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Retrovirus maturation — an extraordinary structural transformation

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Retroviruses such as HIV-1 assemble and bud from infected cells in an immature, non-infectious form. Subsequently, a series of proteolytic cleavages catalysed by the viral protease leads to a spectacular structural rearrangement of the viral particle into a mature form that is competent to fuse with and infect a new cell. Maturation involves changes in the structures of protein domains, in the interactions between protein domains, and in the architecture of the viral components that are assembled by the proteins. Tight control of proteolytic cleavages at different sites is required for successful maturation, and the process is a major target of antiretroviral drugs. Here we will describe what is known about the structures of immature and mature retrovirus particles, and about the maturation process by which one transitions into the other. Despite a wealth of available data, fundamental questions about retroviral maturation remain unanswered.

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Introduction

Retrovirus assembly is mediated by the viral polyprotein Gag, the major structural component of retroviruses. Gag consists of several independently folded domains connected by flexible linkers. Three of these domains are conserved throughout the retrovirus family: the membrane binding N-terminal matrix domain (MA), the bipartite capsid domain (CA) involved in Gag oligomerization, and the nucleic acid-binding nucleocapsid domain (NC). Between these conserved domains additional species-specific domains exist (Figure 1a).

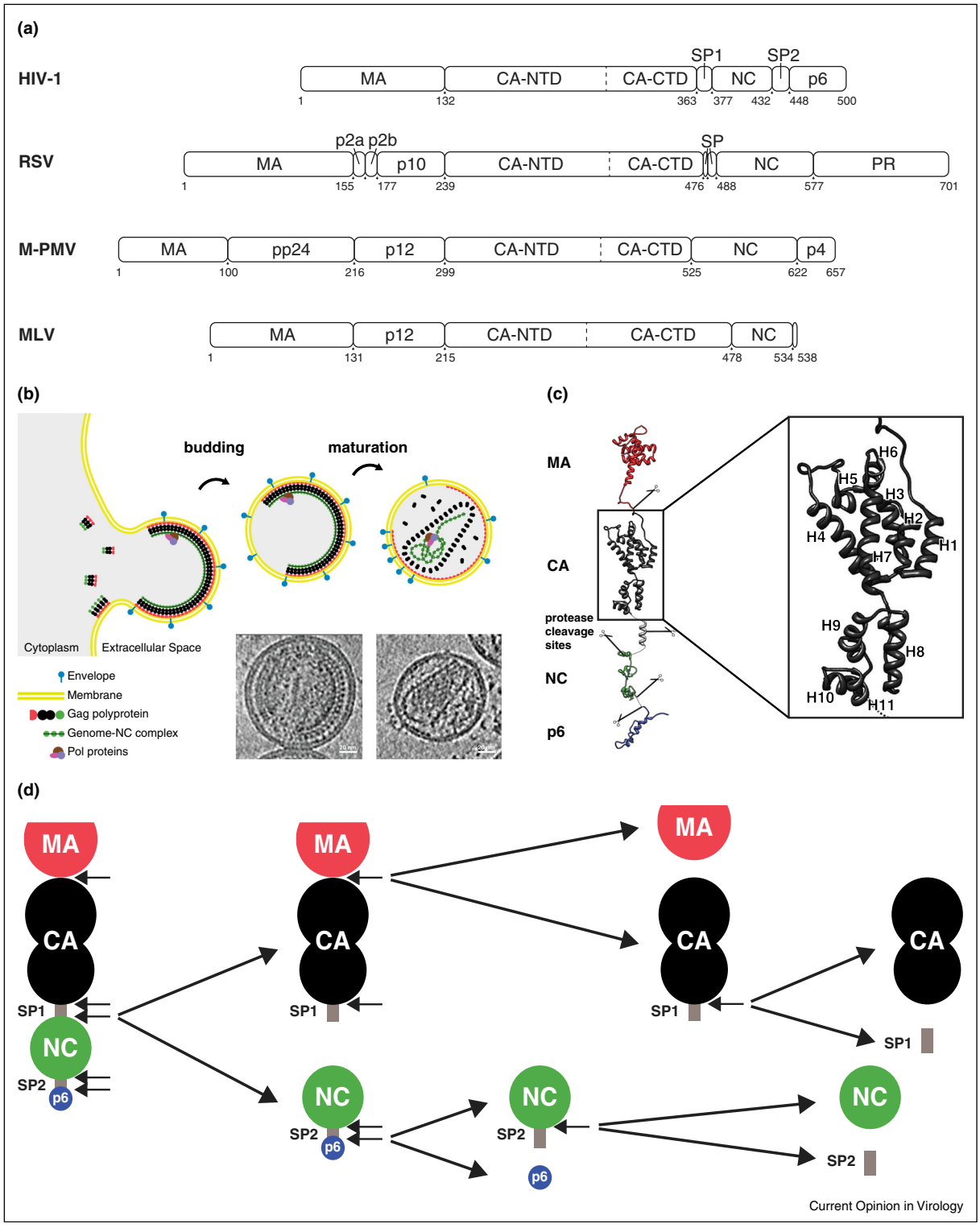
The formation of immature retrovirus particles within an infected cell is driven by the self-assembly of Gag using nucleic acid and/or the membrane as a scaffold (Figure 1b). Gag assembles into a curved hexameric lattice with the overall shape of a truncated sphere. In most of the retroviral genera the formation and growth of the hexameric Gag lattice occurs directly at the plasma membrane, though in betaretroviruses and spumaviruses Gag self-assembly occurs in the cytoplasm before the almost-spherical Gag shell is trafficked to the plasma membrane prior to envelopment and release. Concomitant with or after budding, activation of the viral protease leads to cleavage of Gag at specific positions, inducing major structural rearrangements of the individual Gag domains and a dramatic change in virus morphology (reviewed in [1–5]).

Our understanding of structural aspects of retrovirus maturation derives from: studies of isolated protein domains, mostly by X-ray and nuclear magnetic resonance (NMR) methods; studies of oligomeric assemblies of proteins or domains, mostly by X-ray and electron microscopy (EM) methods; and studies of virus particles, mostly by EM. In this review we will describe our current understanding of the structures of immature and mature retrovirus particles. We will then discuss current models describing how proteolytic cleavage induces local structural changes that lead to large-scale structural rearrangements, and how the complex mature viral architecture is attained. We will in particular focus on CA, the driver of oligomerization, and the impact of cryo-EM in understanding its behaviour.

The domains of Gag and their structures

Despite low sequence conservation between Gag proteins in different retroviruses, the canonical structural domains (Figure 1a and c) display strong tertiary structure conservation [6–11]. Crystal and solution structures of the membrane-binding domain MA reveal an alpha-helical fold with a propensity to form trimers with a basic membrane binding surface [12]. In HIV, MA is post-translationally modified by addition of a myristic acid moiety [13,14]. CA consists of two independently folded parts, CA-NTD and CA-CTD, connected by a flexible hinge region. The CA-NTD contains six to seven alpha-helices and in some viruses a flexible loop region implicated in binding of cellular host factors [15]. The CA-CTD consists of four alpha-helices and contains the major homology region (MHR), a stretch of approximately 20 well conserved residues [16]. The NC-domain harbours positively charged regions and one or two zinc-finger like motifs necessary for recruiting and binding of viral RNA.

Figure 1



Retrovirus assembly and maturation. **(a)** A schematic depiction of Gag domain architecture in human immunodeficiency virus type 1 (HIV-1), Rous sarcoma virus (RSV), Mason-Pfizer monkey virus (M-PMV) and murine leukemia virus (MLV). The three canonical domains MA, CA and NC are conserved. Outside of these domains additional domains and peptides are present in the different retroviruses. **(b)** A graphical representation of HIV-1 assembly, budding and maturation. MA is colored red, CA black, NC green and the membrane in yellow. Representative sections through cryo-electron tomograms of immature and mature HIV-1 particles are illustrated. **(c)** A structural cartoon of the HIV-1 Gag polyprotein marking the sites at which proteolytic cleavage occurs during maturation. In the enlarged panel on the right individual alpha-helices of the HIV-1 CA domain are numbered. **(d)** HIV-1 Gag proteolytic processing depicted in the order as it is believed to occur based upon relative cleavage rates in solution [63].

Assembly of immature retrovirus particles

In the absence of nucleic acid, Gag shows weak protein-protein interactions and flexibly adopts different conformations; in HIV these include a compact arrangement in which MA folds back to interact with NC [17]. Upon interaction with nucleic acid (RNA) in the cytoplasm, NC-RNA-NC and CA–CA interactions mediate Gag oligomerization. Gag oligomers then shuttle to the plasma membrane where the basic patch in MA mediates binding to raft-like membrane domains containing phosphatidylinositol-4,5-bisphosphate (PIP2) [18,19]. Assembly of a curved Gag lattice at the membrane leads to formation of a membrane bud. Membrane scission at the bud neck is mediated by the cellular ESCRT system, which is recruited through linear motifs in Gag [20]. Alongside virus-encoded proteins, several host proteins are packaged into the virus [21].

Recombinant expression of Gag is sufficient to assemble and release immature virus-like particles from cells in the absence of further retroviral proteins. Various Gag derived proteins can also assemble immature virus-like particles (VLPs) *in vitro* in the presence of nucleic acid. The C-terminal domain of CA and a stretch of downstream residues including part of the spacer peptide between CA and NC represents the minimal construct able to assemble virus-like particles *in vitro* [22] (for a detailed review on *in vitro* assembly of retroviruses see [23]).

Cryo-electron tomography studies of the immature Gag structure

The use of cryo-electron tomography (cryo-ET) has heavily influenced our current understanding of the arrangement of the Gag protein within immature virus particles. Cryo-ET has been applied both to authentic immature virus particles, and to *in vitro* assembled Gag-derivatives from different retroviruses that mimic the structure of immature particles. Early cryo-ET and subtomogram averaging work revealed that the immature HIV-1 Gag lattice within virus particles adopts a wide range of curvatures and incorporates regions where no ordered Gag protein is seen [24]. Subsequent work showed that within immature virions, Gag forms a continuous patch of hexagonal order containing randomly distributed defects and larger gaps [25]. These studies also generated the first 3D structural data on the immature Gag lattice. The CA-NTD was observed to form rings around the hexameric center, with what appeared to be extensive inter-hexamer interactions. The CA-CTD appeared as dimers similar to published dimeric crystal structures of the CA-CTD [26]. Below the CA-CTD there is a column of density at the six-fold symmetry axis that has a size able to accommodate a bundle of six alpha helices — it had previously been proposed that the spacer peptide SP1 forms a six-helix bundle in the immature virus [27].

Recent improvements in cryo-ET acquisition and data processing protocols [28] allowed the structure of CA within native immature HIV-1 virus particles to be determined to subnanometer resolution [29••]. At this resolution alpha-helices can be individually resolved, and available high-resolution structures for the CA domain could be flexibly fitted into the structure to give a detailed view on immature capsid lattice interactions (Figure 2a). Extensive inter-hexameric and intra-hexameric CA-NTD contacts were observed. Inter-hexameric CA-NTD contacts involved homo-dimeric and homo-trimeric interfaces in helices 1 and 2, respectively. Intra-hexameric interactions were formed by the top of helix 4 and the region spanning helix 5 and 6 in the adjacent monomer. Dimeric inter-hexamer interactions at the CA-CTD level were in agreement with earlier studies and involved hydrophobic residues in helix 9 (see Figure 1c for information on helix nomenclature and Figure 2a for illustrations of the lattice). The MHR was in a position where it could contribute contacts stabilizing the hexamer ring around the central 6-fold. As suggested by hydrogen-deuterium exchange experiments no extensive CA-NTD/CA-CTD interfaces were formed [30]. Downstream of CA, rod-like densities were consistent with the presence of a 6-helix bundle formed by residues of CA and SP1.

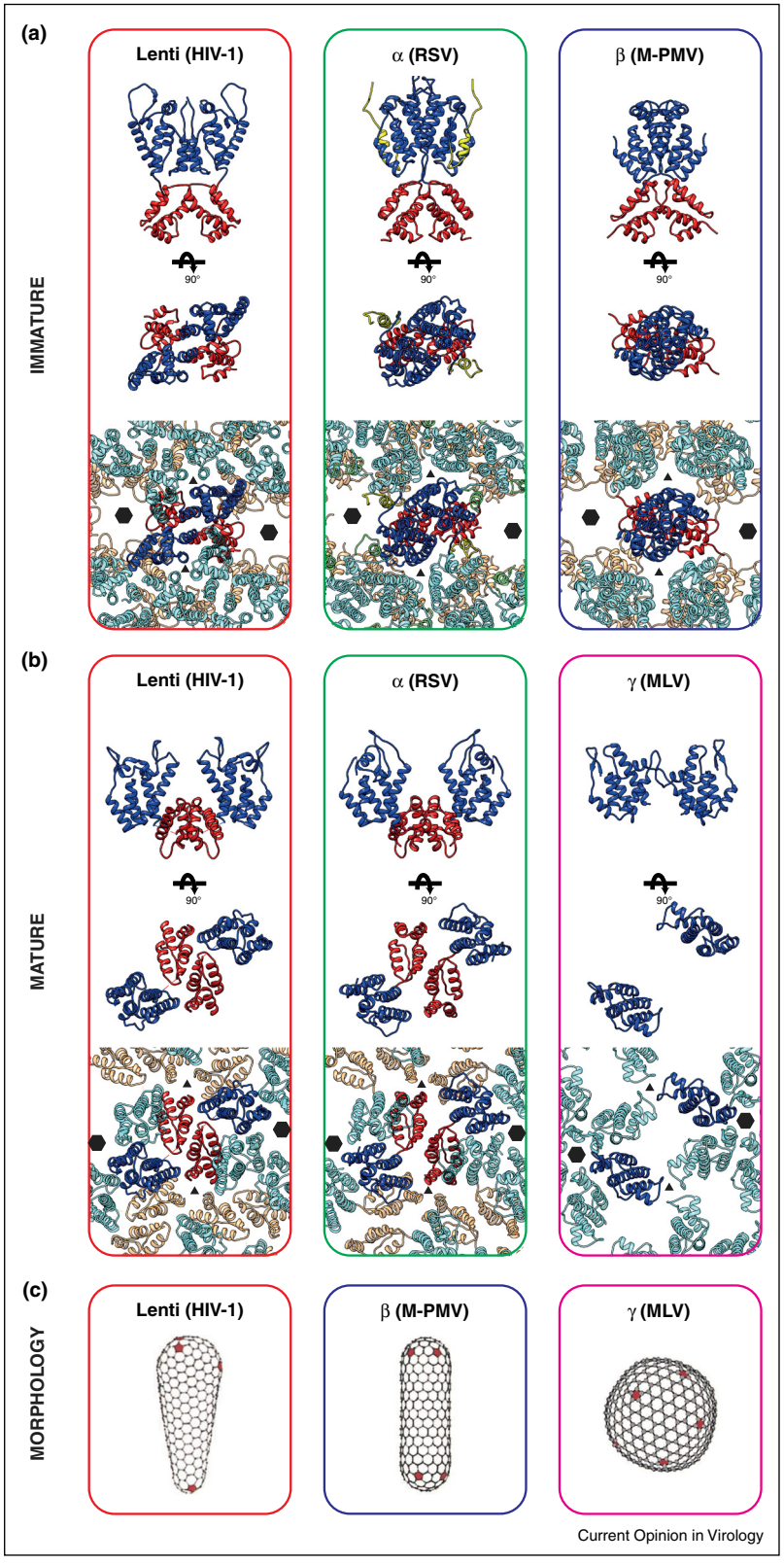
Using the same cryo-ET and subtomogram averaging approach, the capsid lattices in *in vitro* assembled immature virus-like Rous-sarcoma virus (RSV) Gag particles [31] and native immature Mason-Pfizer monkey virus (M-PMV) particles were also solved [29••]. The results obtained for M-PMV were consistent with a previous structure of *in vitro* tubular assemblies of an M-PMV capsid mutant [32]. Comparison of capsid quaternary arrangements found in all three viruses revealed that it is conserved for the CA-CTD domains (Figure 2a). Unexpectedly, although previous low-resolution structures had showed a similar immature lattice spacing of ~80 Å and suggested a conserved CA-NTD arrangement [33,34], the arrangement of the CA-NTD domains was in fact different in all three viruses when observed at higher-resolution. In contrast to HIV-1, M-PMV and RSV CA-NTDs form extensive dimeric contacts to ensure hexamer-hexamer interaction. RSV employs part of p10, a domain upstream of CA, as an additional domain to stabilize the immature CA lattice [31,35,36].

In none of the structures solved did the MA domain or NC-RNA complex show organized structures relative to CA, meaning there seems to be no fixed structural arrangement between different Gag domains.

Determinants of immature Gag lattice structure

A number of observations indicate that the essential interfaces for assembly and stabilization of the immature

Figure 2



The arrangement and interactions of the CA domains in immature and mature retroviruses. **(a)** A comparison of immature CA arrangements of HIV-1 (PDB 4USN [29••]), RSV (PDB 5A9E [31]), and M-PMV [29••]. HIV-1 and M-PMV were determined 'in virus', RSV *in vitro*. Immature CA dimers are shown in an orthogonal view (top panel), in a top view (middle panel), and as part of the hexameric immature lattice (lower panel).

lattice reside in the CA-CTD and immediately downstream residues. Firstly, as described above, the arrangement of the CA-NTD is not conserved between retroviruses, while that of the CA-CTD is largely conserved [29••,31,32]. Secondly, a mutated HIV-1 CANC construct has been shown to assemble arrays *in vitro* in which the CA-CTD arrangement matches that seen in native immature HIV-1 virus particles, while the CA-NTD adopts an arrangement that would be unable to form a hexameric lattice in spherical virions [37]. Thirdly, the minimal construct for assembly of virus-like particles *in vitro* does not include the CA-NTD [38]. Fourthly, extensive mutagenesis studies locate the critical assembly residues in the CA-CTD [39,40•]. During immature virus assembly, the CA-NTD is therefore likely only to play a modulating or regulatory role, while it plays a more critical role in mature virus assembly (see below).

The structure of the mature capsid core

Activation of the protease, and cleavage of Gag into its individual Gag domains leads to a major structural rearrangement that results in the formation of the mature capsid core encasing the condensed genome [1] (Figure 1b). The retroviral mature CA core is pleomorphic and has been proposed to be a fullerene structure in which a hexameric lattice is closed by the incorporation of 12 pentameric Gag assemblies [41].

The structure of the mature CA hexameric arrangement has been intensively studied over the last few decades. CA and CA-NC constructs can be assembled into mature-like tubular assemblies *in vitro* for analysis by cryo-electron microscopy [42,43••], and CA derived proteins have also been crystallized in hexameric arrangements [44,45••,46•]. These assemblies have dimensions consistent with the ~100 Å hexameric lattice spacing observed in cores isolated from mature virions [47]. Using *in vitro* systems, the mature-like arrangements of CA have been resolved for HIV-1 [42,43••,44,45••], RSV [48] and Bovine leukemia virus (BLV) [46•], while the CA-NTD arrangement has also been studied in Murine leukemia virus (MLV) [11,49]. While in-virus structures are currently lacking, these *in vitro* studies indicate that the arrangement of CA in mature retroviruses is well conserved (Figure 2b).

The mature CA lattice has a hexamer-hexamer spacing larger than that seen in the immature virus particle [47,50,51]. The CA–CA interactions that mediate mature core assembly involve different surfaces of the protein than those in immature particles. In HIV six CA-NTDs form a hexameric ring with a central 18 helix bundle formed by helices 1, 2, and 3, providing the main intra-hexamer stability [44,45••,46•,52]. The CA-CTDs are located beneath the hexameric ring and form dimeric inter-hexamer interactions involving mainly hydrophobic interactions between residues on helix 9 at the local two-fold [6,53] and residues on helix 10 at the local three-fold [43••,45••,54]. An interface is also present between each CA-CTD and the CA-NTD of the neighbouring CA molecule in the hexameric ring [55].

In order to accommodate spherical curvature within hexameric lattices, either larger defects and cracks must be present, as in the immature virus, or the lattice must contain pentameric vertices, as is thought to be the case in the mature virus. According to the fullerene model of the core, forming a closed structure from a hexameric lattice requires the incorporation of 12 pentamers [41]. Cardone *et al.* determined a low-resolution cryo-EM structure of the pentamer in an *in vitro* assembled icosahedral RSV capsid structure [56]. The fitting of high-resolution crystal structures of the individual CA domains suggested that the hexameric and pentameric assemblies were quasi-equivalent. A crystal structure of a cross-linked pentameric assembly of HIV-1 CA confirmed that a pentamer could be obtained by removal of one copy of CA from a hexamer while keeping essentially the same protein-protein interactions [57]. An arginine (residue 18 in CA) in CA-NTD points into the center of the hexameric and pentameric rings. It has been proposed that electrostatic repulsion between arginine 18 residues favours hexamer formation over pentamer formation (where the proximity between charges would be higher), and that this may provide a mechanism to regulate pentamer formation. To date, there is no published structural data on hexamers or pentamers within retroviral particles.

Different retroviruses adopt distinct core geometries, and the cores within a single virus preparation can also vary in morphology [47,58,50,59]. Different core geometries are proposed to differ in the distribution of the pentamers within the hexameric lattice [41,60,61] (Figure 2c). The cores are typically conical in HIV (5 pentamers at the tip and 7 at the base), tubular in M-PMV (6 pentamers at each end), and polyhedral in RSV and MLV (12 pentamers homogenously distributed). Within the core, there are variable local curvatures of the hexameric CA lattice (for example the lattice is more tightly curved at

(Figure 2 Legend Continued) CA-NTD and CA-CTD are colored blue/cyan and red/orange, respectively. The p10-domain in immature RSV is colored yellow/green. Six-fold and three-fold symmetry axes are labeled with black hexagons or triangles, respectively. While the CTD quaternary structure is conserved between the retroviruses, the NTD arrangement is not. **(b)** A comparison of mature CA arrangements of HIV-1 (PDB 4XFX [45••]), RSV (PDB 3TIR [48]), and MLV (PDB 1U7K [11]) based on *in vitro* assemblies. The mature CA quaternary structure is conserved between these retroviruses. **(c)** A schematic illustration of different mature core morphologies. All cores are formed from hexagonal lattices, containing 12 pentamers to accommodate curvature and closure. Cores of different morphologies are assumed to vary in the relative positions of pentamers within the lattice.
Panel C adapted with permission from [60].

the narrow end of the HIV-1 cone than at the broad end). This requires considerable structural flexibility of the CA lattice that has been suggested to be accommodated by the flexible linker between CA-NTD and CA-CTD [44,45••], by rotations around the dimeric CA-CTD interface [43••], and by structural changes at the trimeric CA-CTD interface [46•].

The maturation switch

How is the transition from immature to mature virus morphology triggered and controlled? There are three aspects to this question. Firstly, how is proteolytic cleavage activated, and how is the timing of cleavage at the different sites within Gag regulated? Secondly, what is the structural mechanism by which proteolytic cleavage induces a switch in the CA structure from an immature packing to a mature one? Thirdly, how is the complex architecture of the mature virus built, that is, how do the viral proteins successfully assemble a pleiomorphic core containing the condensed RNP?

The mechanism of timing of viral protease activation is poorly understood, but it seems likely to be linked to viral budding. Premature proteolytic cleavage can apparently occur in a subset of cells infected with adenoviral vectors expressing HIV-1 Gag and Gag-Pol, leading to cleavage between CA and NC before budding, and preventing RNP incorporation into particles [62]. Once the protease is activated, the rate of cleavage differs between each cleavage site in Gag. This determines a kinetically regulated, ordered release of the different domains [63] (Figure 1d). Abolishing or altering the rates of cleavage at individual sites leads to maturation and core morphology defects [64–67] and loss of infectivity [63,68,69]. We attempted to synchronize maturation using a washout procedure to remove a protease inhibitor [70•]. Although Gag was successfully cleaved, this approach resulted in aberrant core morphologies for the vast majority of the particles after maturation, suggesting that timing of cleavage may be important. Together, these observations point to a requirement for kinetic regulation of Gag and Gag-Pol proteolysis for ordered maturation.

Upon proteolytic cleavage, the CA domains rearrange to form the mature core. CA–CA interactions in the immature lattice are broken and new interactions, involving different parts of the CA surface, form to mediate assembly of the mature lattice. Why one set of interactions is destabilised by proteolytic cleavage, and why another set of interactions becomes favoured, is unclear. Two regions have been proposed to function as structural switches in HIV-1. A beta-hairpin is formed by the N-terminal residues in mature-like CA, stabilized by a salt bridge between the N-terminal proline and a conserved aspartate in helix 3 [10,71–73]. This conformation cannot be present in the immature virus since the N-terminal proline is part of the CA-MA linker. In order to act as a structural switch, hairpin formation presumably either destabilises the immature lattice or stabilises the mature lattice. Current low-resolution structures of the immature lattice suggest, however, that the hairpin could be accommodated in the immature lattice [29••], and high-resolution structures of the mature hexamer do not reveal a clear stabilizing role for the hairpin. The second region proposed to act as a structural switch is the SP1 spacer peptide downstream of CA [74]. Cleavage upstream of CA and downstream of SP1 leads to disruption of the immature lattice, while cleavage between CA and SP1 is additionally required to assemble the mature lattice [64,66]. There is no published high-resolution structural data on SP1 within assembled lattices, so the possible mechanism of such a switch is unclear.

The architectural transition

Upon maturation, the truncated-sphere arrangement of the immature virus particle is converted to a conical (or other polygonal) mature capsid structure. CA can be induced to assemble *in vitro* into conical structures with some similarity to the viral core [41]. However, *in vivo* other viral components modulate mature core assembly, including integrase [75,76•], the RNP [77], and the viral membrane [78]. *In vivo*, the core must encapsidate the RNP rather than assemble adjacent to it. Disruption of RNP condensation interferes with core assembly, but removal of the RNP completely by exchanging NC with a leucine zipper (LZ) moiety, still permits core assembly, indicating it is not essential for nucleation of core growth [77].

Various models have been proposed to explain how the mature capsid architecture is achieved in the virus [78–80,81•,82]. The correct model must be consistent with two striking experimental observations. Firstly, only approximately two thirds of the CA molecules within immature lattice are used to assemble the mature core [47,83,84]. Secondly, the distribution of defects in the immature hexameric lattice, and the distribution of pentamers within the mature hexameric core are not the same: transition between the two therefore requires the breaking of CA–CA interactions as well as the formation of new CA–CA interactions. The majority of authors have favoured a model in which the immature CA lattice largely disassembles upon cleavage, into monomers, dimers, hexamers or other small oligomers of CA, which reassemble to form the mature core [51,78–80,81•]. Core growth has been proposed to be initiated at the narrow end [78], the broad end [79], or the sides of the cores [81•]. An alternative non-diffusional phase transition model has also been proposed in which the immature CA layer remains largely intact and curls to form the mature core, leading to the formation of strained cores with lattice defects as have been observed in some tomograms [82]. In this alternative model it is more difficult to understand

how the defects in the immature lattice are repositioned and the pentamers appropriately arranged.

Understanding the architectural transitions during maturation will require imaging of maturation intermediates. This is challenging because the process is rapid and asynchronous. It is difficult to identify maturation intermediates, and to distinguish intermediates that are on a productive assembly pathway from defective intermediates. This problem is further complicated because interfering with the timing of proteolytic cleavage, which would be essential in synchronizing or arresting maturation, may also cause defects in core morphology.

Conclusions

Many fundamental questions related to retrovirus capsid maturation therefore remain unanswered. What is the structure of the SP1 region that is so essential in immature virus assembly? How is proteolytic cleavage activated? What are the structural switches triggered by proteolytic cleavage and how do these modulate CA–CA interactions? Does the CA lattice disassemble into small oligomers or curl up to form the mature core? Is there a template for core growth, and how does growth proceed? Are viral cores perfect closed fullerene structures, or do they contain structural defects?

Answering these questions will require advances in our understanding of retrovirus structure. In particular, new insights can be expected from better structures of CA within immature and mature virus particles, and from methods that allow the study of intermediate stages in maturation.

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- •• of outstanding interest

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