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Maturation of retroviruses

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During retrovirus maturation, cleavage of the precursor structural Gag polyprotein by the viral protease induces architectural rearrangement of the virus particle from an immature into a mature, infectious form. The structural rearrangement encapsidates the viral RNA genome in a fullerene capsid, producing a diffusible viral core that can initiate infection upon entry into the cytoplasm of a host cell. Maturation is an important therapeutic window against HIV-1. In this review, we highlight recent breakthroughs in understanding of the structures of retroviral immature and mature capsid lattices that define the boundary conditions of maturation and provide novel insights on capsid transformation. We also discuss emerging insights on encapsidation of the viral genome in the mature capsid, as well as remaining questions for further study.

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Introduction

The architectures of immature and mature retroviral particles are defined by the viral structural protein Gag and its maturation products (Figure 1) (reviewed in Refs. [1–3]). All retroviral Gag proteins consist of three major domains: MA, which mediates binding to membranes and targets Gag to appropriate assembly sites in the cell; CA, which mediates lattice-forming protein–protein interactions in both the immature and mature capsids; and NC, which contains one or two zinc knuckles that bind and package the viral RNA genome. These three domains are connected by spacer peptides or additional domains, which vary across different species.

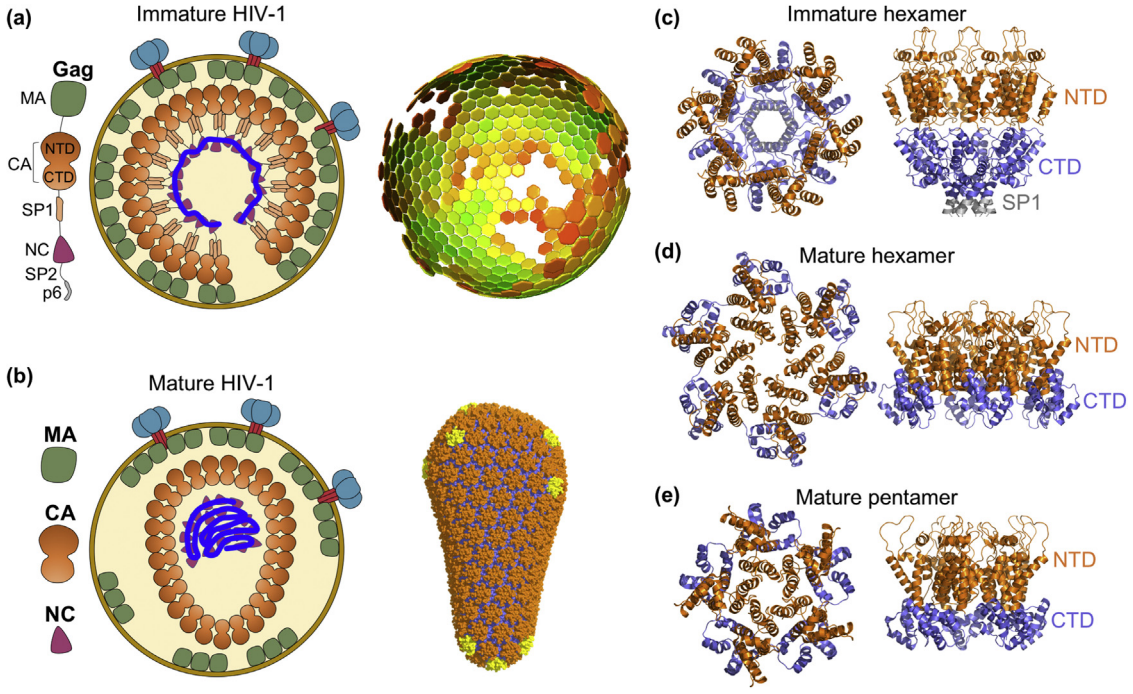
Retroviruses initially assemble in an immature form, in which Gag makes a spherical protein shell comprising up to 4000 subunits (Figure 1a). The Gag shell is attached to the viral membrane by the MA domain, which contains a positively charged patch that binds to phospholipid headgroups and, in some species, an N-terminal acyl chain modification that inserts into the inner leaflet of the lipid bilayer. During or immediately after budding, the viral protease (PR) auto-activates and cleaves Gag at specific sites to initiate maturation. This results in disassembly of the Gag lattice and condensation of the released NC–RNA complex into a compact ribonucleoprotein particle, which presumably prepares the genome for reverse transcription and integration. Around 1500 copies of the new CA proteins then assemble into the mature capsid that re-encapsulates the genomic complex and its associated replicative enzymes. This generates the retroviral core, which consists of the mature capsid and its contents (Figure 1b). In practical terms, retrovirus maturation can be viewed as the transformation of the membrane-bound immature particle into a diffusible particle – the mature core – in which the viral genome is primed to initiate infection upon entry into the cytoplasm of a host cell.

In this review, we summarize studies that provide detailed views of the molecular structures of the immature and mature lattices, and how these structures inform understanding of capsid transformation during retrovirus maturation. In particular, we highlight molecular switches that drive CA self-association and assembly of both types of capsid. We also discuss emerging models of viral protease activation, which regulates the onset of maturation, as well as recent insights on how the viral RNA is encapsidated in the mature capsid.

Structures of the immature and mature capsid shells

The transformation of the immature shell into the mature capsid underlies the dramatic change in virion morphology that occurs during retroviral maturation. Electron microscopy of *in vitro* model systems [4–13,14••,15] and authentic virions [12,14••,16–20,21••,22•], crystallography of capsid protein oligomers [23–30,31••], and structure-based modeling studies [10,27,32,33] have now revealed the detailed structures of the assembled subunits (Figure 1c–e). Each is organized with hexagonal symmetry and made of interlinked CA hexamers, but the lattice spacings and detailed protein–protein interactions differ considerably. The architectural transformation of the capsid requires breaking of essentially all the immature

Figure 1



Structural organization of immature and mature HIV-1 particles. **(a)** The immature virion is organized by the Gag polyprotein and its multiple domains. Gag assembles into a spherical immature lattice made up of interlinked Gag hexamers. **(b)** The mature virion is organized by the mature structural proteins – MA, CA, and NC – which are derived from Gag. The genome is encapsidated in a fullerene capsid made up of CA hexamers and pentamers. **(c)** Structure of the immature hexamer, made of the CA subdomains (NTD colored in orange and CTD in blue) and downstream SP1 spacer (colored in gray). **(d)** Structure of the mature hexamer. **(e)** Structure of the mature pentamer. Gag lattice map in panel (a) adapted with permission [19]. Mature capsid model in panel (b) adapted with permission [27].

CA–CA interactions before formation of the mature interactions. Various models have been proposed to explain how this transition occurs, based on comparisons of the two lattices, analyses of maturation intermediates, and simulations [34–40,41•]. On balance, the available data support a disassembly-and-reassembly mechanism, in which proteolysis induces disassembly of the immature lattice into CA monomers, dimers, hexamers, or other small oligomers that then reassemble to form the mature capsid (reviewed in Ref. [3]).

CA is composed of two independently folding subdomains, called NTD and CTD (N-terminal and C-terminal domains). In the mature lattice, the hexamers are made by the NTD, which makes a ring held together by a central 18-helix barrel comprising the first three α-helices of this subdomain [5,6,23,30] (Figure 1d). The CTD makes a 'belt' around this central ring, and connects the hexamers to each other through dimeric and trimeric interactions [7,25]. Mature retroviral capsids are organized as fullerene structures, and also contain pentamers [42] (Figure 1e). The CA pentamer is quasi-equivalent to the hexamer; that is, it is generated by essentially the same protein–protein interfaces but with fivefold instead of sixfold rotational symmetry [6,21••,22•,27]. High-resolution structures of various retroviral CA hexamers and pentamers are now known, and these reveal a remarkable degree of structural conservation despite highly divergent protein primary sequences and capsid morphologies. The mature capsid morphologies of different retroviral genera are explained by differing distributions of the pentamers in the mature hexagonal lattice [42,43]: conical HIV-1 (lentivirus) capsids have five pentamers at the narrow end and seven at the broad end, tubular MPMV (Mason-Pfizer monkey virus, betaretrovirus) capsids have six pentamers at each end, and polyhedral or 'spherical' capsids in RSV (Rous sarcoma virus, alpharetrovirus) and MLV (murine leukemia virus, gammaretrovirus) have the pentamers more randomly distributed. A completely closed fullerene shell contains exactly 12 pentamers, as found predominantly in HIV-1 [21••]. However, MLV appears to form mostly open capsids with seams and multiple layers (having more than 12 pentamers) as well as multiple capsids per virion [22•]. Why some retroviruses produce predominantly closed capsids while others form predominantly open ones remains an outstanding and intriguing question. It was suggested that this difference may reflect species-specific requirements [22•] – for example, having a closed capsid may be essential

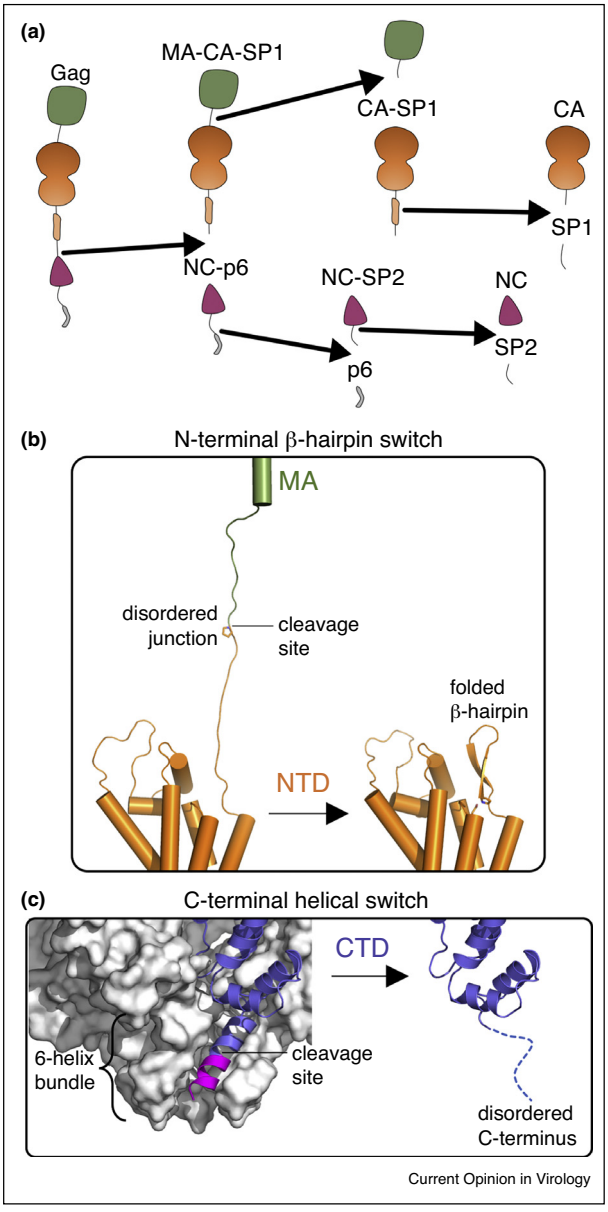
for proper nuclear targeting of HIV-1 and lentivirus cores, which infect terminally differentiated or non-dividing cells.

In contrast to the mature hexamer, the immature hexamer is organized by the CTD, again in a manner that is conserved across all retroviruses examined to date [9,11–13,14••,22•,31••] (Figure 1c). Hexamerization interactions are provided in part by the major homology region (MHR), a highly conserved sequence of around 20 amino-acid residues that is found in all retroviruses and retrotransposons. However, it is important to note that the most highly conserved MHR residues do not directly participate in intermolecular interactions [44] – instead, these appear to promote hexamerization indirectly by stabilizing the configurations of the MHR loops that come together in the middle of the hexamer. The most critical hexamer-forming contacts are provided by the C-terminal CTD residues, which in HIV-1 form part of an α-helix (the 'junction helix') that extends to the downstream SP1 spacer and makes a six-helix bundle [14••,31••]. Similar six-helix bundles are observed in RSV [13] and MLV [22•], but not in MPMV [9] (although a functionally equivalent 'SP-like domain' is reported in this virus as well [45]). Unlike the CTD, the immature configurations of the NTD vary considerably across retroviruses. For example, in HIV-1 the NTD is rotated and displaced relative to its mature configuration and mediates dimerization instead of hexamerization contacts [14••], whereas in MLV the NTD makes a hexameric ring that is more similar to the mature ring [22•]. This dramatic contrast between the conserved immature CTD and divergent immature NTD arrangements indicate that the major protein–protein interactions that drive immature retrovirus assembly are provided by the CTD, which is consistent with the observation that the minimal construct for assembly of immature HIV-1 virus-like particles does not include the NTD [46,47]. Finally, unlike the mature fullerene capsid, the immature lattice does not incorporate pentamers. Instead, curvature is accommodated by gaps or discontinuities in the immature hexagonal lattice.

Structural switching from immature to mature

Since CA directs the assembly of both the immature and mature lattices, an important aspect of the molecular mechanism of capsid transformation concerns how the structural and assembly properties of CA are modulated during maturation (Figure 2). In HIV-1, both the N-terminal and C-terminal cleavage sites that flank CA have been proposed to function as structural switches, because they undergo localized changes in secondary structure upon proteolysis and mutagenesis of these regions cause severe defects in virus infectivity. Cleavage of the MA–CA junction induces folding of the first 12 residues of the newly mature CA protein into a β-hairpin, which is stabilized by a buried salt bridge between the new proline N-terminus and a conserved aspartate sidechain in helix 3 [48,49] (Figure 2b). This conformation requires protonation of the N-terminal proline, which cannot occur in the unprocessed MA–CA junction. *In vitro* assembly and hydrogen/deuterium exchange studies have shown that the β-hairpin structure and its dynamics are important

Figure 2



Proteolytic processing of HIV-1 Gag. **(a)** Cleavage of Gag sites occur at different rates, giving rise to a number of processing intermediates. **(b)** Secondary structural change that occurs in the N-terminal end of CA during maturation. Upon cleavage of the MA–CA junction, the first 12 amino-acid residues of CA fold into a β-hairpin that is absent in the immature form. **(c)** Structural change in the C-terminal end of CA. The last seven residues of CA and the first seven residues of the SP1 spacer fold into an α-helix that makes a six-helix bundle that is critical for stability of the immature lattice. Upon cleavage of the CA–SP1 junction, these residues become disordered.

determinants of the assembly kinetics of the mature lattice [50]. Furthermore, the β-hairpin was shown to affect the dynamics of helix 1, which forms part of the 18-helix barrel in the mature hexamer core. However, analyses of HIV-1 particles with mutated protease sites indicate that a mature CA lattice can form without a folded β-hairpin when processing of the MA–CA site is prevented, and conversely, an intact β-hairpin can be accommodated within the immature lattice that is retained when the CA–NC processing sites are rendered non-cleavable [41•,51]. Consistent with these studies, recombinant HIV-1 Gag proteins harboring unprocessed MA–CA junctions and CA proteins lacking the first 12 amino-acid residues retain the ability to assemble into mature capsid-like particles [52,53]. These indicate that β-hairpin formation in HIV-1 is neither sufficient to destabilize the immature CA lattice nor required to assemble the mature lattice. Interestingly, studies of MPMV CA constructs suggest that this assembly system is much more responsive to mutations that disrupt the β-hairpin [54–56]. As with the *in vitro* HIV-1 studies [50], folding of the β-hairpin is proposed to modulate flexibility of the NTD helices and helps to lock the NTD helical bundle in its mature conformation [54,56]. Thus, β-hairpin formation may have the general function of promoting a CA configuration that is primed to assemble into the mature lattice, although its quantitative contribution to capsid assembly efficiency could vary across different retroviruses.

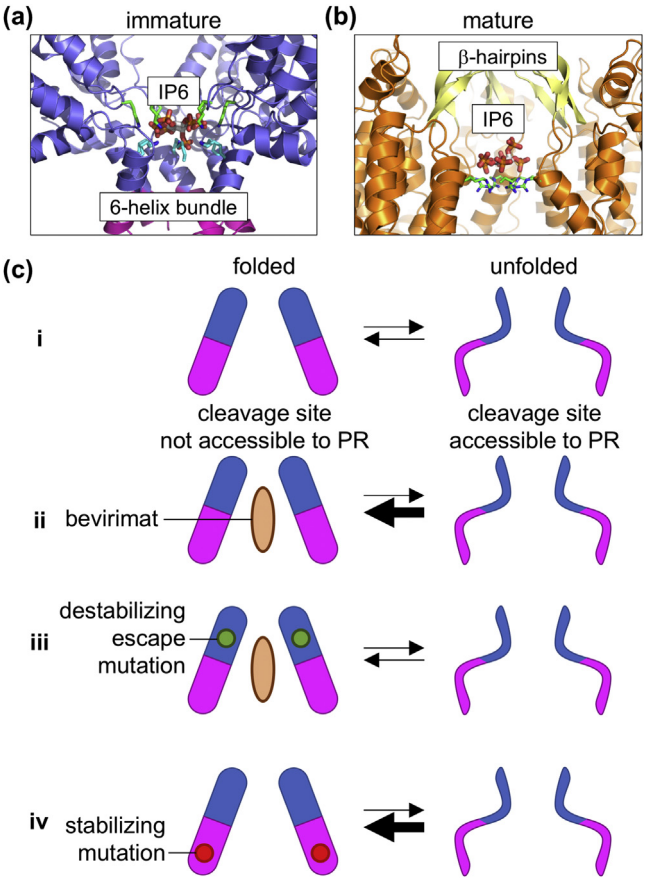
At the C-terminal end, an intact CA–SP1 junction is required in order to generate the six-helix bundle that holds together the immature hexamer [14••,31••,53,57] (Figure 2c). Proteolysis of the CA–SP1 junction in the middle of the helix irreversibly disrupts the six-helix bundle and destabilizes the immature hexamer, leading to disassembly of the immature lattice. In contrast to the β-hairpin, the folding status of the C-terminal CA–SP1 junction more cleanly correlates with the assembly status of HIV-1 ca. *In vitro*, an intact junction is absolutely required to assemble immature virus-like particles from recombinant HIV-1 Gag constructs, and the assembly efficiency is directly related to the efficiency by which the six-helix bundle folds [31••,53,58•,59••]. Correspondingly, mutations that prevent folding of the six-helix bundle favors assembly of mature lattices *in vitro*, even with a non-processed CA–SP1 junction [31••,53]. Both *in vitro* and in virions, HIV-1 particles with non-cleavable CA–SP1 junctions can form mature lattices, and in these lattices the junction helix is unfolded [41•,60]. Thus, folding of the CA–SP1 junction helix and formation of the six-helix bundle are required to assemble the immature HIV-1 lattice, whereas unfolding during maturation (or failure to fold *in vitro*) leads to assembly of the mature lattice. In HIV-1 and RSV, the six-helix bundle is stabilized by hydrophobic 'knobs-in-holes' interactions similar to classical coiled-coil proteins [14••,31••,61]. In contrast, MLV contains a 'charged assembly helix' that also forms a six-helix bundle but is primarily stabilized by electrostatic interactions [22•,62]. Furthermore, the MLV helix does not harbor a cleavage site, indicating that proteolysis is not required in this case to trigger conformational switching.

The importance of structural switches in HIV-1 assembly and maturation is underscored by the recent finding that inositol hexakisphosphate (IP6), a highly negatively charged small molecule that is abundant in mammalian cells, potently promotes folding of the CA–SP1 six-helix bundle and assembly of the immature lattice (Figure 3a) [59••]. IP6 binds just above the six-helix bundle though ionic contacts with positively charged lysine sidechains. During maturation, disruption of the six-helix bundle by proteolysis releases IP6. Proteolysis also unmasks an alternative binding site in mature CA, this time composed of arginine residues (Figure 3b) [59••,63••]. IP6 binding to this new site promotes formation of mature CA hexamers and assembly of the fullerene cone capsid. This second site is located near the base of the β-hairpin in mature CA, and so it is tempting to speculate that IP6 modulates both types of structural switches in HIV-1. Both the immature and mature IP6 binding sites appear conserved in lentiviruses; it remains to be seen whether equivalent sites exist in other retrovirus genera.

Small molecule inhibition of the HIV-1 CA–SP1 maturation switch

Previous studies have established that proteolytic maturation of retroviruses is temporally regulated, because PR processes the various Gag and Gag-Pol cleavage sites at different relative rates (Figure 2a) and altering these rates or preventing cleavage at essentially any site results in aberrant capsid reorganization and loss of infectivity (reviewed in Refs. [64,65]). In HIV-1 Gag, the CA–SP1 boundary elicits the slowest rate of proteolysis and is the final trigger for maturation. As described above, comparison between the immature and mature lattice structures indicates that this rate very likely reflects the unfolding kinetics of the six-helix bundle. Since the scissile bond is located in the middle of the junction helix and is sequestered by the six-helix bundle, it has been proposed that helical bundle must 'breathe' or transiently unwind to allow access by PR (Figure 3c–i) [31••,60]. This model is further supported by studies of a class of experimental drugs called 'maturation inhibitors' (MI). Biochemical and structural studies indicate that MI bind to the six-helix bundle and induce aberrant architectural rearrangement of the capsid, similar to mutations that prevent CA–SP1 processing (Figure 3c-ii) [36,60,66,67,68••]. MI escape mutations map primarily to regions in the CTD and SP1 that are important for hexamerization, and share the general property of destabilizing the immature hexamer [69,70–74]. Thus, MI disrupt the CA–SP1 switch by stabilizing the six-helix bundle and inhibiting its unwinding, whereas escape mutations destabilize the bundle and restore the switch (Figure 3c-iii). Interestingly, some escape mutations destabilize the

Figure 3



Small molecule modulation of HIV-1 maturation. **(a)** Inositol hexakisphosphate (IP6) binds just above the six-helix bundle to two rings of lysine residues (green and cyan) and promotes immature Gag lattice assembly. **(b)** Upon maturation, IP6 binds to a new site in mature CA, this time composed of a ring of arginine residues (green) located just below the N-terminal β-hairpins (yellow) and promotes assembly of the mature capsid. **(c)** Mechanism of 'maturation inhibitors' such as bevirimat. i – Model of stochastic 'breathing' or unwinding of the CA–SP1 six-helix bundle allows access of the viral protease. ii – Bevirimat binds at the center of the six-helix bundle and prevents its unwinding. iii – Escape mutations destabilize the six-helix bundle and allows unwinding even in the presence of bevirimat. iv – Mutations that stabilize the six-helix bundle phenocopy the effect of bevirimat.

immature hexamer to the point where assembly of the mutant virions become dependent on the presence of inhibitor [74]. Conversely, mutations that stabilize the six-helix bundle phenocopy the effect of MI (Figure 3c-iv) [75].

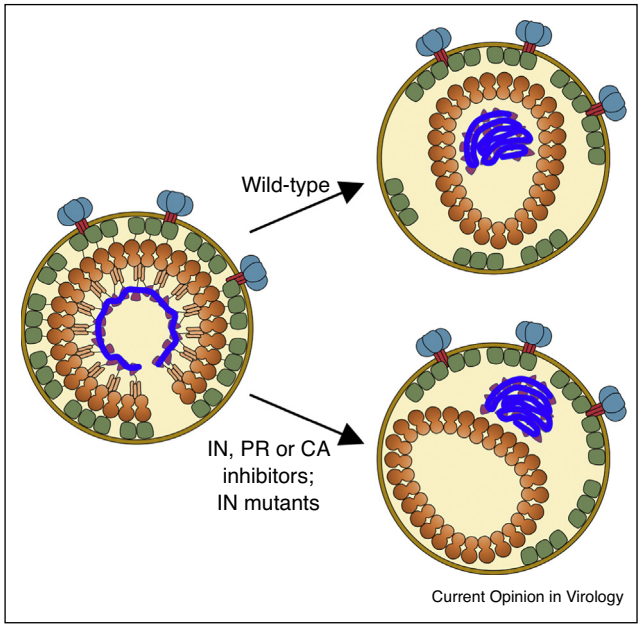
Protease activation and the timing of maturation

Temporal regulation of maturation manifests not only in Gag processing but also in activation of PR itself, which marks the onset of maturation. PR and the other essential viral enzymes, reverse transcriptase (RT) and integrase (IN), are expressed as Gag-Pol precursors and packaged into the assembling virion through incorporation of the Gag moiety into the immature lattice. PR functions as a dimer [76], and so activation requires interaction of two Gag-Pol subunits. Biochemical and structural studies indicate that PR dimers in context of Gag-Pol are unstable and exhibit low enzymatic activity, which favors self-processing that release the PR N-terminus [77–79]. Upon self-proteolysis, the N-terminal amino-acid residues of PR fold with C-terminal residues into a four-stranded β-sheet [77], which greatly stabilizes the PR dimer and promotes more efficient intermolecular cleavages. In context of actual virions, the mechanism of PR activation has been more challenging to analyze, in part because purified virions mature asynchronously. Synchronous maturation has been recently achieved by first preparing immature virions in the presence of PR inhibitors, and then initiating proteolysis by using an inhibitor washout procedure or photodeactivation [80,81]. Interestingly, these experiments showed that the resulting particles had aberrant core morphologies and were non-infectious, despite successful Gag cleavage. These observations were interpreted to mean that protease activation has to occur in concert with other replication steps such as virus budding [80], as also suggested by a number of other studies [82,83]. It appears that understanding of this important step will require the ability to track and visualize single virions at high spatial and temporal resolution as they assemble at the plasma membrane, bud from the cell surface, and become mature.

RNA re-encapsidation

During maturation, the viral RNA condenses with the new NC proteins into a compact ribonucleoprotein complex. In HIV-1, the genome appears as an electron-dense object within the capsid, typically near the broad end of the fullerene cone (Figure 4) [16,17]. Early studies have implicated IN in HIV-1 assembly and release [84], and have shown that IN mutations can induce aberrant HIV-1 core assembly [85]. These IN mutant virions display a characteristic phenotype of having electron-dense material outside an assembled yet apparently empty capsid shell; that is, the condensed genome is not re-encapsidated [85]. More recent studies have confirmed that these 'eccentric condensates' consist of the viral RNA in complex with NC, and that the same phenotype occurs when aberrant IN multimerization is induced by mutagenesis or allosteric integrase inhibitors, or when IN binding to the viral RNA is abrogated [86•,87•,88•]. Importantly, the maturation defect can be partially rescued when wildtype IN is supplied in *trans*, that is, outside the normal route of Gag-Pol packaging into the assembling virion [86•]. These observations have led to the proposal that HIV-1 IN plays an active role in promoting viral RNA re-encapsidation into the mature core by initiating capsid formation around the condensed ribonucleoprotein complex [87•]. This step of maturation appears particularly vulnerable to drugs, because apart from IN inhibitors,

Figure 4



The genomic ribonucleoprotein complex is normally re-encapsidated in the fullerene capsid upon maturation. In the presence of various inhibitors to integrase (IN), protease (PR) or the capsid protein (CA), the genome is excluded from the capsid interior as an 'eccentric condensate'. A number of IN mutations also produce the same ' eccentric condensate' phenotype.

suboptimal inhibition of PR and a capsid-targeted compound can also induce the same 'eccentric condensate' phenotype [89,90]. Developing a mechanistic understanding of how viral RNA condensation and capsid assembly are coupled during maturation is an important area of further research.

Concluding remarks

Our understanding of the process of retrovirus maturation has improved dramatically over the past several years. Elucidation of the structural endpoints of retrovirus maturation – the immature Gag spherical capsid and the mature CA fullerene capsid – have illuminated the conformational states of CA that direct the architectural transformation of the virion, and helped to clarify mechanisms of small molecule inhibition. Remaining challenges include understanding how efficient re-encapsidation of the genomic ribonucleoprotein complex occurs, and how this process prepares the genome for reverse transcription and delivery into a new host cell. As before, answering these questions will require the development and application of novel techniques.

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