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FIV Gag: Virus Assembly and Host-cell Interactions

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Abstract

Infection of domestic cats with virulent strains of the feline immunodeficiency virus (FIV) leads to an acquired immunodeficiency syndrome (AIDS), similar to the pathogenesis induced in humans by infection with human immunodeficiency virus type 1 (HIV-1). Thus, FIV is a highly relevant model for anti-HIV therapy and vaccine development. FIV is not infectious in humans, so it is also a potentially effective non-toxic gene therapy vector. To make better use of this model, it is important to define the cellular machinery utilized by each virus to produce virus particles so that relevant similarities can be identified. It is well understood that all replication-competent retroviruses encode *gag*, *pol*, and *env* genes, which provide core elements for virus replication. As a result, most antiretroviral therapy targets *pol*-derived enzymes (protease, reverse transcriptase, and integrase) *or env*-derived glycoproteins that mediate virus attachment and entry. However, resistance to drugs against these targets is a persistent problem, and novel targets must be identified to produce more effective drugs that can either substitute or be combined with current therapy. Elements of the *gag* gene (matrix, capsid, nucleocapsid, and "late" domains) have yet to be exploited as antiviral targets, even though the Gag precursor polyprotein is self-sufficient for the assembly and release of virus particles from cells. This process is far better understood in primate lentiviruses, especially HIV-1. However, there has been significant progress in recent years in defining how FIV Gag is targeted to the cellular plasma membrane, assembles into virions, incorporates FIV Env glycoproteins, and utilizes host cell machinery to complete virus release. Recent discoveries of intracellular restriction factors that target HIV-1 and FIV capsids after virus entry have also opened exciting new areas of research. This review summarizes currently known interactions involving HIV-1 and FIV Gag that affect virus release, infectivity, and replication.

Keywords

FIV; HIV-1; Gag; viral late domains; ESCRT; virus-cell interactions

1. Introduction: FIV is a relevant model for AIDS and lentiviral gene therapy

FIV is endemic in the wild and has evolved for millennia, along with its ancestral hosts (Pedersen et al., 1987; Troyer et al., 2005). FIV infection of its native host induces feline AIDS, characterized by a progressive decline in $CD4^+$ T-cells that is clinically asymptomatic for years

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unless challenged with an opportunistic pathogen (Burkhard and Dean, 2003). Unlike SIV-induced simian AIDS, development of feline AIDS does not require a cross-species transmission, which provides some clear advantages for studying both horizontal and vertical transmission in relatively inexpensive cat colonies and feral cats (Coats, 2005; Willett et al., 1997). FIV pathology is similar to that of HIV-1 and is best described in domestic cats (*Felis catus*) infected with FIV_{fca}, due to a high incidence of infection (~1 to 30%) with apparently no evolution of host resistance (Troyer et al., 2005; Winkler et al., 1999). Despite centuries of close contact, there is no evidence of FIV transmission to humans, possibly due to poor recognition of the FIV promoter (5'-LTR) in human cells (Mustafa et al., 2005). This block can be overcome for FIV-based gene therapy, applicable to both dividing and non-dividing cells of virtually any type, by substitution of the FIV 5'-LTR with a CMV promoter (Johnston et al., 1999). Thus, understanding FIV biology in human cells is also potentially relevant to clinical applications. FIV is the only lentivirus for which a vaccine is readily available (Hohdatsu et al., 1997), which is protective against a subset of known FIV subtypes. Mechanisms of protection appear to involve both humoral and cellular immunity (Pu et al., 1997). Thus, FIV is clearly a useful model for the development of AIDS vaccines, antiretroviral drugs, and non-pathogenic gene therapy vectors. Compared to primate lentiviruses, many fundamental aspects of FIV cellular biology are not well understood. However, significant progress has been made in recent years in identifying molecular mechanisms of infection, in part based on comparative studies with HIV-1 and other lentiviruses (Elder et al., 2008; Luttge et al., 2008).

2. FIV and HIV-1 genome homology

Both FIV and HIV-1 contain essential elements found in all retroviruses (*gag*, *pol*, *env*), possibly derived from a common ancestral lentivirus (Katzourakis et al., 2007), with the addition of accessory factors that enhance replication *in vivo* but are often dispensable in cell culture models [reviewed in (Elder et al., 2008)]. Since FIV has evolved independently in cats, it has virtually no sequence similarity to primate lentiviruses in homologous open reading frames and the collection of accessory factors is the least conserved (Olmsted et al., 1989; Pecon-Slattery et al., 2008). With the exception of Rev and Vif, most accessory factors encoded by HIV-1 (including Tat, Nef, Vpr, and Vpu) have not been clearly identified in FIV, although FIV Orf2/OrfA may have functions partially related to both Tat and Vpr (Sundstrom et al., 2008). Earlier studies with FIV Vif suggested a possible analogy with HIV-1 Vif, but their functional similarity has only recently been clearly demonstrated (Münk et al., 2008).

3. The Gag protein is a self-contained virus assembly “machine”

Expression of retroviral Gag polyprotein precursors alone, within a suitable host cell, is sufficient for the production of virus-like particles (VLPs) (Fig. 1A) [reviewed in (Adamson and Freed, 2007;Ganser-Pornillos et al., 2008)]. All retroviral Gag proteins contain domains required for membrane targeting, Gag-Gag interaction, and virus release (Fig. 1B). Through interactions and modifications of the membrane-targeting domain, lentiviral assembly typically occurs at the plasma membrane (PM) while budding away from the cytoplasm of the infected cell. Other steps in virus assembly and release are driven by interactions of Gag with itself and with host cell factors via “late” domains (Fig. 1C). After these domains have exerted their functions in assembly and release, the Gag precursor is cleaved by the virally encoded protease (PR), if present, into the final mature Gag proteins. For HIV-1 and FIV these include matrix (MA), capsid (CA), nucleocapsid (NC), spacer peptides (SP1, SP2) flanking NC, and a C-terminal peptide (p6 in HIV-1, p2 in FIV) (Elder et al., 1993;Ganser-Pornillos et al., 2008;Lin et al., 2006). Activation of PR, and the resulting cleavage of Gag, appears to coincide with final events in virus release. However, retrovirus release itself does not depend upon PR function; immature VLPs containing unprocessed Gag are released efficiently in the absence

of PR (Calistri et al., 2009;Fu et al., 2006;Huang et al., 1995;Ono et al., 2004;Ono et al., 2000;Ono et al., 2005;Peng et al., 1989;Tomonaga et al., 1998).

3.1 Matrix (MA)

Lentiviral MA domains play important roles in the assembly of infectious particles by directing Gag to the PM, mediating the association between Gag and the inner leaflet of the PM lipid bilayer, and recruiting the viral envelope (Env) glycoproteins into virions (Freed, 1998; Freed and Martin, 1995, 1996) (Fig. 1B).

Membrane targeting—The ability of most retroviral MA domains to direct Gag-membrane binding requires the covalent modification of the MA N-terminus with myristic acid and is also promoted by a highly basic patch of amino acid residues (Dalton et al., 2007; Freed et al., 1994; Hill et al., 1996; Yuan et al., 1993; Zhou et al., 1994). For both HIV-1 and HIV-2, Gag targeting to the PM is facilitated by a direct interaction between some of these basic residues and the host factor phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$], which is concentrated on the inner leaflet of the PM (Fig. 1B) (Chukkapalli et al., 2008; Ono et al., 2004; Ono et al., 2005; Saad et al., 2008; Saad et al., 2006). FIV MA appears to utilize a similar mechanism for targeting FIV assembly, since perturbation of $PI(4,5)P_2$ levels inhibits FIV release (Luttge and Freed, unpublished results) and basic patches in FIV MA are required for particle production (Manrique et al., 2004a). SIV also relies on basic residues in MA for membrane-targeted assembly (González et al., 1993), and it has been shown that SIV MA can substitute for FIV MA to produce infectious virus (Manrique et al., 2004a). FIV MA, however, is generally less basic than SIV or HIV-1 MA (Fig. 1B), and cannot substitute for SIV MA in SIV to produce infectious particles. Interestingly, this block is readily overcome by site-directed mutagenesis of FIV MA to restore two critical lysine residues found in SIV and HIV-1 MA. Thus, as observed with many other domains of FIV Gag, these data suggest a high degree of conserved structure and function in FIV MA with MA from primate lentiviruses, despite the absence of significant amino acid sequence identity (Burkala and Poss, 2007; Elder et al., 1993).

Env incorporation—The N-terminal region of HIV-1 MA also functions to enhance incorporation of the Env glycoprotein into virions by a putative interaction with the cytoplasmic tail of HIV-1 Env (Env-CT, Fig. 1A) (Davis et al., 2006;Freed and Martin, 1996;Lambelé et al., 2007;Murakami and Freed, 2000). It has recently been proposed that the 47-kDa tail-interacting protein (TIP47; also known as mannose-6-phosphate receptor binding protein 1, M6PRBP1) may be an important adaptor protein that bridges this interaction by binding both the HIV-1 Env-CT and the N-terminal domain of HIV-1 MA (Lopez-Vergès et al., 2006). The requirement of the FIV Env-CT for virion incorporation of FIV Env is likely to be similar to that of HIV-1; however, a direct role for FIV MA has not yet been determined (Celma et al., 2007).

3.2 Capsid (CA)

Retroviral CA domains perform important functions both in promoting Gag-Gag interactions during assembly and in forming the outer shell of the viral core that houses the viral genome and *pol*-encoded enzymes in the mature virion. CA folds into two structural and functional domains, the N-terminal and C-terminal domains (NTD and CTD, respectively, Fig. 1B). The HIV-1 CTD contains a major interface for CA-CA dimerization that promotes Gag multimerization (Gamble et al., 1997). Both the NTD and the CTD bear interaction interfaces that are crucial for the assembly of the hexameric CA lattice that ultimately gives rise to the conical core characteristic of mature lentiviral particles (Ganser-Pornillos et al., 2007).

Structure—Compared to HIV-1 and several other retroviruses, very little is known about FIV CA (p24/p25) in terms of its structure, dimerization interface, and interactions with host

cell factors. Several lines of evidence suggest that non-primate lentiviral CA proteins (from FIV and EIAV) retain a conserved fundamental structure that is similar to that of HIV-1 CA, despite sequence divergence. For example, vaccination of cats with HIV-1 CA provides protection against FIV infection, most likely through one or more cross-reactive T-cell epitopes (Coleman et al., 2005). Antibodies raised against CA from EIAV or HIV-1 are cross-reactive with FIV CA, and *vice versa* in the case of EIAV (Egberink et al., 1990; Egberink et al., 1991; Nath and Peterson, 2001). A three-dimensional structure for FIV CA is not yet available. However, secondary structural predictions of FIV CA NTD and CTD have been modeled against the crystal structures for EIAV and HIV-1 CA, and highlight many similarities (Burkala and Poss, 2007). Conserved structural features in each NTD include an N-terminal β-hairpin, tightly packed α-helices (seven in HIV-1 and five in both FIV and EIAV) arranged into an arrowhead shape, and an exposed flexible loop (Ganser-Pornillos et al., 2007; Jin et al., 1999). A highly conserved feature of this extended loop in each CA NTD is the exposure of proline residues, at least one of which binds peptidylprolyl isomerase A (cyclophilin A or CypA) (Gamble et al., 1996; Lin and Emerman, 2006). Structural highlights of the CTD include a strand-turn-helix motif known as the major homology region (MHR), which is found in many divergent retroviruses, and four highly conserved α-helices, one of which (helix 9 in HIV-1, helix 7 in FIV and EIAV) forms a critical dimerization interface between HIV-1 CA monomers in solution that is apparently retained in the mature capsid structure (Gamble et al., 1997; Ganser-Pornillos et al., 2007). Overall, these studies suggest that many elements of CA structure are highly conserved among lentiviruses.

Interactions with CypA and TRIM-family proteins—CA binding to CypA, described above, is a conserved feature of several retroviruses, including FIV and HIV-1 (Franke et al., 1994; Lin and Emerman, 2006). The precise role for this interaction in virus replication remains enigmatic. However, inhibition of CypA-CA binding by treatment with cyclosporin A inhibits the replication of both FIV and HIV-1 (Karpas et al., 1992; Mortola et al., 1998). Interestingly, factors expressed in monkey cells that naturally restrict HIV-1 infection have been shown to exploit the CypA-binding loop to target incoming viral capsids shortly after virus entry [reviewed in (Luban, 2007; Towers, 2007)]. Recent studies have shown that these restriction factors are based on derivatives of the tripartite motif 5 protein (TRIM5), which is part of a large family of TRIM proteins (~70 in humans) that possibly constitute a broad innate immune response (Ozato et al., 2008). The first example of a TRIM protein that restricts HIV-1, TRIM5α_{rh}, was discovered in rhesus macaques (Stremlau et al., 2004), and is a natural inhibitor of HIV-1 infection that is stimulated by type 1 interferon. TRIM5α homologs, formerly referred to as Lv1 and Ref1 activities, have since been identified in African green monkey (AGM) and human (hu) cells, respectively (Keckesova et al., 2004). Perhaps not surprisingly, human TRIM5α has relatively weak anti-HIV-1 activity (Sokolskaja et al., 2006; Stremlau et al., 2004). The TRIM5α homologs described above (rh, hu, AGM) have also been shown to restrict FIV and EIAV, which suggests that this family of restriction factors may have evolved as broad-based lentiviral inhibitors (Hatziioannou et al., 2003; Hatziioannou et al., 2004; Saenz et al., 2005). Divergence of the TRIM-family proteins arises partly from alternative splicing of the RBCC tripartite motif (RING domain, B-Box domains, and coiled-coil motifs), and a variable C-terminal domain (CTD). Together these domains incorporate functions of E3 ubiquitin ligase activity (RING), TRIM protein multimerization (CC), and target-binding specificity (CTD). The antiretroviral activity of TRIM5α_{rh} appears to require the RING and CC domains, resulting in a ubiquitin-mediated proteasomal degradation of the bound target, although TRIM multimerization may also be required at an earlier step to prematurely accelerate virion uncoating (Javanbakht et al., 2007; Rold and Aiken, 2008; Stremlau et al., 2006). Specificity for TRIM5α targeting to the CypA-binding loop of lentiviral capsids lies in the C-terminal B30.2/SPRY domain. In recent years, several laboratories have independently made similar discoveries of a TRIM5-related restriction factor in owl monkeys and Asian

macaques (pig-tailed and rhesus), in which the B30.2/SPRY domain of TRIM5α is missing and contains a CypA domain instead, creating the TRIMCyp protein (Brennan et al., 2008; Newman et al., 2008; Nisole et al., 2004; Ribeiro et al., 2005; Sayah et al., 2004; Virgen et al., 2008; Wilson et al., 2008). Each of these TRIMCyp factors evolved entirely independently from each other and TRIM5α. Like HIV-1, FIV is sensitive to both owl monkey and macaque TRIMCyp proteins, which again argues that relatively broad-based restriction factors may have evolved through host resistance against ancient retroviral pathogens (Diaz-Griffero et al., 2007; Kratovac et al., 2008; Nath and Peterson, 2001; Virgen et al., 2008).

Interactions with the cytoskeleton and associated cellular motors—Retroviral CA proteins have also been shown to interact with the host cell cytoskeleton, possibly to exploit retrograde cytoskeletal motors for directional transport of incoming intracellular viral cores, reverse transcription complexes, and pre-integration complexes toward the nucleus [reviewed in (Fackler and Krausslich, 2006; Naghavi and Goff, 2007)]. Similar interactions may also be involved with anterograde trafficking of virus assembly intermediates toward the plasma membrane. Early studies suggested direct interactions of HIV-1 Gag with filamentous actin (Bukrinskaya et al., 1998; Rey et al., 1996). Actin and actin-binding proteins (e.g., ezrin-radixin-moesin (ERM) family members, and cofilin) have also been shown to be incorporated into HIV-1 particles; however, attempts to define the role of actin in retrovirus release by treatment with actin-disrupting agents (latrunculin, cytochalasin D) have been complicated by the cytotoxic side effects of these drugs (Ott et al., 2000; Ott et al., 1996; Sasaki et al., 1995). The cortical actin layer just below the PM provides one of the first intracellular barriers to viral infection. Moesin, which is a cortical actin crosslinker, acts as a natural restriction factor against retroviral infection if highly expressed (Naghavi et al., 2007). The antiviral activity of moesin may be due to its ability to regulate the formation of stable microtubules (MT), which has also recently been shown for ezrin (Haedicke et al., 2008). Other reports suggest an indirect interaction of Gag with MT by binding MT-associated motors. For example, HIV-1 Gag binds KIF-4, an MT-associated anterograde cellular motor (Tang et al., 1999). Consistent with this finding, it has recently been shown that disruption of the function of endogenous KIF-4, either through siRNA-mediated knockdown of KIF-4 or expression of dominant-negative KIF-4, decreases HIV-1 virus-like particle production and Gag protein stability (Martinez et al., 2008). The relationship between Gag precursors, CA, cytoskeleton, and cellular motors still remains poorly understood for HIV-1, FIV, and most other retroviruses.

3.3 Nucleocapsid (NC)

NC domains serve several major roles during retroviral replication (Freed, 1998): 1) together with CA they mediate Gag-Gag interactions that lead to the assembly of the virus particle, 2) they bind the viral RNA genome and recruit it into the assembling virus particle, and 3) they serve as a nucleic acid chaperone that facilitates a variety of steps early in the virus replication cycle. FIV NC contains essentially all known structural components and functions of HIV-1 NC (Moscardini et al., 2002). Specifically, NC packages the FIV RNA genome through recognition of a specific packaging signal,Ψ, which partially overlaps the *gag* open reading frame (Kemler et al., 2002). Through this interaction, the cellular trafficking of viral RNA and Gag polyprotein precursors, as a ribonucleoprotein complex, are likely to be intimately linked; however, the precise cellular location at which Gag-RNA association begins is poorly understood and may differ between retroviruses. It has been well demonstrated that interactions between Gag and RNA, through the NC domain, are required for assembly of retrovirus particles (Campbell and Rein, 1999; Campbell and Vogt, 1995; Muriaux et al., 2001; Rulli et al., 2007). It is not yet clear why lentiviral NCs have two zinc (Zn) fingers, since most NC functions apparently rely on the proximal Zn finger. Nonetheless, the tandem arrangement of two CCHC-type Zn fingers in HIV-1 is conserved in FIV NC. The amino acid sequence of EIAV NC is more similar to that of FIV NC than that of HIV-1 NC; however, there is an

unusually high degree of sequence similarity in the tandem Zn finger region among all lentiviruses. In particular, the spacing of Zn-coordinating Cys and His residues in each Zn finger is identical among NCs from FIV, HIV-1, and EIAV, with only a slightly longer basic linker between Zn fingers in HIV-1 NC. Three-dimensional structures for NC from both HIV-1 and EIAV are now available (Amodeo et al., 2006; De Guzman et al., 1998), and it remains to be seen what the structure of FIV NC will be.

3.4 Late (L) domains and FIV p2

Retroviral late domains—L domains are short peptide motifs found within the retroviral Gag protein that are required for efficient virus release. These motifs bind directly to components of the cellular endosomal sorting complexes required for transport (ESCRT-I, II, III), or bind cellular factors associated with this machinery. As the name implies, ESCRTs are required for the sorting and transport of ubiquitinated cargo proteins (associated with endosomal membranes) for delivery into late endosomes. The final action of ESCRT and associated proteins results in the budding of intraluminal vesicles (ILVs) away from the cytoplasm into late endosomes, hence these compartments are also known as multivesicular bodies (Fig. 2). The complete ESCRT and associated machinery is highly complex, and will not be described fully here, but has been detailed extensively in the literature, especially with regards to its role in virus release (Bieniasz, 2006;Demirov and Freed, 2004;Hurley and Emr, 2006;Morita and Sundquist, 2004;Williams and Urbe, 2007). Most if not all retroviruses, and many other enveloped viruses, utilize this machinery to drive the budding of virions from the PM, especially at the crucial “pinching off” step that completes virus release. There are currently three known retroviral L domain motifs (PT/SAP, YPX_nL, and PPXY). The PT/SAP motif in HIV-1 and FIV Gag (Fig. 1C) binds Tsg101, one of four components of the human ESCRT-I (Garrus et al., 2001;Luttge et al., 2008;Martin-Serrano et al., 2001;VerPlank et al., 2001). The YPX_nL motif in HIV-1 and EIAV Gag (Fig. 1C) binds Alix, which associates with ESCRT-I and III (Martin-Serrano et al., 2003;Strack et al., 2003). The PPXY motif (not found in FIV, HIV-1, or most other lentiviral Gag protein) binds Nedd4 or Nedd4-like ubiquitin E3 ligases (Kikonyogo et al., 2001). This motif is common in simple retroviruses (e.g., murine leukemia virus and Rous sarcoma virus), mouse mammary tumor virus, human T-cell lymphotropic virus, and some non-retroviruses like Ebola, often in combination with a PT/SAP motif. The mechanism by which viral proteins are able to divert ESCRT machinery for a budding process at the PM, rather than into late endosomes, is still unknown and somewhat controversial. Indeed, initial reports suggested that the initial release of HIV-1 may occur into an intracellular compartment in some cell types (Joshi and Freed, 2007). However, further studies now suggest that in fact these compartments, which were seemingly intracellular by two-dimensional electron microscopy, may actually be deeply invaginated extensions of the PM, from which virus is later released to the extracellular space formed between adjacent cells (Deneka et al., 2007;Groot et al., 2008). This form of release may be stimulated by cell-cell contact, resulting in a “virological synapse” with similarities to an immunological synapse between antigen-presenting cells and T-lymphocytes, to achieve a “cell-mediated” rather than “cell-free” infection (Sattentau, 2008).

Requirements for ESCRT in lentiviral release—The role of ESCRT and associated host factors in enhancing virus release can be shown by exogenous expression of either full-length or fragments of ESCRT-related proteins. These often have a dominant-negative effect on either ESCRT function in general or the association of Gag with ESCRT machinery. In either case, the ability of endogenous host factors to enhance virus release is abrogated. In some instances, inhibition is achieved simply by competing for a direct interaction with Gag. For example, overexpressing the N-terminal, ubiquitin E2-variant (UEV) domain of Tsg101 (TSG-5') has a dominant-negative effect on release of viruses that contain a PT/SAP motif (*e.g.* HIV-1, FIV, Fig. 1C), because the UEV domain binds to the PT/SAP motif and prevents it from interacting

with endogenous Tsg101 (Fig. 2) (Demirov et al., 2002;Luttge et al., 2008). Similarly, overexpression of the central, Gag-binding V domain of Alix alone (Fig. 2) inhibits release of viruses that contain a YPXnL motif (HIV-1, EIAV) (Fig. 1C), because critical Bro1 and proline-rich domains of Alix are absent (Lee et al., 2007;Luttge et al., 2008;Munshi et al., 2007) [reviewed in (Fujii et al., 2007)]. Conversely, overexpression of ESCRT-associated factors that do not bind Gag late domains can also inhibit retroviral release by a variety of mechanisms. For example, overexpression of the C-terminal half of Tsg101 (TSG-3', Fig. 2) results in an aberrant aggresome-like accumulation of TSG-3' in the cytoplasm, within which other ESCRT components and possibly ubiquitin are sequestered (Goila-Gaur et al., 2003;Johnson et al., 2005;Luttge et al., 2008;Shehu-Xhilaga et al., 2004).

FIV p2—Most lentiviruses have a PT/SAP motif in the C-terminal domain of Gag (Fig. 1C). The PSAP motif in the p2 domain of FIV Gag is essential for FIV release in feline and human cells and for FIV replication in feline cell lines (Calistri et al., 2009;Luttge et al., 2008;Manrique et al., 2004b). Peptides derived from the sequence of FIV p2 bind directly to the UEV domain of human Tsg101 *in vitro*. Tsg101 is also required for efficient FIV release in human cells, as shown by siRNA-mediated knockdown of Tsg101 (Luttge et al., 2008). As is the case for HIV-1, FIV release is sensitive to ESCRT-associated dominant-negative inhibitors, and TSG-5' can be stably expressed in CrFK cells to constitutively inhibit FIV release and replication (Luttge et al., 2008). Unlike HIV-1 and EIAV, the ESCRT-associated protein Alix appears to have no role in facilitating FIV release in human cells. Specifically, the Alix V domain targets YPXnL late domains in HIV-1 and EIAV Gag (Fig. 1C) *in vitro*, but there is no apparent interaction with FIV Gag p2 (Luttge et al., 2008). Initial studies of site-directed mutants in FIV p2 suggested that a C-terminal LxxL motif was required for FIV release, using the FIV-14 molecular clone expressed in CrFK cells (Manrique et al., 2004b). However, this phenotype was not reproducible with the FIV-34TF10 clone in CrFK cells or with an FIV Gag-Pol expression vector in either feline or human (HeLa) cells (Luttge et al., 2008), even though Gag sequences used in each study were apparently identical.

4. Role of Gag ubiquitination in retroviral release

Covalent attachment of individual ubiquitin molecules (monoubiquitination or multiubiquitination) to lysine residues of intracellular proteins is a signal for recruitment of ESCRT and associated machinery, which results in the sorting of ubiquitinated cargo into MVBs (Fig. 2) [reviewed in (Hicke and Dunn, 2003;Stuffers et al., 2008)]. In contrast, attachment of a chain of ubiquitin molecules (polyubiquitination) to a single lysine residue is associated with targeting to the proteasome for protein degradation. Ubiquitination follows a pathway that involves a series of enzymes known as E1, E2, and E3. E1 activates individual ubiquitin molecules, which are then attached to an E2 ubiquitin-conjugating enzyme. E3 ligases transfer the activated ubiquitin from E2 to a bound substrate. Specificity for the E3 step is determined by adaptor proteins in a multimeric SCF complex (Skp1-Cullin-F box protein) [reviewed in (Ho et al., 2008)]. Some components of the ESCRT pathway, including Tsg101, utilize ubiquitin interacting motifs (UIMs) to facilitate interactions as ubiquitinated cargo is handed from one ESCRT complex to another (Hurley et al., 2006). The precise role of mono/multi-ubiquitination in retroviral release is not entirely clear [reviewed in (Martin-Serrano, 2007)]. However, most retroviral Gag proteins, including that of FIV, are ubiquitinated (Calistri et al., 2009). It has been speculated that ubiquitination of Gag enhances its interaction with ESCRT, through UIMs. For example, primary targets of the retroviral PPxY late domain motif (described above) are E3 ubiquitin ligases (Nedd4 and Nedd4-like proteins), yet viruses with this late domain still ultimately rely on ESCRT for release. Overexpression of Nedd4-like proteins has been shown to enhance ubiquitination of HIV-1 and FIV Gag, which appears to rescue virus release defects in PT/SAP-defective mutants (Calistri et al., 2009;Chung et al., 2008). One explanation for this phenomenon is that the ubiquitin moiety itself, attached to Gag,

may be able to directly recruit ESCRT components that enhance virus release through UIMs, if the normal late domain is absent. Consistent with this hypothesis, release of EIAV Gag mutants that lack a late domain is rescued when Gag is artificially fused to ubiquitin (Joshi et al., 2008). Alternatively, the ubiquitination of unidentified host factors could enhance virus release, and ubiquitination of Gag may simply be a byproduct of its association with ESCRT machinery and proximal E3 Ub-ligases. Nedd4-like protein overexpression results in the monoubiquitination of many cellular proteins, in addition to Gag. This global increase in ubiquitination may enhance interactions between proteins with UIMs, such as the ESCRT machinery, which would likely favor virus release. In support of this hypothesis, foamy virus Gag naturally has very few lysine residues available for monoubiquitination, yet it can still be released efficiently in a ubiquitin-dependent manner when all sites for Gag ubiquitination are removed by site-directed mutagenesis (Zhadina et al., 2007).

5. Conclusions

Due to the fundamental importance of Gag and its many functions in the retroviral life cycle, the processes of virion assembly, release, and post-entry events remain attractive targets for antiviral intervention. Endogenous restriction factors appear to inhibit FIV or HIV-1 by targeting Gag or virions at each of these stages, some of which are counteracted by virally encoded accessory proteins. For example, APOBEC3G (a member of the APOBEC family of cytosine deaminases) is incorporated into virions during virus assembly, inhibits infectivity, and is counteracted by HIV-1 Vif (Goila-Gaur and Strebel, 2008; Münk et al., 2007). Recently it has been shown that feline APOBEC3CH inhibits the infectivity of HIV-1, SIV, and Vif-deficient FIV, which is counteracted by FIV Vif (Münk et al., 2008). Bst-2/tetherin, which inhibits fully infectious particles at the level of virus release, is counteracted by HIV-1 Vpu (Jouvenet et al., 2009; Neil et al., 2008; Van Damme et al., 2008) [reviewed in (Wolf and Goff, 2008)]. TRIM5-related proteins (TRIM-5α, TRIM-Cyp, described above) target the viral CA at a post-entry step (Ozato et al., 2008). Similarly, since Gag mediates many of its functions (membrane targeting, virus assembly, Env incorporation) while it is still relatively accessible prior to virus release and maturation, there may be an important window of opportunity for antiretroviral intervention during assembly. In this regard, CA assembly and virion maturation seem to be especially promising targets [reviewed in (Adamson and Freed, 2007; Ganser-Pornillos et al., 2008; Salzwedel et al., 2007) and (Adamson et al., 2009)]. FIV infectivity, like that of HIV-1, is highly sensitive to the efficiency and order of Gag processing during virion maturation, which encourages the continued use of FIV as a model for developing PR and maturation inhibitors (Lin et al., 2006).

Although this review is focused primarily on HIV-1 and FIV Gag, many other retrovirus-encoded components required for virus replication are still active targets for antiviral drug development. For example, enzymes encoded by the *pol* gene must be present for infectivity to be achieved. These enzymes include PR, reverse transcriptase (RT), integrase (IN), and an additional enzyme, dUTPase (DU), found only in non-primate lentiviruses. HIV-1 *pol*-encoded enzymes have been successfully targeted by PR, RT, and IN inhibitors, and many of these have recently been modeled against homologous FIV pol-encoded enzymes: PR (Heaslet et al., 2007; Lin et al., 2006; Norelli et al., 2008), RT (Martins et al., 2008), and IN (Savarino et al., 2007). Thus, FIV continues to be a useful model for understanding the cellular and molecular biology of HIV-1 replication and in developing more effective antiretroviral therapeutics.

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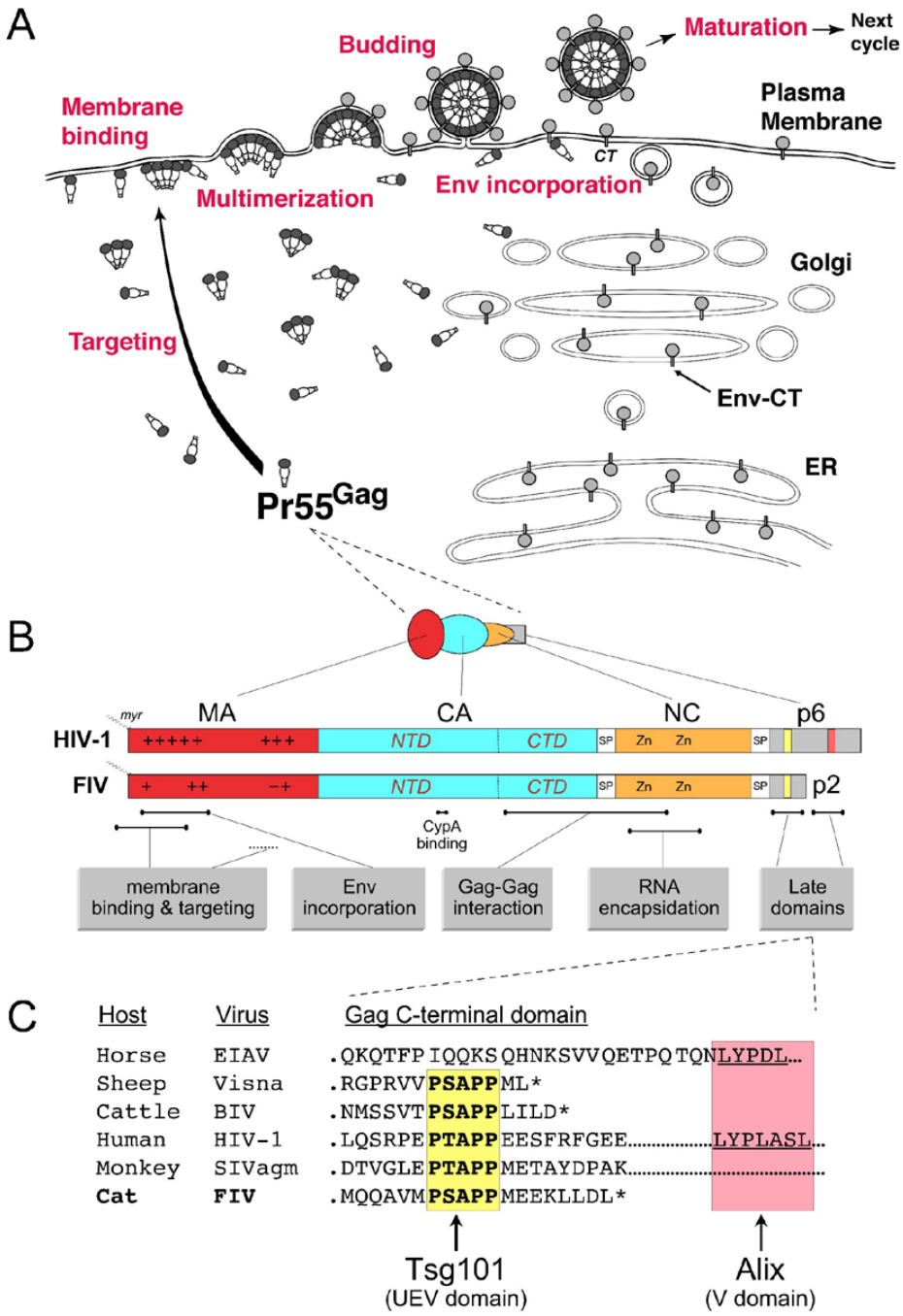

Figure 1. Overview of HIV-1 and FIV Gag domains and the virus assembly and release pathway

A) Gag is synthesized as a precursor protein (HIV-1 $Pr55^{Gag}$ = FIV $Pr50_{Gag}$) that exists in both monomeric and oligomeric forms in the cytoplasm. Interactions between Gag and host factors target Gag to the plasma membrane (PM) in most cell types. Membrane binding facilitates higher-order Gag multimerization that leads to virus assembly and budding. Release of virus particles is driven by interactions with host cell factors. Maturation of the virus occurs during or shortly after virus release and is required for virus infectivity. Env is a highly glycosylated protein, synthesized in the endoplasmic reticulum (ER), that follows the secretory pathway through the trans-Golgi network to the PM. Env is incorporated into budding virus particles via interactions between the matrix (MA) domain of Gag and the Env cytoplasmic tail (Env-

CT). Adapted from (Joshi and Freed, 2007) with permission of Future Medicine Ltd. **B)** Domains of the Gag precursor protein from HIV-1 and FIV are shown, including known or predicted functions and interactions. The MA domain is covalently modified with myristate (myr) at the N-terminus. Two patches of basic residues (+) in MA are modestly conserved. Capsid (CA) folds into an N-terminal domain (NTD), which contains a conserved cyclophilin A (CypA) binding loop, and a C-terminal domain (CTD). Nucleocapsid (NC) contains two conserved zinc-coordinating motifs (Zn), which are important for interaction of Gag with viral RNA. NC is flanked by two spacer peptides (sp) in both HIV-1 and FIV, which must be cleaved from CA and NC during maturation for full viral infectivity. C-terminal domains of Gag in HIV-1 (p6) and FIV (p2) contain late domains important for virus release. **C)** An alignment of C-terminal domains of several lentiviral Gag proteins is shown, highlighting a highly conserved PSAPP motif that binds the ubiquitin E2-variant (UEV) domain of tumor-susceptibility gene 101 (Tsg101). Equine infectious anemia virus (EIAV) and HIV-1 share an $LYPX_nL$ motif that binds the V domain of apoptosis-linked-gene-2-interacting protein X (Alix). BIV, bovine immunodeficiency virus; SIVagm, simian immunodeficiency virus from African green monkey.

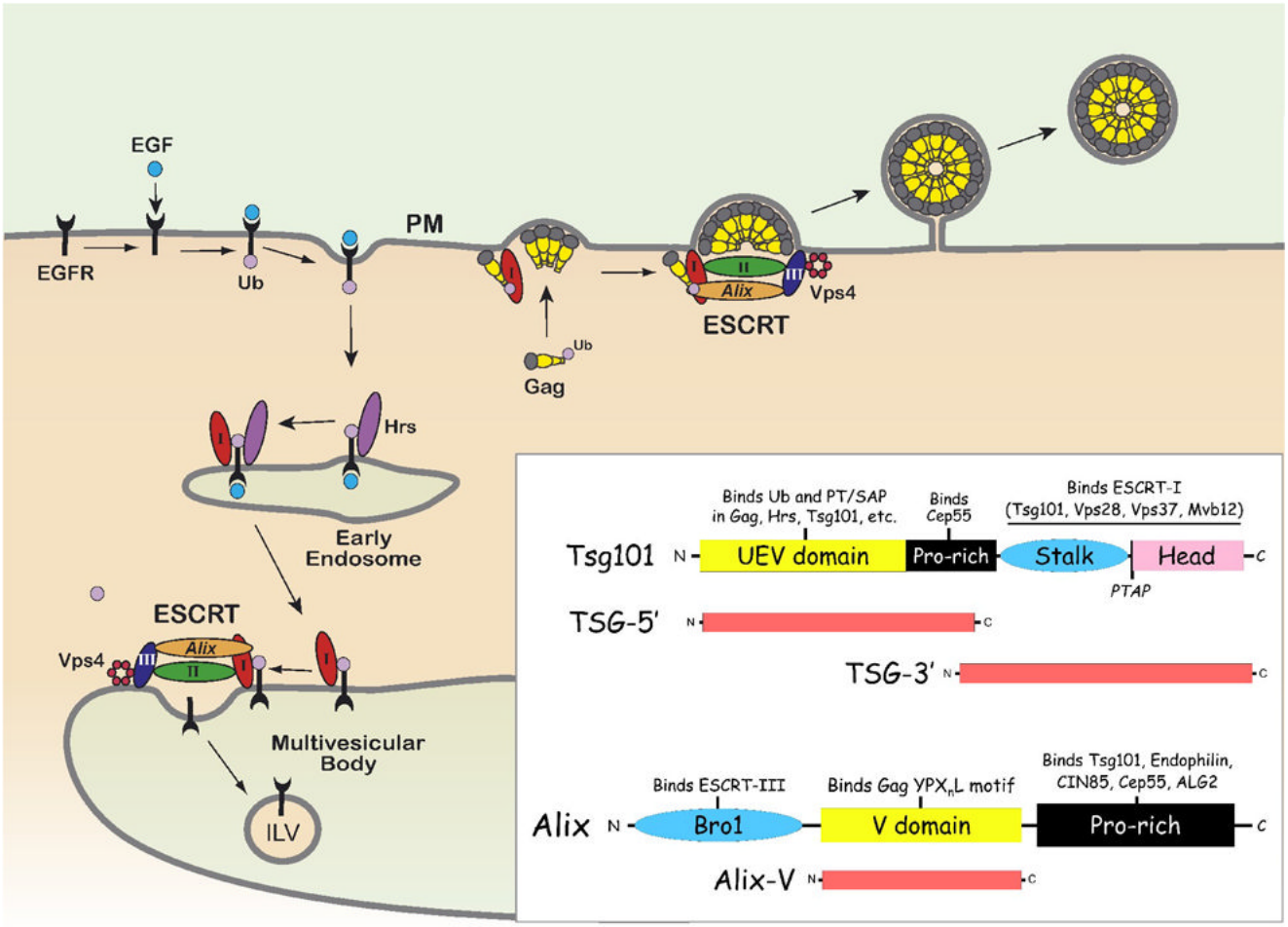

Figure 2. Relationship between retrovirus release and ESCRT machinery

Retroviruses utilize late domains in Gag to recruit endosomal-sorting complexes required for transport (ESCRT-0, I, II, III), which facilitate virus release from the plasma membrane (PM). An example of the normal function of ESCRT is demonstrated by the downregulation of epidermal growth factor receptor (EGFR). Upon binding its ligand (EGF) at the PM, the EGFR is monoubiquitinated and internalized by endocytosis. Attachment of ubiquitin (Ub) to EGFR targets Ub-EGFR to the multivesicular body (MVB) through an endosomal sorting process, and results in the degradation of EGFR in lysosomes. First, Ub-EGFR bound to an endocytic vesicle is internalized into early endosomes by ESCRT-0 through a direct interaction with hepatocyte growth factor regulated tyrosine kinase substrate (Hrs). ESCRT-I (I) is recruited to Ub-EGFR through a PSAP motif in Hrs, which binds the UEV domain of Tsg101. The Tsg101 UEV domain binds Ub-EGFR to complete the transfer from Hrs. Tsg101 itself is bound to ESCRT-I through C-terminal Stalk and Head domains. ESCRT-I bound to Ub-EGFR then traffics from the early endosome to the MVB. Through a series of interactions with ESCRT-II, III, Alix, and Vps4, an intraluminal vesicle (ILV) containing EGFR is formed, which buds into the MVB. HIV-1 and FIV Gag are each ubiquitinated proteins with a PT/SAP motif, which mimic Hrs and recruit ESCRT machinery to stimulate virus release from the PM. TSG-5' and TSG-3' constructs are dominant-negative inhibitors of Tsg101 that inhibit HIV-1 and FIV release, because each lacks components found in the endogenous full-length protein. Alix is an ESCRT-associated protein that binds many cellular factors in the domains indicated, including Tsg101 and ESCRT-III. HIV-1 and EIAV Gag each contain a YPX_nL motif that binds the Alix V domain. Association of Gag with Alix recruits ESCRT machinery, which

enhances virus release. Expression of Alix-V alone, without ESCRT-(I,III)-binding domains, inhibits HIV-1 and EIAV Gag release but has no effect on FIV. Adapted from (Demirov and Freed, 2004) with permission from Elsevier.