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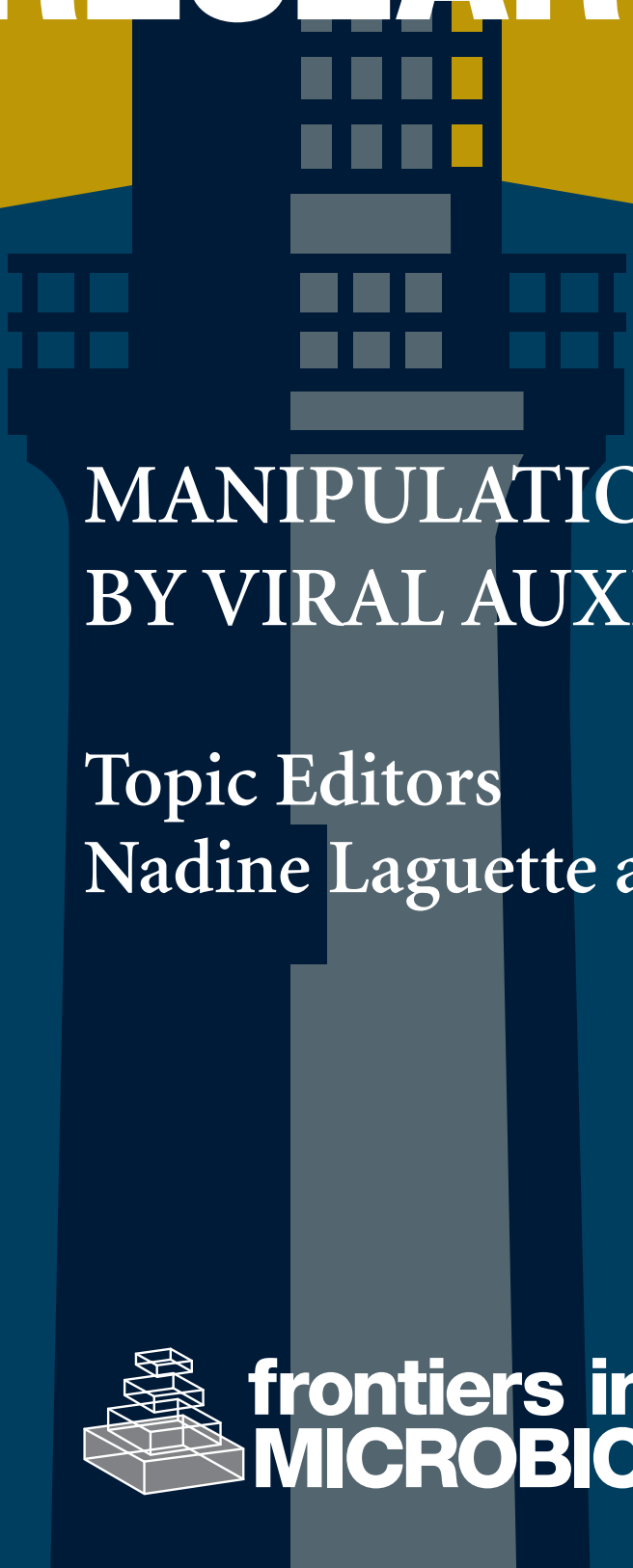

MANIPULATION OF THE HOST CELL BY VIRAL AUXILIARY PROTEINS

Topic Editors
Nadine Laguette and Monsef Benkirane

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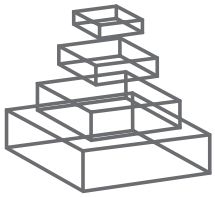

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MANIPULATION OF THE HOST CELL BY VIRAL AUXILIARY PROTEINS

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Productive HIV infection requires completion of all the steps of the replication cycle, the success of which largely relying on the multiple interactions established by viral proteins with cellular partners. Indeed, cellular and viral fates are intertwined and this interplay may involve rerouting of cellular factors/pathways to the benefit of the viral life cycle. To gain a foothold into host cells, HIV has to take advantage of available cellular factories and overcome the numerous potential blocks opposed to its replication while ensuring cellular survival. Viral auxiliary proteins are a perfect paradigm to illustrate the complexity of the relationship between HIV and its host. Although these accessory proteins are mostly unnecessary for viral replication in permissive cells in vitro, they play a crucial role in regulating viral spread ex vivo in non-permissive cells and in vivo in hosts. Most accessory proteins are pleiotropic and instrumental in the counteraction of restriction factors and proteins involved in innate immune response.

Several proteins of the "intrinsic" immune system that detect the presence of the assailant and initiate a subsequent immune response, as well as restriction factors that are directly devoted to arresting the replication cycle at precise steps have been characterized. Despite the numerous cellular mechanisms dedicated to preventing viral replication, HIV is able to efficiently replicate in humans. Indeed, as a master regulator of cellular machineries and processes, not only has HIV evolved strategies to avoid triggering of pattern recognition receptors, but HIV has also elaborated ways to counteract host restriction factors, thereby overcoming the hurdles that oppose efficient replication.

This review collection is dedicated to the manipulation of host cells by HIV-1 and HIV-2, with a particular focus on viral accessory proteins.

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Shaping of the host cell by viral accessory proteins

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Keywords: HIV, accessory proteins, restriction factors, intrinsic immunity, sensing

Optimal viral replication relies on the ability of viruses to use cellular resources and to overcome the intracellular defense mechanisms. The human immunodeficiency virus (HIV) is no exception to the basic rules governing viral replication cycles. Indeed, to gain a foothold into host cells and disseminate, HIV needs to efficiently complete a series of steps, including entry, reverse transcription of its RNA genome into DNA competent for integration, and transcription of the integrated provirus into RNA molecules that will either be translated into progeny virion protein components, or constitute viral genomes. At each of these steps, cellular components, such as dNTPs, NTPs and amino acids, as well as cellular pathways are mobilized. Some of the latter passively serve the viral life cycle whereas some are actively high-jacked and sidetracked from their usual cellular function. While the presence of such resources and pathways within host cells facilitates viral replication, they do not suffice to render cells permissive to HIV infection. In fact, cells possess an arsenal of detection mechanisms (pathogen recognition receptors) and proteins that can block specific steps of the viral life cycle (restriction factors) that together constitute the intrinsic immune system.

Due to its limited genome size and the small number of encoded proteins, HIV-derived proteins, including accessory proteins (Vpr, Vpx, Vpu, Vif, and Nef), are highly pleiotropic and can contact numerous cellular partners. These proteins, which were initially coined as "accessories" because they appeared to be virtually unnecessary for *in vitro* viral replication in permissive cells, are encoded by lentiviral genomes in addition to major structural and enzymatic proteins (Gag, Pol, and Env) and regulatory proteins (Tat and Rev). The importance of accessory proteins can be evidenced as inefficient viral spread, delay in replication curves or low viral loads upon disruption of their corresponding open reading frames (ORF) in non-permissive cells and *in vivo*. Because these accessory proteins are key virulence factors, they have been the subject of intense investigation over the past decades. While this has allowed the identification of several cellular pathways and processes they subvert, the outcome of the established interactions remains sometimes elusive. Nonetheless, major functions of these accessory proteins may be grossly classified as: (i) counteraction of cellular restriction factors (ii) escape from innate immune sensing, (iii) disturbance of cellular pathways, and (iv) enhancement of viral infectivity. Importantly, no accessory protein has been shown to date to fit all four categories, but it is consensually admitted that more investigations are needed for full characterization of their function. This review collection aims at discussing recent advances in our understanding of manipulation of host cells by the HIV type 1 (HIV-1) and type 2 (HIV-2), with a particular focus on viral accessory proteins. Importantly, because our understanding of the function of viral accessory proteins frequently requires a better understanding of the antagonized cellular proteins or processes, these are also discussed.

To address these aspects, this review series starts by relating our current understanding of Nef function (Basmaciogullari and Pizzato, 2014), a master regulator of intracellular trafficking pathways. Particular emphasis is put on Nef-dependent enhancement of viral infectivity, an as of today poorly understood phenotype. Interestingly, together with Vpr, Nef is the only other HIV accessory protein for which a *bona fide* antagonized cellular restriction factor has not been identified to date. Similar to Nef, Vpr was documented to disturb several cellular pathways (Guenzel et al., 2014), with a major consequence being a potent cell cycle arrest. However, this major outcome was recently shown to be a mere side effect of Vpr-induced activation of a cellular SLX4 endonuclease complex (Bregnard et al., 2014; Laguette et al., 2014). Activation of the SLX4 complex likely serves to allow the processing of virion-derived reverse transcripts to favor escape from innate immune detection. This function is similar to that proposed for the TREX1 cellular exonuclease (Yan et al., 2010). Importantly, TREX1 and SLX4 complex activation are only two of the many ways by which the HIV virus evades innate immune sensing. While a particular focus is given to TREX1 (Hasan and Yan, 2014), this review also discusses the potential role played by the cyclic GMP-AMP Synthase (cGAS) pathogen recognition receptor (Gao et al., 2013).

To the contrary of Nef and Vpr, the other HIV-1 and HIV-2 accessory proteins, Vpu, Vif, and Vpx, have been shown to directly counteract cellular restriction factors. However, in recent years, a role for these restriction factors in detection of infections has also been described, suggesting that counteraction of these proteins serves in both alleviating the restriction and in escape from innate immune detection. Indeed, Vif, Vpu, and Vpx can directly target APOBEC3G, Tetherin and SAMHD1, respectively. Because of the dual role played by these restriction factors, it is both important to understand the molecular mechanism underlying the antagonism by accessory proteins and the function fulfilled by the cellular protein (Feng et al., 2014; Moris et al., 2014; Roy et al., 2014; Sauter, 2014; Schaller et al., 2014).

This review collection should therefore help the reader have an overview of the conflicting forces that underlie the interactions established by HIV with the host cell, and in particular of the complex relationship between viral accessory proteins and their

cellular partners. This review series highlights how progress made in the understanding of the HIV life cycle has also impacted on our understanding of important cellular processes, especially with regards to the mechanisms underlying molecular aspects of the innate immune system. In fact, while the complexity of the interactions established between viral and cellular proteins precludes definite conclusions as of today, understanding their contribution to HIV-associated pathogenesis is likely to be the next big challenge in the field.

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The activity of Nef on HIV-1 infectivity

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The replication and pathogenicity of lentiviruses is crucially modulated by "auxiliary proteins" which are expressed in addition to the canonical retroviral ORFs *gag, pol,* and *env*. Strategies to inhibit the activity of such proteins are often sought and proposed as possible additions to increase efficacy of the traditional antiretroviral therapy. This requires the acquisition of an in-depth knowledge of the molecular mechanisms underlying their function. The Nef auxiliary protein is expressed uniquely by primate lentiviruses and plays an important role in virus replication *in vivo* and in the onset of AIDS. Among its several activities Nef enhances the intrinsic infectivity of progeny virions through a mechanism which remains today enigmatic. Here we review the current knowledge surrounding such activity and we discuss its possible role in HIV biology.

Keywords: HIV, AIDS, auxiliary proteins, Nef, retrovirus infectivity

A BRIEF HISTORY OF THE DISCOVERY OF THE Nef ORF

The first reports of the entire HIV-1 genome sequences exposed an ORF, partially overlapping with the 3′LTR, which was initially named 3′ORF (Ratner et al., 1985; Sanchez-Pescador et al., 1985). Early studies demonstrated that its gene product was antigenic during the course of natural infection (Arya and Gallo, 1986; Franchini et al., 1986). However, it took several years before its role *in vivo* and its molecular functions began to be understood. Initial studies suggested that 3′ORF encoded for a GTPase (Guy et al., 1987), a finding soon dismissed as a possible consequence of sample contamination with bacterial GTPases (Backer et al., 1991). Subsequent reports suggested that 3′ORF was a negative factor (hence the name *nef*, still in use today), because its over-expression was found to attenuate viral transcription and HIV replication in cell culture (Luciw et al., 1987; Ahmad and Venkatesan, 1988; Niederman et al., 1989). These findings were soon contradicted by later reports (Hammes et al., 1989; Kim et al., 1989) which attributed the negative effect to the LTR sequences maintained in Nef-encoding vectors and interfering with HIV gene expression.

The first evidence demonstrating the requirement for an intact *nef* allele in the maintenance of high viral load and the timely development of immunodeficiency came from Rhesus macaques infected with a mutated strain of $SIVmac_{239}$ lacking the Nef ORF (Kestler et al., 1991). Further evidence came from patients who contracted infection with Nef-deleted viruses and manifested long-lasting low level of virus replication and delayed onset of the disease (Deacon et al., 1995; Kirchhoff et al., 1995). A positive effect of Nef on HIV-1 replication was eventually confirmed *in vitro* using primary cell cultures and, to a lesser extent, in transformed cell lines (Terwilliger et al., 1991; de Ronde et al., 1992; Zazopoulos and Haseltine, 1993; Miller et al., 1994; Spina et al., 1994).

OVERVIEW OF Nef ACTIVITIES

The *nef* gene is only present in the genomes of primate lentiviruses, i.e., HIV-1, HIV-2, and SIV. It is translated from a multiply spliced mRNA which generates a protein of 27–32 KDa highly expressed from the early stages of the infection process. Based on crystal (Lee et al., 1996; Arold et al., 1997; Grzesiek et al., 1997) and NMR (Grzesiek et al., 1996, 1997) structures, we know that Nef is made of a globular core domain flanked by a flexible N-terminal arm and a C-terminal disordered loop. Residues crucial for the interaction with different host factors are located in all three regions of the protein. Nef is myristoylated, which contributes to its association with membranes, together with a stretch of basic aminoacids close to the N-terminus (Bentham et al., 2006). Indeed, a significant fraction of Nef is observed in association with the plasma membrane and perinuclear membrane complexes (Kohleisen et al., 1992; Fujii et al., 1996; Greenberg et al., 1997). Myristoylation may also contribute to prevent Nef from multimerizing (Breuer et al., 2006). The protein is also detected within virion particles (Pandori et al., 1996; Welker et al., 1996, 1998; Bukovsky et al., 1997), a feature which could depend on the ability of Nef to associate with cellular membranes. Packaged Nef has also been reported to undergo cleavage by the viral protease (Bukovsky et al., 1997; Chen et al., 1998). However, as discussed below, the meaning and the specificity of Nef packaging into virions remain unclear.

Perhaps the most remarkable feature of Nef is its multi-functionality. Nef does not contain enzymatic activity, but exerts

several cellular functions resulting from its ability to interact with numerous host factors. The most characterized activities of Nef result from the ability of the protein to connect with the cellular vesicular trafficking machinery and to perturb cell signaling.

MODULATION OF CELL-SURFACE MOLECULES EXPRESSION LEVELS

Nef interacts with several proteins implicated in intracellular trafficking and modulates cell surface expression of several molecules (Landi et al., 2011). Nef down-regulates CD4 (Garcia and Miller, 1991) by enhancing its uptake into the endosome–lysosome compartment (Aiken et al., 1994; Chowers et al., 1994; Rhee and Marsh, 1994; Schwartz et al., 1995a; Bresnahan et al., 1998; Craig et al., 1998; Piguet et al., 1998, 1999; Janvier et al., 2001; Faure et al., 2004), a function conserved and maintained throughout disease progression that increases both virus infectivity and replication, as discussed in Section "Potential Effect of Nef During Virus Biogenesis."

Nef affects the trafficking of many other proteins, which favors virus replication in the host by hiding or protecting infected cells from immune surveillance and by promoting virus dissemination. Because these properties are not strictly related to the ability of Nef to increase virus infectivity, they are mentioned in this chapter but the underlying mechanism will not be discussed further.

The ability of Nef to prevent the elimination of infected cells by the immune system is an important feature that favors virus dissemination in the host. Nef down-regulates molecules of the major histocompatibility complex-I (MHC-I; Schwartz et al., 1996) through a still debated mechanism distinct from that involved in CD4 down-regulation (Piguet et al., 2000; Blagoveshchenskaya et al., 2002; Williams et al., 2002, 2005; Larsen et al., 2004; Roeth et al., 2004; Lubben et al., 2007; Noviello et al., 2008; Dikeakos et al., 2012). This protects infected cells against killing by cytotoxic T cells (Collins et al., 1998), and is maintained under strong selective pressure only during the acute phase of infection, when the host is still fully immunocompetent (Carl et al., 2001). Protection against cell lysis is further achieved by Nef-dependent FasL up-regulation which triggers apoptosis of bysander cytotoxic cells (Xu et al., 1997, 1999). Of note, HIV and SIV Nef also interfere with MHC-II functions by down-regulating MHC-II complexes and up-regulating the MHC-II-associated II invariant chain (Schindler et al., 2003).

Cell infection by HIV or SIV is also characterized by T-cell receptor (TCR) pathway dysfunction. Nef can down-regulate the TCR/CD3 complex (Bell et al., 1998; Schaefer et al., 2000; Munch et al., 2002), a property restricted to alleles derived from SIV isolates non-pathogenic to their natural host, which might explain the higher virulence of HIV compared with SIV (Schindler et al., 2006). In addition, TCR activity can also be inhibited by the Nef-dependent down-regulation of the co-stimulatory molecule CD28 (Bell et al., 2001; Swigut et al., 2001). Finally, SIV Nef down-regulates the restriction factor BST-2 and ensures efficient release of viral particles from infected cells (Jia et al., 2009).

Key to these activities of Nef is the ability to form ternary complexes with cargo molecules and adaptor or coatamer complexes via a ExxxLL acidic di-leucin motif (Aiken et al., 1994) a EE di-acidic sequence located in its C-terminal loop (Piguet et al., 1999), a EEEE acidic cluster (Piguet et al., 2000) and a YxxΦ motif (where Φ represents a hydrophobic residue) on its N-terminal arm (Lock et al., 1999).

MODULATION OF T-CELL ACTIVATION

In addition to modulating receptor expression levels, Nef also hijacks signaling pathways and alters the activation threshold of lymphocytes (Baur et al., 1994; Alexander et al., 1997; Schrager and Marsh, 1999; Simmons et al., 2001) by interacting with Src family tyrosine kinases (Saksela et al., 1995), members of the p21-activated serine/threonine kinases (Sawai et al., 1994, 1996; Khan et al., 1998; Renkema et al., 1999; Agopian et al., 2006) and Vav (Fackler et al., 1999; Rauch et al., 2008). This leads to a transcriptional program resembling that triggered by TCR stimulation, which might create a favorable intracellular milieu for virus replication. Signaling perturbation by Nef also results in the inactivation of cofilin, which inhibits cytoskeleton rearrangement and cell motility (Stolp et al., 2009). Features of the Nef protein, which are reported to contribute to this activity, include a PxxP proline-rich motif (Saksela et al., 1995), an amphipathic α-helix in the N-terminal arm (Baur et al., 1997) and a hydrophobic surface within the C-terminal loop (Agopian et al., 2006). Given that Nef increases cell-free virus infectivity in non-T-cell systems, the effect on primary T-cell activation does not seem to correlate with the activity of Nef on virus infectivity.

THE EFFECT OF Nef ON RETROVIRUS INFECTIVITY

With the term infectivity we here indicate the efficiency with which the virus establishes an infection event within a cell, which culminates with the integration of the virus genome into the host cell genome. This is therefore a parameter which does not depend on steps of the virus life cycle which follow integration, such as virus gene expression or virus release. Infectivity is measured by relating the number or the frequency of the infectious events produced by cell-free virus with the physical number of virus particles. To compare infectivity across different samples, infectious events are therefore normalized to the physical virus content in the inoculum, determined by quantifying the amount of p24 CA protein or the RT-activity of the virus. Most studies investigating HIV-1 infectivity employ infection assays limited to a single round of viral replication by using trans-complemented molecular clones or by the addition of AZT or entry inhibitors at various time points following infection with replication competent viruses, in order to avoid the contributions from successive rounds of replication to the overall phenotype. Steps of the virus life cycle where the effect of Nef on infectivity can be manifest include receptor interaction, entry, uncoating, reverse transcription, nuclear import and integration.

The Guatelli lab was the first to report that HIV-1 lacking the ability to express Nef has lower infectivity compared with the Nef-positive counterpart (Chowers et al., 1994). This observation has then been confirmed by several labs using a variety of experimental systems differing for producer cell type, target cells and viral molecular clones (Aiken and Trono, 1995; Goldsmith et al., 1995; Miller et al., 1995; Tokunaga et al., 1998; Khan et al., 2001; Tobiume et al., 2001; Papkalla et al., 2002). Altogether, the magnitude by which Nef alters HIV-1 infectivity is highly variable,

ranging from 3 to 40. In particular, the infectivity of Nef-defective HIV-1 seems to be the most impaired when virus is produced from lymphoid cell lines (Pizzato, 2010).

Additional observations indicate that the effect of Nef on infectivity plays a major function in the biology of primate lentiviruses. First, a comprehensive analysis including *nef* alleles derived from a large panel of diverse HIV and SIV isolates has demonstrated that the activity on infectivity is phylogenetically highly conserved (Munch et al., 2007). Second, functional analysis of *nef* alleles obtained during different stages of HIV infection revealed that the Nef effect on infectivity is maintained by a strong selective pressure during disease progression (Carl et al., 2001). Although this evidence suggests an important function for the Nef effect on infectivity, its precise role during the pathogenic infection remains to be deciphered. While an increased virus infectivity would logically imply an advantage on virus replication and therefore on viral load, clear evidence demonstrating such a link remains elusive.

THE EFFECT OF Nef ON VIRUS REPLICATION

A positive effect of Nef on virus replication was first observed more than 20 years ago (Kim et al., 1989) and remains today mechanistically unclear. While the role of Nef for efficient virus replication *in vivo* is evident, this is not always the case in spreading infection *in vitro*, for which the effect of Nef is highly variable depending on the experimental system. The most robust requirement for Nef was observed using primary T-cells or macrophages infected before mitogenic stimulation of the cultures (Miller et al., 1994; Spina et al., 1994). An important contribution to this effect could therefore stem from the ability of Nef to alter T-cell activation status and favor preliminary virus replication before massive stimulation following exposure to mitogens.

In contrast, Nef seems to only have a modest effect on the replication of HIV-1 in transformed cell lines and in activated primary human T-cells. Discrepancy is observed between the marked effect of Nef on the infectivity of single round infection competent viruses and the modest Nef requirement for virus replication in the same cell cultures (Haller et al., 2011). One major difference between single round infections using cell-free virus and spreading infection of HIV throughout a cell culture is that in the latter cell-associated virus can be transmitted directly from cell to cell (cell-to-cell transfer; Jolly et al., 2004) which seems to be remarkably efficient [up to 1000-fold more efficient than cell-free virus (Sourisseau et al., 2007)]. A recent report indicates that Nef exerts only a modest positive effect on cell-to-cell transfer using both transformed cell lines and activated primary cells (Malbec et al., 2013), therefore overriding bigger differences from the contribution of cell-free virus (Haller et al., 2011). The role of the effect of Nef on infectivity on virus replication remains therefore to be elucidated.

THE MECHANISTIC DETAILS OF THE EFFECT OF Nef ON INFECTIVITY

The effect of Nef on virus infectivity requires its expression in producer cells rather than target cells (Aiken and Trono, 1995). Nef might thus play a role as a virus-borne protein when virions hit target cells. Alternatively, in the presence of Nef, progeny virus particles might inherit a modification which is required to maintain their full infectious potential. Two sides of the same coin should therefore be considered. (1) What is the nature of the Nef-dependent modification inherited by the virus particle and how is it acquired? (2) In which step of the virus life cycle (ranging from receptor interaction to integration) is the infection of a target cell affected by such Nef-dependent modification? (**Figure 1**).

INFECTIVITY: IS VIRUS-BORNE Nef DOING IT ITSELF?

Conflicting results have been published regarding the effect of Nef on fusion/entry which will be discussed later (Zhou and Aiken, 2001; Tobiume et al., 2003; Cavrois et al., 2004). Nevertheless the literature agrees that Nef+ viruses complete post-entry steps more efficiently than their Nef− counterparts (Aiken and Trono, 1995; Chowers et al., 1995; Schwartz et al., 1995b).The fact that Nef is incorporated into viral particles and cleaved by HIV-1 protease at residues $W_{57}L_{58}$ in the course of virion maturation makes it reasonable to hypothesize a specific role for virus-borne Nef in early steps of viral replication (Pandori et al., 1996; Welker et al., 1996; Bukovsky et al., 1997). Site-directed mutagenesis led to the identification of molecular species with a range of phenotype regarding their incorporation and maturation in virions; however, no correlation could be drawn between incorporation/maturation and infectivity because mutants were also deficient in other known functions of Nef, mostly CD4 or MHC down-regulation (Bukovsky et al., 1997; Miller et al., 1997; Chen et al., 1998; Welker et al., 1998; Bentham et al., 2006). The fact that Nef can be also incorporated into MLV particles (without affecting virus infectivity) further suggests that it is passively and "unpurposely" incorporated into enveloped virions due to its association with cell membranes (Bukovsky et al., 1997). Two papers by Laguette et al. (2009a) and Qi and Aiken (2008) addressed the question of Nef incorporation and its correlation to infectivity by fusing WT Nef proteins with a "viral carrier protein", Vpr and CypA, respectively. These results support the idea that a role of Nef in the course of virus biogenesis, not as a virus-borne factor, likely accounts for its effect on virus infectivity, and are in line with the inability of Nef to complement the infectivity of Nef− viruses when it is expressed in target cells (Aiken and Trono, 1995; Pizzato et al., 2008).

POTENTIAL EFFECT OF Nef DURING VIRUS BIOGENESIS

One of the first effects attributed to Nef is its ability to down-regulate cell surface CD4 expression level in infected cells (Guy et al., 1987). Because CD4 is the primary receptor for HIV (Dalgleish et al., 1984; Klatzmann et al., 1984), its down-regulation from the plasma membrane spares cells from cytotoxic superinfection and favors virus dissemination (Benson et al., 1993; Little et al., 1994). Furthermore CD4 down-regulation prevents the formation of CD4/gp120 complexes in intracellular compartments and at the plasma membrane, which has been shown to interfere with envelope glycoproteins (Env) incorporation into nascent virions and to decrease virus infectivity (Lama et al., 1999; Cortes et al., 2002; Arganaraz et al., 2003; Lundquist et al., 2004; Schiavoni et al., 2004). This phenotype appears to be of particular importance for primate lentiviruses since HIV and SIV Nef alleles, but also Vpu and Env, down-regulate cell surface CD4 through distinct mechanisms (Garcia and Miller, 1991; Benson et al., 1993;

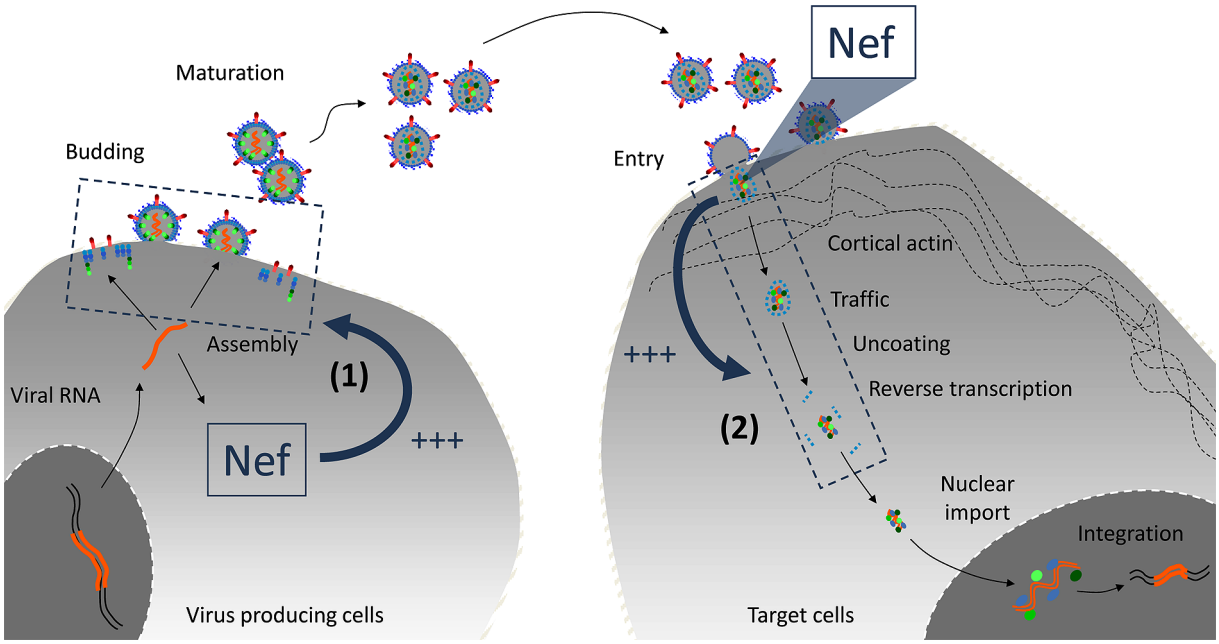

FIGURE 1 | Cellular or virus-borne: localization of Nef activity on virus infectivity. Nef is expressed in infected cells and incorporated into virions. Given the profound imprint of Nef on the biology of infected cells, it may regulate a cellular function in virus-producing cells that favors virus infectivity (1). The fact that virus-borne Nef molecules are processed during maturation also suggests that cleaved molecules may play a role when virions hit target cells (2). Recent findings tend to favor the former hypothesis.

Sanfridson et al., 1994; Chen et al., 1996; Fujita et al., 1997; Hua and Cullen, 1997; Margottin et al., 1998; Schubert et al., 1998; Wildum et al., 2006; Laguette et al., 2009b; Magadan et al., 2010). CD4 down-regulation by Nef thus seems to favor HIV infectivity and replication as correlated by the Aiken and Kirchhoff groups (Glushakova et al., 2001; Lundquist et al., 2002). However, the sole ability of Nef to down-regulate CD4 cannot explain its effect on virus infectivity since Nef remains capable of increasing virus infectivity when virions are produced from CD4-negative cells or when virions are pseudotyped with the MLV-A envelope glycoprotein that does not interact with CD4 (Aiken and Trono, 1995; Miller et al., 1995; Aiken, 1997; Pizzato et al., 2008). In addition, Nef does not seem to modulate Env incorporation into the viral membrane when virions are produced from CD4-negative cells (Miller et al., 1995; Lai et al., 2011). The effect on infectivity is therefore an independent activity of Nef.

The hypothesis of a role of Nef in the course of virus biogenesis was further investigated in a comparative proteomic analysis of WT and Nef-defective NL4-3 viral particles intended to identify differences in their composition that might explain the higher infectivity of WT viruses (Bregnard et al., 2013). This study revealed that Ezrin and EHD4 are more abundant in Nef-defective than in WT NL4-3 virions but failed to demonstrate a direct inhibitory effect of these virus-borne proteins on Nef-defective virus infectivity. On the contrary, Ezrin and EHD4 depletion decreased WT viruses infectivity but not that of Nef-defective viruses, which supports their roles as possible co-factors in the Nef-mediated increase of virus infectivity. A similar comparative analysis has been conducted to identify a specific signature of Nef on the lipid composition of the viral membrane (Brugger et al., 2007). Although differences could be identified between Nef+ and Nef− derived membranes, none was found to account for the Nef-dependent increase of virus infectivity. Nef-dependent post-translational modification of viral proteins could also account for the higher infectivity of Nef+ over Nef− viruses. Although Nef-associated kinases were found to induce matrix phosphorylation on serine residues (Swingler et al., 1997), matrix was later found dispensable not only for virus replication, but also for Nef responsiveness, which ruled out its contribution to the phenotype (Reil et al., 1998; Dorfman et al., 2002). Of note, matrix deletion from Gag requires mutation or deletion of HIV-1 Env cytoplasmic tail to ensure the incorporation of the retroviral glycoprotein into the viral membrane and the biogenesis of infectious virions (Ono et al., 1997; Reil et al., 1998; Murakami and Freed, 2000; Ono et al., 2000; Tedbury et al., 2013). Simultaneous mutations might thus interfere and hide the requirement for matrix phosphorylation in the Nef-dependent increase of virus infectivity. Further investigation is thus required to formally identify Nef-dependent modifications of viral proteins, or incorporation/exclusion of cellular factors into/from virions, that might directly affect virus infectivity.

EFFECT OF Nef (OR MODIFICATIONS INHERITED FROM PRODUCER CELLS) IN TARGET CELLS

Although Nef does not seem to affect the concentration of Env on the virus surface, its ability to increase virus infectivity is somewhat dependent on the mechanism that promotes fusion between the virus and the cell membrane and was long thought to be linked

to the route of viral entry. While Nef increases the infectivity of viruses decorated with Env that allow for fusion at neutral pH such as that of HIV and A-MLV (referred to as responsive Env), no effect of Nef is observed with Env that requires virus endocytosis and endosome acidification to promote fusion such as that of VSV and RSV-A (Miller et al., 1995; Aiken, 1997; Luo et al., 1998; Pizzato et al., 2008). This has suggested the existence of a post-entry block encountered by incoming capsids when fusion takes place at the plasma membrane and counteracted by Nef (or its effects inherited from the virus producing cell, see below) or bypassed by incoming virions traveling through the endocytic network, out of reach of cytoplasmic factors. The cortical actin network has been suggested to account for such post-entry block, based on the relieving effect of actin-targeting drugs on the poorly infectious Nef− viruses but not on the fully infectious Nef+ viruses (Campbell et al., 2004). However, although Nef increases the infectivity of HIV-1, regardless of its tropism for CXCR4 or CCR5 receptors, not all HIV-1 Envs are equally responsive to Nef (Chazal et al., 2001; Papkalla et al., 2002; Lai et al., 2011; Usami and Gottlinger, 2013). Furthermore, the correlation between Nef responsiveness and entry at the plasma membrane is at odds with evidence of HIV entry into target cells in early endosomes, after endocytic vesicles have passed the cortical actin network but before endosome acidification (Miyauchi et al., 2009; van Wilgenburg et al., 2014). In addition, Nef responsiveness can be observed even when fusion takes place in acidic endosomes, in experimental setting where cells express RSV-A Env and virions are pseudotyped with the cognate Tva receptor (Pizzato et al., 2008).

Nef might thus have an effect on fusion that correlates neither with the route of entry, nor with Env dependence on pH. Such effect has been investigated with responsive and non-responsive Envs and led to contradictory results. While a Nef-dependent increase of the fusion process extrapolated from virus/cell or intravirion fusion assays has been reported by some groups (Schaeffer et al., 2001; Zhou and Aiken, 2001), others have failed to detect any difference between Nef+ and Nef− viruses in the completion of the fusion step, including groups that used the most quantitative βlam-Vpr fusion assay developed in the Greene laboratory (Miller et al., 1995; Tobiume et al., 2003; Campbell et al., 2004; Cavrois et al., 2004; Basmaciogullari, personal communication). Such conflicting results led Cavrois et al. (2004) to cautiously interpret the sensitivity of the βlam-Vpr assay and the hypothetical effect of Nef on fusion. The authors suggested that Nef might assist the enlargement of the pore arising from the fusion between the virus and cell membranes, and promote translocation of the viral capsid into the cell cytoplasm. Small pores arising from the fusion between Nef− viruses and cell membranes would restrict capsid translocation but allow for full diffusion of the fluorogenic substrate or βlam-Vpr. This would explain why Nef− viruses are less infectious than Nef+ viruses yet induce identical βlam-Vpr readouts. However, this model is based on the assumption that pore size does not limit substrate or βlam-Vpr diffusion, which needs to be demonstrated. Alternatively, if small pores restrict diffusion, hence βlam-Vpr positivity, identical βlam-Vpr readouts obtained with Nef+ and Nef− viruses can only be achieved if large pores are underrepresented and have a negligible contribution to the overall βlam-Vpr signal (i.e. Nef+ viruses: 99% small pores and 1% large pores/full entry; Nef− viruses, 99.9% small pores and 0.1% large pores/full entry). The fact that fusion inhibitors clearly decrease infectivity and the Vpr-βlam signal does not support this hypothesis. It thus seems that the most likely explanation for identical βlam-Vpr readouts is that Nef has no effect on fusion/capsid delivery but affects post-fusion steps of the virus life cycle. Of Note, Day et al. (2004), who also used this assay, reported a fusion advantage of Nef+ viruses over Nef− viruses, which highlights the need for a more robust and sensitive fusion assay in order to clarify the possible effect of Nef on fusion.

Besides these discrepancies on fusion, it has been shown that Nef affects the accessibility of neutralizing antibodies directed against the MPER region of gp41 in a cell/virus fusion context (Lai et al., 2011). Although this did not fully correlate with the ability of Nef to increase virus infectivity, it nevertheless demonstrates that Nef might affect Env proteins conformation or the lipid environment adjacent to the MPER region and thus the fusion capacity of Env glycoproteins. The difference between responsive and non-responsive HIV-1 Env was recently mapped to an epitope within the V2 region of gp120 (Usami and Gottlinger, 2013). Yet, given the divergence between Nef responsive HIV-1 and MLV-A Env glycoprotein sequences and the responsiveness of Tva-pseudotyped viruses (Pizzato et al., 2008), the common parameter that allows for Nef responsiveness remains unknown. It thus seems that proteins found on the virus surface that mediate fusion, whether they be viral Envs or cognate receptor(s), are major determinants of Nef responsiveness but most likely not through their role in fusion.

The effect of Nef has also been documented at the level of cDNA synthesis in target cells. Although early experiments could not discriminate between effects of Nef on fusion or post-fusion steps, they nevertheless demonstrated that Nef+ viruses generate more early reverse transcription products than Nef− viruses, supporting an effect of Nef operating anywhere between fusion and viral DNA translocation to the nucleus (Aiken and Trono, 1995; Schwartz et al., 1995b). Of note, intravirion stimulation of reverse transcription was shown to compensate for the effect of Nef on virus infectivity (Khan et al., 2001). Given the interdependence between uncoating and RT, these results suggest that Nef might assist either of the mechanisms (Hulme et al., 2011; Yang et al., 2013). Although Nef does not show any effect on uncoating *in vitro*, an effect of Nef *in vivo* cannot be ruled out (Forshey and Aiken, 2003).

CONVERGING MECHANISMS

Three papers published recently describe a striking parallel between HIV-1 Nef and MLV glycoGag (Pizzato, 2010; Usami and Gottlinger, 2013; Usami et al., 2014). This protein arises from the translation of the unspliced MLV RNA from a CUG initiation codon upstream from the conventional initiation codon of Gag, which results in the addition of 88 residues in frame and N terminal to Gag, responsible for the type II orientation of the corresponding protein where the added N terminal residues constitute the transmembrane domain and extend into the cytoplasm of the cell. It has been shown that ectopic expression of Nef or glycoGag similarly increases the infectivity of viruses produced from cells transfected with a Nef− provirus. Interestingly, simultaneous co-expression

of Nef and glycoGag has no synergistic effect and glycoGag has no effect on VSV-G pseudotyped viruses, suggesting that these proteins are involved in the same mechanism that eventually leads to the increase of virus infectivity (Pizzato, 2010). In addition the V2 region of HIV-1 Env, which dictates Nef responsiveness, also dictates responsiveness to glycoGag (Usami and Gottlinger, 2013). Finally, although the ability of Nef to increase virus infectivity and its effect on the accessibility of MPER epitopes on HIV-1 Env partially overlap, glycoGag expression also affects the accessibility of similar MPER epitopes (Lai et al., 2011). This further confirms the converging functions of these unrelated proteins.

MECHANISTIC HYPOTHESES

THE BROAD PICTURE

Several hypotheses can be put forward in a two-act scenario responsible for the differential infectivity of Nef+ and Nef− viruses. Nef might hijack cellular pathways in virus-producing cells that ultimately optimize infectivity (modification of viral proteins or viral content beyond its known elements). Cellular pathways in target cells might then specifically assist Nef+ viruses in the early steps of the virus life cycle (**Figures 2A,C**). Both sides of this same coin can then either involve cofactors recruited by Nef or inhibitory factors counteracted by Nef. So far, the lentiviral auxiliary proteins Vif, VpU, and VpX have been involved in the neutralization of an inhibitory factor (or restriction factor) such as Apobec3, Bst2, and SAMHD1. In the case of Nef both possibilities remain open. The Nef requirement for reaching optimal infectivity is highly variable and depends on the cell types from which virions are produced (Pizzato, 2010), it is thus plausible that such variability is conferred by differential expression of one or more cellular genes involved in the scenario depicted above.

WHAT IS Nef DOING TO THE VIRUS, HOW AND WHERE?

Comparative analysis of Nef+ and Nef− viral particles have clearly revealed differences in the lipid and protein composition, confirming that Nef expression in virus-producing cells has an impact on the virus biogenesis. Although the modification of the viral lipid bilayer by Nef did not seem to account for the higher infectivity of Nef+ viruses over Nef− viruses, further investigation with more sensitive fusion assays that are yet to be developed might reveal a direct link between the viral membrane lipid composition, membrane fusion and infectivity. Differences in the protein composition of viral particles also revealed partial depletion or enrichment in particular proteins, depending on the expression of Nef in virus-producing cells. Ezrin and EHD4 were found in higher concentration in Nef− viruses and their involvement in the infectivity phenotype confirmed; however, their relative excess did not seem to account for the poor infectivity of Nef− viruses. Rather, it was interpreted that Ezrin and EHD4 are hijacked by Nef in the process of increasing virus infectivity, thus preventing their passive incorporation into virions.

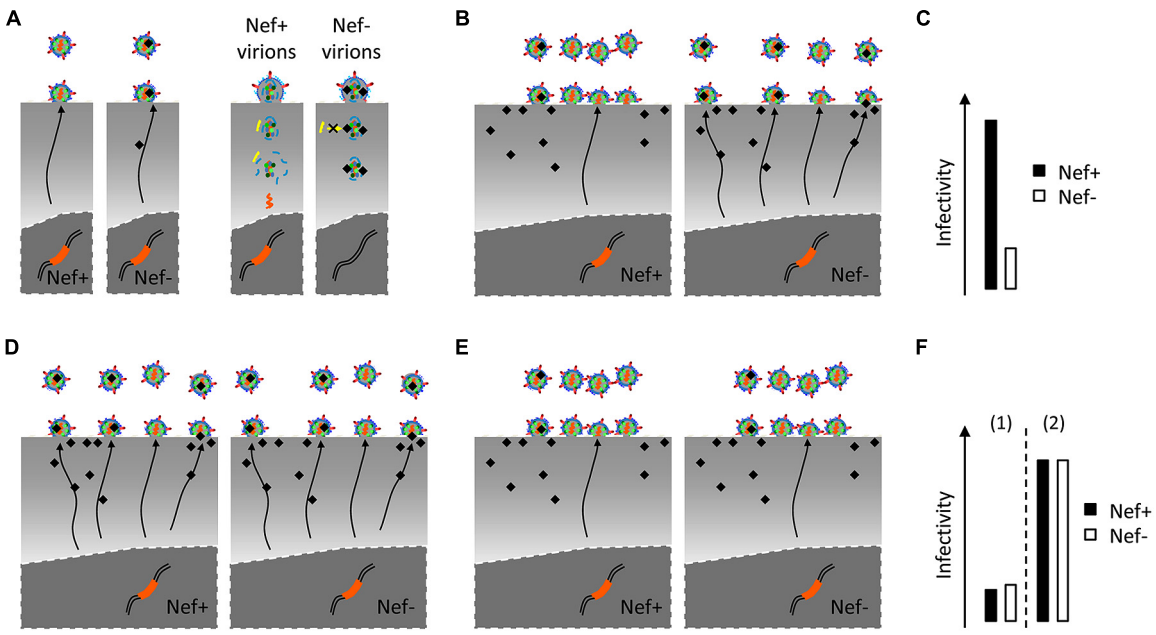

FIGURE 2 | Possible mechanisms responsible for the differential infectivity of Nef+ and Nef− viruses. (A), Nef− virions may acquire a defect during biogenesis which could be either the packaging of an inhibitor into virions (black shape), or the exclusion of a cofactor (not shown). This defect might then prevent the recruitment of cofactor (yellow shape) or be the target of an inhibitor (not shown) in target cells. **(B,C)** The effect of Nef on virus infectivity is evident when virus is produced from Nef-responsive cells, in which Nef regulates such inhibitor or promote virus assembly to subcellular locations where the defect is not acquired **(B)**. **(D–F)** In contrast, Nef-non-responsive producer cells generate Nef+ and Nef− viruses with similar infectivities **(F)**. Two possibilities may explain this phenotype: Nef fails to protect the virus from the defect [**D,F(1)**, Virions have suboptimal infectivity even in the presence of Nef]; alternatively, producer cells lack the cause of the defect or target virus assembly away from inhibitors [**E,F(2)**, Nef- virus already has optimal infectivity].

It is interesting to note the converging phenotype of the *nef*− mutation and mutations of p24CA on serine residues: both mutants are poorly infectious but their infectivity is restored by VSV-G pseudotyping (Brun et al., 2008). Although this is far from explaining how Nef increases virus infectivity, it nevertheless confirms that post-translational modification by Nef is an interesting line of investigation to follow. Additional work is thus required in order to identify the differences between Nef+ and Nef− responsible for the differential behavior of viruses when they hit target cells.

As presented in this review, a main functional feature which characterizes Nef alleles and is required for several Nef activities, is the ability to intersect with the vesicular trafficking machinery of the cell. As we discussed earlier, Nef is capable of interacting with AP1, AP2, and βCOP1 via distinct and discrete motifs present in HIV and SIV Nef molecules (ExxxLL, EE, EEEE, YxxL). Many observations converge toward a fundamental role of vesicular trafficking for the activity of Nef for infectivity. In particular: (1) HIV-1 Nef requires the integrity of the AP2 interacting motif ExxxLL in virus producing cells (Chowers et al., 1994). (2) Similarly, SIV Nef requires the YxxL motif (Lock et al., 1999). (3) Mutant HIV-1 Nef proteins, which do not interact with dynamin 2 (crucial for intracellular vesicles biogenesis), are no longer capable of increasing HIV infectivity (Pizzato et al., 2007). (4) The activity of Nef on infectivity requires functional clathrin-mediated endocytosis, because it is impaired by silencing clathrin gene expression and by over-expressing transdominant-negative dynamin 2 and AP180. (Pizzato et al., 2007). (5) Glycogag requires and interacts with AP2 via a YxxL motif to rescue the infectivity of Nef-defective HIV-1 (Usami et al., 2014). 5) EHD4 and Ezrin silencing render Nef+ viruses as poorly infectious as Nef− viruses (Bregnard et al., 2013).

As highlighted earlier, doubts remain on the possibility that Nef affects cytoplasmic delivery of HIV. In the hypothesis where Nef enhances the efficiency of fusion between the virus and the cell membranes, it might do so by promoting the endocytosis of a membrane-bound fusion inhibitor in virus producing cells otherwise incorporated into viral membrane. More generally, factors with potential inhibitory effect, or responsible for the recruitment of co-factors in target cells, might also be rerouted away from or to the viral assembly platforms through the deregulation of protein trafficking by Nef in infected cells.

Another interesting challenge is the identification of the cellular compartment where Nef+ virions acquire their phenotype. Nef might drive the viral components to the appropriate assembly platforms where virions acquire specific features responsible for their higher infectivity and, as suggested earlier, the nature of the protein that decorates virions might play a role in this sorting mechanism, independently of their involvement in membrane fusion. Despite some flexibility in virus pseudotyping, much evidence suggests that the acquisition of Env by budding virions is somewhat regulated (Johnson et al., 1998; Jorgenson et al., 2009; Muranyi et al., 2013; Roy et al., 2013). Env might be targeted by Nef and rerouted to ensure assembly at specific sites (**Figure 2B**). Of note, similar infectivities of Nef– and Nef+ viruses might either reflect the inability of Nef to drive virus assembly to such compartments (**Figures 2D,F**) or indicate that optimal assembly does not require the presence of Nef (**Figures 2E,F**). Because cytoplasmic tail-deleted Envs are still Nef responsive, specific determinants might lie in the extracellular domain, and interact with a chaperone along its secretion and delivery to the plasma membrane. Although this awaits formal evidence, it is compatible with the recent identification of a V2 region in HIV-1 Env as a major determinant of responsiveness to Nef/glycoGag.

CONSEQUENCES IN TARGET CELLS

Tracking virions in target cells is particularly challenging, especially when virions cannot be pseudotyped with VSV-G. For this reason, not much is known about possible functions of virus-borne molecules in early steps of the life cycle. Whether Nef itself or the modifications it brings to viral particles impact on the completion of early steps, this most likely relies on the recruitment of co-factors or the neutralization of inhibitory factors in target cells. A cutting edge genetic approach based on the screening of a human siRNA library has allowed for the identification of cellular factors that modulate HIV-1 replication (HDFs, HIV-1 dependency factors). However, virions used in such HTS were either Nef− or VSV-G pseudotyped, excluding *de facto* the possibility to identify HDFs involved in the ability of Nef to increase virus infectivity (Brass et al., 2008; Konig et al., 2008; Zhou et al., 2008). A similar screening performed in experimental settings where Nef+ and Nef− viruses could be compared such as that described by Bregnard et al. (2013), would shed light on the pathways usurped by Nef to increase virus infectivity.

CONCLUSION

Biochemistry-based approaches have been used in order to identify cellular factors involved in the many functions of Nef. Affinity tagging or immunoprecipitation followed by mass spectrometry (Pizzato et al., 2007; Jager et al., 2012; Mukerji et al., 2012) and conventional or ubiquitin-split yeast two-hybrid screening (Benichou et al., 1997; Kammula et al., 2012) have identified Nef binding partners. Omics methods have also been used to identify how HIV infection or Nef expression alone modifies the biology of the cell (Schrager and Marsh, 1999; Wang et al., 2000; Simmons et al., 2001; Kramer-Hammerle et al., 2005; van't Wout et al., 2005; Berro et al., 2007; Ringrose et al., 2008; Kramer et al., 2012). Given the many Nef partners and cellular processes affected by Nef identified so far, a strategy focused on the analysis of viral particles might better address the question of the effect of Nef on virus infectivity. Mass-spectrometry based techniques and computing tools have strikingly improved, which makes possible the analysis of Nef-induced post-translational modification of HIV-1 proteins in order to reveal specific differences between Nef+ and Nef− virions. In addition, due to the convergence of Nef and glycoGag on the infectivity phenotype, comparing the results of experiments carried out with these proteins might narrow down the list of modifications relevant for virus infectivity.

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HIV-1 Vpr—a still "enigmatic multitasker"

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Like other HIV-1 auxiliary proteins, Vpr is conserved within all the human (HIV-1, HIV-2) and simian (SIV) immunodeficiency viruses. However, Vpr and homologous HIV-2, and SIV Vpx are the only viral auxiliary proteins specifically incorporated into virus particles through direct interaction with the Gag precursor, indicating that this presence in the core of the mature virions is mainly required for optimal establishment of the early steps of the virus life cycle in the newly infected cell. In spite of its small size, a plethora of effects and functions have been attributed to Vpr, including induction of cell cycle arrest and apoptosis, modulation of the fidelity of reverse transcription, nuclear import of viral DNA in macrophages and other non-dividing cells, and transcriptional modulation of viral and host cell genes. Even if some more recent studies identified a few cellular targets that HIV-1 Vpr may utilize in order to perform its different tasks, the real role and functions of Vpr during the course of natural infection are still enigmatic. In this review, we will summarize the main reported functions of HIV-1 Vpr and their significance in the context of the viral life cycle.

Keywords: HIV-1 Vpr, reverse transcription, cell cycle, apoptosis, nuclear import

INTRODUCTION

The *vpr* gene is conserved among human (HIV-1 and HIV-2) and simian immunodeficiency viruses (SIV) and encodes the regulatory viral protein R (Vpr), a small basic protein (14 kDa) of 96 amino acids (Ogawa et al., 1989; Hattori et al., 1990; Steffy and Wong-Staal, 1991; Tristem et al., 1992). The importance of Vpr has been initially shown in macaque rhesus monkeys that were experimentally infected with a *vpr*-mutated SIVmac, and exhibited a decrease in virus replication and a delay in disease development progression (Lang et al., 1993; Hoch et al., 1995). *In vitro*, in the absence of Vpr, HIV-1 replicates less efficiently in macrophages, a cell type that represents an important viral reservoir by harboring the virus over long periods of time (Connor et al., 1995). Despite its small size, HIV-1 Vpr has been shown to have several roles during the viral life cycle. Due to its specific incorporation into the viral particle by interaction with the Pr55Gag-derived p6 protein, Vpr is readily present upon entry of the virus into the cell, which speaks in favor for enrollment during early steps of viral replication (see **Figure 1**). In this regard, Vpr has been shown to influence the reverse transcription of HIV-1 via the interaction and recruitment of the human uracil DNA glycosylase 2, an enzyme of the DNA repair machinery (Guenzel et al., 2012). A relationship that is not without controversy since different research reports argue whether UNG2 might rather have a negative impact or even no impact on HIV-1 replication (Schrofelbauer et al., 2005; Kaiser and Emerman, 2006; Yang et al., 2007). Furthermore, Vpr also affects the nuclear import of the viral DNA within the pre-integration complex (PIC), the cell cycle progression, the regulation of apoptosis and the transactivation of the HIV-1 LTR as well as host cell genes.

This review will be focused on the Vpr protein of HIV-1 and will give a summary of the multifunctional nature of Vpr during the viral life cycle in order to integrate previous and more recent studies.

THE STRUCTURE OF Vpr

HIV-1 Vpr is a relatively small protein composed of 96 amino acid residues (**Figure 2A**) (Checroune et al., 1995; Ramboarina et al., 2004; Kamiyama et al., 2013). The secondary and higher-order structures of Vpr have been investigated by nuclear magnetic resonance (NMR), circular dichroism (CD), and fluorescence spectroscopy (Zhao et al., 1994; Wang et al., 1996; Mahalingam et al., 1997; Kichler et al., 2000; Bruns et al., 2003; Morellet et al., 2003; Kamiyama et al., 2013). According to NMR studies on the full-length Vpr protein dissolved in acidic aqueous-organic solvents (**Figure 2B**) (Morellet et al., 2003), the central region of the Vpr polypeptide chain folds into three amphiphilic helices (Sherman et al., 2002; Bruns et al., 2003; Kamiyama et al., 2013). These bundled α-helices span residues 17–33, 38–50, and 55–77 and are flanked by unstructured flexible N- and C-terminal domains that are negatively or positively charged, respectively (Morellet et al., 2003). Four conserved proline residues at position 4, 10, 14, and 35 which are subjected to *cis/trans* isomerization are found in the N-terminal domain (reviewed in Bruns et al., 2003; Le Rouzic and Benichou, 2005). It was indeed reported that the cellular peptidyl-propyl isomerase cyclophilin A was able to interact with Vpr via prolines (position 14 and 35) for correct folding of the viral protein (Zander et al., 2003). The carboxy-terminal domain of Vpr contains six arginine residues between positions 73 and 96 (see **Figure 2A**), and this domain shows similarity to those of arginine-rich protein transduction domains. This might potentially explain the transducing properties of Vpr and its ability to cross the cell membrane lipid bilayer (Kichler et al., 2000; Sherman et al., 2002; Coeytaux et al., 2003). Additionally, the third helix of Vpr is rich in leucine residues (Schüler et al., 1999),

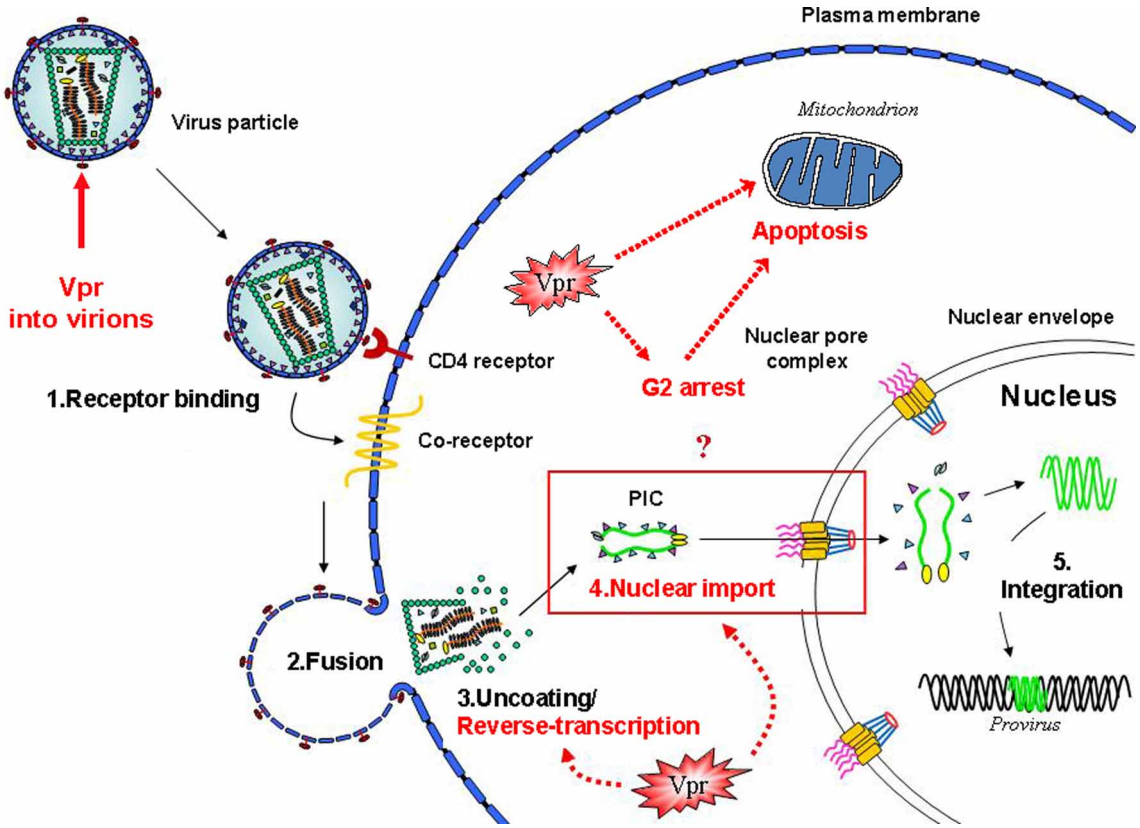

FIGURE 1 | Vpr functions and early steps of the HIV-1 life cycle. Schematic view of the early steps of the HIV-1 infection of a target cell. The functional events in which the Vpr protein is involved are highlighted. Vpr has been shown to play multiple functions during the virus life cycle, including an effect on the accuracy of the reverse-transcription process, the nuclear import of the viral DNA as a component of the pre-integration complex, cell cycle progression, regulation of apoptosis, and the transactivation of the HIV-LTR as well as host cell genes.

where one side of the helix presents a stretch of hydrophobic side chains that can form a leucine-zipper like motif (**Figure 2**). This region was proposed to account for the formation of Vpr oligomers (Wang et al., 1996; Mahalingam et al., 1997; Schüler et al., 1999; Fritz et al., 2008) and for interaction with certain cellular partners (reviewed in Planelles and Benichou, 2009).

Vpr has been shown to exist as dimers, trimers, tetramers and higher order multimers (Zhao et al., 1994), however it is still not completely elucidated how the dimeric or multimeric states of the protein affect the different functions of Vpr. A real-time study using a flow cytometry fluorescence resonance energy transfer has confirmed that Vpr self-associates within live cells (Bolton and Lenardo, 2007). Self-association was dependent on the hydrophobic patch that is located on the third α-helix and mutations in this region did not impair the ability of Vpr to induce G2 arrest, suggesting that oligomerization of Vpr is not absolutely required for the functions of the protein. In addition, mutations in the arginine-rich domain, such as R80A and R87/88A, did not impair self-association but were unable to induce G2 arrest (Bolton and Lenardo, 2007). Therefore, it appears that Vpr does not require oligomerization toward induction of the cell cycle blockage but the exposed hydrophobic amino acids in the amino-terminal helix-1 are important for the cell cycle arrest and cytopathogenic functions of Vpr (Barnitz et al., 2011). A more recent study reports that oligomerization of Vpr is essential for incorporation into virus particles (Venkatachari et al., 2010). Moreover, it has been recently proposed that Vpr may assume an antiparallel helical dimer with the third α-helices of the two subunits facing each other, and the His71 and Trp54 play a crucial role in this dimer formation (Kamiyama et al., 2013).

Vpr IS INCORPORATED INTO VIRUS PARTICLES

Vpr is expressed at a late stage of the virus life cycle, but it is present during the early steps of infection in target cells since it is packaged into virions that were released from the producing cells. The incorporation of Vpr occurs through a direct interaction with the carboxy-terminal p6 region of the *gag*-encoded Pr55Gag precursor (Bachand et al., 1999; Paillart and Göttlinger, 1999; Selig et al., 1999; Jenkins et al., 2001a,b). The integrity of the α-helices of Vpr is required for efficient packaging into virions (Singh et al., 2000), and a leucine-rich (LR) motif found in the p6 region of the Pr55Gag precursor is directly involved in the interaction with Vpr (Kondo and Göttlinger, 1996; Selig et al., 1999; Jenkins et al., 2001a,b; Fritz et al., 2010). The Pr55Gag p6 region has also been found to be phosphorylated during

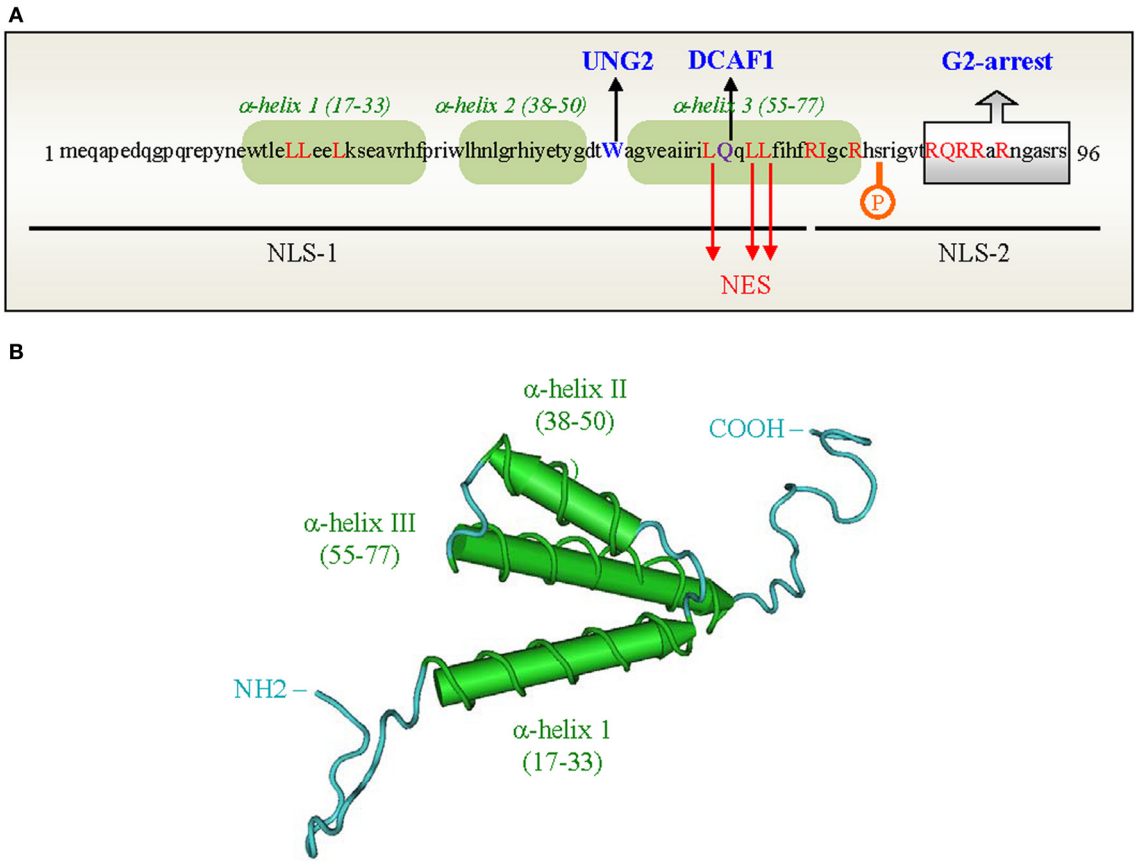

FIGURE 2 | Primary sequence and three-dimensional structure of the HIV-1 Vpr protein. (A) Primary sequence of the Vpr protein from the HIV-1Lai strain. The 3 α-helices are boxed in green. Domains and Leu residues of Vpr involved in the nuclear import (black lines) and nuclear export (Leu in red) of proteins are indicated. The Trp residue in position 54 as well as the Gln residue critical for Vpr binding to UNG2 and DCAF1 are highlighted in blue and purple, respectively. **(B)** Three-dimensional structure of the HIV-1 Vpr protein (adapted from Morellet et al., 2003). The three α-helices (17–33, 38–50, 55–77) are colored in green, respectively; the loops and flexible domains are in blue.

HIV-1 infection by atypical protein kinase C (Hemonnot et al., 2004) regulating the incorporation of Vpr into HIV-1 virions and thereby supporting virus infectivity (Kudoh et al., 2014). After assembly and proteolytic cleavage of Pr55Gag in matrix, capsid, nucleocapsid (NCp7), and p6 mature proteins, Vpr is recruited into the conical core of the virus particle (Accola et al., 2000; Welker et al., 2000) where it is tightly associated with the viral RNA (Zhang et al., 1998; De Rocquigny et al., 2000). Interestingly, Vpr displays a higher avidity for NCp7 than for the mature p6 protein (Dong et al., 1997; Selig et al., 1999; Jenkins et al., 2001a,b). Since p6 is excluded from the virion core (Accola et al., 2000; Welker et al., 2000), Vpr could switch from the p6Gag region of the precursor to the mature NCp7 protein in order to gain access to the core of the infectious virus particle budding at the cell surface. In any case, p6 has been reported to show a high affinity for membrane bilayers which substantially increases the interaction between p6 and Vpr (Salgado et al., 2009). It was estimated that Vpr is efficiently incorporated in HIV-1 virions with a Vpr/Pr55Gag ratio of ∼1:7 (Knight et al., 1987; Cohen et al., 1990; Welker et al., 2000), which may represent 275 molecules of Vpr per virion. More recently it has been shown that the HIV-1 Pr55Gag precursor induces the recruitment of Vpr oligomers to the plasma membrane (Fritz et al., 2010). Vpr oligomerization has been found to be essential for binding of Vpr to Pr55Gag and for its accumulation at the plasma membrane early during Pr55Gag assembly, but the exact role of these oligomers is not certain yet (Fritz et al., 2010).

The incorporation of Vpr has also been used as a unique tool to target cargoes such as cellular and viral proteins or drugs into viral particles (Wu et al., 1995; Yao et al., 1999; Fritz et al., 2010). This property found extensive use in studies that evaluated the respective functions of integrase (IN) and reverse transcriptase (RT) during virus replication by expressing Vpr-IN and Vpr-RT fusions in *trans* in virus-producing cells (Wu et al., 1997, 1999; Liu et al., 1999). Furthermore, Vpr fused to the green fluorescence protein (GFP) has been used to tag HIV particles in order to follow intracellular virus behavior during the early intracellular steps of infection in target cells (Loeb et al., 2002; Steffens and Hope, 2003; Fritz et al., 2010).

Vpr AND THE CELL CYCLE

Among the range of functions of the Vpr protein, the Vpr-dependent G2 arrest activity was extensively explored since it was described for the first time in 1995 (He et al., 1995; Jowett et al., 1995; Re et al., 1995; Rogel et al., 1995). The Vpr protein encapsided into HIV-1 virions is able to block proliferation of newly infected T lymphocytes. Following infection, these cells accumulate at the G2-M phase and show a 4N DNA content. The first studies proposed that the presence of Vpr leads to the accumulation of the hyperphosphorylated form of the cyclin-dependent kinase CDC2 (the complex p34 cdc2/cyclin B). This inactive form of the complex would be able to block the cell cycle before the mitosis.

This cytostatic function of Vpr is well conserved among primate lentiviruses (Planelles et al., 1996; Stivahtis et al., 1997), and could be a strategy used by HIV and SIV to improve viral replication and protein expression, and even to reactivate the virus through an epigenetic control of the LTR promoter (Yao et al., 1998; Thierry et al., 2004). The biological significance of this cell cycle arrest during the natural infection is not well understood, but the HIV-1 LTR seems to be more active in the G2 phase, implying that the G2 arrest may confer a favorable cellular environment for efficient transcription of HIV-1 (Goh et al., 1998). In agreement, the Vpr-induced G2 arrest correlates with high level of viral replication in primary human T cells. Overexpression of dominant negative mutant of the p34 cdc2 kinase shows that Vpr-induced G2 arrest correlates with HIV-1 activation (Goh et al., 1998). Vpr might also be involved in virus activation through other interactions such as the formation of a complex with p53 and the transcription factors Sp1 (Wang et al., 1995; Sawaya et al., 1998). This complex could lead to the activation of the p21/WAF promoter resulting in the transactivation of the viral LTR (Cui et al., 2006). Using a human hematopoietic stem cell-transplanted humanized mouse model, it was recently shown that Vpr causes G2 cell cycle arrest and apoptosis predominantly in proliferating CCR5+ CD4+ T cells, which mainly consist of regulatory CD4+ T cells (Tregs), resulting in Treg depletion and enhanced virus production during acute infection *in vivo* (Sato et al., 2013). In addition, recent results just published by Laguette et al. (2014) show that the interaction of Vpr with the structure-specific endonuclease (SSE) regulator SLX4 complex (SLX4com) is crucial for the G2-arrest activity but also for escape of HIV-1 from innate immune sensing in infected cells.

Some studies try to correlate the Vpr structure with cell cycle regulation. Historically, this function of Vpr was associated with the helix-3 and the flexible C-terminal part of the protein (Marzio et al., 1995; Mahalingam et al., 1997; Chen et al., 1999). Some key phosphorylations of the C-terminus part have also been associated with the G2 arrest, such as phosphorylation of the Ser79 residue (see **Figure 2A**) (Zhou and Ratner, 2000). Vpr is mainly localized in the nucleus and at the nuclear envelope where previous reports indicated it could induce herniations and burstings of the nuclear membrane and even defects in the nuclear lamina (de Noronha et al., 2001; Sörgel et al., 2012). These morphological modifications could impact several nuclear factors and redistribute a large range of proteins from the nucleus to the cytoplasm leading to alteration of the cell cycle. Indeed, the cyclins involved in the cell cycle regulation are closely regulated and their spatio-temporal distribution is primordial for the continuity of the cell cycle. More recently, interactions between Vpr and chromatin have been reported (Belzile et al., 2010; Shimura et al., 2011). Vpr can cause epigenetic disruption of heterochromatin by inducing the displacement of heterochromatin protein 1-α (HP1-α) through acetylation of the histone H3 and causes premature chromatids separation and consequently G2 arrest (Shimura et al., 2011). The interaction between Vpr and the chromatin should target and activate the ataxia telangiectasia mutated and Rad3-related kinases ATM/ATR, two of the main sensors of the cell cycle (Koundrioukoff et al., 2004). The link between ATR and the Vpr-dependent G2 arrest was initially reported by Roshal et al. (Roshal et al., 2003) (for review on ATR pathway, see Sørensen and Syljuåsen, 2012). The ATR and ATM proteins control the G2 arrest provoked by DNA damage but it is controversial if Vpr really causes DNA damage or just mimics this damage and activates some sensors involved in this process (Cliby et al., 2002). It was reported that the inhibition of ATR abrogates the Vpr-dependent G2 arrest. Following ATR activation by Vpr, Chk1 is activated through phosphorylation and required for the G2 arrest (Li et al., 2010). Clearly, Vpr acts on the cell cycle by a cascade of reversible phosphorylations. The expression of Vpr correlates with inactivation of the p34/cdc2 CDK1 kinase associated with cyclin B. Cdc2 is normally activated by the cdc5 phosphatase which is inactive in its hypophosphorylated form in Vpr-expressing cells (He et al., 1995; Re et al., 1995), whereas Wee1 inhibits the cdc2 kinases (Sørensen and Syljuåsen, 2012). Vpr seems to be able to directly activate the Wee1 protein by binding to its "N" lobe but this interaction is not sufficient for induction of the G2 arrest (Kamata et al., 2008). However, other key regulators of the cell cycle interacting with Vpr could be members of the 14-3-3 protein family (Kino et al., 2005) which bind phosphorylated serine/threonine proteins such as the cell cycle regulators Wee1, Cdc25, and Chk1. Consequently, 14-3-3 could regulate activities and distribution of these proteins (Lopez-Girona et al., 1999; Hermeking and Benzinger, 2006). These authors revealed that overexpression of 14-3-3 leads to an increase of the cell cycle arrest in the presence of Vpr while the absence of this scaffolding protein reduces the Vpr-induced activity. Another study revealed how Vpr disrupts 14-3-3θ from centrosome and increases its association with the importin β, Cyclin B1, and Cdk1 (Bolton et al., 2008).

Today, almost all the new studies about the Vpr-induced G2 arrest try to identify the potential target of Vpr degraded by the proteasome machinery. Indeed, several groups clearly showed that Vpr connects the DCAF1 adaptor of the Cul4A ubiquitin ligase to a so far unidentified host target protein linked to the G2 arrest (Belzile et al., 2007; DeHart et al., 2007; Le Rouzic et al., 2007; Schrofelbauer et al., 2007). First, the interactions between Vpr and cullins 1 and 4 (Cul1, Cul4), belonging to the ubiquitin ligase complex, were reported (Schrofelbauer et al., 2007). Then, the Vpr-binding protein (VprBP) was described as a substrate specificity module in Cul4 and DDB1 (damaged-DNA specific binding protein 1)-based ubiquitine ligase E3 complexes (Angers et al., 2006; He et al., 2006; Higa et al., 2006a; Jin et al., 2006). Furthermore, other teams described a

larger complex where Vpr was associated with Cul4A, DDB1, Rbx2/Roc1 and an ubiquitin-conjugating enzyme or E2. At the same time VprBP was renamed DDB1-and Cul4-associated factor (DCAF)-1 (Belzile et al., 2007; DeHart et al., 2007; Hrecka et al., 2007; Le Rouzic et al., 2007; Schrofelbauer et al., 2007; Tan et al., 2007; Wen et al., 2007). The Cul4-DDB1-E3 ligase complex can bind several DCAFs and seems involved in the maintenance and control of the genome stability, DNA replication and cell cycle check point control (Sugasawa et al., 2005; Higa et al., 2006b; Wang et al., 2006). From these studies, a model where Vpr binds the Cul4-DDB1-DCAF1 E3 ligase to trigger the degradation of a putative protein responsible for the G2 arrest has emerged (Dehart and Planelles, 2008). In this model, Vpr uses two distinct interfaces for binding, one for the attachment to VprBP/DCAF1 and the other for the putative substrate protein. Vpr binds DCAF1 through the LR motif found between amino acids 60 and 68 while the C-terminal basic flexible region binds to the substrate to be ubiquitinylated and degraded and responsible for G2 arrest (Zhao et al., 1994; DeHart et al., 2007; Le Rouzic et al., 2007). Recently, Belzile et al. (2010) proposed that Vpr is present in the nucleus and more specifically inside nuclear foci where it is associated with VprBP and the DDB1-CUL4A-E3 ubiquitine ligase. These foci colocalize with DNA repair foci containing proteins such as γH2AX and RPA2. This association may lead to the recruitment and the degradation of a chromatin-bound substrate via a K48-linked polyubiquitinylation (Belzile et al., 2010) which activates the key protein ATR and the G2 arrest. Finally, a new essential actor of the Vpr-dependent G2 arrest, the SSE regulator SLX4com has been recently identified by proteomic analysis (Laguette et al., 2014). Vpr activates SLX4com through direct interaction with SLX4 leading to the recruitment of VprBP and the kinase-active PLK1. This association would lead to the cleavage of DNA by SLX4-associated MUS81-EME1 endonucleases. Vpr activation of premature MUS81-EME1 induces accumulation of FANCD2 foci and consequently DNA intermediates cleavage and replication stress.

Vpr AND APOPTOSIS

Acute phase of AIDS is characterized by a net decrease of CD4+ T cells, and the hallmark of the chronic phase is a gradual decrease of the peripheral CD4+ T cells. While the virus mainly targets lymphocytes and macrophages, no depletion of macrophages has been reported and these terminally-differentiated cells may rather serve as virus reservoirs. The reason why infected macrophages were not susceptible to apoptosis has been recently explored. Using macrophage-like cells derived from differentiated THP1 CD4+ myeloid cells, a recent report showed that Vpr is not able to downregulate the anti-apoptotic protein cIAP1/2 (Busca et al., 2012). However, Mishra et al. (2007) previously revealed the possibility that the C-terminal part of Vpr could induce apoptosis in monocytes via a JNK-dependant pathway.

Although different HIV-1-induced pathways for apoptosis induction have been described, Vpr appears as one of the main actors of the cell death observed during HIV-1 infection. However, it is still controversial how Vpr induces apoptosis and/or necrosis. Moreover, uninfected bystander T cells can be also targeted by Vpr, since Vpr can get access to the extracellular compartment like a soluble protein (Reiss et al., 1990; Cummins and Badley, 2010; Abbas, 2013). A previous model for Vpr-induced apoptosis proposed that Vpr would be able to bind the WxxF motif of the transmembrane adenine nucleotide transporter (ANT) protein exposed in the inner membrane of mitochondria. Jacotot et al. (2000, 2001) were the first to detect this interaction and found that Vpr could also bind to another member of the permeability transition pore complex (PTPC), the voltage-dependent anion channel (VDAC). This team showed the capacity of a synthetic Vpr polypeptide to trigger permeabilization of the mitochondrial membrane resulting in the collapse of the mitochondrial transmembrane potential. Following permeabilization of both inner and outer mitochondrial membranes (Ghiotto et al., 2010), the release of pro-apoptotic proteins like the cytochrome c forms the apoptosome with the caspase 9 and Apaf-1 and allows recruitment of caspase 3. Bax, another pore forming complex protein should also be involved in the Vpr-induced cell death since a conformational change and activation of Bax was detected in apoptotic cells expressing Vpr (Andersen et al., 2006). In this study, the authors characterized cell death in mice, and described that ANT may promote a necrotic cell death rather than apoptosis.

It was indeed discussed whether the Vpr-induced G2 arrest was linked to the observed apoptosis in Vpr expressing cells. Earlier, some studies concluded that Vpr-induced apoptosis was independent of the G2 arrest activity (Nishizawa et al., 2000a,b) showing that a C-terminal truncated form of Vpr still induced apoptosis but did not induce G2 arrest. However, others and more recent studies found a correlation between both Vpr activities and suggested that apoptosis was a consequence of the prolonged G2 arrest (Andersen et al., 2006). According to Stewart and colleagues, apoptosis would happen in cells after the G2 arrest as a consequence of the blockage, and this was observed in human fibroblasts, T cell lines, as well as primary peripheral blood lymphocytes (Stewart et al., 1997, 1999). Accordingly, Zhu et al. (2001) showed that treating cells with caffeine, an inhibitor of both ATM and ATR, which are key proteins involved in cell cycle control, abrogated both G2 arrest and apoptosis. Trying to understand this ATR-dependent mechanism, subsequent studies from the same team described an activation of the DNA repair enzyme BRCA1 leading to the regulation of the growth arrest and DNA damage protein 45α (GADD45α) involved in the cell death process (Zimmerman et al., 2004; Andersen et al., 2005). Moreover, some cell cycle regulators such as Wee1 and Chk1 could also be involved in the Vpr-dependent apoptosis pathway. The Vpr-dependent phosphorylation of Chk1, an event that begins during S phase of the cell cycle, could also trigger apoptosis (Li et al., 2010).

Furthermore, it was reported that Vpr may impact the immune system homeostasis by stimulating the secretion of TNF-α by dendritic cells, resulting in apoptosis of CD8+ T cells (Majumder et al., 2007). Vpr could also increase expression of the NKG2D stress ligand in CD4+ T cells promoting their destruction by the Natural Killer (NK) cells (Ward et al., 2009; Richard et al., 2010). According to Ward et al. (2009), this mechanism causes a link between the G2 arrest and the apoptosis since

they showed NKG2D expression is dependent of ATR activation by Vpr.

Finally, some studies revealed that 40–50% of HIV-1 seropositive patients have neurocognitive disorders (Ances and Ellis, 2007; Jones et al., 2007; Harezlak et al., 2011), and different theories have been proposed to explain these neurological disorders. Among the Vpr effects, some hypothesized that extracellular Vpr might be able to enter into neurons (Rom et al., 2009) where it can cause apoptosis. Jones et al. (2007) tested the effect of soluble Vpr in neurons and detected apoptosis involving cytochrome c release, p53 induction, and activation of caspase-9. This study also suggested that Vpr triggers the release of the inflammatory IL-6 cytokine by astrocytes which could affect neuron survival. More recently, it was shown that Vpr could also act on the glycolytic pathway of astrocytes leading to secretion of stress molecules (Ferrucci et al., 2013).

Vpr AND THE REVERSE TRANSCRIPTION

After virus entry, the viral core is released into the cytoplasm of the target cell where the reverse transcription of the viral RNA takes place within a large nucleoprotein complex (Farnet and Haseltine, 1991; Bukrinsky et al., 1993; Miller et al., 1997; Fassati and Goff, 2001; Nermut and Fassati, 2003; Lyonnais et al., 2013). This reverse transcription complex (RTC) contains the two copies of viral RNA and the viral RT, IN, NCp7, Vpr and a few molecules of the matrix protein. It is generally believed that the reverse transcription process is initiated in virus particles and then completed in the cytoplasm after the virus has entered into the target cell (**Figure 1**). The reverse transcription process is likely to take place in parallel during both virus uncoating and trafficking through the cytoplasm (for reviews, see Goff, 2001; and Pomerantz, 2000). Several studies confirmed that Vpr co-localizes with the viral nucleic acids and IN within purified HIV-1 RTCs (Fassati et al., 2003; Nermut and Fassati, 2003; Steffens and Hope, 2003), and remains associated with the viral DNA within 4–16 h after infection (Fassati and Goff, 2001). Interestingly, Vpr has recently been reported to be essential for unintegrated HIV-1 gene expression and *de-novo* virus production in a virus replication pathway utilizing RT DNA products that failed to integrate (Poon and Chen, 2003; Trinité et al., 2013).

In addition to a potential role in the initiation step of the reverse transcription process (Stark and Hay, 1998), it has been shown that Vpr modulated the *in vivo* mutation rate of HIV-1 by influencing the accuracy of the reverse transcription. The HIV-1 RT is an error-prone RNA-dependent DNA polymerase, and quantification of the *in vivo* rate of forward virus mutations per replication cycle revealed that the mutation rate was 4-fold higher in the absence of Vpr expression when measured in dividing cells using a genetically engineered system (Mansky and Yemin, 1995; Mansky, 1996). Furthermore, analysis in non-dividing cells showed that this phenotype is even more pronounced in primary monocyte-derived macrophages (MDMs) leading to a 16-fold increase of the HIV-1 mutation frequency (Chen et al., 2004). Strikingly, this activity correlates with the interaction of Vpr with the nuclear form of uracil DNA glycosylase (UNG2) (Mansky et al., 2000), an enzyme of the base excision repair system that specifically removes the RNA base uracil from DNA. The inclusion of uracil in DNA can occur either by misincorporation of dUTP or by cytosine deamination. While the Trp residue in position 54 located in the exposed loop connecting the second and the third α-helix of HIV-1 Vpr has been shown to be critical to maintain the interaction with UNG2, the Vpr-binding site was mapped within the C-terminal part of UNG2, and occurs through a WxxF motif. So far, three distinct cellular partners of Vpr were known to contain a WxxF motif: the TFIIB transcription factor, the adenosine-nucleotide translocator (ANT) and UNG2 (as reviewed in Planelles and Benichou, 2009). However, the WxxF motif is not sufficient for Vpr binding, since other cellular Vpr-interacting proteins, such as DCAF1 or DICER for example, still bind to Vpr independently of the presence of a WxxF motif within their primary sequence (Belzile et al., 2007; DeHart et al., 2007; Le Rouzic et al., 2007; Schrofelbauer et al., 2007; Casey Klockow et al., 2013).

Some authors suggested that the association of Vpr with UNG2 in virus-producing cells allows the incorporation of a catalytically active enzyme into HIV-1 particles where UNG2 may directly influence the reverse transcription accuracy (Mansky et al., 2000; Chen et al., 2004), and this plays a specific role in the modulation of the virus mutation rate. The model supporting the direct contribution of incorporated UNG2 in the reverse transcription process was demonstrated by using an experimental system in which UNG2 was recruited into virions independently of Vpr. UNG2 was expressed as a chimeric protein fused to the C-terminal extremity of the VprW54R mutant, a Vpr variant that fails to recruit UNG2 into virions and to influence the virus mutation rate, even though it is incorporated as efficiently as the wild-type Vpr protein. The VprW54R-UNG2 fusion is also efficiently packaged into HIV-1 virions and can restore a mutation rate equivalent to that observed with wild-type Vpr, both in actively dividing cells and in MDMs. In agreement with this phenotype on the virus mutation frequency, it was finally documented that the Vpr-mediated incorporation of UNG2 into virus particles contributed to the ability of HIV-1 to replicate in primary macrophages. When the VprW54R variant was introduced into an infectious HIV-1 molecular clone, virus replication in macrophages was both reduced and delayed. Although it was proposed that the viral integrase was also able to mediate interaction with UNG2 (Priet et al., 2003), Vpr seems to be the main viral determinant that allows for the incorporation of UNG2 into virus particles. However, further analyses are required to document the nature of interactions between UNG2, Vpr, IN as well as RT both in virus-producing cells and in target cells.

Other studies also confirmed that UNG2 was efficiently recruited into virus particles (Priet et al., 2005; Kaiser and Emerman, 2006; Yang et al., 2007; Jones et al., 2010), indicating that this recruitment might influence the accuracy of the reverse transcription process and has a positive influence on viral replication (Chen et al., 2004; Priet et al., 2005; Jones et al., 2010). Interestingly, it has been recently reported that HIV-1 DNA generated in infected macrophages and CD4-positive T cells is heavily uracilated (Yan et al., 2011). However, the specific role of UNG2 incorporation into virions was challenged by other studies (Schrofelbauer et al., 2005; Kaiser and Emerman, 2006; Yang et al., 2007). While the specificity of the interaction

between Vpr and UNG2 was not questioned, these studies suggested a detrimental (Schrofelbauer et al., 2005; Yang et al., 2007; Eldin et al., 2013) or dispensable (Kaiser and Emerman, 2006) effect of UNG2 on virus replication. In the model suggesting a detrimental effect on UNG2 on virus replication, Vpr was shown to induce the proteasomal degradation of UNG2 in virus-producing cells in order to prevent its recruitment into virus particles (Schrofelbauer et al., 2005, 2007; Eldin et al., 2013). It has also been reported that the Vpr-UNG2 interaction temporarily impairs the uracil excision activity of UNG2 in infected cells (Eldin et al., 2013). However, other data have indicated that the Vpr-induced reduction of endogenous UNG2 observed in HIV-1 infected cells was not solely related to proteasomal degradation (Langevin et al., 2009; Nekorchuk et al., 2013) and that UNG2 might not be responsible for the degradation of HIV-1 DNA containing misincorporated dUTP which prevents viral integration (Weil et al., 2013). More recently, it has been argued that incorporation of UNG2 into HIV-1 particles may not be detrimental for virus infection in target cells but rather has a positive impact on virus replication and virus infectivity achieved through a non-enzymatic mechanism mapping within a 60-amino-acid long domain located in the N-terminal region of UNG2 (Guenzel et al., 2012). Interestingly, this domain is also required for interaction of UNG2 with the p32 subunit (RPA2) of the replication protein A complex (Nagelhus et al., 1997; Otterlei et al., 1999; Mer et al., 2000; De Silva and Moss, 2008). It was observed that enforced virion recruitment of UNG2, through UNG2 overexpression in virus producing cells, similarly influenced infectivity of X4 and R5 HIV-1 strains in transformed cell lines and MDMs, respectively (Guenzel et al., 2012), which stands in contrast to another report suggesting that UNG2 was exclusively required for efficient infection of primary cells by R5-tropic viruses (Jones et al., 2010). Strikingly, viruses produced from cells depleted of endogenous UNG2 and RPA2 resulted in significantly reduced infectivity and replication, the latter evidenced by a reduced amount of viral transcripts measured during the reverse transcription process (Guenzel et al., 2012). These new intriguing findings are not yet completely understood and further investigations are needed to clarify the mechanism.

HIV-1 and other lentiviruses are unusual among retroviruses in their ability to infect resting or terminally differentiated cells. While Vpr has been shown to facilitate the nuclear import of viral DNA in non-dividing cells (see below), the virion incorporation of UNG2 via Vpr may also contribute to the ability of HIV-1 to replicate in primary macrophages. This implies that UNG2 is a cellular factor that plays an important role in the early steps of the HIV-1 replication cycle (i.e., viral DNA synthesis). This observation is in agreement with a report showing that the misincorporation of uracil into minus strand viral DNA affects the initiation of the plus strand DNA synthesis *in vitro* (Klarmann et al., 2003). This observation suggests that UNG2 is likely to be recruited into HIV-1 particles to subsequently minimize the detrimental accumulation of uracil into the newly synthesized proviral DNA. While further works are needed to explain the precise mechanism for how the UNG2 catalytic activity may specifically influence HIV-1 replication in macrophages, it is worth noting that non-dividing cells express low levels of UNG and contain relatively high levels of dUTP (Chen et al., 2002). Similarly, most non-primate lentiviruses, such as feline immunodeficiency virus (FIV), caprine-arthritis-encephalitis virus (CAEV) and equine infectious anemia virus (EIAV), have also developed an efficient strategy to reduce accumulation of uracil into viral DNA. These lentiviruses encode and package a viral-encoded dUTP pyropshophatase (dUTPase) into virus particles, an enzyme that hydrolyzed dUTP to dUMP, and thus maintains a low level of dUTP. Interestingly, replication of FIV, CAEV, or EIAV that lack functional dUTPase activity is severely affected in non-dividing host cells (e.g., primary macrophages). Taken together, these results indicate that uracil misincorporation in viral DNA strands during reverse transcription is deleterious for the ongoing steps of the virus life cycle. The presence of a viral dUTPase or a cellular UNG will prevent these detrimental effects for replication of non-primate and primate lentiviruses in macrophages, respectively.

Finally, it is intriguing to note that two viral auxiliary proteins from HIV-1, Vpr and Vif, can both influence the fidelity of viral DNA synthesis. The Vif protein forms a complex with the cellular deaminase APOBEC3G thereby preventing its encapsidation into virions (Sheehy et al., 2002; Lecossier et al., 2003; Mangeat et al., 2003; Mariani et al., 2003; Stopak et al., 2003), while Vpr binds the DNA repair enzyme UNG2. In this context it was suggested that incorporation of UNG2 into viral particles would have a detrimental effect on reverse transcription by introducing a basic sites into viral DNA in regards to uracil residues resulting from cytosine deamination by the cytidine deaminase APOBEC3G (Schrofelbauer et al., 2005; Yang et al., 2007). While the specific role of UNG2 in the antiviral activity of APOBEC3G was not directly questioned (Schrofelbauer et al., 2005), others reported data indicating that the antiviral activity of overexpressed APOBEC3G was partially affected when viruses were produced in UNG2-depleted 293T cells (Yang et al., 2007). However, these data are in apparent contradiction with results from other reports in which viruses were produced in UNG2-depleted cells which expressed or did not express APOBEC3G (Priet et al., 2005; Jones et al., 2010; Guenzel et al., 2012), but also from reports showing that APOBEC3G-mediated restriction of HIV-1 was independent of UNG2 (Kaiser and Emerman, 2006; Langlois and Neuberger, 2008). More recently, and in favor for a correlative positive impact of UNG2, it has been shown that the detrimental hypermutation of Hepatitis B virus DNA induced by either APOBEC3G or interferon treatment was enhanced in a human hepatocyte cell lines when UNG2 activity was inhibited (Kitamura et al., 2013; Liang et al., 2013). Additional investigations are thus required to further understand this apparent contradiction regarding the role of UNG2 for the antiviral activity of APOBEC restriction factors. However, it is tempting to speculate that the action of both viral proteins may influence the mutation rate during the course of HIV-1 infection, and their balance may play a key role during disease progression and antiretroviral treatment susceptibility in infected individuals (Fourati et al., 2010, 2012).

Vpr AND THE VIRAL DNA NUCLEAR IMPORT

Like other retroviruses, HIV-1 has the capacity to infect and integrate its genomic DNA into dividing cells like T lymphocytes, but lentiviruses are also remarkable by their capacity to infect non-dividing cells, in contrast to onco-retroviruses which need the disintegration of the nuclear envelope to allow access of their genome for integration in the host genome (Greber and Fassati, 2003). Indeed, HIV-1 can infect terminally-differentiated macrophages and produces new virions after integration of its DNA into the cell genome. The Vpr protein has been described as a potential enhancer of HIV-1 replication especially in macrophages whereas it does not impact on virus replication in proliferating T cells (Balliet et al., 1994; Connor et al., 1995; Eckstein et al., 2001). In macrophages, the viral DNA needs to be transported into the interphasic nucleus by an active mechanism (Vodicka et al., 1998). After virus entry into the cell, the viral genome is reverse-transcribed in full length viral double-strand DNA which is associated with viral and host cell proteins into the so-called PIC. Among the protein components of the PIC, four viral proteins have been detected (e.g., the reverse-transcriptase and integrase enzymes, the matrix protein and Vpr) (Heinzinger et al., 1994; Jenkins et al., 1998; Eckstein et al., 2001; Le Rouzic et al., 2002; Schang, 2003; Suzuki et al., 2009).

Despite the absence of a basic canonical or a M9-dependant nuclear localization signal (NLS) in the protein sequence, Vpr shows evident karyophilic properties (Gallay et al., 1996; Jenkins et al., 1998; Depienne et al., 2000). Finally, Vpr seems to use a non-classical pathway to be transferred in the nucleus through direct binding to importin-α (Gallay et al., 1996; Nitahara-Kasahara et al., 2007). However, it was largely shown that Vpr is able to shuttle between the cytoplasm and the nuclear compartments and could play a potential role in transport of the viral DNA (Jenkins et al., 2001a,b; Sherman et al., 2001, 2003; Le Rouzic et al., 2002). By using a photobleaching strategy on living cells, Le Rouzic and colleagues revealed that Vpr-GFP has shuttling properties (Le Rouzic et al., 2002). This activity was linked to the distal LR region, a classical nuclear export signal (NES) recognized by the CRM1-dependent pathway (Sherman et al., 2001, 2003). This NES could be involved in the release of Vpr back into the cytoplasm, making it available for virion packaging through interaction with the Pr55Gag precursor (Jenkins et al., 2001a,b).

The role of Vpr within the PIC has been studied in living cells through tracking of a GFP-tagged form of Vpr (McDonald et al., 2002). These authors evidenced a tight association between the PIC and the cytoplasmic microtubules, targeting the viral DNA toward the nucleus. The PIC moves along the cytoskeletal microtubule filaments using the dynein/dynactin complex as a motor, leading to its accumulation in the perinuclear region close to the centrosome. So far, it is not known if Vpr plays an active role in the intracytoplasmic transport of the PIC; it may be only associated with the complex and play a role later for nuclear membrane anchoring and translocation of the viral DNA into the nucleus (for review, Le Rouzic and Benichou, 2005).

The nuclear envelope contains two concentric membranes with nuclear pore complexes (NPC) consisting of aqueous channels which allow for selective transport between the cytoplasmic and nuclear compartments. The NPC corresponds to a 125 MDa structure consisting of 30 distinct nuclear pore proteins, named nucleoporins (Nups) (Cronshaw et al., 2002). Most of these Nups have filamentous structures containing FG or FxFG motif repeats emanating from both sides of the NPC and able to dock transport factors (Rout and Aitchison, 2001). It was initially reported that Vpr was able to recognize these FG motifs in Nups such as p54 and p58 leading to the docking of Vpr to the nuclear membrane (Fouchier et al., 1998; Popov et al., 1998). Another interaction with the human CG1 (hCG1) nucleoporin has been described by Le Rouzic et al. (2002). However, Vpr associated with the N-terminal region of hCG1 while the FG repeats of this Nup were located in the C-terminal part of the protein. This interaction results in Vpr accumulation at the nuclear envelope, which is believed to be involved in active nuclear import of the PIC in non-dividing cells, such as macrophages (Jacquot et al., 2007). Through these interactions with components of NPC, Vpr may be responsible for the first step of viral DNA import by targeting the PIC to the nuclear pore complex while other components of the PIC could trigger the next step of the nuclear translocation. As mentioned above, it was also reported that Vpr can induce herniations and the dissociation of lamina and nuclear envelope which provoke a blend of nuclear and cytoplasmic proteins (de Noronha et al., 2001). The exact mechanism inducing these membrane perturbations is not understood, but some authors hypothesize that the interaction of Vpr with the NPC proteins could impact nuclear membrane stability. Consequently it may also facilitate the entry of the PIC through a non-conventional pathway (Segura-Totten and Wilson, 2001).

CONCLUSIONS AND FUTURE DIRECTIONS

Like other HIV-1 auxiliary proteins, Vpr is a small but multifunctional protein which is potentially able to interact with plenty of cellular partners. During the last two decades, several groups looked for such partners but the importance of such interactions often needs to be better documented to support their real impact on HIV-1 propagation, immune and antiretroviral treatment evasion and disease progression. While major efforts have been made during the last years to better define the molecular mechanisms and cellular targets of Vpr, additional works are needed for the complete understanding of its wide range of activities in key processes during the early steps of the viral life cycle (i.e., reverse transcription, intra-cytoplasmic routing and nuclear import of the viral DNA). However, precise characterization of Vpr interactions leading to the proteasomal degradation of some host cell factors is certainly the main challenge for a better understanding of the Vpr contribution to the overall pathogenesis of HIV-1 infection.

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DNA damage repair machinery and HIV escape from innate immune sensing

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Viruses have been long known to perturb cell cycle regulators and key players of the DNA damage response to benefit their life cycles. In the case of the human immunodeficiency virus (HIV), the viral auxiliary protein Vpr activates the structure-specific endonuclease SLX4 complex to promote escape from innate immune sensing and, as a side effect, induces replication stress in cycling cells and subsequent cell cycle arrest at the G2/M transition. This novel pathway subverted by HIV to prevent accumulation of viral reverse transcription by-products adds up to facilitating effects of major cellular exonucleases that degrade pathological DNA species. Within this review we discuss the impact of this finding on our understanding of the interplay between HIV replication and nucleic acid metabolism and its implications for cancer-related chronic inflammation.

Keywords: DNA damage response, innate immunity, HIV, Vpr, G2/M arrest, SLX4 complex

INTRODUCTION

Efficient human immunodeficiency virus (HIV) replication in target cells relies on its ability to use cellular resources and to overthrow host defense mechanisms. Indeed, viral fitness is defined by both the availability of cellular dependency factors and the ability to escape cellular blocks. One of the most challenging steps of HIV life cycle is the delivery of its single stranded RNA (ssRNA) genome and its conversion into double stranded DNA (dsDNA) in the host cell without inducing innate immune responses. Indeed, cellular "sensors" specialized in the recognition of foreign or pathological nucleic acids are present in different compartments through which viruses enter target cell. These nucleic acid sensors belong to the pattern recognition receptors (PRRs) family that recognize pathogen associated molecular patterns (PAMPs) and subsequently trigger a signaling cascade that culminates in the production of pro-inflammatory cytokines, including antiviral interferon (IFN; for review Kawai and Akira, 2006). Once recognition established, a signaling cascade is triggered, endowing an antiviral state and a cellular response aiming to clear the infection. In the course of reverse transcription of the HIV ssRNA genome into dsDNA, several intermediate, and sometime abortive, nucleic acid species are generated, including DNA:RNA hybrids, DNA flap structures and dsDNA. Exposure of these in the cytoplasm engages various sensors. Those include Toll like receptor 7 (TLR7) that detects viral RNA in endosomes (Beignon et al., 2005), Gamma-interferon-inducible protein 16 (IFI16) that recognizes virus-derived DNA in the cytoplasm of lymphoid quiescent CD4 T cells (Monroe et al., 2013) and the cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS; Gao et al., 2013).

To avoid recognition of reverse-transcription intermediates, viral genomes are protected within the capsid core. If uncoating is correctly orchestrated, this ensures delivery of fully reverse-transcribed viral genomes into the nucleus that are subsequently integrated into the host cell genome, a pre-requisite for the establishment of productive infection. However, as reviewed in Yan and Hasan within this issue, while few viruses efficiently undergo these steps, abortive infection events also occur, leading to accumulation of "junk" nucleic acid species in the host cell cytoplasm that may be detected and thereby have adverse effects on the infection. The frailty of viral reverse-transcription is highlighted by the plethora of antiviral factors that target this specific step. Those include three of the prototypical restriction factors. Although their mechanism of action being beyond the scope of this review, it is worthy to mention that Tripartite Motif 5 alpha (TRIM5α) causes untimely uncoating, leading to premature exposure of virus-derived nucleic acid species in the host cell cytoplasm, apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G (APOBEC3G) induces hypermutations in the viral genome generating non-functional unstable genomes and SAM domain and HD domain 1 (SAMHD1) deprives the viral reverse transcriptase of the deoxynucleoside building blocks required for its action (Malim and Bieniasz, 2012 and this review series).

Escape from innate immune sensing is therefore paramount to the establishment of productive viral infections. In recent years, several lines of evidence have shown that HIV has evolved highly specialized mechanisms to elude cellular blocks. For example, blocks imposed by restriction factors are mostly overcome through the use of viral accessory proteins (Vpx, Vpr, Nef, Vif, Vpu). Accessory proteins, initially qualified as such because unrequired for *in vitro* replication in permissive cells, are encoded by lentiviral genomes in addition to the essential structural and enzymatic proteins required for mature viral particles

production (Gag, Pol, Env, Tat, and Rev). Additional mechanisms deployed by viruses to avoid innate immune sensing include a direct action on the IFN signaling cascade: inhibition of IFN synthesis, IFN receptor decoy and inhibition of IFN signaling (for review Stetson and Medzhitov, 2006; Broz and Monack, 2013). HIV also takes advantage of pre-existing cellular processes. Importantly, while cellular nucleic acid sensors recognize virus-derived nucleic acids and thereby detect incoming virions, they also play crucial roles in cellular metabolism and are usually constitutively expressed. They may therefore detect the presence of nucleic acid species resulting from DNA damage-associated repair mechanism or endogenous retroelement life cycle. Thus, cellular processes co-exist to prevent accumulation of abnormal self-nucleic acids, thereby preventing auto-initiation of pro-inflammatory responses (for review Yan and Hasan). These include major cellular exonucleases that have also been shown to positively impact of HIV life cycle: ribonuclease H2 (RNaseH2) and three prime repair exonuclease 1 (TREX1) (Yan et al., 2010; Genovesio et al., 2011). Importantly, these proteins involved in nucleic acid metabolism belong to a family of genes that, when mutated, lead to the Aicardi-Gouttière syndrome (AGS). This rare autosomal recessive genetic encephalopathy is characterized by neurological dysfunctions, intracranial calcifications, brain atrophy, psychomotor retardation and increased plasma levels of IFN that lead to chronic inflammation (Lebon et al., 1988). We recently established that the SLX4 structure-specific endonuclease regulator complex also acts as a facilitator of HIV infection (Laguette et al., 2014). This finding bears substantial similarities with what was shown for the TREX1 exonuclease. Indeed, similar to TREX1, the SLX4 complex is involved in nucleic acid metabolism and plays crucial roles in the repair of DNA lesions. In addition, the core component of this complex is the SLX4 molecular scaffold that assembles structure-specific endonuclease modules. Biallelic mutations in *SLX4* are involved in the onset of Fanconi anemia (FA), a cancer predisposition syndrome characterized by congenital malformations, hypersensitivity to DNA interstrand cross-linking agents and progressive bone marrow failure (Sasaki and Tonomura, 1973). In addition, FA patients experience heightened pro-inflammatory cytokines levels (Whitney et al., 1996; Rathbun et al., 1997; Dufour et al., 2003; Briot et al., 2008). The latter is a feature shared with AGS patients and supports a potential link between proteins involved in DNA damage response and the development of inflammatory responses. These recent findings also shed a new light on the implication on proteins involved in the maintenance of genomic stability and the HIV life cycle.

CROSS-TALK BETWEEN CELL CYCLE REGULATION MACHINERY AND VIRAL INFECTIONS

Viruses have been long known to keep a privileged relationship with cell cycle regulatory mechanisms. Indeed, an estimated 20% of human cancers arise from infection with DNA or RNA viruses. Malignancy frequently results from side-tracking of cell cycle regulatory elements. A stricking example is virus-driven oncogenesis that results from subversion of the boundaries between the DNA replication step (S), segregation of sister chromatids (mitosis) and gap phases (G1 and G2). This is frequently achieved through viral non-structural proteins that modulate cell cycle regulators. Transforming viruses essentially subvert the G1/S boundary, thereby pushing cells into proliferation. In the case of the retrovirus HTLV-I, that encodes several potential oncogenes, the well-studied Tax protein is necessary and sufficient to initiate cellular transformation, while the HBz protein is required for its maintenance (Gatza et al., 2003). Similarly, HBx from the DNA virus HBV has the ability to transform immortalized cell lines and to provoke liver cancer in mice (Kim et al., 1991). Small DNA tumor viruses often encode potent oncoproteins that can cause cellular transformation *in vitro* [for example E6/E7 from HPV – for review (Howley and Livingston, 2009)]. In contrast certain viruses such as EBV require the concerted action of several proteins to achieve cellular transformation (Kutok and Wang, 2006).

Importantly, manipulation of the cell cycle or of cell cycle regulators is not solely confined to transforming viruses. Indeed, several DNA and RNA viruses are able to cause cell cycle arrest at the G2/M transition, including HIV (Davy and Doorbar, 2007). The molecular mechanisms underlying virus-induced G2/M arrest vary widely and have been extensively studied. Nonetheless, the understanding of the biological end-point of G2/M arrest remains poor despite suggestions that G2/M arrest may decrease propensity to secrete IFN (Lee and Rozee, 1970), increase RNA production rates (Lee and Rozee, 1970), and overall boost early step of the HIV life cycle (Groschel and Bushman, 2005). Subversion of the host cell cycle by HIV-1 relies on the highly conserved viral protein regulatory (Vpr) protein that causes a potent G2/M arrest in most cycling eukaryotic cells (Di Marzio et al., 1995; He et al., 1995; Jowett et al., 1995; Re et al., 1995; Rogel et al., 1995).

From a mechanistic stand point, Vpr-induced G2/M arrest is a well-documented phenotype. To understand how G2/M arrest is achieved, it is necessary to recapitulate the mechanism underlying this cell cycle check-point (for more details see Guenzel et al, this issue). Indeed, in healthy cells, the G2/M transition is controlled by Cyclin-dependent kinase1:CyclinB1 (CDK1:CCNB1). As cells progress through G2, CDK1:CCNB1 is progressively activated and once the G2/M boundary crossed, the complex is inactivated. The G2/M check-point serves as a quality-control step during the replication of the cellular genome that ensures the transmission to daughter cells of a complete unaltered set of chromosomes. Thus, when genotoxic stress is incurred, entry into mitosis is prevented to provide an opportunity to repair genomic lesions (Stark and Taylor, 2006). This is achieved through preventing CDK1:CCNB1 activation (Sanchez et al., 1997). This response is regulated through a signaling cascade that involves detection of the DNA lesion by the key DNA damage response regulators ataxia-telangiectasia-mutated kinase (ATM), ATM and Rad3-related kinase (ATR), and DNA-dependent protein kinase [DNA-PK; for review (Smith et al., 2010; Sirbu and Cortez, 2013)]. When damaged DNA or unreplicated regions of the genome are detected, these kinases activate downstream CHK1 or CHK2 that in turn inactivate CDK1:CCNB1 (Lopez-Girona et al., 1999).

Similar to what is observed following genotoxic stress, Vpr expression activates ATR, ATM, and the downstream CHK1/CHK2

kinases (Roshal et al., 2003; Munoz et al., 2009), thereby inactivating CDK1:CCNB1. In agreement, treatment of Vpr-expressing cells with caffeine, which inhibits ATR and ATM, relieves the cell cycle block (Poon et al., 1997; Shostak et al., 1999). Intriguingly, although mobilization onto sub-regions of the chromatin of breast cancer susceptibility protein 1 (BRCA1) and γH2ax have been reported upon Vpr expression, it remains unclear whether actual DNA breaks occur in the presence of Vpr and whether these lesions would be the trigger for cell cycle arrest. Rather, the prevailing view is that Vpr mediates ATR-dependent replication stress. Importantly, since the only consensual cellular partner of Vpr for the induction of G2/M arrest is the VPRBP-DDB1-CUL4 E3-ligase complex (Belzile et al., 2007; DeHart et al., 2007; Hrecka et al., 2007; Le Rouzic et al., 2007; Tan et al., 2007; Wen et al., 2007), it was assumed that Vpr would provoke the proteasomal degradation of a cell cycle regulatory element governing the G2/M transition. We recently identified the SLX4 complex as being the Vpr partner required for G2/M arrest (Laguette et al., 2014). Indeed, the structure specific endonucleases ERCC4^{XPF}-ERCC1 and MUS81-EME1 together with the SLX4^{FANCP} scaffold protein co-purified with Vpr as well as the poorly characterized TSPYL1 and C20orf94^{SLX4IP} subunits. Vpr binds to the C-terminus of SLX4, inducing the recruitment of VPRBP and kinase-active PLK1. This leads to VPRBP-induced ubiquitination of MUS81 and hyperphosphorylation of EME1, the consequence of which being activation of SLX4-associated MUS81-EME1. Vpr-induced untimely activation of SLX4-bound MUS81-EME1 results in replication stress, ultimately leading to G2/M cell cycle arrest (Laguette et al., 2014).

VIRAL PROTEIN REGULATORY AND SLX4 COMPLEX REGULATION

Vpr AND THE FANCONI ANEMIA PATHWAY

SLX4, also as known as FANCP, together with the fifteen additional identified FA or FA-like proteins, is involved in the FA DNA repair pathway. This pathway has been extensively described in reviews (Garner and Smogorzewska, 2011; Su and Huang, 2011; Constantinou, 2012). Briefly, FANCM binds chromatin at damage sites and recruits the E3 ubiquitin ligase activity-containing FA core complex (Kim et al., 2008). The FA core complex monoubiquitinates FANCD2–FANCI and stabilizes them at sites of damage (Garcia-Higuera et al., 2001). FANCD2–FANCI subsequently activate DNA repair proteins, including the SLX4 complex (Yamamoto et al., 2011). The latter is involved in the repair of double strand breaks, interstrand cross-links (ICL), and collapsed/damaged replication forks by homologous recombination (HR; Munoz et al., 2009; Svendsen et al., 2009; Kim et al., 2011; Stoepker et al., 2011). HR allows accurate repair by using the sister chromatid as a template and leads to the formation of four-way DNA structures, Holliday junctions (HJ), that must be removed prior to chromosome segregation. It is important to note that in somatic cells, the favored pathway to remove HJ relies on non-endonucleolytic dissolution by Bloom (BLM)-related helicases, a process that prevents sister chromatid exchanges (Wu and Hickson, 2003). Sister chromatid exchanges are particularly disfavored in somatic cells because they may engender loss of heterozygosity thereby predisposing cells to cancer (Matos et al., 2011, 2013; Gallo-Fernandez et al., 2012; Dehe et al., 2013; Saugar et al., 2013; Szakal and Branzei, 2013). However, in certain cases, for example in the absence of BLM or when the levels of damage incurred are above those that can be salvaged through dissolution, structure-specific endonucleases activities associated with the SLX4 complex may be mobilized (Schwartz and Heyer, 2011; Garner et al., 2013). *In vitro* studies have shown that SLX4–SLX1 (Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009) and MUS81-EME1 have 5′ and 3′ endonuclease activities, respectively (Boddy et al., 2001; Doe et al., 2002; Ciccia et al., 2003; Gaillard et al., 2003). However, SLX4-associated resolvase activity requires interaction of SLX1 and MUS81-EME1 with the SLX4 scaffold (Castor et al., 2013; Garner et al., 2013; Wyatt et al., 2013).

Because activation of MUS81-EME1 during S phase may cause pathological processing of healthy replication forks (Dehe et al., 2013; Matos et al., 2013; Saugar et al., 2013) and replication stress (Blais et al., 2004; Matos et al., 2013; Szakal and Branzei, 2013), under physiological conditions and when DNA damage is incurred, acquisition of MUS81-EME1 endonuclease activity is under tight regulatory circuits. Those ensure that MUS81-EME1 activity is mostly confined to late G2-early M, when bulk DNA synthesis has been completed (Dehe et al., 2013; Saugar et al., 2013). The molecular mechanism underlying Mus81-Mms4^{EME1} regulation has been extensively studied, and is achieved through phosphorylation of EME^{Mms4} by PLK1^{Cdc5} in budding yeast and in mammalian cells (Matos et al., 2011; Gallo-Fernandez et al., 2012; Saugar et al., 2013) or Cdc2^{CDK1} in fission yeast (Dehe et al., 2013). Importantly, work in mammalian cells has recently shown that MUS81-EME1 is regulated through phosphorylation of EME1 within the SLX4 complex (Castor et al., 2013; Garner et al., 2013; Wyatt et al., 2013).

Importantly, interaction of Vpr with the SLX4 scaffold protein induces recruitment of VPRBP and kinase-active PLK1, thereby activating the MUS81-EME1 endonuclease module independently of the cell cycle stage (**Figure 1**). This results in replication stress as visualized by accumulation of FANCD2 on sub-regions of the chromatin that likely mark the sites of abnormal processing of replication intermediates (Naim and Rosselli, 2009). Thus, as supported by previous work, Vpr causes cell cycle arrest through a S phase-dependent mechanism (Li et al., 2010), which is congruent with activation of the ATR pathway in Vpr expressing cells (Roshal et al., 2003; Lobrich and Jeggo, 2007). Interestingly, SLX4 has also been identified as an ATR substrate (Matsuoka et al., 2007; Mu et al., 2007) and in yeast, phosphorylation of Eme1 requires Rad53^{ATR} activation (Dehe et al., 2013). Thus, aberrant processing of stalled replication forks by Vpr-activated SLX4-associated MUS81-EME1 would cause replication stress, ATR-CHK1 pathway activation, resulting in inhibition of CDC25C. This signaling cascade will ultimately lead to inability of CDC25C to activate CCNB1:CDK1 and thus result in G2/M arrest (**Figure 1**).

Vpr AND GENOMIC INSTABILITY

The MUS81-EME1 endonuclease module plays an important role in the removal of ultrafine DNA bridges (UFBs). These DNA

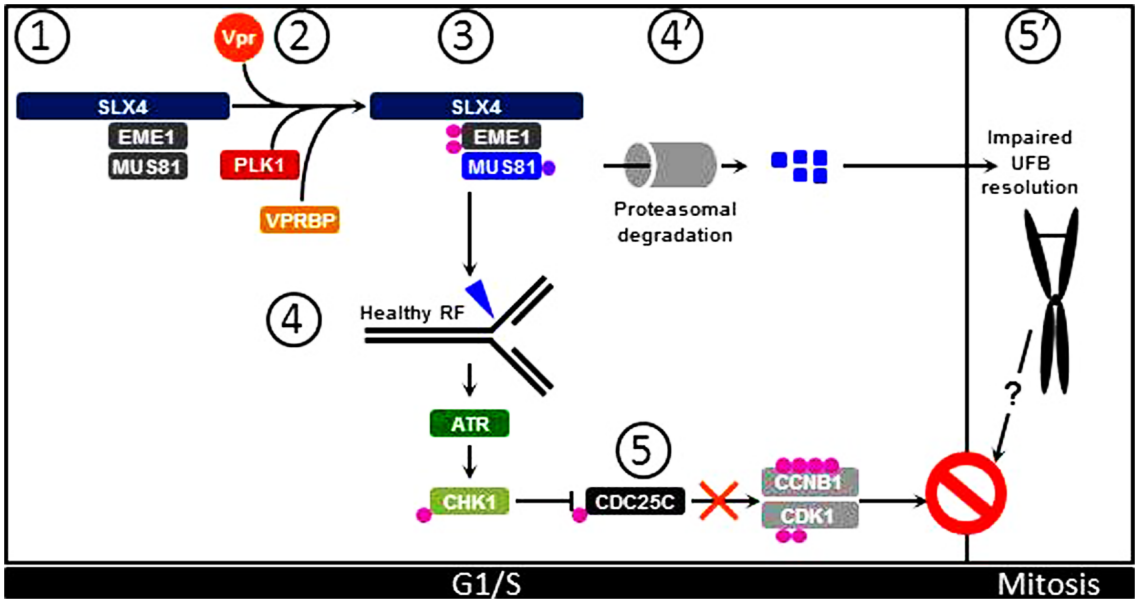

FIGURE 1 | Vpr induces G2/M arrest through activation of the SLX4 complex. (1) Under physiological conditions, inactive MUS81-EME1 interact with the SLX4 scaffold. (2) Upon Vpr expression, PLK1 and VPRBP are recruited to SLX4. (3) PLK1 phosphorylates EME1 while VPRBP causes ubiquitination of MUS81. (4) These posttranslational modifications contribute to activation of SLX4-bound MUS81-EME1 that can process healthy replication forks (RF) in cycling cells. (5) This leads to activation of the ATR signaling pathway and subsequent activation of CHK1. Activated CHK1 provokes inhibitory phosphorylation of CDC25C, leading to inhibition of CCNB1:CDK1 and cell cycle arrest at the G2/M transition. (4′) In addition, ubiquitinated MUS81 molecules are degraded by the proteasome machinery, leading to decreased steady-state levels of MUS81. (5′) Consequently, UFBs are not processed and persist in Mitosis. This possibly contributes to overall G2/M arrest in Vpr expressing cells.

structures that arise from regions of the genome that replicate at slower rates, such as centromeres and common fragile sited (CFS), are formed during the S phase and can be visualized after chromosome condensation in mitotic cells. They form bridges between sister chromatids that must be removed prior to chromosome segregation. Absence of MUS81-EME1 results in non-processing of UFBs and thus leads to CFS-associated chromosomal instability and mitotic catastrophe (Chan et al., 2009; Chan and Hickson, 2011; Wechsler et al., 2011; Naim et al., 2013; Ying et al., 2013). Accumulation of UFBs therefore causes cell cycle arrest at the G1/S transition. Intriguingly, Vpr targets MUS81 for ubiquitination by VPRBP, leading to decreased levels of MUS81 prior to G2/M arrest (Laguette et al., 2014). Since a stark increase of FANCD2 twin foci that mark the edges of UFBs (Chan et al., 2009) occurs in the presence of Vpr, this indicates that, although not complete, decreased MUS81 levels in Vpr-expressing cells may be sufficient to impair UFBs untangling prior to mitosis (Chan et al., 2009; Wechsler et al., 2011; Naim et al., 2013; Ying et al., 2013). However, Vpr-associated replication stress prevents completion of G2. This likely prevents the occurrence of mitotic catastrophe. While it is possible that additional Vpr-associated functions may prevent cells from exiting mitosis, one may also speculate that steric hindrance imposed by UFBs tying together sister chromatids may also contribute to the extent of G2/M arrest witnessed in Vpr-expressing cells (**Figure 1**). Thus, the complete sequence of events leading from SLX4 complex premature activation to cell cycle arrest by Vpr requires further investigations.

VIRAL PROTEIN REGULATORY AND INNATE IMMUNITY

One interesting feature of Vpr is that disruption of the corresponding open reading frame (ORF) results in inefficient viral spread *ex vivo* particularly in primary macrophages (Connor et al., 1995) while its most studied molecular function is to halt cell cycle progression. This conundrum has puzzled the HIV field for several years but little was understood about how these two observations can be reconciled until recent work (Laguette et al., 2014).

Infection with an HIV-1 molecular clone harboring a deletion of the Vpr ORF causes an increase of IFN production as compared to infection with wild type HIV-1 (Okumura et al., 2008; Doehle et al., 2009; Laguette et al., 2014). This HIV-induced IFN production is augmented following SLX4 complex subunits (SLX4, VPRBP, and MUS81) knock-down, suggesting that the presence of the SLX4 complex is required for inhibition of HIV-dependent type 1 IFN production. Furthermore, the SLX4 complex binds HIV-1-derived reverse transcribed DNA in presence of Vpr, suggesting that Vpr is required for this interaction. In addition, in the absence of SLX4, there is an increase of HIV DNA in infected cells. This further suggests that the SLX4 complex is required to degrade excess HIV-derived nucleic acids susceptible of triggering innate immune responses. While the MUS81-EME1 endonuclease module appears to be required for this process one cannot exclude contribution of additional SLX4-bound endonucleases. Indeed, SLX4–SLX1 interaction is required for SLX4 complex associated resolvase activity and SLX1 expression is required for Vpr-mediated cell cycle

arrest (Laguette et al., 2014). Overall, similar to TREX1 and RNaseH2, the SLX4 complex would prevent sensing of excess nucleic acids derived from HIV reverse transcription (**Figure 2**). Importantly, RNaseH2 and TREX1 preferentially degrade DNA within DNA:RNA hybrids (Haruki et al., 2002) and ssDNA substrates, respectively (Mazur and Perrino, 2001) while SLX4-bound MUS81-EME1 presumably target dsDNA structures (Fadden et al., 2013). While all these nucleic acid species arise in the course of HIV reverse transcription, the relative contribution of these nucleases remains to be evaluated. Furthermore, SLX4-mediated nucleic acids processing would lead to the generation of DNA fragments that likely require further processing to avoid recognition. This leaves several questions open amongst which are: what is the sensor triggered in the absence of SLX4 and what are the nucleases mobilized to clear SLX4-processed DNA fragments? These processes may rely on previously identified key players in viral life cycles. However, one must bear in mind that the SLX4 scaffold can bind to additional proteins involved in DNA metabolism and is as such involved in several additional pathways, including Telomere maintenance and DNA mismatch repair (Svendsen et al., 2009). Whether these may intervene in the degradation of virus derived nucleic acids is also to be explored.

As previously mentioned, FA is associated with high production of pro-inflammatory cytokine in patients. This can be recapitulated *in vitro* in *SLX4*-deficient patient cells (Kim et al., 2011) and also in mouse embryonic fibroblasts knocked-out of *MUS81* (McPherson et al., 2004) through the activation of NF-κB pathway (Laguette et al., 2014). This leads to the establishment of an antiviral state that likely accounts for the inability of these cells to support efficient HIV replication. While this bears similarities with what is observed in TREX1 deficiency, the endogenous trigger for SLX4 complex activation remains unknown. Those may include, nucleic acids derived from processing of aberrant replication intermediates or endogenous retroviruses. Elucidating the trigger for spontaneous upregulation of pro-inflammatory cytokines in FA is likely to be the next horizon in the field. This would possibly provide insight into the molecular basis of FA-associated chronic inflammation. In addition, Vpr has been shown to modulate immune responses at additional levels. This includes impairment of DC/macrophage maturation, disruption of natural killer T cells effector functions, increased apoptosis of cytotoxic T cells and disruption of T cell activation pathways (reviewed in Ayinde et al., 2010). Thus, Vpr compromises the establishment of adaptive immune responses. How inhibition of pro-inflammatory cytokines by Vpr through

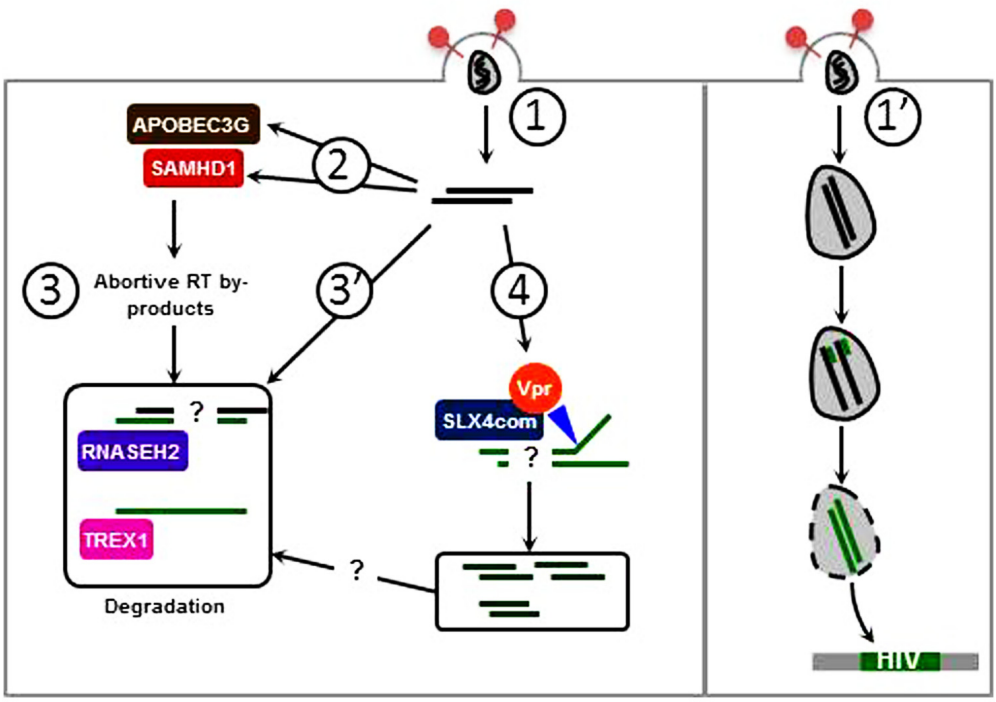

FIGURE 2 | Vpr-induced SLX4 complex activation promotes escape from innate immune sensing. (1) Premature delivery of viral genomes in the cytoplasm of host cells may lead to recognition by nucleic acid sensors. (2) Editing by APOBEC3G and dNTP hydrolysis by SAMHD1 induce viral DNA instability and impair reverse transcription. Of note, a nuclease activity has been described for SAMHD1 which may target viral nucleic acids. (3) APOBEC3G and SAMHD1 contribute to generate abortive RT by-products which are taken care of by cellular exonucleases: RNaseH2 degrades DNA:RNA hybrids while TREX1 degrades ssDNA preferentially. (3′) Abortive nucleic acid intermediates may also be directly degraded by RNaseH2 and TREX1. (4) The SLX4 structure specific endonuclease complex is activated by Vpr and likely cleaves dsDNA. Whether these dsDNA fragments are further degraded by cellular exonucleases or may be recognized by nucleic acid sensors remains questioned. (1′) When uncoating is correctly orchestrated, viral nucleic acids are protected from mechanisms described in 1–4, and ensures delivery of viral genomes in the nucleus.

activation of SLX4 complex contributes to this process remains to be explored.

DNA DAMAGE RESPONSE AND INNATE IMMUNITY

Initiation of the DNA damage response usually requires recognition of abnormal nucleic acid species in the nucleus and the triggering of a signaling cascade that orchestrates repair. This process bears similarities with what is witnessed when virus-derived nucleic acids are delivered into host cells. Those are recognized by sensors that trigger a signaling cascade aiming at clearing the infection. It has been widely speculated that the nucleic acid-based repository of the information required for *de novo* virus production is difficultly modified by viruses to promote escape; it constitutes a prime target for cellular sensors. Recent work has placed key players of the DNA damage response on the front line of pathogen recognition. For example DNA-PK has been shown to act as a PRR for DNA and RNA viruses (Zhang et al., 2011; Ferguson et al., 2012). Indeed, DNA-PK is involved in DNA damage response, more particularly in the repair of double-strand breaks and these functions are related to its nuclear localization (for review Davis and Chen, 2013). However, this complex is also found in the cytoplasm where it can bind nucleic acids and activates the production of IFN. This highlights how overlapping mechanisms have evolved for the recognition of pathological nucleic acid species. Furthermore, inflammation impacts every step of tumorigenesis, from initiation to metastatic progression. Tumor-promoting inflammation may either result from environmental factors, as clearly identified in the case of exposure to asbestos for example, or from chronic viral infections and attempts of the immune system to eliminate those. This results in a feed-forward regulatory loop that favors chronic production of pro-inflammatory cytokines, supporting tumorigenesis. Indeed, it is recognized that subclinical, often undetectable, inflammation increases cancer risk (reviewed in Grivennikov et al., 2010). Thus, persistent DNA damage or inability to repair broken DNA may lead to tumor-promoting chronic inflammation (Zheng et al., 2007; Rodier et al., 2009).

Cellular mechanisms exist that serve to avoid the accumulation of pathological nucleic acid species susceptible of triggering innate immune responses. These include the previously mentioned TREX1 exonuclease and the SLX4 complex. Intriguingly, TREX1 that was initially described to be involved in DNA base excision repair (Mazur and Perrino, 2001), also degrades ssDNA derived from aberrant replication intermediates and thus similar to the SLX4 complex is involved in DNA damage response (Yang et al., 2007; Gehrke et al., 2013). Thus, like in TREX1 deficiency, absence of the SLX4 complex may lead to accumulation of pathological nucleic acids in the cytoplasm. Recognition of those by a yet to be identified sensor, activates the immune system.

Importantly, HIV is not the sole virus affected by cellular enzymes involved in DNA metabolism. Indeed, several DNA viruses can be targeted by cellular factors involved in DNA damage response. For example, the genome of the Adenovirus or Herpes Simplex Virus type 1 (HSV-1) can be targeted by protein complexes that control the non-homologous end-joining DNA repair pathway [reviewed in Weitzman et al. (2010)]. These viruses have evolved potent ways of counteracting these proteins that operate as potential restriction factors. This can be compared to what is witnessed during HIV infection in the presence of SAMHD1. Indeed, this HIV restriction factor has been shown, in addition to depleting the dNTP pool (Lahouassa et al., 2012), to have *in vitro* nuclease activity (Beloglazova et al., 2013).

While there is accumulating evidence that proteins involved in DNA repair are involved in viral life cycles, recent work has also shown that proteins previously identified as counteractors of HIV infection are in fact involved in the DNA damage response. This is the case for APOBEC proteins where mutation patterns were found in human cancers (Leonard et al., 2013; Roberts et al., 2013) and SAMHD1. In the case of SAMHD1, recent work has highlighted that SAMHD1 may qualify as a tumor suppressor gene, and thus play roles in DNA damage response, through its ability to regulate the dNTP pool (Clifford et al., 2014; Kretschmer et al., 2014), the levels of these being important for genome stability (Mathews, 2006). Although this SAMHD1 function has been essentially described to be involved in HIV restriction, it may also be related to increased IFN production in AGS. Moreover, RNaseH2, as previously mentioned, is also involved in AGS and degrades RNA in DNA:RNA hybrids and thus may also prevent chronic inflammation (Rice et al., 2007). Importantly, both SAMHD1 and APOBEC3G have been shown to control endogenous retrotransposition (Esnault et al., 2006; Zhao et al., 2013). Since absence of SAMHD1 also correlates with increased pro-inflammatory cytokine production, one may also speculate about the trigger for this response and whether there is a correlation between inefficient DNA repair or endogenous retroelement retrotransposition in the absence of SAMHD1 and chronic inflammation. Similarly, the origin of chronic inflammation in *SLX4*-deficiency remains to be identified and may include residual nucleic acids resulting from processing of aberrant replication intermediates or endogenous retroelements.

CONCLUSION

Although host cells oppose numerous blocks to HIV replication, several mechanisms have evolved to counteract those. While defective viruses may elicit pro-inflammatory responses, viruses that establish productive infections remain mostly protected from cellular defenses. Viral accessory proteins are specialized in mediating this escape, in part through counteraction of cellular restriction factors. Interestingly, restriction factors have also been recently reported to play a role in detection of viral infections (PRR). Thus, viral accessory proteins, though their degradation simultaneously achieve escape from recognition and overthrowing of mediators of the antiviral responses. This complex array of interactions between innate immune responses and viral replication is still poorly understood. While new insight into the role of the DNA damage response machinery in this process may add a further layer of complexity, this may also provide with an additional way to identify sensors that detect incoming viruses and escape mechanisms.

In addition to the described role of Vpr in arresting the cell cycle and promoting escape from innate immune sensing, this

HIV accessory protein has been shown to play several additional roles in HIV life cycle. Indeed, Vpr contributes to fidelity of reverse transcription (Stark and Hay, 1998), nuclear transport of the pre-integration complex (Heinzinger et al., 1994). Vpr also promotes the transactivation of LTR promoter (Felzien et al., 1998), and induction of apoptosis (Stewart et al., 1997). Do these also result from Vpr-induced activation of the SLX4 complex or do they rely on additional interactions established by Vpr? Importantly, in addition to interacting with structure-specific endonucleases, the SLX4 molecular toolkit also recruits MSH2–MSH3 and TRF2–RAP1 (Svendsen et al., 2009) and is involved in additional cellular functions, including Telomere maintenance, which may be altered upon binding to Vpr. For example, since SLX4 inhibits over-lengthening of telomeric ends, Vpr-induced activation of the SLX4 complex may lead to Telomere shortening and cell death. Whether this is related to increased apoptosis witnessed in Vpr-expressing cells is yet to be explored.

The discovery of the SLX4 complex as being involved in inhibition of pro-inflammatory responses opens new avenues in the understanding of the interplay between innate immune responses and HIV infection. This work also opens new perspectives in the understanding of the molecular mechanism underlying cancer related chronic inflammation. Based on the fact that pro-inflammatory cytokine production is witnessed in all cancers, one may anticipate that additional DNA damage repair mechanisms may be involved in pathogen recognition and inhibition of spontaneous pro-inflammatory cytokine production.

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Safeguard against DNA sensing: the role of TREX1 in HIV-1 infection and autoimmune diseases

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Innate immune recognition is crucial for host responses against viral infections, including infection by human immunodeficiency virus 1 (HIV-1). Human cells detect such invading pathogens with a collection of pattern recognition receptors that activate the production of antiviral proteins, such as the cytokine interferon-type I, to initiate antiviral responses immediately as well as the adaptive immune response for long-term protection. To establish infection in the host, many viruses have thus evolved strategies for subversion of these mechanisms of innate immunity. For example, acute infection by HIV-1 and other retroviruses have long been thought to be non-immunogenic, signifying suppression of host defenses by these pathogens. Studies in the past few years have begun to uncover a multifaceted scheme of how HIV-1 evades innate immune detection, especially of its DNA, by exploiting host proteins. This review will discuss the host mechanisms of HIV-1 DNA sensing and viral immune evasion, with a particular focus on TREX1, three prime repair exonuclease 1, a host 3′ exonuclease (also known as DNase III).

Keywords: HIV, innate immunity, DNA sensing, Trex1, autoimmune diseases

THE INTERFERON RESPONSE TO HIV DNA

Human immunodeficiency virus (HIV) enters T cells and macrophages by first interacting with host receptor CD4 then with co-receptor chemokine (C-C motif) receptor 5 (CCR5) or chemokine (C-X-C motif) receptor 4 (CXCR4) on the target cell plasma membrane, triggering viral envelope fusion. HIV can also bind to cell surface lectins (sugar-binding proteins) and enter cells by endocytosis. This is the predominant mode of entry into dendritic cells (DCs), which play important roles in progression and pathology of HIV infection (Luban, 2012; Manches et al., 2014), although HIV-1 does not replicate efficiently in DCs. Regardless of the mode of viral entry, the viral core containing its RNA is released into the cytosol, and HIV-encoded reverse transcriptase begins to convert viral RNA into DNA while still encapsulated in the capsid core. After completion of reverse transcription, viral integrase binds to both ends of full-length HIV-1 DNA to form pre-integration complex, which delivers functional HIV DNA to the host nucleus for chromosomal integration (Goff, 2007). Since only a few copies of HIV DNA integrate, the bulk of HIV DNA remains in the cytosol unless cleared by host enzymes (Yan et al., 2010).

Although abundant, the HIV-encoded cytosolic DNA produced by reverse transcription does not trigger a cell-autonomous interferon (IFN) or inflammatory response in activated CD4 T cells and macrophages, its primary targets (Goldfeld et al., 1991; Yan et al., 2010). HIV achieves immune evasion in these target cells by exploiting the host DNase TREX1 (Yan et al., 2010), the most abundant exonuclease in mammalian cell (Hoss et al., 1999; Mazur and Perrino, 1999), to clear its DNA. This action of TREX1 diminishes HIV DNA products in the cytosol below the threshold of immune activation. This is strikingly demonstrated in *Trex1*−/− or knockdown cells. In the absence of TREX1, HIV infection triggers a robust type I IFN response strictly dependent upon the cytosolic DNA sensing pathway, including the endoplasmic reticulum (ER) localized adaptor stimulator of interferon genes (STING), TANK-binding kinase 1 (TBK1), and the transcription factor interferon regulatory factor 3 (IRF3) (Yan et al., 2010). The HIV-stimulated IFN response in *Trex1*−/− cells can be dampened by reverse transcriptase inhibitor (e.g., nevirapine) but not by integrase inhibitor (e.g., raltegravir), consistent with HIV DNA being the main pathogen associated molecular pattern (PAMP) detected by an innate immune sensor (Yan et al., 2010). The HIV DNA is sensed by binding to cGAMP synthase (cGAS), which then synthesizes the unique second messenger dinucleotide cyclic GMP-AMP (cGAMP) that binds to STING to activate downstream IFN signaling (Gao et al., 2013; **Figure 1A**). This "competition" between TREX1 (proviral) and cGAS (antiviral) for cytosolic DNA also applies to murine leukemia virus (MLV) and simian immunodeficiency virus (SIV; Gao et al., 2013), and likely many other retroviruses.

Thus, the absence of an immunogenic response to acute retroviral infection of target immune cells can be at least partially explained by TREX1, which is ubiquitously expressed and at high levels in immune cells (Pereira-Lopes et al., 2013), and may synergize with other host factors (see below). In one population study comparing cohorts of healthy control and HIV-positive individuals, *TREX1* polymorphisms associated with susceptibility to HIV infection (Pontillo et al., 2013), although another study using different cohorts of patients failed to detect such an association (Sironi et al., 2012), suggesting that further investigation is needed before genetic linkage is established. From an evolutionary standpoint, it is intriguing that TREX1 is only found in mammals that

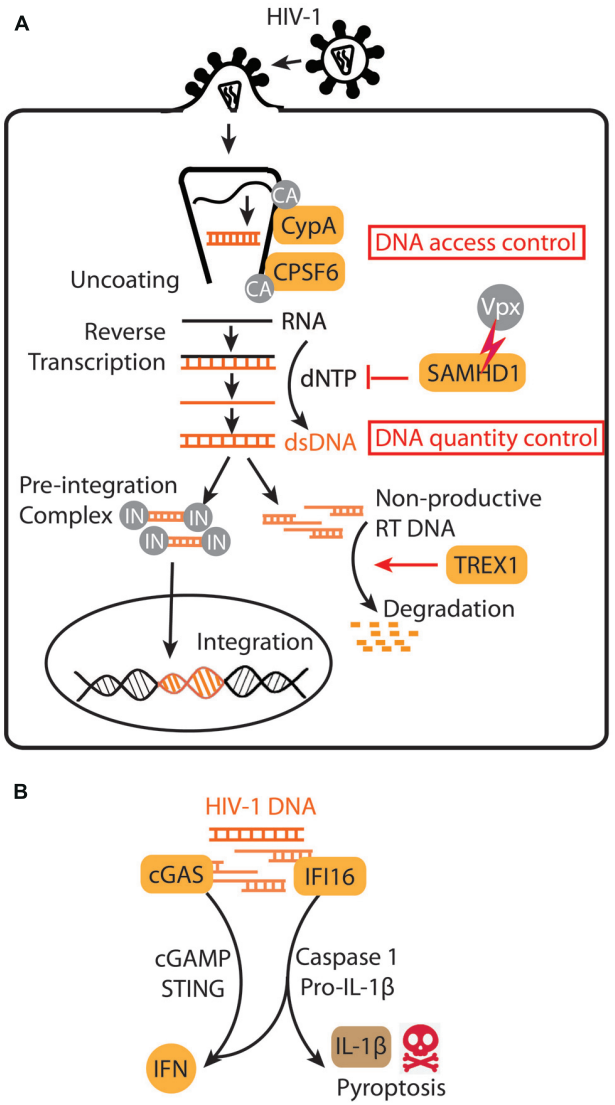

FIGURE 1 | HIV-1 subversion of innate immune DNA sensing. (A) A schematic diagram of early stages of HIV-1 life cycle. HIV-1 capsid (CA) and associated host factors, CypA and CPSF6, regulate HIV DNA access to innate immune sensing. TREX1 and SAMHD1 regulate HIV DNA quantity. IN, integrase. **(B)** HIV-1 DNA sensing by cGAS in many target cells activates a STING-mediated IFN response, whereas HIV-1 DNA sensing by IFI16 in "bystander" quiescent CD4+ T cells trigger IFN signaling, caspase-1 activation, IL-1β secretion and pyroptosis.

have co-evolved with retrovirus, suggesting that retroviruses have adapted to exploit TREX1 for survival. Such an adaptation may be particularly essential for HIV, which does not appear to encode its own factors to antagonize intracellular innate immune sensing systems like many other DNA and RNA viruses do. Indeed, these findings have engendered a new paradigm for HIV-host interactions – that HIV not only exploits many host factors for the successful completion of the life cycle (Brass et al., 2008; König et al., 2008), it also exploits several key host factors that are critical for subversion of innate immune responses in target cells (Doitsh et al., 2010, 2014; Manel et al., 2010; Yan et al., 2010).

PROINFLAMMATORY RESPONSE TO HIV DNA

HIV DNA can also trigger a proinflammatory response in non-productively infected "bystander" CD4+ T cells and promote T cell killing (Doitsh et al., 2010). HIV replication is restricted in these "bystander" CD4+ T cells due to the action of SAM domain and HD domain containing protein 1 (SAMHD1) that depletes the dNTP pool, as well as other unknown restrictive factors (Baldauf et al., 2012). As a result, HIV replication in these cells arrests early in the reverse transcription stage, although the limited amount of DNA produced can be recognized by another cytosolic DNA sensor interferon-inducible protein 16 (IFI16) (Monroe et al., 2014; **Figure 1B**). IFI16 was initially identified as a sensor that recognizes viral DNA or exogenous double-stranded DNA (introduced by transfection) and signals via STING to activate the IFN response (Unterholzner et al., 2010). A recent study found that 'bystander" CD4+ T cells harboring abortive HIV DNA products trigger IFI16-mediated IFN signaling and inflammasome response, including activation of caspase-1, secretion of IL-1β, and death of the host cell by pyroptosis (Doitsh et al., 2014). This series of discoveries reveals another exciting example of how HIV takes advantage of DNA sensing as well as SAMHD1 restriction. In this case, instead of avoiding DNA sensing, HIV stalls DNA replication early in the reverse transcription stage to trigger inflammation and cell death in "bystander" CD4+ T cells. Since CD4+ T cell depletion is a highly diagnostic clinical feature of AIDS, these studies raise an exciting possibility of reversing CD4+ T cell depletion by blocking the inflammasome response with caspase-1 inhibitors (Doitsh et al., 2014). Both cGAS and IFI16 sense HIV DNA, yet they seem to function in distinct cell types and lead to different consequences. Also, it was unclear why TREX1 is not able to inhibit IFI16-mediated detection of HIV DNA. Further investigation is needed to show what determines which innate immune signaling pathway HIV DNA triggers or avoids, and how that influences the overall fitness of the virus.

QUANTITY CONTROL OF HIV DNA: TREX1 AND SAMHD1

As discussed above, the amount of HIV-1 DNA in the cytosol depends upon the rates of synthesis and degradation by two host factors (**Figure 1A**): TREX1 mediates HIV DNA degradation in several immune cell types, and SAMHD1 limits HIV-1 DNA synthesis by depleting the dNTP pool in resting CD4+ T cells as well as several other cell types of myeloid linage (Hrecka et al., 2011; Laguette et al., 2011; Baldauf et al., 2012). In DCs, SAMHD1 also prevents innate immune activation (Manel et al., 2010; Sunseri et al., 2011). SAMHD1 restriction can be overcome by treating cells with virus-like particles (VLPs) containing the SAMHD1-antagnist protein Vpx, found in SIVmac and HIV-2 viruses (Goujon et al., 2006). HIV-1, which does not encode Vpx, Vpx-deficient SIVmac, and HIV-2 all fail to replicate efficiently in DCs (Hrecka et al., 2011; Laguette et al., 2011). Detailed reviews on SAMHD1/Vpx can be found elsewhere (Daugherty and Malik, 2012; Luban, 2012; Sze et al., 2013). Here, we highlight features of SAMHD1 as in comparison with TREX1.

TREX1 is expressed in most mammalian cell types (Pereira-Lopes et al., 2013), whereas SAMHD1 expression is more restricted (Laguette et al., 2011). Since a defect in either enzyme triggers

HIV-1 DNA sensing, they are thought to play non-redundant roles. In cell culture, TREX1 deficiency leads to accumulation of total HIV-1 DNA, but not integrated proviral DNA (Yan et al., 2010). It therefore appears that TREX1 does not degrade full-length integration-competent HIV-1 DNA, perhaps because it is protected by integrase in the pre-integration complex. In contrast, TREX1 appears able to degrade non-productive partial-length DNA generated by error-prone reverse transcription that are not incorporated into integrase complexes. This is consistent with enzymatic properties of TREX1, a 3′ to 5′ exonuclease. Although it has some activity toward any form of DNA, it is most efficient with single-stranded DNA or double-stranded DNA with a single-stranded overhang (Mazur and Perrino, 1999). Consequently, overall HIV-1 replication in which TREX1 expression is suppressed or ablated is reduced compared replication in normal cells, because DNA sensing results in expression of antiviral IFNs (Yan et al., 2010). In contrast, SAMHD1 depletion results in increased HIV DNA synthesis, integration and overall replication (Hrecka et al., 2011; Laguette et al., 2011). It appears confusing at first glance that although both TREX1 and SAMHD1 control the quantity of cytosolic HIV DNA, the overall outcome of HIV-1 replication is quite the opposite. Also, both proteins attenuate autoimmunity, and patients carrying defective TREX1 or SAMHD1 develop similar autoimmune and sterile inflammatory phenotypes (Crow et al., 2006; Crow and Rehwinkel, 2009; Rice et al., 2009). Therefore, important questions remain. Is the antiviral role of SAMHD1 in HIV-1 pathogenesis in the complex human immune setting different from current understanding of its role as a restrictive factor? Has HIV-1 evolved to exploit SAMHD1 restriction to prevent replication in certain cell types, such as DCs, in order to avoid cell-autonomous innate immune activation, or to abort the reverse transcription stage in "bystander" CD4+ T cells to activate inflammasomes and trigger pyroptosis?

Comparison of HIV-1 with HIV-2 offers some insights into these questions. HIV-2 (containing Vpx) is far less pathogenic compared to HIV-1 (lacking Vpx) in humans, so one possible contributing factor to HIV-1 pathogenesis could be its inability to antagonize SAMHD1 or ability to avoid SAMHD1 expressing cells. One should also consider other important differences between HIV-1 and HIV-2, such as Vpu, which is only encoded by HIV-1, that antagonizes restriction factor Tetherin (Neil et al., 2008). Taken together, the opposing influences of TREX1 and SAMHD1 upon overall HIV-1 fitness suggests that it will be important to further evaluate these immunomodulators in combination as well as in isolation in immune systems.

ACCESS CONTROL OF HIV DNA: CAPSID AND ASSOCIATED HOST FACTORS

Aside from quantity, another factor in HIV-1 DNA sensing is encapsulation of the DNA by the viral capsid, which encapsulates the incoming genomic RNA and, later on, the reverse transcribed DNA. It remains unclear whether or not the HIV-1 capsid core disassembles during or after reverse transcription, and where in the cell it occurs. In any case, capsid core uncoating and reverse transcription are closely related. Several capsid assembly mutants affect reverse transcription (Tang et al., 2001; Ambrose et al., 2012). Conversely, inhibition of reverse transcription increases stability of the HIV-1 capsid core (Yang et al., 2013). Therefore, stability of the capsid core may play a role in HIV-1 DNA access or sensing. In the case of MLV, the glycosylated Gag protein (glyco-Gag) from the incoming virus enhances viral core stability and reduces DNA sensing in TREX1 knock-down cells, so a similar relationship may apply for HIV-1 (Stavrou et al., 2013). Moreover, host cofactor proteins that bind the capsid also modulate sensing of cytosolic HIV DNA. In one study (Lahaye et al., 2013), mutations of HIV-1 and HIV-2 capsid proteins that enhance cyclophilin A (CypA) binding triggered more robust DNA sensing in monocyte-derived dendritic cells (MDDCs). In another study (Rasaiyaah et al., 2013), mutations N74D and P90A in the HIV-1 capsid protein impaired interaction with host factors cofactors cleavage and polyadenylation specificity factor sub-unit 6 (CPSF6) and CypA, and also promoted more DNA sensing in monocyte-derived macrophages (MDMs). Although different cell types were examined, both studies revealed an unexpected new dimension in sensing of HIV DNA by the host – access to the viral DNA dependent upon the viral capsid and factors encoded by the host. It remains to be determined whether the incoming capsid core simply "shields" HIV DNA from cytosolic sensing machinery by physical or steric means, or whether a more complex mechanism might be in play that regulates the dynamic connections of uncoating, reverse transcription, and nuclear import.

SELF-DNA SENSING AND AUTOIMMUNE DISEASE

Remarkable similarity in clinical conditions between infectious and autoimmune diseases has been recognized for many years. And yet, molecular mechanisms are much better defined in infectious diseases, whereas cell-intrinsic causes of autoimmune disease remain largely mysterious. TREX1 is an excellent example of a protein that may bridge this gap in our knowledge by playing important roles in both infectious and autoimmune diseases. In addition to its role in innate immunity against HIV discussed above, TREX1 is also a critical suppressor of self-recognition that safeguards the host from erroneous autoimmune activation. Mutations in *TREX1* in humans are associated with the autoimmune and autoinflammatory disorders (Kavanagh et al., 2008; Crow and Rehwinkel, 2009) Aicardi-Goutières syndrome (AGS), familial chilblain lupus (FCL), systemic lupus erythematosus (SLE), and retinal vasculopathy with cerebral leukodystrophy (RVCL). In fact, *TREX1* represents one of the highest monogenic linkages of SLE (Moser et al., 2009). *Trex1*–/– mice develop multiple-organ inflammation, as well as autoantibodies, and succumb to inflammation burden early in age (Gall et al., 2012). Self-DNA from endogenous retroelements (Stetson et al., 2008) or DNA replication debris (Yang et al., 2007) are among the likely causes of inflammation in *Trex1*–/– mice. Inflammatory phenotypes can be rescued by eliminating essential components of the known DNA sensing pathway (e.g., *Irf3*–/–, *Sting*–/–), functionally linking the DNase function of TREX1 to disease (Stetson et al., 2008; Gall et al., 2012). Moreover, chemically modified self-DNA that resists degradation by TREX1 can cause immune activation even in the presence of TREX1. One example is oxidized DNA that contains 8-hydroxyguanosin (8-OHG), which can be formed in UV-exposed skin lesions or neutrophil extracellular traps (networks of extracellular fibers composed of mostly DNA from

neutrophils). The oxidized DNA elevates cGAS/STING-mediated immune activation compared to unmodified DNA in a variety of cell types (Gehrke et al., 2013).

TREX1: BEYOND DNase FUNCTION

TREX1 mutations that disrupt its DNase activity were mostly found in AGS, and many disease-associated mutations of TREX1 do not affect its DNase activity, especially the ones associated with SLE and RVCL (Lee-Kirsch et al., 2007; Richards et al., 2007). TREX1 is a single exon gene that encodes an exonuclease domain at its amino terminus, and an ER localization domain at its carboxyl terminus. The ER localization domain consists of a hydrophilic linker region of unknown function [although this region harbors many SLE mutations (Crow and Rehwinkel, 2009)] and a small hydrophobic segment at the extreme carboxyl-terminus that sorts TREX1 to the cytosolic leaflet of the ER membrane by serving as an ER tail-anchor (rather than a classical transmembrane span; Lee-Kirsch et al., 2007; Lindahl et al., 2009). In overexpression studies, the C-terminal domain of TREX1 mediated interaction with ubiquilin-1, leading to monoubiquitination at multiple lysine residues of TREX1 (Orebaugh et al., 2013). These modifications did not lead to TREX1 degradation, rather, they are possibly able to regulate TREX1 function or localization (Hicke, 2001). Notably, several disease-causing mutations of TREX1 exhibit altered ubiquitination patterns when co-expressed with ubiquitin (Orebaugh et al., 2013), but do not affect DNase activity. Clearly, the biological significance of monoubiquitination on native or disease-causing forms of TREX1 for viral DNA sensing and autoimmunity requires further investigation.

Abnormal accumulation of self-DNA likely promotes TREX1 AGS, since these alleles impair DNase activity, but the molecular cause for TREX1 SLE that is strongly associated with mutations in the C-terminal localization domain rather than the DNase domain remains unclear. We recently found that TREX1-deficient cells have expanded lysosomal compartments, and that TREX1 deficiency promotes lysosomal biogenesis through mTORC1 and transcription factor TFEB (Hasan et al., 2013). It remains to be seen whether this new function of TREX1 is dependent upon its DNase activity, and how it relates to various autoimmune diseases associated with *TREX1*. Interestingly, although arising from the same genetic locus, clinical conditions of TREX1 AGS and TREX1 SLE are highly distinct. TREX1 AGS mutations are autosomal recessive. Patients develop severe neurological brain diseases with excess IFNα in cerebral spinal fluid resembling intrauterine infections, affecting mostly infant and young children, many of whom die early in age (disease onset usually by 4 months; Kavanagh et al., 2008). In contrast, TREX1 SLE mutations are mostly autosomal dominant, with two exceptions where compound heterozygous missense mutations were found (Lee-Kirsch et al., 2007). Unlike TREX1 AGS, which is a pediatric disorder, TREX1 SLE disease onset is usually between 15–40 years of age as is typical for lupus-type autoimmune disorders. Similarly, TREX1 SLE patients have high titers of a wide array of autoantibodies and IFN signatures (Kavanagh et al., 2008). Despite some phenotypic overlap between SLE and AGS, distinct disease mechanisms are therefore likely.

CONCLUSIONS AND PERSPECTIVES

Extensive effort in the past two decades provided us with incredible details on HIV-1 virology, life cycle, interactions with host factors, and antagonism of host intrinsic restriction factors with accessory proteins (Goff, 2007). In more recent years, with the rapidly expanded understanding of innate immunity, studies have begun to uncover mechanistic details of how HIV affects host innate immunity (Luban, 2012). Like many other viruses, evasion of host innate immunity is one of the most essential requirements for overall fitness of HIV-1. However, what is remarkable about HIV-1 compared to other DNA or RNA viruses is how it achieves immune evasion by exploiting host proteins. Most DNA or RNA viruses encode viral proteins that target specific intracellular innate immune sensing pathways involved in recognizing DNA or RNA, or immune activation. HIV-1 does it by exploiting host negative regulators of innate immunity to subvert sensing of its own DNA. The focus of this review, DNase III/TREX1, normally safeguards the host from autoimmune activation by self-DNA, but is exploited by HIV-1 to evade sensing of its own DNA. In a seemingly counterintuitive move, HIV-1 does not encode Vpx that antagonizes a potent restriction factor SAMHD1. Interestingly, *vpx* deletion during the evolution of SIVcpz (that eventually gave rise to HIV-1) resulted in the creation of a unique *vif* that can antagonize hominid restriction factor APOBEC3 (Etienne et al., 2013). Whether this curious omission of Vpx contributes to overall HIV-1 fitness, and how much of that advantage is contributed from SAMHD1, remain to be seen. This could be an unprecedented way for a virus to subvert immune activation – by keeping its replication low in professional IFN producing cells such as DCs. Along the same line, abortive HIV DNA products in bystander quiescent CD4+ T cells (due to the SAMHD1 block) activate IFI16-mediated inflammasome response and pyroptosis – an effective way to paralyze the host immune system.

These discoveries of unique HIV immune evasion strategies may mark the beginning of an exciting new era on studies of HIV innate immunity, with important new avenues to be explored. Further studies in this emerging field will certainly open our eyes on how HIV navigates the human innate immune system. This knowledge could also be harnessed for novel strategies of HIV vaccine design that specifically target HIV immune evasion. We look forward to seeing these studies unfold.

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Suppression of APOBEC3-mediated restriction of HIV-1 by Vif

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The APOBEC3 restriction factors are a family of deoxycytidine deaminases that are able to suppress replication of viruses with a single-stranded DNA intermediate by inducing mutagenesis and functional inactivation of the virus. Of the seven human APOBEC3 enzymes, only APOBEC3-D, -F, -G, and -H appear relevant to restriction of HIV-1 in CD4+ T cells and will be the focus of this review. The restriction of HIV-1 occurs most potently in the absence of HIV-1 Vif that induces polyubiquitination and degradation of APOBEC3 enzymes through the proteasome pathway. To restrict HIV-1, APOBEC3 enzymes must be encapsidated into budding virions. Upon infection of the target cell during reverse transcription of the HIV-1 RNA into (−)DNA, APOBEC3 enzymes deaminate cytosines to form uracils in single-stranded (−)DNA regions. Upon replication of the (−)DNA to (+)DNA, the HIV-1 reverse transcriptase incorporates adenines opposite to the uracils thereby inducing C/G to T/A mutations that can functionally inactivate HIV-1. APOBEC3G is the most studied APOBEC3 enzyme and it is known that Vif attempts to thwart APOBEC3 function not only by inducing its proteasomal degradation but also by several degradation-independent mechanisms, such as inhibiting APOBEC3G virion encapsidation, mRNA translation, and for those APOBEC3G molecules that still become virion encapsidated, Vif can inhibit APOBEC3G mutagenic activity. Although most Vif variants can induce efficient degradation of APOBEC3-D, -F, and -G, there appears to be differential sensitivity to Vif-mediated degradation for APOBEC3H. This review examines APOBEC3-mediated HIV restriction mechanisms, how Vif acts as a substrate receptor for a Cullin5 ubiquitin ligase complex to induce degradation of APOBEC3s, and the determinants and functional consequences of the APOBEC3 and Vif interaction from a biological and biochemical perspective.

Keywords: HIV, mutagenesis, deaminase, Vif, APOBEC3, restriction factor, ubiquitination

OVERVIEW

Retrotransposons and endogenous retroviruses have been genomic parasites in organisms throughout evolution and have contributed to both species evolution and disease (Hancks and Kazazian, 2012). The APOBEC (*Apo*lipoprotein *B* mRNA-editing *e*nzyme-*c*atalytic polypeptide) family of enzymes present in their earliest form in bony fish acted as a defense to retroelements (MacDuff et al., 2009). Due to expansion of retroelements through evolution, there was a corresponding expansion in the APOBEC family (Jern and Coffin, 2008; LaRue et al., 2008). The most recent expansion in placental mammals formed the APOBEC-like *3* (APOBEC3) family in response to ancient pathogenic retroviruses (LaRue et al., 2008; Munk et al., 2012). Humans contain seven APOBEC3 (A3) enzymes (A3A, A3B, A3C, A3D, A3F, A3G, and A3H, Jarmuz et al., 2002; LaRue et al., 2009). The A3 enzymes act as host restriction factors to inhibit retroelement replication through either RNA binding ability or activity as single-stranded (ss) DNA cytosine deaminases that catalyze the formation of promutagenic uracils (Esnault et al., 2005; Bogerd et al., 2006; Chiu et al., 2006; Jonsson et al., 2006; Armitage et al., 2008; OhAinle et al., 2008; Khatua et al., 2010; Duggal et al., 2011; Koyama et al., 2013). Currently, A3 enzymes are primarily studied for their ability to restrict the replication of retroviruses (such as HIV-1, Sheehy et al., 2002; Harris et al., 2003; Mangeat et al., 2003; Zhang et al., 2003; Liddament et al., 2004; Wiegand et al., 2004; Zheng et al., 2004; Dang et al., 2006, 2008; OhAinle et al., 2008; Richardson et al., 2014) and other viruses with an ssDNA intermediate (such as Hepatitis B Virus, Kock and Blum, 2008; Lucifora et al., 2014). Restriction of the replication of these present day viruses occurs primarily through the deoxycytidine deamination activity of A3 enzymes which results in hypermutated and inactivated viral genomes. The gene duplications that resulted in the human A3 repertoire formed two general groups of deaminases with different Zinc (Z) coordinating domains: A3A, A3C, and A3H are enzymes with a single Z-domain and A3B, A3G, A3D, and A3F enzymes with two Z-domains (LaRue et al., 2008, **Figure 1**). For A3 enzymes with two Z-domains, only one domain is catalytically active, except for A3B, which may have two catalytically active domains (Hache et al., 2005; Navarro et al., 2005; Bogerd et al., 2007; Bonvin and Greeve, 2007, **Figure 1**).

For HIV-1 (referred to as HIV) to successfully infect humans, it must overcome numerous physical and immunological barriers (Harris et al., 2012; Shaw and Hunter, 2012; Xu et al., 2013). Within

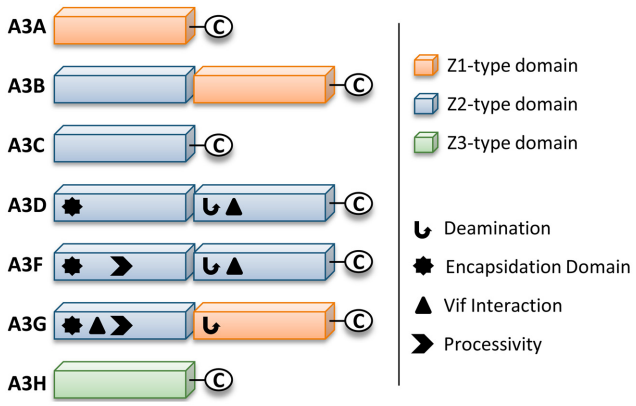

FIGURE 1 | Zinc (Z) coordinating-type domains of human A3 enzymes. A3 enzymes coordinate zinc through the motif H-X-E-X_{23-28}-P-C-X_{2-4}-C. The glutamate activates a water molecule to enable zinc-hydroxide-mediated nucleophilic attack to complete the deamination reaction. Deamination activity has been demonstrated for all A3 enzymes. For the enzymes with two Z-type domains that restrict HIV in CD4+ T cells (A3D, A3F, and A3G), a legend depicts known biochemical functions of each Z-type domain. A common feature of A3 enzymes with two Z-type domains is the segregation of functions in the N-terminal domain (NTD) and C-terminal domain (CTD). The NTD is responsible for encapsidation and the CTD is responsible for deamination activity. Both domains can bind nucleic acids. The binding site of Vif is in the NTD for A3G and in the CTD for A3D and A3F. The determinants for enzyme processivity have only been studied for A3G and A3F. A3G and A3F processivity is imparted by the NTD.

cells, HIV must overcome a network of restriction factors that are able to block specific replication steps of the virus, including A3 enzymes (Harris et al., 2012; Rahm and Telenti, 2012). HIV can overcome these restriction factors through mutations or encoding accessory proteins that specifically block the restriction factor function (Harris et al., 2012; Rahm and Telenti, 2012). HIV uses the viral infectivity factor (Vif) to overcome A3 enzymes (Sheehy et al., 2002; Conticello et al., 2003; Harris et al., 2003; Mangeat et al., 2003; Mariani et al., 2003). The Vif protein of simian immunodeficiency virus (SIV), the non-human primate form of the virus, has co-evolved with species-specific A3s for millions of years (Compton and Emerman, 2013). The HIV-1 predecessor, SIV_{cpz} from chimpanzees underwent a key evolutionary event that altered the 3′ region of the *vif* gene that was essential for antagonism of human A3 function, along with further evolutionary changes in chimpanzees that adapted SIV_{cpz} for improved infection of humans (Etienne et al., 2013). To antagonize A3 enzymes, HIV Vif must maintain the ability to physically interact with relevant A3s, host protein CBFβ for stability (Jager et al., 2012; Zhang et al., 2012), and components of the host ubiquitin ligase assembly (Yu et al., 2003, 2004; Kobayashi et al., 2005; Xiao et al., 2006). Ultimately Vif thwarts A3s by inducing their polyubiquitination and degradation through the proteasome (**Figures 2A,B**). It is thought that by disrupting the Vif–host cell interactions through novel pharmaceuticals, A3 enzymes can be used to suppress HIV. However, the natural balance of A3 enzymes and HIV must be first understood since there is evidence that HIV can take advantage of A3 enzymes to accelerate its quasispecies evolution (Simon et al., 2005; Kim et al., 2010).

From cell culture studies, it appears that only A3A, A3D, A3F, A3G, and A3H are relevant to HIV restriction (Berger et al., 2011; Hultquist et al., 2011; Koning et al., 2011; Refsland et al., 2012; Chaipan et al., 2013). It is not surprising that not all seven A3 members restrict HIV replication since they likely evolved to restrict different retroelement pathogens (LaRue et al., 2008). There are two paradigms of how A3 enzymes can suppress HIV. A3A present in HIV target myeloid cells can restrict replication of incoming virions through low levels of deamination and possibility another mechanism that is not yet fully elucidated (Berger et al., 2011; Koning et al., 2011). In CD4+ T cells, A3D, A3F, A3G, and A3H restrict HIVΔ*vif* by becoming virion encapsidated in the HIV producer cell and traveling with the virion to the next susceptible cell where they catalyze promutagenic deaminations of cytosine to uracil in nascent single-stranded HIV (−)DNA (Hultquist et al., 2011, **Figure 2A**). Although A3B can also restrict HIV in this manner in 293T or HeLa cells, it is unable to become virion packaged and restrict HIV in T cell lines, has low expression in activated T cells, and is not antagonized by Vif, suggesting that restriction by A3B is not physiologically relevant (Doehle et al., 2005; Koning et al., 2009; Refsland et al., 2010; Hultquist et al., 2011; Pak et al., 2011). This review will focus on the restriction of HIV by virion encapsidated A3D, A3F, A3G, and A3H and how Vif antagonizes their function.

A3-MEDIATED RESTRICTION OF HIV

The A3D, A3F, A3G, and A3H molecules that escape Vif-mediated inhibition can restrict HIV by entering the assembling virus particle by binding RNA (HIV genome or cellular RNA such as 7SL or Y) that also interacts with the nucleocapsid (NC) portion of the Gag polyprotein (Alce and Popik, 2004; Cen et al., 2004; Douaisi et al., 2004; Svarovskaia et al., 2004; Burnett and Spearman, 2007; Khan et al., 2007; Bach et al., 2008; Bogerd and Cullen, 2008; Strebel and Khan, 2008; Wang et al., 2008; Ooms et al., 2010; Zhen et al., 2012, **Figure 2A**). After the virus enters the next target cell A3 enzymes exert their anti-viral function during the reverse transcription process (Suspene et al., 2004; Yu et al., 2004; Zheng et al., 2004; Dang et al., 2006; OhAinle et al., 2008, **Figures 2A,C**). Although A3D, A3F, A3G, and A3H are localized to the cytoplasm they require encapsidation to restrict HIV and are not able to access the (−)DNA of an incoming virus (Hultquist et al., 2011, **Figure 2A**). This may be due to the HIV capsid structure or that A3 enzymes can reside in regions of RNA processing, e.g., stress granules or P-bodies, where they may have a role in sequestering human retrotransposon RNA to prevent transposition (Chiu et al., 2006; Kozak et al., 2006; Stopak et al., 2007; Gallois-Montbrun et al., 2008). Since A3 enzymes are ssDNA deaminases, deamination activity is restricted to the (−)DNA strand (Yu et al., 2004, **Figure 2C**). The cytosine (C)⟶uracil (U) deaminations catalyzed on the (−)DNA strand become guanine (G)⟶adenine (A) mutations when reverse transcriptase (RT) uses U as a template during (+)DNA strand synthesis (Yu et al., 2004, **Figure 2C**). The resulting "hypermutation" of the provirus leads to inactivation of HIV (Harris et al., 2003; Mangeat et al., 2003; Zhang et al., 2003, **Figure 2A**). Although it is known that many proviral genomes

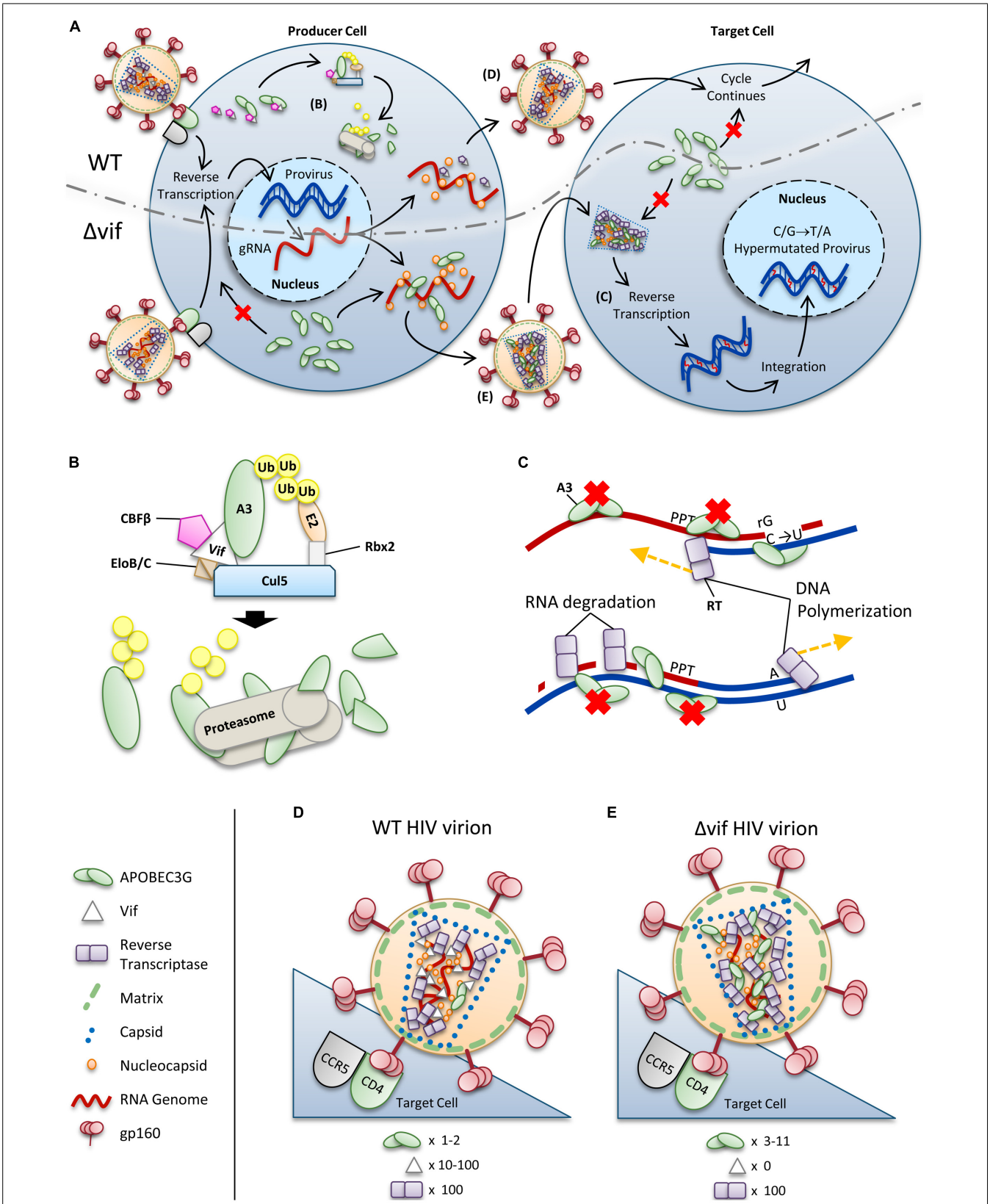

FIGURE 2 | Continued

FIGURE 2 | Continued
Overview of HIV restriction by A3 enzymes. (A) Sketch depicting lifecycles of wild-type (WT) and ΔVif HIV (ΔVif). Each virion enters a cell that expresses A3 enzymes. In the WT virus, Vif is expressed in the cell and recruits host cell CBFβ for stability and CRL5 E3 ubiquitin ligase complex composed of Elongin B/C (EloB/C), Cullin5 (Cul5) and Rbx2 (**B**). In this complex, Vif acts as the substrate receptor to induce degradation of A3 enzymes. As a result, assembling virions do not encapsidate high levels of A3 enzymes and upon infection of a target cell the HIV lifecycle continues. The ΔVif HIV encapsidates A3 enzymes through an RNA and Gag interaction. In the target cell the A3 enzymes within the capsid of HIV can deaminate cytosines to uracils in nascent single-stranded (−)DNA during reverse transcription (**C**). These uracils induce G→A transition mutations upon synthesis of (+)DNA (**C**). The resulting hypermutated virus can be integrated into the host genome but is functionally inactivated. A3 enzymes in the target cell cannot enter the HIV capsid and are unable to restrict virus replication unless encapsidated into budding virions. **(B)** Detailed sketch of Vif-mediated polyubiquitination of A3G. Vif interacts with Elongin C (EloC), which forms an obligate heterodimer with Elongin B (EloB), and Cul5. The transcription cofactor CBFβ stabilizes Vif. Cul5 binds to Rbx2and subsequently recruits an E2 ubiquitin conjugating enzyme. Vif is the substrate receptor that recruits A3 enzymes. The ^{48}K-linked ubiquitin chains result in proteasomal degradation of the A3. **(C)** Sketch demonstrating the limited vulnerability of single-stranded (−)DNA to A3-mediated deamination that is imposed from the dynamics of reverse transcription. Reverse transcriptase is abbreviated as RT. HIV contains two polypurine tracts (PPT) that are used as primers for (+)DNA synthesis. In the figure, only one PPT is depicted. **(D,E)** Sketches depicting the stoichiometry of major virion components for a **(D)** WT and **(E)** ΔVif HIV virion. Figures correspond to **(D)** and **(E)** in **(A)**. **(D)** Low amounts of A3 may escape Vif-mediated degradation and become virion encapsidated (approximately one to two molecules of A3G/virion). **(E)** A ΔVif HIV cannot induce degradation of A3 enzymes and that results in the encapsidation of A3 enzymes through an interaction with RNA and Gag. Approximately 3–11 molecules of A3G can become virion encapsidated. **(D,E)** Stoichiometry values for virions were obtained from Camaur and Trono (1996), Fouchier et al. (1996), Coffin et al. (1997), Xu et al. (2007), Nowarski et al. (2008).

undergo successful integration with these hypermutations (Russell et al., 2009a), some preintegration complexes containing U may be degraded by host DNA repair mechanisms, although there is no consensus regarding the extent to which this occurs in cells (Kaiser and Emerman, 2006; Yang et al., 2007). In cell culture, it has been found that the mutated HIV proteins that may be produced from these proviral genomes can act as a source of HIV antigens due to their misfolding and processing through the proteasome, which can facilitate immune recognition of HIV (Casartelli et al., 2010).

Each ssDNA deaminase acts within a preferred di- or trinucleotide substrate motif. For A3G, this is 5′CCC or 5′CC (Yu et al., 2004, 3′-end C preferred for deamination). A3D, A3F, and A3H deaminate 5′TTC or 5′TC motifs and A3D can also deaminate 5′GC motifs in proviral DNA (Liddament et al., 2004; Dang et al., 2006; Ooms et al., 2013a). Although the majority of A3 actions are repressed by Vif in HIV-infected individuals (**Figures 2A,B,D**), clinical studies have found that individuals with an inherent ability to express a high level of A3G mRNA are less likely to become infected with HIV or progress from HIV to AIDS and that the presence of hypermutated proviral genomes correlates with high CD4+ T-cell counts (Jin et al., 2005; Pace et al., 2006; Biasin et al., 2007; Land et al., 2008; Vazquez-Perez et al., 2009). Other A3 enzymes have not been extensively examined in this regard (Albin and Harris, 2010; Ooms et al., 2013a). However, there is evidence of deaminations in HIV genomes recovered from infected individuals due to C/G⟶T/A mutations in a sequence context that indicates deaminations by A3 enzymes other than A3G do occur (Pace et al., 2006; Land et al., 2008; Vazquez-Perez et al., 2009; Ooms et al., 2013a). For example, a study found that in HIV-infected individuals there is approximately an equal split between mutations occurring in the 5′TC and 5′CC contexts on (−)DNA (Ooms et al., 2013a). However, there is difficulty parsing out the effect of A3F, A3D, and A3H based on their mutation patterns since they all recognize the minimal dinucleotide 5′TC and are more promiscuous than A3G in regard to trinucleotide target site preference (Liddament et al., 2004; Dang et al., 2006; Hultquist et al., 2011). Nonetheless, it has become clear that despite some evidence that A3G has more mutagenic potential than other A3 deaminases, it is not acting alone against HIV (Refsland et al., 2012; Chaipan et al., 2013; Ooms et al., 2013a). The HIV genomes mutated through A3 catalytic activity are also subject to the pressure of purifying selection. This selection pressure results in mutated and inactivated genomes being highest in integrated proviral DNA and lowest in circulating viral RNA (Russell et al., 2009a). Furthermore, integrated proviruses that are inactivated by stop codons in the *gag* gene may still be rescued by dual infection of a cell by HIV quasispecies and complementation of Gag function (Russell et al., 2009a). Recombination within virions by RT template switching can result in "reactivation" of inactivated viral genomes (Mulder et al., 2008; Russell et al., 2009a). As a result, A3-mediated mutagenesis is effective, but the complete inactivation of HIV in an infected individual is potentially a long-term process that is likely to require multiple rounds of exposure to viruses.

A3G-MEDIATED RESTRICTION OF HIV

Deamination-dependent HIV restriction by A3G

Since A3G was the first A3 enzyme discovered A3G has been the most widely studied for how it enacts its role as a restriction factor (over 700 publications in PubMed). There are two key steps that A3G must complete to be an efficient restriction factor. First, the enzyme must be available for binding RNA that will become virion encapsidated through an interaction with the NC portion of Gag (Alce and Popik, 2004; Cen et al., 2004; Douaisi et al., 2004; Svarovskaia et al., 2004; Burnett and Spearman, 2007; Khan et al., 2007; Bach et al., 2008; Bogerd and Cullen, 2008; Strebel and Khan, 2008; Wang et al., 2008). Second, it must have a mechanism to search the nascent HIV (−)DNA, that is available for a finite period of time, for potential cytosines that it can deaminate (Chelico et al., 2006; Nowarski et al., 2008; Ara et al., 2014, **Figure 2C**).

A3G exists in cells as a high molecular mass that is bound to RNA and other proteins in stress granules and P-bodies (Chiu et al., 2006; Kozak et al., 2006; Wichroski et al., 2006; Gallois-Montbrun et al., 2008). However, only newly synthesized A3G that has not associated with host RNAs in these cytoplasmic structures appears to bind the RNA that is also bound by HIV Gag and therefore encapsidated into virions (Soros et al., 2007). A3G requires oligomerization to bind these RNAs effectively in cells and become virion encapsidated (Wang et al., 2007; Bulliard et al., 2009; Huthoff et al., 2009), but *in vitro* oligomerization mutants of A3G can bind many RNAs with less than a threefold difference

from wild-type (WT) A3G (Chelico et al., 2010; Feng and Chelico, 2011). The RNA binding and oligomerization of A3G is primarily mediated by the N-terminal domain (NTD) and the NTD is solely responsible for virion encapsidation of A3G (Hache et al., 2005; Navarro et al., 2005; Huthoff et al., 2009; Chelico et al., 2010, **Figure 1**). The NTD residues ^{124}YYFW^{127} on predicted loop 7 mediate the dimerization of A3G (Huthoff et al., 2009; Chelico et al., 2010, **Figure 3A**). A3G is primarily a dimer in solution and when A3G binds RNA or DNA it oligomerizes into tetramers and higher order structures through C-terminal domain (CTD) residues ^{313}RIYDDQ^{318} on loop 7 (Chelico et al., 2008, 2010; Shlyakhtenko et al., 2011, **Figure 3B**). It is essential for A3G to enter the inner capsid of the virion to restrict HIV. Within the capsid A3G can associate with the ribonucleoprotein complex and access the (−)DNA as it is synthesized. A3 enzymes that cannot encapsidate within the HIV capsid, e.g., A3A and A3C, are unable to restrict HIV replication in CD4+ T cells (Goila-Gaur et al., 2007; Hultquist et al., 2011). However, accessing the ribonucleoprotein complex does not guarantee the ability to restrict HIV. Since the (−)DNA is only available for a finite period of time due to HIV containing two polypurine tracts (PPT) that are used to prime (+)DNA synthesis (Suspene et al., 2006; Hu et al., 2010), A3 enzymes require an efficient mechanism to search for cytosines (**Figure 2C**). Complicating the search is that the (−)DNA contains pieces of annealed RNA due to the endonuclease activity of the RT-associated RNaseH (**Figure 2C**). A3G binds RNA/DNA hybrids less well than ssDNA and encountering these obstacles on the substrate can induce A3G to dissociate from DNA (Iwatani et al., 2006; Chelico et al., 2008).

Unraveling the mechanism by which A3G locates and catalyzes its deamination motif is of pivotal importance for understanding the mechanistic basis of proviral hypermutation. The mechanism with which an A3 enzyme scans non-target DNA in search of its deamination motif is a determinant in its catalytic efficiency (Feng and Chelico, 2011; Ara et al., 2014). DNA scanning is described by the term processivity and is defined as the ability of an enzyme to catalyze multiple events in a single enzyme–DNA substrate encounter. Enzymes that do not use an energy source for movement on DNA use a mechanism termed facilitated diffusion to efficiently search DNA (Berg et al., 1981; von Hippel and Berg, 1989). This is a mechanism where the enzymes, subject to Brownian motion, move randomly on DNA. Since DNA-binding proteins are usually positively charged, the negative charge of the DNA facilitates the enzyme movement through electrostatic interactions (Berg et al., 1981; von Hippel and Berg, 1989). A3G is a positively charged enzyme (charge of +6.5 at pH 7) and processively scans ssDNA by facilitated diffusion (Chelico et al., 2006; Nowarski et al., 2008, **Figure 4**). This mode is distinct from an enzyme that acts on DNA distributively, where only one catalytic event occurs before the enzyme disengages from the substrate (Chelico et al., 2009). Facilitated diffusion can involve a variety of movements such as 1-dimensional (D) sliding (**Figure 4B**) or 3-D movements such as hopping/jumping (**Figure 4C**) or intersegmental transfer (**Figure 4D**, Halford and Marko, 2004). Hopping and jumping describe small microdissociations and reassociations with the same DNA strand without diffusion into the bulk solution (von Hippel and Berg, 1989, **Figure 4C**). Intersegemental transfer involves a two-step mechanism where an enzyme with two DNA-binding sites binds a second site before releasing the first site (von Hippel and Berg, 1989, **Figure 4D**). Facilitated diffusion works best when both 1- and 3-D movements are used to enable local scanning of a small segment of DNA by sliding (<20 nt) and movement to distal regions to restart the local scanning process (Halford and Marko, 2004; Feng and Chelico, 2011). These distal movements do not cause the enzyme to leave the DNA and enter into the bulk solution because the charged surface of the DNA keeps the enzyme within the domain of the DNA (von Hippel and Berg, 1989, **Figure 4C**). Using different methods A3G has been found to scan ssDNA by 1-D sliding motions and 3-D jumping motions (Chelico et al., 2006; Senavirathne et al., 2012; Shlyakhtenko et al., 2012). However, one study has found that A3G moves by 3-D intersegmental transfers (Nowarski et al., 2008). The efficiency imparted by a 3-D movement in the specific case of A3G during reverse transcription is that it provides a means of overcoming the DNA/RNA hybrid barrier (Nowarski et al., 2008; Feng and Chelico, 2011). Clusters of A3G-induced deaminations indicative of processive sliding movements have been found in integrated proviral genomes (Browne et al., 2009) and A3G mutants unable to undergo a local searching process by 1-D sliding, such as H186R and A3G with an ^{195}NPM^{197} insertion have decreased mutagenesis during *in vitro* reverse transcription or in HIV proviral genomes (Feng and Chelico, 2011; Ara et al., 2014). Furthermore, an A3G F126A/W127A mutant that cannot jump has decreased mutagenesis during *in vitro* reverse transcription (Feng and Chelico, 2011). These data demonstrate that neither movement alone can enable high levels of A3G-induced mutagenesis. Interestingly, the F126A/W127A mutant is monomeric, suggesting that the oligomeric state of A3G plays a role in efficient restriction of HIV not only by facilitating virion encapsidation but also by facilitating the DNA scanning process (Huthoff et al., 2009; Chelico et al., 2010; Feng and Chelico, 2011). The processivity determinants of A3G reside on predicted loop 7 and helix 6 of the non-catalytic NTD (Feng and Chelico, 2011; Ara et al., 2014, **Figure 3A**). Thus, despite a lack of catalytic activity, the NTD contributes to A3G deamination activity by mediating the processive scanning mechanism (Feng and Chelico, 2011).

Deamination-independent HIV restriction by A3G

A3G primarily restricts HIV replication through its deamination activity. However, there have been numerous reports of an ability of A3G to physically inhibit other processes of HIV such as RT polymerization (Iwatani et al., 2007; Bishop et al., 2008; Wang et al., 2012; Adolph et al., 2013; Belanger et al., 2013; Gillick et al., 2013), NC strand annealing (Guo et al., 2006, 2007), and proviral DNA integration (Luo et al., 2007; Belanger et al., 2013). These processes do not occur in isolation from deamination, nor do they restrict HIV better than deamination alone (Belanger et al., 2013; Gillick et al., 2013). We will focus on inhibition of RT polymerization since this is the most prevalent mechanism studied.

Early studies of A3G-mediated restriction of HIV proposed that transiently overexpressed WT A3G and deamination null mutants

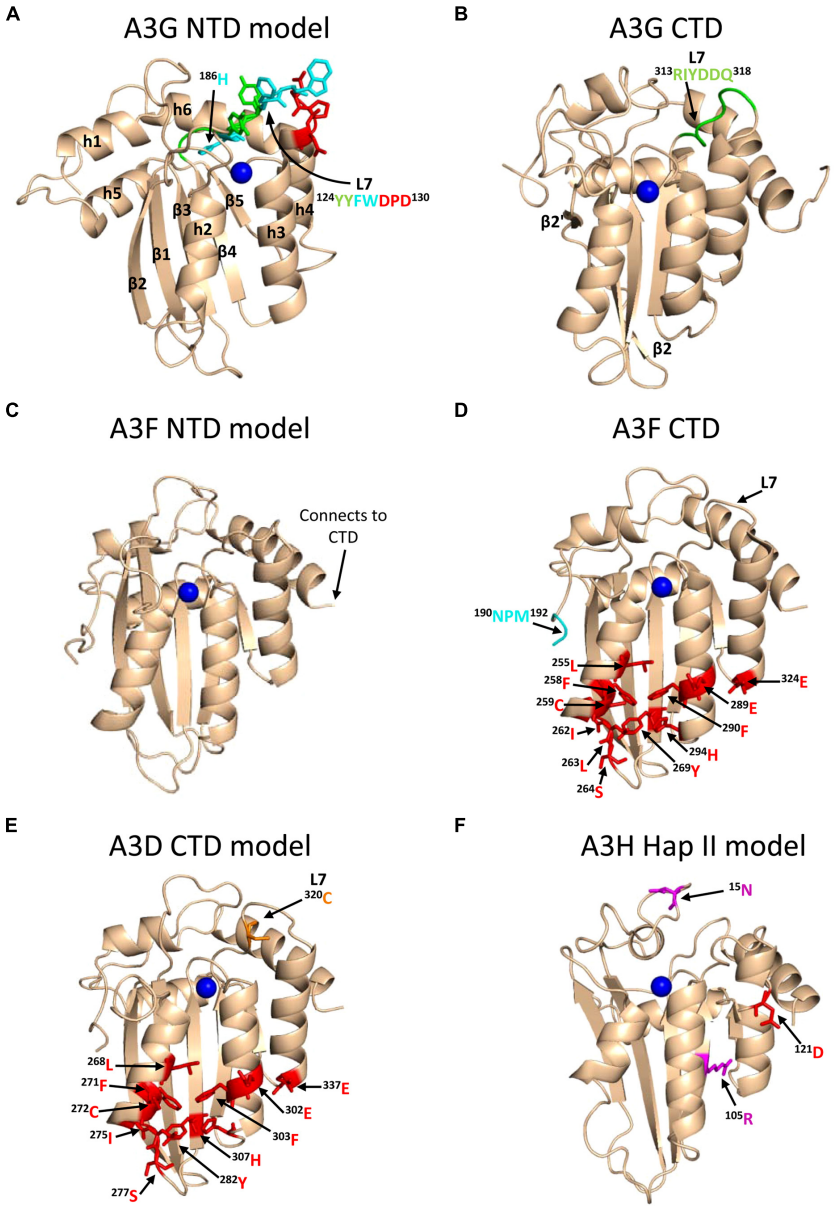

FIGURE 3 | Structures of A3 enzymes. A3 enzymes have a basic structure in each Z-type domain that is composed of a five-stranded β-sheet core surrounded by six α-helices. Numerical assignments to β-strands and α-helices are superimposed in **(A)**. Zinc atoms are shown as blue spheres. **(A)** Model of the N-terminal domain (NTD) of A3G. Loop 7 (L7) of the A3G NTD is a central structure in its anti-HIV function. Highlighted on L7 are the residues important for interaction with Vif (red, ^{128}DPD^{130}), oligomerization/virion encapsidation (green and cyan, ^{124}YYFW^{127}), and jumping component of A3G processivity (cyan, ^{126}FW^{127}). Helix 6 (h6) is adjacent to L7 and contributes to the sliding component of A3G processivity, particularly ^{186}H (cyan). The model of the A3G NTD was obtained by using the automated SWISS-MODEL program using the homologous A3G C-terminal domain structure (CTD, PDB: 3IQS). **(B)** The A3G CTD (PDB: 2KEM) is the catalytic domain of A3G. The A3G CTD has a discontinuous β2 strand forming a loop-like bulge between the β2 and β2′ strands. A3G L7 residues ^{313}RIYDDQ^{318} (green) mediate tetramerization and determine the preferred deamination motif. **(C)** The model of the A3F NTD was obtained by using the automated SWISS-MODEL program using the homologous A3C structure (PDB: 3VM8). The end of h6 connects the NTD to the CTD and contains an ^{190}NPM^{192} motif. This NPM motif is found only in A3D and A3F. **(D)** The A3F CTD (PDB: 4IOU) is the catalytic domain of A3F and interacts with Vif. Residues that interact with Vif across Helix 2, 3, 4, and β-strand 4 are shown in red. Also shown on this structure is the deamination motif specificity loop (L7) and the ^{190}NPM^{192} motif. The structure illustrates the kinked orientation introduced by the Pro in the ^{190}NPM^{192} motif, which blocks the sliding function of A3F. **(E)** The model of the CTD of A3D was obtained by using the SWISS-MODEL program using the homologous A3F structure (PDB: 4IOU). Residues that interact with Vif across Helix 2, 3, 4, and β-strand 4 are shown in red. The ^{320}C residue on L7 that influences A3D activity is shown in orange. **(F)** Model of A3H Hap II showing residues that interact with Vif and cause haplotype instability. In A3H Hap II, ^{121}D (red) on predicted h4 mediates an interaction with Vif. In A3H Hap I the R105G mutation induces protein instability (magenta). In A3H Hap III and IV, the deletion of ^{15}N induces protein instability (magenta). The model of the A3H Hap II was obtained by using the automated SWISS-MODEL program using the homologous APOBEC2 structure (PDB: 2NYT). Figures were made using PyMOL (The PyMOL Molecular Graphics System, Version 1.5.05, Shrödinger, LLC.).

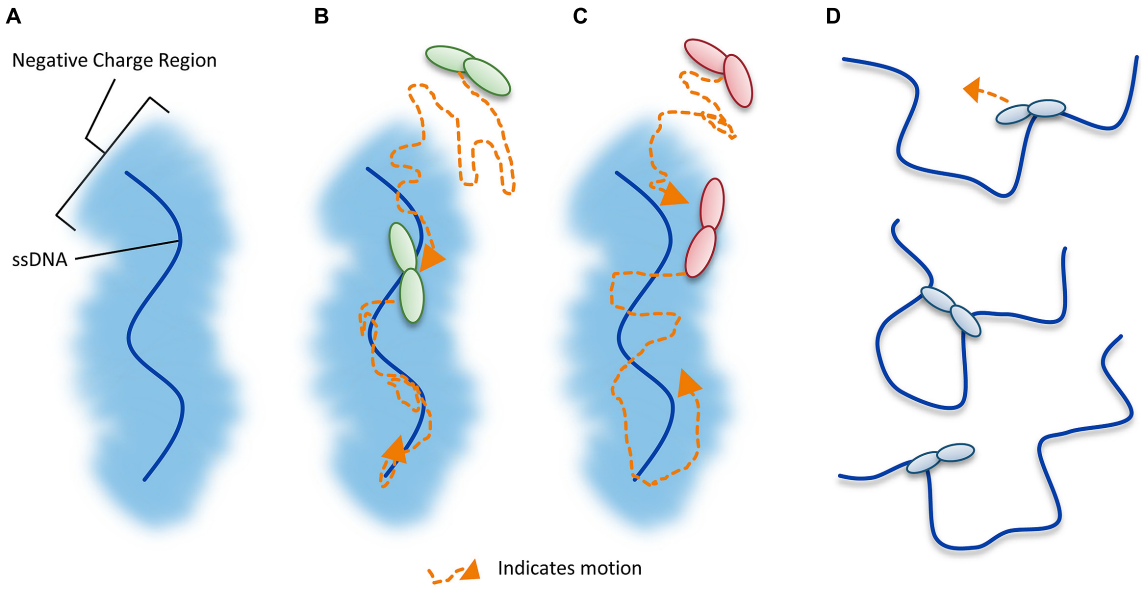

FIGURE 4 | Illustration of DNA scanning by facilitated diffusion. **(A)** Sketch of DNA showing the negatively charged region of DNA important for facilitated diffusion of A3 enzymes. **(B–D)** Enzyme in sketches is shown as a dimer, although the oligomerization state may vary with different A3 enzymes. **(B)** Sketch depicting a 1-dimensional DNA scanning path by sliding. Dotted line indicates path of enzyme (orange). Sliding enables an in depth search of local areas of a substrate. **(C)** Sketch depicting a 3-dimensional DNA scanning path by jumping. Jumping enables larger translocations on DNA substrates, but lacks a local search process. The microdissociations of the enzyme from the DNA that occur when the enzyme jumps does not leave the negatively charged domain of the DNA so the enzyme has a higher likelihood of reassociating with the same DNA substrate than diffusion into the bulk solution. **(D)** Sketch depicting a 3-dimensional DNA scanning path by intersegmental transfer. Intersegmental transfer enables larger translocations on DNA substrates, but lacks a local search process. An enzyme with two DNA-binding domains binds two regions of DNA simultaneously before dissociating from one region to move to another.

of A3G could inhibit HIV proviral DNA formation (Mangeat et al., 2003; Newman et al., 2005). The initial mechanism proposed was that A3G binds the HIV genomic RNA which impedes RT (Iwatani et al., 2007). This has been confirmed in multiple reports using cellular and biochemical experiments (Adolph et al., 2013; Belanger et al., 2013; Chaurasiya et al., 2014). However, the physiological significance of these processes is difficult to reconcile since results from cell-based experiments using transiently expressed A3G cannot be replicated when A3G is stably expressed, suggesting that overexpression of A3G induced experimental artifacts due to excessive packaging of A3G in virions (Mbisa et al., 2007; Miyagi et al., 2007; Schumacher et al., 2008; Browne et al., 2009). Importantly, studies that used deamination null mutants of A3G to show that deamination ability is required for restriction of HIV (Mbisa et al., 2007; Miyagi et al., 2007; Schumacher et al., 2008) should be considered in conjunction with data showing that the A3G E259Q catalytically inactive mutant is not a true proxy for A3G (Bishop et al., 2008; Adolph et al., 2013). A3G E259Q binds RNA less well than A3G and this results in less inhibition of RT *in vitro* and in cells (Bishop et al., 2008; Adolph et al., 2013).

Nonetheless, it is clear that the ability of A3G to inhibit RT is highly dependent on A3G concentration and the primer/template (Mbisa et al., 2007; Miyagi et al., 2007; Schumacher et al., 2008; Browne et al., 2009; Adolph et al., 2013). The initiation of DNA synthesis from an RNA primer on an RNA template is the least efficient type of polymerization activity of RT (Liu et al., 2010). Accordingly, *in vitro*, low levels of A3G can best inhibit RT-mediated primer initiation at this step by competing for substrate (Iwatani et al., 2007; Adolph et al., 2013). In contrast, on a DNA primer and DNA template, A3G could at most inhibit *in vitro* RT-mediated initiation of synthesis by twofold under single turnover conditions and could not block RT from binding the primer/template, but merely lengthened the time RT required to find the free 3′OH (Adolph et al., 2013). These data are in agreement with a computational study that suggests an A3G-mediated deamination-independent mode of HIV restriction contributes <1% of the restriction capability of A3G (Kobayashi et al., 2014). Although studies have shown that a peptide of A3G can interact with RT and inhibit RT-mediated DNA synthesis, it is unlikely that this mechanism is a physiological way to inhibit reverse transcription since in ΔVif virions, only 3–11 A3G molecules are encapsidated whereas there are approximately 100 RT molecules (Coffin et al., 1997; Xu et al., 2007; Wang et al., 2012, **Figure 2E**). In the presence of Vif, there is only an estimated one to two molecules of A3G per virion (Nowarski et al., 2008, **Figure 2D**), emphasizing the importance of a deamination-dependent mechanism over a deamination-independent mechanism. A single molecule of A3G could inactivate an HIV provirus through cytosine deamination whereas the deamination-independent mechanism is much more concentration dependent (Browne et al., 2009; Adolph et al., 2013). Single-molecule studies have brought forth the model that A3G oligomers can act as a road-block for HIV (Chaurasiya et al., 2014). Notably, existence of a deamination-independent

mode of HIV inhibition has been observed *ex vivo* in primary cells (Gillick et al., 2013), but it requires further research as to the significance of this mode of inhibition during an HIV infection.

A3F-MEDIATED RESTRICTION OF HIV

Deamination-dependent HIV restriction by A3F

Approximately 2 years after the discovery of A3G, A3F was discovered to also exhibit restriction activity against HIV (Liddament et al., 2004; Wiegand et al., 2004; Zheng et al., 2004). Sequenced HIV proviral genomes were known to contain G/C⟶A/T transition mutations in 5′CC and 5′TC contexts in the (−)DNA (Pathak and Temin, 1990; Li et al., 1991; Vartanian et al., 1991, 2002; Fitzgibbon et al., 1993) and A3F was found to contribute to transition mutations in the 5′TC context. Of these initial studies demonstrating A3F activity active against HIV (Bishop et al., 2004; Liddament et al., 2004; Wiegand et al., 2004; Zheng et al., 2004), all except one (Zennou and Bieniasz, 2006) found that A3F restriction activity was equivalent to A3G restriction activity. It has since been shown that overexpression of A3 enzymes can result in excessive packaging into HIV virions and result in artifacts of HIV restriction (Miyagi et al., 2007; Xu et al., 2007; Schumacher et al., 2008). Yet even after 10 years of studying A3F, multiple groups still find different activities of A3F against HIV that cannot be attributable to overexpression, but perhaps different experimental systems and techniques (Miyagi et al., 2010; Mulder et al., 2010; Hultquist et al., 2011; Chaipan et al., 2013; Ara et al., 2014). However, A3F must exert a restriction pressure on HIV since Vif maintains an interaction interface with A3F that is distinct from A3G in order to induce A3F degradation (Russell et al., 2009b). As with A3G, for A3F to effectively restrict HIV, it must be encapsidated with the ribonucleoprotein complex within the capsid (Wang et al., 2008) and effectively search for cytosines on the heterogeneous (−)DNA substrate (Ara et al., 2014).

A3F encapsidates into HIV virions through an interaction with RNA, but packages more efficiently than A3G into the core of HIV particles (Zennou and Bieniasz, 2006; Wang et al., 2008; Song et al., 2012). By resolving HIV capsids on a sucrose gradient to observe whether A3F and A3G partition with the RNA and enzymes or the p24 capsid protein, Song et al. (2012) found that more A3F specifically associated with the ribonucleoprotein complex, in comparison to A3G. A3F binds nucleic acids with sevenfold higher affinity than A3G (Ara et al., 2014), which may enable it to package more specifically within the capsid (Song et al., 2012). Furthermore, A3F has been shown to bind double-stranded DNA with a higher affinity than A3G and maintain an association with the pre-integration complex of HIV as it enters the nucleus through its high-affinity nucleic acid binding (Mbisa et al., 2010; Burdick et al., 2013). Despite the quantity of A3F being at an equal or greater amount to A3G, A3F restricted HIV approximately fourfold less than A3G in a single round of infection (Song et al., 2012). Although some reports show A3F can be less effective than A3G in restricting HIV (Miyagi et al., 2010; Mulder et al., 2010; Song et al., 2012; Chaipan et al., 2013; Ara et al., 2014), it cannot be concluded that it does not suppress HIV or impose selective pressure on HIV. Not only because of data showing A3F can effectively restrict HIV in spreading infections (Hultquist et al., 2011; Refsland et al., 2012), but also because an HIV lab strain with tandem stop codons in Vif (from HIV NL4-3) will revert back to expressing a functional Vif in the presence of A3F (Albin et al., 2010a). This does not occur when A3G is used in the same type of forced evolution experiments (Hache et al., 2008, 2009). The HIV evolves to overcome A3G restriction, but does so by acquiring a 5′UTR mutation to make HIV RNA transcription more efficient and altering the cell cycle through a Vpr mutation (Hache et al., 2008, 2009). Together these mutations result in more virus particles being produced. Presumably since A3G has less specific packaging in the capsid than A3F, this strategy titrates out the ribonucleoprotein-packaged A3G enabling the HIV to escape high levels of mutagenesis. These data illustrate that A3G and A3F exert a distinct selective pressure on HIV due to distinct biochemical properties and that A3 packaging into virions is a necessary but insufficient step to ensure efficient HIV restriction (Ara et al., 2014).

To further understand why A3F-mediated restriction of HIV may be different than for A3G, Ara et al. (2014) undertook a biochemical study of A3F in comparison to A3G to identify biochemical differences between these enzymes that could account for differences in restriction efficiency. They found that in contrast to A3G, A3F used only 3-D jumping motions to scan ssDNA. This made the DNA scanning mechanism inefficient since A3F could translocate between many ssDNA regions and overcome intervening RNA/DNA hybrid regions, but lacked a local search mechanism to examine ssDNA regions for its 5′TC motif (**Figure 4C**). The A3F sliding movement is blocked by a ^{190}NPM^{192} motif in the connection domain between the NTD and CTD (**Figures 3C,D**) since mutagenesis of this motif to ^{190}NGM^{192}enabled A3F to slide (Ara et al., 2014). The Bohn et al.'s (2013) A3F CTD structure includes the ^{190}NPM^{192} sequence and shows that it is a kinked region of the loop structure (**Figure 3D**). Since an A3F ^{190}NGM^{192} mutant was able to slide, the data suggest that the rigid ^{191}P residue primarily blocks sliding. However, imparting sliding movement to A3F through the ^{190}NGM^{192} mutant did not increase A3F HIV restriction efficiency because the jumping movements of A3F differed from A3G and were dominant over sliding which maintained an inefficient search of ssDNA (Ara et al., 2014). The differences in DNA scanning between A3F and A3G were shown to be relevant to HIV restriction since A3F was fourfold less effective in restriction of HIV than A3G in a single-cycle infectivity assay (Ara et al., 2014). Of note, A3F was also shown to have a 100-fold lower specific activity than A3G (Ara et al., 2014), but this was not thought to contribute to differences in restriction efficiency since studies with different A3G and A3F mutants showed that mutagenesis efficiency correlated with the efficiency of the ssDNA scanning mechanism, not the specific activity. This is likely because RT polymerization and RNaseH activity limit the (−)DNA substrate available (Feng et al., 2013). The study by Ara et al. (2014) is in agreement with studies where A3F has not been as effective as A3G in restriction of HIV (Miyagi et al., 2010; Mulder et al., 2010; Chaipan et al., 2013), although A3F was found to be as restrictive to HIV replication as A3G in other reports (Hultquist et al., 2011; Refsland et al., 2012). Despite A3F being considered in some reports to be less efficient than A3G as an

HIV restriction factor when considered side by side, this is far from the natural mechanism of these enzymes in which they act in concert (Refsland et al., 2012; Ooms et al., 2013a) and further studies examining how these enzymes work together are needed.

It is of note that Zennou and Bieniasz (2006) noticed that per mutation, A3F was less likely to inactivate HIV than A3G. This was later found to be because the 5′CC motif of A3G overlaps with the only Trp codon (5′TGG/ACC) and results in a stop codon upon deamination of either cytosine in the motif (Yu et al., 2004). In contrast, A3F-induced mutations largely result in missense mutations which may or may not inactivate the encoded protein (Ara et al., 2014). The A3G 5′CC motif also overlaps with Gly codons and in the HIV *prot* mutations at these Gly results in more non-conservative mutations and gene inactivation than A3F-induced missense mutations that primarily cause the conservative mutation of Glu to Gln (Ara et al., 2014). The determinant for motif specificity is loop 7 in the CTD (Langlois et al., 2005; Kohli et al., 2009; Carpenter et al., 2010; Rathore et al., 2013, **Figures 3B,D**). This loop can be grafted into different A3 enzymes to change site specificity (Kohli et al., 2009). However, the consequences of deamination mediated restriction can be independent from inducing amino acid changes. A3F and A3G may be able to block proviral integration through deoxycytidine deaminations that result in aberrant processing of the proviral DNA ends by HIV integrase and inhibition of plus-strand DNA transfer by reducing the efficiency of primer tRNA removal (Mbisa et al., 2007, 2010).

Deamination-independent HIV restriction by A3F

For many years, A3F was thought to have a stronger deamination-independent mode of inhibiting HIV than A3G (Bishop et al., 2006; Holmes et al., 2007). Unlike A3G, the mechanism of deamination-independent "activity" was not extensively studied, but was presumed to be due to inhibition of RT polymerization. A computational study has found that A3G and A3F rely differentially on their deamination-independent modes of HIV restriction with A3G only having the deamination-independent mode contributing to <1% of its restriction activity whereas for A3F this value was approximately 30% (Kobayashi et al., 2014). However, two studies using stably expressed A3F and A3F catalytic mutants C280S/C283A and E251Q demonstrated no inhibition of RT, suggesting that previous results were influenced by A3F overexpression artifacts (Miyagi et al., 2010; Albin et al., 2014). Another study showed that A3F can inhibit HIV integration by reducing 3′ processing of viral DNA at the U5 and U3 ends by integrase (Mbisa et al., 2010). Using a catalytic mutant of A3F (E251Q), the study found that inhibition of integration was decreased approximately twofold from that of WT A3F suggesting that catalytic activity is in part required to produce the aberrant U5 and U3 ends (Mbisa et al., 2010). Thus there appears to be consensus that despite the potentially inefficient mutagenic activity of A3F in some studies, the deamination activity of A3F is still dominant over the deamination-independent activity. Furthermore, if a deamination-independent mode of HIV inhibition functions in cells, it may be the inhibition of integration rather than reverse transcription (Mbisa et al., 2010).

A3D-MEDIATED RESTRICTION OF HIV

A3D was first characterized in 2006 to restrict HIV replication in single-cycle infectivity assays and to be suppressed by Vif, suggesting that it posed a restriction pressure on HIV (Dang et al., 2006). Further evidence of this was that HIV proviral genomes showed evidence of deaminations in the contexts of 5′CC, 5′TC, and 5′GC (Dang et al., 2006). A3D was found to deaminate in the 5′TC and 5′GC contexts which were unique from A3G and A3F that maintain less promiscuous deamination motif preferences (Dang et al., 2006). A3D also forms multimers through an RNA intermediate in cells with a similar profile as A3G (Li et al., 2014). In a clinical study of HIV-infected individuals, A3D was found to be upregulated in both Elite Controllers and in Non-Controllers but was down-regulated in response to successful anti-retroviral treatment, indicating that A3D is part of a virological immune response to HIV (Abdel-Mohsen et al., 2013). However, the restrictive activity of A3D appears less than A3G and A3F in single-cycle infectivity assays in cell lines (Dang et al., 2006, 2011; Hultquist et al., 2011) and spreading infections in primary human cells (Chaipan et al., 2013). Furthermore, A3D represents the most divergent A3 enzyme in the lineage of chimpanzee to humans and the activity of A3D has decreased from chimpanzees to humans (Duggal et al., 2011). Other chimpanzee and human A3 enzymes are more commonly found to have similar restriction potentials (Duggal et al., 2011). Chimpanzee A3D induces more hypermutation of HIV than human A3D, despite equivalent packaging into virions (Duggal et al., 2011). This was attributed to differences in loop 7 of the CTD (Dang et al., 2011; Duggal et al., 2011). One report found a single amino acid in the CTD loop 7, C320, that suppressed A3D antiviral activity (Dang et al., 2011, **Figure 3E**). If the C320 was replaced with a Tyr, as in A3F, the activity of A3D could be increased by more than 20-fold (Dang et al., 2011). In contrast, endogenous A3D from the T cell line CEM2n appears to have activity against HIV-1 that is similar to A3F (Refsland et al., 2012). Using a series of *A3 null* backgrounds or A3 knockdowns, Refsland et al. (2012) found that the HIV-1 proviral hypermutation pattern at 5′CC and 5′TC sites was induced at comparable levels by the combined action of A3G and A3F or A3G and A3D, suggesting a redundancy in the HIV-1 restriction mechanism.

A3H-MEDIATED RESTRICTION OF HIV

A3H was originally identified as not being able to restrict HIV replication due to low steady-state protein levels in mammalian cells, despite normal mRNA expression (Dang et al., 2006; OhAinle et al., 2006). However, when A3H was recombinantly expressed in *Escherichia coli* it could mutate the *E. coli* genomic DNA (OhAinle et al., 2006). In later studies, it was realized that A3H exists as multiple haplotypes in the human population (Hap I-VII) with different stabilities in cells and HIV restriction capabilities (**Table 1**) and the original A3H tested was an unstable form (Hap I, OhAinle et al., 2008; Harari et al., 2009). The unstable Hap I is the most prevalent form of A3H in the population (**Table 1**), but is able to restrict HIV infection by approximately twofold when transiently overexpressed in cell lines (OhAinle et al., 2008; Harari et al., 2009; Li and Emerman, 2011; Wang et al., 2011a). Two amino acid

Table 1 | Summary of A3H haplotype features.

A3H haplotypes	Polymorphic amino acid residues					Antiviral activity in cell culture	Protein stability	Haplotype frequency	Reference
	Δ15	18	105	121	178				
Hap I	N	R	G	K	E	Partial	No	0.526[a] 0.308[b]	OhAinle et al. (2006), OhAinle et al. (2008), Dang et al. (2008), OhAinle et al. (2008), Harari et al. (2009), Ooms et al. (2010, 2013a), Li and Emerman (2011), Wang et al. (2011b), Zhen et al. (2012)
Hap II	N	R	R	D	D	Yes	Yes	0.061[a] 0.265[b]	OhAinle et al. (2008), Ooms et al. (2010), Hultquist et al. (2011), Li and Emerman (2011), Wang et al. (2011b), Zhen et al. (2012), Ooms et al. (2013a)
Hap III	Δ	R	R	D	D	No	No	0.070[a] 0.114[b]	OhAinle et al. (2008), Wang et al. (2011b), Ooms et al. (2013a)
Hap IV	Δ	L	R	D	D	No	No	0.088[a] 0.178[b]	OhAinle et al. (2008), Harari et al. (2009), Wang et al. (2011b)
Hap V	N	R	R	D	E	Yes	Yes	0.202[a] 0.054[b]	OhAinle et al. (2008), Wang et al. (2011b)
Hap VI	Δ	L	G	K	D	No	No	0.026[a] 0.0004[b]	OhAinle et al. (2008), Wang et al. (2011b)
Hap VII	N	R	R	K	E	Yes	Yes	0.009[a] Not detected[b]	OhAinle et al. (2008), Wang et al. (2011b)

[a] *Wang et al. (2011b).*
[b] *OhAinle et al. (2008).*

polymorphisms, ^{105}G and Δ^{15}N, can independently contribute to the instability of A3H (**Table 1**). A3H Hap I is unstable due to a Gly at position 105 (OhAinle et al., 2008). An A3H Hap I G105R mutant (later identified as Haplotype VII, **Table 1**) renders the A3H stable in cells and imparts strong anti-HIV activity (OhAinle et al., 2008; Harari et al., 2009). Other unstable A3H haplotypes (III and IV) have the Δ^{15}N in combination with another polymorphism (OhAinle et al., 2008; Harari et al., 2009, **Table 1**). It is not known biochemically why these A3H haplotypes are unstable, but comparative modeling of A3H with the structure of a related family member APOBEC2 shows that amino acid 105 is in a β-strand within the central five-stranded β-sheet, suggesting that an R105G mutation could destabilize the core structure (**Figure 3F**). The Δ^{15}N is predicted to be within a loop structure (**Figure 3F**) so it is difficult to predict the reason for the instability in this undefined region, but it is known from studies with A3F that deletions to a loop that connect the NTD and CTD cause protein instability (Ara et al., 2014), suggesting that the A3H loop may need to be of a specific length for proper protein folding. Although different haplotypes (II, V, and VII) have been reported to exist in the population as stable forms that are able to restrict HIV (**Table 1**), in this review we focus only on A3H Hap II (A3H Hap II), which has been the most highly studied. Notably, A3H Hap II has some variability in its restriction ability which is dependent on alternative mRNA spliced forms (Harari et al., 2009). A3H Hap II is primarily found in Africans/African Americans (∼50%) and to a much lesser extent within other cultural populations (prevalence of approximately 0–8%, OhAinle et al., 2008; Wang et al., 2011b). It has been proposed that A3H evolved to become unstable due to a combination of the loss of an ancient pathogen and the ability of an ancestral A3H to induce mutagenesis of genomic DNA (Jern and Coffin, 2008; OhAinle et al., 2008).

A3H is the only A3 enzyme with highly diversified antiviral activities based on sequence polymorphisms (Li and Emerman, 2011; Duggal et al., 2013) and appears to be in a category of its own in relation to other A3 enzymes regarding two other aspects. First, A3D, A3F, and A3G that also restrict HIV replication have two Z-type domains, whereas A3H has only one Z-domain (LaRue et al., 2008, **Figure 1**). Phylogenic analyses have shown that the A3 Z-type domains have three distinct categories (Z1, Z2, and Z3) and A3H is the only A3 enzyme with an Z3 (LaRue et al., 2008, **Figure 1**). A3D and A3F have two Z2 domains and A3G has an Z1 (CTD) and Z2 (NTD) domain (LaRue et al., 2008, **Figure 1**). Second, A3H is the only single Z-type domain A3 (others are A3A and A3C) that forms oligomers and multimers. Structural and biochemical studies have found that A3A and A3C are largely monomeric (>90%) in solution and do not multimerize in cells through an RNA intermediate (Kitamura et al., 2012; Love et al., 2012; Byeon et al., 2013; Li et al.,

2014; Logue et al., 2014; Shlyakhtenko et al., 2014). In contrast, A3H Hap II was found to multimerize in cells (Li et al., 2014). The A3H Hap II multimerization in cells was shown by fluorescence fluctuation spectroscopy and determined that multiple A3H Hap II molecules could closely associate on RNA, not that A3H Hap II oligomerized through a protein–protein interaction (Li et al., 2014). It remains to be determined if A3H Hap II can form a dimer in solution in the absence of RNA or DNA. A3G and A3F form oligomers in the absence of nucleic acid suggesting that A3 oligomerization ability facilitates the multimerization of A3 enzymes with RNA in cells (Chelico et al., 2008; Shlyakhtenko et al., 2011; Ara et al., 2014). It has been shown that similar to A3G and A3F, A3H Hap II interacts with cellular RNA and the NC portion of Gag to facilitate its encapsidation into HIV particles (Wang et al., 2011a; Zhen et al., 2012). Studies on A3H Hap II and Hap I have also shown that cytoplasmic localization correlates with restriction efficiency since mutation of A3H Hap I to make it cytoplasmic (G105R) increases its restriction capacity despite other amino acid differences from A3H Hap II (Harari et al., 2009; Li et al., 2010). Additionally, virion mislocalization of certain A3H haplotypes may render them less active against HIV (Ooms et al., 2010). For example, despite nuclear localization of A3H Hap I, it can be encapsidated into HIV particles, but through an association with the matrix and capsid region of Gag, which leads to its primary localization outside the capsid (Ooms et al., 2010). These data suggest that both cellular and virion localization play a role in restriction efficiency. There has been limited information in the literature on the biochemical properties of A3H and how different haplotypes bind and scan ssDNA in search for deamination targets. A3H Hap II prefers to deaminate ssDNA at 5′TC sites, similar to A3F and A3D, and appears have a high mutagenic potential and ability to restrict HIV in both single-cycle and spreading infection experiments and in HIV-infected individuals (Harari et al., 2009; Hultquist et al., 2011; Wang et al., 2011a; Ooms et al., 2013a).

RESTRICTION OF HIV BY COORDINATELY EXPRESSED A3 ENZYMES

Vif-deficient HIV showed replication defects when produced from cell lines such as CEM and H9, resulting in their classification as non-permissive cell lines (Gabuzda et al., 1992; Blanc et al., 1993; Sakai et al., 1993; von Schwedler et al., 1993; Madani and Kabat, 1998; Simon et al., 1998). After many years of investigating the function of Vif and trying to understand the dichotomous phenomenon of permissive and non-permissive cell lines for ΔVif HIV, Sheehy et al. (2002) found that the non-permissive CEM cell line expressed A3G. Thereafter, many groups discovered that Vif was required to induce degradation of A3G to enable HIV replication (Sheehy et al., 2002, 2003; Conticello et al., 2003; Kao et al., 2003; Mariani et al., 2003; Marin et al., 2003; Stopak et al., 2003; Yu et al., 2003). Later, it was realized by analyzing the mRNA expression levels of A3s using quantitative PCR in permissive (CEM-SS, SupT1) and non-permissive (CEM and H9) T cell lines that the classical non-permissive CEM T cell line expressed not only A3G but also A3F and A3D, albeit with lower mRNA levels (Refsland et al., 2010). It is interesting to speculate whether more data would be available on the inhibition of HIV by the combined action of multiple A3s if they were discovered at the same time as A3G.

In primary CD4+ T cells A3 enzymes relevant to HIV restriction are expressed and further induced by mitogens, rather than interferon, indicative of their function in restricting retrotransposons (Koning et al., 2009; Refsland et al., 2010). In contrast, in macrophages, monocytes and dendritic cells expression of select A3 enzymes is induced by interferon (Koning et al., 2009; Refsland et al., 2010). Although A3 enzymes are not individually expressed in cells as in many laboratory experiments (Refsland et al., 2010, 2012), there is an advantage of individual expression of each A3. Individual expression of A3 enzymes enables mechanistic information to be learned about enzyme function and mutational footprints established. However, A3s with perhaps a lesser restriction efficiency would not be expressed alone during an HIV infection suggesting that it may not matter *per se* which enzyme is most effective since they may each contribute cooperatively to HIV restriction. Gillick et al. (2013) found that in primary human CD4+ T cells the majority of proviral mutations were in a sequence context that indicated A3G-induced mutations are dominant (5′CC), but A3F- and A3D-induced mutations (5′TC context) were evident at ninefold less frequency than the 5′CC context in ΔVif HIV. This is in contrast to a study by Ooms et al. (2013a) that used peripheral blood mononuclear cells to examine the hypermutation of HIV in the absence or presence of A3H Hap II. In the absence of A3H Hap II, it was found using a deep sequencing approach that there was approximately an equal number of mutations originating in 5′CC and 5′TC contexts, suggesting that A3F and A3D cooperate to induce an equivalent number of mutations to A3G (Ooms et al., 2013a), in agreement with results from a CEM2n T cell line (Refsland et al., 2012). In the presence of Vif that could induce degradation of all A3s except A3H Hap II, there was a large number of mutations in the 5′TC context demonstrating that A3H when present in a stable form is highly active against HIV (Ooms et al., 2013a).

Although the use of spreading infections in primary cells or T cell lines supports the idea that A3s cooperate, there still may be a question of whether they induce HIV evolution. It has been proposed that if there is an insufficient amount of A3-induced hypermutation this may benefit HIV and contribute to sequence variation by induction of sublethal levels of mutagenesis which results in HIV evolution (Mulder et al., 2008; Kim et al., 2010; Sadler et al., 2010; Monajemi et al., 2014). There is evidence that A3G and A3F hotspots are enriched in immunogenic CTL epitopes and that HIV may utilize A3s to induce immune escape (Monajemi et al., 2014). In addition, A3G may be able to induce resistance to the RT inhibitor lamivudine (3TC) because its deamination motif overlaps with a codon for Met and results in an M146I mutation in the *pol* gene (Mulder et al., 2008; Kim et al., 2010). However, the frequency of this mutation being induced by A3G versus RT activity has been questioned (Jern et al., 2009). It is also not known if A3F/A3D/A3H Hap II induce this evolution any more than A3G, due to differences in inactivation potential derived from their sequence specificities (Yu et al., 2004; Zennou and Bieniasz, 2006; Love et al., 2012; Ara et al., 2014) and if this impacts disease progression in infected individuals. On the other hand, Vif has been shown to adapt within HIV-infected individuals and be less

effective in inducing A3 degradation (Simon et al., 2005; Fourati et al., 2010). It is thought that HIV can utilize Vif as a mutational rheostat in times of viral stress by allowing low amounts of A3s into viral particles to induce sublethal mutagenesis (Simon et al., 2005; Fourati et al., 2010). These types of studies have raised the idea that perhaps inducing hypomutation or shutting off A3 enzymes may benefit HIV-infected individuals (Harris, 2008; Hultquist and Harris, 2009).

HIV Vif

GENERAL PROPERTIES

The main function of Vif remained elusive at the beginning of HIV research, except for the finding that Vif made some cell lines permissive for producing HIV particles capable of undergoing another round of infection (Fisher et al., 1987; Strebel et al., 1987). Non-permissive cells allowed a ΔVif HIV to produce virus particles, but they were rendered non-infectious upon infection of fresh cells. Two laboratories discovered that Vif repressed a host factor (Madani and Kabat, 1998; Simon et al., 1998). It was later identified by subtractive hybridization that A3G (originally called CEM15) was the host factor that was highly packaged into virions in the absence of Vif and blocked infection in the next target cell (Sheehy et al., 2002, **Figure 2A**). Although this is clearly a primary role for HIV infectivity, Vif was also shown to influence HIV particle morphology and this may relate to its potential role as a nucleic acid chaperone (von Schwedler et al., 1993; Hoglund et al., 1994; Henriet et al., 2007; Batisse et al., 2012).

Vif AS AN E3 UBIQUITIN LIGASE SUBSTRATE RECEPTOR

In 2012 it was discovered that Vif interacts with the host transcription cofactor CBFβ for stability in cells (Jager et al., 2012; Zhang et al., 2012). The interaction is mediated through Vif amino acids ^{84}GxSIEW^{89} and ^{102}LADQLI^{107} (Matsui et al., 2014b; Wang et al., 2014, **Figures 5A,B**). The Vif/CBFβ complex is also required for *in vitro* stability of Vif and enables recombinant expression of Vif in a largely soluble form in *E. coli* that can be purified for biochemical studies (Zhou et al., 2012). In contrast, Vif alone expressed in *E. coli* accumulates in inclusion bodies and must be purified under denaturing conditions (Yang et al., 1996). To suppress A3 action Vif interacts directly with A3 enzymes and mimics the human protein suppressor of cytokine signaling-2 (SOCS2) to become the substrate recognition subunit of a Cullin RING ligase-5 (CRL5) E3 ligase complex (**Figure 2B**).

Vif interacts with host proteins Elongin C, which forms an obligate heterodimer with Elongin B (EloB/C) and Cullin 5 (Cul5, Marin et al., 2003; Yu et al., 2003, 2004; Mehle et al., 2004; Luo et al., 2005; Xiao et al., 2007; Stanley et al., 2008; Bergeron et al., 2010). The interaction of Vif with EloB/C increases the stability of Vif in cells and *in vitro* and promotes recruitment of CBFβ (Wang et al., 2013). The interaction of Vif with EloC is mediated through an SLQ motif in Vif termed the Elongin B/C (BC) box (Yu et al., 2003, 2004, **Figures 5A,B**, ^{144}SLQYLA^{149}), similar to human SOCS proteins (Kamura et al., 1998; Iwai et al., 1999). Distinct from human proteins is that Vif does not have the highly conserved Cys in the BC box and instead has a ^{149}A (Kamura et al., 1998, 2004, **Figures 5A,B**). The data with Vif suggest that it is the short side chain of the amino acid at position 149 rather than the Cys that is required for the interaction with EloC (Yu et al., 2004; Stanley et al., 2008). Vif also does not contain a canonical Cul5 box (Luo et al., 2005; Xiao et al., 2007). In search of the conserved Cys in the BC box, two other Cys (^{114}C, ^{133}C) were identified in Vif upstream of the BC box and were found to be involved in binding with Cul5 (Mehle et al., 2004; Yu et al., 2004). These Cys were found to be part of a novel Zinc binding HCCH motif (**Figures 5A,B**, ^{108}Hx$_2$YFxCFx$_4$IRx$_2$LxGx$_6$CxYx$_3$H^{139}). The Zinc coordination in the HCCH was predicted to stabilize a small domain of Vif and indirectly support Cul5 binding (Luo et al., 2005). The primary Vif amino acids that contact Cul5 are at positions 120–121 and 124 in a helix that is adjacent to the HCCH residues (Xiao et al., 2006; Guo et al., 2014, **Figures 5A,B**, ^{120}IRxxL^{124}). The Vif/CBFβ/EloB/C heterotetramer undergoes a conformational change that promotes binding to Cul5, suggesting that there is a prescribed order in the assembly of the E3 CRL5 ligase complex (Fribourgh et al., 2014). Accordingly, Cul5 binds less well to EloB/C in the absence of Vif/CBFβ (Guo et al., 2014).

A recent structural study of Vif bound to CBFβ/EloB/C/Cul5 shows that Vif has an overall elongated cone structure and contains two domains with a Zinc binding domain in the center of the two domains (Guo et al., 2014, **Figure 5C**). CBFβ binds the N-terminal α/β-domain and EloC and Cul5 bind the C-terminal α-domain of Vif (Guo et al., 2014, **Figure 5C**). Both EloC and Cul5 interact with Vif through hydrophobic interfaces on distinct α-helices (Xiao et al., 2006; Guo et al., 2014). The crystal structure also emphasizes the stability that CBFβ imparts to Vif since they have a total interaction surface area of 4797 Å^2 and form an antiparallel β-sheet with a β-strand from each protein (Guo et al., 2014). The side of CBFβ that is bound by Vif is the same side that the human CBFβ binding partner, the RUNX1 transcription factor binds to suggesting a mutually exclusive binding (Kim et al., 2013; Guo et al., 2014), although other reports show CBFβ can bind Vif and RUNX1 on genetically distinct surfaces (Hultquist et al., 2012; Zhang et al., 2012; Du et al., 2013a). Functionally, Vif appears to exclude CBFβ from binding RUNX1 because expression of Vif can alter the RUNX1-dependent transcriptional profile of cells and suggests that Vif may have multimodal effects in HIV-infected cells (Kim et al., 2013).

CBFβ interacts with a hydrophobic region of the Vif α/β-domain, but the rest of the exposed α/β-domain surface is highly positively charged and is thought to mediate electrostatic interactions with A3 enzymes (Aydin et al., 2014; Guo et al., 2014). To target A3s for proteasomal degradation, Cul5 interacts with RING finger protein 2 (Rbx2, Jager et al., 2012) and this results in the assembly of a hexameric complex (**Figure 2B**). Furthermore, an E2 ubiquitin conjugating enzyme interacts with the hexamer through Rbx2 and causes ^{48}K-linked polyubiquitination of the A3 enzyme, on multiple lysine residues signaling it for degradation through the proteasome pathway (**Figure 2B**). Current data for A3G and A3F suggest that the Lys residues that become conjugated to ubiquitin are random (Albin et al., 2013).

Vif amino acids that interact with A3s

Alanine scanning mutagenesis of Vif or comparison of different Vif variants from HIV subtypes has enabled the identification of

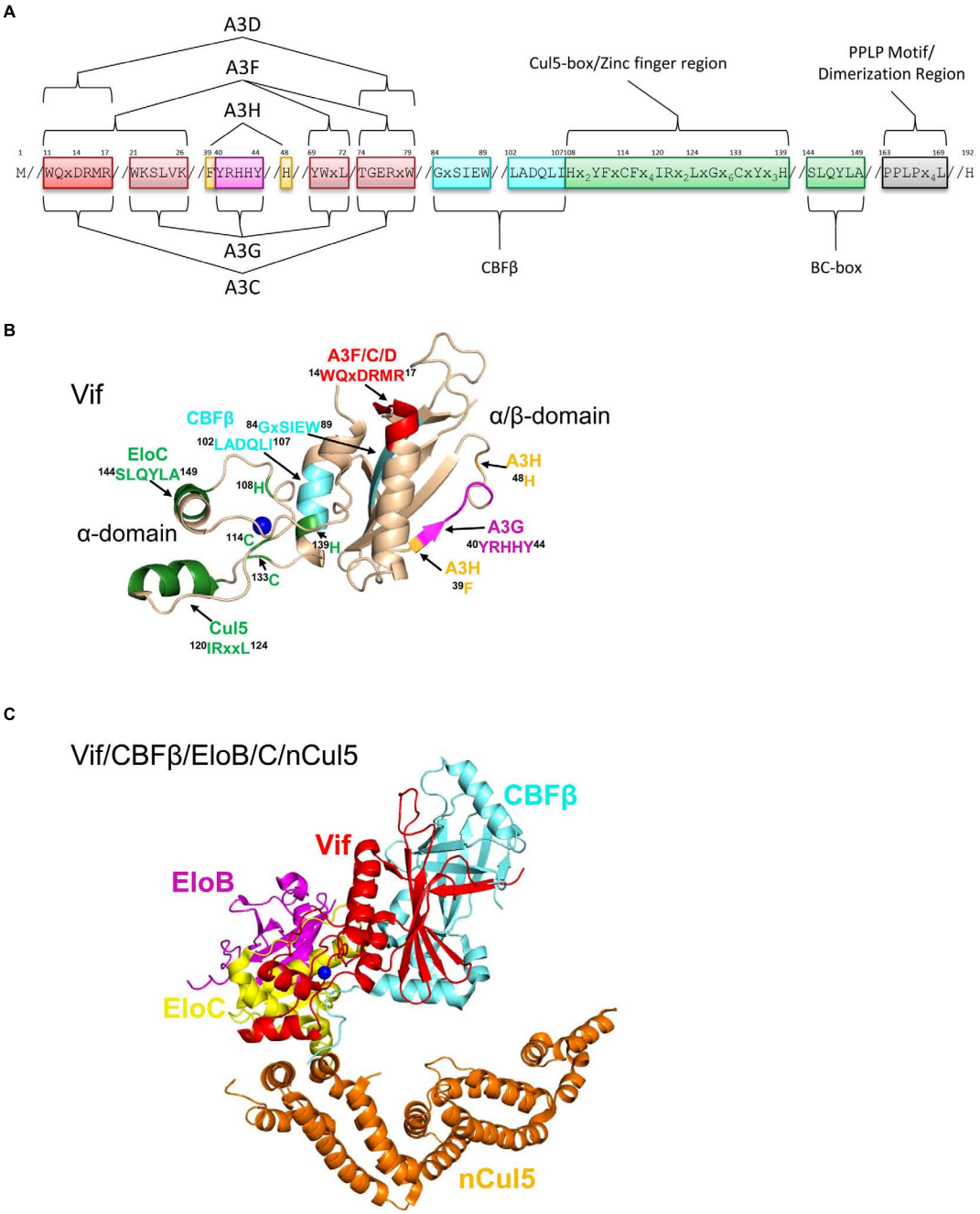

FIGURE 5 | Structure of Vif and host interacting partners. (A) Domain organization of Vif. Vif uses specific motifs to interact with A3G (magenta, ^{40}YRHHY^{44}), A3F/A3C/A3D (red, ^{11}WQxDRMR^{17} and ^{74}TGERxW^{79}), and A3H (orange, ^{39}F and ^{48}H). In conjunction with these specific motifs, there are shared interaction motifs for A3F and A3G with Vif (pink, ^{21}WKSLVK^{26} and ^{69}YWxL^{72}). CBFβ interacts with Vif through two adjacent motifs (cyan, ^{84}GxSIEW^{89} and ^{102}LADQLI^{107}). The Zinc finger region (green, amino acids 108-139) coordinates the Zinc through an ^{108}H^{114}C^{133}C^{139}H motif and stabilizes the Vif structure, which indirectly enables an interaction with Cullin 5 (Cul5). Direct interaction of Vif with Cul5 is through amino acids ^{120}IRxxL^{124}. The BC box mediates an interaction with Elongin C (green ^{144}SLQYLA^{149}). Vif oligomerizes through a PPLP motif (gray, ^{163}PPLPx$_4$L^{169}). Slanted lines are used to indicate intervening amino acids between the domains. **(B)** The crystal structure of Vif (PDB: 4N9F) shows that it has two domains on either side of a bound Zinc (blue). The N-terminal α/β-domain consists of a five stranded β-sheet, a discontinuous β-strand and three α-helices. The α/β-domain contains the binding interface for CBFβ (cyan, ^{102}LADQLI^{107}, ^{84}GxSIEW^{89}) and A3 enzymes. The ^{11}WQxDRMR^{17} motif (red) is used to interact with A3F, A3C, and A3D, the ^{40}YRHHY^{44} motif (magenta) is used to interact with A3G, and residues ^{39}F and ^{48}H (orange) are used to interact with A3H. The α-domain contains two alpha helices that mediate two separate interactions with EloC (green, ^{144}SLQYLA^{149}) and Cul5 (green, ^{120}IRxxL^{124}). **(C)** Structure HIV Vif (red) in complex with CBFβ (cyan), Elongin C (EloC, yellow), and the N-terminal domain of Cullin 5 (nCul5, amino acids 12–386, orange, PDB: 4N9F). Elongin B (EloB, magenta) dimerizes with EloC. Figures were made using PyMOL (The PyMOL Molecular Graphics System, Version 1.5.05, Shrödinger, LLC.).

three distinct regions of Vif that interact with A3G, A3F/A3D/A3C, or A3H (Huthoff and Malim, 2007; Russell et al., 2009b; Binka et al., 2012, **Figure 5A**). Vif interacts with A3G through two positively charged regions on Vif, ^{21}WxSLVK^{26} and ^{40}YRHHY^{44} (Mehle et al., 2007; Russell and Pathak, 2007; Yamashita et al., 2008; Chen et al., 2009; Dang et al., 2009, **Figures 5A,B**). Similarly, various domains in Vif have been identified to interact with A3F, specifically ^{11}WQxDRMR^{17} and ^{74}TGERxW^{79} (Tian et al., 2006; Russell and Pathak, 2007; He et al., 2008; Yamashita et al., 2008, **Figures 5A,B**). In addition, the ^{69}YWxL^{72} motif is a region of Vif that interacts with both A3G and A3F (He et al., 2008; Pery et al., 2009, **Figure 5A**). However, for both A3G and A3F mutation of the ^{40}YRHHY^{44} and ^{14}DRMR^{17} motifs to all alanines are necessary and sufficient to block Vif-induced A3G and A3F degradation, respectively, suggesting the other domains provide a secondary stabilizing interaction (Russell and Pathak, 2007). Of note, A3C and A3D share a common binding site on Vif as A3F with ^{14}DRMR^{17} shown to be of importance (Pery et al., 2009; Kitamura et al., 2011, 2012, **Figures 5A,B**). Vif interacts with A3H through another unique site that involves amino acid ^{39}F and ^{48}H (Binka et al., 2012; Ooms et al., 2013b, **Figures 5A,B**).

The Vif amino acids that interact with A3H are not highly conserved among HIV subtypes, in contrast to the motifs of Vif that interact with A3G and A3F/A3D. It has been suggested that since HIV rarely encounters a host with an active A3H allele, there has been evolutionary drift of Vif to not maintain an interaction site with A3H (Ooms et al., 2013a). As a result, A3H is differentially sensitive to Vif variants. For example, A3H Hap II is not sensitive to HIV NL4-3 Vif (^{39}F, ^{48}N), but is sensitive to HIV LAI Vif (^{39}F, ^{48}H, Ooms et al., 2013b). The inability of some Vif variants to induce degradation of A3H Hap II enabled Ooms et al. (2013a) to test whether A3H could act as an infection barrier to HIV. Ooms et al. (2013a) found that Vif will adapt in infected individuals to induce degradation of A3H Hap II (Li et al., 2010). Importantly, this evolution of Vif affects only A3H and Vif maintains the ability to induce degradation of A3G and A3F (Ooms et al., 2013a), confirming that Vif indeed uses three distinct interfaces to interact with A3 enzymes and supports the idea that multiple A3 enzymes coordinately exert a restriction pressure on HIV. Importantly, treatment naïve HIV-infected individuals at the early or primary infection stage that had at least one active A3H allele (Hap II) had higher levels of mutations in proviral genomes in a 5′TC context, lower viral loads and higher CD4+ T cell counts (Ooms et al., 2013a). Gourraud et al. (2011) similarly reported that early stage, untreated HIV-infected individuals that were homozygous for a stable A3H allele demonstrated lower HIV RNA over time, but this did not correlate with increased hypermutation of HIV proviral genomes. This difference in mutational load between these reports is likely due to the different sequencing strategies used between the two studies (Gourraud et al., 2011; Ooms et al., 2013a). These data are similar to clinical data obtained with A3G and A3F that demonstrate in a number of cohorts (but not all), A3G or A3F mRNA expression or hypermutation levels correlate with high CD4+ T cell counts and low viremia (reviewed in Albin and Harris, 2010).

Vif–A3G interaction

The Vif–A3G interaction was the first Vif–A3 interaction to be studied and it established that Vif inhibits the antiviral activity of A3 enzymes in a species-specific manner (Bogerd et al., 2004; Mangeat et al., 2004; Schrofelbauer et al., 2004; Xu et al., 2004; Etienne et al., 2013; Letko et al., 2013). This means that HIV Vif cannot neutralize A3G from African green monkey (AGM), and AGM SIV Vif cannot neutralize A3G from humans and this has been recognized as a cross-species infection barrier (Bogerd et al., 2004; Mangeat et al., 2004; Schrofelbauer et al., 2004; Xu et al., 2004; Etienne et al., 2013; Letko et al., 2013). Initially, to identify the residues HIV Vif uses to interact with human A3G the human A3G amino acids were replaced with those of AGM or rhesus macaque A3G. Mutation of human A3G ^{128}D to ^{128}K as found in AGM and rhesus macaque A3G abrogated the interaction of HIV Vif with human A3G and its ability to induce degradation of human A3G (Bogerd et al., 2004; Mangeat et al., 2004; Schrofelbauer et al., 2004). However, when the ^{128}D was mutated to ^{128}A, HIV Vif could still interact with and degrade human A3G demonstrating that the charged interface was more important than the amino acid identity (Schrofelbauer et al., 2004). Since mutation of solely ^{128}D to ^{128}K can abrogate the interaction between A3G and Vif in co-immunoprecipitation studies it is clearly a determining residue. However, Vif-mediated degradation can be influenced by mutation of A3G ^{129}P and ^{130}D and Vif also interacts with A3G on surrounding motifs such as helix 6 (Huthoff and Malim, 2007; Lavens et al., 2010; Feng et al., 2013, **Figure 3A**). The loop 7 and helix 6 regions contain more positively charged and neutral amino acids than negatively charged amino acids which may explain why ^{128}D is such an important contact point for the positively charged Vif, despite a larger A3G interface predicted from biochemical studies. These studies with A3G established the principle that a lack of Vif-induced degradation correlates with a lack of an interaction between the A3 and Vif.

Vif–A3F/A3D interaction

A3F and A3D share the same structural motif in the CTD that interacts with Vif (Smith and Pathak, 2010; Kitamura et al., 2012, **Figures 3D,E**). A3F has been studied more extensively than A3D in this regard and will be discussed here. In contrast to A3G, there was no specific single amino acid determinant identified for A3F that clearly mediated both the primary interaction with Vif and was a determinant for Vif-mediated degradation. Rather, different groups identified different amino acids in A3F that altered its susceptibility to Vif. Smith and Pathak (2010) reported that A3F interacts with Vif through CTD amino acids ^{289}EFLARH^{294} and that ^{289}E was critical for A3F sensitivity to Vif. Albin et al. (2010b) identified another residue, ^{324}E, as the key determinant of A3F to Vif-mediated degradation, but mutation of ^{324}E to other amino acids, even those of opposite charge, did not disrupt the interaction between A3F and Vif under stringent co-immunoprecipitation conditions. Although other groups have found that the interaction of A3F and Vif could be disrupted at least partially by mutating ^{324}E, there was a wider region of A3F that appeared to be important for Vif-mediated degradation in comparison to what was identified for A3G (Albin et al., 2010b; Kitamura et al., 2012; Siu et al., 2013). A combination of mutagenesis, structural modeling and a

crystal structure of A3C, that shares the same Vif binding interface with A3F and A3D, identified a novel type of A3 and Vif interaction (Smith and Pathak, 2010; Kitamura et al., 2012). Rather than Vif interacting with a loop as in the case of A3G (**Figure 3A**, loop 7), Vif interacted with a negatively charged surface of A3F/A3D/A3C that spanned helix 2, 3, and 4 and β-strand 4 (Kitamura et al., 2012; Aydin et al., 2014, **Figures 3D,E**). This negatively charged surface supports the hypothesis that it is primarily electrostatic interactions that mediated the A3 and Vif interaction and provides an explanation for why the A3F and Vif interaction may be more difficult to disrupt than the primarily neutral surface present in A3G. It is not known if this would mediate a tighter interaction of Vif with A3F than A3G since there are no quantitative data available for both A3G and A3F using the same experimental conditions (Feng et al., 2013; Siu et al., 2013). Studies with A3F have shown that a lack of Vif-induced degradation does not necessarily correlate with a lack of a Vif-A3F interaction, suggesting that the binding orientation or other factors contribute to successful Vif-mediated degradation rather than only the presence of an interaction (Albin et al., 2010b).

Vif–A3H interaction

A3H sensitivity to Vif is haplotype dependent (OhAinle et al., 2008; Harari et al., 2009; Tan et al., 2009; Li et al., 2010; Hultquist et al., 2011; Binka et al., 2012). The A3H Hap I is not sensitive to HIV LAI Vif-mediated degradation whereas A3H Hap II is sensitive to HIV LAI Vif-mediated degradation (Harari et al., 2009; Li et al., 2010; Zhen et al., 2010; Ooms et al., 2013b). The A3H haplotype polymorphisms only occur at three locations (amino acids 105, 121, and 178, **Table 1**). A3H Hap I encodes GKE at these three positions and A3H Hap II encodes RDD at these positions. It was shown that at position 105, the Arg is required for stable expression in cells and that the 178 position had little effect on Vif-mediated degradation (OhAinle et al., 2008; Harari et al., 2009; Li et al., 2010). Therefore, a single amino acid homologous to A3G ^{128}D at position 121 in A3H Hap II was determined to control sensitivity to Vif-mediated degradation (Li et al., 2010; Zhen et al., 2010, **Figure 3F**). An A3H Hap II mutant with a ^{121}K is not sensitive to Vif-mediated degradation and does not interact with Vif (Zhen et al., 2010). From the A3H structural model (**Figure 3F**), it appears that the ^{121}D of A3H is not located on loop 7 as in A3G, but is on helix 4 and on a different face of the molecule (compare **Figures 3A,F**). Yet, similar to A3G the region surrounding ^{121}D is mainly neutral or positively charged residues, in contrast to the negatively charged interface that Vif uses to interact with A3F and A3D (Aydin et al., 2014).

DEGRADATION INDEPENDENT INHIBITION OF A3G

Although Vif primarily inhibits A3G by inducing its proteasomal degradation, there have been other ways in which Vif can inhibit A3G encapsidation or function through a degradation-independent route. Vif may not be able to completely degrade the A3G in the virus-producing cell and these degradation-independent mechanisms may be another line of defense against A3G virion encapsidation. In particular, Vif can become the target of A3-mediated hypermutation (Simon et al., 2005; Jern et al., 2009), which may result in a Vif unable to interact with the E3 CRL5 ligase complex, but still able to inhibit A3G through a degradation-independent mechanism. It is not known if Vif can act in this manner for other A3 enzymes.

Vif decreases translation of A3G mRNA

Vif can decrease A3G mRNA translation in order to lower the steady-state levels of A3G through a Vif and A3G mRNA interaction, but the exact mechanism is not understood (Kao et al., 2003; Stopak et al., 2003; Mercenne et al., 2010). It is known that Vif can decrease the mRNA levels of A3G by 15–40% and this requires that Vif interact with the 5′UTR of the A3G mRNA (Stopak et al., 2003; Mercenne et al., 2010). Since Vif has been shown in an immunofluorescence study to co-localize with A3 enzymes and P-bodies (Marin et al., 2008), it is possible that Vif shuttles A3G mRNA to P-bodies to delay or prevent mRNA translation.

Vif inhibits virion encapsidation of A3G

Studies by Goila-Gaur et al. (2008) have shown that A3G synthesized *in vitro* using a rabbit reticulocyte lysate translation system would become immunoprecipitation and packaging incompetent in the presence of Vif. Vif was not associated with these high molecular mass A3G forms, but was required for their formation (Goila-Gaur et al., 2008). Although A3G regularly forms high molecular mass complexes in cells, which are less likely to be packaged into virions, Vif can induce an even higher molecular weight form of A3G (Soros et al., 2007; Goila-Gaur et al., 2008). Moreover, studies with an A3G C97A mutant that is resistant to Vif-mediated degradation suggested that Vif-mediated degradation and inhibition of packaging are two distinct properties of A3G since the A3G C97A mutant was encapsidated less well in the presence of Vif (Opi et al., 2007). A molecular mechanism for this effect has not been described.

Vif inhibits deamination of deoxycytidine by virion-encapsidated A3G

A3 enzymes are mainly studied with HIVΔ*vif* in order to observe restriction in single-cycle infectivity assays, but in infected individuals A3 enzymes must contend with Vif. Despite multiple mechanisms that Vif uses to block A3G, it has been shown that A3G is encapsidated in the presence of Vif, albeit in lesser amounts (Nowarski et al., 2008). However, per molecule of A3G there is less deamination activity (Britan-Rosich et al., 2011, **Figure 2E**). This decrease in A3G deamination activity even occurs when A3G and Vif are coexpressed in *E. coli* and mutations are detected with a Rifampicin reversion assay or *in vitro* with purified A3G and Vif, demonstrating that other viral components are not required for the inhibition to take place (Santa-Marta et al., 2005; Britan-Rosich et al., 2011; Feng et al., 2013). Enzymatic studies have shown that Vif can cause a decrease in the specific activity of A3G and that this is due to a combination of Vif competitively binding to the ssDNA substrate and Vif binding directly to A3G (Britan-Rosich et al., 2011; Feng et al., 2013). These are separable functions of Vif since ssDNA-binding studies of the Vif–A3G complex in comparison to each of the components binding ssDNA alone support the hypothesis that Vif bound to A3G is unable to bind ssDNA with high affinity (Feng et al., 2013). Another consequence of Vif binding to A3G is that it disrupts how A3G scans ssDNA in search of cytosines to deaminate (Feng et al., 2013). Vif interacts with the A3G NTD on loop 7, which is required for processive jumping

movements (Feng and Chelico, 2011, **Figure 3A**). In a study that used two Vif variants to examine the effect of Vif/CBFβ on the deamination activity of A3G it was found that HIV HXB2 Vif inhibited A3G jumping movements, consistent with an interaction of Vif on loop 7 (Feng et al., 2013). In contrast, HIV NL4-3 Vif inhibited A3G sliding, which is mediated by helix 6, providing functional evidence that beyond the key loop 7 contact residues, ^{128}DPD^{130}, Vif variants can interact with different regions of A3G (Feng et al., 2013). This appears to have no functional consequence for A3G-mediated degradation (Binka et al., 2012), but provides insights on how variable the Vif variants can be in their extended binding sites on A3 enzymes. This may affect strategies that aim to use small molecule inhibitors of the Vif-A3 interaction as an HIV therapy. Importantly, it has been shown that the specific activity of an A3 enzyme is not of primary importance for high levels of deoxycytidine deamination during reverse transcription (Feng et al., 2013; Ara et al., 2014). Rather, the method of efficiently searching for the cytosines, i.e., the enzyme's processive mechanism appears to be of more importance (Feng et al., 2013; Ara et al., 2014). All together the data suggest that the mechanism by which Vif inhibits A3G deamination activity in virions is by altering the searching mechanism used to find cytosines on ssDNA. Inhibition of A3G deaminase activity by Vif is likely to result in sublethal mutagenesis of HIV and could contribute to the generation of viral quasispecies and HIV evolution (Sadler et al., 2010; Feng et al., 2013).

DEVELOPMENT OF SMALL-MOLECULE INHIBITORS FOR A3-BASED HIV THERAPEUTICS

The A3-HIV host and pathogen relationship creates the possibility of developing novel therapeutics (Greene et al., 2008; Albin and Harris, 2010) and high-throughput screening approaches for small-molecule inhibitors have uncovered positive results. There are strategies to induce either A3G-mediated viral hypermutation by disrupting the Vif-A3G interaction (Nathans et al., 2008; Cen et al., 2010; Nowotny et al., 2010; Ejima et al., 2011; Ali et al., 2012; Mohammed et al., 2012; Matsui et al., 2014a) or viral hypomutation by blocking A3G catalytic activity (Li et al., 2012; Olson et al., 2013). For the "therapy by hypermutation" strategy, the rationale is to find small-molecule inhibitors that antagonize Vif function and increase the cellular level of A3G available for virus restriction. A few candidate molecules that recover A3G expression levels and enable HIV restriction in the presence of Vif have been discovered (Nathans et al., 2008; Cen et al., 2010; Nowotny et al., 2010; Ejima et al., 2011; Ali et al., 2012; Mohammed et al., 2012; Matsui et al., 2014a), although there are little biochemical data to understand the mechanism of action. For the "therapy by hypomutation" strategy, small-molecule inhibitors have been designed that target a key residue in A3G (C321) that inhibits the catalytic activity of A3G (Li et al., 2012; Olson et al., 2013). It is thought that decreasing the viral quasispecies that may arise due to A3-mediated mutagenesis can assist in immune clearance of the virus and decrease resistance to antivirals (Harris, 2008; Hultquist and Harris, 2009).

Inhibitors targeted to Vif may only be successful if administered in a cocktail to cycle their use and prevent the development of drug resistance, a long-standing therapy regimen for HIV-1 drugs (Greene, 2007). One strategy to avoid selection of resistant Vif variants is to utilize the anti-HIV potential of each A3 enzyme and design inhibitors that bind different regions of Vif, based on the unique interactions that Vif has with A3G, A3F/D, and A3H (**Figure 5A**). However, more study is required to determine if all A3 enzymes function equally well as individual restriction factors otherwise, the strategy may need to involve inhibiting degradation of all A3 enzymes together to enable a strong restriction pressure on HIV. Development of inhibitors that target the A3 enzymes may be a problematic route when considering A3G since Vif interacts with A3G near the amino acid residues needed for virion incorporation, oligomerization, and processivity (Huthoff and Malim, 2007; Huthoff et al., 2009; Feng et al., 2013, **Figure 3A**). As a result, the inhibitor molecule may decrease A3G anti-HIV activity. It is unknown whether the activity of A3D, A3H, or A3F would be affected by this type of strategy. Furthermore, it has been shown in the case of A3G, that HIV can overcome the restriction pressure of A3G by acquiring mutations in genetic sequences other than Vif in order to indirectly avoid A3G encapsidation (Hache et al., 2008, 2009).

If Vif were unable to interact with CRL5 E3 ligase complex components, the accelerated degradation of A3G would be blocked. This strategy has been raised as a potential option (Greene et al., 2008; Bergeron et al., 2010; Zuo et al., 2012) and current structural data on the EloB/C-, Cul5-, and CBFβ- Vif interfaces could facilitate development of inhibitors (Stanley et al., 2008; Guo et al., 2014). However, the consequence of targeting the host proteins with small molecules remains unknown. In addition, this approach has potential drawbacks since Vif may remain bound to the A3 enzymes. For A3G, this has been shown to lead to a decrease in mutagenic activity (Britan-Rosich et al., 2011; Feng et al., 2013). There are no published studies investigating whether Vif would affect the mutagenic activity of other A3s. If Vif were unable to interact with CBFβ it would become unstable in the host cells and degradation of A3 enzymes would be circumvented (Jager et al., 2012; Zhang et al., 2012). However, targeting a small molecule to CBFβ may be problematic if this prevents CBFβ from functioning as the transcription cofactor for RUNX proteins. Although some reports show that Vif and RUNX1 interact with CBFβ on distinct surfaces (Hultquist et al., 2012; Zhang et al., 2012; Du et al., 2013b), Kim et al. (2013) demonstrated that Vif recruitment of CBFβ alters the transcriptional profile of the cell by preventing RUNX1 and CBFβ association.

PERSPECTIVES

A3 enzymes have the potential to be manipulated as a therapeutic mechanism to suppress HIV replication. Over the past decade, an immense amount of information has been learned regarding each individual A3 enzyme. Cellular, biochemical, and structural data have provided insights on how A3 enzymes interact with nucleic acids and Vif and these data can be strategically applied to develop novel therapies. Critical to predicting the success of an A3-based strategy requires long-term culture of the virus with the potential small molecules to identify tactics HIV could use to overcome the suppression. Another critical facet is understanding if it is necessary for A3 enzymes to work together to restrict HIV *in vivo* in order to invoke the most restrictive pressure on the virus and prevent sublethal mutagenesis.

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AID and APOBECs span the gap between innate and adaptive immunity

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The activation-induced deaminase (AID)/APOBEC cytidine deaminases participate in a diversity of biological processes from the regulation of protein expression to embryonic development and host defenses. In its classical role, AID mutates germline-encoded sequences of B cell receptors, a key aspect of adaptive immunity, and APOBEC1, mutates apoprotein B pre-mRNA, yielding two isoforms important for cellular function and plasma lipid metabolism. Investigations over the last ten years have uncovered a role of the APOBEC superfamily in intrinsic immunity against viruses and innate immunity against viral infection by deamination and mutation of viral genomes. Further, discovery in the area of human immunodeficiency virus (HIV) infection revealed that the HIV viral infectivity factor protein interacts with APOBEC3G, targeting it for proteosomal degradation, overriding its antiviral function. More recently, our and others' work have uncovered that the AID and APOBEC cytidine deaminase family members have an even more direct link between activity against viral infection and induction and shaping of adaptive immunity than previously thought, including that of antigen processing for cytotoxic T lymphocyte activity and natural killer cell activation. Newly ascribed functions of these cytodine deaminases will be discussed, including their newly identified roles in adaptive immunity, epigenetic regulation, and cell differentiation. Herein this review we discuss AID and APOBEC cytodine deaminases as a link between innate and adaptive immunity uncovered by recent studies.

Keywords: restriction factors, CTL, HIV, correlate of protection, APOBEC1, APOBEC2, APOBEC3

INTRODUCTION

Higher eukaryotes have developed multiple strategies to counteract viral infections. A first line of defense is based on the recognition of pathogen-associated molecular patterns (PAMPs) such as viral replication intermediates that are molecules not commonly found in uninfected host cells. PAMPs were originally defined as molecular patterns specific to microbes, highly conserved and required for microbial function, and thus, are self–nonself discriminating molecules for higher eukaryotic organisms. After the engagement of PAMPs with the subsequently identified PAMP receptors, activation of a cascade of events leads to the expression and, in some cases, secretion of antiviral molecules and chemokines. Some of these molecules have been defined as "restriction factors" meaning host factors that have been evolutionarily selected for based on their capacity to restrict microbial infections. The receptors and effectors of this innate immunity are germline-encoded and mediate key aspects of host defense. However, viruses can also evade host defenses. It is the second arm of the immune system, adaptive immunity, which provides flexible antigen recognition based on somatic modification of antigen receptor genes in immune cells. This process involves selection of immune cells that includes a step of deletion of antigen receptors that are self-reactive, thus preventing autoimmunity, while allowing adaptation to diverse pathogens and the establishment of rapid and robust memory responses. There is evidence that communication between innate and adaptive immunity is required to clear pathogen infections that are otherwise deleterious to the host (Iwasaki and Pillai, 2014). Innate and adaptive immunity are thus considered interdependent.

Activation-induced deaminase (AID) and APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) enzymes are important in both innate and adaptive immune responses. AID/APOBEC family members originate from a large gene superfamily encoding for zinc-dependent deaminases involved in the metabolism of purine and pyrimidine bases (Conticello, 2008). The appearance of AID/APOBECs is thought to be concurrent with the divergence of the vertebrate lineage and the evolution of adaptive immunity (Conticello, 2008). AID/APOBECs have a unique capacity to mutate DNA and/or RNA of both host and pathogen as a result of their ability to deaminate cytidine to uridine. This activity, referred to as nucleic acid "editing," is involved in various immune functions, including restriction of viral replication, antigen presentation, and maturation of host immune receptors. The structure of AID/APOBEC proteins in relation with their editing activities has been recently reviewed (Conticello et al., 2007; Desimmie et al., 2014). AID is

thought to be the oldest member of the family and is essential for antigen-driven B cell terminal differentiation and antibody (Ab) affinity maturation and diversification (Muramatsu et al., 2000). In humans, genetic deficiency of AID leads to Type-2 Hyper-IgM Syndrome (HIGM2), an immunodeficiency characterized by the absence of antibodies other than the IgM class (Revy et al., 2000). APOBEC1, the first family member to be identified, plays an important role in lipid metabolism due to its ability to edit the apopoliprotein B (ApoB) pre-mRNA (Navaratnam et al., 1993; Teng et al., 1993). APOBEC1 might also participate in the restriction of viral infections (Ikeda et al., 2008; Gonzalez et al., 2009). APOBEC3s include seven members (A–C, DE, and F–H) that are involved in the restriction of viral infection and propagation affecting viruses such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV). APOBEC2 and APOBEC4 functions remain poorly understood although a role of APOBEC2 in embryogenesis has been recently proposed (Vonica et al., 2010). Highlighting the crucial role of AID/APOBECs in host defense, viruses have developed mechanisms to interfere with AID/APOBEC biogenesis and/or functions, and in fact, APOBEC3G was originally discovered due to its interaction with the HIV Vif protein (Sheehy et al., 2002). Here, we will review the cellular functions of AID/APOBEC family members and discuss recent work investigating their contribution in innate and adaptive antiviral immunity.

CELLULAR FUNCTIONS OF AID AND APOBEC FAMILY MEMBERS

APOBEC1

In humans, APOBEC1 (A1) is uniquely expressed in the gastrointestinal tract and participates in plasma lipid metabolism. In other species, such as mice, rats, horses, and dogs, A1 is also present in the liver (Greeve et al., 1993). Until recently, ApoB pre-mRNA was thought to be the single cellular target of A1 (Teng et al., 1993). ApoB protein has two isoforms, ApoB-100 and ApoB-48, encoded by a single gene in the liver and small intestine, respectively. The shortest form, ApoB-48, is the product of A1 editing activity and corresponds to the N-terminal portion of ApoB. A1 converts a unique cytidine to uridine (at position 6666 in Apo pre-mRNA) leading to a glutamine to STOP codon substitution and ApoB-48 translation (Navaratnam et al., 1993). ApoB-100 and ApoB-48 have different biological properties and control the homeostasis of plasma cholesterol. The editing activity of A1 is therefore an important determinant for plasma concentrations of ApoB-containing lipoproteins that are implicated in development of hyperlipidemia and atherosclerosis. Overexpression of A1 in the liver of mice or rabbits reduces the concentration of low-density lipoproteins. However, A1 overexpression also induces hepatocellular carcinoma in transgenic animals (Yamanaka et al., 1995), most likely due to its capacity to edit DNA (Harris et al., 2002; Petersen-Mahrt and Neuberger, 2003). A1 is indeed expressed in the nucleus where ApoB pre-mRNA editing also occurs (Lau et al., 1991).

More recently, using a transcriptome-wide RNA sequencing screen comparing wild type and A1-deficient mice, Papavasilou et al. discovered that, in small intestine, many mRNA transcripts other than apoB are edited by A1 (Rosenberg et al., 2011). The targets of A1 are 3′-untranslated regions (3′ UTR) of mRNA transcripts, suggesting additional roles for APOBEC1 beyond its function in ApoB regulation.

APOBEC2

A2 was cloned based on its sequence homology with A1 (Liao et al., 1999). A2 is well-conserved in the vertebrate lineages and can be traced back to bony fish (Liao et al., 1999; Etard et al., 2010). Using *in vitro* models (e.g., *Escherichia coli*), A2 has been shown to exhibit intrinsic cytidine deaminase activity (Liao et al., 1999). Although the A2 structure has been solved (Prochnow et al., 2007), its functions remained elusive until recently. In humans, A2 is exclusively expressed in heart and skeletal muscles (Liao et al., 1999). In mice, A2 KO was reported to have no major effect on animal viability and fertility (Mikl et al., 2005). This is in contrast to recent studies that implicate A2 in embryonic development of fish and xenopus (Etard et al., 2010; Pennings et al., 2010; Vonica et al., 2010). The lack of A2 expression causes a dystrophic muscle phenotype in zebrafish embryos (Etard et al., 2010). A2 seems to inhibit TGFβ-signaling, thus promoting muscle fiber differentiation both *in vivo* (in zebrafish and xenopus embryos) but also *in vitro* using a mammalian myoblastic cell line (Vonica et al., 2010). The mechanism of action and the targets of A2 action during embryogenesis are not defined, however, the ability of A2 (and other deaminases such as AID) to deaminate methylated cystidines suggests a possible role in epigenetic regulation (Rai et al., 2008).

AID

Activation-induced deaminase was cloned in a subtractive cDNA library screen comparing activated and resting B cell lymphomas (Muramatsu et al., 1999). AID is a key determinant in the generation of protective Ab-mediated adaptive immune responses. The cytidine deaminase activity of AID initiates the introduction of double stranded DNA breaks (DSB) in the immunoglobulin heavy chain (IgH) gene locus allowing Ab diversification, referred to as class switch recombination (CSR; Muramatsu et al., 2000). In addition, AID produces point mutations at the V(D)J region of Ig loci, a mechanism referred to as somatic hypermutation, (SHM), allowing B cell maturation (Muramatsu et al., 2000). These functions require a rigorous targeting of AID activities to SHM and CSR substrates (Kohli et al., 2010). Targeting might involve several complementary mechanisms such as AID binding to replication protein A, a ssDNA-binding protein involved in DNA repair (Basu et al., 2005), and/or association with a non-encoding RNA-processing/degradation complex (Basu et al., 2011). The editing activity of AID is not restricted to Ig loci and AID can act on a wide spectrum of genomic targets in B cells (Yamane et al., 2011). As a consequence, aberrant expression of AID promotes cancer development in animal models and humans (Okazaki et al., 2007). Dysregulated expression of AID facilitates DNA translocations that require DSB such as *c-myc/IgH* found in Burkitt's lymphoma and *c-myc/miR-142* found in B cell leukemia (Robbiani et al., 2008, 2009; Hasham et al., 2010). Constitutive or ubiquitous AID expression also leads to cancer development that is characterized by point mutations in oncogenes as well as passenger mutations

(those mutations that do not contribute to cancer growth; Okazaki et al., 2003). AID can therefore produce mutations in many genes other than Ig genes. While most of these mutations are rapidly repaired by the cellular DNA-repair machinery, those that are not successfully repaired, can destabilize the genome of cells.

Although AID expression is at its highest levels in germinal center B cells that undergo CSR and SHM, it is also found in other cell types such as oocytes, embryonic stem (ES) cells, and in estrogen-induced breast tissue (Fritz and Papavasiliou, 2010). The function of AID expression in these cells or tissues remains to be elucidated. However, the study of lower vertebrates including zebrafish suggests that AID expression is involved in epigenetic reprogramming of germ cells during early development (Rai et al., 2008). Using an AID knockout mouse model, Popp et al. (2010) revealed a role of AID in DNA demethylation during primordial germ cell reprogramming. DNA cytosine methylation is associated with gene silencing and plays a key role in development and genomic imprinting. The removal of 5-methyl group on cytosine (5-mC) contributes to epigenetic reprogramming required for the restoration of pluripotency of germ cells. Several lines of evidence suggest that AID, but also A1 and A2, might participate in this process of demethylation: AID and A1 can deaminate 5-methylcytosine *in vitro* and in *E. coli* (Morgan et al., 2004), and germ cells from AID-deficient mouse exhibit a hypermethylation pattern (Popp et al., 2010). AID (and A2) might contribute to the conversion of 5-mC to thymidine (T) later replaced by cytosine (C) by the DNA-repair machinery (Rai et al., 2008). In summary, AID function is not limited to Ab diversification, and evidence is accumulating to suggest a role in epigenetic reprogramming.

APOBEC3s

Sheehy et al. (2002) initially discovered the first family member of APOBEC3, A3G, in ground-breaking studies with HIV infection. Since that original identification, seven human A3 genes clustered in tandem on chromosome 22 have been identified, namely, A3A, A3B, A3C, A3DE, A3F, A3G, and A3H, which most likely arose through gene duplication of a single-copy primordial gene (Jarmuz et al., 2002). A3E was thought to be a pseudogene but in fact, A3D and A3E form one unique protein (A3DE; Dang et al., 2006). All A3 genes encode one or two conserved zinc-coordinating deaminase domain (ZDD), which contains a His/Cys-Xaa-Glu-Xaa$_{23-28}$-Pro-Cys-Xaa$_{2-4}$-Cys signature motif [X denotes any amino acid (aa)]. Regions of human A3 mRNAs share between 30 and 100% homology. Interestingly, depending on the species, the A3 genes expanded and/or contracted. As a result, A3 gene number ranges from one (mice, rats, pigs) to three (cats) and six (horses; LaRue et al., 2008). In humans, A3 genes are also highly polymorphic most likely due to the fact that they have been under strong and continuing selective pressure during primate evolution (Conticello et al., 2005; Henry et al., 2012). As discussed later on in this review, A3 polymorphisms might influence their specific antiviral activity.

A3 gene expression has been mainly documented in immune cells and these results have been determined based on mRNA levels in cells, using quantitative PCR (Koning et al., 2009; Refsland et al., 2010). This approach is particularly difficult since A3 genes are highly homologous and polymorphic. Apart from A3G and A3A, most antibodies to A3s are not very specific and endogenous A3 proteins are often difficult to detect. Nonetheless, several studies indicate a strong correlation between mRNA level and protein expression (Refsland et al., 2010). There is a general consensus that most A3s are highly expressed in T cells [memory or naïve (Refsland et al., 2010)] but also in B cells and phagocytic cells. A3A and A3B are predominantly expressed in monocytes (Peng et al., 2007; Thielen et al., 2010) and B lymphocytes (Koning et al., 2009), respectively. A3G and A3F are expressed in T cells, monocytes and dendritic cells (DC; Sheehy et al., 2002; Pion et al., 2006; Peng et al., 2007; Pido-Lopez et al., 2007; Stopak et al., 2007; Trapp et al., 2009). However, there is no consensus regarding their relative abundance. A3s expression is not confined to immune cell populations, and are highly expressed in human testis and ovary (A3G and A3F) (Koning et al., 2009) as well as ES cells (A3B, A3C, A3DE, A3F, and A3G; Wissing et al., 2011). A3G, A3F, A3B, and A3C are expressed in primary hepatocytes (Bonvin et al., 2006; Tanaka et al., 2006). The true breadth of basal A3 expression in human tissues remains difficult to estimate as leukocytes infiltrate tissues and no suitable specific immunohistochemistry antibodies are presently available. Nevertheless, different observations are in favor of a broad and constitutive A3 expression profile in human tissues. For instance, various cancer cell lines of non-immune origin - colorectal adenocarcinoma, melanoma and lung carcinoma lines - express multiple human A3s. It is possible that A3 expression is induced during oncogenesis, but given the abundance of A3s in different cell types it might also reflect their normal expression profile prior to cell transformation.

The cellular expression of A3s clearly indicates a role of A3s in immunity. The broad distribution of A3s also points toward a putative role in cellular maintenance. A3G and A3F localize in cytoplamic microdomains and stress granules that are sites of RNA storage and metabolism also called mRNA-processing bodies (or P-bodies; Wichroski et al., 2006; Gallois-Montbrun et al., 2008). Within P-bodies, A3G and A3F interact with effectors of the RNA silencing machinery (such as Argonaute 1 and 2) and translation suppressor (RCK/p54), suggesting that A3G-F participate in RNA metabolism and fate determination (Wichroski et al., 2006; Gallois-Montbrun et al., 2008). However, Phalora et al. (2012) found no evidence that A3s participate in specific regulation of miRNA. In addition, the manipulation of P-bodies using siRNA inhibition had no impact of A3 antiviral functions and HIV replication (Phalora et al., 2012). The reason why A3G and A3F localize to these P-bodies remains unclear. More recently, a role of A3s in DNA catabolism has also been proposed. Reminiscent of AID capacity to deaminate B cell genomes (during SHM and CSR), A3A edits host nuclear and mitochondrial DNA leading to the introduction of uridine (Suspene et al., 2011a). In the presence of functional DNA repair machinery, most mutations are likely fixed. In contrast, in uracil DNA-glycosylase (UNG)-deficient cells, (UNGs are enzymes required for excision of uracil bases), cytidine deaminations are readily detected using differential DNA-denaturation PCR (3D-PCR) (Suspene et al., 2011a). The significance of nuclear DNA editing by A3A is rather enigmatic as hyperediting is

synonymous with cell death and aberrant editing and/or repair might contribute to tumorigenesis (Mussil et al., 2013). On the other hand, phagocytic cells that express predominantly A3A may use cytidine-deamination to mark foreign DNA for degradation. In this model, the deamination of multiple cytidines on foreign DNA might lead to uracil excision by UNG, creating nuclease-sensitive abasic sites, and subsequent degradation by cellular nucleases (Stenglein et al., 2010). The nucleases involved have not been characterized, but as discussed by Stenglein et al. (2010) might include the IFN-inducible APEX or TREX1, though a contribution of DNAse I and II cannot be ruled out. This mechanism might represent an intrinsic immune defense reminiscent of bacteria that evolved endonucleases to prevent DNA transmission and bacteriophage infection (Stenglein et al., 2010). To this regard it is interesting to note that A3A and other A3s are induced upon inflammation (as described further, below).

Much remains to be learned regarding the cellular functions of A3s. Depending on cell type and tissue environment, A3s differently contribute to DNA/RNA deamination and their overarching biological roles are still being elucidated.

AID, APOBEC1, AND APOBEC2 IN ANTIVIRAL IMMUNITY

APOBEC1

The sequence homology between A1 and A3G prompted researchers to investigate a potential role of A1 in viral infection (Bishop et al., 2004a,b). In a pioneering work, Bishop et al. (2004b) demonstrated that human A1 (hA1) incorporated into HIV particles had no effect on HIV replication. In contrast, rat A1 had a strong suppressive effect on HIV regardless of Vif expression (Bishop et al., 2004b). Later work confirmed that in contrast to hA1, A1 from small animals (e.g., rabbit, hamster, mouse) inhibited the replication of retroviruses such as SIV (simian immunodeficiency virus), FIV (feline immunodeficiency virus), and murine leukemia virus (MLV), and the activation of autonomous retroelements in a deaminase-dependent manner, thus suggesting a putative role for A1 in the restriction of viral replication (Ikeda et al., 2008). The demonstration that A1 is a restriction factor in the course of viral infections in natural hosts came from the study of MLV and hepadnaviruses by the group of Wain-Hobson and Vartanian (Petit et al., 2009; Renard et al., 2010). Analyzing viral sequences in HBV-infected chimpanzees, woodchucks chronically infected with the natural woodchuck hepatitis virus (WHV) as well as ducks infected with duck hepatitis virus (DHV), the authors provided evidence that A1 edits hepadnaviral genomes and restricts replication *in vivo* (Renard et al., 2010). Analyzing human serum from two HBV chronically infected carriers, the same group also suggested that A1 edits HBV genomes *in vivo* (Gonzalez et al., 2009). These results were somehow surprising due to the fact that in humans A1 is not normally expressed in the liver. However, viral infection might lead to ectopic expression of A1. During the course of viral infections, the influence of IFN induction (or treatment) on A1 expression has not been investigated thus far. Nonetheless, the function of A1 is most likely not limited to the regulation of lipid metabolism. In vertebrates, A1 likely participates in intrinsic defenses against some viral infections.

APOBEC2

Though A2 exhibits deaminase activities (Liao et al., 1999), it has not been assigned a role in the restriction of viral replication thus far. However, it is interesting to note that in hepatocytes, A2 expression is enhanced by pro-inflammatory cytokines such as TNFα and IL-1β (Matsumoto et al., 2006). A2 contains functional NF-kB response elements in the 5′ untranslated region, suggesting a possible involvement in immune responses (Matsumoto et al., 2006). In the tonsils of patients with Immunoglobulin A nephropathy (IgAN) a disease characterized by IgA deposition to glomerular mesangial cells and glomerulonephritis, A2 expression is up-regulated around B cell germinal centers (where B cells undergo CSR and SHM with the "help" of follicular T cells). However, a direct role of A2 in IgAN pathology or IgA production has not been established (Iio et al., 2010).

AID

As discussed earlier, AID is required for CSR and, as a result, is critical for the generation of B cells that secrete Abs with various effector functions and tissue distribution in the organism (Muramatsu et al., 2000). For instance, immunoglobulins of the IgA isotype are found at the portal of pathogen entry in the mucosa and can be transported across the epithelium to neutralize pathogens. IgG is the principal isotype in the blood and extracellular fluid and is involved in pathogen neutralization, opsonization, and complement activation. *AID* $^{-/-}$ mice harbor a complete defect of CSR with a hyper-IgM phenotype and present enlarged germinal centers containing activated B cells (Muramatsu et al., 2000). In addition, AID involvement in SHM allows the generation of B cells with the potential to secrete Abs with higher affinities (Imai et al., 2003). Interestingly, mice carrying a mutated allele of AID with reduced capacity to perform SHM but with normal amounts of CSR, exhibit an impaired gut homeostasis and inefficient mucosal defenses (Wei et al., 2011). In humans, genetic deficiencies of AID are responsible for the development of a rare immunodeficiency, HIGM2 (Revy et al., 2000). HIGM2 is characterized by the absence of antibodies other than IgM and a profound susceptibility to bacterial infections (Revy et al., 2000). AID is therefore a key determinant in protective immunological responses, and the most well-documented mechanism of this protection is through the generation of protective Ab-mediated immune responses.

The action of AID is not limited to B cell differentiation and maturation as there is accumulating evidence that AID contributes to innate defenses against viruses. For example, HCV, Epstein-Barr virus (EBV), and Kaposi's sarcoma-associated herpesvirus (KSHV) have been shown to induce AID expression in B cells residing outside the germinal centers (Machida et al., 2004; Rosenberg and Papavasiliou, 2007; Bekerman et al., 2013). It is unclear so far whether AID up-regulation is beneficial or deleterious to HCV and EBV, however, in the case of KSHV, AID has a direct impact on viral fitness by inhibiting lytic reactivation and by reducing infectivity of virions. Further reinforcing the role of AID in antiviral responses, KSHV encodes microRNAs that dampen AID expression (Bekerman et al., 2013). Whether the deaminase activity of AID is required for KSHV restriction [as described

for A3G (see below)] remains to be determined. In hepatocytes, AID expression also correlates with reduced susceptibility to HBV infection (Watashi et al., 2013), a mechanism that might be dependent on deamination of the HBV genome by AID (Liang et al., 2013). AID might also participate in responses against transforming retroviruses. AID-deficient mice have been shown to be more susceptible to Abelson murine leukemia virus (A-MuLV), a defective virus that causes pro-B cell leukemia *in vivo* (Gourzi et al., 2006). In this case, the action of AID does not involve direct editing of the viral genome. Instead, AID might cause damage in the host cell genome, resulting in cell cycle arrest and/or up-regulation of stress-inducible factors, leading to natural killer (NK) cell activation and thus slower tumor growth (Gourzi et al., 2006). AID expression also correlates with the induction of aberrant SHM that might contribute to B cell transformation and tumorigenesis (Machida et al., 2005; Epeldegui et al., 2007).

APOBEC3s AND ANTIVIRAL IMMUNITY

A3G was the first member of APOBEC family to be assigned a role in antiviral immunity by demonstration of its activity against HIV infectivity (Sheehy et al., 2002). Since then, it has been demonstrated that human A3 cytidine deaminases affect the replication of a variety of viruses (Chiu and Greene, 2008) and impact the activation of adaptive immunity (Casartelli et al., 2010). A3G restricts the replication of retroviruses such as HIV, Foamy virus (FV; Delebecque et al., 2006), human T-cell leukemia virus type-1 (HTLV-1; Mahieux et al., 2005) as well as DNA viruses such as HBV (Turelli et al., 2004; Suspene et al., 2005) and also affects endogenous retroviruses (Esnault et al., 2005). A3A, A3C, and A3H deaminate human papillomavirus (HPV) genomes (Vartanian et al., 2008) and A3C acts on herpes viruses [e.g., herpes simplex-1 (HSV-1) and EBV viruses (Suspene et al., 2011b)]. Human A3G also acts on viruses infecting rodents (MLV) or avian species (Rous sarcoma virus and alpharetroviruses). A3 family members can play redundant roles in antiviral immunity (Albin and Harris, 2010). For instance, A3G and A3F restrict HIV and like A3C, A3G also acts on herpes viruses, although to a lesser extent (Suspene et al., 2011b). Intrinsic specificities of A3 proteins, but also their tissue distribution and cellular expression levels most likely determine the impact of each A3 family member on viral replication and on the activation of antiviral immunity.

INTRINSIC ANTIVIRAL FUNCTION OF A3s

The characterization of mutant HIV defective for the accessory protein Vif led to the discovery of A3G. Vif is essential for HIV-1 replication in a variety of cells including primary human T cells, monocytes, macrophages, DC and lymphoid T cell lines such as CEM, HUT78, also called "non-permissive" cells. However, Vif is dispensable in "permissive" T cell lines such as CEM-SS (a variant of CEM), Jurkat and supT1 cells [see for an in depth review (Henriet et al., 2009)]. A subtractive cDNA library screen – using CEM and CEM-SS T cell lines - led to the identification of human A3G that is strongly expressed by non-permissive CEM-SS cells (Sheehy et al., 2002). In non-permissive cells, A3G is incorporated into budding virions and acts on HIV replication in a post-fusion event in newly infected cells (**Figure 1**). Transfection of A3G in permissive cells leads to abrogated replication of Vif-deficient HIV (HIV Δ*Vif)* (Sheehy et al., 2002) and sequencing of HIV Δ*Vif* DNA revealed a hypermutated pattern with enriched G to A transitions (Lecossier et al., 2003), strongly suggesting that A3G deaminase activity is required for the restriction of HIV replication (Zhang et al., 2003). It is now established that Vif counteracts the antiviral functions of A3G and other A3 family members such as A3F and a certain allele of A3H (Henriet et al., 2009). The action of Vif on A3s has been extensively reviewed (Henriet et al., 2009), but in sum, in infected cells, Vif targets A3G for proteasomal degradation, reducing the amount of A3G incorporated into budding virions (**Figure 1**). Indeed, as a post-fusion event, A3G catalyzes cytosine to uracil deamination on the nascent minus DNA strand of the HIV reverse transcribed genome (Harris et al., 2003; Mangeat et al., 2003; Yu et al., 2004). The presence of uracil on the minus strand of HIV DNA might target HIV DNA for degradation by the cellular DNA repair machinery thus reducing viral replication (Mariani et al., 2003). As exemplified by the existence of hyper-edited sequences retrieved from HIV proviruses *in vivo* (Kieffer et al., 2005), the action of Vif on A3G incorporation/degradation is not absolute and deaminations also lead to G to A transitions in HIV DNA (Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Yu et al., 2004). Editing patterns are dominated by GG to AG hypermutations leading to a high frequency of amino acid substitutions and to the introduction of premature STOP codons (Vartanian et al., 1991). These crippled proviruses express aberrant (i.e., misfolded or truncated) viral proteins that are unable to produce infectious particles (Simm et al., 1995).

Shortly after the discovery of A3G, A3F was shown to restrict HIV replication. A3G and A3F editing is not random. A3G and A3F preferentially introduce mutations in TGG or TGA sequences, respectively. There is a lack of consensus on the ability of other A3 family members to edit HIV [see for a review (Albin and Harris, 2010)]. For instance, over-expression of A3B and A3DE exerts anti-HIV activity in a single cycle assay but insignificant antiviral activity in a spreading infection system (Hache et al., 2008). A3D, F, G, and H but not A3B, C, and DE restrict infection of human CD4+ T lymphocytes by Vif-deficient viruses (Hultquist et al., 2011). A3DE restricts HIV replication in macrophages but to a lower extent than A3G (Chaipan et al., 2013). In myeloid cells, APOBEC3A blocks early steps of reverse transcription but acts when expressed in the target cell of infection (Berger et al., 2011).

There is a general agreement that cytidine deamination plays an important role in the capacity of A3s to restrict viral replication (Schumacher et al., 2008; Browne et al., 2009). However, several reports also suggest that A3G and A3F restrict viral replication to a significant extent through deaminase-independent mechanisms (Newman et al., 2005). A3G and A3F affect reverse transcription priming and extension (Mangeat et al., 2003; Mariani et al., 2003; Guo et al., 2006; Anderson and Hope, 2008; Malim, 2009; Wang et al., 2012; Gillick et al., 2013) and HIV DNA integration into the host genome (Luo et al., 2007; Mbisa et al., 2007; Vetter and D'Aquila, 2009). As proposed by Henriet et al. (2009) interference of A3G and A3F with the viral core assembly might be responsible for these deaminase-independent impairments of HIV replication.

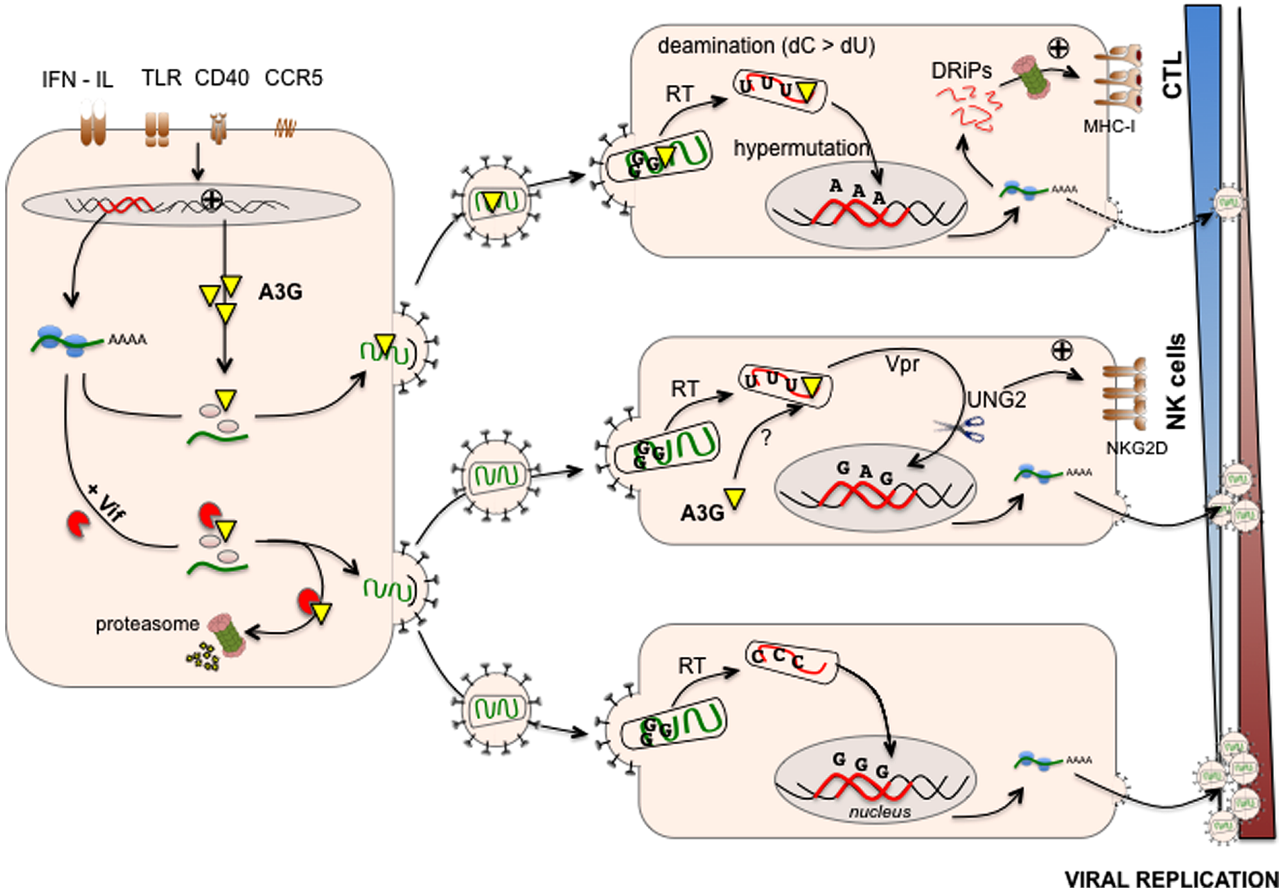

FIGURE 1 | APOBEC3G links innate/intrinsic immunity with adaptive cellular immunity. A3G is expressed at various levels in human immunodeficiency virus (HIV) infected cells and can be induced upon viral sensing by Toll-like receptors (TLR), triggering of CD40 or CCR5 and IFN production **(left)**. A3G is incorporated in budding virions and exerts its antiviral activities in newly infected cells **(right)**. During reverse transcription, A3G catalyzes cytosine to uracil deaminations on the nascent minus DNA strand of HIV. The presence of uracil and the expression of HIV Vpr recruit the cellular DNA repair machinery (UNG2, **right middle**) leading to either HIV DNA restoration or degradation, the latter reducing viral replication. The cellular DNA-repair machinery trying to cope with uracil-rich HIV DNA also activates the DNA-damage- and stress–response pathways leading to the up-regulation of activating natural killer (NK) cell ligands (NKG2D ligands) and killing of infected cells **(right middle)**. In addition, deaminations lead to G to A transitions in HIV DNA leading to the integration of proviruses with a high frequency of amino acid substitutions and/or premature STOP codons. These proviruses express aberrant viral proteins that are unable to produce infectious particles. Infected cells can use these misfolded or truncated viral proteins (called DRiPs, defective ribosomal products) to generate MHC-I epitopes leading to activation of anti-HIV cytotoxic T lymphocyte (CTL) responses **(right upper)**. However, in infected cells, Vif counteracts the antiviral functions of A3G by targeting newly synthetized A3G for proteasomal degradation, thus reducing the amount of A3G incorporated into budding virions **(left)** and facilitating HIV replication in newly infected cells **(right bottom)**. Viral replication is therefore the result of a balance between anti-viral innate and adaptive (cellular) immunity promoted by A3G and Vif-mediated escape mechanisms developed by HIV.

The balance of editing and non-editing-dependent effects of A3G and A3F varies depending on the experimental system and might be affected by their cellular expression levels (Miyagi et al., 2007; Knoepfel et al., 2008; Browne et al., 2009).

INDUCTION OF A3 EXPRESSION

In humans, A3s are not only expressed in a variety of tissues, but their expression is also induced by mediators of inflammation, possibly reflecting their role as a first line of defense against invading viruses (**Figure 1**). IFN-α was reported to enhance A3G and A3A expression in monocytes and macrophages. IFN-γ and -β also induce A3G up-regulation in macrophages (Sarkis et al., 2006; Peng et al., 2007; Stopak et al., 2007; Koning et al., 2009; Refsland et al., 2010; Berger et al., 2011). IFN-α secreted by plasmacytoid dendritic cells (pDC) enhances the expression of A3A, A3C, A3G, and A3F within pDC, indicating that pDC might be armed against viral infection by an autocrine IFN-α loop (Wang et al., 2008). Pathogen sensors such as Toll-like receptors (TLR) also influence A3 gene expression. TLR-3 stimulation by the double stranded RNA analog [poly(I:C)] induces type I IFN responses in DC and subsequent A3G expression (Trapp et al., 2009). Overall, the induction of DC maturation using stimuli such as LPS (a TLR-4 ligand), CCR5 and CD40 ligands correlates with the up-regulation of A3G (Pion et al., 2006; Pido-Lopez et al., 2007; Stopak et al.,

2007). The effect of IFN-α on A3 expression in primary CD4+ T cells is controversial, but in most reports no induction was observed (Rose et al., 2005; Chen et al., 2006; Sarkis et al., 2006; Stopak et al., 2007; Ying et al., 2007; Koning et al., 2009; Refsland et al., 2010). In contrast, IL-2, IL-7, IL-15, and IL-27 and mitogens such as phytohemagglutinin (PHA) and phorbol myristate acetate (PMA) induced modest and strong activation of A3G expression, respectively, (Stopak et al., 2007). Combined with IL-2, PHA induces expression of all A3s except A3A (Greenwell-Wild et al., 2009; Koning et al., 2009; Refsland et al., 2010). Triggering of the T cell receptor (TcR) also induces A3G expression in effector memory T cells and interferes with HIV replication *in vitro* (Pido-Lopez et al., 2009).

The induction of A3 expression is not limited to immune cells. IFN-α secretion, for instance, can induce A3G, A3F, and A3B expression in hepatocytes (Bonvin et al., 2006; Sarkis et al., 2006; Tanaka et al., 2006). In contrast, other factors reduce A3 protein expression. The nerve growth factor (NGF), an essential factor for survival and activation of monocytes/macrophages, is released by HIV infected macrophages and dampens the synthesis of A3G, overriding the IFN-γ-induced upregulation of A3G (Souza et al., 2011).

Overall, the transcriptional regulation of A3 genes seems to play a major role in A3-dependent defenses against viruses. Remarkably, following mucosal immunization of nonhuman primates, two studies reported the up-regulation of A3G in CCR5+ CD4+ memory T cells (Wang et al., 2009) as well as in mucosal DC and CD14+ cells (Sui et al., 2010), all potential cellular targets of HIV replication. A3G mRNA upregulation was maintained over several weeks after immunization and upon challenge with SIV, and correlated inversely with viral loads and positively with a better preservation of CD4+ T cells in the gut (Sui et al., 2010). These studies strongly suggest that A3G provides an antiviral effect *in vivo*. They also demonstrate that mucosal immunization triggers an innate signature that promotes adaptive immune responses. The exact mechanism underlying vaccine-induced A3G expression with protection from SIV infection is not clear. As observed in the murine model of Friend retrovirus (FV) infection (Santiago et al., 2010), by limiting early viral replication, A3G might delay virus-induced immune dysfunction and thus might favor the establishment of humoral [e.g., generation of FV-neutralizing antibodies (Santiago et al., 2010)] and cellular immunity.

A ROLE OF A3G IN THE ACTIVATION OF CELLULAR AND ADAPTIVE IMMUNITY

There is growing evidence that A3G bridges intrinsic and cellular immunity as is the case with AID, that shapes the Ab repertoire, blocks virus-induced cancer by inducing cell cycle arrest and activates NK cells (Gourzi et al., 2006). A3G has been implicated in enhancing NK-cell killing of HIV-infected T cells (Norman et al., 2011). This process requires A3G-editing of HIV DNA (Norman et al., 2011). In this study, the authors propose that the cellular DNA-repair machinery activates DNA-damage- and stress–response pathways in response to uracil-edited HIV DNA, leading to the up-regulation of activating NK cell ligands (NKG2D ligands) and killing of HIV infected cells. The authors also implicate the HIV Vpr protein and its capacity to recruit the DNA-repair machinery as a key factor in NKG2D ligand expression. In contrast, HIV Vif counteracts the action of A3G and the NK cell-mediated elimination of HIV-infected cells (Norman et al., 2011). Interestingly, in the work of Norman et al. (2011), cytoplasmic A3G (expressed by the target cell) seems responsible for HIV editing and thus NK cell recognition. Cytoplasmic A3G has been shown to impact HIV genome integration but has not been assigned a role in HIV genome editing, to our knowledge (Vetter and D'Aquila, 2009). Whether the accumulation of unintegrated HIV genomes or the editing of HIV genomes *per se* might be responsible for the induction of stress responses and thus NK cell recognition remains to be clarified (Norman et al., 2011). Nonetheless, this study suggests that A3G antiviral functions allow triggering of danger signals that, in turn, activate effectors of cellular immunity.

A3G-mediated editing also contributes to the activation of effectors of adaptive immunity (Casartelli et al., 2010), namely, CD8+ cytotoxic T cells (CTLs). CTLs, whose function is critical in the control of HIV infection, are triggered by viral peptides presented by MHC class-I molecules on the surface of infected cells. These antigenic peptides originate from proteasomal degradation of native viral proteins and of defective/abortive proteins generated in the course of translation (Yewdell and Nicchitta, 2006). We made the assumption that hyper-edited proviruses that express aberrant – misfolded or truncated – viral proteins might represent a source of HIV antigens. We demonstrated that A3G enhances cytotoxic T lymphocyte (CTL) recognition of HIV-infected cells (Casartelli et al., 2010). This process requires A3G-editing of HIV DNA as editing–deficient A3G mutants failed to promote CTL activation. Mimicking A3G-editing, we also showed that truncated HIV proteins represent a major source of HIV antigen for CTL activation (Casartelli et al., 2010). However, and as expected, Vif as well as Nef (that down-regulated MHC-I expression) counteracts the action of A3G in antigen generation. Therefore, recognition of HIV-infected cells by CTLs results from a balance between the efficacy of antigen generation enhanced by A3G editing and the immune-escape mechanisms mediated by the virus. Overall, these studies demonstrate that A3G-mediated viral restriction contributes to the immunogenicity of HIV-infected cells and to NK cell as well as CTL activation, thus linking innate and adaptive immunity (**Figure 1**). Whether other APOBEC3 family members enhance NK cell and CTL activation remains to be determined.

IN VIVO INTERACTIONS OF APOBEC3s AND HIV-1

A3s AND CORRELATES OF DISEASE PROGRESSION

Accumulating evidence suggests that A3s play an important role in limiting viral replication *in vivo*. APOBEC3 genes have been subject to strong positive selection throughout the history of primate evolution (Sawyer et al., 2004). In persistent HBV infection, A3 polymorphisms seem to impact liver disease progression and viral loads (Ezzikouri et al., 2013). In the course of HIV infection, A3G plays a critical role in limiting the viral reservoir. A major barrier to an effective cure of HIV infection is the maintenance of a latent viral reservoir in the memory CD4 T cell compartment within HIV-infected, successfully treated

(ST) individuals (Chomont et al., 2009). Examining the state of the latent proviruses in these cells from ST subjects, several studies have established that approximately 30% of HIV proviruses that can not be reactivated *in vitro* carry the hallmark of A3G editing (Ho et al., 2013). Thus, *in vivo*, A3G plays a significant role in rendering the persistent viral reservoir defective, inactive, or "non-inducible." However, whether A3G antiviral functions play a critical role in HIV progression is less clear (Albin and Harris, 2010). Analyzing the mutation patterns of proviral sequences isolated from various cohorts, several studies showed a correlation between hypermutation mediated by either A3G or A3F and reduced viral loads (Pace et al., 2006; Vazquez-Perez et al., 2009; Kourteva et al., 2012) or increased CD4 counts (Land et al., 2008). Consistent with these observations, proviral sequences from HIV-infected long-term non-progressors (LTNP) or viral controllers (elite controllers) seem to harbor an elevated level of hypermutation compared to antiretroviral therapy (ART)-treated or naïve non-controllers (Kourteva et al., 2012; Eyzaguirre et al., 2013). In contrast, others observed no correlation between hypermutation with markers of disease progression (Piantadosi et al., 2009; De Maio et al., 2012) nor with the clinical status such as being elite controllers (Gandhi et al., 2008). These contrasting results might be due to technical issues or the size of the cohorts studied.

However, owing to the editing-independent antiviral activities of A3s, other studies analyzed the association between A3G or A3F expression levels (mostly at the mRNA level) and markers of disease progression. But, again, there is no clear-cut answer. Some studies observed that the mRNA levels of A3G correlate positively with CD4 cell counts (Jin et al., 2005; Vazquez-Perez et al., 2009; Zhao et al., 2010) and inversely with viremia (Jin et al., 2005; Zhao et al., 2010; Kourteva et al., 2012) or the viral set point (that is predictive of disease progression; Ulenga et al., 2008). In addition, exposed uninfected individuals showed greater A3G mRNA levels (Biasin et al., 2007; Vazquez-Perez et al., 2009) and controllers with high A3G protein expression in CD4 T cells seem to harbor fewer HIV proviruses (De Pasquale et al., 2013). However, others observed no correlation between mRNA expression levels of either A3G or A3F and markers of disease progression (Cho et al., 2006; Amoedo et al., 2011). Also, during primary infection no association between A3G expression and viral loads was observed (Reddy et al., 2010) and others did not find greater A3G mRNA levels in exposed uninfected individuals (Mous et al., 2012). Larger cohort studies analyzing side-by-side hypermutation patterns and expression levels of all A3 family members might help in establishing a correlation with clinical parameters.

However, variants of A3 at the genetic levels might also account for limiting disease progression. In a pioneering study, An et al (An et al., 2004) identified a variety of A3G polymorphisms within introns and exons that correlate with clinical parameters. A3G H186R identified in this study was shown in various cohort studies to correlate with disease progression (An et al., 2004; Reddy et al., 2010; Singh et al., 2013). The variant H186R of A3G is also associated with low CD4 counts (Bunupuradah et al., 2012) and a polymorphism within an A3G intron (C40693T) was shown to correlate with increased risk of infection (Valcke et al., 2006).

Other members of the APOBEC3 family present common polymorphisms such as A3H and A3B. In contrast to A3G, A3H anti-HIV activity is strongly influenced by its polymorphisms (Ooms et al., 2013) with some specific alleles associated with lower viremia (Gourraud et al., 2011). A3B is deleted in ∼20% of the world's population. An early study based on a large number of U.S. patients showed that a homozygous deletion of A3B was associated with increased susceptibility to HIV acquisition and progression to AIDS (An et al., 2009). However, a more recent study on Japanese individuals did not observe such a correlation (Imahashi et al., 2014).

Whether A3 family members influence viral transmission and disease progression remains an open question. In addition, the underlying mechanisms are not clear, such as whether A3G H186R and C40693T differentially impact HIV replication, and to date, this has not been experimentally demonstrated.

A3-MEDIATED EDITING AND VIRAL DIVERSIFICATION/ADAPTATION

A3 antiviral activities, and, in particular, editing, might also facilitate HIV survival by introducing sub-lethal mutations that, in turn, favor HIV diversification and adaptation to ART and/or immune responses. The action of Vif on A3 degradation is not absolute thus allowing A3 incorporation and subsequent sub-lethal editing (Sadler et al., 2010). Evidence of this phenomenon was provided by the demonstration that a Vif allele carrying the K22H mutation, less effective in counteracting A3-mediated editing, was prevalent in a cohort of ART-treated patients experiencing virological failure (Fourati et al., 2010). In this study, several drug resistance mutations in reverse transcriptase (RT) and in the protease, were significantly more common in patients harboring elevated levels of K22H-mutated viruses (Fourati et al., 2010). The expression of K22H-Vif might favor adaptation to antiviral drugs by allowing residual A3-mediated deamination and introduction of mutations into HIV genome. A parallel might be drawn here with hypermutator strains of bacteria that adapt more rapidly to antibiotic. These hypermutator strains of bacteria have mutations in genes affecting DNA repair and replication fidelity and exhibit elevated mutation rates (Woodford and Ellington, 2007). Cytidine deamination might directly promote mutations that generate resistance to drugs. For example, the common M184V mutation of RT that causes resistance to 3TC is located in an A3G editing hotspot and is produced *in vitro* by A3G during HIV replication (Mulder et al., 2008; Kim et al., 2010). A3G-mediated sub-lethal editing might also favor mutations within sequences encoding HIV CTL epitopes thus allowing the virus to escape CTL-recognition (Wood et al., 2009; Monajemi et al., 2014). In contrast, cytidine deamination outside HIV CTL epitopes (especially downstream of the epitope) might favor the expression of unstable aberrant or truncated HIV proteins and thus HIV-specific CTL activation (Casartelli et al., 2010).

On the other hand, A3G-editing at physiological levels might be simply lethal to HIV. Armitage et al. (2012) showed, in an *in vitro* study, that even a single A3G molecule within one HIV particle is likely to cause extensive and inactivating levels of HIV hypermutation (Armitage et al., 2012). The authors

suggest that A3-mediated hypermutation might be a discrete "all or nothing" phenomenon (Armitage et al., 2012). The overall impact of A3-mediated hypermutation on HIV survival and adaptation might also vary depending on the anatomical site (Fourati et al., 2014).

CONCLUDING REMARKS

The AID-APOBEC family members evolved from a family of cytidine deaminases to fight viral infection. They constitute an important arm of innate immunity by restricting replication and spread of a plethora of viral infections. Although establishing correlates of disease progression with hypermutation or expression levels as a surrogate of APOBEC antiviral functions has proven to be complex in HIV infection, *in vivo* defective proviral sequences clearly bear the hallmark of APOBEC editing. AID-APOBEC family members also participate in long-term adaptive immunity to viral infections. AID is critical for the generation of B cells that secrete high affinity Abs with various effector functions. A3G contributes in activating NK cell and CTL responses leading to the elimination of HIV-infected cells. Remarkably, the expression levels of AID-APOBEC family members are increased upon the sensing of infection and a tight regulation is required to avoid deleterious editing of host genomes.

Manipulating AID-APOBECs with drugs might constitute promising approaches to fight infections. Strategies have been proposed to increase A3 expression to favor encapsidation within viral particles thus overriding the antagonistic activity of Vif and controlling HIV-1 infection [reviewed in (Albin and Harris, 2010)]. The antiviral properties of Vif inhibitors are currently being evaluated (Ali et al., 2012). A3G expression can be manipulated either by limiting its proteasomal degradation (Ejima et al., 2011), or exacerbated to supraphysiologic levels. Treatment of HIV/HCV co-infected patients with IFN-α, for instance, increases mRNA expression of A3G/F and correlates with the degree of HIV hypermutation (Pillai et al., 2012). Upon vaccination, A3 expresssion can also be induced promoting adaptive immune responses (Wang et al., 2009; Sui et al., 2010). Taken together, these findings further highlight the complex and intriguing interactions of AID-APOBEC family members with the immune system.

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New insights into an X-traordinary viral protein

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Vpx is a protein encoded by members of the HIV-2/SIVsmm and SIVrcm/SIVmnd-2 lineages of primate lentiviruses, and is packaged into viral particles. Vpx plays a critical role during the early steps of the viral life cycle and has been shown to counteract SAMHD1, a restriction factor in myeloid and resting T cells. However, it is becoming evident that Vpx is a multifunctional protein in that SAMHD1 antagonism is likely not its sole role. This review summarizes the current knowledge on this X-traordinary protein.

Keywords: vpx, HIV-2, SIVsmm, SAMHD1, myeloid cells, HIV-1, restriction factor, interferon type I

Vpx was initially identified as an HIV-2 (human immunodeficiency virus type 2)/SIVsmm (simian immunodeficiency virus infecting sooty mangabey monkey) protein of 12–16 kDa, which is incorporated into viral particles (Franchini et al., 1988; Henderson et al., 1988; Kappes et al., 1988; Yu et al., 1988). In addition to viruses from the HIV-2/SIVsmm lineage of primate lentiviruses, this gene is also found in viruses from the SIVrcm (infecting red-capped mangebey)/SIVmnd-2 (infecting mandrill) lineage (Beer et al., 2001; Hu et al., 2003). It is homologous to the *vpr* gene, found in every lineage of primate lentiviruses (Tristem et al., 1990). Vpx was rapidly shown to be dispensable for viral replication in immortalized lymphocytic cell lines, such as HUT78, CEM, or SupT1 (Yu et al., 1988; Guyader et al., 1989; Hu et al., 1989; Shibata et al., 1990; Gibbs et al., 1994; Park and Sodroski, 1995), and in the monocytic cell lines HL60 and U937 (Guyader et al., 1989; Hu et al., 1989). In contrast, *vpx* deletion led to a strong replication defect in monocyte-derived macrophages (MDMs) (Yu et al., 1991; Gibbs et al., 1994; Park and Sodroski, 1995; Fletcher et al., 1996; Ueno et al., 2003). In addition, *vpx* deletion led to SIVmac (infecting rhesus monkey) and HIV-2 replication defects in activated peripheral blood mononuclear cells (PBMCs) or primary T cells, especially at low viral inputs (Guyader et al., 1989; Kappes et al., 1991; Yu et al., 1991; Akari et al., 1992; Gibbs et al., 1994; Kawamura et al., 1994; Park and Sodroski, 1995; Ueno et al., 2003). Vpx was shown to be important for HIV-2 replication in HSC-F cells, a simian lymphocytic cell line (Ueno et al., 2003). Vpx is packaged into viral particles *via* an interaction with the p6 domain of Gag (Wu et al., 1994; Accola et al., 1999; Selig et al., 1999) and is associated with mature viral cores (Kewalramani and Emerman, 1996). This suggested that Vpx could participate in the early steps of infection. Comparisons of virus associated proteins suggested that Vpx from SIVmac and HIV-2 are packaged in equimolar amounts to Gag (Henderson et al., 1988), although the exact number of molecules packaged per virion has not been determined.

Vpx localizes to the nucleus in transfected cells (Depienne et al., 2000; Mahalingam et al., 2001; Belshan and Ratner, 2003), and this is conferred by a C-terminal non-canonical nuclear localization signal (NLS) (65-SYTKYRYL-72) (**Figure 1**) (Belshan and Ratner, 2003; Rajendra Kumar et al., 2003), as well as a potential second N-terminal NLS (Singhal et al., 2006a). Whether Vpx shuttles between the cytoplasm and nucleus due to a nuclear export signal remains controversial (Belshan and Ratner, 2003; Singhal et al., 2006b). Likewise Vpx phosphorylation has been proposed to regulate its nuclear import (Rajendra Kumar et al., 2005) but other studies failed to detect this post-translational modification (Franchini et al., 1988; Belshan et al., 2006). By virtue of its karyophilic properties, Vpx was proposed to play a critical role in the nuclear import of viral reverse transcription complexes in non-dividing cells, such as MDMs and arrested U937 cells (Pancio et al., 2000; Mahalingam et al., 2001; Rajendra Kumar et al., 2003). Indeed the replication defect of viruses lacking Vpx (or bearing non-karyophilic mutated versions of Vpx) correlated with the absence of 2-LTR circles (a surrogate marker for viral DNA nuclear entry) (Fletcher et al., 1996; Pancio et al., 2000; Ueno et al., 2003; Belshan et al., 2006).

Later studies using lentiviral vectors and single-round infections confirmed a cell-type dependent effect of Vpx and a role in the early events of infection. Vpx is essential for transduction of monocyte-derived dendritic cells (MDDCs) with SIVmac based-lentiviral vectors (Mangeot et al., 2002). Surprisingly, when brought *in trans via* virus-like particles (VLPs), Vpx increases HIV-1 transduction of MDDCs and MDMs but not activated T cells (Goujon et al., 2006). This positive effect of Vpx in MDDCs was directly correlated with an increase in viral DNA accumulation, which was observed not only with SIVmac but also with heterologous retroviral vectors, derived from HIV-1, feline immunodeficiency virus (FIV) and murine leukemia virus (MLV) (Goujon et al., 2007). Of note, in the case of MLV, Vpx rescued viral DNA accumulation but not 2-LTR circle formation

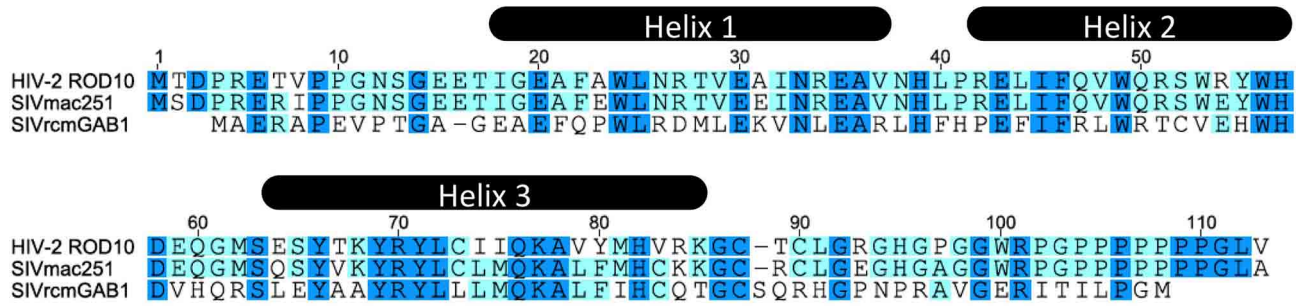

FIGURE 1 | HIV-2 ROD, SIVmac, and SIVrcm Vpx aminoacid sequence alignment.

(Goujon et al., 2007; Gramberg et al., 2013), consistent with a nuclear-entry block to MLV infection in non-dividing cells (Roe et al., 1993; Lewis and Emerman, 1994). Vpx was later shown to favor HIV-2/SIVsmm DNA accumulation in MDMs (Fujita et al., 2008; Srivastava et al., 2008; Bergamaschi et al., 2009).

Animal studies showed that Vpx is crucial for SIVsmm PBj and SIVmne (infecting pig-tailed macaque) replication and spread in a pig-tailed macaque model. SIVsmm PBj *vpx* mutant virus replicated to a considerably lower extent and showed much reduced kinetics as compared to wild-type virus (Hirsch et al., 1998). In addition, the mutant virus was outcompeted when inoculated together with wild-type SIVsmm PBj (Hirsch et al., 1998). *In vitro*, SIVmne *vpx* mutants infected pig-tailed macaque PBMCs to comparable levels to that of wild-type virus, but showed significantly reduced infectivity in MDMs (Belshan et al., 2012). Another study reported a less prominent effect of Vpx in rhesus monkeys infected with SIVmac239, with delayed viral kinetics for *vpx* deficient virus but AIDS development nonetheless (Gibbs et al., 1995).

The fact that Vpx could act *in trans* on other retroviruses strongly suggested at the time that Vpx modulates the cellular environment to increase cell permissivity to infection, possibly by preventing the action of an inhibitory factor. The first evidence of the existence of such a dominant inhibitory factor in myeloid cells came from the use of heterokaryons generated between permissive (COS) cells and restrictive cells (MDMs) (Sharova et al., 2008). Unlike COS cells, both MDMs and COS-MDMs heterokaryons restricted SIVsmm PBj in the absence of Vpx at the level of viral DNA accumulation. The same authors also observed a dominant restriction phenotype in heterokaryons formed between primary monocytes and HeLa cells (Kaushik et al., 2009).

HIJACKING THE DCAF1/DDB1/CUL4A E3 UBIQUITIN LIGASE

In parallel with the aforementioned viral and cell biology experiments, proteomic studies yielded some fundamental insights. Initially, Le Rouzic et al. discovered that HIV-1 Vpr recruits the damage-specific DNA binding protein 1 (DDB1)-Cullin 4A (CUL4A) E3 ubiquitin ligase complex through the binding of a known interactor of Vpr, VprBP (Zhao et al., 1994; Le Rouzic et al., 2007). VprBP had previously been identified in a screen as the substrate recruiting module of DDB1-CUL4A-RBX1/ROC1 complexes, and renamed DDB1-CUL4A-associated factor 1 (DCAF1) (Angers et al., 2006). Using yeast two-hybrid, Le Rouzic et al. also showed that Vpx from SIVmac, similarly to HIV-1 Vpr, was able to bind VprBP/DCAF1 (Le Rouzic et al., 2007). This interaction was soon confirmed in mammalian cells (Goujon et al., 2008; Sharova et al., 2008; Srivastava et al., 2008; Bergamaschi et al., 2009). RNAi-mediated depletion of DCAF1 or DDB1 profoundly reduced SIVmac as well as HIV-2 infection of macrophages (Sharova et al., 2008; Srivastava et al., 2008; Bergamaschi et al., 2009). This invoked a model by which Vpx was hijacking a DCAF1-DDB1-CUL4A E3 ubiquitin ligase to induce the degradation of a myeloid cell specific restriction factor that prevents viral DNA accumulation (Sharova et al., 2008; Srivastava et al., 2008; Bergamaschi et al., 2009).

SAMHD1 ANTAGONISM

To isolate the cellular factor(s) limiting HIV-1 infection in myeloid cells, the groups of Monsef Benkirane and Jacek Skowronski exploited similar proteomic approaches, using tandem affinity purification combined with mass spectrometry to identify Vpx binding partners (Hrecka et al., 2011; Laguette et al., 2011). Both labs identified sterile alpha motif (SAM) and HD-domain-containing protein 1 (SAMHD1) as one of the major binding partners of SIVmac Vpx. SAMHD1 silencing phenocopied the effect of Vpx-containing VLPs in that it rescued HIV-1 infection of both differentiated THP-1 monocytic cells and MDDCs (Laguette et al., 2011). Both studies showed that HIV-2/SIVmac Vpx induced SAMHD1 proteasomal degradation (Hrecka et al., 2011; Laguette et al., 2011). Importantly, SAMHD1 antagonism is a function conserved in all clades of Vpx proteins, in a species specific way (Lim et al., 2012). In certain SIVs devoid of a Vpx protein, Vpr can antagonize SAMHD1. For instance, Vpr from SIVdebCM5 (infecting De Brazza's monkeys), SIVagm (African green monkeys), and SIVmus1 (mustached monkeys) can degrade SAMHD1 of their natural host when expressed in human cells, whereas Vpr from HIV-1 and SIVcpz (chimpanzee) cannot (Lim et al., 2012).

SAMHD1 is a 626 amino acid protein that consists of an amino-terminal SAM domain, a central HD domain and a C-terminal uncharacterized domain (Li et al., 2000; Liao et al., 2008). SAMHD1 is a deoxynucleoside-triphosphate (dNTP) phosphohydrolase (Goldstone et al., 2011; Powell et al., 2011; Yan et al., 2013), which reduces the pool of dNTPs available for reverse transcription both in myeloid cells (Lahouassa et al., 2012;

St Gelais et al., 2012) and in resting T cells (Baldauf et al., 2012; Descours et al., 2012). These observations therefore explain why the provision of exogenous deoxyribonucleosides (dN) to resting T cells, which have long been known to restrict lentiviral infection at several steps of the life cycle (Pan et al., 2013), increases the accumulation of viral DNA (Korin and Zack, 1999).

Crosslinking experiments show that SAMHD1 forms oligomers in cells and it has been proposed that the enzymatically active form of SAMHD1 is a tetramer and that tetramerization is driven by dGTP binding to the allosteric sites (Ji et al., 2013; Yan et al., 2013). Knock-out mice analysis have recently confirmed that SAMHD1 functions as a dNTPase *in vivo* as these mice show elevated levels of intracellular dNTPs in DCs isolated from bone marrow (Behrendt et al., 2013; Rehwinkel et al., 2013). Therefore, SAMHD1 reduces cellular dNTP levels and this impacts reverse transcription most significantly in situations where dNTP levels are naturally lower, such as post-mitotic or non-dividing cells.

Mutations in SAMHD1 are associated with the genetic neurodegenerative disorder Aicardi-Goutières syndrome (AGS), which is characterized by the excessive production of type 1 interferon (IFN) in the cerebrospinal fluid and resembles congenital infection (Rice et al., 2009; Chahwan and Chahwan, 2012). Interestingly, CD14+ cells from AGS patients with mutations in *SAMHD1* are more susceptible to HIV-1 infection than cells from healthy controls (Berger et al., 2011a). This shows the importance of SAMHD1 for preventing HIV-1 infection in monocytes, well known to be naturally refractory to HIV-1 infection *in vitro* (Sonza et al., 1996; Neil et al., 2001; Triques and Stevenson, 2004).

In addition to a variety of retroviruses (Gramberg et al., 2013; Sze et al., 2013), SAMHD1 blocks replication of DNA viruses, such as vaccinia virus and herpex simplex virus 1 (Hollenbaugh et al., 2013; Kim et al., 2013). SAMHD1 is also active against retroelements (Zhao et al., 2013), suggesting that increased retrotransposition might be leading to immune sensing of DNA and activation of signaling pathways in AGS patients with *SAMHD1* mutations, as proposed for the 3′-5′ exonuclease TREX1 (three prime repair exonuclease) (Stetson et al., 2008; Beck-Engeser et al., 2011). Indeed, mutations in *TREX1* can cause AGS (Crow et al., 2006) and in *Trex1*-deficient mice, reverse transcribed DNA from endogenous retroelements accumulated and stimulated intrinsic immune responses (Stetson et al., 2008). Interestingly, Zhao et al. showed that both endogenous and overexpressed SAMHD1 prevented LINE-1 (long interspersed element-1) retrotransposition in 293T cells (Zhao et al., 2013). This effect of SAMHD1 on retrotransposition was not dependent on its catalytic activity and was counteracted by Vpx. Therefore, infection by lentiviruses encoding a SAMHD1 antagonist (either Vpx or Vpr) might lead to increased replication of endogenous retroelements and a possible impact on host genome stability.

SAMHD1 is expressed at similar levels in MDMs, resting CD4+ T cells and in activated CD4+ T cells (Baldauf et al., 2012; Descours et al., 2012), but does not block HIV-1 infection in the latter. The discrepancy of SAMHD1's antiviral function in cycling vs. non-cycling cells led several groups to investigate cell cycle dependent determinants for both the antiviral and dNTPase activities of SAMHD1. SAMHD1 was found to interact with and be phosphorylated by the cell cycle regulator cyclin-dependent kinase 1 (CDK1) in proliferating cells, and phosphorylation at the residue T592 has been shown to prevent lentiviral restriction (Cribier et al., 2013; White et al., 2013). CDK1 is inactive in resting cells, suggesting that the cell cycle progression correlates with SAMHD1's antiviral activity. In line with this, a phosphorylation-defective mutant of SAMHD1 was antiviral both in resting and in dividing U937 cells (Cribier et al., 2013). In addition, the phosphomimetic SAMHD1 mutant T592E was unable to restrict HIV-1 infection (Welbourn et al., 2013; White et al., 2013). However, phosphorylation did not affect the ability of SAMHD1 to hydrolyse dNTPs in an *in vitro* dNTPase assay (Welbourn et al., 2013) or in differentiated U937 monocytic cells (White et al., 2013). Although phosphorylated and lacking antiviral activity in cycling cells, SAMHD1's role in dNTP metabolism in these cells remains unclear. Cycling cells contain high levels of dNTPs (Diamond et al., 2004), therefore *de novo* dNTP synthesis likely compensates for any potential effect of SAMHD1. The fact that SAMHD1 mutant T592E lacks antiviral activity while being an active dNTPase suggests the existence of a potential dNTPase-independent restriction mechanism. Supporting the notion that SAMHD1's influence on infection may be more complex, it has been suggested that SAMHD1 interacts with nucleic acids, specifically ssRNA and ssDNA (Goncalves et al., 2012; Tungler et al., 2013) and that it possesses a nuclease activity (Beloglazova et al., 2013).

SAMHD1 is localized in the nucleus of differentiated cells. However, disruption of its NLS does not affect antiviral activity (Rice et al., 2009; Brandariz-Nunez et al., 2012; Hofmann et al., 2012), yet results in a relative resistance to SIVmac Vpx-mediated degradation (Brandariz-Nunez et al., 2012; Hofmann et al., 2012; Wei et al., 2012; Guo et al., 2013). Given that Vpx efficiently interacts with both nuclear and cytoplasmic SAMHD1 (Hofmann et al., 2012), it is difficult to understand why cytoplasmic SAMHD1 is less sensitive to Vpx-induced degradation. It is possible for example that factors of the DCAF1-DDB1-CUL4A E3 ubiquitin ligase machinery are limiting in the cytoplasm, or that differences in SAMHD1's post-translational modifications prevent cytoplasmic SAMHD1 degradation. Alternatively there might be differences in ubiquitination or deubiquitination processes between nuclear and cytoplasmic SAMHD1.

Intriguingly, in contrast to what is observed in MDDCs, wild-type SAMHD1 from myeloid and plasmacytoid DCs (mDCs and pDCs, respectively), was shown to be resistant to Vpx-induced degradation (Bloch et al., 2014). The sensitivity to Vpx-induced degradation of SAMHD1 as well as the Vpx effect on HIV-1 infectivity could be partially restored by blocking IFN signaling using neutralizing antibodies (Bloch et al., 2014). This suggests that IFN-induced factors might prevent SAMHD1 degradation in mDCs and pDCs or that SAMHD1 localization might be modified following IFN exposure, rendering it resistant to Vpx-mediated degradation. In line with this, Dragin et al. observed a reduced sensitivity of SAMHD1 to Vpx-mediated degradation in IFN-treated THP-1, suggesting that IFN-stimulated genes may participate in this process (Dragin et al., 2013). In addition, IFN treatment did not modify SAMHD1 localization (Dragin et al., 2013). In contrast, we have observed an efficient degradation of SAMHD1 in IFN-treated THP-1 cells (Goujon et al., 2013), but

the reasons for these differences are currently unknown. Type 1 IFN treatment reduced SAMHD1 phosphorylation levels of residue T592 in MDMs and MDDCs, suggesting the existence of IFN-inducible phosphatase(s) activating SAMHD1 (Cribier et al., 2013). The identification of this/these phosphatase(s) would improve our understanding of the regulation of SAMHD1's antiviral activity.

STRUCTURAL INSIGHTS

Vpx recruits the DCAF1-DDB1-CUL4A E3 ubiquitin ligase complex to induce SAMHD1's proteasomal degradation *via* an interaction with its C-terminal domain (Ahn et al., 2012) (**Figure 2**). The crystal structure of a complex between SIVsmm Vpx, the C-terminal domain of the ubiquitin-ligase adaptor DCAF1 (DCAF1-CtD) and the Vpx-binding, C-terminal domain of SAMHD1 (SAMHD1-CtD, residues 582-626) was revealed recently (**Figure 3**) (Schwefel et al., 2014). This structure shows that Vpx is composed of a three-helix bundle stabilized by a zinc finger motif (formed of residues H39, H82, C87 and C89) (**Figure 4A**) and extensively interacts with DCAF1-CtD (**Figure 4B**), the latter forming a seven-bladed β-propeller disc-shaped molecule. Both proteins offer a shared interface used to bind SAMHD1-CtD. A model of the hijacked DCAF1-DDB1-CUL4A complex in association with the RBX1 RING module (RING-box protein 1), generated using previously determined structures, clearly showed that SAMHD1 is positioned in the vicinity of the RBX1 RING module, allowing accessibility to ubiquitination (Schwefel et al., 2014) (**Figure 2**). The SAMHD1 target sites for Vpx-induced ubiquitination have not been determined yet. In addition to ubiquitination, neddylation is required for SAMHD1 degradation, consistent with the importance of NEDD8 transfer to CUL4A through UBC12 (Hofmann et al., 2013). It would be of interest to understand the structural consequences of Vpx interaction with tetrameric SAMHD1. There is some evidence from surface plasmon resonance experiments that Vpx interaction with SAMHD1 causes the disassembly of enzymatically active SAMHD1 oligomers and interferes with SAMHD1 enzymatic activity prior to its degradation (Delucia et al., 2013). This suggests a model where proteasomal degradation may be the consequence and not the initiating event in Vpx-mediated SAMHD1 antagonism.

Extensive mutagenesis of Vpx has shed light into the critical residues of Vpx (**Table 1** and **Figure 4**). Notably, N-terminal Vpx amino acids are essential to rescue virus infectivity in myeloid cells and SAMHD1 degradation (Goujon et al., 2008; Gramberg et al., 2010; Ahn et al., 2012; Fregoso et al., 2013). Of major interest, the phenotypes of some well characterized Vpx mutants are explained by the structure (Schwefel et al., 2014). For instance, the Vpx Q76A mutant is unable to bind DCAF1 and degrade SAMHD1 (Srivastava et al., 2008; Bergamaschi et al., 2009; Hrecka et al., 2011). The structure shows that this defect may be due to disrupted hydrogen bonds between Q76 and residues N1135 and W1156 of DCAF1 (**Figure 4B**) (Schwefel et al., 2014). Similarly, residue K77, critical for DCAF1 binding and Vpx activity (Bergamaschi et al., 2009), is integral to an extensive salt-bridge network that links residues E1091-D1092-E1093 of DCAF1 with residues R70, Y69 and Y66 in helix 3 of

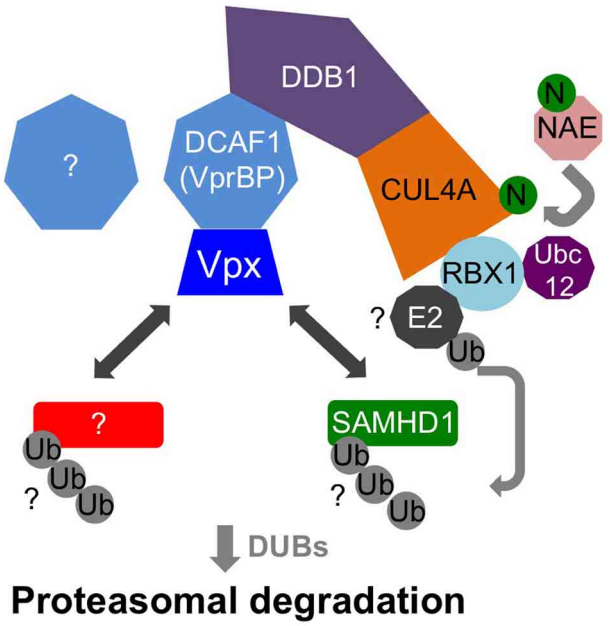

FIGURE 2 | Model of DCAF1-DDB1-CUL4A recruitment by Vpx. Vpx hijacks the DDB1-CUL4A-RBX1 E3 ubiquitin ligase complex through recruitment of DCAF1 (also known as VprBP). Vpx targets (i.e., SAMHD1 and possibly unknown proteins) are ubiquitinated through the activity of RBX1 and an E2 ligase that interacts with RBX1. In addition, RBX1 interacts with the ubiquitin-conjugating enzyme UBC12, which is important for neddylation of CUL4A through transfer of the NEDD8 group from NEDD8 activating enzyme (NAE).

Vpx (**Figure 4B**). In addition, Vpx W24A mutant was shown to have lost SAMHD1 antagonism while being able to bind to DCAF1 (Wei et al., 2012). This phenotype is explained by a stacking between residues W24 of Vpx and R617 of SAMHD1 (**Figure 4C**) (Schwefel et al., 2014). In line with this, SAMHD1 R617 mutants completely lost sensitivity to Vpx-mediated degradation while maintaining antiviral activity (Schwefel et al., 2014).

ADDITIONAL ROLES OF Vpx?

The fact that *vpx* deletion leads to some replication defects in activated PBMCs or primary T cells (Guyader et al., 1989; Kappes et al., 1991; Yu et al., 1991; Akari et al., 1992; Gibbs et al., 1994; Kawamura et al., 1994; Park and Sodroski, 1995; Ueno et al., 2003) strongly suggests that Vpx might have other functions beyond counteracting SAMHD1, which is only relevant in myeloid and resting T cells. As mentioned above, it has been proposed that Vpx participates in the nuclear import of viral reverse transcription complexes (Fletcher et al., 1996; Pancio et al., 2000; Ueno et al., 2003; Belshan et al., 2006). Possibly in favor of such an additional role of Vpx, some Vpx mutants have a more profound effect on MDDCs transduction with SIVmac lentiviral vectors than with HIV-1 (e.g., T17A, T28A, or GC86,87A Vpx mutants which fail to rescue SIVmac infection but improve HIV-1 infection by more than one order of magnitude Goujon et al., 2008; **Table 1**). Moreover, a Vpx mutant devoid of the C-terminus proline-rich

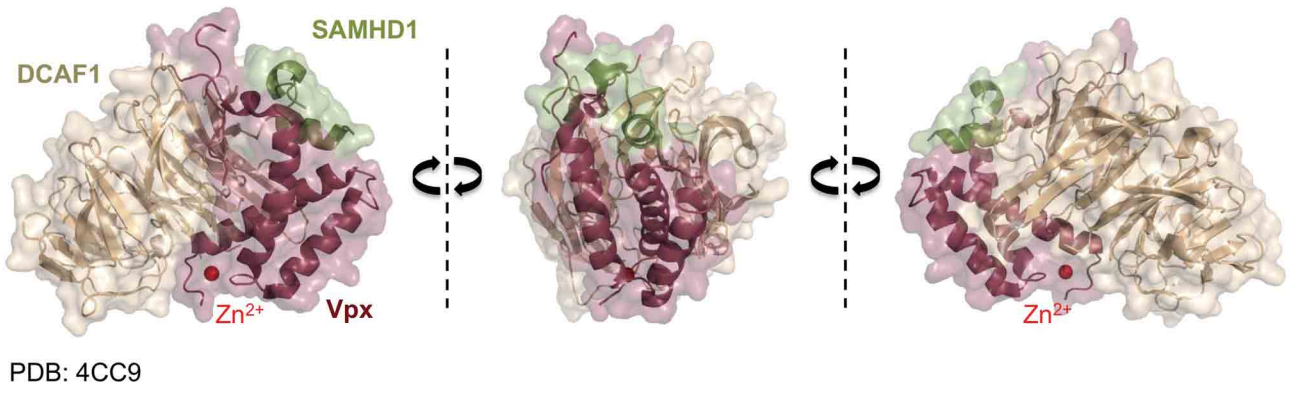

FIGURE 3 | Vpx/SAMHD1 CtD/DCAF1 CtD complex structure. Shown is the co-crystal structure of DCAF1 CtD (brown) with SAMHD1 CtD (green) in complex with Vpx from SIVsmm (dark red) (Schwefel et al., 2014) (PDB:4CC9) from three different sites. The zinc ion coordinated by Vpx is shown in red.

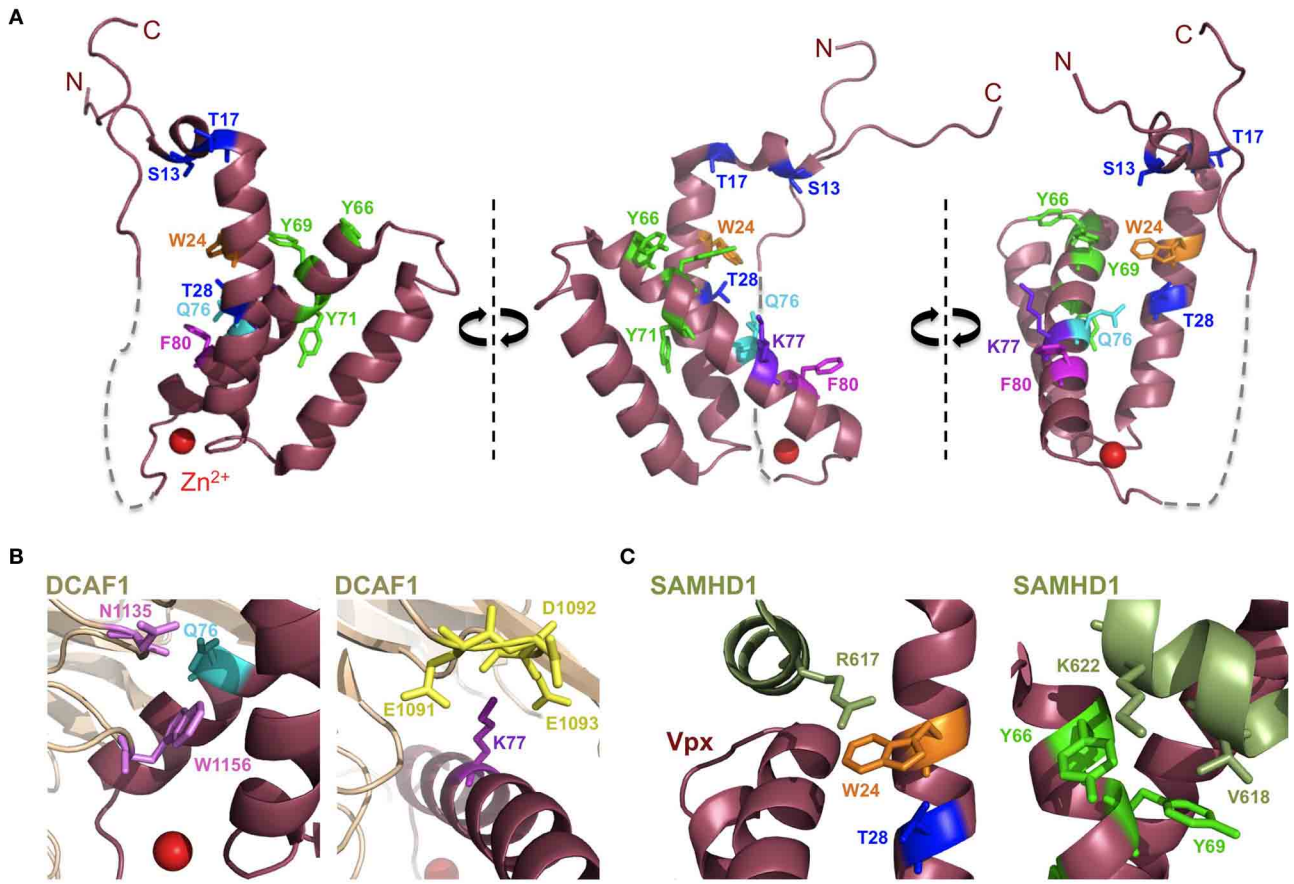

FIGURE 4 | Vpx structure. (A) Depicted is the structure of Vpx from SIVsmm as obtained in the co-crystallization with the C-terminus of DCAF1 and the C-terminus of SAMHD1 (both not shown) with critical amino acids highlighted (PDB:4CC9) (Schwefel et al., 2014). SIVsmm Vpx comprises an anti-parallel bundle of three helices, resembling the shape of a V. Vpx coordinates a zinc ion (red) with amino acids H39, H82, C87, and C89. The structure between Vpx residues 90–100 is absent (dashed gray line). **(B)** Critical aminoacid residues in the interface between DCAF1 and Vpx are highlighted. Vpx Q76 residue (cyan) may form hydrogen bonds with N1135 and W1156 residues (lilac) of DCAF1. The acidic region in DCAF1 comprising E1091, D1092 and E1093 residues (yellow) may interact with positively charged K77 residue (purple) of Vpx. **(C)** Critical amino acid residues for the interaction of SAMHD1 and Vpx are highlighted. SAMHD1 R617 residue (light green) stacks with the indole ring of Vpx W24 residue (orange). Vpx Y66 and Y69 residues (fluorescent green) are in close proximity to SAMHD1 K622 and V618 residues (light green), the latter being positively selected (Laguette et al., 2012).

Table 1 | Phenotype of Vpx mutants.

Mutant	Virus	Phenotype	References
N12A	SIVmac	No infection rescue of HIV-1 in MDMs Partial SAMHD1 degradation	Gramberg et al., 2010; Ahn et al., 2012
S13A	SIVmac	Rescue SIVmac and HIV-1 in MDMs and MDDCs SAMHD1 degradation	Goujon et al., 2008; Gramberg et al., 2010; Berger et al., 2012
E15A	SIVmac	No rescue of HIV-1 in MDMs Partial SAMHD1 degradation	Gramberg et al., 2010; Ahn et al., 2012
E16A	SIVmac	No rescue of HIV-1 in MDMs Partial SAMHD1 degradation	Gramberg et al., 2010; Ahn et al., 2012
T17A	SIVmac	No rescue of SIVmac in MDDCs but partial activity on HIV-1, no rescue in MDMs Partial SAMHD1 degradation	Goujon et al., 2008; Gramberg et al., 2010; Ahn et al., 2012
E20A	SIVmac	Rescue of HIV-1 in MDMs	Gramberg et al., 2010
W24A	SIVmac	No infection rescue, interaction with DCAF1	Wei et al., 2012
N26A	SIVmac	Rescue of SIVmac and HIV-1 in MDDCs	Goujon et al., 2008
R27A	SIVmac	Rescue of HIV-1 in MDMs	Gramberg et al., 2010
T28A	SIVmac	No rescue of SIVmac in MDDCs but partial activity on HIV-1	Goujon et al., 2008
V29S	SIVmac	Reduced interaction with DCAF1 Interaction with SAMHD1 but no degradation, no infection rescue	Wei et al., 2012
STT13,17,28A	SIVmac	No infection rescue in MDDCs with SIVmac or HIV-1, no SAMHD1 degradation	Goujon et al., 2008
I32S	SIVmac	No infection rescue, no interaction with DCAF1, no degradation of SAMHD1	Wei et al., 2012
H39A	SIVmac, HIV-2 GH-1	No infection rescue, no interaction with DCAF1, no degradation of SAMHD1	Goujon et al., 2008; Wei et al., 2012
W49,53,56A	SIVmac	No infection rescue in MDDCs with SIVmac or HIV-1 No degradation of SAMHD1	Goujon et al., 2008; Berger et al., 2012
S52A	SIVmac HIV-2 GH-1	Infection rescue in MDDCs with SIV or HIV-1, HIV-2GL-AN replication in MDDCs SAMHD1 degradation	Goujon et al., 2008; Berger et al., 2012
S63,65A	SIVmac	Infection rescue in MDDCs with SIV or HIV-1	Goujon et al., 2008
KK84,85A	SIVmac, HIV-2 GH-1	Infection rescue in MDDCs with HIV-1, SAMHD1 degradation Slightly reduced activity for SIVmac and HIV-2GL-AN replication in MDDCs	Goujon et al., 2008; Berger et al., 2012
KK84,85R	SIVsm PBj1.9	Defect in DNA accumulation in MDMs	Sharova et al., 2008
K77A	SIVsmPBj	No infection rescue, no interaction with DCAF1	Bergamaschi et al., 2009
KK68,77A or R	SIVmac SIVsm PBj1.9	No infection rescue in MDDCs or MDMs infection with SIVmac, SIVSM PBj1.9 or HIV-1 No degradation of SAMHD1	Goujon et al., 2008; Sharova et al., 2008; Berger et al., 2012
KKKR68,77,84,85A or R	HIV-2 GH-1, SIVsm PBj1.9	No infection of MDMs, no replication of HIV-2 GL-AN in MDDCs	Goujon et al., 2008; Sharova et al., 2008
Y66,69,71A	SIVmac, SIVmne	No infection rescue, no interaction with DCAF1, no degradation of SAMHD1	Goujon et al., 2008; Belshan et al., 2012; Berger et al., 2012
Q76A or R	SIVmac, HIV-2 ROD, HIV-2 GH-1	No infection rescue, no interaction with DCAF1, no degradation of SAMHD1 BUT rescue of HIV-1 from IFN block	Srivastava et al., 2008; Hrecka et al., 2011; Berger et al., 2012 Bergamaschi et al., 2009; Laguette et al., 2011; Pertel et al., 2011 Wei et al., 2012
F80A	SIVmac	No infection rescue, no interaction with DCAF1, no degradation of SAMHD1 BUT rescue of HIV-1 from IFN block	Srivastava et al., 2008; Laguette et al., 2011; Pertel et al., 2011
GC86,87A	SIVmac	No rescue of MDDCs infection with SIVmac but partial activity on HIV-1	Goujon et al., 2008
ΔPro	SIVmac, HIV-2 GH-1, HIV-2 ROD	Rescue of HIV-1 infection, slight activity loss on SIVmac, SAMHD1 degradation No replication of HIV-2GL-AN or HIV-2rod in MDDCs and MDMs	Pancio et al., 2000; Goujon et al., 2008; Berger et al., 2012

Non-exhaustive list of characterized Vpx mutants, summarizing their activity either in single-cycle infection with SIVmac and HIV-1 or in replication (in MDMs and/or MDDCs), and their ability to interact with DCAF1 and SAMHD1, and to degrade the latter.

region is still able to efficiently degrade SAMHD1 and to rescue HIV-1 in single-cycle infection of MDDCs (Goujon et al., 2008; Berger et al., 2012), but does not support HIV-2 replication in MDMs or MDDCs (Pancio et al., 2000; Goujon et al., 2008). Finally, Vpx was proposed to increase HIV-1 infectivity in a DCAF1-independent way both in IFN-treated MDDCs and in THP-1 cells, suggesting additional function(s) beyond SAMHD1 degradation (Goujon et al., 2008; Pertel et al., 2011; Reinhard et al., 2014).

APOBEC3A ANTAGONISM?

In addition to the well-documented interaction with SAMHD1, Vpx interacts with a member of the apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3 (APOBEC3) family of cytidine deaminases, namely APOBEC3A. APOBEC3 family members, exemplified by APOBEC3G, are potent restriction factors (reviewed in Malim and Bieniasz, 2012). HIV-1 prevents APOBEC3G/F/D/H action through proteasomal degradation induced by the Vif accessory protein. APOBEC3A affects various viruses and retroelements, such as human papilloma virus (Vartanian et al., 2008), adeno-associated virus (AAV) (Chen et al., 2006), and retrotransposons (Bogerd et al., 2006; Chen et al., 2006; Muckenfuss et al., 2006). Interestingly, APOBEC3A expression levels have been linked to myeloid cell restriction of HIV-1 infection (Peng et al., 2007). In line with this, APOBEC3A silencing increased the ability of HIV-1 to replicate in MDMs (Peng et al., 2007; Berger et al., 2011b). Two independent groups have shown that HIV-2/SIVsmm Vpx interacts with human APOBEC3A using co-immunoprecipitation of overexpressed proteins (Berger et al., 2010, 2011b). Both groups reported a decreased stability of APOBEC3A in the presence of Vpx. Similarly, wild-type SIVsmm replication led to slightly decreased APOBEC3A expression levels in monocytes compared to Vpx-deficient viruses (Berger et al., 2010). These data led to the hypothesis that Vpx might enhance infection of myeloid cells by inducing APOBEC3A degradation. However, the precise contribution of APOBEC3A to the resistance of HIV-1 infection in myeloid cells is difficult to evaluate, as SAMHD1 imposes a strong barrier to infection in these cells and is counteracted by Vpx. It would be crucial to separate, if possible, the two activities of Vpx, for instance by identifying mutants of Vpx retaining the ability of interacting with APOBEC3A but not SAMHD1 (or vice versa), and evaluating their effect in myeloid cells.

ANTAGONISM OF INTERFERON-INDUCED FACTOR(S)?

It has been known for decades that type 1 IFN treatment potently decreases HIV-1 infection in some immortalized cell lines and in primary cells, such as MDMs and MDDCs (Kornbluth et al., 1989; Shirazi and Pitha, 1992; Baca-Regen et al., 1994; Cheney and McKnight, 2010; Goujon and Malim, 2010). Interestingly, this IFN-induced block is primarily exerted at the level of viral DNA accumulation (Shirazi and Pitha, 1993; Baca-Regen et al., 1994; Cheney and McKnight, 2010; Goujon and Malim, 2010), which is exactly the step at which SAMHD1 acts (Hrecka et al., 2011; Laguette et al., 2011). Strikingly, Vpx is still capable of enhancing HIV-1 infection in IFN-treated myeloid cells and the magnitude of the infectivity enhancement is increased in this context (Gramberg et al., 2010; Pertel et al., 2011; Goujon et al., 2013). SAMHD1 was reported to be IFN-inducible in some cell types, such as primary monocytes (Berger et al., 2011a). This suggested that SAMHD1 might be playing a role in the IFN block to HIV-1 infection. But neither SAMHD1 expression nor dNTP intracellular concentrations seem to be regulated by IFN treatment in MDMs or MDDCs (St Gelais et al., 2012; Dragin et al., 2013; Goujon et al., 2013). Furthermore, RNAi-mediated SAMHD1 silencing fails to rescue myeloid cell infection in the presence of IFN (Dragin et al., 2013; Goujon et al., 2013). In addition, Q76A Vpx mutant was still able to substantially rescue IFN-treated MDDC infection (Pertel et al., 2011; Reinhard et al., 2014), although it was unable to bind DCAF1 (Pertel et al., 2011) and induce SAMHD1 degradation (Hrecka et al., 2011). Whereas exogenous dN and SIVmac Vpx increased the accumulation of HIV-1 late reverse transcription products to the same extent in IFN-treated myeloid cells, Vpx had a higher impact on 2-LTR circle and proviral DNA formation (Reinhard et al., 2014). This feature was not observed with SIVmus and SIVdeb Vpr, despite their ability to degrade human SAMHD1 (Reinhard et al., 2014). Taken together, these data strongly suggest that HIV-2/SIVmac Vpx may be counteracting other myeloid specific, IFN-inducible anti-HIV-1 factor(s). It could be tempting to speculate that APOBEC3A may be one of these. Indeed, APOBEC3A is highly induced by IFN treatment in myeloid cells (Peng et al., 2006; Koning et al., 2009; Refsland et al., 2010) and Vpx is known to interact with APOBEC3A (Berger et al., 2010, 2011b). However, APOBEC3A was not degraded in the presence of Vpx-VLPs following IFN treatment, contrary to SAMHD1, which is potently and rapidly degraded (Dragin et al., 2013). It remains possible that APOBEC3A is sequestered by Vpx rather than degraded in these conditions. Single-genome sequencing of viral DNA following HIV-1 infection of IFN-treated MDMs showed only infrequent editing and no sign of hypermutation, arguing against a major role for APOBEC3 proteins in the IFN block (Koning et al., 2011), though this does not exclude a deamination-independent mechanism. Indeed, APOBEC3A is known to inhibit AAV *via* a deamination-independent mechanism (Narvaiza et al., 2009). Further work will be required to address the exact role of APOBEC3A in the IFN-induced block to HIV-1 infection, and to determine whether Vpx is counteracting additional IFN-induced anti-HIV-1 factors in myeloid cells. Of note, SIVmac lentiviral vectors are still sensitive to the antiviral action of IFN in human primary cells despite the presence of Vpx (Goujon and Malim, 2010; Reinhard et al., 2014). This may reflect the existence of species-specific IFN-induced genes to which SIVmac is sensitive in human cells contrary to HIV-1, and which are not counteracted by Vpx (Bitzegeio et al., 2013; Cordeil et al., 2013).

ORIGIN AND EVOLUTION OF *vpx*

The HIV-2/SIVsmm and SIVrcm/SIVmnd-2 lineages possess 2 homologous genes to HIV-1/SIVcpz *vpr*: *vpr* and *vpx*. The *vpr* and *vpx* genes are paralogs and are the result of complex duplication and/or recombination of their precursor throughout the diversification of primate lentiviruses. *vpr* has most likely diverged through cross-species transmission, rather than co-evolution with their hosts following ancient infection. To

illustrate this, Wertheim and Worobey studied mitochondrial sequences of several species of the African green monkey lineage and their SIVagm and have rejected the hypothesis of co-divergence between SIVagm and its host. Their data support the hypothesis of a host-switching model following a geographical pattern of transmission (Wertheim and Worobey, 2007). Whilst *vpr* is found in all lentiviral groups, *vpx* is found only in two lineages: HIV-2/SIVsmm/SIVmac and SIVrcm/SIVmnd-2 (Beer et al., 2001; Hu et al., 2003). These viruses infect sooty mangabeys, macaques, mandrills and red-capped mangabeys, all of which belong to the same primate family.

The *vpx* genes from the SIVrcm/SIVmnd-2 lineage are quite divergent from other *vpx* genes (only about 30–40% identity at the amino acid level with Vpx from HIV-2/SIVsmm). Nonetheless, the monophyletic nature of the *vpx* clade supports the hypothesis of a single event leading to the birth of *vpx*, rather than multiple events (Hu et al., 2003).

Two hypotheses have been proposed so far to explain the birth of *vpx*. It has first been suggested that *vpx* arose by duplication of the *vpr* ancestor (Tristem et al., 1990). This was supported by sequence similarity and by the fact that both genes are always adjacent to each other in the genome. Alternatively, because *vpx* genes are closely related to *vpr* from SIVagm.Sab (infecting African green monkeys) (**Figure 5**), it has been proposed by another group that a recombination event between SIVagm and SIVsmm could have led to the acquisition of SIVagm.Sab *vpr* by the latter (Sharp et al., 1996; Hu et al., 2003). In agreement with this, recombination events between those two viruses have been shown before (Jin et al., 1994) and the habitats of African green monkeys and sooty mangabeys are partly overlapping. As the Vpr proteins of the HIV-2/SIVsmm/SIVmac and SIVrcm/SIVmnd-2 lineages are inactive against SAMHD1 (Lim et al., 2012) and as this function was acquired by the other *vpr* genes before the birth of *vpx*, it seems likely that the former Vpr proteins were never able to antagonize SAMHD1. Therefore, the acquisition of *vpr* from SIVagm would have been beneficial in this respect. Along these lines, it has been shown that the Vpr protein of SIVagm.Ver is able to antagonize a broad range of SAMHD1 proteins from primates (Lim et al., 2012). It is tempting to speculate that this may also be the case for SIVagm.Sab Vpr. This would favor the recombination (rather than the duplication) hypothesis, but would need to be assessed experimentally.

The rate of evolution of *vpx* seems to be slower than that of *vpr*, as shown by shorter branch lengths in the *vpx* lineage compared to the *vpr* one (see the HIV-2/SIVsmm/SIVmac lineage in **Figure 5**) (Tristem et al., 1992). This could be explained by the fact that *vpx* is overlapping with other genes to a greater degree than *vpr*, inducing a stronger constraint. In HIV-1 HXB2 for instance, 28% of the *vpr* gene is overlapping with other reading frames (*vif* and *tat*), whereas in HIV-2 BEN, 50% of *vpx* is overlapping (with *vpr*). A difference in selective pressures could also explain this disparity in evolution rate.

SIVcpz, which gave rise to the four known groups of HIV-1 after cross-species transmissions, is the result of recombination events between ancestors of SIVs from red-capped mangabeys and *Cercopithecus* species such as greater spot-nosed, mustached and mona monkeys (Bailes et al., 2003; Sharp and Hahn, 2011). Each of these viruses has the ability to antagonize SAMHD1 from the species they target (SIVrcm with *vpx* and SIVgsn/SIVmus/SIVmon with *vpr*) (Lim et al., 2012). However, this function was lost in SIVcpz (Etienne et al., 2013). As *vpx* from SIVrcm was unable to antagonize chimpanzee SAMHD1 (Lim et al., 2012), the loss of *vpx* might have had minimal consequences for the transmission of SIV to chimpanzees. It was hypothesized that the loss of *vpx*—*vpr* remained intact—led to the reconstruction of *vif* by overprinting and the acquisition of full antagonism of APOBEC3 proteins from chimpanzee (Etienne et al., 2013). One might speculate that evolution of *vif* to fully counteract chimpanzee APOBEC3G was more important for SIVcpz than evolution of *vpx* as a SAMHD1 antagonist.

Evolutionary analysis of SAMHD1 revealed strong signatures of positive selection in the N- and C-terminal parts of the protein (Laguette et al., 2012; Lim et al., 2012), suggesting that both termini may contribute to the interaction with Vpx. It has been shown recently that the separation of the two clades of *vpx* (HIV-2/SIVsmm/SIVmac and SIVrcm/SIVmnd-2) correlates with the domain of SAMHD1 targeted by these Vpx proteins. Vpx proteins from the HIV-2/SIVsmm/SIVmac lineage recognize the C-terminal domain of SAMHD1 (Ahn et al., 2012; Fregoso et al., 2013), whereas Vpx proteins from the SIVrcm/SIVmnd-2 lineage interact with the N-terminal domain of SAMHD1 (Fregoso et al., 2013; Wei et al., 2014). The model presented to explain this involves the particular head-to-tail dimer conformation of SAMHD1 and a Vpx (or Vpr) protein binding to both N-terminal and C-terminal domains of SAMHD1 with a high affinity for one of these domains and a lower affinity for the other. When mutations occurring in SAMHD1 lead to a decreased affinity and escape from Vpx, mutants of Vpx leading to higher affinity on the other domain might be selected (Fregoso et al., 2013). This evolutionary arms-race likely led to species specificity (Fregoso et al., 2013; Wei et al., 2014) and emphasizes the importance of the conservation of Vpx (Vpr) mediated SAMHD1 antagonism for lentiviruses. In agreement with this notion, SAMHD1 antagonism is actively maintained in natural infections, as exemplified by SIVagm *vpr* adaptations to SAMHD1 polymorphisms found in the African green monkey population (Spragg and Emerman, 2013).

HIV-1, A PANDEMIC VIRUS LACKING THE X-TRAORDINARY PROTEIN

Although essential for certain primate lentiviruses, it is somewhat surprising that the ability to antagonize SAMHD1, as well as any other functions borne by *vpx*, were lost during the genesis of SIVcpz (Etienne et al., 2013). They were dispensable for the establishment of SIVcpz in chimpanzees and its cross-species transmission to humans, which gave rise to HIV-1. Whereas the other potential functions of *vpx* might be provided by other genes in HIV-1/SIVcpz, it seems that HIV-1 does not need to antagonize SAMHD1 to replicate efficiently in humans. It is still not understood why SAMHD1 antagonism is crucial in some primate lentiviruses but dispensable in others. HIV-1 mainly targets cycling CD4+ T cells in which dNTP concentrations are high (Diamond et al., 2004), hence SAMHD1 antagonism may not be needed. Of note, HIV-1

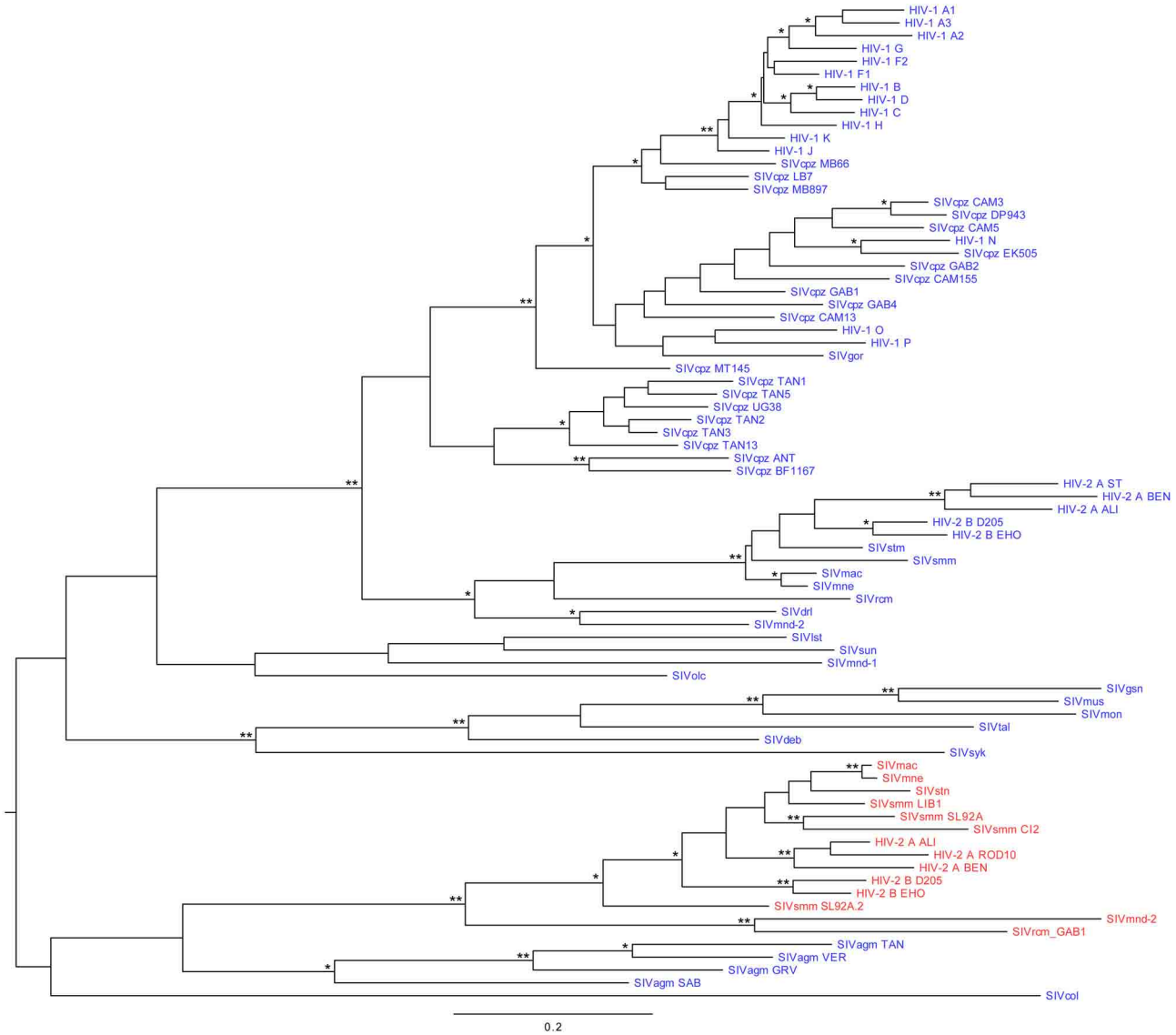

FIGURE 5 | Maximum likelihood phylogeny of primate lentiviruses *vpr* and *vpx* genes. *vpr* are represented in blue and *vpx* in red. Branch lengths represent nucleotide substitutions per site, as indicated in the scale. Branch supports are indicated by one asterisk (≥90% confidence) or two asterisks (≥99%). The tree was rooted using the midpoint rooting method. Abbreviations: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; cpz, chimpanzee (*Pan troglodytes*); gor, gorilla (*Gorilla gorilla*); stm, stump-tailed macaque (*Macaca arctoides*); smm, sooty mangabey monkey (*Cercocebus atys*); mac, rhesus macaque (*Macaca mulatta*); mne, pig-tailed macaque (*Macaca nemestrina*); rcm, red-capped mangabey (*Cercocebus torquatus*); drl, drill monkey (*Mandrillus leucophaeus*); mnd, mandrill (*Mandrillus sphinx*); lst, L'Hoest's monkey (*Cercopithecus lhoesti*); sun, sun-tailed monkey (*Cercopithecus solatus*); olc, olive Colobus (*Procolobus verus*); gsn, greater spot-nosed monkey (*Cercopithecus nictitans*); mus, mustached monkey (*Cercopithecus cephus*); mon, mona monkey (*Cercopithecus mona*); tal, northern talapoins (*Miopithecus ogouensis*); deb, De Brazza's monkey (*Cercopithecus neglectus*); syk, Sykes' monkey (*Cercopithecus mitis*); agm TAN, tantalus African green monkey (*Chlorocebus tantalus*); agm VER, vervet African green monkey (*Chlorocebus pygerythrus*); agm GRV, grivet African green monkey (*Chlorocebus aethiops*); agm SAB, sabaeus African green monkey (*Chlorocebus aethiops sabaeus*); col, guereza colobus monkey (*Colobus guereza*).

reverse transcriptase (RT) has a relatively high binding affinity for dNTPs, as compared to MLV (Weiss et al., 2004), which facilitates replication in cells with reduced dNTP content such as MDMs (Diamond et al., 2004). In line with this, HIV-1 RT mutant V148I, which has reduced dNTP binding affinity (Diamond et al., 2003, 2004), decreases HIV-1's ability to infect MDMs (Diamond et al., 2004; Lahouassa et al., 2012). Whether the RTs of HIV-2/SIVsmm have lower dNTP binding affinities than HIV-1 RT is currently unknown, but this could contribute to their dependence upon *vpx*. Alternatively viruses coding *vpx* might rely more on myeloid cell infection to disseminate *in vivo* than the ones lacking this gene. Of note, most non-primate lentiviruses, such as maedi-visna or caprine arthritis-encephalitis virus, tend toward myeloid tropism (though the mechanisms

used by these viruses to cope with low dNTP levels are currently unknown).

It would be of major interest to determine whether there is a connection between SAMHD1 degradation and an altered equilibrium between pathogen replication and host survival. Indeed primate lentiviruses able to antagonize SAMHD1 seem better tolerated by their natural hosts. This is exemplified by the difference observed in pathogenicity between HIV-1 and SIVmnd-1 compared to HIV-2, SIVsmm and SIVmnd-2.

The *in vitro* infection of MDDCs with HIV-2 causes their activation through detection of viral cDNA by the DNA sensor cyclic GMP-APM synthase (cGAS) (Lahaye et al., 2013). In contrast, HIV-1 is normally unable to infect MDDCs and therefore does not activate them (Manel et al., 2010). However, HIV-1 can be artificially rendered able to infect DCs through the provision of Vpx, leading to potent MDDC activation and secretion of cytokines including type 1 IFN (Manel et al., 2010; Lahaye et al., 2013). Under these artificial conditions HIV-1 seems to cause a stronger MDDC activation than HIV-2 (Yu et al., 2013). This strongly suggests that the absence of Vpx may actually be beneficial for HIV-1 as this would help avoiding infection and activation of DCs and therefore prevent initial innate (and then adaptive) immune responses detrimental to the virus. This would not be a unique example of a virus benefiting from limiting myeloid cell tropism. Indeed mosquito-borne North American eastern equine encephalitis virus (EEEV) is potently restricted by a specific miRNA in myeloid cells, whereas the sequence targeted by this miRNA is crucial for mosquito vector infection (Trobaugh et al., 2013). By limiting myeloid cell tropism and consequent innate immunity induction, the restriction directly promotes neurologic disease manifestations characteristic of EEEV infection in humans. In line with this, it is tempting to speculate that HIV-2 might be less pathogenic because it induces innate immune responses, possibly through myeloid cell infection. However, *vpx* isolated from viremic and long-term aviremic HIV-2 infected individuals display similar abilities to antagonize SAMHD1 and enhance virus infection (Yu et al., 2013), presumably reflecting the complexity of viral pathogenesis.

Interestingly, there is some evidence that individuals presenting HIV-1/HIV-2 co-infections have better long-term outcomes and slower progression to AIDS, compared to HIV-1 mono-infected patients (Esbjornsson et al., 2012). HIV-2 co-infection seems to protect from HIV-1 pathogenesis for a certain period of time. It would be of high interest to understand the basis for this and whether Vpx plays a role in it.

CONCLUSION

Vpx is a protein uniquely encoded by the HIV-2/SIVsmm and SIVrcm/SIVmnd-2 lineages, which has the property to favor myeloid cell infection through inducing the degradation of SAMHD1. It is absolutely required for efficient HIV-2/SIVsmm viral replication *in vivo*, suggesting an important role of myeloid cells as target cells for these viruses and/or that additional roles, such as APOBEC3A antagonism, may be important. In addition, Vpx is able to render DCs more permissive to HIV-1 infection, and this may promote the induction of stronger innate immune responses. These major findings might open the way for the prospective development of novel HIV-1 vaccine and treatment strategies based on the use of Vpx. In theory, treatment with a vaccine using Vpx-VLPs may stimulate immune responses, potentially leading to improved protection against (or control of) HIV-1.

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Mechanisms underlying HIV-1 Vpu-mediated viral egress

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Viruses such as lentiviruses that are responsible for long lasting infections have to evade several levels of cellular immune mechanisms to persist and efficiently disseminate in the host. Over the past decades, much evidence has emerged regarding the major role of accessory proteins of primate lentiviruses, human immunodeficiency virus and simian immunodeficiency virus, in viral evasion from the host immune defense. This short review will provide an overview of the mechanism whereby the accessory protein Vpu contributes to this escape. Vpu is a multifunctional protein that was shown to contribute to viral egress by down-regulating several mediators of the immune system such as CD4, CD1d, NTB-A and the restriction factor BST2. The mechanisms underlying its activity are not fully characterized but rely on its ability to interfere with the host machinery regulating protein turnover and vesicular trafficking. This review will focus on our current understanding of the mechanisms whereby Vpu down-regulates CD4 and BST2 expression levels to favor viral egress.

Keywords: Vpu, CD4, BST2/Tetherin, HIV-1, ESCRT, degradation, cell surface down-regulation, NF-κB

INTRODUCTION

Viral egress and replication rely on a complex interplay between viral and cellular proteins. During their replication cycle, viruses, notably lentiviruses, have to face several levels of the host immune defense mechanisms and must counteract these barriers to persist and disseminate in the host. Understanding the mechanisms underlying lentiviruses evasion from host antiviral activities has been the focus of many studies of the past decades. Not only did they contribute to major advances in the characterization of host strategies to repress viral replication, notably by the identification of antiviral cellular proteins such as APOBEC3G, SAMHD1, TRIM5α, and BST2 referred to as restriction factors (Sheehy et al., 2002; Stremlau et al., 2004; Neil et al., 2008; Van Damme et al., 2008; Laguette et al., 2011), but also they unraveled the importance of the accessory proteins of viruses in this process (Malim and Bieniasz, 2012).

The genome of lentiviruses encodes for several accessory proteins such as Nef, Vif, Vpr, Vpx, and Vpu, in addition to the structural and enzymatic proteins Gag, Pol, and Env and the regulatory proteins Tat and Rev (Malim and Bieniasz, 2012). These accessory proteins are, however, not common to all lentiviruses: Nef and Vpr are specific of primate lentiviruses (HIV-1, HIV-2, and SIV), Vpx is expressed by HIV-2 and its closely related SIV_{smm} and SIV_{mac}, Vpu is expressed by HIV-1 strains and a few strains of SIV (described later; Malim and Emerman, 2008). These proteins are not strictly required for viral replication *in vitro*. However, much evidence has highlighted their importance in the pathogenesis of the infection, as they contribute to modify the cell environment to facilitate viral replication and evasion from the host antiviral immune response (Malim and Emerman, 2008).

This review will provide an overview of our current understanding of the mechanisms whereby the accessory protein Vpu exploits the host cell machineries to counteract two components of the adaptive and innate host immune system: the protein CD4 and the restriction factor BST2.

CHARACTERISTICS AND FUNCTIONS OF THE ACCESSORY PROTEIN VPU

The accessory protein Vpu is an 81-amino acid type I integral membrane phosphoprotein, expressed by the genome of HIV-1, the related SIV_{cpz} (Strebel et al., 1988) and the SIV_{gsn} lineages including the greater spot-nosed monkey (SIV_{gsn}), the mona monkey (SIV_{mon}), the mustached monkey (SIV_{mus}) and Dent's mona monkey (SIV_{den}) isolates (Gao et al., 1999; Courgnaud et al., 2002, 2003; Bailes et al., 2003). Vpu contains a short luminal N-terminal domain, a 23-amino acid transmembrane domain and a large cytoplasmic C-terminal domain (Strebel et al., 1988; Maldarelli et al., 1993). Vpu localizes mainly in the endoplasmic reticulum (ER), the *trans*–Golgi network (TGN) and endosomal compartments. Two major functions have been attributed to Vpu during HIV-1 replication cycle. Firstly, Vpu targets the newly synthesized CD4 receptor for proteasomal degradation (Willey et al., 1992a). Secondly, it favors the release of viral particles from most human cell types through counteracting the inhibitory effect of BST2 (Neil et al., 2008; Van Damme et al., 2008). More recently, Vpu was shown to down-regulate cell surface expression of two additional mediators of the immune response: the lipid-antigen presenting protein CD1d expressed by antigen-presenting cells (Moll et al., 2010) and the natural killer cells ligand NTB-A (Shah et al., 2010). Vpu appears therefore as a key factor for HIV evasion from the host immune system.

VPU-INDUCED DOWN-REGULATION OF CD4

CD4 constitutes the major component of the receptor complex used by primate lentiviruses to infect the cells. It is a 54 kDa

type I integral glycoprotein expressed at the surface of helper T-lymphocytes, cells of the monocyte/macrophage lineage and hematopoietic progenitor cells.

Infection of $CD4^+$ cells by primate lentiviruses results in a rapid and constant down-modulation of cell surface CD4 expression level (Ray and Doms, 2006). CD4 down-regulation was proposed to prevent lethal superinfection of cells by additional virions (Wildum et al., 2006), contribute to the escape of infected cells from the immune system and favor viral fitness (Willey et al., 1992b). CD4 depletion in infected cells is achieved by the concerted, though mechanistically distinct, action of three viral proteins: Nef, Vpu and, to a lower extent, Env (Wildum et al., 2006). Nef, produced shortly after infection, enhances internalization of pre-existing CD4 from the cell surface and targets the receptor for lysosomal degradation (Chaudhuri et al., 2007). Env precursor gp160 binds CD4 in the ER and blocks its transport to the cell surface (Crise et al., 1990; Jabbar and Nayak, 1990). Vpu targets CD4 molecules present in the ER for proteasomal degradation (Willey et al., 1992a).

Vpu induces degradation of newly synthesized CD4 by a multistep process involving binding of Vpu with CD4 *via* their transmembrane domains (TMD), retention and poly-ubiquitination of CD4 in the ER, followed by its delivery to the ER-associated degradation pathway (ERAD) for further proteasomal degradation (Magadan et al., 2010; Magadan and Bonifacino, 2012; **Figure 1**). Vpu-induced degradation of CD4 requires the integrity of two phosphoserines S_{52}/S_{56} present in a canonical DSGXXS motif within the cytoplasmic tail of Vpu and involved in an interaction with the β-transducin repeat-containing protein 1 or 2 (β-TrCP1; β-TrCP2), two adaptors for the SKP1-cullin1-F-Box (SCF) E3 ubiquitin ligase complex (Margottin et al., 1998). Recruitment of the $SCF^{\beta\text{-}TrCP}$ complex by Vpu results in poly-ubiquitination of the cytoplasmic tail of CD4 on lysine, serine and threonine residues (Magadan et al., 2010). Interestingly, Vpu-induced SCF-mediated poly-ubiquitination of CD4 contributes to retain the receptor in the ER and enables the recruitment of the ERAD VCP-UFD1L-NPL4 dislocase complex, leading to the extraction of CD4 from the ER membrane and its subsequent degradation by the proteasome (Magadan et al., 2010; **Figure 1**).

VPU-MEDIATED ANTAGONISM OF THE RESTRICTION FACTOR BST2

A major breakthrough in understanding how Vpu promotes the release of HIV-1 particles was made by the identification of BST2 as a restriction factor for HIV-1 release (Neil et al., 2008; Van Damme et al., 2008).

CHARACTERISTICS OF BST2

BST2 is a 30–36 kDa highly glycosylated type II integral membrane protein, constitutively expressed in several cell types and can be up-regulated by type-I interferon and pro-inflammatory stimuli (Neil, 2013). BST2 is composed of a short N-terminal cytoplasmic tail, linked to a transmembrane domain and an extracellular domain anchored to the membrane through a C-terminal glycosyl-phosphatidylinositol (GPI) moiety (Kupzig et al., 2003). Recently, a short isoform of BST2 produced by an alternative translation initiation from the methionine residue at position 13 has been identified (Cocka and Bates, 2012). BST2 is localized at the plasma membrane (PM) in cholesterol–rich microdomains (rafts) and in intracellular compartments such as the TGN as well as early and recycling endosomes (Kupzig et al., 2003; Masuyama et al., 2009). BST2 was proposed to assemble as a "picket fence" around the lipid rafts, playing a role in organizing membrane microdomains (Billcliff et al., 2013). BST2 was shown to physically trap the *de novo* formed mature viral particles at the surface of infected cells, thereby considerably reducing virus release (Neil et al., 2008; Van Damme et al., 2008; Perez-Caballero et al., 2009; Hammonds et al., 2010). This activity relies on BST2 ability to form parallel disulfide–bond homo-dimers and to bridge virions and cellular membranes *via* its N– and C–terminal membrane anchoring domains (Iwabu et al., 2009; Perez-Caballero et al., 2009; Schubert et al., 2010), with a preference for an "axial" configuration in which the GPI anchors are inserted into virions, and the N-termini transmembrane anchors remain in the infected cells membrane (Venkatesh and Bieniasz, 2013).

Although initially identified as the factor responsible for defective release of HIV-1 mutants lacking the accessory gene *vpu* (Neil et al., 2008; Van Damme et al., 2008), it is now well established that BST2 restricts the release of nearly all enveloped viruses (retroviruses, herpes viruses, filoviruses, rhabdoviruses, paramyxoviruses, and arenaviruses) (Neil, 2013). BST2 therefore appears as a major mediator of the innate immune defense against viral dissemination. Primates lentiviruses deploy three proteins to antagonize BST2 antiviral activity: Vpu in HIV-1 (Neil et al., 2008; Van Damme et al., 2008); Env in HIV-2 ROD10, HIV-2 RODA, SIV_{agm}Tan and $SIV_{mac239}\Delta nef$ isolates (Gupta et al., 2009b; Le Tortorec and Neil, 2009; Hauser et al., 2010; Serra-Moreno et al., 2011) and Nef in most isolates of SIV (Jia et al., 2009; Sauter et al., 2009; Zhang et al., 2009). HIV-1 Vpu, HIV-2 Env, and SIV Nef were shown to down-regulate the cell surface expression level of BST2 to favor its removal from viral budding sites and further viral release (Neil, 2013). To date, the precise mechanism involved in this process has not been fully characterized.

BINDING OF VPU WITH BST2

Binding of Vpu with BST2 through their respective TMD was shown to be essential to counteract BST2 antiviral activity. Mutagenesis analyzes have unraveled the critical role of residues I_{34}, L_{37}, L_{41} of BST2 and A_{14}, W_{22} and to some extent A_{18} of Vpu in this interaction (Vigan and Neil, 2010; Kobayashi et al., 2011). These residues were proposed to form an anti-parallel helix-helix interface (Skasko et al., 2012). Interestingly, recent studies have identified additional residues, located at the periphery of the TMD of BST2 and Vpu respectively, required for the interaction between both proteins and antagonism of BST2 (McNatt et al., 2013; Pickering et al., 2014).

VPU-INDUCED CELL SURFACE DOWN-REGULATION OF BST2

The mechanism whereby Vpu decreases cell surface BST2 expression appears to rely on interference with BST2 intracellular trafficking. BST2 was thought to cycle between the PM, the TGN and the endosomes, with a fraction sorted for lysosomal degradation through an Endosomal Sorting Complexes Required

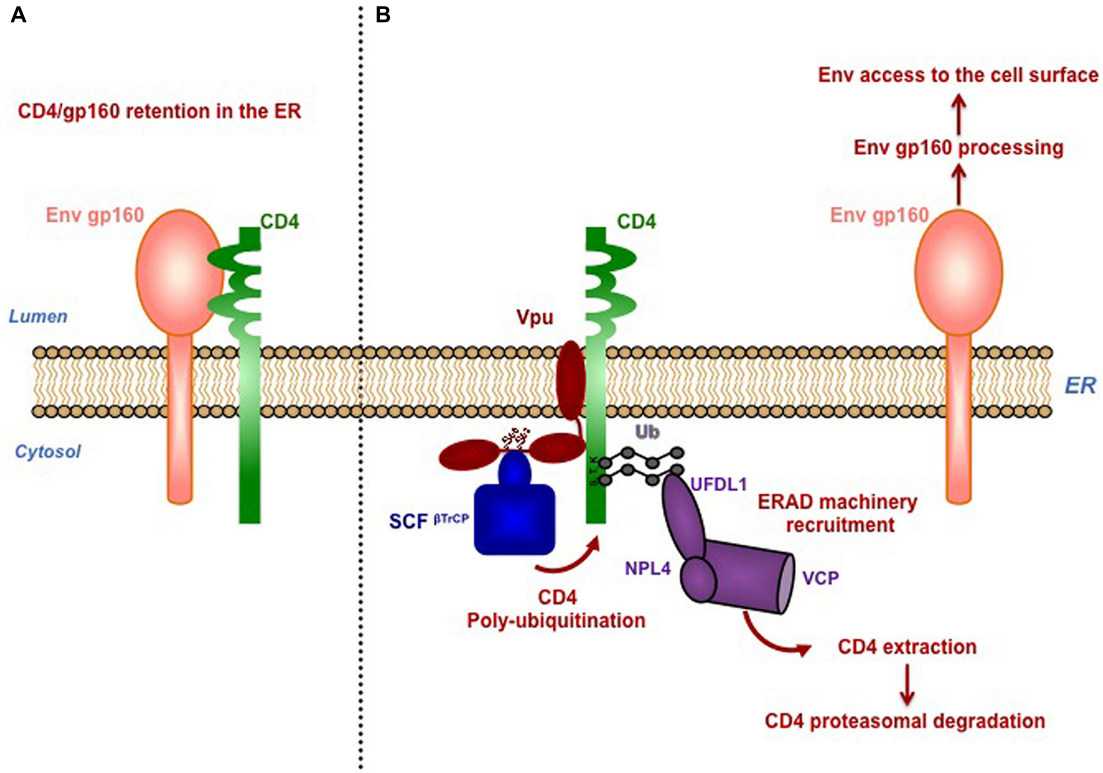

FIGURE 1 | Vpu mediates proteasomal degradation of CD4 to favor viral fitness. (A) Newly synthetized CD4 and HIV-1 envelope precursor gp160 interact in the ER through their luminal domain, preventing Env trafficking to the cell surface. **(B)** Vpu induces retention of CD4 in the ER through interaction *via* their transmembrane region and connects CD4 to SKP1-cullin1-F-Box (SCF) E3 ubiquitin ligase through binding with the SCF subunits β-TrCP1 and β-TrCP2. Interaction of Vpu with β-TrCP involves the conserved phophorylated serines S_{52} and S_{56}, located in the cytoplasmic tail of Vpu. Recruitment of the $SCF^{ß\text{-}TrCP}$ complex by Vpu induces poly-ubiquitination of CD4 on lysine, serine and threonine residues in its cytoplasmic tail. Poly-ubiquitination of CD4 partially contributes its retention in the ER, through a yet-to-be-determined mechanism. Vpu-induced SCF-mediated poly-ubiquitination of CD4 enables the recruitment of the ERAD VCP-UFD1L-NPL4 dislocase complex, leading to the extraction of CD4 from the ER membrane and its subsequent degradation by the proteasome. Degradation of CD4 by Vpu was proposed to dissociate CD4 from newly synthesized viral Env present in the ER, allowing Env maturation and trafficking to the cell surface for its subsequent incorporation in the forming virions.

for Transport (ESCRT)-mediated pathway (Rollason et al., 2007; Masuyama et al., 2009; Habermann et al., 2010; Janvier et al., 2011). Internalization of BST2 from the PM occurs through clathrin-coated vesicles, *via* direct binding of the clathrin adaptor complexes AP2 with non-canonical dual tyrosine residues (Y_6XY_8) in the cytoplasmic tail of BST2 (Rollason et al., 2007; Masuyama et al., 2009). Binding of BST2 with AP1 complexes regulates its retrieval from the early endosomes back to the TGN (Rollason et al., 2007). Vpu does not increase the rate of BST2 endocytosis but rather slows down the recycling of internalized BST2 back to the PM and inhibits the access of *de novo* synthetized BST2 to the cell surface, thereby decreasing the resupply of BST2 to the PM (Mitchell et al., 2009; Dube et al., 2011; Lau et al., 2011; Schmidt et al., 2011; **Figure 2**).

In some cell types such as T-cells and primary macrophages, antagonism of BST2 by Vpu is not associated with decreased expression of cell surface BST2 (Miyagi et al., 2009; Chu et al., 2012), consistent with the view that Vpu promotes viral release by displacing BST2 from viral budding sites at the PM. Using a sophisticated approach, it has been recently suggested that this function relies on the integrity of a "dileucine"-like motif $E_{59}VSAL_{63}V$ in the cytoplasmic tail of Vpu (McNatt et al., 2013), first reported to be essential for Vpu to down-regulate CD4 and BST2 expression and counteract BST2 antiviral activity (Hill et al., 2010; Kueck and Neil, 2012). How this motif contributes to these functions is, however unclear. Vpu EXXXLV motif fits the consensus dileucine – based sorting signal (D/E)XXXL(L/I/M) that mediates binding to the AP complexes. However, evidence for an interaction of Vpu with the AP complexes or a direct contribution of these complexes in Vpu's functions could not be demonstrated (Kueck and Neil, 2012).

VPU-MEDIATED DEGRADATION OF BST2

In some cell types, Vpu-induced down-regulation of cell surface BST2 is associated with enhanced targeting of the cellular protein to the degradation pathway (Gupta et al., 2009a; Mitchell et al., 2009). This process is ubiquitin-dependent and requires the recruitment of the $SCF^{ß\text{-}TrCP}$ complex by Vpu *via* its $DS_{52}GxxS_{56}$ motif (Douglas et al., 2009; Goffinet et al., 2009; Mangeat et al., 2009; Mitchell et al., 2009; Tokarev et al., 2010). The importance

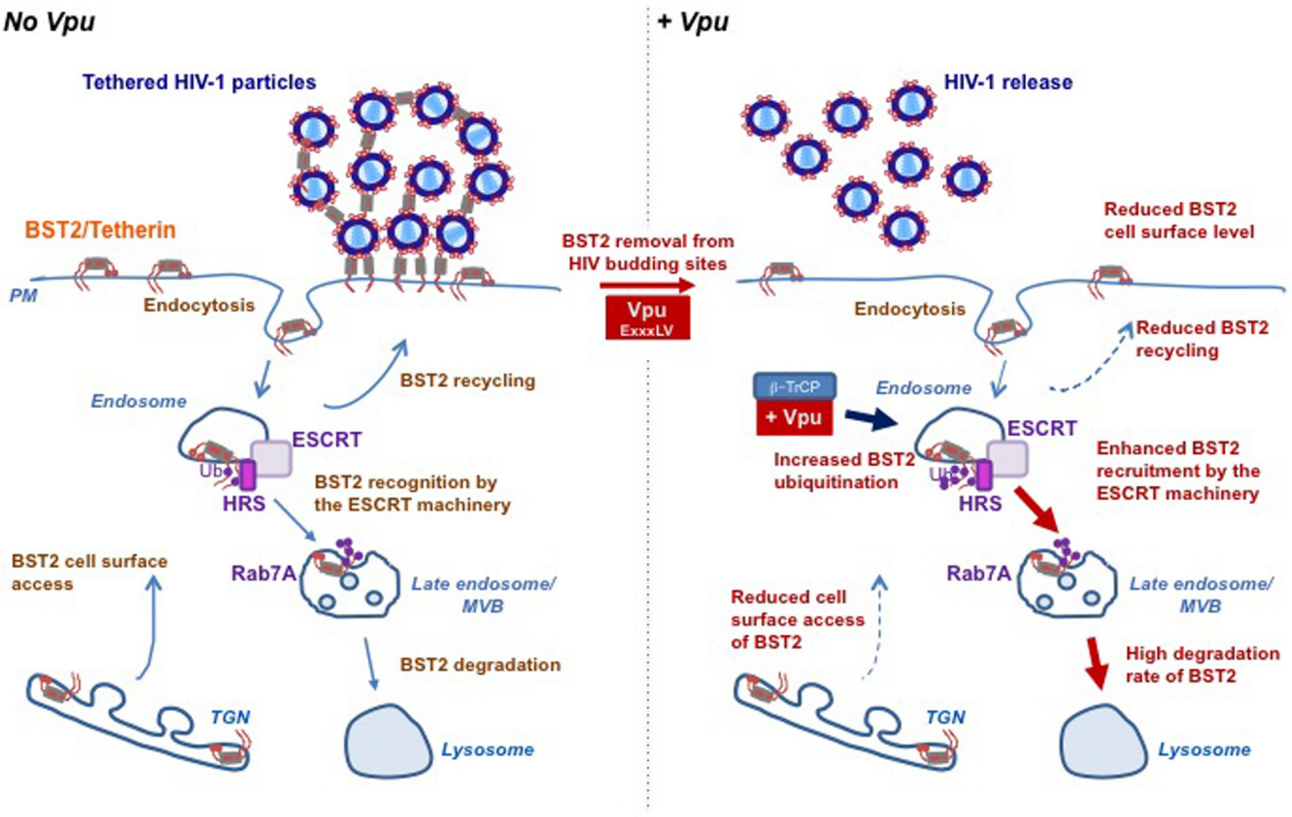

FIGURE 2 | Vpu subverts the host cell trafficking and ubiquitin machineries to counteract BST2 antiviral activity. BST2 physically tethers HIV-1 particles on the surface of infected cells preventing their release and further dissemination. The accessory protein Vpu counteracts this restriction by hijacking the host machineries involved in BST2 trafficking and turnover. BST2 cycles between the plasma membrane, the TGN and the endosomes, with a fraction sorted for lysosomal degradation through an ESCRT-mediated pathway (left panel). Vpu counteracts BST2 restriction activity through (i) displacing it from viral assembly sites by a yet-to-be-determined mechanism involving a "dileucine"-like motif located in its cytoplasmic tail and (ii) down-regulating BST2 cell surface expression level and as such restores viral release. Vpu down-regulates cell surface BST2 by slowing down the PM access of internalized and newly synthesized BST2. Vpu-induced BST2 cell surface down regulation is associated with targeting of the restriction factor for lysosomal degradation through an ubiquitin-dependent mechanism that involves the recruitment of $SCF^{\beta\text{-TrCP}}$ E3 ubiquitin ligase complex and the ESCRT machinery. Indeed, through binding to HRS, Vpu enhances the affinity of BST2 for HRS thus accelerating its sorting by the ESCRT machinery for lysosomal degradation. One might propose that through binding with the ESCRT machinery, Vpu may favor the sorting of BST2 at the level of endosomes, thereby reducing its recycling to the plasma membrane (right panel). This figure is adapted from Janvier et al. (2012).

of β-TrCP in Vpu-mediated antagonism of BST2 antiviral activity remains controversial to date (Douglas et al., 2009; Iwabu et al., 2009; Mangeat et al., 2009; Mitchell et al., 2009; Miyagi et al., 2009; Tervo et al., 2011). BST2 undergoes ubiquitination on lysine residues located in its cytoplasmic tail (Pardieu et al., 2010). Interestingly, Vpu increases BST2 ubiquitination on lysine/serine and threonine residues located in its cytoplasmic tail, as is also observed with CD4 (Tokarev et al., 2010). Mutation of these residues reduces Vpu-induced antagonism of BST2. Consistent with this observation, the short isoform of BST2 lacking these residues shows decreased sensitivity to Vpu antagonism (Tokarev et al., 2010; Cocka and Bates, 2012). One study challenged the requirement of the $S_3T_4S_5$ residues in Vpu-induced ubiquitination of BST2 (Gustin et al., 2012) but no explanation has been proposed for this discrepancy.

Despite the similarity in the molecular mechanisms underlying Vpu-induced ubiquitination and degradation of CD4 and BST2, the fate of both proteins differs. Indeed, it is now well established that Vpu does not target BST2 for proteasomal degradation but induces β-TrCP-dependent lysosomal sorting of BST2 (Douglas et al., 2009; Iwabu et al., 2009; Mitchell et al., 2009). In agreement with this notion, we highlighted a major role of Rab7, a regulator of the endo/lysosomal trafficking, in this process (Caillet et al., 2011), and revealed that Vpu enhances ESCRT-mediated sorting of BST2 for degradation (Janvier et al., 2011). The ESCRT machinery is a set of four hetero-oligomeric protein complexes involved in the sorting of ubiquitinated membrane proteins into vesicles budding into endosomes for their subsequent degradation in the lysosomes. The ESCRT-0 protein HRS (hepatocyte growth factor-regulated tyrosine kinase substrate) coordinates this process by linking ubiquitinated cargoes and the ESCRT-I component TSG101 (Raiborg and Stenmark, 2009). We showed that Vpu-mediated down-regulation of BST2 and viral release require HRS, and unveiled an increased affinity of BST2 for HRS upon Vpu

expression (Janvier et al., 2011). One might propose that through binding with HRS and BST2, Vpu accelerates ESCRT-mediated sorting of BST2 to the lysosomes, thereby reducing its recycling to the PM (Janvier et al., 2012; **Figure 2**). Further evidence of the requirement of the ESCRT machinery in Vpu-mediated BST2 degradation was obtained by the characterization of a new component of the ESCRT-I machinery: the protein UBAP1 (Agromayor et al., 2012). Depletion of UBAP1 abolishes Vpu-induced degradation of BST2, but has no impact on Vpu antagonism of BST2 antiviral activity (Agromayor et al., 2012), consistent with the notion that degradation of BST2 is not strictly required for Vpu-mediated antagonism of BST2 (Miyagi et al., 2009; Goffinet et al., 2010).

VPU AND BST2 VIRAL SENSING ACTIVITY

In addition to its activity as a restriction factor of viral release, BST2 has been recently characterized as an innate immune sensor for HIV (Cocka and Bates, 2012; Galao et al., 2012; Tokarev et al., 2013). In 2003, BST2 was first reported to stimulate the activity of the NF-κB family of transcription factors, using a whole-genome cDNA screen (Matsuda et al., 2003). Recent studies further revealed that tethered HIV particles increase BST2 signaling activity, resulting in enhanced production of pro-inflammatory stimuli, consistent with a role of BST2 as a sensor for assembled viruses (Cocka and Bates, 2012; Galao et al., 2012; Tokarev et al., 2013). This function seems separable from its activity as an inhibitor of viral release and relies on the integrity of the non-canonical dual tyrosine residues (Y_6XY_8) regulating its trafficking (Galao et al., 2012; Tokarev et al., 2013). Whether BST2 trafficking is relevant for its signaling activity is unclear. BST2 was proposed to activate the "canonical" NF-κB pathway through engaging mediators of this pathway *via* its tyrosine motif (Galao et al., 2012; Tokarev et al., 2013). Regulation of this pathway depends on the activation of the transforming growth factor-β-activated kinase-1 (TAK1)/TAK1-binding protein 1 and 2 (TAB1 and TAB2) complex, through poly-ubiquitination by E3 ligases of the TNF receptor-associated factors (TRAFs) family (Skaug et al., 2009). Interestingly, BST2 was shown to co-immunoprecipitate with TRAF2, TRAF6 as well as the TAK1/TAB1 complex (Galao et al., 2012; Tokarev et al., 2013).

Vpu was shown to counteract BST2 signaling activity through a β-TrCP-dependent mechanism (Tokarev et al., 2013). Interestingly, over a decade ago, Vpu was shown to sequester β-TrCP away from its substrates and inhibit NF-κB activation by interfering with β-TrCP-mediated degradation of the NF-κB inhibitor IκB (Bour et al., 2001; Besnard-Guerin et al., 2004). Whether this mechanism accounts solely for Vpu-induced inhibition of BST2 signaling activity requires further investigation. Furthermore, many questions remain regarding how viral expression triggers BST2 sensing activity.

CONCLUDING REMARKS

Over the past decade, Vpu has emerged as an important asset for viral egress and evasion from the host antiviral mechanisms. Although tremendous progress has been made toward understanding the mechanisms underlying Vpu's functions, many questions remain regarding how this protein contributes to viral pathogenesis. Vpu's contribution to viral immune evasion relies on its ability to alter the trafficking of its targets by subverting cellular machineries involved this process. However, despite some similarities in the mechanisms involved, major differences have been reported regarding the site of action of Vpu as well as the fate of its targets in cells. Further characterization of the mechanisms controlling Vpu expression and distribution in cells as well as the interplay with its targets and the host cell machineries might contribute to explain these pleiotropic effects. In keeping with this line of thought, another fascinating aspect worthy of further investigation is the role of Vpu with regards to BST2, in cell-to-cell transmission of HIV through virological synapses. So far, conflicting results have been obtained regarding the impact of both proteins on this process, but intriguingly, they have underlined a multifaceted role of BST2 in HIV pathogenesis (Schubert et al., 1995; Gummuluru et al., 2000; Casartelli et al., 2010; Jolly et al., 2010). Adding to this complexity, BST2 was recently described to act as a host sensor of assembled viruses. Therefore, a more detailed characterization of BST2's functions in cells as well as its interplay with Vpu would contribute to better comprehend the role of both proteins in viral egress and dissemination. Addressing all these questions might provide important insights into AIDS pathogenesis and contribute to the future development of therapeutic strategies.

AUTHOR CONTRIBUTIONS

Nicolas Roy and Grégory Pacini conceived the figures and contributed to the writing of the manuscript; Clarisse Berlioz-Torrent edited the manuscript; Katy Janvier wrote the manuscript and contributed to the drawing of the figures.

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Counteraction of the multifunctional restriction factor tetherin

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The interferon-inducible restriction factor tetherin (also known as CD317, BST-2 or HM1.24) has emerged as a key component of the antiviral immune response. Initially, tetherin was shown to restrict replication of various enveloped viruses by inhibiting the release of budding virions from infected cells. More recently, it has become clear that tetherin also acts as a pattern recognition receptor inducing NF-κB-dependent proinflammatory gene expression in virus infected cells. Whereas the ability to restrict virion release is highly conserved among mammalian tetherin orthologs and thus probably an ancient function of this protein, innate sensing seems to be an evolutionarily recent activity. The potent and broad antiviral activity of tetherin is reflected by the fact that many viruses evolved means to counteract this restriction factor. A continuous arms race with viruses has apparently driven the evolution of different isoforms of tetherin with different functional properties. Interestingly, tetherin has also been implicated in cellular processes that are unrelated to immunity, such as the organization of the apical actin network and membrane microdomains or stabilization of the Golgi apparatus. In this review, I summarize our current knowledge of the different functions of tetherin and describe the molecular strategies that viruses have evolved to antagonize or evade this multifunctional host restriction factor.

Keywords: ISG, restriction factor, tetherin, BST-2, HIV, Vpu, Nef, moonlighting proteins

INTRODUCTION

In the late 1960s, researchers estimated that the human genome may contain up to two million protein-coding genes (Kauffman, 1969). Today, we know that the actual number is much lower. In 2012, an *in vitro* gene expression analysis yielded a number of 20,687 protein-coding genes (Pennisi, 2012). Interestingly, several hundred of them are interferon-stimulated genes (ISGs) that are upregulated during viral infections (de Veer et al., 2001; Lanford et al., 2006; Fernandez-Suarez et al., 2013). Although an antiviral effector function has been described for some of these factors, the role of most ISGs during viral infections remains obscure. Three extensively studied proteins induced by type I interferons (IFN) are the restriction factors APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G), TRIM5α (tripartite motif 5-alpha), and tetherin (BST-2, CD317, or HM1.24). These host proteins are key players of the innate immune response and part of the first line of defense against lentiviruses. Like other IFN-inducible proteins, they target specific steps of the viral life cycle: APOBEC3G is a cytidine deaminase that inhibits reverse transcription and introduces G-to-A substitutions in the viral genome (Sheehy et al., 2002), TRIM5α binds incoming viral capsids and interferes with the uncoating process (Stremlau et al., 2004), and tetherin inhibits the release of budding virions from infected cells (Neil et al., 2008; Van Damme et al., 2008).

Surprisingly, the number of human genes is not significantly larger than that of many less complex organisms such as *Caenorhabditis elegans* or *Drosophila melanogaster*. Yet, the number of proteins can of course not be directly inferred from the number of genes. Alternative pre-mRNA splicing, DNA rearrangement, post-translational modifications, RNA editing, and the use of alternative start codons or reading frames are means to increase the coding potential of genes. Another possibility of coping with a limited number of genes is the evolution of multifunctional proteins, a phenomenon called "gene sharing" or "moonlighting" (Jeffery, 2003). ISGs may be especially prone to moonlighting since viruses exert a substantial selection pressure on the genomes of their host species. Antiviral proteins frequently have to acquire novel functions to cope with rapidly evolving or newly emerging viruses. Thus, viral infections may drive the evolution of antiviral activities in proteins that initially only exerted functions unrelated to immunity. Another common feature of many host restriction factors is their counteraction by viral antagonists. Whereas HIV-1 is resistant against human TRIM5α due to mutations in its capsid protein (Stremlau et al., 2004), APOBEC3G and tetherin are directly targeted by the accessory proteins Vif and Vpu, respectively (Sheehy et al., 2002; Neil et al., 2008; Van Damme et al., 2008). Notably, the combination of an antiviral function with activities beyond immunity within one protein may be a means to impede counteraction by viruses. Antiviral moonlighting proteins that also exert essential cellular functions cannot simply be degraded as this might be detrimental for the host cell and thus terminate viral replication.

The host restriction factor tetherin is such a moonlighting protein fulfilling all characteristics of known restriction factors: it is induced by IFNs, inhibits a specific step of the viral replication cycle, shows signatures of positive selection and is counteracted by viral proteins. In this review, I will summarize our current knowledge of the different activities of tetherin and discuss the strategies

evolved by different viruses to antagonize or evade this restriction factor.

STRUCTURE, TOPOLOGY, AND POST-TRANSLATIONAL MODIFICATIONS

The structural topology of tetherin is almost unique among mammalian proteins. Tetherin is a type II transmembrane protein consisting of a short N-terminal domain followed by an alpha-helical transmembrane domain, a labile coiled-coil ectodomain and a C-terminal glycosyl-phosphatidylinositol (GPI) anchor (**Figures 1A** and **2A**; Kupzig et al., 2003). This unusual topology with two membrane anchors is only shared with a special form of the prion protein (Hegde et al., 1998, 1999; Stewart et al., 2001), ponticulin from the slime mold *Dictyostelium discoideum* (Hitt et al., 1994a,b), Sm23 from *Schistosoma mansoni* (Köster and Strand, 1994), and NcSRS2 from *Neospora caninum* (Nishikawa et al., 2002).

The N-terminal intracellular domain of tetherin contains an evolutionarily conserved tyrosine motif (YxY; **Figure 1A**), which mediates clathrin-dependent internalization by recruitment of AP2 (**Figure 2A**; Ohtomo et al., 1999; Blasius et al., 2006; Rollason et al., 2007; Masuyama et al., 2009). Mature tetherin recycles between the plasma membrane, endosomes and the trans-Golgi-network (TGN) with a mean surface half-life of a few

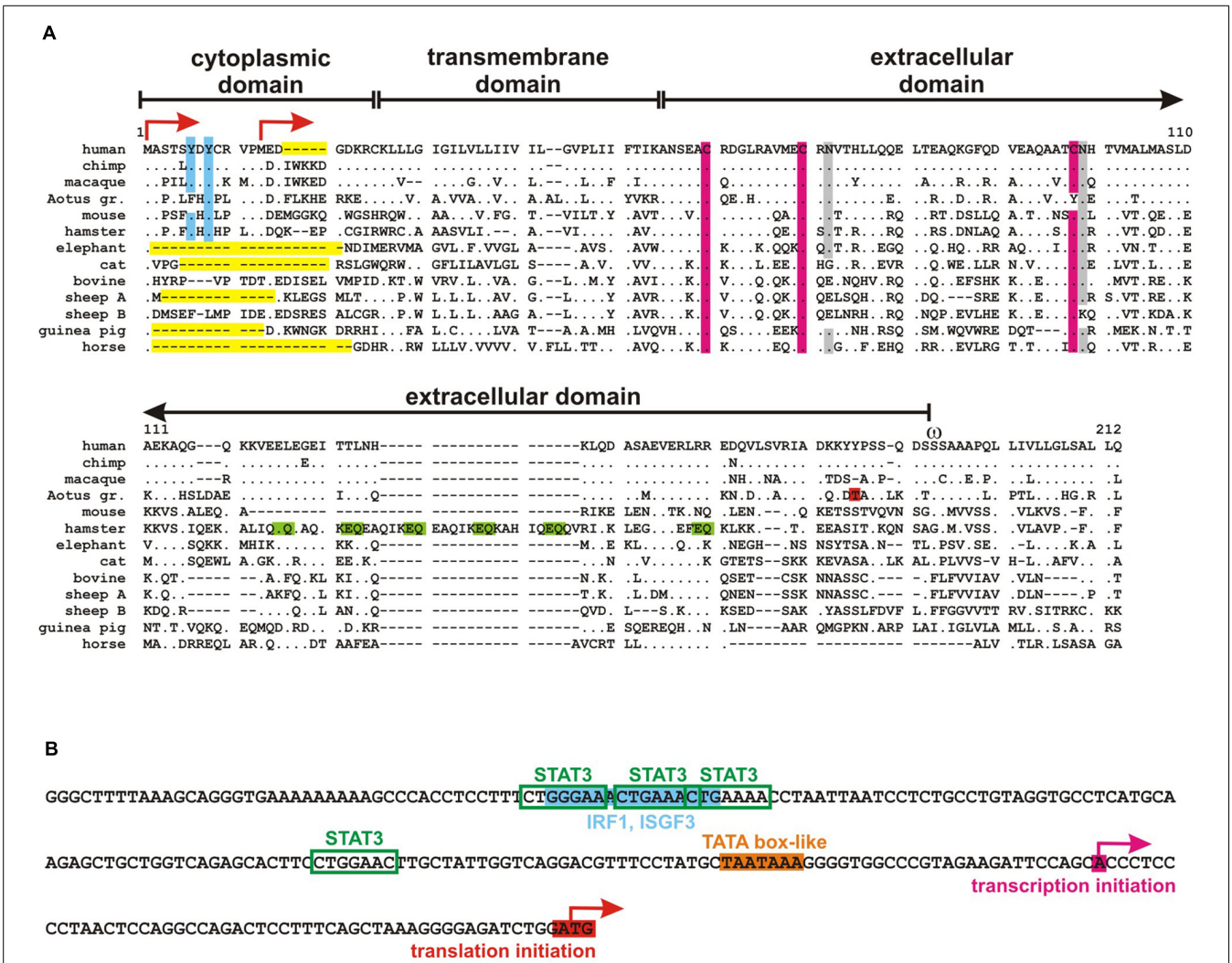

FIGURE 1 | Tetherin sequences. (A) Alignment of tetherin orthologs from different mammalian species. Dots indicate identity and gaps (dashes) were introduced to improve the alignment. *Aotus gr.* designates the Gray-handed night monkey *Aotus griseimembra*, sheep A and B the two tetherin proteins encoded by sheeps. A dual tyrosine motif that has been shown to be important for NF-κB activation as well as RICH2 and adapter protein binding is highlighted in blue, conserved cystein residues that form disulfide bridges are shown in pink and glycosylation sites in gray. Threonine at position 164 of *Aotus* tetherin is responsible for the lack of virion retention in this species and is highlighted in red. A unique stretch of EQ repeats in the hamster ortholog that may determine Golgi localization is marked in green. Deletions and truncations in the N-terminal cytoplasmic domain including the human-specific five amino acid deletion are shown in yellow. Two alternative start codons are indicated by arrows and ω marks the omega site of GPI-anchor addition. **(B)** Partial sequence of the human tetherin promoter. Transcription and translation start sites are indicated by pink and red arrows, respectively. A TATA box-like sequence is highlighted in orange and STAT3 and IRF1/ISGF3 binding sites are marked in green and blue, respectively.

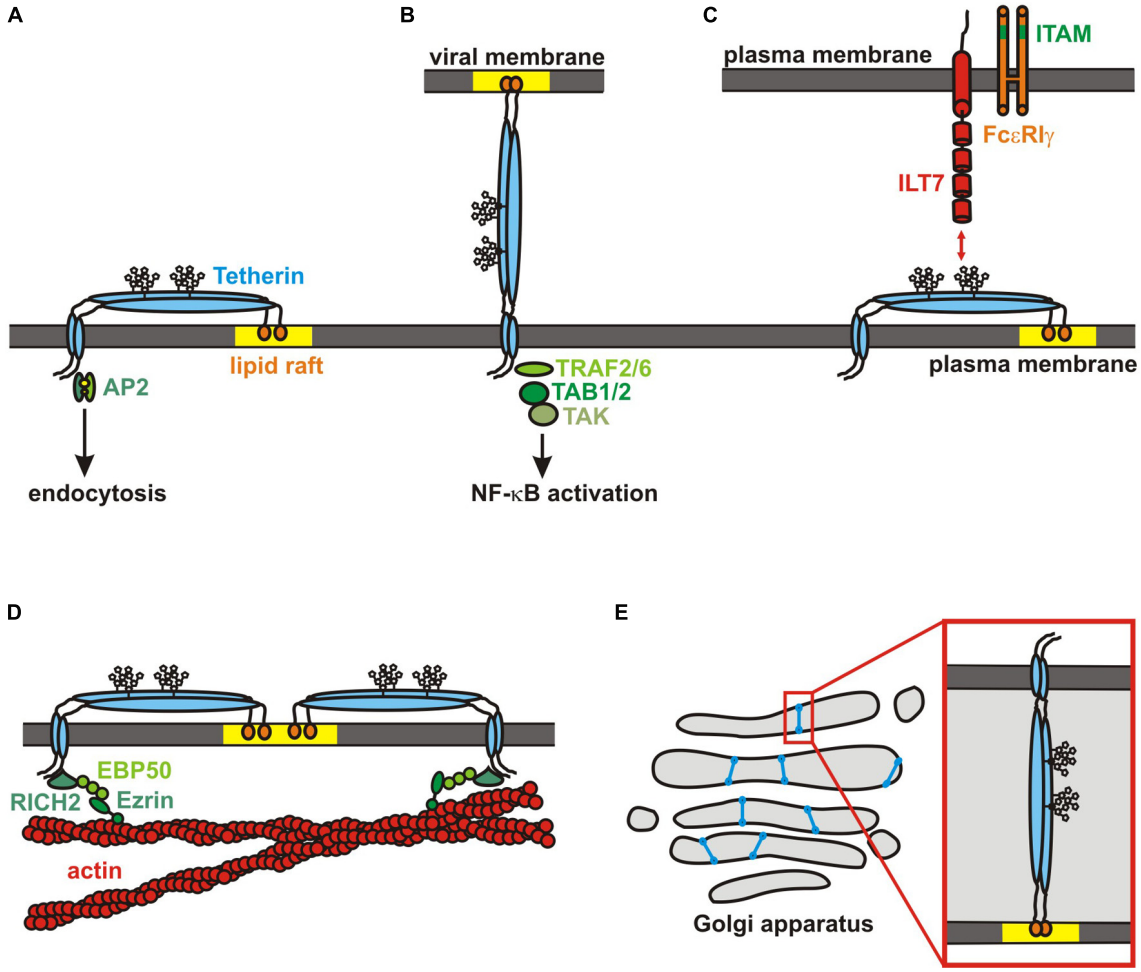

FIGURE 2 | Tetherin functions. (A) Topology of a tetherin dimer: the N-terminal cytoplasmic domain is followed by an alpha-helical transmembrane domain, a glycosylated coiled-coil extracellular domain and the GPI-anchor that is located in lipid rafts (yellow). Adapter proteins bind to a dual tyrosine motif in the N-terminal domain to induce clathrin-mediated endocytosis. **(B)** A tetherin dimer restricting the release of a budding virion is shown. Whereas the GPI-anchor is incorporated into the viral membrane, the N-terminal transmembrane domain remains attached to the host cell. TRAFs are recruited to the N-terminus upon virion binding. Subsequent recruitment of TABs and activation of TAK1 induces an NF-κB-dependent antiviral immune response. **(C)** Tetherin interacts with ILT7 on pDCs. ILT7 forms a complex with FcεRIγ, which contains a cytoplasmic ITAM. **(D)** Tetherin forms a picket fence around lipid rafts and links them to the underlying actin cytoskeleton via RICH2/EBP50/Ezrin. **(E)** Hamster tetherin is required for maintenance of the Golgi apparatus. It may connect and stabilize opposite membranes in the Golgi cisternae or sense their curvature and distance.

hours (Masuyama et al., 2009; Skasko et al., 2011; Sauter et al., 2013). Notably, methionine at position 13 of human tetherin has been shown to serve as alternative start codon, resulting in the expression of a shorter isoform (**Figure 1A**; Blasius et al., 2006; Cocka and Bates, 2012). Although both isoforms are expressed at comparable levels, the short isoform may preferentially localize to the plasma membrane due the lack of the YxY endocytosis motif (Cocka and Bates, 2012).

Mature tetherin is characterized by complex N-linked glycosylation at two conserved asparagine residues in its extracellular domain (**Figure 1A**; Ohtomo et al., 1999; Kupzig et al., 2003). Glycosylation occurs in the endoplasmic reticulum (ER) and Golgi apparatus and is required for entry of tetherin into the secretory pathway (Kupzig et al., 2003). Nevertheless, tetherin has never been shown to be secreted. Interestingly, glycosylated tetherin binds to various lectins with different selectivities (Ohtomo et al., 1999). However, a functional role of lectin binding by tetherin *in vivo* has not been demonstrated yet. In addition to the conserved asparagine residues, the ectodomain of human tetherin contains three cysteine residues that mediate disulfide-linked homodimer formation of the mature protein (**Figure 1A**; Kupzig et al., 2003). Recently, it has been suggested that interaction of the transmembrane domains may also be involved in homodimerization of tetherin (Cole et al., 2012). Tetherin homodimers are characterized by a parallel dimeric coiled-coil over the C-terminal two-thirds of the ectodomain. The N-terminal third may form an antiparallel four-helix bundle with another dimer, creating a tetherin tetramer (Schubert et al., 2010; Yang et al., 2010a).

During maturation, the C-terminus of tetherin is cleaved off in the ER to enable the addition of a GPI-anchor to serine at position

161 (**Figure 1A**). Tetherin is trapped in the ER in cells that have defects in the GPI-biosynthetic pathway (Perez-Caballero et al., 2009). The GPI-anchor serves as subcellular localization signal and targets tetherin to cholesterol-rich microdomains (lipid rafts; Kupzig et al., 2003). In contrast, the N-terminal transmembrane domain is most likely located outside lipid rafts with the cytosolic domain being a membrane microdomain exclusion motif (Billcliff et al., 2013). Thus, it has been suggested that parallel tetherin dimers form a picket fence like structure at the boundary of lipid rafts (**Figure 2D**; Kupzig et al., 2003).

EXPRESSION AND INDUCTION

The tetherin core promoter has a size of about 2000 base pairs. Transcription starts 51 nucleotides upstream of the start codon and a TATA box-like sequence is located 81 nucleotides upstream of the start codon (**Figure 1B**; Ohtomo et al., 1999). The promoter contains consensus binding sites for the transcription factors STAT3, IRF1, and ISGF3 (**Figure 1B**), suggesting that tetherin is an IFN-inducible gene (Ohtomo et al., 1999; Kawai et al., 2006; Ge et al., 2006). Indeed, it has been shown that IFN-α, -β, -γ, -τ, -λ3, and -ω induce the expression of tetherin in various cell types from different species (Blasius et al., 2006; Arnaud et al., 2010; Dietrich et al., 2011; Cobos Jiménez et al., 2012; Liu et al., 2012; Amet et al., 2014). Like other ISGs, tetherin expression is upregulated upon viral infection and protein levels correlate with viral loads both, in HIV-infected humans and simian immunodeficiency viruses (SIV)-infected macaques (Homann et al., 2011; Mous et al., 2012; Rahmberg et al., 2013). Retroviral infection is sensed by various pattern recognition receptors (PRR) such as TLRs, RIG-I, or IFI16 thereby inducing the release of type I IFNs and subsequent upregulation of tetherin (Blasius et al., 2006; Zhou et al., 2010; Jakobsen et al., 2013; Tavano et al., 2013; Wang et al., 2013). Interestingly, several IFN-independent stimulants of tetherin have also been reported. Bego et al. (2012) for example showed that TLR3 and TLR8 stimulation are able to induce the expression of tetherin independently of IFNs. Similarly, IL-27 has been identified as a potent inducer of tetherin in the absence of IFN (Guzzo et al., 2012). In contrast, IL-4, IL-6, IL-10, IL-12, TNFα, and CD40L have no or only marginal effects on tetherin expression levels (Blasius et al., 2006; Tavano et al., 2013) and its expression is downmodulated by TGFβ (Sayeed et al., 2013).

Although tetherin expression levels are markedly upregulated upon IFN stimulation, many cell types constitutively express tetherin in the absence of viral infections. Tetherin was originally identified as a marker for bone marrow stromal cells and various tumor cells (Goto et al., 1994; Ishikawa et al., 1995; Ohtomo et al., 1999; Walter-Yohrling et al., 2003; Grützmann et al., 2005; Capurso et al., 2006; Cai et al., 2009). More recently, it has become clear that tetherin is more widely expressed and can be detected to high levels in hepatocytes, pneumocytes, activated T cells, monocytes, pDCs, ducts of major salivary glands, pancreas and kidney cells, vascular endothelium, and many other cell types (Vidal-Laliena et al., 2005; Blasius et al., 2006; Kawai et al., 2006; Erikson et al., 2011). This constitutive expression in many organs and tissues suggests that tetherin is a key player of the early innate immune response. Alternatively, constitutive expression may indicate that tetherin performs cellular functions beyond immunity that require expression also in the absence of IFN and viral infections.

FUNCTIONS

INHIBITION OF VIRUS RELEASE

Although tetherin had already been described as an interferon-inducible gene in the 1990s, it took until 2008 to discover the potent antiviral activity of this cellular protein. In this year, two groups reported the ability of tetherin to inhibit the release of budding HIV virions from infected cells (Neil et al., 2008; Van Damme et al., 2008). Electron microscopic analyses revealed that tetherin appears to tether virions to each other as well as to the plasma membrane (**Figure 2B**; Neil et al., 2007, 2008). This antiviral activity depends on the unusual topology of tetherin. Budding virions incorporate one of the two membrane anchors of the restriction factor, whereas the other one remains attached to the plasma membrane of the host cell (Neil et al., 2008; Van Damme et al., 2008; Perez-Caballero et al., 2009). Using sophisticated modified tetherin variants, Venkatesh and Bieniasz (2013) could show that tetherin dimers adopt a parallel configuration with a three- to five-fold preference for the insertion of the GPI-anchor rather than the transmembrane domain into virions. This is in agreement with the observation that HIV progeny virions bud from cholesterol-rich microdomains (Nguyen and Hildreth, 2000; Bhattacharya et al., 2006). Microscopic analyses showed that tetherin accumulates at HIV budding sites with around four to seven molecules per assembly cluster (Habermann et al., 2010; Lehmann et al., 2011). A quantitative Western blotting approach yielded slightly higher numbers suggesting that a few dozen tetherin dimers are used to tether a single virion to the plasma membrane (Venkatesh and Bieniasz, 2013).

Perez-Caballero et al. (2009) showed that an artificial tetherin molecule consisting of the transmembrane domain of the transferrin receptor, the coiled-coil ectodomain of the dystrophia myotonica protein kinase (DMPK), and the GPI modification signal from the urokinase plasminogen activator receptor (uPAR) was able to restrict the release of budding virions. The fact that this artificial protein lacks any sequence homology with tetherin strongly argues against the requirement of any viral or cellular cofactors for viral restriction. Instead, the overall configuration with two membrane anchors and a flexible ectodomain seems to be sufficient to inhibit virion release. As mentioned above, a naturally occuring prion protein form also consists of an N-terminal transmembrane domain, a glycosylated ectodoamin and a C-terminal GPI-anchor. This prion protein variant is expressed at the cell surface and it would certainly be interesting to test whether it displays similar antiviral activities. In agreement with a direct tethering model involving an axial configuration of the dimer, tetherin mutants lacking either the GPI-anchor or the transmembrane domain are non-functional (Neil et al., 2008). Interestingly, several hereditary diseases affect enzymes of the GPI-anchor biosynthetic pathway. The best described probably being paroxysmal nocturnal hemoglobinuria (PNH), a syndrome that is most commonly caused by mutations in phosphatidylinositol glycan A (PIGA). This protein is part of an enzyme complex that catalyzes the first step of the GPI-anchor synthesis, the addition of *N*-acetylglucosamine (GlcNAc) to phosphatidylinositol

(PI). Subsequently, GlcNAcPI is deacetylated by the ER-resident enzyme phosphatidylinositol glycan anchor biosynthesis, class L (PIGL). Mutations in PIGL cause the CHIME syndrome that is characterized by colobomas, heart defects, ichthyosiform dermatosis, mental retardation, and ear anomalies (Ng et al., 2012). Although PIGL has been shown to be required for the transport of tetherin to the cell surface (Perez-Caballero et al., 2009), viral infections are not a major symptom of PNH or CHIME syndrome patients.

In agreement with a direct tethering mechanism of the viral membrane to the plasma membrane of the host cell, it has been demonstrated that tetherin is able to inhibit the release of a large number of enveloped viruses. Studies using either virus-like particles or replication competent viruses revealed that tetherin restricts budding of members of alpha-, beta-, gamma-, and deltaretroviruses, lentiviruses, and spumaviruses (Neil et al., 2008; Jouvenet et al., 2009; Groom et al., 2010; Goffinet et al., 2010b), arena- and filoviruses (Jouvenet et al., 2009; Sakuma et al., 2009; Radoshitzky et al., 2010), as well as paramyxo- and rhabdoviruses (Radoshitzky et al., 2010; Weidner et al., 2010; Sarojini et al., 2011; Kong et al., 2012). Interestingly, viruses that bud from intracellular membranes such as HSV-1 or HCoV-229E are also restricted by tetherin (Blondeau et al., 2013; Wang et al., 2014). The effect of tetherin on HCV replication is still controversial (Dafa-Berger et al., 2012; Ye et al., 2012; Pan et al., 2013; Amet et al., 2014). Notably, restriction of virion release is not the only antiviral activity of tetherin. Thus, seemingly discrepant results may be explained by the fact that some of the studies analyzed several rounds of viral replication whereas others focused on the release of viral particles.

INNATE SENSING AND SIGNALING

Tetherin was identified in an over-expression screening for activators of NF-κB (Matsuda et al., 2003). This raised the possibility that tetherin may also act as signaling molecule in addition to its role as an inhibitor of virion release. Subsequent studies demonstrated that antibody-mediated crosslinking of surface tetherin and – most importantly – virion budding induces the activation of NF-κB (Galão et al., 2012; Tokarev et al., 2013). Thus, tetherin may indeed act as a PRR inducing an antiviral immune response upon binding of budding progeny virions. Notably, however, activation of NF-κB and restriction of virion release are genetically separable functions of tetherin (Tokarev et al., 2013). The presence of the GPI-anchor, for example, is essential for inhibition of virion release but dispensable for signaling. Conversely, disruption of the tetramerization motif specifically disrupts signaling (Tokarev et al., 2013). It remains to be determined whether intrinsic tetherin-mediated activation of NF-κB in the absence of tethered viral particles is just an *in vitro* artifact due to over-expression or plays an important role *in vivo*. In both scenarios (over-expression and sensing of budding virions), tetherin seems to recruit TRAF2 and/or TRAF6 as well as the mitogen-activated kinase TAK1 and TAB, thereby activating the canonical NF-κB pathway (**Figure 2B**; Galão et al., 2012; Tokarev et al., 2013). It is still unclear how exactly these signaling molecules are recruited to tetherin. Although human tetherin contains a putative TRAF binding site [PxExx(Ar/Ac)] in its N-terminal cytoplasmic domain, mutational analyses revealed that this motif is most likely dispensable for TRAF6 recruitment (Ye et al., 2002; Galão et al., 2012). In contrast, mutation of the dual tyrosine motif YxYxxφ abrogated the signaling activity of tetherin. Depletion of AP2 and the analysis of a naturally occurring Y8H variant of tetherin, however, revealed that endocytosis is not required for efficient NF-κB activation (Galão et al., 2012; Sauter et al., 2013). The short isoform of tetherin fails to act as an innate sensor since it lacks the dual tyrosine motif. As this isoform acts in a dominant-negative manner on NF-κB activation, it is probably only homodimers of the long isoform that activate NF-κB (Cocka and Bates, 2012). It is tempting to speculate that the two N-terminal tyrosine residues in homodimers are phosphorylated upon virion sensing to recruit the first components of the NF-κB signaling cascade. Interestingly, a rare single nucleotide polymorphism (SNP) changing arginine at position 19 to histidine also abrogates the signaling activity of tetherin without affecting surface expression or its ability to restrict virion release (Sauter et al., 2013). Unfortunately, this SNP is probably too rare to assess a possible association with disease progression.

Remarkably, tetherin has also been shown to directly interact with the immunoglobulin-like transcript 7 (ILT7, LILRA4, CD85g) on pDCs (Cao et al., 2009; **Figure 2C**). ILT7 forms a complex with FcεRIγ which contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic tail and induces a calcium-dependent signaling cascade that inhibits the release of type I interferons and other proinflammatory cytokines from pDCs (Cao et al., 2006; Cho et al., 2008). Thus, it has been suggested that binding of tetherin to ILT7 may induce a negative feedback signaling to prevent an uncontrolled prolonged inflammatory response (Cao et al., 2009). A recent study by Tavano et al. (2013), however, has challenged this hypothesis. Whereas antibody-mediated crosslinking of ILT7 significantly suppressed IFNα production by pDCs a modulation of IFN production by tetherin was not observed in their experimental setup (Tavano et al., 2013). It is tempting to speculate that binding of ILT7 to tetherin may also activate tetherin-mediated NF-κB signaling.

STRUCTURAL ORGANIZATION OF THE CELL

In addition to its roles in antiviral immunity tetherin has also been reported to be an organizer of different cellular structures and organelles. It has early been shown that tetherin enters the secretory pathway and is thus mainly transported to the apical membrane of polarized cells (Kupzig et al., 2003). Knockdown experiments revealed that tetherin is required for the maintenance of the apical actin network and microvilli in such cells (Rollason et al., 2007). The protein RICH2 binds to the cytoplasmic dual tyrosine motif of tetherin and to EBP50, thereby linking it to Ezrin and the apical actin cytoskeleton (**Figure 2D**; Rollason et al., 2007). Interestingly, the adaptor protein binding site is masked in this process and RICH2 binding prevents clathrin-mediated endocytosis of tetherin (Rollason et al., 2007). RICH2 is a Rho-type GTPase-activating protein that inhibits the activation of Rac and Rho which is involved in the remodeling of the actin cytoskeleton. The activation of these Rho-GTPases is increased in tetherin-depleted cells (Rollason et al., 2007). Thus, tetherin does not only act as an anchor and stabilizer of the apical actin network

but also seems to be involved in the regulation of Rho-GTPases. Interestingly, the *Dictyostelium* protein ponticulin that also contains an N-terminal transmembrane domain and a C-terminal GPI-anchor has also been shown to link the plasma membrane to the cortical actin network (Hitt et al., 1994a,b).

With the GPI-anchor being localized in a lipid raft and the transmembrane domain just adjacent to it, tetherin may also serve as a picket fence stabilizing and organizing membrane microdomains (**Figure 2D**; Kupzig et al., 2003). This hypothesis is supported by the observation of Billcliff et al. (2013) that the N-terminal cytoplasmic tail of tetherin serves as a microdomain-exclusion motif. Thus, tetherin would link membrane rafts to the underlying actin cytoskeleton, a role that has previously been ascribed to the tetraspanin CD82 (**Figure 2D**; Delaguillaumie et al., 2004).

Another interesting observation comes from the hamster ortholog of tetherin called Golgi-resident GPI-anchored protein (GREG). As the name suggests, hamster tetherin is preferentially localized to the Golgi apparatus rather than the plasma membrane. GREG is characterized by a stretch of unique EQ tandem repeats serving as a putative Golgi-retention signal that is absent from all other tetherin orthologs (**Figure 1A**; Li et al., 2007). Nevertheless, the overall topology of the protein is conserved and a certain amount of GREG can also be detected at the plasma membrane (Perez-Caballero et al., 2009). Thus, it is very likely that hamster tetherin is still able to inhibit the release of budding virions despite the presence of a putative Golgi-retention signal. Interestingly, many circular and ring-like structures rather than the classical Golgi cisternae were observed in GREG-depleted cells, suggesting an essential role of GREG in the maintenance of the Golgi complex (**Figure 2E**; Li et al., 2007). A similar phenotype was observed in cells lacking PIGL which is required for the GPI-anchor synthesis (Li et al., 2007). A model has been proposed in which opposing membranes within a cisterna are linked by GREG dimers (**Figure 2E**). These dimers may either stabilize the Golgi structure or act as a sensor, surveilling the distance between opposing membranes. In this context, Swiecki et al. (2011) made the interesting observation that tetherin ectodomain dimers are similar to that formed by BAR-domains. BAR-domains have been shown to bind and stabilize membrane curvatures (Frost et al., 2009). It is tempting to speculate that the extracellular part of tetherin may perform similar activities that are involved in the sensing of budding virions and/or Golgi structure.

SPECIES-SPECIFIC DIFFERENCES

Tetherin orthologs have been described in many mammalian species (**Figure 1A**). The only non-mammalian tetherin has been identified in the Chinese alligator *Alligator sinensis* (accession numbers: XP_006017475, XP_006017476). The sequence homology to mammalian tetherins is, however, only very limited and it remains to be clarified whether this reptile protein really represents a tetherin ortholog.

Like most antiviral genes, tetherin is under high selection pressure and residues in all three domains have been shown to be under positive selection (Gupta et al., 2009a; McNatt et al., 2009; Lim et al., 2010). Seven very rare non-synonymous SNPs have been described in human tetherin (Y8H, R19H, N49S, D103N, E117A, D129E, and V146L), one of which specifically abrogates the sensing and signaling activity of tetherin (Sauter et al., 2013). A unique characteristic of the human ortholog is the deletion of five amino acids in the cytoplasmic N-terminal tail (**Figure 1A**). Since these five amino acids are also absent from the genomes of the Denisova and Neanderthal, it probably emerged at least 800,000 years ago, before the separation of these ancient hominin species but after the divergence of humans from non-human primates (Sauter et al., 2011b). The methionine residue at position 13 of human tetherin is conserved in many orthologs, suggesting that a long and a short isoform of tetherin are expressed in many mammalian species (**Figure 1A**). Some species, however, such as cats, guinea-pigs, horses, or elephants encode only the short isoform or a variant with deletions in the N-terminal part (**Figure 1A**). One well-characterized example is the cat ortholog, which expresses only the short isoform due to a mutation in the upstream start codon (Celestino et al., 2012). Since this isoform lacks the dual tyrosine motif that is required for activation of NF-κB signaling and binding of RICH2, feline tetherin is probably deficient in these two functions. The short isoform may, however, restrict virion release more efficiently because it is expressed to higher levels at the cell surface due to the lack of the endocytosis signal. Furthermore, a short N-terminal cytoplasmic tail may confer a selective advantage because it reduces the number of target sites for potential cytoplasmic viral antagonists.

Interestingly, most mammalian tetherin orthologs seem not to be able to sense viral particles although they express the long isoform and contain the dual tyrosine motif. Only human tetherin and (to a lesser extent) chimpanzee tetherin have been shown to perform this function (Galão et al., 2012). Although most experiments have been performed in human cells, it has been suggested that the deletion of the five amino acid patch in the cytoplasmic tail of tetherin during human evolution may have led to an increased signaling capacity (Galão et al., 2012). Tetherin orthologs from other species may perform additional functions. As mentioned above, hamster tetherin contains unique EQ tandem repeats that may determine its preferentially intracellular localization and its involvement in the maintenance of the Golgi apparatus (**Figures 1A** and **2E**; Li et al., 2007). The Gray-handed night monkey *Aotus (lemurinus) griseimembra* encodes a tetherin variant that is not able to inhibit virion release. This lack of restriction could be ascribed to a S164T mutation in the extracellular domain of tetherin (**Figure 1A**; Wong et al., 2009). To my knowledge, this is the only naturally occurring tetherin variant that fails to restrict virion release.

Sheep, goats, and cows encode two tetherin variants implying a gene duplication event before the divergence of these ruminants (Arnaud et al., 2010). At least in sheep, both proteins (BST-2A and BST-2B) are able to inhibit the release of budding virions, although they may be differentially expressed in various cell types (Arnaud et al., 2010). Interestingly, BST-2A is characterized by a truncated N-terminal domain and appears to restrict retroviral release more efficiently than BST-2B (**Figure 1A**; Arnaud et al., 2010).

Thus, some tetherin functions such as maintenance of the Golgi structure or innate sensing may have evolved relatively recently during evolution and may hence only be found in few species. In contrast, the inhibition of virion release is conserved among

diverse mammals suggesting that this is an ancient function of tetherin.

VIRAL ANTAGONISTS

SIV Nef

Simian immunodeficiency viruses are primate lentiviruses that have been identified in more than forty different African primate species. Most of these viruses use their accessory protein Nef to counteract tetherin in their respective host species (Jia et al., 2009; Sauter et al., 2009; Zhang et al., 2009; Schmökel et al., 2011). Like many other viral antagonists, Nef enhances virion release by decreasing the surface expression levels of tetherin (**Figure 3A**; Götz et al., 2012; Serra-Moreno et al., 2013). However, total cellular tetherin levels remain unaffected suggesting that Nef sequesters it to intracellular compartments rather than inducing its degradation. Indeed, it has been shown that Nef induces clathrin, AP2- and dynamin2-dependent endocytosis of the restriction factor (**Figure 3A**). Mutational analyses revealed that residues within and adjacent to a highly conserved [D/E]xxxLL motif in the C-loop of Nef are critical for its anti-tetherin activity (Zhang et al., 2011; Serra-Moreno et al., 2011; Götz et al., 2012). Although recruitment of AP2 via this motif is required for Nef-mediated downmodulation of CD4 (Garcia and Miller, 1991; Lindwasser et al., 2008), these mutations specifically disrupted the downmodulation of tetherin (Götz et al., 2012; Serra-Moreno et al., 2013). Thus, residues surrounding the AP2-binding site may be involved in the direct binding of tetherin rather than AP2 recruitment. In agreement with this hypothesis, Serra-Moreno et al. (2013) verified a direct physical interaction between Nef and the N-terminal cytoplasmic tail of tetherin. Interestingly, the sensitivity of tetherin toward Nef maps to the DIWKK motif that is missing in the human ortholog (Jia et al., 2009; Sauter et al., 2009; Zhang et al., 2009). Thus, human tetherin is resistant against Nef-mediated counteraction and may thus represent a hurdle for successful cross-species transmissions of SIV to humans (Sauter et al., 2009, 2010).

HIV-1 M AND N Vpu

The current AIDS pandemic is a sinister example for the enormous plasticity and adaptability of primate lentiviruses. HIV-1 groups M, N, O, and P are the result of four independent cross-species transmission events from apes to humans (Sharp and Hahn, 2011). SIVcpz and SIVgor, the direct precursors of HIV-1 use their Nef proteins to antagonize the tetherin ortholog of their respective host species. Although human tetherin is resistant to Nef due to a five amino acid deletion in its cytoplasmic tail, pandemic HIV-1 group M strains mastered this species barrier by switching from Nef- to Vpu-mediated counteraction (**Figure 3B**; Sauter et al., 2009). This evolution of Vpu as an effective tetherin antagonist may have been an important prerequisite for the pandemic spread of HIV-1 group M (Sauter et al., 2010). Several residues in the transmembrane domain of Vpu have been shown to directly interact with the transmembrane domain of tetherin (Kobayashi et al., 2011; McNatt et al., 2013). As a consequence, Vpu-mediated counteraction of tetherin is often species-specific (Goffinet et al., 2009; Gupta et al., 2009a; McNatt et al., 2009; Rong et al., 2009). Notably, an AxxxAxxxAxxxW face in the transmembrane domain of HIV-1 group M Vpu is essential for the counteraction of human tetherin (Vigan and Neil, 2010). It remains, however, unclear whether these residues directly interact with the transmembrane domain of tetherin or rather confer stability to the alpha-helical structure of the transmembrane domain (Kleiger et al., 2002; Schneider and Engelman, 2004).

Although an interaction of Vpu with tetherin at the cell surface has been suggested (Iwabu et al., 2009), it is now quite well established that both proteins interact in the TGN (Dubé et al., 2009) and that Vpu inhibits the anterograde transport of tetherin to the plasma membrane (Dubé et al., 2009; Hauser et al., 2010; Andrew et al., 2011; Schmidt et al., 2011). Although this interaction and sequestration to the TGN may be sufficient for a partial relief of restriction, full counteraction activity depends on the presence of a di-serine motif (DSGxxS) in the cytoplasmic part of Vpu that is phosphorylated by casein kinase II (Mangeat et al., 2009; Goffinet et al., 2010a; Schindler et al., 2010). This motif recruits the SCF E3 ubiquitin ligase complex via the adaptor protein βTrCP thereby inducing the subsequent ubiquitination of tetherin. The exact residues in the cytoplasmic tail of tetherin that become ubiquitinated are still unclear (Tokarev et al., 2011; Gustin et al., 2012). The absence of putative ubiquitination sites in the short isoform of tetherin may, however, explain its relative resistance against Vpu (Cocka and Bates, 2012). Although some reports propose an ERAD- and proteasome-dependent degradation of tetherin (Goffinet et al., 2009; Mangeat et al., 2009; Petris et al., 2014), it seems more likely that tetherin enters the ESCRT-dependent endolysosomal pathway upon ubiquitination (Douglas et al., 2009; Iwabu et al., 2009; Mitchell et al., 2009; Janvier et al., 2011; Agromayor et al., 2012; Gustin et al., 2012; Kueck and Neil, 2012; Rollason et al., 2013). Interestingly, the presence of a βTrCP-consensus sequence (DSGxxS) is not absolutely required for efficient anti-tetherin activity (Kluge et al., 2013) and some studies suggest that the di-serine itself rather than βTrCP recruitment is required for Vpu-mediated counteraction of tetherin (Schmidt et al., 2011; Tervo et al., 2011). Whereas Tervo et al. (2011) propose the binding of an as-yet unknown factor to the di-serine it is also conceivable that the presence of this motif is required for the structural integrity of the second alpha-helical domain of Vpu (Coadou et al., 2002, 2003). Notably, this second alpha-helix contains a putative ExxxLV trafficking motif that is required for efficient anti-tetherin activity (Kueck and Neil, 2012) and fusion of this domain to tetherin was sufficient to remove it from the sites of budding (McNatt et al., 2013). A similar DxxxLV motif evolved in the Vpu of a recently isolated highly pathogenic HIV-1 group N strain which counteracted human tetherin as efficiently as pandemic HIV-1 group M Vpus (Sauter et al., 2012). In contrast, most of the previously characterized HIV-1 N Vpus do not contain a DxxxLV motif in their cytoplasmic domain and counteract tetherin only inefficiently (Sauter et al., 2009, 2012). This poor anti-tetherin activity of HIV-1 group N viruses could be a reason for their very limited spread in the human population. Similarly, Vpu proteins of non-pandemic HIV-1 groups O and P have not evolved efficient anti-tetherin activity either (Sauter et al., 2009, 2011a; Yang et al., 2010b; Petit et al., 2011). Thus, only pandemic HIV-1 M strains mastered the tetherin hurdle "perfectly" by switching from Nef to Vpu to antagonize the human ortholog of this restriction factor.

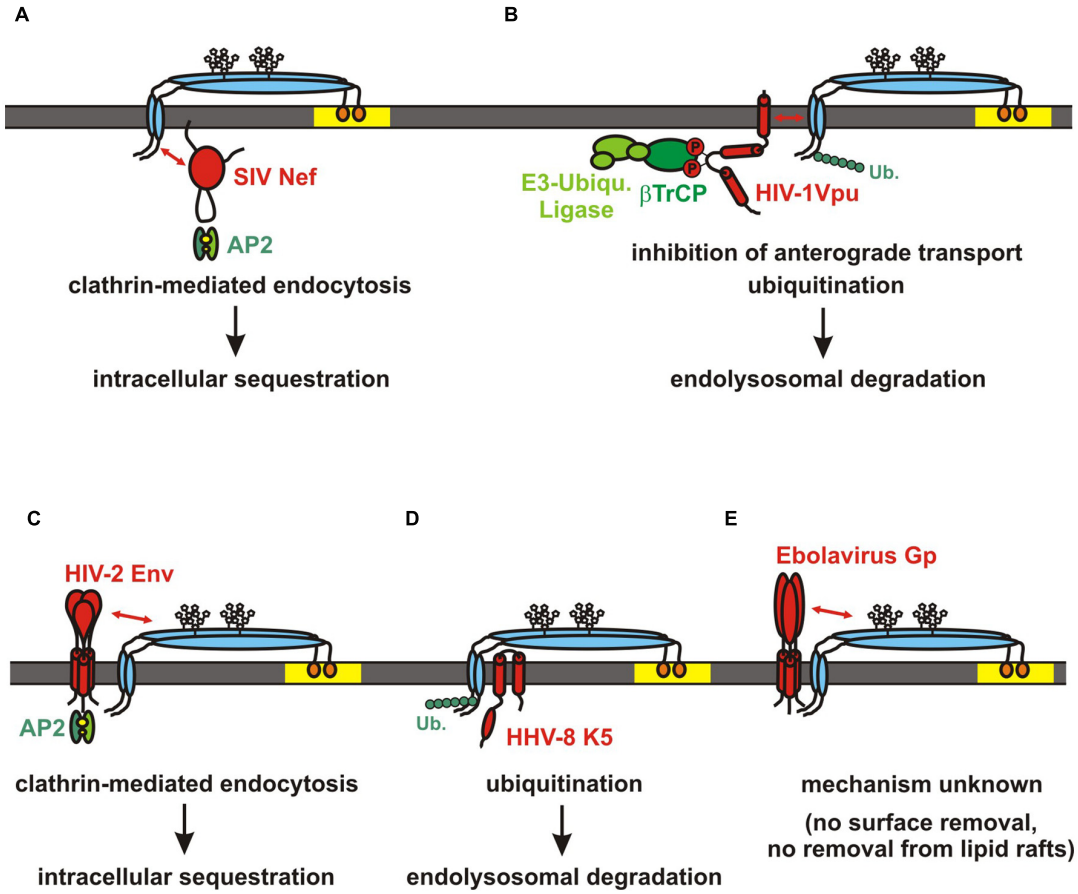

FIGURE 3 | Viral antagonists of tetherin. Schematic structure and mode of action are shown for **(A)** SIV Nef, **(B)** HIV-1 Vpu, **(C)** HIV-2 Env, **(D)** HHV-8 K5, **(E)** Ebolavirus Gp. Tetherin is indicated in blue, the viral antagonists are shown in red. SIV Nef and HIV-2 Env sequester tetherin to intracellular compartments without affecting total cellular tetherin levels. In contrast HIV-1 Vpu and HHV-8 K5 induce the ubiquitination and subsequent degradation of the restriction factor. The mechanism of Ebola Gp-mediated tetherin antagonism is still unclear. Interactions between domains of tetherin and its antagonists are indicated by red arrows.

HIV-2 AND SIV Env

The second human immunodeficiency virus HIV-2 is the result of at least nine independent cross-species transmissions of SIVsmm infecting sooty mangabeys to humans (Sharp and Hahn, 2011; Ayouba et al., 2013). These transmission events gave rise to HIV-2 groups A–I. HIV-2 does not encode a *vpu* gene and switched from Nef to its envelope protein (Env) to antagonize human tetherin (Le Tortorec and Neil, 2009). Similar to Nef-mediated counteraction of tetherin, Env does not induce the degradation of this restriction factor but rather sequesters it to intracellular compartments, probably the TGN (**Figure 3C**; Le Tortorec and Neil, 2009; Hauser et al., 2010). The interaction with tetherin occurs very likely via the ectodomain of Env (Lopez et al., 2010) and depends on an endocytic motif in gp41 (Le Tortorec and Neil, 2009). Notably, most assays were performed with Env alleles from HIV-2 group A strains and it remains unclear whether other HIV-2 groups also evolved Env-mediated anti-tetherin activity and/or whether the ability to antagonize tetherin correlates with the spread of the respective HIV-2 group in the human population.

OTHERS

The broad antiviral activity of tetherin is reflected by the fact that a substantial number of enveloped viruses have evolved antagonists of this restriction factor. Similar to the retroviral proteins mentioned above, most antagonists reduce tetherin levels at the sites of budding to enable efficient release of progeny virions.

The Herpes simplex virus 1 (HSV-1) glycoprotein M (gM), the Chikungunya virus non-structural protein 1 (Nsp1) and the Env of SIVtan and equine infectious anemia virus (EIAV), for instance, all reduce the surface expression levels of tetherin (Gupta et al., 2009b; Blondeau et al., 2013; Jones et al., 2013; Yin et al., 2014). Neuraminidase (N1 and N2) has been suggested to be the tetherin antagonist of influenza viruses (Yondola et al., 2011; Mangeat et al., 2012; Leyva-Grado et al., 2013) and Sendaivirus uses the fusion (F) and hemagglutinin-neuraminidase proteins (HN) in concert to induce the degradation of the restriction factor (Bampi et al., 2013).

In 2006, tetherin was identified in a screen for factors that are downmodulated by the RING-CH ubiquitin ligase K5 of the Kaposi's sarcoma-associated herpesvirus (KSHV, HHV-8; Bartee

et al., 2006). It soon became clear that HHV-8 utilizes K5 to ensure efficient virion release by inducing the degradation of tetherin. K5 ubiquitinates lysine 18 in the cytoplasmic tail of tetherin thereby targeting it for proteasomal or ESCRT-dependent endo-lysosomal degradation (**Figure 3D**; Mansouri et al., 2009; Pardieu et al., 2010; Agromayor et al., 2012). In agreement with an ongoing coevolution between tetherin and its viral antagonists, K5 efficiently counteracts human tetherin but fails to antagonize the rhesus macaque and mouse orthologs (Pardieu et al., 2010). Bartee et al. (2006) made the interesting observation that MARCH-VIII, the cellular homolog of K5 is also able to induce the degradation of tetherin.

In contrast to other tetherin antagonists, the Ebolavirus glycoprotein (Gp) is able to enhance virion release without decreasing the surface levels of the restriction factor (**Figure 3E**; Lopez et al., 2010; Kühl et al., 2011). Interestingly, removal of tetherin from lipid rafts is not involved either (Lopez et al., 2010) and Ebolavirus Gp fails to rescue the release of arenaviruses (Radoshitzky et al., 2010). Although the exact mechanism of Gp-mediated counteraction of tetherin remains unclear, it has been suggested that tetherin interacts directly with the GP2 subunit (Kühl et al., 2011). A similar mechanism has also been proposed for FIV Env. Morrison et al. (2014) showed that FIV Env incorporation is required to antagonize feline tetherin but does not involve a reduction of total or surface tetherin levels. Like Ebola Gp, FIV Env was not able to rescue the release of non-cognate particles (Celestino et al., 2012; Morrison et al., 2014). However, another study suggested that FIV does not encode a direct antagonist but rather overcomes restriction by direct cell-to-cell spread (Dietrich et al., 2011).

Thus, viruses may also evolve evasion strategies without directly targeting tetherin. It has for example been suggested that some viruses do not induce IFN production *in vivo* or have evolved means to inhibit the IFN-mediated expression of tetherin (Lim and Emerman, 2009; Mangeat et al., 2012). Influenza virus for instance does not only use its Neuraminidase protein to directly antagonize tetherin but may also impede the induction of tetherin via the viral protein NS1 (Mangeat et al., 2012). To evade restriction some viruses such as HCV may also bud from internal membranes that contain no or only low levels of tetherin. Similarly, direct cell-to-cell spread has been suggested to be used by some viruses to overcome restriction (Jolly et al., 2010; Dietrich et al., 2011; Ilinskaya et al., 2013). Furthermore, the antiviral activity of tetherin is simply saturated if a large number of virions is budding (Yadav et al., 2012). Some viruses may even exploit tetherin for their own benefit: HTLV-1 infected cells produce tetherin-containing extracellular viral assemblies that are transferred to neighboring cells and are required for efficient spread of infection (Pais-Correia et al., 2010). Tetherin has also been suggested to enhance the entry of human cytomegaloviruses and to be required for efficient FIV particle release (Viswanathan et al., 2011; Morrison et al., 2014). In summary, viruses have evolved a multiplicity of mechanisms to counteract, evade or even hijack the restriction imposed by tetherin.

SUMMARY AND CONCLUDING REMARKS

The continuous arms race between viruses and their hosts has certainly driven the evolution of the host restriction factor tetherin. Whereas the ability of tetherin to restrict virion release seems to be an ancient function that is highly conserved among all mammalian orthologs, some species have evolved unique features of this protein. Bovids, for instance, express two tetherin homologs that probably differ in their expression pattern and may thus facilitate adaptation to emerging viral infections (Arnaud et al., 2010). Human and (to a lesser extent) chimpanzee tetherin are apparently the only tetherin orthologs that act as innate sensors activating the NF-κB signaling cascade upon binding of viral particles (Galão et al., 2012). Conversely, tetherin also exerted a substantial selection pressure on viral evolution. Diverse enveloped viruses have evolved effective ways of escaping restriction. Whereas some viral proteins directly target tetherin and sequester it away from the sites of budding and/or induce its degradation, other viruses may overcome restriction via cell-to-cell spread or prevent the mounting of an IFN response to inhibit expression of tetherin and other ISGs.

The AIDS pandemic is an impressive example of this ongoing coevolution between viruses and tetherin. Analyses of different HIV-1 groups revealed that counteraction of tetherin may be a prerequisite for the efficient spread of lentiviruses in the human population (Sauter et al., 2010): in contrast to pandemic HIV-1 group M viruses, non-pandemic HIV-1 groups N, O, and P strains failed to evolve efficient tetherin antagonists after cross-species transmissions of SIV to humans (Sauter et al., 2009, 2011a). Notably, however, patients infected with non-pandemic HIV-1 strains develop high viral loads and ultimately progress to AIDS although tetherin is not efficiently counteracted (Ayouba et al., 2000; Plantier et al., 2009; Vessière et al., 2010; Delaugerre et al., 2011). This strongly suggested that the evolution of a specific tetherin antagonist is required for efficient viral transmission and spread of the virus in the population rather than for efficient replication within an infected individual. In agreement with this, the presence of Vpu did not or only slightly enhance the cytopathicity of HIV-1 in humanized mouse models (Aldrovandi and Zack, 1996; Sato et al., 2012). Furthermore, Vpu boosted the initial phase of R5- and X4-tropic HIV-1 replication but was less important for viral dissemination during late stages of infection (Aldrovandi and Zack, 1996; Sato et al., 2012; Dave et al., 2013). These observations would be in agreement with a model in which cell-free virions are involved in transmission of the virus, whereas spread within patients occurs mainly via direct cell-to-cell spread that overcomes tetherin restriction (Jolly et al., 2010; Sato et al., 2012).

A recent publication by Pickering et al. (2014), however, challenged this hypothesis revealing that potent anti-tetherin activity is not only a characteristic of transmitted/founder viruses but preserved throughout infection. Thus, efficient counteraction of tetherin may confer a selective advantage during all stages of viral infection. One possible explanation for this observation is that tetherin may still restrict direct cell-to-cell spread of HIV-1. In contrast to Jolly et al. (2010), Kuhl et al. (2010) reported that tetherin reduces the transfer of infectious material via virological synapses. Similarly, Casartelli et al. (2010) also showed that tetherin is able to restrict direct cell-to-cell spread. They suggest that tetherin induces the transfer of large patches of cross-linked viruses with reduced infectivity from producer to target cells (Casartelli

et al., 2010). Alternatively, the contribution of cell-free virions to persistant HIV-1 infection may be higher than initially thought. This hypothesis is supported by the successful use of broadly neutralizing antibodies in recent vaccine trials (Klein et al., 2013). Furthermore, different functions of tetherin may exert selection pressure at different stages of the viral replication cycle. Thus, even if restriction of virion release primarily affects transmission efficiency and early viral dissemination, the sensing activity of tetherin (Galão et al., 2012) may still exert a high selection pressure on Vpu function during late stages of infection. In addition to its restriction and signaling functions, tetherin seems to enhance the antibody opsonization of infected cells by increasing the accessibility of epitopes on the cell surface (Alvarez et al., 2014; Pham et al., 2014). As a result, Vpu-mediated down-modulation of tetherin may confer a selective advantage throughout all stages of infection because it reduces the susceptibility of infected cells to NK-cell-mediated antibody-dependent cellular cytotoxicity (Alvarez et al., 2014; Pham et al., 2014).

Several additional mouse studies affirmed a crucial role of tetherin in restricting viral infection *in vivo*. In contrast to the humanized mouse models described above, these studies focused on the infection of immunocompetent mice with murine pathogens. In a study by Liberatore and Bieniasz (2011), viral loads were significantly increased in tetherin knockout mice infected with Moloney MLV and these mice progressed to disease faster than their wild type littermates. Increased replication of Moloney MLV in tetherin-deficient mice was also confirmed by a study of Swiecki et al. (2012). Paradoxically, however, viral titers in the lungs were reduced upon infection with vesicular stomatitis virus in this mouse model (Swiecki et al., 2012). A third *in vivo* study took advantage of a polymorphism that disrupts the first start codon of tetherin (Barrett et al., 2012). As a consequence, these mice express only the short form of tetherin which lacks the tyrosine-based endocytosis signal and is therefore expressed to higher levels at the cell surface. In agreement with the other two studies, the increased tetherin expression resulted in decreased viral loads and reduced the pathogenic effects of Friend MLV (Barrett et al., 2012).

The observation that tetherin knockout mice do not show any obvious developmental or functional defects (Liberatore and Bieniasz, 2011) argues against essential cellular roles of tetherin beyond antiviral immunity. Some activities of tetherin such as the ability to induce the NF-κB signaling cascade may, however, be unique for the human ortholog. Thus, mouse models cannot fully recapitulate all characteristics of human tetherin and further studies are warranted to characterize the relative contribution of different tetherin functions in antiviral activity and beyond.

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