryotic cells employ to retain unspliced RNA in their nuclei. In the case of HIV-1 infection in human cells, the viral Rev protein achieves this by mediating nuclear export of unspliced HIV genomic RNA via the Crm1 export pathway (reviewed[24,78]), while other retroviruses utilize different mechanisms and export pathways. Notably, a study by Swanson, et al.[99] demonstrated that the pathway used for nuclear export dictates the fate of Gag polypeptides that are translated from these exported RNA (reviewed[98]). When murine cells were transfected with an HIV-RNA construct that used the Crm1 pathway for nuclear export, Gag synthesis occurred but membrane targeting and assembly failed. In contrast, when the native Rev-response element in HIV genomic RNA construct was replaced with an RNA element from Mason-Pfizer monkey virus (the constitutive transport element) that mediates nuclear export via the NXF1/Tap pathway, Gag synthesis occurred at equivalent levels and, surprisingly, Gag was also able to bind to membranes, assemble, and release mature virus[99]. The authors of this study proposed that nuclear export pathways "mark" RNA differently. Proper marking of genomic RNA during export could lead either to trafficking of genomic RNA to a specific cytoplasmic microdomain for translation, and/or to recruitment to genomic RNA of specific factors, which in turn act directly or indirectly to facilitate assembly of newly synthesized Gag. It should be noted that this study did not assess whether restoration of targeting, assembly, and release of Gag in murine cells through altering nucleocytoplasmic transport of HIV-RNA results in production of infectious virus particles[99]. Thus, it is possible that additional factors, besides those acquired during RNA export through the NXF1 pathway, are required to overcome all the late defects in virus production in murine cells. Nevertheless, these studies suggest that factors acquired during HIV-RNA transport may specify a series of events that ultimately influence events of immature capsid assembly.

Once in the cytoplasm, retroviral genomic RNA acts both as a template for translation of the Gag and Gag-Pol proteins and as a source of genomic RNA for packaging into newly formed viral capsids. Studies of murine leukemia virus (MLV) and other retroviruses demonstrated that the genomic RNA of these viruses are present in two distinct cytoplasmic pools, one that undergoes translation and another that is packaged. In contrast, studies of HIV-1 suggest that one pool of HIV-1 genomic RNA is available both for packaging and translation (reviewed[11]). In the case of HIV-2, Gag appears to preferentially associate with the genomic RNA template used for its translation[46], and this may occur before Gag is transported to the site of assembly. However, such a requirement for translation from an mRNA prior to packaging of that RNA has not been demonstrated for HIV-1[12]. Thus, HIV-1 may employ another mechanism to ensure that enough Gag is synthesized and that genomic RNA is accessible for packaging, both of which are required for efficient virion production. One such mechanism could involve preferential use of viral RNA as a template for translation when concentrations of Gag are low, followed by inhibition of translation at higher Gag concentrations in order to make genomic RNA available for packaging. Consistent with this model, recent studies in a cell-free system showed that when Gag concentrations reach an upper threshold, protein synthesis from a reporter construct containing the HIV-1 5'-untranslated region (UTR) was inhibited, and this inhibition was dependent on the presence of the packaging signal in the RNA and RNA binding regions in Gag[2]. Additionally, a previous study found that a glutathione S-transferase-matrix (GST-MA) construct inhibited cell-free translation of a reporter construct containing the HIV-1 leader sequence[21]. Thus, Gag itself might help regulate how HIV-1 genomic RNA is utilized. However, additional studies in cellular systems will be needed to determine if negative feedback on Gag translation is important for packaging or assembly during HIV infection.

Are microdomains important for genomic RNA encapsidation?

As described above, data from assembly defective murine cells raise the possibility that Gag synthesis needs to occur in a specific cellular location or microdomain of the cytoplasm in order for proper assembly to follow. However, a recent report by Perlman, et al. found that newly synthesized Gag is first seen throughout the cytoplasm before it localizes to perinuclear sites[77]. While these data suggest that Gag translation occurs diffusely in the cytoplasm[77], it remains possible

that Gag is targeted posttranslationally to a distinct cellular location for proper encapsidation of the HIV-1 genomic RNA. A study by Poole, et al. showed that upon overexpression, both Gag and genomic RNA were present at the pericentriolar region of the cell[79]. Because pericentriolar localization of Gag preceded membrane binding of Gag to some extent and was also influenced by the presence of a packaging signal in the genomic RNA, the authors proposed that the pericentriolar region might be the site at which Gag selectively associates with HIV-1 genomic RNA. Trafficking of genomic RNA to the pericentriolar region may also be dependent on heterogeneous nuclear ribonucleoprotein A2 (HnRNP A2), which belongs to a family of proteins that coat mRNA and are involved in many posttranscriptional events including RNA trafficking and enhancement of translation (reviewed[93]). The HIV-1 contains two HnRNP A2 response elements[63] that appear to be important for trafficking genomic RNA into and/or from the pericentriolar region, and also for specific packaging of HIV genomic RNA[5,54]. While all these findings raise the possibility that cellular factors may direct HIV-1 genomic RNA to a specific microdomain for RNA encapsidation, more will need to be done to test this model. For example, it will be important to demonstrate that the Gag and RNA transported to the pericentriolar region undergo assembly and encapsidation, and do not represent pools of Gag or genomic RNA that are slated for degradation or other fates.

Another HnRNP was recently shown to play a role in trafficking HIV-1 RNA out of the nucleus. Dominant-negative constructs of HnRNP U eliminated cytoplasmic accumulation of HIV transcripts and gene expression[103]. However, because Gag translation was also eliminated by the dominant-negative constructs, it was not possible to assess whether HnRNP U also has effects on assembly.

Additionally, the host protein Staufen1 has been implicated in HIV genomic RNA trafficking and packaging[62]. Staufen1 is an RNA binding protein that is present in RNA granules and is important for RNA trafficking in drosophila and human neuronal cells[34,45,49] (reviewed[89]). Staufen1 specifically associates with genomic RNA, and the amount of Staufen1 that is packaged into wild-type and mutant virus correlates with the amount of genomic RNA that is packaged[62]. Moreover, Staufen1 has been found in a Gag-containing complex, and knockdown of Staufen1 reduces HIV infectivity by 60%[18]. Nevertheless, the mechanism by which Staufen1 acts remains unclear. A recent study demonstrated that both siRNA-mediated depletion of Staufen1 and overexpression of Staufen1 led to small increases in Gag multimerization at membranes and VLP release[17]. While the authors suggested possible mechanisms to explain these contradictory observations, such paradoxical findings raise questions about whether Staufen1 acts specifically during HIV packaging or multimerization, or instead acts during maintenance and turnover of HIV genomic RNA.

Membrane targeting of Gag is influenced by Gag multimerization and one or more cellular factors

After its synthesis, Gag traffics from the cytoplasm to membranes and also undergoes multimerization. Taken together, most studies support a model in which some degree of lower-order Gag oligomerization occurs in the cytoplasm before Gag targets to membrane sites of assembly, where more extensive higher-order Gag multimerization occurs[28,68,72,76,88,100]. Two recent studies that used novel approaches to monitor Gag multimerization (epitope masking[72] and fluorescence resonance energy transfer of tagged Gag polypeptides[28]) confirmed that extensive Gag multimerization occurs at membranes. As discussed below, cytoplasmic oligomerization of Gag followed by multimerization at membranes is also the basis for the myristoylation switch model of membrane targeting.

Targeting of Gag from the cytoplasm to the cytoplasmic face of a host membrane requires a bipartite motif in the M domain, consisting of an N-terminal myristate (C-14 fatty acid) and a cluster of basic residues at the N-terminus of MA. The myristate is thought to insert into the lipid bilayer, while the positive charge stabilizes association with the membrane via electrostatic interactions with the negatively charged phosphate backbone of lipids in the membrane. Mutations that disrupt either of these features impair membrane binding and assembly[10,38,41,97,101,106,107]. As described above, the MA domain serves multiple functions throughout the HIV-1 life cycle. When MA is part of full-length Gag during the late part of the viral lifecycle, it directs Gag from the cytoplasm to host membranes at the onset of assembly. In contrast, during the post-entry phase of the lifecycle, cleaved MA is part of the pre-integration complex that transports the newly reverse-transcribed viral DNA from the cytoplasm to the nucleus. A decade ago, the myristoylation switch model put forth an explanation for how Gag is capable of being either cytoplasmic or membrane bound[108]. In this model, which is supported by extensive mutational studies[69,74,76,88,95,97,106], the N-terminal myristate moiety is concealed within the globular head of MA, and membrane insertion occurs upon oligomerization of Gag because of a resulting conformational change that exposes the hydrophobic myristate. A nuclear magnetic resonance (NMR) spectroscopy study demonstrated that in the context of MA, which is predominantly monomeric, the N terminal myristate is largely sequestered, but in MA-CA polypeptides, which trimerize, the equilibrium shifts towards exposure of the myristate[100].

Thus, these data support the notion that oligomerization is required for membrane binding of Gag and acts by promoting exposure of the myristate that is largely concealed in the context of monomeric Gag.

A role for the cellular phosphoinositide $PI(4,5)P_2$ in promoting myristoylation exposure is also supported by a recent NMR study. Saad, et al. demonstrated that a soluble, truncated form of $PI(4,5)P_2$ binds specifically to a hydrophobic cleft in MA and triggers a conformation change exposing the myristate moiety[86]. The early experiments that initially led to interest in $PI(4,5)P_2$ found that in the presence of inositol phosphate (a phosphoinositide that is structurally related to $PI(4,5)P_2$), recombinant Gag forms spherical particles that have the same radius of curvature and size as capsids produced in cells[13] rather than the inappropriately small particles produced by recombinant Gag in the presence of RNA alone[14]. A region in MA is important for this size change, suggesting that inositol phosphates interact with the MA domain[13]. Subsequently, Ono, et al. found that altering levels and localization of $PI(4,5)P_2$, which is a component of the cytoplasmic leaflet of the plasma membrane (PM), can redirect the subcellular localization of Gag[67]. Overproduction of $PI(4,5)P_2$ in cells resulted in enrichment of $PI(4,5)P_2$ at CD63-containing intracellular vesicles, with consequent localization and assembly of Gag at these intracellular vesicles, and failure of virus to be released[67]. These data demonstrated that $PI(4,5)P_2$ can direct the subcellular targeting of assembling Gag. Additionally, the finding that Gag can be engineered to largely bypass the need for $PI(4,5)P_2$ using a myristoylation signal from Fyn in place of the myristoylation signal from Gag suggested that $PI(4,5)P_2$ acts on targeting of wild-type Gag[67] and most likely does not act directly on Gag multimerization. Notably, while subsequent structural studies separately showed that both Gag multimerization[100] and $PI(4,5)P_2$ binding[86] are important for myristate exposure of MA, their relative contributions to myristate exposure remain unknown. Specifically, do multimerization and $PI(4,5)P_2$ act additively or cooperatively on myristate exposure of Gag? And can they substitute for each other in a cellular context?

Evidence that other factors may be critical for membrane binding of Gag

The data outlined above suggest a model in which Gag multimerization, perhaps acting in concert with binding of $PI(4,5)P_2$, exposes the N-terminal myristate, which in turn functions with N-terminal basic amino acids in Gag to promote binding of full-length Gag to acidic membrane phospholipids during assembly. However, new structural data raise the possibility that this model is too simplistic. Some years ago, mutagenesis studies identified a mutation in the N-terminus of the MA domain that increases basic charge but eliminates membrane binding (Val to Arg substitution at position 6; 6VR[69]), suggesting the presence of an additional determinant of membrane binding within MA. Second site compensatory mutations in MA were found to restore membrane binding of 6VRGag[47,69,71]. Surprisingly, a very recent structural study of recombinant MA constructs found that binding of a soluble, truncated form of $PI(4,5)P_2$ did not cause myristate exposure in either the 6VR-containing MA mutant, which fails to bind membranes, or in a second site compensatory mutant that restores membrane binding[85]. These authors noted that the MA constructs encoding the compensatory mutations bound the soluble, truncated form of $PI(4,5)P_2$ with twofold higher affinity than the nonbinding mutants, raising the possibility that $PI(4,5)P_2$ binding in the absence of myristate exposure may be sufficient to target Gag to membranes[85]. Notably, all the structural studies of myristate exposure have been performed on wild-type or mutant MA polypeptides using a soluble form of $PI(4,5)P_2$. Whether the myristate moiety remains concealed during assembly when the compensatory mutations are expressed in the context of full-length Gag in cells is not known and warrants examination, given these surprising results obtained with MA constructs and soluble, truncated $PI(4,5)P_2$. Together, these data raise additional questions about how Gag binds to membranes. Specifically, is $PI(4,5)P_2$ binding necessary and/or sufficient for exposure of the myristate within full-length Gag? Is myristate exposure an absolute requirement for membrane binding? And do additional factor(s) influence membrane binding of Gag and/or myristate exposure in infected cells? If other factors are involved, the compensatory mutations that enable 6VRGag to target to membranes could act by restoring recruitment of these unidentified cellular factor(s), which in turn rescues membrane binding.

The possibility that other cellular factors in addition to $PI(4,5)P_2$ are required for the proper membrane targeting of Gag during assembly is supported by data obtained using murine cells. As described above, a defect in targeting and assembly of Gag expressed in murine cells can be overcome by redirecting genomic RNA targeting through an alternate nuclear export pathway[99], suggesting that factors acquired during RNA transport act directly or through recruitment of other factors to facilitate Gag targeting. Notably, mutant Gag constructs containing a deletion or alteration of the globular head of MA that conceals the hydrophobic myristate are not defective for targeting and assembly in murine cells[19,43,81]. This finding would make sense if the unidentified factors that are absent or defective in murine cells facilitate exposure of the concealed myristate, since such factors would not be required to target Gag mutants lacking the MA globu-

lar head. Thus, existing data on membrane targeting can be reconciled by a model in which exposure of the concealed myristate is promoted by multimerization of Gag, $PI(4,5)P_2$ at the PM, and other unidentified cellular factors, possibly acting in concert.

Other specific factors besides $PI(4,5)P_2$ have already been implicated in facilitating Gag targeting to membranes. A recent study demonstrated that the δ subunit of the AP-3 complex is important for Gag targeting in human cells[30]. The AP-3 complex is known to mediate the sorting of intracellular cargo[8,64,82], including Lamp-1, Lamp-2, and CD63 to the lysosome and late endosomes [25,26,51,83]. Dong, et al. hypothesized that AP-3δ acts in an analogous manner on Gag, transporting it to the late endosomal compartment. However, AP-3δ knockdowns and dominant-negative inhibitors appeared to eliminate Gag targeting to all membranes in this study, suggesting that the AP-3 complex may have a more general role in membrane targeting[30]. Thus, while it is clear that AP-3δ binds to the MA region of Gag[30], exactly how and when AP-3δ acts on Gag remains unanswered. Other groups have reported similar effects on Gag targeting and assembly upon disrupting the function of the Golgi-associated ubiquitin ligase named hPOSH[1], and annexin 2[84]. However, as in the case of AP-3δ, more studies will be required to determine how and where these factors act in the pathway from Gag synthesis to capsid assembly, especially in light of recent advances in our understanding of Gag trafficking, described below.

Defining the route: does newly synthesized Gag target only to the plasma membrane?

Although it is clear that membrane targeting of Gag is critical for proper assembly, the identity of the target membranes has been controversial. Electron micrographs produced over decades of studying HIV have documented electron-dense capsid structures assembling and releasing from the PM, and led to the notion that HIV targets to the PM for assembly. However, in recent years investigators also documented examples of HIV virions within intracellular compartments in macrophages and other cells[65,66,70,75,80,92], resurrecting findings from early HIV literature[37,73]. These virus-containing intracellular compartments were CD63 positive, which suggested that they represented late endosomes (LE) and/or multivesicular bodies (MVB)[75,80]. Not only are mature virions found within the lumen of these compartments, but ultrastructural studies also revealed actively assembling and budding immature virus at the limiting membrane of MVB[75,80]. These data raised the possibility that Gag can target to the MVB/LE, allowing assembly to occur at these intracellular sites.

A number of models were proposed to explain accumulation of virus at the MVB/LE. In one possibility, the membrane at which Gag assembles could differ depending on cell-type, with PM being the target in T-cells and MVB/LE being the predominant target in macrophages. The finding that Gag targeting, and therefore the site of assembly, could be altered by mutations in Gag[70] supported the plausibility of this model. Such differential targeting could be achieved by expression of different cellular adaptors or receptors in specific cell types that would direct Gag either to the PM or to the MVB/LE. While evidence indicates that cellular factors play a role in Gag targeting, to date there is no definitive evidence in support of a model in which specific cellular factors direct Gag to specific membranes.

A second model proposed that Gag might first target to the MVB/LE in all cells. This model was attractive given that ESCRT proteins, which act on and are located at the MVB/LE, are required for retroviral budding. In this model, Gag could take advantage of ESCRT proteins present at the MVB and would not need to recruit them to other cellular sites. This model was supported by immunofluorescence and electron microscopic studies demonstrating assembling HIV at MVB/LE even in epithelial-derived cell lines and in T-cells that were thought to solely support assembly and budding at the PM[42,66,92]. Notably, assembly and budding were seen both at the PM and MVB/LE in many of these studies. A more recent study that used biarsenical/tetracysteine labeling to follow newly-synthesized Gag microscopically over time also supported a version of this model[77]. Taken together, data in support of this model suggested that in all cell types, Gag initially targets to the MVB/LE. In some cell types, such as macrophages, Gag could assemble and bud at the MVB/LE, while in T-cells and other cell types, it could traffic on vesicles to the PM for assembly and budding. However, studies to date have not shown direct targeting of newly syntehsized Gag to the MVB for productive assembly, or subsequent trafficking of Gag from the MVB/LE to the PM for assembly in some cell types. In the absence of such definitive temporal studies, a third possibility could not be ruled out: that Gag found at the MVB/LE was originally targeted to the PM and arrived at the MVB/LE through the process of endocytosis.

This third model proposed that endocytosis could account for all trafficking of Gag to the MVB/LE. In this scenario, Gag targets to the PM in all cells, but is endocytosed from the surface of some cell types during or after assembly (Fig. 1A). Using drugs and dominant-negative constructs that inhibit endocytosis, Jouvenet, et al.[44] recently provided compelling evidence for this model. In the absence of endocytosis, wild-type Gag

assembles exclusively at the PM, with subsequent virion release. In contrast, Gag constructs that were rationally engineered to target to intracellular sites assembled at the MVB/LE even in the presence of endocytosis inhibitors, but failed to undergo release. Thus, this study argues that in all cells, Gag initially targets to the PM, and that cells proficient in phagocytosis and endocytosis, such as macrophages (but not T-cells), subsequently internalize assembling or assembled virions from the PM[44]. This results in the appearance of all stages of virus assembly and budding at the limiting membrane and within the lumen of intracellular vesicles. If the rate of assembly and release at the PM is slower than the rate of endocytosis, as might be expected in phagocytic cells, then internalization of assembling Gag from the PM into the endocytic pathway will be favored over release of assembling Gag from the PM. Moreover, point mutations previously thought to alter the site of assembly[70] could be explained if they simply delay assembly so that Gag remains at the PM longer. This would cause the rate of assembly and release to be slower than the rate of endocytosis, thereby favoring the appearance of these Gag mutants at MVB/LE.

Two very recent studies have convincingly supported the notion that assembly occurs at the PM in macrophages[27,104], which were previously thought to support assembly primarily at MVB-like structures. Both of these studies used markers that are endocytosed by live cells to label the MVB/LE compartments, and separately labeled the entire PM[27,104]. Their results demonstrate that the PM of macrophages is a complex, interconnected system, with adjacent membranes closely apposed so that virions that assemble at the PM can become sequestered in surface-connected, intracellular compartments that resemble the MVB/LE morphologically but in fact are extensions of the PM (Fig. 1A). In macrophages, virus accumulates at these compartments, suggesting that in these cells assembly does indeed occur at the PM not at the MVB/LE. However, contrary to the findings of Jouvenet, et al.[44], these studies found little or no accumulation of Gag at the MVB[27,104], raising questions about whether endocytosis of Gag occurs in macrophages. Nevertheless, together the recent studies suggest that wild-type HIV-1 assembles and releases at the PM in macrophages, and perhaps in all cell types. Additional studies will be needed to resolve the question of whether Gag can be endocytosed from the PM in macrophages.

Notably, if endocytosis of Gag is prominent in many cell types, this could lead to trafficking of Gag on endosomal vesicles. Studies have suggested that MLV Gag may traffic to sites of assembly on endosomal vesicles along with MLV RNA and the MLV Env protein[3,4]. Similar findings have not been published for HIV-1, although investigators have raised the possibility that HIV Gag could be transported on endosomal vesicles before or during assembly[77]. However, the recent data discussed above demonstrating endocytosis of assembling or assembled HIV Gag from the PM[44] support a model in which HIV-1 Gag is transported on endosomal vesicles after, rather than before, targeting and initiation of assembly at the PM (Fig. 1A). Experiments in which HIV-1 Gag is followed over time in the presence of endocytosis inhibitors will be needed to clarify whether the putative vesicular population of Gag originates before or after assembly.

Regardless of how virus arrives at the MVB/LE or surface-connected compartments, such cell-associated virus accumulations could have important implications for cell-to-cell spread of HIV if exocytic release of virus from intracellular sites can occur (Fig. 1A). Macrophages are antigen-presenting cells that intimately interact with T-cells, delivering co-stimulatory signals. A macrophage capable of mobilizing virus accumulated in internal compartments upon contacting a T lymphocyte could deliver a large dose of infectious virus to the susceptible target cell, ensuring a protected and highly efficient means of virus spread[16]. However, whether such a mode of delivery occurs during infection *in vivo* remains unclear. Moreover, it has not yet been shown even in cultured cells that compartments containing virus within macrophages can migrate to the cell surface, fuse with the PM, and release infectious virus. Such a possibility is supported by the demonstration that HIV was transmitted from macrophages to peripheral blood mononuclear cells (PBMC) *in trans* weeks after macrophages were treated with indinavir to prevent *de novo* production of infectious virus[91]. Furthermore, whether intracellular virus in macrophages undergoes exocytosis or degradation (Fig. 1A) could depend on signaling pathways or other modulators that have yet to be identified. While Ca^{+2} ionophores have been used to ask whether signaling pathways can promote virus release from MVB in epithelial cell lines[77], it will be necessary to perform studies in macrophages using physiologically relevant stimuli to demonstrate a role for signaling pathways in exocytotic release of virus. Thus, it remains possible that cell-associated virus in macrophages is relevant for spread of infection *in vivo.*

Markers for the intracellular Gag population undergoing assembly

While membrane targeting of Gag can be scored easily in cells using immunofluorescence microscopy or membrane flotation, the process of Gag assembly is much more difficult to track and quantify by these

techniques alone. Fluorescence microscopy has been used by a variety of groups to follow Gag trafficking[40,77] and fluorescence resonance energy transfer assays have been used to study Gag multimerization[28]. However, tracking the pool of Gag that is undergoing productive assembly is complicated by the possibility that most intracellular Gag polypeptides may not be part of the assembling pool. For example, as described above, much of the intracellular Gag in some cells may represent Gag that has been endocytosed or that remains stably associated with the PM after assembly[44]. Even more problematic is the finding that a very large fraction (30-80%) of newly synthesized Gag undergoes rapid degradation in transfected cells[33,90,102]. Importantly, it remains to be determined whether similar rates of degradation are seen in other cell types and in infected cells. Moreover, while much of the degradation is likely to be proeasomeal, a role for lysosmal degradation has not yet been ruled out (Fig. 1A). Interestingly, a recent report found that rhesus tripartite motif-5α (TRIM5α), but not human TRIM5α, promotes degradation of newly synthesized Gag and inhibits particle production[87]. Although currently there is no evidence that human TRIM proteins promote degradation of Gag, these findings raise the possibility that some mechanisms of Gag degradation during virus production could be cell-type specific. Nevertheless, considerable Gag degradation may occur in most cell types, given that degradation could constitute a host defense against the virus that offers the added benefit of priming the immune system through generation of peptides for major histocompatibility complex presentation. While the exact numbers need to be determined in specific cell types, the presence of Gag populations undergoing degradation or accumulated in cellular compartments after assembly suggests that the pool of assembling Gag that is present in cells at any one time may be relatively small. Tracking a small number of assembling Gag polypeptides against a large background of Gag that is slated for degradation or remains cell-associated after assembly poses a difficult technical challenge for investigators.

For these and other reasons, new approaches will be needed to distinguish between different populations of intracellular Gag based on their fates. One method for doing this involves defining cellular proteins that mark specific functional pools of Gag and then using these to track specific Gag subpopulations. Some progress has been achieved in using cellular factors to define the subpopulation of assembling Gag in cells. A number of studies suggest that Gag forms distinct complexes that chase into completed capsids, and are therefore termed assembly intermediates[31,33,52,53,56,102,109]. A complete analysis of all cellular factors present in each of these specific assembly intermediates and how they promote HIV assembly has not been performed yet. However, our group has identified one cellular protein associated with Gag in high molecular weight assembly intermediates, the ATPase ABCE1 (ATP-binding cassette protein in the E subfamily), and demonstrated that it appears to be critical for assembly[32,33,109]. In primate cells expressing the HIV provirus, we have demonstrated that Gag progresses through a pathway of ABCE1-containing intermediates, culminating in formation of the 750S, fully assembled immature capsid[31,33,56,109]. We have used a variety of approaches to demonstrate that the pool of Gag present in ABCE1-containing assembly intermediates, while small, represents intracellular Gag that is actively undergoing assembly. These include pulse-chase analyses in cells, showing ABCE1 associates with Gag as it assembles and dissociates from Gag at the onset of budding[33], mutational analyses showing that ABCE1 fails to associate with assembly incompetent Gag constructs[31,33,55,109], and a morphologic approach demonstrating recruitment of ABCE1 to sites of Gag assembly at the PM using quantitative double immuno-gold electron microscopy[33]. Thus, in addition to playing a role in capsid assembly, ABCE1 appears to be a marker for the pool of intracellular Gag that is undergoing assembly into immature capsids.

In uninfected eukaryotic cells, ABCE1 (previously referred to as RNase L inhibitor and HP68) is important for nucleocytoplasmic export of ribosomes and assembly of the ribosome pre-initiation complex[20,29,48,105]. In mammalian cells, ABCE1 also appears to act as an RNase L inhibitor that can protect viral RNA from degradation[7,59,60]. Our group has demonstrated that ABCE1 is required posttranslationally in a cell-free system during HIV Gag multimerization to form a protease-resistant, completely assembled immature capsid[109], but the exact mechanism by which ABCE1 acts during this process and how this function relates to the normal cellular function of ABCE1 remain unclear. Unfortunately, siRNA knockdowns cannot be used to identify the exact role of ABCE1 since depleting ABCE1 results in rapid cell death[20,33]. Ultimately, a mechanistic understanding of how different domains of ABCE1 act in all of these cellular processes will be required for a complete understanding of how ABCE1 promotes HIV-1 virion production.

Another marker for assembling Gag was defined in a study by Ono, et al.[72]. These authors demonstrated that Gag becomes inaccessible to immunoprecipitation with HIV antiserum when it multimerizes at membranes. Thus, masking of Gag epitopes appears to constitute a biochemical feature that can be used to distinguish and identify assembling Gag[72]. It is likely that markers for other subpopulations of Gag will be identified in the future. Of particular interest is the subpopulation of assembling Gag that traffics from the

cytoplasm to the PM, which may be marked by unique cellular factors. It would also be useful to identify specific cellular factors that mark Gag destined for degradation or endocytosis. When additional markers or antibodies specific for each intracellular Gag pool have been identified and validated, it should be possible to distinguish between separate Gag subpopulations that are simultaneously undergoing degradation, assembly, or endocytosis. This would allow half-lives, trafficking, and the final destination of each subpopulation to be tracked in parallel, biochemically. Moreover, such biochemical markers could ultimately be used in fluorescence microscopy studies to track specific subpopulations of Gag in real time.

Gag targeting and assembly: unanswered questions and future directions

Studies in recent years have altered our view of intracellular Gag considerably. It is increasingly evident that Gag consists of different subpopulations that coexist in the cell but are destined for very different fates, including proteasomal degradation, capsid assembly, and endocytosis. A lack of adequate tools for distinguishing each of these subpopulations biochemically and microscopically and tracking them over time has led to limitations in our understanding of late events in the virus lifecycle as well as misperceptions of the subcellular site where assembly occurs. Recent data suggest that Gag is targeted to and assembles at the PM[27,44,104]. Given new awareness of the many subpopulations of intracellular Gag, it will be important for investigators in the future to define the exact population of Gag that they are studying, especially when studying the function of cellular factors that influence assembly and release.

Additionally, the exact role of specific cellular factors in the process of targeting and assembly needs to be addressed. To date, manipulation of a number of cellular factors has been found to have effects on assembly. Identifying the exact step(s) at which these host factors facilitate virion formation and the mechanisms by which they are acting will allow for better insights into how Gag progresses from a single polypeptide to a structured immature capsid poised for release from an infected cell at the proper location. Additionally, it will be important to identify new factors that act in the pathway from Gag synthesis to immature capsid formation in human cells. Thus, the shift from studying Gag in isolation to examining Gag in the context of the host cell is one that has complicated the field, leading to controversies as described above, but it also holds promise for future advances in our understanding of HIV-1 infection *in vivo*.

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References

1. Alroy I, Tuvia S, Greener T, et al. The trans-Golgi network-associated human ubiquitin-protein ligase POSH is essential for HIV type 1 production. Proc Natl Acad Sci USA 2005;102:1478-83.
2. Anderson E, Lever A. HIV-1 Gag polyprotein modulates its own translation. J Virol 2006;80:10478-86.
3. Basyuk E, Boulon S, Skou Pedersen F, Bertrand E, Vestergaard Rasmussen S. The packaging signal of MLV is an integrated module that mediates intracellular transport of genomic RNA. J Mol Biol 2005;354:330-9.
4. Basyuk E, Galli T, Mougel M, Blanchard J, Sitbon M, Bertrand E. Retroviral genomic RNAs are transported to the plasma membrane by endosomal vesicles. Dev Cell 2003;5:161-74.
5. Beriault V, Clement J, Levesque K, et al. A late role for the association of hnRNP A2 with the HIV-1 hnRNP A2 response elements in genomic RNA, Gag, and Vpr localization. J Biol Chem 2004; 279:44141-53.
6. Bieniasz P, Cullen B. Multiple blocks to HIV-1 replication in rodent cells. J Virol 2000;74:9868-77.
7. Bisbal C, Martinand C, Silhol M, Lebleu B, Salehzada T. Cloning and characterization of a RNAse L inhibitor. A new component of the interferon-regulated 2-5A pathway. J Biol Chem 1995;270:13308-17.
8. Boehm M, Bonifacino J. Genetic analyses of adaptin function from yeast to mammals. Gene 2002;286:175-86.
9. Briggs J, Simon M, Gross I, et al. The stoichiometry of Gag protein in HIV-1. Nat Struct Mol Biol 2004;11:672-5.
10. Bryant M, Ratner L. Myristoylation-dependent replication and assembly of HIV-1. Proc Natl Acad Sci USA 1990;87:523-7.
11. Butsch M, Boris-Lawrie K. Destiny of unspliced retroviral RNA: ribosome and/or virion? J Virol 2002;76:3089-94.
12. Butsch M, Boris-Lawrie K. Translation is not required to generate virion precursor RNA in HIV-1-infected T cells. J Virol 2000;74:11531-7.
13. Campbell S, Fisher R, Towler E, et al. Modulation of HIV-like particle assembly *in vitro* by inositol phosphates. Proc Natl Acad Sci USA 2001;98:10875-9.
14. Campbell S, Rein A. *In vitro* assembly properties of HIV-1 Gag protein lacking the p6 domain. J Virol 1999;73:2270-9.
15. Campbell S, Vogt V. Self-assembly in vitro of purified CA-NC proteins from Rous sarcoma virus and HIV-1. J Virol 1995;69:6487-97.
16. Carr J, Hocking H, Li P, Burrell C. Rapid and efficient cell-to-cell transmission of HIV infection from monocyte-derived macrophages to peripheral blood lymphocytes. Virology 1999;265:319-29.
17. Chatel-Chaix L, Abrahamyan L, Frechina C, Mouland A, DesGroseillers L. The host protein Staufen1 participates in HIV-1 assembly in live cells by influencing pr55Gag multimerization. J Virol 2007;81:6216-30.
18. Chatel-Chaix L, Clement J, Martel C, et al. Identification of Staufen in the HIV-1 Gag ribonucleoprotein complex and a role in generating infectious viral particles. Mol Cell Biol 2004;24:2637-48.
19. Chen B, Rousso I, Shim S, Kim P. Efficient assembly of an HIV-1/MLV Gag-chimeric virus in murine cells. Proc Natl Acad Sci USA 2001;98:15239-44.
20. Chen Z, Dong J, Ishimura A, Daar I, Hinnebusch A, Dean M. The essential vertebrate ABCE1 protein interacts with eukaryotic initiation factors. J Biol Chem 2006;281:7452-7.
21. Cimarelli A, Luban J. Translation elongation factor 1-alpha interacts specifically with the HIV-1 Gag polyprotein. J Virol 1999;73:5388-401.
22. Coskun A, van Maanen M, Janka D, et al. Isolation and characterization of mouse-human microcell hybrid cell clones permissive for infectious HIV particle release. Virology 2007;362:283-93.
23. Coskun A, van Maanen M, Nguyen V, Sutton R. Human chromosome 2 carries a gene required for production of infectious HIV-1. J Virol 2006;80:3406-15.
24. Cullen B. Nuclear mRNA export: insights from virology. Trends Biochem Sci 2003;28:419-24.

25. Dell'Angelica E, Aguilar R, Wolins N, Hazelwood S, Gahl W, Bonifacino J. Molecular characterization of the protein encoded by the Hermansky-Pudlak syndrome type 1 gene. J Biol Chem 2000;275:1300-6.
26. Dell'Angelica E, Shotelersuk V, Aguilar R, Gahl W, Bonifacino J. Altered trafficking of lysosomal proteins in Hermansky-Pudlak syndrome due to mutations in the beta 3A subunit of the AP-3 adaptor. Mol Cell 1999;3:11-21.
27. Deneka M, Pelchen-Matthews A, Byland R, Ruiz-Mateos E, Marsh M. In macrophages, HIV-1 assembles into an intracellular plasma membrane domain containing the tetraspanins CD81, CD9, and CD53. J Cell Biol 2007;177:329-41.
28. Derdowski A, Ding L, Spearman P. A novel fluorescence resonance energy transfer assay demonstrates that the HIV-1 Pr55Gag I domain mediates Gag-Gag interactions. J Virol 2004;78:1230-42.
29. Dong J, Lai R, Nielsen K, Fekete C, Qiu H, Hinnebusch A. The essential ATP-binding cassette protein RLI1 functions in translation by promoting preinitiation complex assembly. J Biol Chem 2004; 279:42157-68.
30. Dong X, Li H, Derdowski A, et al. AP-3 directs the intracellular trafficking of HIV-1 Gag and plays a key role in particle assembly. Cell 2005;120:663-74.
31. Dooher J, Lingappa J. Conservation of a step-wise, energy-sensitive pathway involving HP68 for assembly of primate lentiviral capsids in cells. J Virology 2004;78:1645-56.
32. Dooher J, Pineda M, Overbaugh J, Lingappa J. Characterization of virus infectivity and cell-free capsid assembly of SIVmneCL8. J Med Primatol 2004;33:262-71.
33. Dooher J, Schneider B, Reed J, Lingappa J. Host ABCE1 is at plasma membrane HIV assembly sites and its dissociation from Gag is linked to subsequent events of virus production. Traffic 2007;8:195-211.
34. Ferrandon D, Elphick L, Nusslein-Volhard C, St Johnston D. Staufen protein associates with the 3'UTR of bicoid mRNA to form particles that move in a microtubule-dependent manner. Cell 1994;79:1221-32.
35. Freed E. HIV-1 replication. Somat Cell Mol Genet 2001;26:13-33.
36. Gelderblom H. Assembly and morphology of HIV: potential effect of structure on viral function. Aids 1991;5:617-37.
37. Gendelman H, Orenstein J, Martin M, et al. Efficient isolation and propagation of HIV on recombinant colony-stimulating factor 1-treated monocytes. J Exp Med 1988;167:1428-41.
38. Gheysen D, Jacobs E, de Foresta F, et al. Assembly and release of HIV-1 precursor Pr55gag virus-like particles from recombinant baculovirus-infected insect cells. Cell 1989;59:103-12.
39. Goff S. Host factors exploited by retroviruses. Nat Rev Microbiol 2007;5:253-63.
40. Gomez C, Hope T. Mobility of HIV-1 Pr55Gag in living cells. J Virol 2006;80:8796-806.
41. Gottlinger H, Sodroski J, Haseltine W. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of HIV-1. Proc Natl Acad Sci USA 1989;86:5781-5.
42. Grigorov B, Arcanger F, Roingeard P, Darlix J, Muriaux D. Assembly of infectious HIV-1 in human epithelial and T-lymphoblastic cell lines. J Mol Biol 2006;359:848-62.
43. Hatziioannou T, Martin-Serrano J, Zang T, Bieniasz P. Matrix-induced inhibition of membrane binding contributes to HIV-1 particle assembly defects in murine cells. J Virol 2005;79:15586-9.
44. Jouvenet N, Neil S, Bess C, et al. Plasma membrane is the site of productive HIV-1 particle assembly. PLoS Biol 2006;4:e435.
45. Kanai Y, Dohmae N, Hirokawa N. Kinesin transports RNA: isolation and characterization of an RNA-transporting granule. Neuron 2004; 43:513-25.
46. Kaye J, Lever A. HIV types 1 and 2 differ in the predominant mechanism used for selection of genomic RNA for encapsidation. J Virol 1999;73:3023-31.
47. Kiernan R, Ono A, Englund G, Freed E. Role of matrix in an early postentry step in the HIV-1 life cycle. J Virol 1998;72:4116-26.
48. Kispal G, Sipos K, Lange H, et al. Biogenesis of cytosolic ribosomes requires the essential iron-sulphur protein Rli1p and mitochondria. Embo J 2005;24:589-98.
49. Kohrmann M, Luo M, Kaether C, DesGroseillers L, Dotti C, Kiebler M. Microtubule-dependent recruitment of Staufen-green fluorescent protein into large RNA-containing granules and subsequent dendritic transport in living hippocampal neurons. Mol Biol Cell 1999; 10:2945-53.
50. Koito A, Shigekane H, Matsushita S. Ability of small animal cells to support the postintegration phase of HIV-1 replication. Virology 2003;305:181-91.
51. Le Borgne R, Alconada A, Bauer U, Hoflack B. The mammalian AP-3 adaptor-like complex mediates the intracellular transport of lysosomal membrane glycoproteins. J Biol Chem 1998; 273:29451-61.
52. Lee Y, Liu B, Yu X. Formation of virus assembly intermediate complexes in the cytoplasm by wild-type and assembly-defective mutant HIV-1 and their association with membranes. J Virol 1999; 73:5654-62.
53. Lee Y, Yu X. Identification and characterization of virus assembly intermediate complexes in HIV-1-infected CD4+ T cells. Virology 1998;243:78-93.
54. Levesque K, Halvorsen M, Abrahamyan L, et al. Trafficking of HIV-1 RNA is mediated by heterogeneous nuclear ribonucleoprotein A2 expression and impacts on viral assembly. Traffic 2006;7:1177-93.
55. Lingappa J, Dooher J, Newman M, Kiser P, Klein K. Basic residues in the nucleocapsid domain of Gag are required for interaction of HIV-1 gag with ABCE1 (HP68), a cellular protein important for HIV-1 capsid assembly. J Biol Chem 2006;281:3773-84.
56. Lingappa J, Hill R, Wong M, Hegde R. A multistep, ATP-dependent pathway for assembly of HIV capsids in a cell-free system. J Cell Biol 1997;136:567-81.
57. Mariani R, Rasala B, Rutter G, Wiegers K, Brandt S, Krausslich H, Landau N. Mouse-human heterokaryons support efficient HIV-1 assembly. J Virol 2001;75:3141-51.
58. Mariani R, Rutter G, Harris M, Hope T, Krausslich H, Landau N. A block to HIV-1 assembly in murine cells. J Virol 2000;74:3859-70.
59. Martinand C, Montavon C, Salehzada T, Silhol M, Lebleu B, Bisbal C. RNase L inhibitor is induced during HIV-1 infection and down regulates the 2-5A/RNase L pathway in human T cells. J Virol 1999;73:290-6.
60. Martinand C, Salehzada T, Silhol M, Lebleu B, Bisbal C. RNase L inhibitor (RLI) antisense constructions block partially the down regulation of the 2-5A/RNase L pathway in encephalomyocarditis-virus-(EMCV)-infected cells. Eur J Biochem 1998;254:248-55.
61. Morita E, Sundquist W. Retrovirus budding. Annu Rev Cell Dev Biol 2004;20:395-425.
62. Mouland A, Mercier J, Luo M, Bernier L, DesGroseillers L, Cohen E. The double-stranded RNA-binding protein Staufen is incorporated in HIV-1: evidence for a role in genomic RNA encapsidation. J Virol 2000;74:5441-51.
63. Mouland A, Xu H, Cui H, et al. RNA trafficking signals in HIV-1. Mol Cell Biol 2001;21:2133-43.
64. Nakatsu F, Ohno H. Adaptor protein complexes as the key regulators of protein sorting in the post-Golgi network. Cell Struct Funct 2003;28:419-29.
65. Nguyen D, Hildreth J. Evidence for budding of HIV-1 selectively from glycolipid-enriched membrane lipid rafts. J Virol 2000;74:3264-72.
66. Nydegger S, Foti M, Derdowski A, Spearman P, Thali M. HIV-1 egress is gated through late endosomal membranes. Traffic 2003;4:902-10.
67. Ono A, Ablan S, Lockett S, Nagashima K, Freed E. Phosphatidylinositol (4,5) bisphosphate regulates HIV-1 Gag targeting to the plasma membrane. Proc Natl Acad Sci USA 2004;101:14889-94.
68. Ono A, Demirov D, Freed E. Relationship between HIV-1 Gag multimerization and membrane binding. J Virol 2000;74:5142-50.
69. Ono A, Freed E. Binding of HIV-1 Gag to membrane: role of the matrix amino terminus. J Virol 1999;73:4136-44.
70. Ono A, Freed E. Cell-type-dependent targeting of HIV-1 assembly to the plasma membrane and the multivesicular body. J Virol 2004;78:1552-63.
71. Ono A, Huang M, Freed E. Characterization of HIV-1 matrix revertants: effects on virus assembly, Gag processing, and Env incorporation into virions. J Virol 1997;71:4409-18.
72. Ono A, Waheed A, Joshi A, Freed E. Association of HIV-1 gag with membrane does not require highly basic sequences in the nucleocapsid: use of a novel Gag multimerization assay. J Virol 2005;79:14131-40.
73. Orenstein J, Meltzer M, Phipps T, Gendelman H. Cytoplasmic assembly and accumulation of HIV types 1 and 2 in recombinant human colony-stimulating factor-1-treated human monocytes: an ultrastructural study. J Virol 1988;62:2578-86.
74. Paillart J, Gottlinger H. Opposing effects of HIV-1 matrix mutations support a myristyl switch model of gag membrane targeting. J Virol 1999;73:2604-12.
75. Pelchen-Matthews A, Kramer B, Marsh M. Infectious HIV-1 assembles in late endosomes in primary macrophages. J Cell Biol 2003;162:443-55.
76. Perez-Caballero D, Hatziioannou T, Martin-Serrano J, Bieniasz P. HIV-1 matrix inhibits and confers cooperativity on gag precursor-membrane interactions. J Virol 2004;78:9560-3.

77. Perlman M, Resh M. Identification of an intracellular trafficking and assembly pathway for HIV-1 gag. Traffic 2006;7:731-45.
78. Pollard V, Malim M. The HIV-1 Rev protein. Annu Rev Microbiol 1998;52:491-532.
79. Poole E, Strappe P, Mok H, Hicks R, Lever A. HIV-1 Gag-RNA interaction occurs at a perinuclear/centrosomal site; analysis by confocal microscopy and FRET. Traffic 2005;6:741-55.
80. Raposo G, Moore M, Innes D, et al. Human macrophages accumulate HIV-1 particles in MHC II compartments. Traffic 2002;3:718-29.
81. Reed M, Mariani R, Sheppard L, Pekrun K, Landau N, Soong N. Chimeric HIV-1 containing murine leukemia virus matrix assembles in murine cells. J Virol 2002;76:436-43.
82. Robinson M, Bonifacino J. Adaptor-related proteins. Curr Opin Cell Biol 2001;13:444-53.
83. Rous B, Reaves B, Ihrke G, et al. Role of adaptor complex AP-3 in targeting wild-type and mutated CD63 to lysosomes. Mol Biol Cell 2002;13:1071-82.
84. Ryzhova E, Vos R, Albright A, Harrist A, Harvey T, Gonzalez-Scarano F. Annexin 2: a novel HIV-1 Gag binding protein involved in replication in monocyte-derived macrophages. J Virol 2006;80:2694-704.
85. Saad J, Loeliger E, Luncsford P, et al. Point mutations in the HIV-1 matrix protein turn off the myristyl switch. J Mol Biol 2007;366:574-85.
86. Saad J, Miller J, Tai J, Kim A, Ghanam R, Summers M. Structural basis for targeting HIV-1 Gag proteins to the plasma membrane for virus assembly. Proc Natl Acad Sci USA 2006;103:11364-9.
87. Sakuma R, Noser J, Ohmine S, Ikeda Y. Rhesus monkey TRIM5alpha restricts HIV-1 production through rapid degradation of viral Gag polyproteins. Nat Med 2007;13:631-5.
88. Sandefur S, Varthakavi V, Spearman P. The I domain is required for efficient plasma membrane binding of HIV-1 Pr55Gag. J Virol 1998;72:2723-32.
89. Saunders L, Barber G. The dsRNA binding protein family: critical roles, diverse cellular functions. Faseb J 2003;17:961-83.
90. Schubert U, Anton L, Gibbs J, Norbury C, Yewdell J, Bennink J. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. Nature 2000;404:770-4.
91. Sharova N, Swingler C, Sharkey M, Stevenson M. Macrophages archive HIV-1 virions for dissemination in trans. Embo J 2005;24:2481-9.
92. Sherer N, Lehmann M, Jimenez-Soto L, Visualization of retroviral replication in living cells reveals budding into multivesicular bodies. Traffic 2003;4:785-801.
93. Shyu A, Wilkinson M. The double lives of shuttling mRNA binding proteins. Cell 2000;102:135-8.
94. Sokolskaja E, Luban J. Cyclophilin, TRIM5, and innate immunity to HIV-1. Curr Opin Microbiol 2006;9:404-8.
95. Spearman P, Horton R, Ratner L, Kuli-Zade I. Membrane binding of HIV-1 matrix protein in vivo supports a conformational myristyl switch mechanism. J Virol 1997;71:6582-92.
96. Spearman P, Ratner L. HIV-1 capsid formation in reticulocyte lysates. J Virol 1996;70:8187-94.
97. Spearman P, Wang J, Vander Heyden N, Ratner L. Identification of HIV-1 Gag protein domains essential to membrane binding and particle assembly. J Virol 1994;68:3232-42.
98. Swanson C, Malim M. Retrovirus RNA trafficking: from chromatin to invasive genomes. Traffic 2006;7:1440-50.
99. Swanson C, Puffer B, Ahmad K, Doms R, Malim M. Retroviral mRNA nuclear export elements regulate protein function and virion assembly. Embo J 2004;23:2632-40.
100. Tang C, Loeliger E, Luncsford P, Kinde I, Beckett D, Summers M. Entropic switch regulates myristate exposure in the HIV-1 matrix protein. Proc Natl Acad Sci USA 2004;101:517-22.
101. Tashiro A, Shoji S, Kubota Y. Antimyristoylation of the gag proteins in the HIV-infected cells with N-myristoyl glycinal diethylacetal resulted in inhibition of virus production. Biochem Biophys Res Commun 1989;165:1145-54.
102. Tritel M, Resh M. Kinetic analysis of HIV-1 assembly reveals the presence of sequential intermediates. J Virol 2000;74:5845-55.
103. Valente S, Goff S. Inhibition of HIV-1 gene expression by a fragment of hnRNP U. Mol Cell 2006;23:597-605.
104. Welsch S, Habermann A, Jager S, Muller B, Krijnse-Locker J, Krausslich H. Ultrastructural analysis of ESCRT proteins suggests a role for endosome-associated tubular-vesicular membranes in ESCRT function. Traffic 2006;7:1551-66.
105. Yarunin A, Panse V, Petfalski E, Dez C, Tollervey D, Hurt E. Functional link between ribosome formation and biogenesis of iron-sulfur proteins. Embo J 2005;24:580-8.
106. Yuan X, Yu X, Lee T, Essex M. Mutations in the N-terminal region of HIV-1 matrix protein block intracellular transport of the Gag precursor. J Virol 1993;67:6387-94.
107. Zhou W, Parent L, Wills J, Resh M. Identification of a membrane-binding domain within the amino-terminal region of HIV-1 Gag protein which interacts with acidic phospholipids. J Virol 1994;68:2556-69.
108. Zhou W, Resh M. Differential membrane binding of the HIV-1 matrix protein. J Virol 1996;70:8540-8.
109. Zimmerman C, Klein K, Kiser P, et al. Identification of a host protein essential for assembly of immature HIV-1 capsids. Nature 2002;415:88-92.