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RETROVIRUSES, RETROELEMENTS AND THEIR RESTRICTIONS

Topic Editors

Atsushi Koito and Yukihiro Ishizaka



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RETROVIRUSES, RETROELEMENTS AND THEIR RESTRICTIONS

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Human retroviruses, HIV and HTLV have been recognized as important pathogens because of their association with lethal diseases such as AIDS and ATL. Considerable resources and efforts have been directed at understanding the interaction between these retroviruses and their host which may provide clues as to how the infection can be controlled or prevented. Among the key scientific successes is the identification of intracellular “restriction factors” that have evolved as obstacles to the replication of pathogens including infectious retroviruses. The discovery of APOBEC, which are strong mutagens of retroviral genomes and intracellular retroelements, began a new era of intense research activities into the spectrum of intrinsic anti-HIV activity, leading to the identification of TRIM5 α , BST2/Tetherin, and SAMHD1. In response, HIV has evolved several accessory genes as weaponries to evade these intracellular restriction activities. The intracellular antiretroviral defenses evolved in response to endogenous retroelements that make up more than 40% of the entire mammalian genome, and which are regarded as ancestors of infectious retroviruses. LTR-type retroelements are present in all higher eukaryotes, representing about 8% of the human genome. Non-LTR retroelements can be found at extremely high copy numbers also, with a significant portion of mammalian genomes consisting of LINEs. Mammalian genomes are modified by LINEs through insertions, but also by the indirect replication of non-autonomous retrotransposons such as SINEs. LINEs insertion was shown to have played, and continue to play important roles in genomic evolution and somatic genome mosaicism-mediated physiology. And, because retrotransposition can confer genetic diversity that is beneficial to the host, the vertebrate intrinsic immunity has evolved to support a balance between retroelement insertions that confer beneficial and those that cause deleterious gene disruptions. The articles published in this Research Topic should serve not only as valuable references for the field, but provide future topics of research for investigators that should further our understanding of the retrovirus, retroelements and their restrictions.

Table of Contents

- 04 *Retroviruses, Retroelements and their Restrictions***
Atsushi Koito and Yukihiro Ishizaka
- 06 *Antiviral Mechanism and Biochemical Basis of the Human APOBEC3 Family***
Mayumi Imahashi, Masaaki Nakashima and Yasumasa Iwatani
- 13 *HIV-1 Vif: A Guardian of the Virus that Opens Up a New Era in the Research Field of Restriction Factors***
Akifumi Takaori-Kondo and Keisuke Shindo
- 18 *Vpu and BST2: Still not there Yet?***
Kei Sato, Peter Gee and Yoshio Koyanagi
- 27 *SAMHD-1 Dependent and –Independent Functions of HIV-2/SIV Vpx Protein***
Mikako Fujita, Masako Nomaguchi, Akio Adachi and Masami Otsuka
- 34 *Role of Human TRIM5 α in Intrinsic Immunity***
Emi E. Nakayama and Tatsuo Shioda
- 47 *Role of Host-Encoded Proteins in Restriction of Retroviral Integration***
Yasutsugu Suzuki, Mun Lok Chew and Youichi Suzuki
- 60 *Role of Nucleocytoplasmic RNA Transport During the Life Cycle of Retroviruses***
Hisatoshi Shida
- 71 *Animal Models on HTLV-1 and Related Viruses: What did we Learn?***
Hiba El Hajj, Rihab Nasr, Youmna Kfoury, Zeina Dassouki, Roudaina Nasser, Ghada Kchour, Olivier Hermine, Hugues de Thé and Ali Bazarbachi
- 89 *The Role of Genes Domesticated From LTR Retrotransposons and Retroviruses in Mammals***
Tomo koKaneko-Ishino and Fumitoshi Ishino
- 99 *Modes of Retrotransposition of Long Interspersed Element-1 by Environmental Factors***
Yukihiro Ishizaka, Noriyuki Okudaira, Masato Tamura, Kenta Iijima, Mari Shimura, Motohito Goto and Tadashi Okamura
- 107 *Retroelements Versus APOBEC3 Family Members: No Great Escape From the Magnificent Seven***
Juan F. Arias, Takayoshi Koyama, Masanobu Kinomoto and Kenzo Tokunaga
- 119 *Intrinsic Immunity Against Retrotransposons by APOBEC Cytidine Deaminases***
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Retroviruses, retroelements and their restrictions

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HIV and HTLV have been recognized as important pathogens because of their association with lethal diseases in human: HIV causes Acquired Immunodeficiency Syndrome (AIDS) and HTLV is the etiological agent for adult T-cell leukemia. Considerable resources and efforts therefore have been directed at understanding the interaction between these human retroviruses and their host which may provide clues as to how the infection can be controlled or prevented. Among the key scientific successes is the identification of intracellular “restriction factors” that have evolved as obstacles to the replication of pathogens including infectious retroviruses. The discovery of APOBEC3 cytidine deaminases, which are strong mutagens of retroviral genomes and intracellular retroelements, opened a new era of intense research activities into the spectrum of intrinsic anti-HIV activity, leading to the identification of TRIM5 α , BST2/Tetherin, and SAMHD1. In response, HIV has evolved several accessory genes as weaponries to evade or antagonize these intracellular restriction activities. This issue of Research Topic covers several reviews of the mechanisms of these restriction factors and their counterbalance by HIV-1 gene products. The anti-HIV-1 control mechanism by APOBEC3 cytidine deaminase is explained in the paper by Imahashi et al. (2012), while the article by Takaori-Kondo and Shindo (2013) discusses the functions of the viral infectivity factor (Vif) in the HIV life cycle and its integral role in antagonizing APOBEC3. In Sato et al. (2012) review, the interaction between HIV-1 Vpu and other viral proteins with BST2/Tetherin is presented, and Fujita et al. (2012) describe the functions of HIV-2/SIV encoded Vpx and how it counteracts SAMHD1 to facilitate viral replication. TRIM5 α - and Fv1-mediated restriction activity that targets the incoming viral capsid to prevent uncoating is reviewed by Nakayama and Shioda (2012), and Suzuki et al. (2012) discuss host factors that restrict retroviral integration. In addition, investigations into the export of HIV and HTLV genomic RNA from the nucleus are summarized by Shida (2012), and animal models on HTLV and related retroviruses are evaluated by Hajj et al. (2012).

The intracellular antiretroviral defenses evolved in response to endogenous retroelements that make up more than 40% of the entire mammalian genome, and which are regarded as ancestors of infectious retroviruses. Long term repeat (LTR)-type retroelements are present in all higher eukaryotes, representing about 8% of the human genome. The critical role played by LTR retrotransposons in “hijacking” retroviral genes to shape mammalian evolution is described by Kaneko-Ishino and Ishino (2012). Non-LTR retroelements can be found at extremely high copy numbers also, with a significant portion of mammalian genomes consisting of long interspersed elements (LINEs). Mammalian genomes are modified by LINEs through insertions, but also by the indirect replication of non-autonomous retrotransposons such as short interspersed elements (SINEs). LINEs insertion was shown to have played, and continue to play important roles in genomic evolution and somatic genome mosaicism-mediated physiology such as brain activity in mammalian species. Despite the impact of LINEs insertion, much of the process of LINEs retrotransposition remains unexplored. The contribution by Ishizaka et al. (2012) describes the cellular factors required for the LINEs retrotransposition. And, because retrotransposition can confer genetic diversity that is beneficial to the host, the vertebrate intrinsic immunity has evolved to support a balance between retroelement insertions that confer beneficial and those that cause deleterious gene disruptions. Arias et al. (2012) summarize our current understanding of the anti-retroelement and antiretroviral activities and functions of human APOBEC3s. The mechanism of action of not only APOBEC3 but also of other APOBEC cytidine deaminases such as APOBEC1, and their role in controlling mobile elements are discussed by Koito and Ikeda (2013).

The papers published in this Research Topic present findings and observations that are informative and insightful. They should serve not only as valuable references for the field, but provide future topics of research for investigators that should further our understanding of the retrovirus, retroelements and their restrictions.

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Antiviral mechanism and biochemical basis of the human APOBEC3 family

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The human APOBEC3 (A3) family (A, B, C, DE, F, G, and H) comprises host defense factors that potentially inhibit the replication of diverse retroviruses, retrotransposons, and the other viral pathogens. HIV-1 has a counterstrategy that includes expressing the Vif protein to abrogate A3 antiviral function. Without Vif, A3 proteins, particularly APOBEC3G (A3G) and APOBEC3F (A3F), inhibit HIV-1 replication by blocking reverse transcription and/or integration and hypermutating nascent viral cDNA. The molecular mechanisms of this antiviral activity have been primarily attributed to two biochemical characteristics common to A3 proteins: catalyzing cytidine deamination in single-stranded DNA (ssDNA) and a nucleic acid-binding capability that is specific to ssDNA or ssRNA. Recent advances suggest that unique property of A3G dimer/oligomer formations, is also important for the modification of antiviral activity. In this review article we summarize how A3 proteins, particularly A3G, inhibit viral replication based on the biochemical and structural characteristics of the A3G protein.

Keywords: antiviral, APOBEC3, APOBEC3G, cytidine deaminase, HIV, retrovirus, reverse transcription, Vif

INTRODUCTION

Productive infections of primary human lymphocytes, monocytes, and certain T-cell lines by HIV-1 require a virally encoded gene product, Vif (originally named “Sor” or “A”; Fisher et al., 1987; Strebel et al., 1987). In early work on Vif, vif-deficient virions produced in non-permissive cells were found to be significantly impaired in their ability to complete reverse transcription (Sova and Volsky, 1993; von Schwedler et al., 1993), and they were 100- to 1000-fold less infectious than wild type (WT) virions (Fisher et al., 1987; Strebel et al., 1987; Fouchier et al., 1996). Sheehy et al. (2002) identified A3G as the cellular enzyme that restricts the replication of vif-deficient HIV-1.

The human A3G protein is a cellular cytidine deaminase that belongs to the APOBEC3 family, which comprises seven members (A3A, B, C, DE, F, G, and H; LaRue et al., 2009). These proteins contain one (A3A, A3C, and A3H) or two (A3B, A3DE, A3F, and A3G) zinc-cluster domains with the consensus sequence (H/C)XE(X)_{23–28}CXXC (Wedekind et al., 2003). Among the APOBEC3 family members, A3G is the most potent inhibitor of HIV-1 but only in the absence of Vif. HIV-1 Vif counteracts A3G by promoting its polyubiquitination through the recruitment of a Cullin5-based E3 ubiquitin ligase complex (Yu et al., 2003), which targets A3G proteins for rapid proteasomal degradation in infected cells.

The specific A3G degradation is determined by the capability of Vif to bind with A3G in the E3 ubiquitin ligase complex (reviewed in Kitamura et al., 2011). The region in A3G responsible for HIV-1 Vif interaction was identified by the studies on the species specificity of Vif-mediated A3G degradation, which is determined by a single amino acid difference in human A3G, D128 versus K128 in

the A3G of African green monkeys (Bogerd et al., 2004; Mangeat et al., 2004; Schrofelbauer et al., 2004; Xu et al., 2004). Subsequent mutational analyses have confirmed that the 128DPD130 motif of A3G, located near the zinc-coordinating residues of NTD, is crucial for direct binding to HIV-1 Vif (Huthoff and Malim, 2007; Russell et al., 2009; Lavens et al., 2010). This motif is just downstream of residues 124YYFW127, which are involved in A3G's ability to bind nucleic acids (Huthoff and Malim, 2007).

The primary mechanism by which A3G inhibits vif-deficient HIV-1 replication requires its expression in virus producer cells and its incorporation into virions (Mariani et al., 2003; Marin et al., 2003; Sheehy et al., 2003; Stopak et al., 2003; Svarovskaia et al., 2004). During reverse transcription in the target cells, the virion-packaged A3G deaminates cytidine to uridine in the viral minus-strand DNA (Harris et al., 2003a; Lecossier et al., 2003; Mangeat et al., 2003; Zhang et al., 2003; Suspène et al., 2004; Yu et al., 2004). Subsequent incorporation of adenines instead of guanines in the plus-strand results in extensive G-to-A hypermutation and inactivation of the viral genome. Shortly after A3G was suggested as the key restriction factor against vif-deficient HIV-1, it was proposed that A3G-mediated deamination might be a lethal trigger, eventually leading to the degradation of reverse-transcribed viral DNA through a base-excision pathway and/or the reduced replication of progeny viruses by introducing premature stop codons and/or amino acid changes (Cullen, 2003; Goff, 2003; Harris et al., 2003a,b; KewalRamani and Coffin, 2003). Indeed, the catalytic center of the A3G protein is clearly essential for its antiviral functions (Mangeat et al., 2003; Navarro et al., 2005; Iwatani et al., 2006; Browne et al., 2009). However, several lines of recent evidence have indicated that the catalytic activity of A3G is not

sufficient to explain its full antiviral activity. What is the fundamental mechanism(s) of A3G antiviral activity that explains the observation by von Schwedler et al. (1993), that the reverse transcription of *vif*-deficient HIV-1 is impaired when produced from A3G-expressing “non-permissive” cells?

BIOCHEMICAL PROPERTIES OF A3G

The zinc coordination of A3 family proteins is mediated by a histidine and two cysteines, which form a catalytic center for cytidine deaminase activity. In A3G, the zinc-binding motif at the C-terminal domain (CTD) is primarily associated with cytidine deaminase catalysis whereas the N-terminal domain (NTD) does not catalyze deamination (**Figure 1A**; Haché et al., 2005; Navarro et al., 2005; Iwatani et al., 2006; Friew et al., 2009). The A3G enzyme converts deoxycytidine (dC) residues to deoxyuridine (dU), and acts preferentially on residues that are preceded by another dC, with a much higher preference for the 5'-CCCA-3' sequence in single-stranded DNA (ssDNA; Beale et al., 2004; Suspène et al., 2004; Yu et al., 2004). During retroviral reverse transcription, A3G deaminates dC to dU in the viral minus-stranded DNA, and the subsequent incorporation of deoxyadenines (dA) instead of deoxyguanines (dG) in the plus-strand results in G-to-A hypermutation of the nascent viral DNA (Harris et al., 2003a; Lecossier et al., 2003; Mangeat et al., 2003; Zhang et al., 2003; Suspène et al., 2004; Yu et al., 2004). The ssDNA-specific deamination by A3G appears to be determined by a structural groove, presumably accommodating ssDNA, that positions the cytosine for deamination (Chen et al., 2008; Holden et al., 2008).

The nucleic acid-binding property of A3G is also a major biochemical feature. The minimum unit of A3G for binding to ssDNA is a monomer (Chelico et al., 2010) and/or a dimer (Bennett et al.,

2008), as illustrated in **Figure 1B**. The apparent equilibrium dissociation constant (K_d) for A3G (to ssDNA) is between 52 and 238 nM (Chelico et al., 2006; Iwatani et al., 2006, 2007), whereas the K_d for HIV-1 nucleocapsid protein (NC) binding to RNA is approximately 23–320 nM (Shubbs et al., 2002; Levin et al., 2005), suggesting that the nucleic acid-binding affinity of A3G is as high as that of NC. A3G binds preferentially to ssDNA or ssRNA (**Figure 1B**; Yu et al., 2004; Iwatani et al., 2006; Shlyakhtenko et al., 2011), especially dT or dU residues of ssDNA and AU-rich regions in ssRNA, respectively (Jarmuz et al., 2002; Iwatani et al., 2006). Interestingly, the substrate specificities and nucleotide preferences of the A3G protein differ for its deaminase and nucleic acid-binding activities, as is the case for APO1 (Anant et al., 1995; Navaratnam et al., 1995; Anant and Davidson, 2000). Because mutations that disrupt zinc coordination at the NTD, such as the substitution of the C100 residue with a serine, abrogate the nucleic acid-binding affinity of A3G (Navarro et al., 2005; Iwatani et al., 2006), some local conformation near the zinc coordination of NTD might be responsible for its recognition of single-stranded nucleotides.

The formation of an A3G homo-multimer is the third unique feature of A3G. The intrinsic propensity of A3G multimerization has been verified by biochemical and structural studies (Jarmuz et al., 2002; Navarro et al., 2005; Burnett and Spearman, 2007; Bennett et al., 2008; Bulliard et al., 2009; Friew et al., 2009; Huthoff et al., 2009; Chelico et al., 2010; McDougall et al., 2011). Because the full-length A3G structure has not been determined, the A3G interface for multimerization remain unclear. However, structural analyses by SAXS (small-angle X-ray scattering), co-immunoprecipitation assays (Wedekind et al., 2006; Bennett et al., 2008), and X-ray crystallography (Shandilya et al., 2010) have

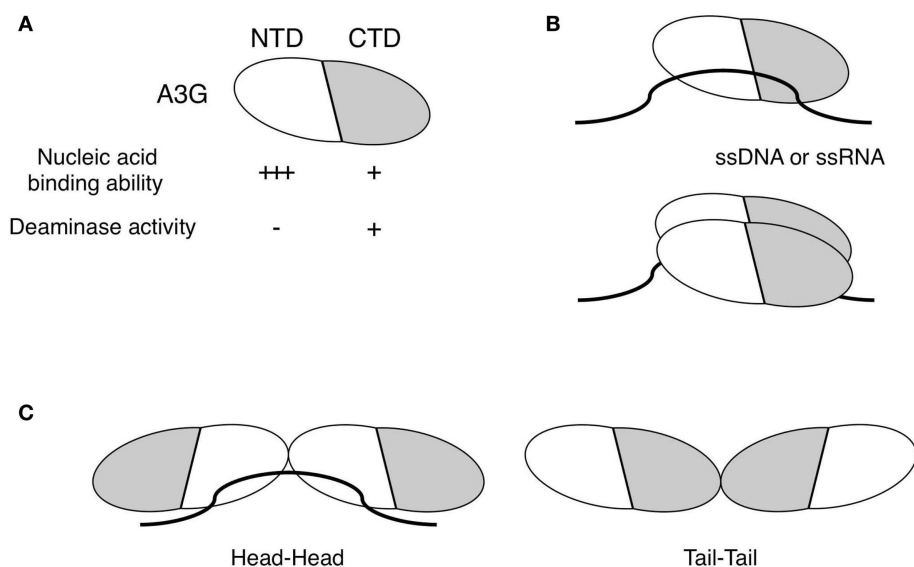


FIGURE 1 | Biochemical characteristics of A3G. (A) A3G consists of an NTD and CTD. The NTD is responsible for the nucleic acid-binding affinity of A3G and has no detectable deaminase activity. In contrast, the CTD is solely involved in deaminase activity and has less affinity for nucleic acids than the NTD. **(B)** A3G specifically binds to single-stranded

DNA (ssDNA) or RNA (ssRNA) but not to double-stranded nucleotides. A monomeric and/or dimeric A3G bind to ssDNA or ssRNA as the minimum unit. **(C)** The A3G protein forms homodimers or higher-order homo-oligomers through interactions between its NTDs (head-head) or CTDs (tail-tail).

demonstrated an interaction between the A3G CTDs (tail–tail), as illustrated in **Figure 1C**. In addition, homo-dimerization through the NTDs also occurs (head–head), as shown in **Figure 1C**, and this interaction appears to depend on the presence of RNA (Frieu et al., 2009; Huthoff et al., 2009). These observations were supported by an analytical ultracentrifugation study that showed a predominant dimer form of A3G in equilibrium with minor monomeric and tetrameric species under RNA-depleted conditions (Salter et al., 2009).

Chelico et al. (2006, 2008, 2010) have used an *in vitro* system to demonstrate that A3G has a 3' to 5' catalytic orientation specificity for the deamination of naked ssDNA (Feng and Chelico, 2011). The preferred asymmetric direction for A3G catalysis likely yields an approximately 30-nt “dead” zone located at the 3' end of ssDNA that is much less efficiently deaminated by A3G (Chelico et al., 2008, 2010). However, we need further investigations on how significant the length of dead zone is because the central CCC motif of ~40-nt ssDNA can be deaminated efficiently by A3G in our or the other *in vitro* deamination assay systems (Beale et al., 2004; Yu et al., 2004; Iwatani et al., 2006). The formation of tetramers and higher-order homo-oligomers of A3G on ssDNA is required for efficient deamination (McDougall et al., 2011).

ANTIVIRAL MECHANISMS OF A3G AGAINST VIF-DEFICIENT HIV-1

A3G exerts its inhibitory activity by being encapsidated into virus particles of *vif*-deficient HIV-1. During the subsequent infection cycle, A3G has been proposed to interfere with reverse transcription and/or integration through one or more molecular mechanisms (**Figure 2**). Based on whether the enzymatic activity is involved or not, there are two separable mechanisms, i.e., deaminase-dependent and -independent mechanisms.

Although the catalytic center of A3G is clearly critical for its antiviral effect (Mangeat et al., 2003; Navarro et al., 2005; Iwatani et al., 2006; Browne et al., 2009), the precise molecular mechanisms underlying the inhibition of the further processing of A3G-deaminated DNA products in cells remain unclear. A3G-mediated hypermutation of viral genomes is clearly detrimental to further spreading the infection because mutations in the viral structural and/or the regulatory genes may trigger defects in the production of infectious progeny virus (“1” in **Figure 2**). For example, because the preferred sequences of A3G include TGG (a codon for tryptophan within the viral *orf*), many G-to-A mutations may incidentally produce premature stop codons, such as TAG (or TGA), resulting in viral inactivation (Simon et al., 2005; Pace et al., 2006).

In its second mechanism, A3G reduces the efficiency and specificity of primer tRNA processing and removal, resulting in proviral DNA ends that are aberrant substrates for integration and plus-strand DNA transfer (Luo et al., 2007; Mbisa et al., 2007; “2” in **Figure 2**). In this mechanism, the presumed deamination sites are located at the plus primer-binding site (PBS), which is annealed by the tRNA. Considering the biochemical characteristics of A3G, it remains unclear how the A3G enzyme deaminates cytidine residues on the DNA/RNA duplex and near the 3' end of the plus-strand transfer donor DNA, which is supposed to be a presumed

“dead” zone for A3G-mediated deamination (Chelico et al., 2008, 2010).

It was hypothesized that the antiviral functions of A3G might be associated with the uracilation of the nascent reverse transcripts (Harris et al., 2003a; Zhang et al., 2003), resulting in their degradation through the activity of cellular DNA glycosylases, e.g., UDG2 (uracil DNA glycosidase 2) and SMUG1 (single-strand selective monofunctional uracil DNA glycosylase). However, several groups have revealed that neither uracil DNA glycosidase affected the antiviral effect of A3G (Kaiser and Emerman, 2006; Mbisa et al., 2007; Langlois and Neuberger, 2008), although one study showed that UDG2 is involved in the degradation of nascent reverse transcripts (Yang et al., 2007). In addition, we cannot exclude the possibility that other unidentified DNA repair enzymes might participate in the degradation mechanism. Therefore, further studies will be required to elucidate the potential factors that precede the degradation of uracilated DNA following A3G-mediated deamination (“4” in **Figure 2**).

Earlier studies on A3G suggested that G-to-A hypermutation resulting in lethal mutations was the sole basis of the A3G antiviral mechanism. However, more recent studies have demonstrated that the catalytic activity of A3G may not wholly determine its molecular mechanism, i.e., a deaminase-independent mechanism might also be involved in A3G antiviral activity: (i) mutations of the catalytic center do not completely abolish antiviral activity against HIV-1 (Navarro et al., 2005; Iwatani et al., 2006; Holmes et al., 2007; Luo et al., 2007; Miyagi et al., 2008); (ii) A3G inhibits replication of hepatitis B virus without detecting significant G-to-A hypermutation (Turelli et al., 2004; Bonvin and Greeve, 2007; Nguyen et al., 2007; reviewed in Bonvin and Greeve, 2008); and (iii) other A3 proteins block the replication of HIV-1 (Luo et al., 2007; Miyagi et al., 2010), mouse mammary tumor virus (Okeoma et al., 2007), murine leukemia virus (Takeda et al., 2008), and parvovirus adeno-associated virus (Narvaiza et al., 2009) and retrotransposition of LINE-1 and Alu (Bogerd et al., 2006; Muckenfuss et al., 2006; Stenglein and Harris, 2006; Wissing et al., 2011) despite the absence of editing activity.

Several groups have reported that the deaminase-independent mechanisms of reverse transcription inhibition would involve interference with tRNA primer annealing, initiation and elongation of DNA synthesis, and minus-/plus-strand transfer reactions (Guo et al., 2006, 2007; Iwatani et al., 2007; Li et al., 2007; Luo et al., 2007; Mbisa et al., 2007; Anderson and Hope, 2008; Bishop et al., 2008; Zhang et al., 2008). Using enzymatically active recombinant A3G and the *in vitro* reconstituted systems of HIV-1 reverse transcription, it has been demonstrated that A3G blocks all RT-catalyzed DNA elongation processes in a deaminase-independent manner, although the protein does not significantly interfere with tRNA primer placement (Iwatani et al., 2007). Moreover, the analysis of endogenous reverse transcription in cell-free HIV-1 particles also indicated that A3G reduces HIV-1 viral DNA levels by inhibiting the elongation of reverse transcription rather than by inducing the degradation of the reverse transcripts (Bishop et al., 2008). The block of RT elongation by A3G might be attributed to A3G's unique nucleic acid-binding ability (Iwatani et al., 2007). More recently, Wang et al. (2012) have observed physiological and functional interactions between RT and A3G, although our group

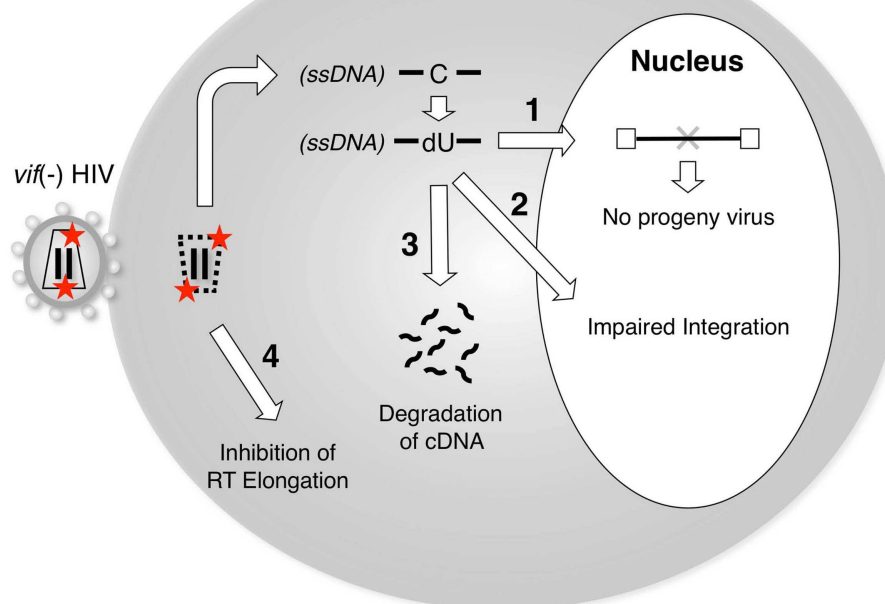


FIGURE 2 | A3G blocks the reverse transcription and/or integration of *vif*-deficient HIV-1. Packaging of A3G proteins into *vif*-deficient virus particles is prerequisite for the inhibition of viral replication by A3G. Upon the infection of target cells, A3G blocks the post-entry step of viral replication by one or more of the following mechanisms: (1) Cytidine deamination of nascent reverse transcripts by A3G enzymes could prevent progeny virus production due to inactivating mutations in viral genes and/or proteins. (2)

A3G-mediated editing might create aberrant structures at viral DNA ends, which might be inefficient substrates for integration. (3) The reverse transcripts containing dU might induce DNA degradation by cellular DNA repair pathways. (4) RT-mediated elongation could be blocked by the presence of A3G on RNA or DNA templates. A3G might exert both deaminase activity-dependent (1–3) and deaminase activity-independent (4) functions to inhibit *vif*-deficient HIV-1 replication.

has been unable to detect direct interactions using recombinant A3G and RT proteins (Iwatani, Y., and Levin, J. G., unpublished observations). It might be interesting to know whether direct interaction is applicable to other A3 proteins and/or retroviral RTs, i.e., how the broad range of A3G's inhibitory effect can be linked to the specific interaction between RT and A3G. Further investigations are required to understand the molecular mechanisms of the deaminase-independent pathway in more detail.

CONCLUSIONS

Studies over the past 10 years have established that human APOBEC3 family proteins potently restrict retroviral replication. However, the molecular mechanisms of the A3 family's antiviral activities remain unclear. Recent biochemical studies of A3G may provide a better understanding of these mechanisms. Currently, it

is possible that the deaminase activity of A3G is largely required for its antiviral activity against *vif*-deficient HIV-1, although it is not known whether A3G-mediated deamination and/or the architecture of the catalytic center in A3G are intrinsically required for its inhibitory activity. Further investigations will provide the fundamental answers to explain the first observation by von Schwedler et al., made when A3G had not yet been discovered.

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HIV-1 Vif: a guardian of the virus that opens up a new era in the research field of restriction factors

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The research on virion infectivity factor (Vif) protein had started in late 1980s right after HIV-1 was cloned, and the function of Vif had been a mystery for a long time. However, the research on Vif has finally lead to the identification of APOBEC3G, which opens up a new era in the research field of host restriction factors in HIV-1 infection followed by TRIM5 α , Tetherin/BST-2, and SAMHD1. This suggests that continuation of basic research on fundamental questions is quite important. We still have many questions on Vif and APOBEC3 and should continue to work on these proteins in the future in order to better regulate HIV-1. We will discuss not only the history but also recent advances in Vif research.

Keywords: HIV-1 Vif, restriction factor, ubiquitin ligase, cell cycle arrest, p53, MDM2

INTRODUCTION

HIV-1 virion infectivity factor (Vif) was identified as an accessory gene right after the HIV-1 genome was sequenced. It is well conserved among lentiviruses except in equine infectious virus and plays a crucial role in the viral life cycle to facilitate viral infectivity as its name indicates (Desrosiers et al., 1998). In the early reports, Strebel and colleagues described that the mutant virus deficient in the *vif* gene produces virion particles normally; however, the particles are ~ 1000 times less infectious than the wild type (Fisher et al., 1987; Strebel et al., 1987). The underlying mechanism of Vif function had been unsolved and a mystery for a long time.

EARLY OBSERVATIONS OF VIF FUNCTION LEAD TO IDENTIFICATION OF APOBEC3G

Virion infectivity factor exerts its function in a cell-type-specific manner. Vif is dispensable for producing infectious viral particles in permissive cells such as all known adherent cells (e.g., HeLa and 293T cells) and some T cell lines (e.g., CEM-SS and SupT1 cells); in contrast, Vif is indispensable in non-permissive cells such as physiologically relevant CD4⁺ T cells and macrophages, and other T cell lines (e.g., CEM and H9 cells; Gabuzda et al., 1992; Sakai et al., 1993; Simon et al., 1998b). These findings raise two possibilities; one is that permissive cells have a vif-like cellular factor which facilitates virion infectivity, another is that non-permissive cells possess an anti-HIV-1 host factor which is antagonized by Vif. Later studies using heterokaryon experiments have shown the latter possibility (Madani and Kabat, 1998; Simon et al., 1998a). In 2002, Malim's group identified this factor using very sophisticated subtraction cloning methods between non-permissive CEM cells and its derivative subclone permissive CEM-SS cells, which was first called as CEM15 and is now known as APOBEC3G (Sheehy et al., 2002). Details of functions of APOBEC3G and other APOBEC3 family members are described and discussed in many

reviews and other chapters of this issue (Goila-Gaur and Strebel, 2008; Wissing et al., 2010; Kitamura et al., 2011).

In addition to the above described main function, early studies also revealed several important Vif functions including dimerization (Yang et al., 2001), virion incorporation (Camaur and Trono, 1996; Simon et al., 1997), and phosphorylation (Yang et al., 1996; Yang and Gabuzda, 1998); however, the significances of these functions are not discussed much recently. Recently, a novel Vif function on cell cycle has been reported, which is discussed in more detail later.

VIF ANTAGONIZES APOBEC3G

As described above, the main function of Vif is to antagonize APOBEC3G. Right after identification of APOBEC3G, many studies have shown that Vif inhibits the virion incorporation of APOBEC3G, which is mainly attributable to degradation of cellular APOBEC3G via the proteasomal pathway (Marin et al., 2003; Sheehy et al., 2003; Stopak et al., 2003; Mehle et al., 2004b). However, some studies have also shown that Vif directly inhibits the virion incorporation of APOBEC3G (Opi et al., 2007) or that Vif inhibits translation of APOBEC3G (Mariani et al., 2003; Stopak et al., 2003).

Yu et al. (2003) have independently shown that Vif forms E3 ligase complexes with cellular proteins including Cullin 5, Elongin B, and C (Vif-Cul5-EloB/C complex) using mass-spectrometry techniques. They and others have also shown that this complex works as the E3 ligase for APOBEC3G to induce polyubiquitination of APOBEC3G and direct it to the 26S proteasome for degradation (Mehle et al., 2004a; Yu et al., 2004; Kobayashi et al., 2005). Iwatani et al. (2009) have identified four critical lysine residues (K²⁹⁷, K³⁰¹, K³⁰³, and K³³⁴) in APOBEC3G which are required for Vif-mediated degradation, although others have reported that Vif can ubiquitinate and degrade a lysine-free APOBEC3G

(Shao et al., 2010). Vif also antagonizes other APOBEC3 proteins from APOBEC3C to H by the same E3 ligase complex (Shirakawa et al., 2006).

Virion infectivity factor binds to the E3 ligase complex through two interaction sites; it binds to Elongin C through its suppressors of cytokine signaling (SOCS) box motif (Mehle et al., 2004a; Yu et al., 2004), S¹⁴⁴LQYLA¹⁴⁹, and to Cullin 5 through a zinc-binding motif (Luo et al., 2005; Mehle et al., 2006), H¹⁰⁸X₅CX_{17–18}CX_{3–5}H¹³⁹ (Figure 1). The SOCS box motif is well conserved among Vif proteins, indicating that this motif is crucial for Vif function, and mutation of S¹⁴⁴, a presumed phosphorylation site in Vif, affects binding of Vif to Elongin C (Mehle et al., 2004a). The zinc-binding motif is also important for Vif function to form the E3 ligase complex. Therefore, a zinc chelating agent can inhibit Vif function in infectivity assays (Xiao et al., 2007).

THE INTERACTION OF Vif WITH APOBEC3 PROTEINS

It is quite important to reveal the interaction sites between Vif and APOBEC3 proteins, because the regulation of this interaction may lead to the development of novel therapeutic strategies for HIV-1 infection. However, their structural information is not fully elucidated yet, because it is quite difficult to produce these proteins as soluble forms. Thus, the information described below is mainly obtained by many studies using site-directed mutagenesis, which sometimes shows different results.

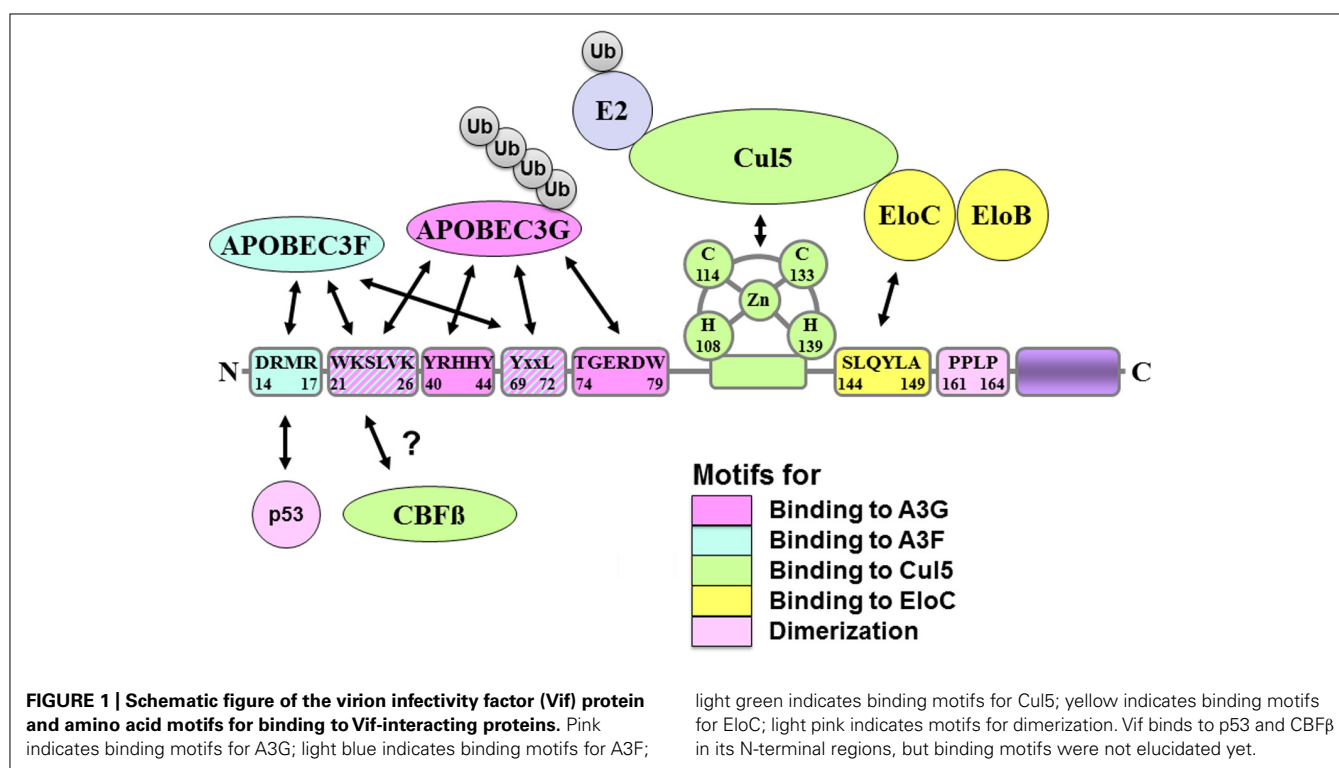
First of all, the most important and confirmed evidence is that the interaction between Vif and APOBEC3G is critically dependent on D¹²⁸PD¹³⁰ in APOBEC3G (Huthoff and Malim, 2007). Many groups have simultaneously reported this evidence by comparing human and African green monkey (agm)

APOBEC3G (Bogerd et al., 2004; Mangeat et al., 2004; Schrefelbauer et al., 2004; Xu et al., 2004). In detail, HIV-1 Vif binds and antagonizes human APOBEC3G, but not agm APOBEC3G. In contrast, SIVagm Vif antagonizes agm APOBEC3G, but not human APOBEC3G. By comparing amino acids residues and preparing chimeric APOBEC3G between human and agm APOBEC3G, they identified D¹²⁸ as the determinant of the species-specific binding of Vif to APOBEC3G (Bogerd et al., 2004; Mangeat et al., 2004; Schrefelbauer et al., 2004; Xu et al., 2004). On the other hand, SIVmac and HIV-2 Vif can antagonize both human and agm APOBEC3G, indicating that the interaction between Vif and APOBEC3G is not restricted by D¹²⁸, in other words, D¹²⁸ is not the sole determinant for species-specific target by Vif (Gaur and Strebel, 2012). Furthermore, the interaction between Vif and APOBEC3G is regulated by phosphorylation of APOBEC3G at T³² by protein kinase A (Shirakawa et al., 2008).

The interaction sites in Vif are reported by many groups and are much more complicated. The binding site only for APOBEC3G is Y⁴⁰RHHY⁴⁴ (Russell and Pathak, 2007), while that only for APOBEC3F is D¹⁴RMR¹⁷ (Russell and Pathak, 2007), and T⁷⁴GERxW⁷⁹ (He et al., 2008). The binding sites for both APOBEC3G and F are W²¹KSLVK²⁶ (Chen et al., 2009; Dang et al., 2009), V⁵⁵xIPLx_{4–5}LxΦx₂YWxL⁷² (He et al., 2008), and Y⁶⁹xxL⁷² (Pery et al., 2009; Figure 1). To identify the real interaction sites, we have to wait a little longer until we will get the structural information of these complexes.

Vif AND CBFβ

Recent mass-spectrometry screening of Vif-binding proteins has identified a T cell transcription factor, core-binding factor subunit



beta (CBF β), as an important Vif-binding protein (Jager et al., 2012; Zhang et al., 2012). CBF β directly binds to Vif and plays a crucial role in forming a stable Vif–Cul5–EloB/C E3 ligase complex. Without CBF β , the Vif–Cul5–EloB/C E3 ligase complex is not stable enough to polyubiquitinate APOBEC3G and its function is severely impaired. The binding sites of Vif with CBF β are identified as W²¹ and W³⁸ (Figure 1). However, the mechanisms by which CBF β regulates the E3 ligase complex are still under investigation. Furthermore, since CBF β is an important T cell transcription factor, it would be very interesting to determine whether Vif affects T cell differentiation.

Vif IS ALSO UBIQUITINATED

Fujita et al. (2004) have reported that expression of the Vif protein in virus-producing cells is maintained at very low levels, which is regulated by the ubiquitin–proteasome pathway. It is because its high expression inhibits viral infectivity by affecting proteolytic processing of Gag protein (Akari et al., 2004). We have identified the E3 ligase for Vif as mouse double minute 2 homolog (MDM2; Izumi et al., 2009; Figure 2). Since Vif is a component of a Cul5–EloB/C complex, one report showed that this complex ubiquitinated Vif (Mehle et al., 2004a). Another report showed that other E3 ligases such as neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4) and atrophin-interacting protein 4 (AIP4) bound to Vif, however, it didn't show the direct evidence of Vif ubiquitination by these ligases (Dusart et al., 2004). The identification of the E3 ligase has lead to elucidation of the mechanisms of Vif-induced G2 cell cycle arrest described below.

A NOVEL Vif FUNCTION: G2 CELL CYCLE ARREST

In early 1990s, viral protein R (Vpr) had been shown to induce G2 cell cycle arrest in HIV-1-infected cells (He et al., 1995; Re et al., 1995; Roshal et al., 2003; Nakai-Murakami et al., 2006). Many groups have extensively worked on Vpr-induced G2 arrest in terms of its molecular mechanisms and published many papers. Although only one paper reported the virological significance of G2 arrest induced by Vpr (Goh et al., 1998), the basic and fundamental questions of why the virus needs to induce G2 arrest still remain unsolved. More than 10 years had passed since then, and two recent reports came out, describing that Vif as well as Vpr induce G2 arrest in HIV-1-infected cells (Sakai et al., 2006; Wang et al., 2007). We have recently shown the molecular mechanisms by which Vif induces G2 arrest (Izumi et al., 2010; Figure 2). Vif activates p53, which is well known as a tumor suppressor gene and the regulator of cell cycle as “a guardian of the genome.” Vif binds and activates p53 by stabilizing and sequestering it to the nucleus. Activation of p53 induces its downstream cascade such as activation of p21 and inactivation of Cdc2/CyclinB, resulting in G2 arrest. Furthermore, we identified the amino acid residues in Vif responsible for its interaction with p53 and a Vif mutant which does not induce G2 arrest. Using a mutant virus which possesses the *vif* mutant, we have demonstrated that Vif-induced G2 arrest facilitates viral replication (Izumi et al., 2010; Figure 2). Thus, HIV-1 needs to have G2 cell cycle arrest to efficiently replicate so that it possesses two accessory genes such as *vif* and *vpr*. Vif induces G2 arrest in a p53-dependent manner, while Vpr accomplishes the same goal in a p53-independent manner.

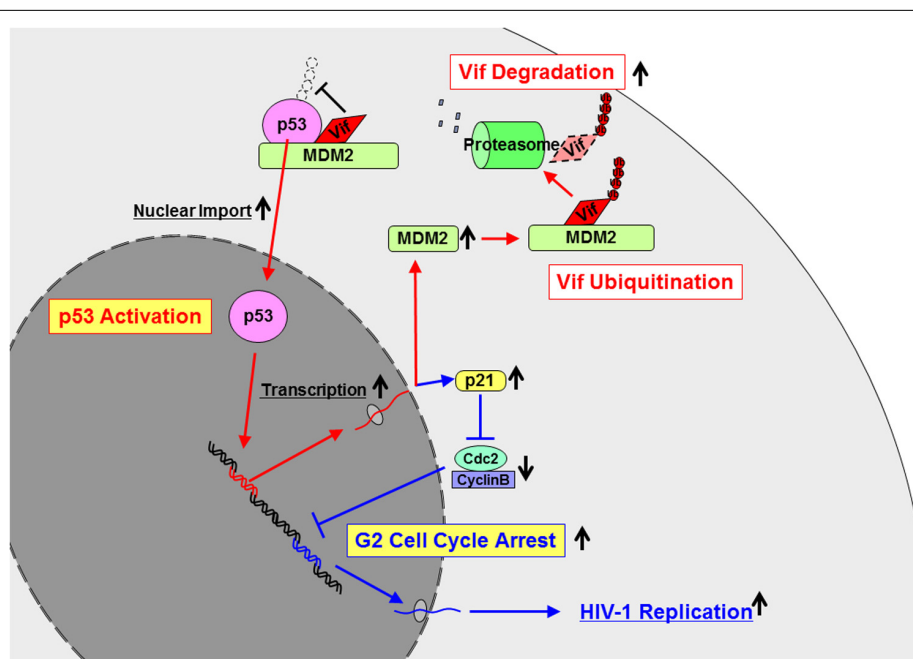


FIGURE 2 | The mechanisms how Vif is ubiquitinated and degraded and how Vif induces G2 cell cycle arrest. Vif is ubiquitinated and degraded by MDM2. On the contrary, Vif inhibits ubiquitination of p53 by MDM2 to induce activation and nuclear import of p53. Activated p53 induces transcription of

several genes including MDM2 and p21. Enhanced expression of MDM2 may lead to more Vif ubiquitination and degradation, which forms the autoregulatory circuit of Vif expression. On the other hand, activation of p21 leads to G2 cell cycle arrest, resulting in more HIV-1 replication.

CONCLUSION

HIV Vif is an intriguing viral protein, not only because it opens up a new era in the research field of host restriction factors, but also because it has a variety of functions for the viral life cycle by interacting several cellular proteins. It suggests that it might be a good target for control of HIV-1 infection.

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Vpu and BST2: still not there yet?

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Extensive investigations have identified two cellular proteins in humans that potentially inhibit HIV type 1 (HIV-1) replication and are widely accepted as “restriction factors.” APOBEC3G was identified as a restriction factor that diminishes HIV-1 replication by inducing G-to-A hypermutation in the viral genome, while BST2 has been identified as another restriction factor that impairs the release of nascent virions by tethering them on the surface of infected cells. To counter these restriction factors, HIV-1 has equipped itself with its own weapons: viral infectivity factor (Vif) degrades APOBEC3G, while viral protein U (Vpu) antagonizes BST2. These findings have allowed us to further our understanding of virus–host interaction, namely, the interplay between viral factors versus host restriction factors. In the first case, the interplay between APOBEC3G and Vif is clear: *vif*-deficient HIV-1 is incapable of replicating in APOBEC3G-expressing cells. This insight directly indicates that APOBEC3G is a *bona fide* restriction factor and has intrinsic immunity against HIV-1, and that Vif is a prerequisite for HIV-1 infection. In other words, the relationship between Vif and APOBEC3G has already “matured,” and Vif has highly evolved to overcome APOBEC3G. On the other hand, although BST2 drastically impairs the release of *vpu*-deficient HIV-1 virions, it is puzzling that *vpu*-deficient HIV-1 is still able to replicate in BST2-expressing cells. These insights imply that BST2-mediated anti-HIV-1 activity is vulnerable, and that Vpu is dispensable for HIV-1 infection. If so, why has Vpu acquired the counteracting potential against BST2? Was it necessary or important for HIV-1? Or is the relationship between Vpu and BST2 still “immature”? In this review, we particularly focus on the interplay between Vpu and BST2. We discuss the possibility that Vpu has evolved as a potent antagonist against BST2, and finally, propose a hypothesis that Vpu has evolved as a promoter of human-to-human HIV-1 transmission. Since the first report of acquired immunodeficiency syndrome patients in 1981, HIV-1 has spread explosively worldwide and is currently a pandemic. This review proposes a concept suggesting that the current HIV-1 pandemic may be partly attributed by Vpu.

Keywords: HIV-1, Vpu, BST2, restriction factor, viral evolution, pandemic

INTRODUCTION

Primate lentiviruses have co-evolved and co-diversified with primate hosts including humans for a myriad of years. During their long history, it is conceivable that hosts have evolved the genes necessary to counteract lentivirus infections, whereas viruses have developed weapons to eliminate these host obstacles. The continuous “cat-and-mouse games” have driven the virus–host co-evolution.

During the last 100 years, human immunodeficiency virus (HIV; particularly HIV type 1, HIV-1), a highly evolved primate lentivirus, has emerged, and has infiltrated the human population (Worobey et al., 2008). HIV-1 was identified as the causative agent of acquired immunodeficiency syndrome (AIDS) in 1983–1984 (Barre-Sinoussi et al., 1983; Gallo et al., 1983; Kitchen et al., 1984), and now, HIV-1 infection is a worldwide pandemic. In 2009, UNAIDS (Joint United Nations Programme on HIV and AIDS) estimated that more than 33 million people worldwide are infected with HIV-1, out of which, more than 2.8 million people are newly infected and approximately 1.8 million people annually die

of AIDS (<http://www.unaids.org/globalreport/>). Since the HIV-1 outbreak has been one of the most urgent issues for mankind, scientists have invested a great deal of effort to reveal the molecular mechanism of HIV-1 replication, which can provide clues to elucidate HIV-1 infection and AIDS.

In the past 10 years, two proteins capable of robustly counteracting HIV-1 infection and encoded in the human genome were identified: apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G), and bone marrow stromal antigen 2 (BST2, also known as tetherin, CD317, and HM1.24). APOBEC3G and its homologs in humans (APOBEC3A, B, C, DE, F, and H; APOBEC3s) are cellular cytidine deaminases and certain APOBEC3s, particularly APOBEC3G and APOBEC3F, induce G-to-A hypermutation in HIV-1 proviral DNA resulting in diminished HIV-1 replication (Sheehy et al., 2002; Harris and Lidament, 2004). BST2 is a type II transmembrane glycoprotein and tethers nascent HIV-1 particles on the plasma membrane of infected cells resulting in the impairment of virus release (Neil et al., 2008; Van Damme et al., 2008). The identification of these

two genes has provided us with a concept of “intrinsic immunity” or “restriction factors” which means that hosts possess innate congenital factors potentially capable of restricting viral replication. In parallel, HIV-1 encodes genes that antagonize cellular restriction factors and have given rise to two proteins, also known as accessory proteins, viral infectivity factor (Vif), and viral protein U (Vpu) which counteract the actions of APOBEC3s and BST2, respectively. Taken together, the battle between HIV-1 and humans can be interpreted as the interplay between viral factors (Vif and Vpu) and host factors (APOBEC3s and BST2), which has been a hot topic in the field of HIV-1 virology.

Here, we first provide a brief introduction on the interplay between Vif and APOBEC3s as an excellent example of virus–host interaction. Then, by comparing the Vpu and BST2 relationship with the Vif–APOBEC3s interaction, we summarize and discuss the significance of their interplay from the views of (1) virology, (2) molecular biology, (3) evolutionary biology, and (4) epidemiology.

VIROLOGY

Vif VERSUS APOBEC3s

Certain cell lines (e.g., CEM-SS cells, 293T cells, and HeLa cells) are permissive to *vif*-deficient HIV-1 replication and are called “permissive cells,” whereas some cell lines (e.g., CEM cells, HUT78 cells, and H9 cells) are not. Two studies using heterokaryons of permissive cells and non-permissive cells suggested that non-permissive cells such as CEM cells express a factor which potentially restricts HIV-1 replication and is counteracted by Vif (Madani and Kabat, 1998; Simon et al., 1998). Sheehy et al. (2002) identified APOBEC3G by a subtraction screening assay using CEM cells and CEM-SS cells. Subsequent investigations revealed that certain APOBEC3s including APOBEC3G are incorporated into released virions and mutate viral complementary DNA in newly infected cells, while Vif impedes the incorporation of certain APOBEC3s into progeny virions by degrading these proteins through the ubiquitin–proteasome pathway (Harris and Liddament, 2004; Izumi et al., 2008). Importantly, endogenous APOBEC3s are expressed in primary human CD4-positive T cells and monocyte-derived macrophages (MDMs), which are the primary targets for HIV-1 infection *in vivo*, and that *vif*-deficient HIV-1 is not able to replicate in *in vitro* cultures of these cells. More strikingly, *vif*-deficient HIV-1 is unable to infect and replicate in a SCID-hu mouse model (Aldrovandi and Zack, 1996) and a humanized mouse model (Sato et al., 2010). These reports indicate that endogenous APOBEC3s are potent restriction factors for HIV-1 infection *in vitro* and *in vivo*, and that Vif is absolutely essential for HIV-1 replication.

Vpu VERSUS BST2

Certain cell lines such as HEK293 cells and Cos-7 cells are able to produce nascent virions of *vpu*-deficient HIV-1, whereas HeLa cells are not. An experiment using heterokaryons of HeLa cells and Cos-7 cells suggested that HeLa cells exclusively express a factor which potentially impairs virus production and is counteracted by Vpu (Varthakavi et al., 2003). Neil et al. (2008) identified BST2 by microarray analyses using HEK293 cells and HeLa cells. The authors revealed that BST2 robustly inhibits the release of budding virions and that Vpu antagonizes the anti-viral action of

BST2, leading to the proposal that BST2 tethers nascent virions (Neil et al., 2008). In addition, Van Damme et al. (2008) revealed that BST2 expressed on the surface of HIV-1-producing cells is down-regulated by Vpu.

The ability of BST2 to restrict the release of nascent virions is not limited to HIV-1. BST2 can impair the release of various enveloped viruses belonging to *Retroviridae* [HIV type 2 (HIV-2), simian immunodeficiency viruses (SIVs), equine infectious anemia virus (EIAV), feline immunodeficiency virus (FIV), prototype foamy virus (PFV), Mason-Pfizer monkey virus (MPMV), human T-cell leukemia virus type 1 (HTLV-1), Rous sarcoma virus (RSV), and murine leukemia virus (MLV; Douglas et al., 2009; Jia et al., 2009; Jouvenet et al., 2009; Le Tortorec and Neil, 2009; Sauter et al., 2009; Zhang et al., 2009; Yang et al., 2010], *Filoviridae* [Ebola virus (EBOV) and Marburg virus; Jouvenet et al., 2009; Kaletsky et al., 2009; Sakuma et al., 2009], *Arenaviridae* (Lassa fever virus; Kaletsky et al., 2009; Sakuma et al., 2009), *Herpesviridae* (Kaposi's sarcoma-associated herpesvirus, KSHV; Mansouri et al., 2009; Pardieu et al., 2010), *Rhabdoviridae* (vesicular stomatitis virus; Weidner et al., 2010), *Orthomyxoviridae* (influenza A virus; Watanabe et al., 2011), and *Paramyxoviridae* (Nipah virus; Radoshitzky et al., 2010). On the other hand, the suppression of virus release can be elicited by not only human BST2 but also its orthologs in several mammals including chimpanzees (McNatt et al., 2009; Sauter et al., 2009), gorillas (Sauter et al., 2009; Lim et al., 2010), rhesus macaques (McNatt et al., 2009), cats (Dietrich et al., 2011; Fukuma et al., 2011), and mice (McNatt et al., 2009; Kobayashi et al., 2011). Since ectopically expressed human BST2 impairs HIV-1 release from cells derived from a broad range of species including potoroo and quail, human BST2 can function without any co-factors exclusively expressed in primates (Sato et al., 2009).

Like HIV-1 Vpu, certain viruses possess their own counterparts to antagonize BST2. For example, HIV-2 envelope glycoprotein (Env), which has been known as the enhancer of virus release (Bour and Strebel, 1996; Bour et al., 1996), counteracts human BST2 (Douglas et al., 2009; Jia et al., 2009; Le Tortorec and Neil, 2009; Hauser et al., 2010). On the other hand, certain SIVs from chimpanzees (SIVcpz), sooty mangabeys (SIVsmm), and gorillas (SIVgor) impair simian BST2 by their commonly shared accessory protein, negative factor (Nef; Douglas et al., 2009; Jia et al., 2009; Sauter et al., 2009; Zhang et al., 2009; Lim et al., 2010). EBOV glycoprotein (GP; Kaletsky et al., 2009) and KSHV K5/MIR2 protein (Mansouri et al., 2009; Pardieu et al., 2010) also possess the potential to counteract human BST2. Given these findings, it is reasonable to assume that the ability of BST2 to impair the release of a broad spectrum of viruses plays a crucial role in host immunity, and that various viruses have evolved a way to counteract the anti-viral action of BST2.

Despite the fact that endogenously and ectopically expressed BST2 proteins are prominent in restricting the release of a broad range of viruses, its capacity to control virus replication remains questionable. In the case of HIV-1, *vpu*-deficient virus is able to replicate in *in vitro* cultures of BST2-expressing cells such as primary human CD4-positive T cells (Schubert et al., 1995, 1996, 1999; Neil et al., 2007) and MDMs (Schubert et al., 1995, 1999; Theodore et al., 1996; Neil et al., 2007) and *in vivo* in a humanized

mouse model (Sato et al., in press). Furthermore, SIVagm [an SIV from African green monkey (AGM)] does not encode any accessory factors to antagonize AGM BST2 (Lim and Emerman, 2009), yet naturally infects AGM. Moreover, although human BST2 can impair the release of influenza A virus and EBOV, it does not suppress the spread of these viruses in *in vitro* cell cultures (Radoshitzky et al., 2010; Watanabe et al., 2011). These findings imply a limitation in BST2's anti-viral function.

Why is BST2 unable to “restrict” the replication of viruses despite its robust ability to impair the release of virions? One possible explanation is the effect of BST2 on cell-to-cell transmission of viruses. Viruses are usually propagated by at least two modes: cell-free virus-mediated transmission and cell-to-cell transmission (Sattentau, 2008; Martin and Sattentau, 2009). In fact, one study has clearly shown that cell-to-cell HIV-1 transmission overcomes BST2-mediated restriction (Jolly et al., 2010), although it remains controversial (Casartelli et al., 2010; Kuhl et al., 2010). In addition, it has been suggested that Vpu is not required for efficient cell-to-cell spread of HIV-1 in the culture of Jurkat cells (Gummuluru et al., 2000), which express endogenous BST2. Interestingly, feline BST2 can impair the release of FIV but enhances the spread of FIV (Dietrich et al., 2011). Moreover, the entry of human cytomegalovirus is promoted by human BST2 (Viswanathan et al., 2011). Therefore, it is conceivable that BST2 does not diminish the cell-to-cell spread of viruses even if the cell-free virus-mediated infection of these viruses is severely restricted.

MOLECULAR BIOLOGY

BST2 directly interacts with various anti-BST2 proteins through distinct manners and is subsequently antagonized. For instance, BST2 and HIV-1 Vpu interact through their transmembrane domains (Dube et al., 2010b; Vigan and Neil, 2010; Kobayashi et al., 2011; Skasko et al., 2012). On the other hand, BST2 interacts with HIV-2 Env (Gupta et al., 2009) and EBOV GP (Kaletsky et al., 2009; Lopez et al., 2010) through an extracellular domain, whereas SIVs Nef interacts with the cytoplasmic domain of BST2 (Sauter et al., 2009; Lim et al., 2010). Since KSHV K5/MIR2 is an E3 ubiquitin ligase, this protein ubiquitinates the lysine residues in the cytoplasmic domain of BST2 (Mansouri et al., 2009; Pardieu et al., 2010). The molecular mechanisms for BST2 antagonization by these viral proteins are dependent on each protein, but most likely occur through (i) degradation by the ubiquitin–proteasome pathway and/or lysosome, (ii) sequestration from the functional site, or (iii) enhancement of down-regulation. However, these mechanisms remain controversial. For more details on this topic, please see the following review papers available elsewhere (Douglas et al., 2010; Dube et al., 2010a; Evans et al., 2010; Arias et al., 2011; Martin-Serrano and Neil, 2011).

Given the role of BST2 in restricting virus release, is BST2 physiologically tethering something other than enveloped viruses? Human BST2 was first cloned as one of the candidates for the surface molecules involved in pre-B cell development (Ishikawa et al., 1995). It is known that BST2 is localized in lipid rafts on the plasma membrane of the apical side and is internalized by clathrin (Kupzig et al., 2003; Rollason et al., 2007, 2009). BST2 provides a physical link between lipid rafts and the apical actin network, which is crucial for the maintenance of microvilli in polarized

epithelial cells (Rollason et al., 2009). However, it is unlikely that BST2 plays a role as a natural “tethering” factor in our cells. If this is the case, it is important to understand what BST2 is doing in addition to its anti-viral function.

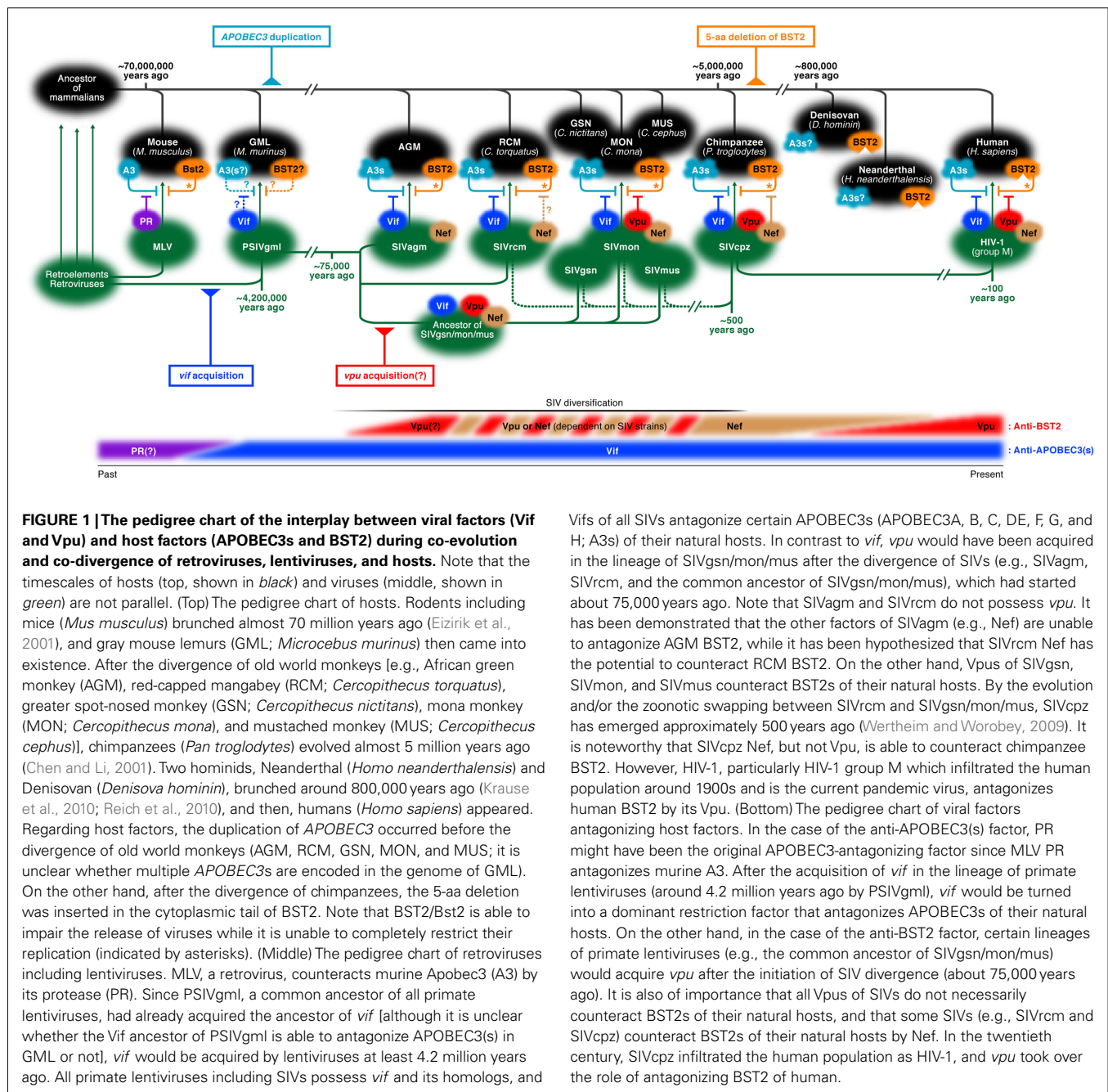
Human BST2 and murine *Bst2* (the ortholog of human BST2 in mouse) are interferon-stimulated genes (ISGs) and their expression is augmented by stimulation with type I interferons (IFNs; Blasius et al., 2006; Neil et al., 2008; Miyagi et al., 2009). However, their expression patterns in cell lineages are different between humans and mice. Human BST2 is expressed in broad lineages of hematopoietic cells (Homann et al., 2011) and certain epithelial cell lines such as HeLa cells (Neil et al., 2008; Van Damme et al., 2008). On the other hand, murine *Bst2* is exclusively expressed in plasmacytoid dendritic cells (pDCs), a potent producer of type I IFNs, in naïve mice, and therefore, is used as a specific marker of pDCs (Blasius et al., 2006). Human BST2 expressed on pDCs negatively regulates the production of type I IFNs and inflammatory cytokines by interacting with immunoglobulin-like transcript 7 (ILT7; Cao et al., 2009). Although murine *Bst2* is expressed in murine pDCs, a direct ortholog of human *ILT7* is absent in mice (Brown et al., 2004). Therefore, the function of BST2 as a negative regulator of type I IFN signaling may have evolved recently (Cao et al., 2009).

EVOLUTIONARY BIOLOGY

So, why has HIV-1 acquired the weapons to counteract the abilities of APOBEC3s and BST2 in its limited genome space (approximately 10 kb)? From an evolutionary point of view, here we shed light on the interplay of cellular factors and viral factors, illustrated in **Figure 1**.

APOBEC3s AND *Vif*

The orthologs of human (*Homo sapiens*) APOBEC3s are encoded in the genomes of chimpanzees (*Pan troglodytes*) and mice (*Mus musculus*). Although mice have a sole ortholog, *Apobec3*, which is located on chromosome 15, it is of interest that there are seven paralogs of murine *Apobec3* in the genomes of humans and chimpanzees (designated to APOBEC3A, B, C, DE, F, G, and H), which are located on chromosome 22 (Harris and Liddament, 2004). Since gene duplication is strong evidence that the duplicated genes have been exposed to selective pressures, these findings indicate that murine *Apobec3* had been subjected to selective pressures and was duplicated during the evolutionary process of rodents and primates. Apobec3/APOBEC3 family proteins have functioned to potentially inhibit the replication of retroviruses and retroelements (Harris and Liddament, 2004) suggesting that they have played a role as the selective pressure on *Apobec3*. On the other hand, although MLV does not encode *vif*, murine *Apobec3* can be degraded by viral protease (Abudu et al., 2006). These findings strongly suggest that the conflict between retroviruses (e.g., the MLV and its ancestors) and their hosts had also taken place in rodents. In the case of monkeys including primates, it has been suggested that primates have been infected by retroviruses for over 30 million years (Belshaw et al., 2004). In addition, it has been suggested that APOBEC3G has been subjected to strong positive selection throughout the history of primate evolution for at least 33 million years (Sawyer et al., 2004) and has rapidly evolved



(Zhang and Webb, 2004). Therefore, it is reasonable to consider that the expansion of *APOBEC3*s in primates was attributed to repeated retrovirus infections and has been further promoted by their antagonists, Vif, and its ancestors.

When was *vif* acquired by primate lentiviruses? In 2008, a unique “molecular fossil record,” an endogenous lentivirus named gray mouse lemur prosimian immunodeficiency virus (PSIVgml), was identified in the genome of gray mouse lemur (*Microcebus murinus*), which is a strepsirrhine primate from Madagascar (Gifford et al., 2008). Gifford et al. (2008) demonstrated that PSIVgml is the putative common ancestor of all known primate lentiviruses. Of particular importance, PSIVgml had already

acquired the putative ancestor gene of *vif*. In addition, one study has estimated that gray mouse lemurs were infected with PSIVgml at least 4.2 million years ago (Mya; Gilbert et al., 2009), suggesting that the *vif* ancestor had already existed in primate lentiviruses during this period. Moreover, it has also been estimated that *Carnivora* introduced the PSIV ancestor(s) from Africa to Madagascar at least 19 Mya, which would lead to the establishment of lentiviruses in primates (Yoder et al., 2003; Gifford, 2012). Although it is still unknown whether the PSIV ancestor(s) had already possessed a putative *vif* ancestor, it seems convincing that primates and their ancestors have co-existed and co-evolved with primate lentiviruses encoding *vif* for more than several million years.

In summary, the interplay between APOBEC3(s) and retroviruses in terms of evolutionary biology, a scenario in which APOBEC3s have co-evolved with retroelements and retroviruses (including lentiviruses) in hosts for over 30 million years, is reasonable. In parallel, Vif has co-evolved as a masterpiece of primate lentiviruses with expertise in counteracting APOBEC3s. In other words, Vif is a consequence of the “Red Queen’s race” with APOBEC3s.

BST2 AND Vpu

As in the case of human APOBEC3s, has human BST2 been exposed to selective pressures by its counteracting agents such as HIV-1 Vpu? In sharp contrast to APOBEC3s, BST2 duplication is not observed. BST2 is encoded in the genomes of human and chimpanzee located on chromosome 19, while murine *Bst2* is located on chromosome 8. Although it has been suggested that BST2 is a consequence of positive selection (McNatt et al., 2009), others have implied that human BST2 is in an intermediate stage in transition to adaptive evolution (Liu et al., 2010). Therefore, it seems feasible to consider that BST2 has been not undergone extensive selective pressure compared to APOBEC3s. However, it is of note that Nefs of SIVcpz and SIVgor are able to antagonize BST2s of both chimpanzees and gorillas but not that of humans (Lim et al., 2010). By comparing the amino acid (aa) sequences of BST2s in these three species, the absence of a 5-aa motif in the cytoplasmic tail of human BST2, where Nefs of certain SIVs (e.g., SIVcpz and SIVgor) target was found (Sauter et al., 2009; Lim et al., 2010). These findings suggest that the 5-aa deletion made human BST2 resistant to the counteraction by Nefs of certain SIVs. Moreover, the 5-aa deletion in BST2 is detected in two hominids, Neanderthal (*Homo neanderthalensis*) and Denisovan (*Denisova hominin*), suggesting that this deletion occurred at least 0.8 Mya (Sauter et al., 2011b). Therefore, it would be possible to assume that the selective pressure by Nefs of SIVs, which can antagonize BST2s of their natural hosts, has resulted in the 5-aa deletion in BST2. However, as mentioned above, other viruses also possess anti-BST2 factors such as EBOV GP (Kaletsy et al., 2009) and KSHV K5/MIR2 (Mansouri et al., 2009; Pardieu et al., 2010). Therefore, a possibility that the resulting 5-aa deletion in BST2 is due to selective pressure by these other factors cannot be excluded.

When was *vpu* acquired by primate lentiviruses? In contrast to *vif*, PSIVgml does not encode a putative ancestor of *vpu* (Gifford et al., 2008), suggesting that *vpu* participated in the evolutionary history of primate lentiviruses after the period of PSIVgml emergence. In addition, although *vif* and its homologs has been encoded by a broad range of lentiviruses including caprine arthritis-encephalitis virus in small ruminants, bovine immunodeficiency virus in ruminants, FIVs in carnivores, all SIVs in monkeys, and HIV-1 in humans (Gifford et al., 2008; Gifford, 2012), *vpu* has only been encoded in certain primate lentiviruses. Out of all primate lentiviruses, *vpu* has only been encoded by SIVcpz, three SIVs from greater spot-nosed monkeys (SIVgsn), mona monkeys (SIVmon), and mustached monkeys (SIVmus; Courgnaud et al., 2002, 2003), and HIV-1. SIVcpz, the origin of HIV-1, emerged in chimpanzees as a hybrid of SIVgsn/mon/mus and SIVrcm (an SIV from red-capped mangabeys; Bailes et al., 2003), meaning that the common ancestor of *vpu* was acquired by the ancestral

lineage of SIVgsn/mon/mus after the initiation of SIV divergence (Sharp et al., 2005; Gifford et al., 2008). Since primate lentiviruses have co-diversified and co-evolved with hosts along with cross-species swapping during the past millions of years, estimating the evolutionary history of SIVs has been challenging and it seems difficult to pinpoint the period when the lineage of SIVgsn/mon/mus acquired the putative *vpu* ancestor. However, one study has estimated that the SIV diversification had started about 75,000 years ago (Worobey et al., 2010), which suggests that *vpu* was acquired by the lineage of SIVgsn/mon/mus after this point. Considering this evidence, it is likely that *vpu* is much younger than *vif* in the evolutionary history of primate lentiviruses (Figure 1). More importantly, although Vpus of SIVgsn, SIVmon, and SIVmus are able to antagonize BST2s of their natural hosts, Vpu of SIVcpz (a chimera of SIVgsn/mon/mus and SIVrcm) appears to have transferred its BST2 antagonizing ability to Nef (Kirchhoff, 2010). These series of events resulted in a restoration of BST2 antagonism by Vpu in the only pandemic group of HIV-1 (for more details, see the next section), which emerged during the past 100 years (estimated in 1908–1933; Worobey et al., 2008). Therefore, in terms of co-evolution of lentiviruses and hosts, the interplay between HIV-1 Vpu and BST2 can be considered to be immature and still in the midst of the “Red Queen’s race.”

BST2 AND OTHER VIRAL FACTORS

As mentioned above, various families of viruses belonging to *Retroviridae* (HIV-1 Vpu, HIV-2 Env, and SIV Nef), *Filoviridae* (EBOV GP), and *Herpesviridae* (KSHV K5/MIR2) respectively possess their own anti-BST2 factor that can antagonize BST2 through different molecular mechanisms of action. Since these viruses are highly divergent, it should be interpreted that each anti-BST2 factor has independently acquired its BST2 counteracting ability at different periods in time. Therefore, in order to further understand the interplay between BST2 and viruses, it is necessary to focus on the evolutionary history of individual viral anti-BST2 factors. For instance, although EBOV GP is able to counteract human BST2 (Kaletsy et al., 2009), it is still unclear whether GP’s function is a common feature in *Filoviridae*. Given that EBOV GP counteracts the ortholog(s) of BST2 in its natural host(s), it would be interesting to explore this possibility. In addition, among eight human herpesviruses, it has been suggested that the gene encoding K5/MIR2 is unique in KSHV (Russo et al., 1996; Haque et al., 2000), indicating that the antagonizing potential of human BST2 has been originally acquired by KSHV after the divergence of human herpesviruses. Moreover, since it has been suggested that bovine herpesvirus 4 (BHV4) possesses the homologous gene to K5/MIR2 in KSHV (Russo et al., 1996), it would be interesting to address the antagonizing ability of the protein transcribed by the homologous gene in BHV4 against bovine BST2. Taken together, revealing these insights would provide clues to understand the evolutionary history of viruses and BST2.

EPIDEMIOLOGY

What is the significance of Vpu’s potential to antagonize BST2? One possible explanation has been provided by Sauter et al. (2009), in which Vpu has contributed to the explosion of HIV-1 infection

resulting in a pandemic. HIV-1 has been classified into four lineages; group M (main/major), group O (outlier), group N, and group P. Only HIV-1 group M is pandemic, while the others are endemic in Africa. Within the four groups, Vpus of HIV-1 groups M and O are able to antagonize BST2's tethering ability, while those of HIV-1 groups N and P are not (Sauter et al., 2009, 2011a; Yang et al., 2011). Although it is well known that HIV-1 Vpu has another ability to rapidly degrade CD4 molecules, the primary receptor of HIV-1, (Willey et al., 1992; Margottin et al., 1998; Schubert et al., 1998), group O Vpu fails to induce the degradation of CD4 (Sauter et al., 2009). Taken together, group M Vpu is the only protein able to antagonize and/or degrade both BST2 and CD4, indicating a clear correlation between the range of virus epidemicity and the degree of Vpu-mediated BST2 and CD4 down-regulation. In other words, these findings suggest that Vpu positively contributes to the efficiency of inter-individual (i.e., human-to-human) spread of HIV-1. HIV-1 is predominantly transmitted sexually, and it has been assumed that HIV-1 transmission can be accomplished by cell-free virions in genital fluids at a low probability (1 in 200–3,000; Gray et al., 2001). Therefore, consistent with a hypothesis proposed by Kirchhoff (2010), it is conceivable that Vpu increases the amount of cell-free virions in genital fluids by antagonizing BST2, which can lead to a rise in the efficiency of human-to-human HIV-1 transmission.

In the field of HIV-1 virology, there is a longstanding unanswered question: why has only group M led to a pandemic – was it just by chance? The hypothesis mentioned above may be the clue to answer this mystery. Moreover, it has been suggested that each HIV-1 group (M, N, O, and P) was independently established through zoonotic transmission of SIVcpz from chimpanzees to the human population (Gao et al., 1999; Heeney et al., 2006). However, it is still unclear whether each HIV-1 group is derived from (1) a single chimpanzee infected with SIVcpz or (2) separate chimpanzees infected with SIVcpz as a respective ancestor of each HIV-1 group (Heeney et al., 2006). Furthermore, as mentioned in the previous section, SIVcpz was derived from a hybrid of SIVgsn/mon/mus and SIVrcm in a chimpanzee (Bailes et al., 2003). Has this recombination event occurred only once in a chimpanzee and the chimpanzee simultaneously transferred the virus to humans as the origin of each HIV-1 group? Or was the hybrid SIV first transmitted

to different chimpanzees and then individually transferred from chimpanzees to humans? Since the phenotype of Vpu is clearly different among HIV-1 groups and SIVs, the difference in Vpu's ability may be an important key to trace the origin of each HIV-1 group.

FUTURE DIRECTIONS

Here, we briefly described the interplay between Vpu and BST2 from four different aspects: virology, molecular biology, evolutionary biology, and epidemiology. It is particularly noteworthy to consider how Vpu has acquired its anti-BST2 activity during HIV-1 evolution. In addition, various viral anti-BST2 factors including HIV-1 Vpu, HIV-2 Env, SIV Nef, EBOV GP, and KSHV K5/MIR2 have respectively procured individual anti-BST2 activity during viral evolution and diversity since the molecular mechanisms of action to antagonize BST2 are different among these proteins. If so, what was/is the advantage for viruses to acquire this activity? On the other hand, why have human BST2 and its orthologs evolved a function to “tether” viral particles? Finally, it would be difficult to conclude that HIV-1, particularly a group of the pandemic HIV-1, has aimlessly acquired the anti-BST2 activity within its limited genome space (one would expect that artificially inserted unnecessary and/or inadequate portions in HIV-1 genome can be easily deleted after several passages even in cell cultures), and *vice versa*, it would be also difficult to assume that BST2 has accidentally acquired its activity to tether virions during evolution of hosts. Understanding how these events occurred will be important and interesting beyond the field of science.

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SAMHD1-dependent and -independent functions of HIV-2/SIV Vpx protein

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Both human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) encode a unique set of accessory proteins that enhance viral replication in the host. Two similar accessory proteins, Vpx and Vpr, are encoded by HIV-2. In contrast, HIV-1 encodes Vpr but not Vpx. Recent studies have indicated that Vpx counteracts a particular host restriction factor, thereby facilitating reverse transcription in myeloid cells such as monocyte-derived macrophages and monocyte-derived dendritic cells. This mechanism of counteraction is similar to that of the accessory proteins Vif and Vpu which antagonize other host factors. In 2011, the protein SAMHD1 was identified as the restriction factor counteracted by Vpx. Studies have since revealed that SAMHD1 degrades deoxynucleoside triphosphates (dNTPs), which are components of viral genomic cDNA, in order to deprive viruses of dNTPs. Although interactions between SAMHD1 and Vpx continue to be a major research focus, Vpx has also been shown to have an apparent ability to enhance nuclear import of the viral genome in T lymphocytes. This review summarizes the current knowledge regarding SAMHD1-dependent and -independent functions of Vpx, and discusses possible reasons why HIV-2 encodes both Vpx and Vpr, unlike HIV-1.

Keywords: Vpx, HIV-2, SIV, SAMHD1, reverse transcription, dNTP, nuclear import, Vpr

INTRODUCTION

Human and simian immunodeficiency viruses (HIV/SIVs) carry a unique set of accessory proteins, Vif, Vpx, Vpr, Vpu, and Nef, which enhance viral replication in the host. Of these accessory proteins, Vpx is unique to HIV-2-type viruses, defined in this paper as the HIV/SIVs carrying both Vpr and Vpx, such as HIV-2, SIV_{smm} (Sooty mangabey), and SIV_{mac} (Rhesus monkey) (Fujita et al., 2010). Vpr and Vpx are small proteins of approximately 100 amino acids and similar sequence (approximately 20–25% similarity). Both Vpr and Vpx are predicted to have a similar structure consisting of three major helices (Khamsri et al., 2006). In contrast, while HIV-1 carries Vpr, it does not carry Vpx. The answer to the question why HIV-2 viruses encode these two similar proteins while HIV-1 carries only one remains elusive, and must await the determination of their functional details.

Extensive research over the past decade has revealed that lentiviruses carry genes for accessory proteins that overcome host antiviral factors. The first such accessory protein identified was Vif, which inactivates APOBEC3 proteins, cellular cytidine deaminases that restrict the replication of retroviruses by hypermutating viral cDNA and/or inhibiting reverse transcription (Sheehy et al., 2002; Goila-Gaur and Strebel, 2008; Kitamura et al., 2011). Vif reduces the amount of APOBEC3 through proteasome-mediated degradation and other degradation-independent mechanisms. The second major finding in this area was that the viral protein Vpu counteracts host BST-2/tetherin, which normally blocks the release of virions by directly tethering viral particles to the membranes of infected cells (Neil et al., 2008; Van Damme et al., 2008; Arias et al.,

2011). The mechanism through which Vpu antagonizes the function of BST-2/tetherin may be proteasome/lysosome degradation or relocation from the cell surface.

Recently, it was reported that the viral accessory protein Vpx inhibits the host restriction factor SAMHD1 in monocyte-derived macrophages (MDMs) and monocyte-derived dendritic cells (MDDCs) (Hrecka et al., 2011; Laguette et al., 2011), stimulating interest in SAMHD1 and Vpx. In addition to inhibiting SAMHD1 in MDMs and MDDCs, Vpx is also capable of enhancing viral replication in T lymphocytes (Guyader et al., 1989; Kappes et al., 1991; Yu et al., 1991; Akari et al., 1992; Gibbs et al., 1994; Kawamura et al., 1994; Tokunaga et al., 1997; Ueno et al., 2003; Doi et al., 2011). In this review, we summarize current research into SAMHD1-dependent and -independent functions of Vpx and discuss the virological significance of this protein.

SAMHD1-DEPENDENT FUNCTIONS OF Vpx

Several studies have shown that while wild-type HIV-2-type viruses grow well in MDMs, growth of these Vpx-deletion mutants is completely suppressed, demonstrating that Vpx is essential for viral replication in MDMs (Ueno et al., 2003; Fujita et al., 2008a). It is known that Vpx is packaged in virions and functions in the target cell. Independent work in our laboratory and that of another group revealed that Vpx is critical for reverse transcription of the viral RNA genome in MDMs (Fujita et al., 2008a; Srivastava et al., 2008), correcting the long-held misconception that Vpx contributes to nuclear import of the viral genome but does not play a role in reverse transcription. Furthermore, Vpx was shown to

induce proteasome-degradation of an unknown restriction factor to facilitate reverse transcription of the viral genome. It was demonstrated that degradation of the unknown factor involves formation of a Cul4-DDB1-DCAF1 E3 ligase complex (Sharova et al., 2008; Bergamaschi et al., 2009; Kaushik et al., 2009). Considerable effort was subsequently directed toward identification of the unknown factor, and in 2011 SAMHD1 was identified as the MDM host factor from co-immunoprecipitation studies of Vpx expressed in THP-1 cells and in 293T cells (Hrecka et al., 2011; Laguette et al., 2011). SAMHD1 has a tandem sterile alpha motif (SAM) and HD domain with potential phosphohydrolase activity. The SAMHD1 protein was initially identified from MDDCs as a homolog of mouse interferon- γ -induced protein (Li et al., 2000), and is upregulated in response to viral infection (Prehaud et al., 2005; Hartman et al., 2007; Zhao et al., 2008). Furthermore, SAMHD1 is believed to be involved in regulating cellular intrinsic antiviral responses (Rice et al., 2009).

The identification of SAMHD1 as a target of Vpx was not sufficient to explain all the related phenomena, suggesting the involvement of another factor (Hrecka et al., 2011; Planelles, 2011). However, based on reports indicating that SAMHD1 is a deoxynucleoside triphosphate (dNTP) triphosphohydrolase (Goldstone et al., 2011; Powell et al., 2011), it was hypothesized that SAMHD1 degrades dNTPs (which are small molecule components of viral genomic cDNA) in order to deprive viruses of dNTPs by keeping their concentration low. Lahouassa et al. (2012) recently demonstrated the validity of this hypothesis (Figures 1 and 2). Thus, the additional factor targeted by Vpx appears to be dNTPs. Although dNTPs are utilized for reverse transcription in the cytosol, they are small enough to freely diffuse through nuclear pores in and out of the nucleus. Since SAMHD1 is a nuclear protein (Rice et al., 2009), it is most likely that the concentration of dNTPs in the cytosol is controlled by SAMHD1 in the nucleus. In fact, it was suggested that Vpx-mediated degradation of SAMHD1 is initiated in the nucleus (Brandariz-Nuñez et al., 2012; Figure 2).

In addition to being components of the viral genome, dNTPs are components of the host genome; thus, proliferative CD4⁺ T cells do not express SAMHD1, and maintain the concentration of dNTPs at an optimal level for cell proliferation (2–4 μ M; Lahouassa et al., 2012). In contrast, since MDMs do not proliferate, they do not require high levels of dNTPs, and the low dNTP levels (20–40 nM) resulting from SAMHD1-mediated degradation are therefore not harmful to MDMs (Lahouassa et al., 2012). HIV-2-type viruses carry Vpx for proteasome-mediated degradation of SAMHD1 in order to facilitate replication in MDMs. In contrast, HIV-1 does not require Vpx in order to replicate in MDMs (Fujita et al., 2010) because its reverse transcriptase (RT) is capable of catalyzing viral cDNA synthesis from very low levels of dNTPs (Diamond et al., 2004; Lahouassa et al., 2012). The activity of HIV-2 RT is probably lower [Michaelis constant (K_m) of HIV-2 RT is higher] than that of HIV-1 RT, and therefore, to overcome this disadvantage, HIV-2-type viruses may have evolved to carry Vpx.

We previously mapped the functional region of Vpx involved in viral replication in MDMs (Fujita et al., 2008a,b; Figure 3). It is known that the region in major helix 3 containing amino acids Q⁷⁶

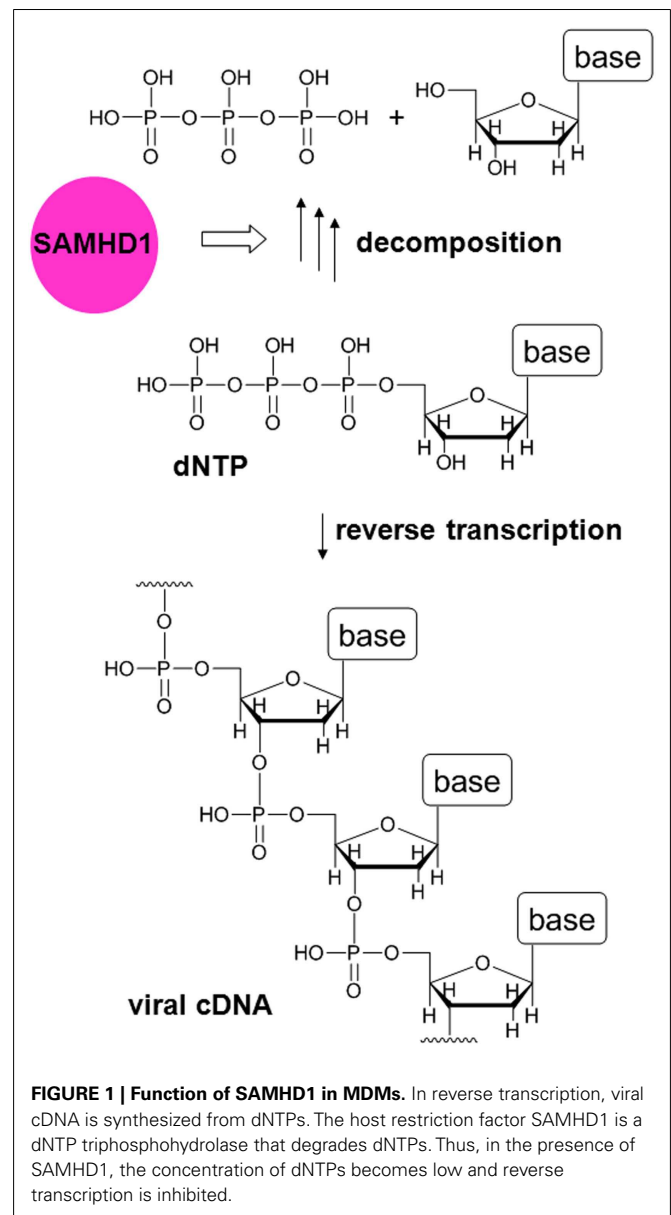


FIGURE 1 | Function of SAMHD1 in MDMs. In reverse transcription, viral cDNA is synthesized from dNTPs. The host restriction factor SAMHD1 is a dNTP triphosphohydrolase that degrades dNTPs. Thus, in the presence of SAMHD1, the concentration of dNTPs becomes low and reverse transcription is inhibited.

and F⁸⁰ interacts with DCAF1, a subunit of the Cullin4-based E3 ubiquitin ligase complex (Srivastava et al., 2008). This region could overlap with a region that is critical for virion incorporation (Park and Sodroski, 1995; Jin et al., 2001). Gramberg et al. (2010) suggested that another region, which includes amino acids P⁹, N¹², E¹⁵, E¹⁶, and T¹⁷ in the N-terminal loop, binds to a restriction factor; this region was later confirmed to be a SAMHD1-binding region (Ahn et al., 2012).

We also identified several other functional regions in Vpx, including a central region located between major helix 1 and major helix 2, and a polyproline motif in a loop in the C-terminus (Fujita et al., 2008a,b). We revealed that the C-terminal polyproline motif is critical for stable expression of Vpx. Although the function of the central region remains unknown, it has been confirmed that this region is not involved in virion incorporation.

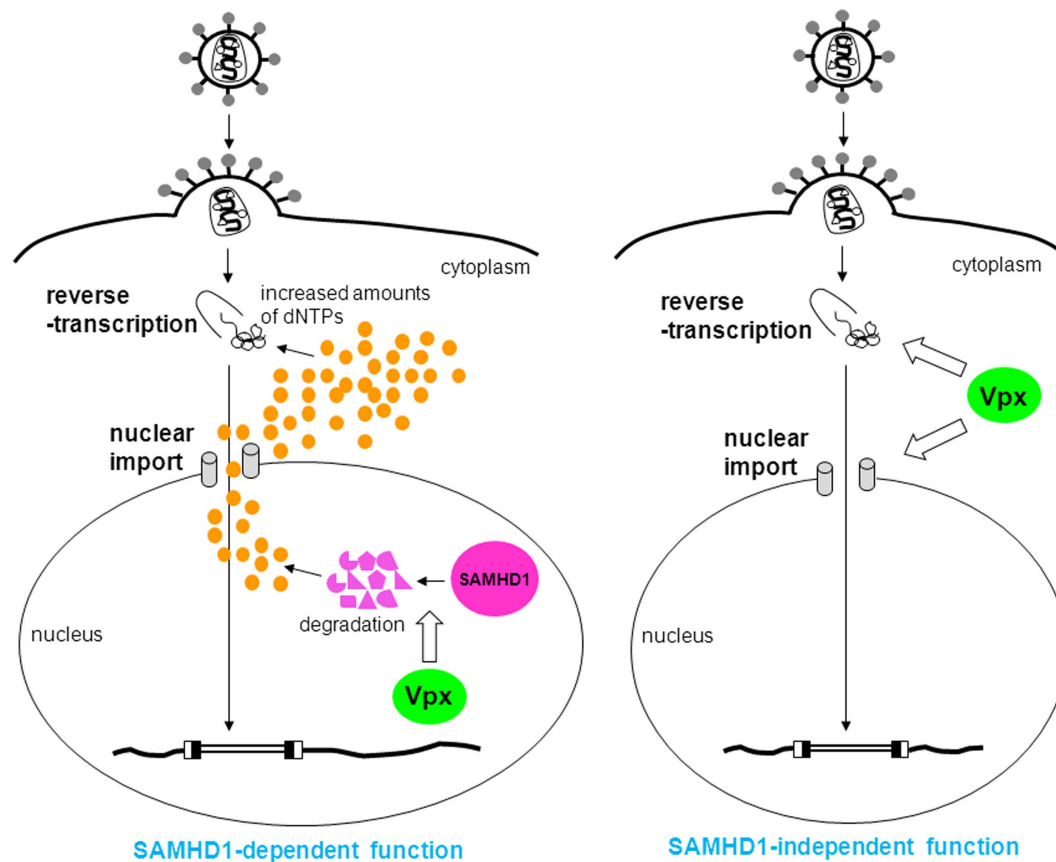


FIGURE 2 | SAMHD1-dependent and -independent functions of Vpx. In the former function, Vpx degrades SAMHD1 to increase amounts of dNTPs (orange circles), thus, reverse transcription proceeds (left). In the latter function, Vpx enhances reverse transcription and nuclear import by an unknown mechanism (right).

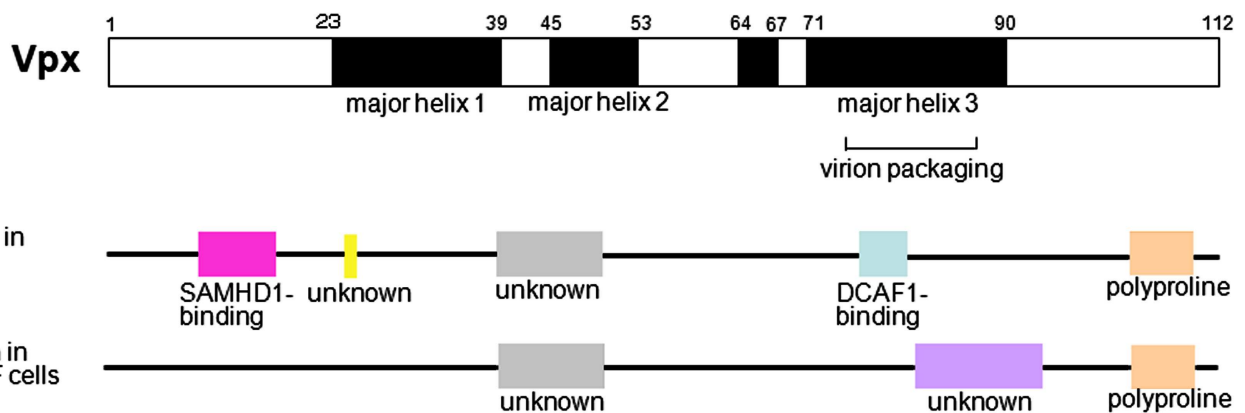


FIGURE 3 | Mapping of the functional region of Vpx. In the upper structure, the positions of predicted helices are shown in black (HIV-2 GLAN clone; Khamsri et al., 2006). The region important for virion packaging is shown. Middle diagram shows regions critical for replication in MDMs: the SAMHD1-binding region (pink), mechanistically unknown amino acid (yellow), mechanistically unknown region (gray), DCAF1-binding region (light blue), and polyproline motif

(pale orange). Lower diagram shows regions involved in replication in HSC-F cells: the mechanistically unknown region (gray), mechanistically unknown and unique region (purple), and polyproline motif (pale orange). The polyproline motif is critical for stable expression of Vpx. All functionally important regions, except those for virion packaging, SAMHD1-binding, and DCAF1-binding, are based on our report (Fujita et al., 2008a,b).

Following the identification of SAMHD1, several investigators showed that although some cells such as undifferentiated THP-1 cells express SAMHD1, both the wild-type HIV-2-type virus and its Vpx mutant infect these cells to an equivalent degree (Hrecka et al., 2011; Planelles, 2011). There are several possible explanations for the similar infectivity of wild-type and Vpx mutant viruses: (1) These cells contain large amounts of dNTPs, and thus, even in the presence of SAMHD1 there are sufficient quantities of dNTPs for viral replication, and (2) SAMHD1 does not function in these cells for some as yet unexplained reason. A plausible explanation may be posttranslational modification (phosphorylation, etc.) of the protein. Further study will be required to uncover the molecular basis for this phenomenon.

Around the time SAMHD1 was identified, it was reported that another host restriction factor, APOBEC3A, inhibits HIV-1 infection of MDMs, and that APOBEC3A is degraded by Vpx (Berger et al., 2010, 2011). In addition, APOBEC3A reportedly decreases the amount of viral cDNA synthesized during reverse transcription. Presumably, degradation of SAMHD1 alone is not sufficient to enable reverse transcription to proceed smoothly, and therefore degradation of APOBEC3A is also required for viral replication in MDMs, suggesting that Vpx functions to counteract the antiviral effects of both APOBEC3A and SAMHD1. A comparative study between APOBEC3A and SAMHD1 must be performed in order to establish each protein's contribution to restricting lentivirus infection in myeloid cells.

SAMHD1-INDEPENDENT FUNCTIONS OF Vpx

Prior to the time that Vpx was found to act on reverse transcription (Fujita et al., 2008a; Srivastava et al., 2008), it was thought that Vpx is critical for nuclear import of the viral genome (Fletcher et al., 1996; Pancio et al., 2000), based on the results of non-quantitative polymerase chain reaction (PCR) studies. This notion was supported by the tendency of Vpx to localize in the nucleus when Vpx is transduced to a cell solely (Pancio et al., 2000; Mahalingam et al., 2001). It has been well established that Vpx is critical for reverse transcription in MDMs, but this does not preclude participation of Vpx in nuclear import in these cells. We previously identified several Vpx mutants that are defective in both reverse transcription and nuclear import (Fujita et al., 2010) in MDMs, which suggests that Vpx also enhances nuclear import in these cells. We hypothesize that this function of Vpx is SAMHD1-independent, since it is plausible that there is no connection between the amount of dNTPs and nuclear import. Further investigations are underway in order to determine if this is indeed the case.

In T cells, such as peripheral blood lymphocytes (PBLs), peripheral blood mononuclear cells (PBMCs), and cultured simian cell lines immortalized by Herpesvirus saimiri such as HSC-F and M1.3S cells, HIV-2-type viruses grow well, but Vpx-deletion mutants exhibit defective replication (Guyader et al., 1989; Kappes et al., 1991; Yu et al., 1991; Akari et al., 1992; Gibbs et al., 1994; Kawamura et al., 1994; Tokunaga et al., 1997; Ueno et al., 2003; Doi et al., 2011). These results indicate that Vpx is also important for viral replication in T cells. Dispensability of Vpx for the infection of T cells has been believed by some researchers (Bergamaschi et al., 2009; Belshan et al., 2012), but this belief was probably based on the results of infectious experiments using high-titer virus.

Our research showed that Vpx enhances nuclear import of the viral genome in HSC-F cells, and that the smaller effect of Vpx on reverse transcription was also observed (Ueno et al., 2003; Fujita et al., 2008a; **Figure 2**). We mapped the region of Vpx involved in viral replication in HSC-F cells (Fujita et al., 2008b; **Figure 3**) and found that as is the case in MDMs, the central region and the C-terminal polyproline motif are critical for replication. There is also a unique functional region spanning from major helix 3 to the C-terminal loop, but how this region influences infectivity is unclear. The apparent SAMHD1- and DCAF-1-binding regions are not necessary for viral replication in HSC-F cells, in contrast to MDMs. Furthermore, in HSC-F and M1.3S cells, expression of SAMHD1 was below the detectable level (Nomaguchi, M. and Adachi, A., in preparation). Thus, in these cells, Vpx enhances reverse transcription and nuclear import of the viral genome through an unknown SAMHD1-independent mechanism. Not only cultured cell lines, but also primary T cells are considered to have SAMHD1-independent functions, since SAMHD1- and DCAF-1-binding regions are dispensable for viral replication in PBLs (Fujita, M. and Adachi, A., unpublished data).

It has been reported that Vpx is important for SIV infection in monkeys, and the predominantly infected cells are the intraepithelial T lymphocytes rather than myeloid cells such as macrophages (Hirsch et al., 1998; Belshan et al., 2012). The Vpx in T cells is considered to play a significant role in infection by HIV-2-group viruses *in vivo*. Thus, we strongly suggest that SAMHD1-independent functions of Vpx are also important, although almost all the recent Vpx research has focused on SAMHD1-dependent functions.

WHY DO HIV-2 VIRUSES HAVE TWO SIMILAR PROTEINS?

Lim et al. (2012) recently revealed that Vpr, a Vpx-related protein found in HIV-1 and HIV-2-type viruses, is not involved in degradation of SAMHD1. Instead, the Vpr carried by HIV-1 and HIV-2 arrests cells in the G₂ phase of the cell cycle, a function not associated with Vpx (Fletcher et al., 1996; Stivahtis et al., 1997; Fujita et al., 2010; **Table 1**). This G₂ arrest is known to be induced via formation of a Cul4-DDB1-DCAF1 E3 ligase complex that includes Vpr as an adaptor. Formation of the complex is followed by proteasomal degradation of an unknown cellular target. This pathway is similar to that involving Vpx, which also functions as an adaptor for the Cul4-DDB1-DCAF1 E3 ligase complex to facilitate proteasomal degradation of SAMHD1 (Ahn et al., 2012). Although the virological significance of the Vpr-mediated G₂ arrest has not been determined, this function is likely to be important since it

Table 1 | The roles of Vpx and Vpr in HIV-1 and HIV-2-type viruses.

	HIV-1	HIV-2 type viruses
Reverse transcription at low dNTP concentrations (in MDMs)	Reverse transcriptase (high activity)	Vpx
Induction of G ₂ arrest	Vpr	Vpr
Enhancement of nuclear import	Vpr (?) ^a	Vpx

^aFurther study is required (see text).

is broadly conserved among HIV/SIV. Since the activity of HIV-2 RT is lower than that of the enzyme found in HIV-1-type viruses, HIV-2 may require SAMHD1 degradation in order to increase the concentration of dNTPs, in addition to induction of G₂ arrest.

Both of these functions, SAMHD1 degradation and G₂ arrest, are mediated via the Cul4-DDB1-DCAF1 E3 ligase complex. SIVagm (African Green Monkey) is known to have only one Vpr, which induces both the degradation of SAMHD1 (Lim et al., 2012) and G₂ arrest (Planelles et al., 1996; Stivahtis et al., 1997; Zhu et al., 2001). Lim et al. proposed that in the evolution of HIV/SIVs, neofunctionalization of Vpr to degrade SAMHD1 resulted in the rapid evolution of the SAMHD1 protein, which induced the birth of a similar protein, Vpx (subfunctionalization), to maximize its SAMHD1-targeting capability. Here, we propose another reason why HIV-2 viruses have similar proteins, Vpr and Vpx. It is known that the region of HIV-1 Vpr spanning from major helix 3 to the C-terminal loop (which includes a cluster of basic amino acids) is critical for induction of G₂ arrest (Di Marzio et al., 1995; Selig et al., 1997; Jacquot et al., 2007). This region corresponds to the mechanistically unknown and unique region of Vpx required for replication in HSC-F cells (Figure 3; Khamsri et al., 2006; Fujita et al., 2008b), but the corresponding region in Vpx does not contain a cluster of basic amino acids in the C-terminal loop. Induction of G₂ arrest and enhancement of replication in T cells may be incompatible functions for one protein. The presence of both Vpr and Vpx may facilitate G₂ arrest and enhancement of HIV-2 replication in T cells, but a full explanation as to why HIV-2 has two proteins that are so similar will require further study.

CONCLUSION

Recent findings indicating that Vpx mediates the degradation of SAMHD1 are intriguing, and reveal yet another example of a virus with a means to counteract host defense mechanisms. Table 1 summarizes the roles played by Vpx and Vpr in HIV-1 and HIV-2. HIV-2/SIV Vpx negates the effect of the unique

host restriction factor SAMHD1 by inducing its degradation, thereby enabling reverse transcription to occur under conditions of low dNTP concentrations. In addition, Vpx enhances reverse transcription and nuclear import of the viral genome in an SAMHD1-independent manner. We are trying to isolate host factor(s) which concern with SAMHD1-independent function. Different regions of the Vpx protein are involved in mediating SAMHD1-dependent and -independent functions. A number of HIV/SIV accessory proteins have multiple functions, including HIV-1 Vpr (Fujita et al., 2010; Sharifi et al., 2012), Vpu (Nomaguchi et al., 2008; Andrew and Strebel, 2010), and Nef (Foster and Garcia, 2008; Laguette et al., 2010). Importance of those two functions of Vpx in the infected individuals should be revealed in the future.

HIV-1 Vpr has a modest effect on replication in MDMs (Fujita et al., 2010), and there have been reports that Vpr enhances nuclear import of the viral genome in these cells (Tsurutani et al., 2000; Agostini et al., 2002). It is possible that HIV-1 Vpr and HIV-2 Vpx function similarly with respect to nuclear import. However, further study is required to elucidate how these proteins impact nuclear import, since the role of HIV-1 Vpr was demonstrated using non-quantitative PCR, and we could not reproduce this result (Fujita et al., 2010).

Expression of Vpx in MDMs results in the degradation of SAMHD1 and a subsequent increase in the concentration of dNTPs, resulting in an increase in the infectivity of HIV-1. To date, studies of Vpx/SAMHD1 have been mainly restricted to HIV-1, even though Vpx is carried by HIV-2-type viruses. Through future studies involving HIV-2-type viruses, we hope to provide a more complete picture of the roles played by Vpx and Vpr.

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Role of human TRIM5 α in intrinsic immunity

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Human immunodeficiency virus (HIV) has a very narrow host range. HIV type 1 (HIV-1) does not infect Old World monkeys, such as the rhesus monkey (Rh). Rh TRIM5 α was identified as a factor that confers resistance, intrinsic immunity, to HIV-1 infection. Unfortunately, human TRIM5 α is almost powerless to restrict HIV-1. However, human TRIM5 α potently restricts N-tropic murine leukemia viruses (MLV) but not B-tropic MLV, indicating that human TRIM5 α represents the restriction factor previously designated as Ref1. African green monkey TRIM5 α represents another restriction factor previously designated as Lv1, which restricts both HIV-1 and simian immunodeficiency virus isolated from macaque (SIVmac) infection. TRIM5 is a member of the tripartite motif family containing RING, B-box2, and coiled-coil domains. The RING domain is frequently found in E3 ubiquitin ligase, and TRIM5 α is thought to degrade viral core via ubiquitin-proteasome-dependent and -independent pathways. The alpha isoform of TRIM5 has an additional C-terminal PRYSPRY domain, which is a determinant of species-specific retrovirus restriction by TRIM5 α . On the other hand, the target regions of viral capsid protein (CA) are scattered on the surface of core. A single amino acid difference in the surface-exposed loop between α -helices 6 and 7 (L6/7) of HIV type 2 (HIV-2) CA affects viral sensitivity to human TRIM5 α and was also shown to be associated with viral load in West African HIV-2 patients, indicating that human TRIM5 α is a critical modulator of HIV-2 replication *in vivo*. Interestingly, L6/7 of CA corresponds to the MLV determinant of sensitivity to mouse factor Fv1, which potently restricts N-tropic MLV. In addition, human genetic polymorphisms also affect antiviral activity of human TRIM5 α . Recently, human TRIM5 α was shown to activate signaling pathways that lead to activation of NF- κ B and AP-1 by interacting with TAK1 complex. TRIM5 α is thus involved in control of viral infection in multiple ways.

Keywords: Fv1, TRIM5 α , TAB2, HIV-1, HIV-2, SIV, capsid, TRIMCyp

INTRODUCTION

The acquired immune response, both humoral and cellular immunity, requires lymphocyte differentiation and education for effective protection of the host from invasive infection. It requires priming and takes time. On the other hand, innate immunity provides antiviral defenses that can be deployed more rapidly. It does not require education, but most innate immune effectors generally require intracellular and intercellular signaling events, including receptor-ligand binding, adaptor protein phosphorylation, and interferon release from infected cells as well as the interferon signaling pathway to induce an antiviral state in bystander cells. Most toll-like receptors (TLRs), which play a critical role in pattern recognition of invaders, such as double-stranded RNA, lipopolysaccharide (LPS), and CpG DNA, are expressed on macrophages and dendritic cells.

Aside from these conventional immunological definitions, many pieces of evidence provide a new concept of potent protection from viral infection designated as intrinsic immunity. It is constitutively expressed and active in many cells, and does not require any virus-triggered signaling or intercellular communication. The molecules involved in intrinsic immunity are called restriction factors. Two major cellular defense mechanisms against retrovirus infection are Fv1 and TRIM5 α that target incoming

retroviral core and the Rfv3/APOBEC3 family that causes viral genome hypermutation. This review focuses on the roles of Fv1 and TRIM5 α in intrinsic and innate immunity.

THE PROTOTYPE RESTRICTION FACTOR Fv1

Mammalian cells show differences in susceptibility to retrovirus infection. The idea that cellular genes could encode constitutive inhibitors of retroviral replication was first suggested in genetic studies of laboratory mice (Odaka and Yamamoto, 1965; Lilly, 1967). Susceptibility of mouse cells to murine leukemia virus (MLV) infection is determined by a restriction factor called Fv1 (Lilly, 1970; Pincus et al., 1971, 1975). The virus resistance induced by Fv1 is genetically dominant over susceptibility, and is evident in cells *in vitro* (Goff, 2004). Two major allelic variants of Fv1, called Fv1ⁿ and Fv1^b, were shown to restrict infection by specific strains of MLV (Pincus et al., 1971). The Fv1^b allele present in BALB/c mice blocks infection by so-called N-tropic MLV (N-MLV). The Fv1ⁿ allele present in NIH/Swiss mice blocks infection by B-tropic MLV (B-MLV). NB-tropic viruses are blocked by neither Fv1^b nor Fv1ⁿ (Hartley et al., 1970). A less common third allele, Fv1^{nr}, restricts B-MLVs and certain strains of N-MLV (Kozak, 1985). N-MLVs that are not restricted by Fv1^{nr} are called NR-tropic MLVs (Jung and Kozak, 2000; Stevens et al., 2004). The inhibition of a

particular virus infection could be abrogated by prior or simultaneous infection by other virus particles. Abrogating particles themselves do not need to be infectious, but they do need to be derived from a restrictive viral strain (Bassin et al., 1978; Boone et al., 1990). These data indicated that Fv1 encodes a unique inhibitor that targets the incoming viral capsid but could be saturated and overwhelmed by simultaneous challenge by multiple virion particles.

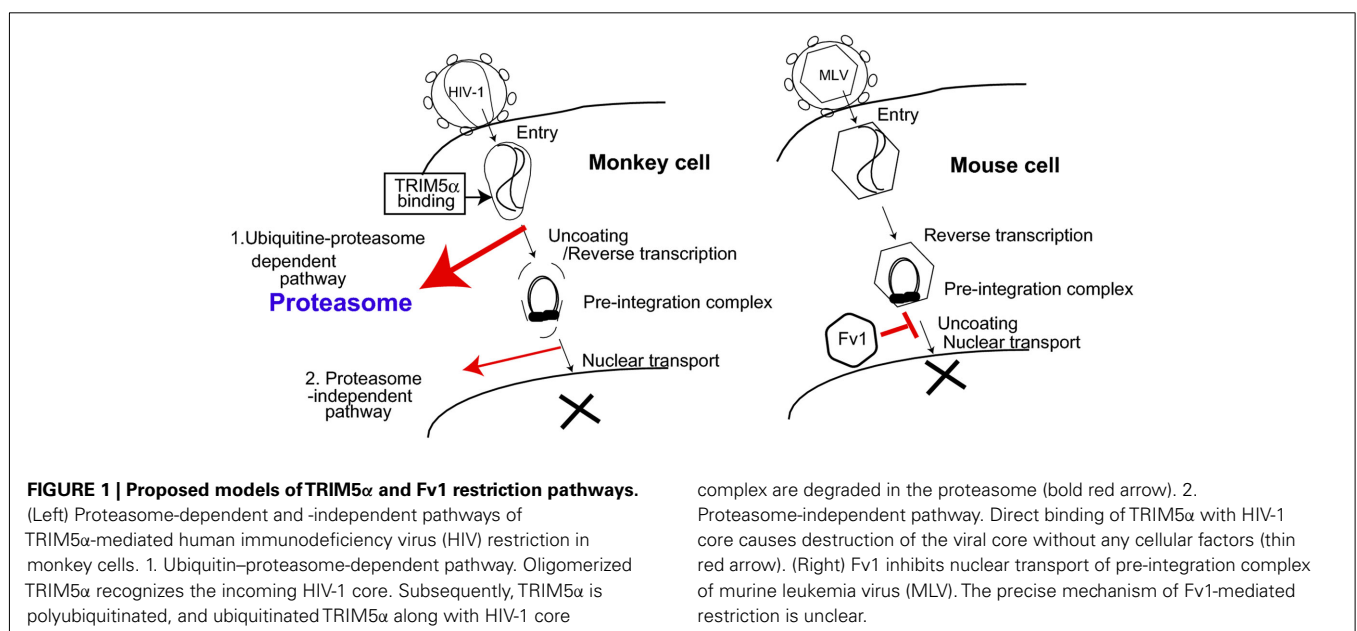
The Fv1 gene was successfully isolated by a positional cloning strategy (Best et al., 1996). The Fv1 gene product is a retroviral Gag-like protein, with sequence similarity to the HERV-L family of endogenous retroviral DNAs in the human genome, and to the MuERV-L family in the mouse (Benit et al., 1997). The B and N alleles differ in positions 358 and 399 and the C-terminal portion, all of which seem to contribute to the phenotype (Bock et al., 2000; Bishop et al., 2001). Fv1^{HR} is identical to Fv1^N, except for a single point mutation at position 352 (Stevens et al., 2004). A predicted coiled-coil region containing a dimerization domain is located in the N-terminus, and there is a second multimerization domain in the C-terminal half of the molecule (Yap and Stoye, 2003; Bishop et al., 2006). It is likely that multimerization is important for Fv1 function.

Infection of non-permissive cells by a restricted virus is blocked after reverse transcription. The virus enters the cell and synthesizes the viral cDNA by reverse transcription, but the DNA does not enter the nucleus and integrated proviral DNAs are not found (Pryciak and Varmus, 1992; **Figure 1**). Genetic studies have shown that the viral target of Fv1 is the MLV capsid protein (DesGroseillers and Jolicoeur, 1983) and subsequent work identified position 110 as the major determinant of susceptibility to Fv1 restriction (Kozak and Chakraborti, 1996). B-MLV has a glutamine (Q) at this position, and N-MLV has an arginine (R). More recently, many other residues in CA have been implicated in NB- and NR-tropism (Jung and Kozak, 2000; Stevens et al., 2004). Direct allele-specific binding between Fv1 and MLV CA has not

been observed. Most recently, Hilditch et al. (2011) developed a method for the ordered assembly of MLV CA protein on the surface of lipid nanotubes and succeeded in showing specific binding between Fv1 and MLV CA protein. However, the mechanism of action remains unclear.

Fv1 LIKE RESTRICTION FACTORS

Cells from several mammalian species, including humans, acted as if they were homozygous for Fv1^b in that they specifically resisted N-MLV infection (Towers et al., 2000). In humans, the postulated inhibitor was designated as Ref1 (for restriction factor 1) and the same capsid residue at the 110th position that controlled sensitivity to Fv1 also controlled sensitivity to Ref1 (Towers et al., 2000). The equine infectious anemia virus (EIAV) was also restricted in human cells, and this was abrogated by both EIAV itself and N-MLV particles (Towers et al., 2002). As analysis of the human genome revealed no intact Fv1 like endogenous retroviral Gag sequences that seemed likely to be responsible for Fv1 like activity (Best et al., 1996), Ref1 was thought to be independent from Fv1. Interest in these restriction systems increased markedly with the finding that several non-human primates restrict human immunodeficiency virus type 1 (HIV-1; Shibata et al., 1995; Himathongkham and Luciw, 1996) in a saturable manner (Hofmann et al., 1999; Towers et al., 2000). HIV-1 infects humans and chimpanzees but not Old World monkeys (OWMs), such as rhesus monkey (Rh) and cynomolgus monkey (CM). HIV-1 efficiently enters cells of OWMs but encounters a block before reverse transcription, and the resistance is dominant over sensitivity in human–monkey heterokaryons (Cowan et al., 2002; Munk et al., 2002). The gene responsible was named Lv1, for lentivirus restriction factor 1. Several primate species were shown to restrict a broader or different range of viruses than just HIV-1. African green monkey (AGM) cells, for example, restrict HIV-1, HIV-2, EIAV, and simian immunodeficiency virus isolated from macaque (SIVmac; Besnier et al., 2002; Hatzioannou et al., 2003).



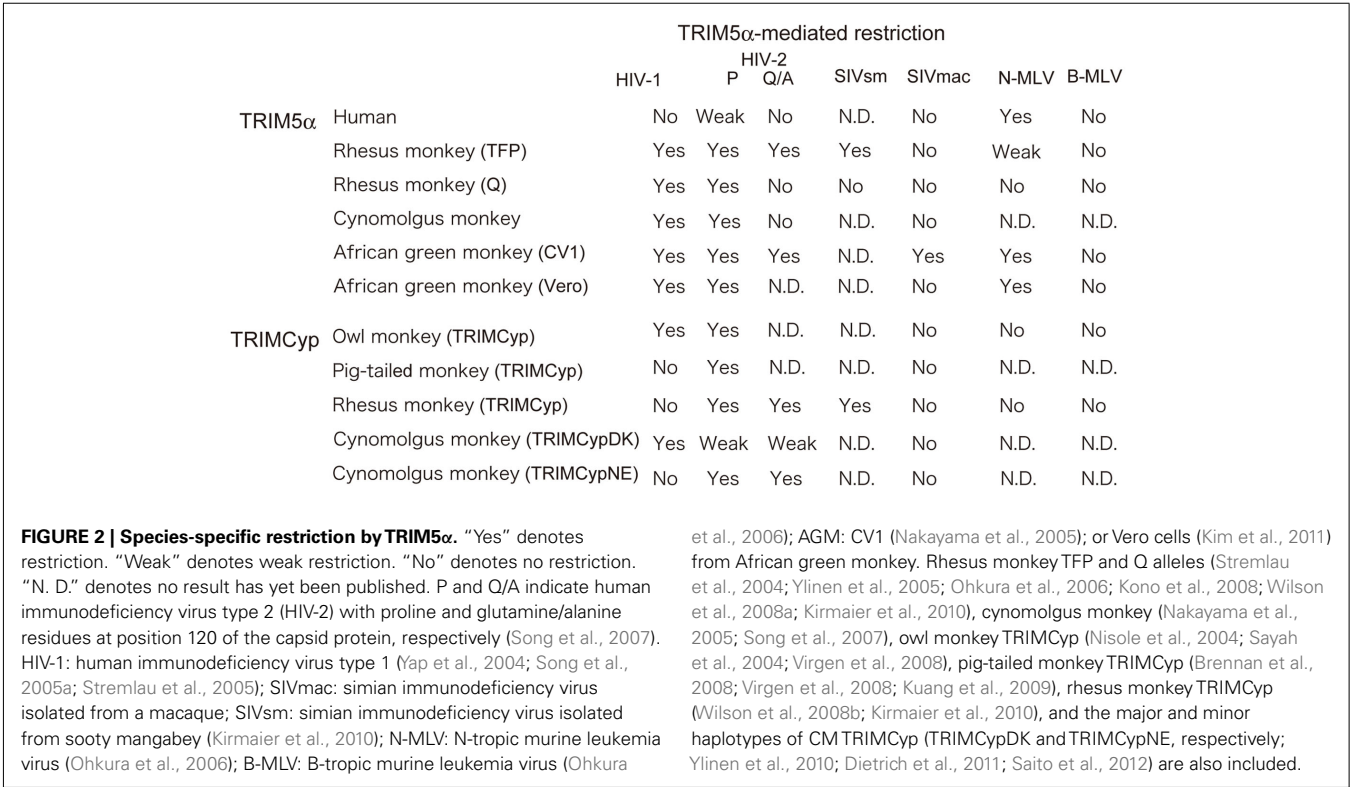
In 2004, the screening of a Rh cDNA library identified TRIM5α as a cellular antiviral factor (Stremlau et al., 2004; **Figure 1**). Rh TRIM5α shows strong restriction of HIV-1, is less effective against SIVmac and N-MLV, and does not restrict B-MLV (Hatzioannou et al., 2004; Stremlau et al., 2004). CM TRIM5α restricts HIV-1 but not SIVmac (Nakayama et al., 2005). Human TRIM5α shows little restriction of HIV-1, has a slight effect against SIVmac, and is potently restrictive against N-MLV but shows no effect on B-MLV. It is now widely accepted that human TRIM5α represents the restriction factor Ref1 (Hatzioannou et al., 2004; Keckesova et al., 2004; Perron et al., 2004; Yap et al., 2004). On the other hand, AGM cells have been shown to possess Lv1, which restricts HIV-1, HIV-2, N-MLV, EIAV, and SIVmac infection, and our group and others identified the factor as AGM TRIM5α (Hatzioannou et al., 2004; Keckesova et al., 2004; Nakayama et al., 2005). AGM TRIM5α fails to restrict SIV isolated from AGM (SIVagm) and B-MLV (Song et al., 2005b; **Figure 2**). It is now known that type I interferon upregulates the transcription of TRIM5α in human (Asaoka et al., 2005) and monkey cells (Carthagena et al., 2008), and this in turn enhances restriction activity against N-MLV (Sakuma et al., 2007a; Carthagena et al., 2008).

TRIM5α

TRIM5α is a member of the tripartite motif family containing RING, B-box2, and coiled-coil domains (**Figure 3**). The RING domain is frequently found in E3 ubiquitin ligase and TRIM5α degrades incoming viral core via the ubiquitin–proteasome-dependent (Stremlau et al., 2006) and -independent pathways leading to potent suppression of HIV-1 reverse transcription (Anderson et al., 2006; Wu et al., 2006; Maegawa et al., 2010; Kim

et al., 2011; **Figure 1**). The levels of HIV-1 late reverse transcription products recovered in the presence of the proteasome inhibitor MG132. However, the resultant HIV-1 cDNA still could not enter the nucleus, suggesting the presence of a proteasome-independent pathway of HIV-1 restriction. The distinct molecular mechanism of the proteasome-independent pathway has yet to be elucidated. TRIM5α has been shown to form a dimer via the coiled-coil region (Kar et al., 2008; Langelier et al., 2008), while the B-box2 domain mediates higher-order self-association of Rh TRIM5α oligomers (Li and Sodroski, 2008; Diaz-Griffero et al., 2009; Ganser-Pornillos et al., 2011). The α-isoform of TRIM5 has an additional C-terminal PRYSPRY (B30.2) domain. The sequence variations in variable regions of the PRYSPRY domain among different monkey species affect species-specific retrovirus infection, while differences in amino acid sequences in the viral capsid protein determine viral sensitivity to restriction (Nakayama and Shioda, 2010). TRIM5α recognizes the multimerized capsid (viral core) of an incoming virus by its PRYSPRY domain and is thus believed to control retroviral infection. Biochemical studies have shown that TRIM5α associates with CA in detergent-stripped N-MLV virions (Sebastian and Luban, 2005) or with an artificially constituted HIV-1 core structure composed of the capsid–nucleocapsid (CA–NC) fusion protein in a PRYSPRY domain-dependent manner (Stremlau et al., 2006). The PRYSPRY domain is thus thought to recognize viral cores.

Studies on human and Rh recombinant TRIM5αs have shown that the determinant of species-specific restriction against HIV-1 infection resides in variable region 1 (V1) of the PRYSPRY domain (Perez-Caballero et al., 2005; Sawyer et al., 2005). We found that 17 amino acid residues and the adjacent 20-amino acid duplication



in the V1 of AGM TRIM5 α determined species-specific restriction against SIVmac (Nakayama et al., 2005). Interestingly, a study comparing human and Rh TRIM5 α showed that a single amino acid change from R to proline (P) at position 332 in the V1 of human TRIM5 α (R332P) conferred potent restriction ability against not only HIV-1 but also SIVmac strain 239 (SIVmac239; Stremlau et al., 2005; Yap et al., 2005). In the case of human immunodeficiency virus type 2 (HIV-2) infection, we found that three amino acid residues of threonine, phenylalanine, and proline (TFP) at positions 339–341 of Rh TRIM5 α V1 are important for restricting particular HIV-2 strains that are still resistant to CM TRIM5 α (Kono et al., 2008).

Furthermore, a comparison of human and Rh TRIM5 α restriction of N-MLV showed that the amino acid residues of human TRIM5 α at positions 409 and 410 in variable region 3 (V3) of the PRYSPRY domain are important for restricting N-MLV (Perron et al., 2006).

TRIM5 α ON VIRAL PRODUCTION

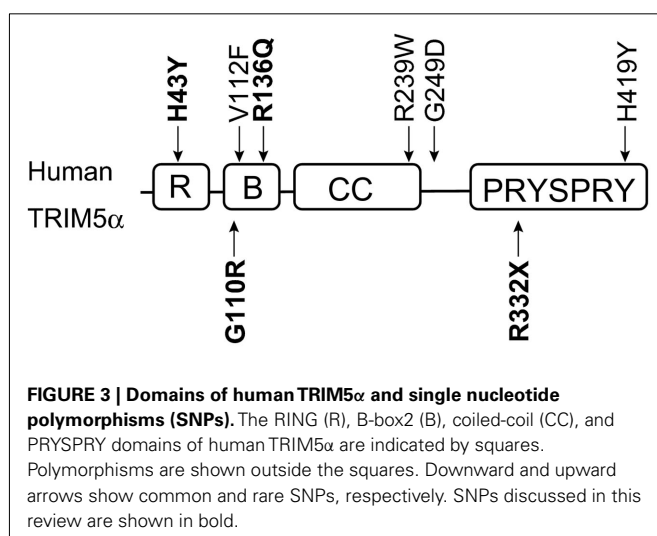
Sakuma et al. (2007b) reported that Rh but not human TRIM5 α blocks HIV-1 production through rapid degradation of HIV-1 Gag polyproteins. They reported that the RING structure was essential for this activity. Subsequently, Zhang et al. (2008) at Aaron Diamond AIDS Research Center argued against this new pathway of TRIM5 α -mediated restriction. Both groups found reduced HIV-1 Gag expression when they cotransfected high levels of Rh TRIM5 α expression plasmid (1 μ g) with HIV-1 proviral plasmids (0.1 μ g) in 293T cells. However, Zhang et al. did not observe increased yield of virus production from TRIM5 α knockdown Rh FRhK4 cells, even though they succeeded in almost complete knockdown of endogenous Rh TRIM5 α by siRNA transfection as shown by Western blotting analysis. They transfected siRNA first and then transfected siRNA again together with plasmid expressing HIV-1 24 h later. In contrast, Sakuma et al. (2007b) showed increased levels of Gag precursor protein in cell lysates and 10-fold increased virus titers in the culture supernatant of siRNA-treated FRhK4 cells, in which TRIM5 mRNA was knocked down by siRNA. The results of these two studies were inconsistent, although both

groups clearly showed TRIM5 knockdown in the same cell line. In the author reply to Zhang et al., Sakuma et al. (2008) suggested that the discrepancies in the results were due to differences in the method of siRNA transfection, in that they transfected HIV-1 plasmid first and siRNAs were transfected 6 h later. However, it is still unclear why the different transfection protocols led to different results in HIV-1 production even though both methods led to complete knockdown of TRIM5 α expression. We feel that the importance of late-phase inhibition by Rh TRIM5 α is limited, as it is widely accepted that Rh TRIM5 α potently inhibits HIV-1 infection at the early phase before HIV-1 particle production (Stremlau et al., 2004, 2006). Consistent with this, Uchil et al. (2008) analyzed 55 TRIM family proteins along with Rh TRIM5 α but failed to find an inhibitory effect of Rh TRIM5 α on the late-phase of HIV-1 infection, while they did detect a potent inhibitory effect of Rh TRIM5 α on the early phase of HIV-1 infection.

Sakuma et al. (2010) speculated that the species specificity for late-phase infection was determined by the coiled-coil region, as introduction of human TRIM5 α -specific amino acid residues to Rh TRIM5 α , M113T, and/or T146A, abrogated late-phase inhibition activity of Rh TRIM5 α , while chimeric Rh TRIM5 α containing PRYSPRY of human TRIM5 α still inhibited HIV-1 production. On the other hand, the same group showed that the effects of CM and AGM TRIM5 α on viral production were lower than that of Rh TRIM5 α (Ohmine et al., 2011), consistent with the fact that AGM derived COS7 cells were widely used to recover HIV-1 stock by transfection with proviral plasmid. The experiment of chimeric TRIM5 α showed that the C-terminal halves of CM and AGM TRIM5 α are responsible for the weakened late-phase inhibition, in contrast to chimeric TRIM5 α between Rh and human described above. Finally, Zhang et al. (2010) at Hokkaido University confirmed that human TRIM5 α used in the first study had no effect on HIV-1 production and demonstrated that this human TRIM5 α contained R437C substitution at the PRYSPRY domain. R437C substitution was not found in the NCBI single nucleotide polymorphism (SNP) database. In addition, they found that a human TRIM5 α with authentic R at position 437 reduced HIV-1 production to the same extent as Rh TRIM5 α in the high-dose cotransfection experiments (Zhang et al., 2010), consistent with the findings of Zhang et al. (2008). Furthermore, Zhang et al. (2008) found that high-level expression of Rh TRIM5 α reduced production of virus with CA derived from SIVmac, while the first and third groups did not. The species specificity of the inhibition of viral production by TRIM5 α is therefore controversial.

VIRAL DETERMINANT OF TRIM5 α SENSITIVITY

To determine the CA region that interacts with TRIM5 α , we focused on HIV-2, which highly resembles SIVmac (Hahn et al., 2000). Previous studies have shown that HIV-2 strains vary widely in their ability to grow in OWM cells such as baboon, Rh, and CM cells (Castro et al., 1990, 1991; Locher et al., 1998, 2003; Fujita et al., 2003), and HIV-2 isolates with various growth capabilities in OWM cells were evaluated for their sensitivity to CM TRIM5 α (Song et al., 2007). We found that viral sensitivity to CM TRIM5 α was inversely correlated with growth capability in OWM cells. Sequence analysis showed that the CM TRIM5 α -sensitive viruses had proline (P) at position 119 or 120 of CA, while the CM

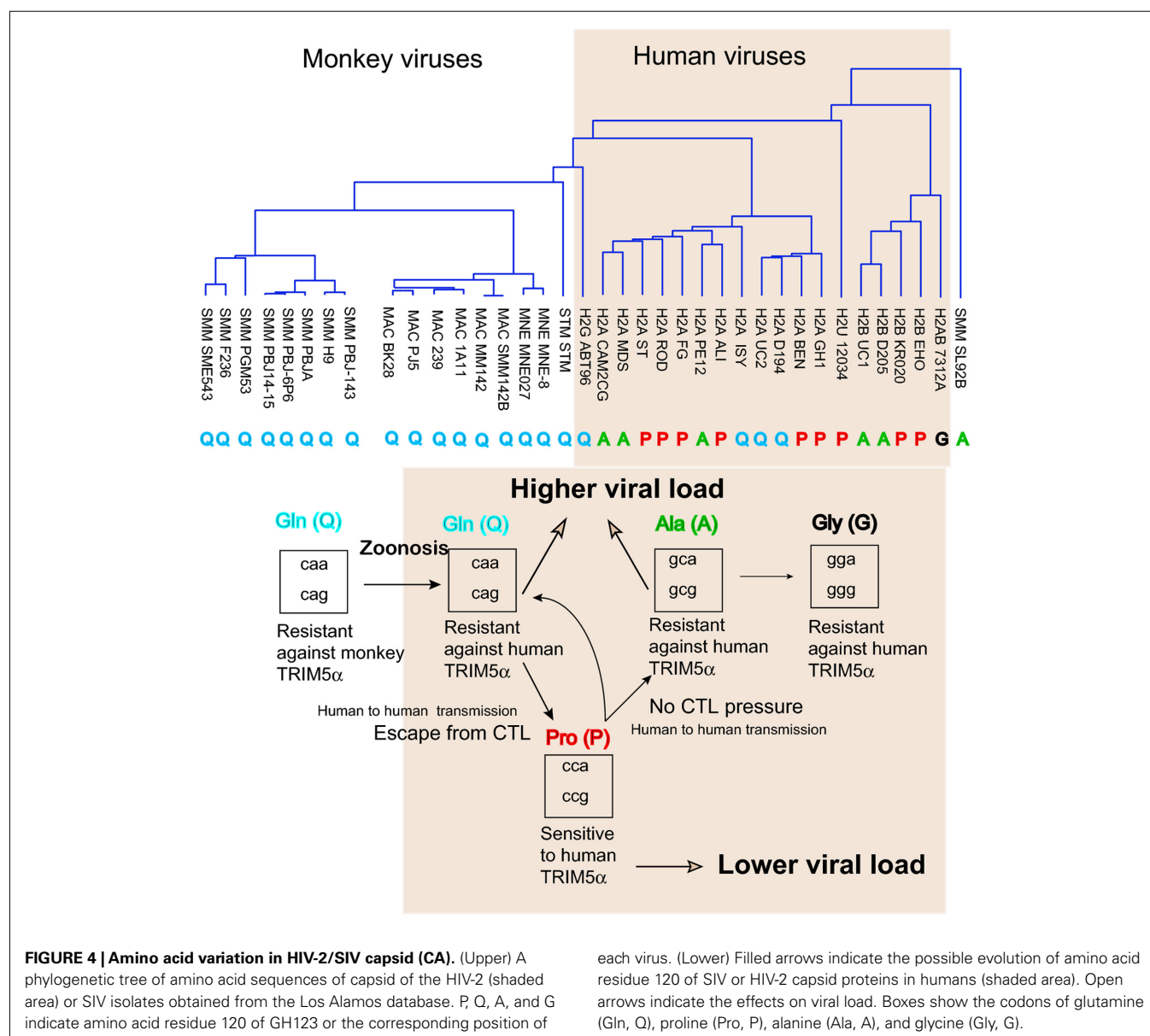


TRIM5 α -resistant viruses had either alanine (A) or glutamine (Q) at the same position (**Figure 4**). Replacing the P of a CM TRIM5 α -sensitive HIV-2 molecular clone with A, Q, or glycine (G) changed the phenotype from sensitive to resistant and the mutant viruses replicated well in the presence of CM TRIM5 α (Song et al., 2007; Miyamoto et al., 2011). Similar results, although to a lesser extent, were observed when human TRIM5 α was used (Song et al., 2007). In the case of Rh TRIM5 α , multiple regions of CA including the N-terminal region, L4/5, and amino acid 120 were shown to affect recognition by Rh TRIM5 α (Ylinen et al., 2005; Lin and Emerman, 2008; Kono et al., 2010; Pacheco et al., 2010; Ohkura et al., 2011).

Positions 119 and 120 are located in the loop between α -helices 6 and 7 (L6/7; **Figure 5**). Previously, a single amino acid substitution at position 110 of MLV CA had been shown to determine viral susceptibility to Fv1 (Kozak and Chakraborti, 1996). The recently published 3-D structure of MLV CA (Mortuza et al., 2004, 2008)

revealed that position 110 of N-MLV CA is located at a position in the surface-exposed loop analogous to position 119 or 120 of HIV-2 CA. HIV-2 is assumed to have originated from SIV isolated from sooty mangabey (SIVsm) as a result of zoonotic events involving monkeys and humans (Hahn et al., 2000). Almost all the SIV isolates in the Los Alamos database contain Q at the position corresponding to position 119 or 120 of HIV-2 CA (**Figure 4**). In contrast, HIV-2 strains possess a mixture of Q, A, and P at the corresponding position.

Does amino acid residue at position 119 or 120 in HIV-2 CA affect HIV diseases in infected individuals? It is known that HIV-1 and HIV-2 have distinct natural histories, levels of viremia, transmission rates, and disease associations despite strong sequence homology between the two viruses (Rowland-Jones and Whittle, 2007). Although some HIV-2-infected patients progress to AIDS as rapidly as HIV-1-infected patients, virus replication is controlled



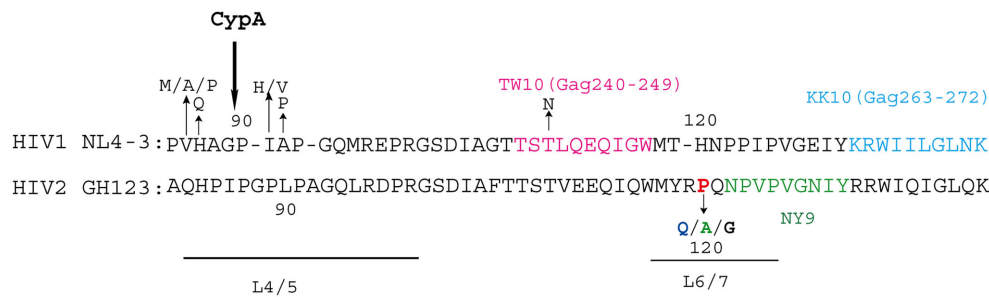


FIGURE 5 | Human immunodeficiency virus (HIV)-1/2 capsid sequence variations and epitopes of cytotoxic T lymphocytes (CTL). The amino acid sequences of the NL4-3 CA (amino acids 85–140) and GH123 CA (amino acids 82–140) are shown. The loop between α -helices 4 and 5 (L4/5) and the loop between α -helices 6 and 7 (L6/7) are underlined. The CTL epitopes are indicated in pink (TW10), sky blue (KK10), and green (NY9). Amino acid

residues that are commonly mutated and were mentioned in this review are indicated. The amino acid residues are numbered both according to the amino acid residues in CA and the whole Gag precursor polyprotein (numbers in parentheses). Cyclophilin A (CypA) catalyzes the 90th proline residue of HIV-1 CA, and alanine residue at the 88th position is critical for CypA binding (Price et al., 2009).

in the majority of HIV-2 patients (Poulsen et al., 1989; Berry et al., 2001) and those with low viral load (VL) achieve much longer survival than those with high VL (Ariyoshi et al., 2000). Detailed sequence analysis of HIV-2 CA variations within a large community cohort in Guinea-Bissau comprised of both high- and low-VL patients indicated that CA from viruses in low-VL patients had P residues at position 119 or 120, but in patients with higher VL, position 119 or 120 was frequently occupied by non-P residues. Stratification of the subjects according to the presence or absence of P at position 119 or 120 showed a threefold difference in the median VL of the two groups. These results indicated that HIV-2 replication in infected individuals can be linked to CA variation and human TRIM5 α sensitivity (Onyango et al., 2010).

CTL ESCAPE, DRUG RESISTANCE, COMPENSATORY MUTATION, AND TRIM5 α RESISTANCE

Recently, Leligdowicz et al. (2010) reported that HLA-B*3501 was associated with HIV-2 with P at position 119 or 120 in the community cohort in Guinea-Bissau. The cytotoxic T cell (CTL) NY9-epitope (NPVPVGNIIY) was located two amino acids downstream of position 119 or 120. It is thus possible that viruses were forced to change Q to P at position 119/120 to escape from HLA-B*3501-specific immune response, even though the virus became more sensitive to human TRIM5 α due to this substitution (Figure 5). After transmission to individuals lacking HLA-B*3501, viruses may have evolved from the P virus to become more resistant to human TRIM5 α (Figure 4). Moreover, several patients with HIV-2 that had a high VL and developed AIDS rapidly have recently been identified in Japan. Sequence analysis of viruses isolated from these patients indicated that they carried G at position 119 or 120. The selection pressure for G substitution is not clear at present but it is worth noting that G was found only in clade A/B recombinants (Ibe et al., 2010).

In the case of HIV-1, Kootstra et al. proposed that a histidine (H)-to-Q substitution at position 87 (H87Q; H219Q in Gag) was a result of escape from human TRIM5 α , as the H87Q mutation occurred in 7 of 30 HIV-1-infected individuals in the late-phase of the asymptomatic period and ultimately became the dominant virus population. They also showed that H87Q mutation was

associated with resistance to human TRIM5 α -mediated inhibition (Kootstra et al., 2007), although the restriction activity of human TRIM5 α is much weaker than that of monkey TRIM5 α . H87Q mutation was previously observed in HIV-1 variants isolated from HLA-B57-positive individuals. In these individuals, escape mutations in the HLA-B57-restricted CTL epitope TW10 (Figure 5) were observed and it was suggested that H87Q was a compensatory mutation to restore replicative capacity of the otherwise attenuated phenotype of the TW10 escape mutant (Leslie et al., 2004). Amino acid residue 87H is located in the L4/5 and H87Q mutation reduces incorporation of cyclophilin A (CypA) into HIV-1 virions (Gatanaga et al., 2006). H87Q was also observed in protease inhibitor-resistant viruses (Gatanaga et al., 2002) as well as non-nucleoside reverse transcriptase inhibitor-resistant viruses (Ibe et al., 2008). It remains to be elucidated whether mutations in CTL escape or drug-resistant viruses and compensatory mutations in revertant viruses affect viral sensitivity to human TRIM5 α . From this point of view, Battivelli et al. (2011) recently reported that some Gag CTL escape mutations indeed increased sensitivity to human TRIM5 α . In addition to the H87Q mutation, valine (V)-to-A or V-to-P at position 86, I-to-H or I-to-V at position 91, and A-to-P at position 92 were frequently found in the CypA binding site of HIV-1 in infected individuals, resulting in decreased binding affinity to CypA (Figure 5). Furthermore, Pacheco et al. (2010) adapted HIV-1 to cells expressing Rh TRIM5 α and found that a mutant with V-to-M at position 86 showed reduced affinity for Rh TRIM5 α but retained the ability to bind CypA efficiently. The relationship between CypA binding and TRIM5 α sensitivity should also be evaluated.

POLYMORPHISMS IN THE HUMAN TRIM5 GENE

Human immunodeficiency virus-1 infection in humans is generally characterized by a long-term chronic disease course gradually progressing to AIDS. Polymorphisms in human CCR5 and other genes have been reported to affect susceptibility to HIV-1 transmission and/or the rate of disease progression to AIDS (O'Brien and Nelson, 2004; Shioda and Nakayama, 2006). Sawyer et al. (2006) reported a common H-to-tyrosine (Y) polymorphism at amino acid residue 43 (H43Y, rs3740996) of the human TRIM5

gene. This SNP is located in the RING domain (**Figure 3**) and was shown to greatly reduce the ability of TRIM5 α to restrict N-MLV (Sawyer et al., 2006). Several studies have indicated that the anti-HIV-1 activity of TRIM5 α with 43Y was lower than that with 43H *in vitro* (Javanbakht et al., 2006; Sawyer et al., 2006; Nakayama et al., 2007), although the difference in anti-HIV-1 activity was very small.

Associations of H43Y with the rate of progression to AIDS have been tested in several studies, but with inconsistent results (Javanbakht et al., 2006; Speelman et al., 2006; Nakayama et al., 2007; van Manen et al., 2008). Despite the lower anti-N-MLV and anti-HIV-1 activities of TRIM5 α with 43Y (Sawyer et al., 2006), Javanbakht et al. (2006) reported a paradoxical protective effect of TRIM5 α with 43Y against HIV-1 transmission in African-Americans. Interestingly, we also found that the 43Y-allele was found less frequently in Japanese and Indian HIV-1-infected subjects than in ethnicity-matched controls (Nakajima et al., 2009). Furthermore, Liu et al. (2011) reported that the frequency of H43Y homozygotes was higher in seronegative intravenous drug users than in HIV-infected drug users. The reasons for the discrepancy between the epidemiological and functional effects of H43Y remain unclear, and further studies are required to clarify the impact of H43Y on susceptibility to HIV-1 transmission and/or rate of progression to AIDS. H43Y polymorphism was frequently found in humans but not in monkey species (Johnson and Sawyer, 2009).

In the B-box2 domain, we recently found a novel and rare G-to-R substitution at position 110 of TRIM5 α (G110R, rs146215995) in Japan, and this 110R allele was observed more frequently in HIV-1-infected subjects than in controls. As observed epidemiologically, this substitution weakened the anti-HIV-1 and anti-HIV-2 activity *in vitro* (Nakajima et al., 2009). Price et al. (2010) sequenced exon 2 of the *TRIM5* gene in 1032 women enrolled in a long-term monitored Pumwani sex worker cohort, and found that women with the R136Q polymorphism (rs10838525) were less likely to seroconvert despite heavy exposure to HIV-1 through active sex work. The B-box2 domain is important for higher-order multimerization, which is required to form the hexagonal structure to stabilize the interaction between TRIM5 α and the capsid (Ganser-Pornillos et al., 2011). It is likely that R136Q substitution affects higher-order multimerization.

Position 332 in the V1 region of the PRYSPRY domain is critical for species-specific recognition of capsid by TRIM5 α (Stremlau et al., 2005; Yap et al., 2005). There is no human SNP in this region except for a rare null allele 332X (**Figure 3**). Torimiro et al. reported that 332R changed to a stop codon in Baka pygmies at an allele frequency of 0.02. This rare allele encoded a truncated form of TRIM5 α lacking part of the PRYSPRY domain and showed a dominant negative effect against authentic TRIM5 α *in vitro* (Torimiro et al., 2009). These findings suggest that anti-HIV-1 activity of human TRIM5 α may affect HIV-1 transmission although it can hardly protect humans from an HIV-1 pandemic.

EVOLUTION OF THE *TRIM5* GENE

TRIM5 homologs have been found in the genomes of primates, mice, rats, rabbits, dogs, cows, and pigs, but not in chickens (Sawyer et al., 2007; Schaller et al., 2007; Tareen et al., 2009). *TRIM5* homolog genes are found in several copies in cows, rats, and

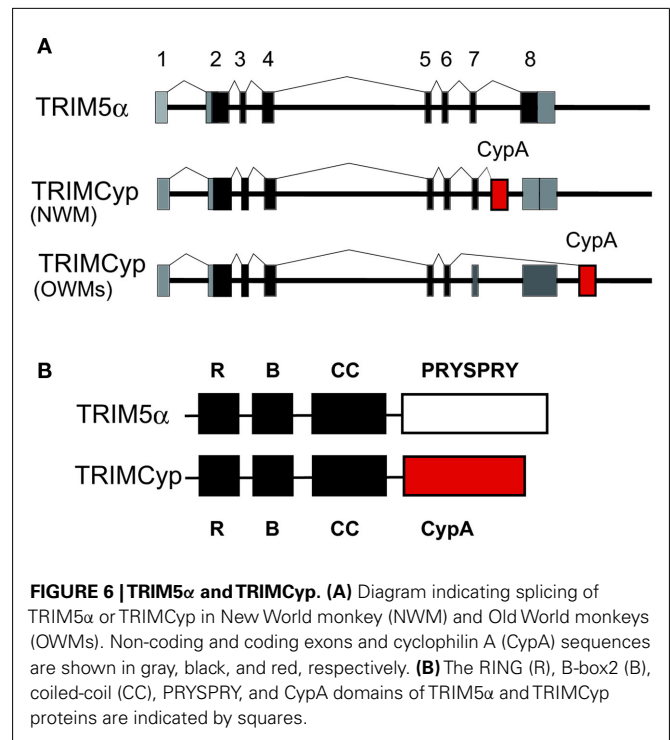


FIGURE 6 | TRIM5 α and TRIMCyp. (A) Diagram indicating splicing of TRIM5 α or TRIMCyp in New World monkey (NWM) and Old World monkeys (OWMs). Non-coding and coding exons and cyclophilin A (CypA) sequences are shown in gray, black, and red, respectively. **(B)** The RING (R), B-box2 (B), coiled-coil (CC), PRYSPRY, and CypA domains of TRIM5 α and TRIMCyp proteins are indicated by squares.

mice, but the human genome contains only a single copy of the *TRIM5* gene, and the canine homolog is inactivated by a transposon (Johnson and Sawyer, 2009). TRIM5 mRNA expressed in cat cells lacks the PRYSPRY domain (McEwan et al., 2009). No antiviral activity against eight retroviruses, i.e., HIV-1, SIVmac, EIAV, N-MLV, B-MLV, NB-MLV, feline immunodeficiency virus (FIV), and Mason-Pfizer monkey virus, has been reported for the mouse TRIM5 homologs (TRIM12 and TRIM30; Tareen et al., 2009) and mouse TRIM30 targets TAK1-binding protein (TAB) 2 for degradation (Shi et al., 2008).

The *TRIM5* gene sequence varies considerably among primate species. The distribution of positively selected amino acid site is located in the PRYSPRY domain and coiled-coil domains (Sawyer et al., 2005; Song et al., 2005a; Newman et al., 2006). It is not surprising that the beginning of the PRYSPRY domain (V1) is highly variable because TRIM5 α interacts with several different retroviral cores through this region, as discussed above. Interestingly, in Rh, there is a 339-threonine–phenylalanine–proline (TFP)-341-to-Q polymorphism in TRIM5 α (Newman et al., 2006), which reduces the anti-HIV-2 (Kono et al., 2008) and anti-SIVsm (Kirmaier et al., 2010) activity. In the case of SIVsm challenge *in vivo*, Rh TRIM5 α ^{TFP/TFP} homozygotes markedly diminished viral replication compared to Rh TRIM5 α ^{Q/Q} homozygotes (Kirmaier et al., 2010; Reynolds et al., 2011; Yeh et al., 2011). Position 332 in human TRIM5 α is arginine (R). Kaiser et al. (2007) showed that a 4-million-year-old endogenous retrovirus from the chimpanzee genome (ptERV1) was suppressed by chimpanzee and human TRIM5 α bearing R at position 332 but not gorilla, gibbon, or orangutan TRIM5 α bearing Q at the same position. Although Perez-Caballero et al. (2008) failed to reproduce the sensitivity of

ptERV1 to human TRIM5 α , the main positive selection pressure for TRIM5 α is likely to be endogenous retroviruses.

Among New World monkeys, owl monkeys possess CypA as a fusion protein with TRIM5 (TRIMCyp) as a result of LINE-1-mediated retrotranspositional insertion in addition to the authentic CypA (Nisole et al., 2004; Sayah et al., 2004; **Figure 6**). CypA can bind to the CA of HIV-1, so that the TRIMCyp expressed in owl monkey cells recognizes the HIV-1 core and shows an anti-HIV-1 effect. Retrotransposition of *CypA* into the *TRIM5* gene also occurred independently in OWM, an ancestor of Rh, CM and the pig-tailed monkey (PM; Brennan et al., 2007; Newman et al., 2008; Virgen et al., 2008; Wilson et al., 2008b; Kuang et al., 2009; **Figure 6**). Dietrich et al. and our group also found major and minor haplotypes of CM TRIMCyp with SNPs in the CypA domain. The major haplotype of CM TRIMCyp bears aspartic acid (D) and lysine (K) at positions 66 and 143 of the CypA domain, respectively. In contrast, the minor haplotype of CM TRIMCyp encodes asparagine (N) and glutamic acid (E) at positions 66 and 143, respectively (Dietrich et al., 2011; Saito et al., 2012). N66 and E143 were also found in PM and Rh TRIMCyps, and the CypA portion of the minor haplotype of CM TRIMCyp has the same amino acid sequence as that of Rh TRIMCyp. Rh, PM, and minor haplotype of TRIMCyp restrict infection of HIV-2, SIVsm, and FIV, but not HIV-1 or SIVmac, while the major haplotype of CM TRIMCyp restricts infection by HIV-1 but not HIV-2 or SIVmac

(Brennan et al., 2007; Virgen et al., 2008; Wilson et al., 2008b; Kuang et al., 2009; Dietrich et al., 2011; Saito et al., 2012; **Figure 2**). As we reviewed recently, genotyping of the monkey *TRIM5* gene is important to control animal experiments (Nakayama and Shioda, 2012).

TRIM5 α AND TAK1 COMPLEX

Ovyannikova et al. genotyped healthy children receiving rubella-containing vaccine for 14 candidate genes, including *TLR3*, *TLR4*, *RIG-I*, *TRIM22*, and *TRIM5*. They measured 6 interleukins, INF- γ , TNF- α , and GM-CSF secretion levels in peripheral blood mononuclear cell culture before and after rubella virus stimulation. An allelic dose-related decrease was observed between H43Y of *TRIM5* and TNF- α secretion in response to stimulation, as the medians of 553 HH homozygotes, 131 HY heterozygotes, and 8 YY homozygotes were 34.7 pg/ml (IQR: -3.6 to 95.6), 16.2 pg/ml (IQR: -15.1 to 65.9), and -13.8 pg/ml (IQR: -37.5 to 61.5), respectively. They concluded that *TRIM5* gene polymorphism could influence adaptive cytokine responses to rubella vaccination (Ovsyannikova et al., 2010).

How does TRIM5 α affect immunological response against non-retroviruses? There have been several reports that TRIM5 α has additional activities that are uncoupled from retroviral capsid recognition (Pertel et al., 2011; Tareen and Emerman, 2011). The observation that mouse TRIM30, one of the mouse TRIM5

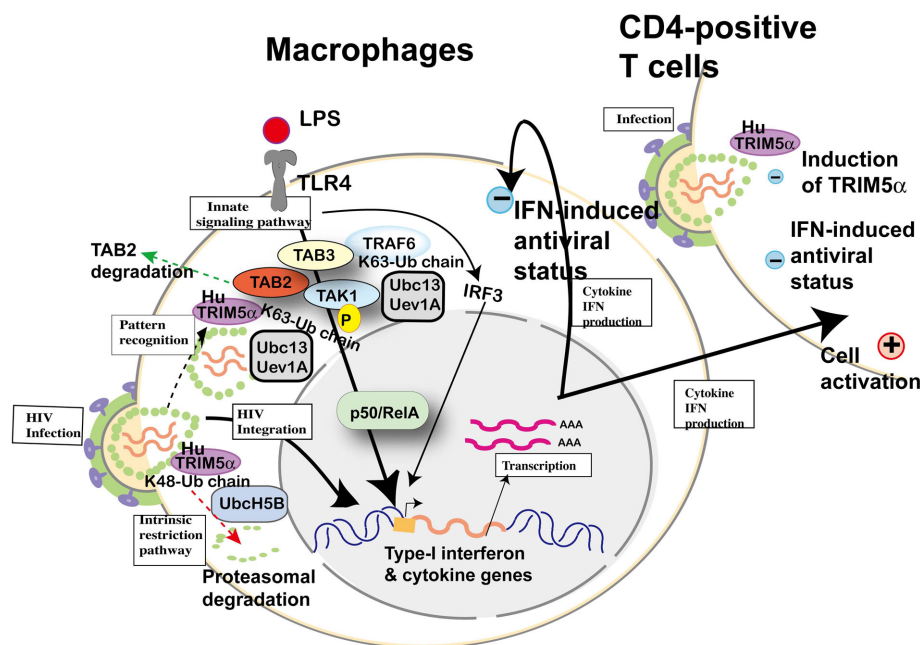


FIGURE 7 | Cellular factors involved in toll-like receptor (TLR) 4-mediated innate signaling and possible involvement of human TRIM5 α in HIV-1 infection. Upon lipopolysaccharide (LPS) stimulation, TLR4 recruits tumor necrosis factor receptor-associated factor 6 (TRAF6) to activate the TGF- β -activated kinase 1 (TAK1) complex (TAK1, TAK1-binding protein (TAB) 2 and TAB3) for NF- κ B (p50/RelA heterodimer) activation. TRAF6 is polyubiquitinated by the ubiquitin-conjugating enzyme UBC13-UEV1A. TRIM5 α is ubiquitinated by UbcH5B (Xu et al., 2003), but the recognition of HIV-1 core by human TRIM5 α and proteasomal

degradation (dotted arrow in red) cannot inhibit HIV-1 integration into the human genome. When human TRIM5 α recognizes an invasive pathogen (dotted arrow in black), human TRIM5 α catalyzes the synthesis of unattached K63-linked ubiquitin chains that activate the TAK1 complex (Pertel et al., 2011). On the other hand, TAB2 is degraded by human TRIM5 α (dotted arrow in green; Tareen and Emerman, 2011). Activation of IRF3 and NF- κ B-dependent gene expression causes both (+) positive status favorable for viral replication and (-) negative status suppressive for viral replication in macrophages and T cells.

homologs described above, inhibits NF- κ B activation by targeting TLR4 signaling intermediates TAB2 and TAB3 for lysosome-mediated degradation (Shi et al., 2008) prompted Tareen and Emerman (2011) to evaluate the interaction between TRIM5 α and TAB2. They showed that human TRIM5 α was able to decrease the expression levels of human, mouse and Rh TAB2, while Rh TRIM5 α was unable to affect the levels of either Rh or human TAB2 (Tareen and Emerman, 2011). Using an NF- κ B-inducible luciferase reporter gene, they assessed the effects of overexpressing human or Rh TRIM5 α in 293T cells; however, they found that the expression of human TRIM5 α by itself resulted in activation of NF- κ B-driven transcription, which was not the case with the mouse TRIM30. At a higher concentration (1.5 μ g DNA of TRIM5 α vs. 0.5 μ g of TAB2), human but not Rh TRIM5 α reached saturation and resulted in a drop in NF- κ B activation, as human but not Rh TRIM5 α degraded TAB2. Both abilities of TRIM5 α to target TAB2 and to upregulate NF- κ B were independent of the PRYSPRY domain, which is critical for capsid recognition. The RING domain of TRIM5 α was necessary to activate NF- κ B, while RING and B-box2 of human TRIM5 α were sufficient to degrade TAB2 (Tareen and Emerman, 2011).

Subsequently, Pertel et al. (2011) reported similar upregulation of NF- κ B and AP-1 activation in human TRIM5 α -transfected HEK-293 cells and further confirmed interaction with TAB2, TAB3, and TAK1 complex by immunoprecipitation; however, they did not mention TAB2 degradation reported by Tareen and Emerman (2011). LPS recognized by TLR4 activates AP-1 and NF- κ B-signaling, and this culminates in the expression of inflammatory genes. The knockdown of human TRIM5 α in THP-1 cells attenuated the induction of AP-1 and NF- κ B-dependent genes, indicating that TRIM5 α makes a major contribution to LPS-signaling. Acting with the ubiquitin-conjugating enzyme UBC13–UEV1A, human TRIM5 α catalyzed the synthesis of unattached K63-linked ubiquitin chains that activate the TAK1 complex. Anti-HIV-1 activity of LPS (Kornbluth et al., 1989) and *Escherichia coli* infection (Ahmed et al., 2010) were previously reported in

macrophages. TRIM5, UBC13, or TAK1 knockdown in THP-1 macrophages rescued HIV-1, SIV, rhabdovirus vesicular stomatitis virus, and paramyxovirus Newcastle disease virus from LPS-induced antiviral state. Finally, they compared induced cytokine levels between stimulation with restricted (e.g., SIVmac) and unrestricted (e.g., HIV-1) virus by human TRIM5 α in THP-1 and concluded that antiviral potency was correlated with TRIM5 α avidity for the retroviral capsid lattice, although it is not clear whether the induced cytokines are sufficient to protect macrophages themselves and bystander T cells from viral infection (Figure 7). Especially in HIV-1 infection, it has been speculated that LPS-signaling caused by microbial translocation stimulates cells non-specifically and chronically, resulting in exhaustion of immunity (Brenchley and Douek, 2008). As HIV-1 prefers stimulated T cells, it is reasonable that H43Y RING mutation of TRIM5 α showed the paradoxical protective effect on HIV-1 transmission described above.

CONCLUSION

The mechanism of antiviral intrinsic immunity via capsid recognition of monkey TRIM5 α has been elucidated, although it is still unclear how the prototype antiviral factor Fv1 in mice suppresses nuclear import of MLV. Many TRIM family members, including TRIM21, TRIM23, TRIM27, and TRIM30 α , were found to be involved in the TLR4 signaling pathway in mice (Kawai and Akira, 2011). Human TRIM5 α has also recently been shown to be involved in this innate immunity (Pertel et al., 2011), and therefore the significance of human TRIM5 α *in vivo* must be clarified in future studies. As the function of mouse TRIM30 α is not identical to that of human TRIM5 α , it would be interesting to perform human genetic association study with other infections, including bacterial infection.

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Role of host-encoded proteins in restriction of retroviral integration

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In retroviral infections, a copy of the viral DNA is first synthesized from genomic RNA by reverse transcription and subsequently integrated into host chromatin. This integration step, executed by the viral enzyme integrase (IN), is one of the hallmarks of retroviral infection. Although an obligate role for IN in retroviral integration has been clearly defined by numerous biochemical analysis of its recombinant protein and genetic analysis of the viral IN gene, several host cellular proteins have also been implicated as key factors involved in the integration step during viral replication. Although studies on integration cofactors have mostly emphasized factors that aid the integration process either through direct or indirect association with IN, it has become apparent that host cells may also harbor proteins that act as inhibitors of retroviral integration. Intriguingly, some of these inhibitory proteins appear to hamper the integration process via posttranslational modifications of the components of the preintegration complex including IN. A better understanding of the molecular mechanisms leading to the inhibition of integration will provide us with clues for the development of new strategies for treating retroviral infections. In this review, we draw attention to recent insights regarding potential host cellular factors that restrict integration, and illustrate how these inhibitory effects are achieved.

Keywords: retrovirus, integration, host factors, RAD51, KAP1, VRK, posttranslational modifications

INTRODUCTION

After entry into a target host cell, retroviruses carry out reverse transcription for the synthesis of double-stranded DNA from their RNA genome in a complex derived from the viral core. Subsequently, the newly synthesized viral DNA remains associated with viral and cellular proteins and forms a high-ordered nucleoprotein complex called the preintegration complex (PIC). The PIC guides nuclear entry and the subsequent integration process of the viral DNA genome (Suzuki and Craigie, 2007). The viral DNA that is integrated into chromosomal DNA, termed the provirus, acts as a template for the efficient transcription of viral RNA and ensures the equal segregation of genetic material to daughter cells upon division. Once established, proviruses cannot be distinguished or excised from the cellular genomic DNA of infected cells. Therefore, integration occupies an important place not only in viral replication but also in the treatment of retroviral infectious diseases (Lewinski and Bushman, 2005).

The viral enzyme catalyzing the integration is integrase (IN). As shall be shown, the chemical mechanism of the integration reaction has been elucidated by biochemical studies using oligonucleotide DNA substrates and purified IN protein produced by the *Escherichia coli* system (Bushman et al., 1990; Craigie et al., 1990; Katz et al., 1990; Sherman and Fyfe, 1990). These studies indicate that IN alone can carry out the DNA breakage and joining reactions during integration (Engelman, 2003; Turlure et al., 2004). However, the reaction by recombinant IN lacks the full fidelity of DNA integration observed in virus-infected cells. In contrast, PICs

extracted from infected cells efficiently insert both viral DNA ends into a target DNA in a concerted manner *in vitro*, a hallmark of the integration reaction *in vivo*, indicating that the PIC harbors additional factors essential for the authentic integration in cells (Fujiwara and Mizuuchi, 1988; Bowerman et al., 1989; Brown et al., 1989; Farnet and Haseltine, 1990; Turlure et al., 2004). Indeed, a number of cellular proteins have since been identified as co-factors of retroviral IN and PICs (Turlure et al., 2004; Al-Mawsawi and Neamati, 2007).

As seen with the integration step, retroviruses need to hijack host biological processes to execute their efficient replication in the cell (Goff, 2007). Under the selection pressure of such viral virulence mechanisms, the host organism has itself evolved cellular antiviral defense strategies called intrinsic immunity, which block virus replication at the post-entry, post-integration, and virion release steps (Wolf and Goff, 2008), and in addition to well-studied antiviral cellular proteins such as APOBEC and TRIM family proteins, intense efforts have been dedicating to identify novel inhibitory factors against retroviruses (Liu et al., 2011; Tyagi and Kashanchi, 2012). Intriguingly, new cellular inhibitory factors for retroviral integration are also beginning to attract attention. Although viruses may be still capable of surmounting the host defense system, further understanding of the host's inhibitory machineries could potentially translate into new treatments for retroviral infectious diseases. Here, we focus on the recent findings that illustrate the involvement and molecular mechanisms of potential restriction factors for retroviral integration.

BIOCHEMICAL FEATURES OF THE INTEGRATION REACTION

Retroviral IN is expressed and incorporated into virions as the C-terminal part of the Gag-Pol polyprotein precursor during the late phase of retroviral infection. IN comprises three structurally and functionally distinct domains, termed the N-terminal domain (NTD), the catalytic core domain (CCD), and the C-terminal domain (CTD; **Figure 1A**; Lewinski and Bushman, 2005). The NTD of IN is relatively well conserved amongst retroviruses especially with the respect to a motif consisting of two histidines and two cysteines (HHCC motif). This HHCC motif makes up the zinc-binding site with an overall helix-turn-helix fold commonly found in IN of all retroviruses and retrotransposons (Craigie, 2001). Further studies utilizing mutants containing amino acid mutations in the HHCC motif also elucidated the importance of the NTD in the key steps of integration as well as its involvement in the multimerization of IN (Zheng et al., 1996; Lee et al., 1997; Craigie, 2001). The CCD is composed of a mixture of α -helices and β -sheets folds that bring a triad of critical acidic residues called the DDE motif into close proximity (Lewinski and Bushman, 2005). Crystal structure studies of HIV-1 and avian sarcoma virus (ASV) IN have shown this motif to be important for the binding of divalent metal cations such as Mg^{2+} (Craigie, 2001). Additionally, the CCD was also shown to possess viral DNA recognition and binding ability (Lewinski and Bushman, 2005). Compared to the NTD and CCD of IN, the CTD displays the greatest degree of variation across the retrovirus family. Despite this, studies on the CTD region of HIV-1 have revealed that it has strong DNA-binding activity and also potentially has multimerization capabilities (Woerner et al., 1992; Engelman et al., 1994; Lutzke and Plasterk, 1998; Gao et al., 2001).

The retrovirus-mediated integration reaction consists of three biochemically distinct steps (**Figure 1B**; Craigie, 2001). In the first step, IN specifically recognizes the viral attachment (*att*) sites on the 5' and 3' long terminal repeats (LTRs) of newly synthesized viral DNA ends. This interaction between IN and DNA allows for the 3' processing of viral DNA, where water serves as the nucleophile for the cleavage of viral DNA. In this 3' processing step, most retroviral IN such as HIV-1 IN catalyze the removal of two nucleotides adjacent to the highly conserved CA dinucleotide from the 3' end of LTR regions. This chemically activates the viral DNA through the formation of 3' hydroxyl radicals at the terminal ends of viral DNA, allowing it to participate in the next reaction.

The second step of integration involves the insertion of 3' processed, activated viral DNA strand into the target DNA through a single transesterification reaction, which is called the strand transfer step (Engelman et al., 1991). IN brings the viral DNA ends into close proximity with the target DNA allowing a nucleophilic attack by the 3' hydroxyl radical on the target DNA. Subsequently, IN ligates both the 3' hydroxyl radical terminal of the viral DNA to the 5' phosphoryl ends of the target DNA and establishes new phosphodiester bond between them. This forms intermediate DNA products with unrepaired gaps between the non-ligated 5' ends of viral DNA as well as the 3' ends of target DNA. Additionally, the unrepaired gaps result in the short duplication of target DNA sequences flanking both strand ends, followed by the formation of imperfect inverted repeats

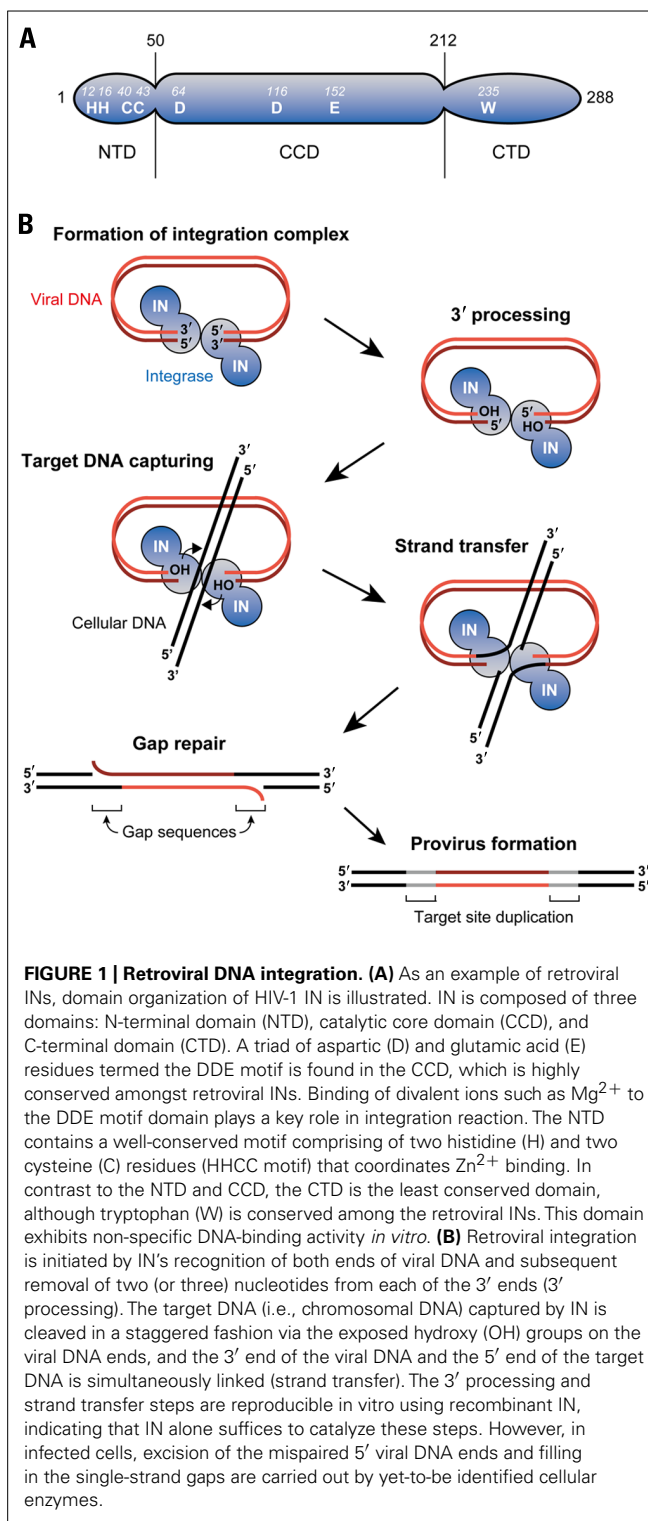


FIGURE 1 | Retroviral DNA integration. (A) As an example of retroviral INs, domain organization of HIV-1 IN is illustrated. IN is composed of three domains: N-terminal domain (NTD), catalytic core domain (CCD), and C-terminal domain (CTD). A triad of aspartic (D) and glutamic acid (E) residues termed the DDE motif is found in the CCD, which is highly conserved amongst retroviral INs. Binding of divalent ions such as Mg^{2+} to the DDE motif domain plays a key role in integration reaction. The NTD contains a well-conserved motif comprising of two histidine (H) and two cysteine (C) residues (HHCC motif) that coordinates Zn^{2+} binding. In contrast to the NTD and CCD, the CTD is the least conserved domain, although tryptophan (W) is conserved among the retroviral INs. This domain exhibits non-specific DNA-binding activity *in vitro*. (B) Retroviral integration is initiated by IN's recognition of both ends of viral DNA and subsequent removal of two (or three) nucleotides from each of the 3' ends (3' processing). The target DNA (i.e., chromosomal DNA) captured by IN is cleaved in a staggered fashion via the exposed hydroxy (OH) groups on the viral DNA ends, and the 3' end of the viral DNA and the 5' end of the target DNA is simultaneously linked (strand transfer). The 3' processing and strand transfer steps are reproducible *in vitro* using recombinant IN, indicating that IN alone suffices to catalyze these steps. However, in infected cells, excision of the mispaired 5' viral DNA ends and filling in the single-strand gaps are carried out by yet-to-be identified cellular enzymes.

upon the sealing of the nick. The length of this inverted repeat sequences can vary from 4 to 6 base pairs (bp) and is highly characteristic of the retrovirus species from which the IN protein is derived. It should be noted that the 3' processing and strand transfer steps have been shown to be catalyzed by IN proteins

through various *in vitro* studies conducted with *E. coli*-expressed IN and DNA substrates harboring viral LTR sequence (Turlure et al., 2004).

Lastly, after the ligation reaction, the gaps in the intermediate DNA products are repaired to yield fully functional integrated proviruses (gag repair step). The previous step generates an intermediate product in which viral DNA is joined to the target DNA solely by the 3' ends of both strands. As such, DNA repair is needed to seal the nick between the 5' end of viral DNA and the 3' end of the target DNA to complete the provirus formation. Unlike the earlier steps which are catalyzed by IN, this step is probably mediated by some cellular enzymes involved in a variety of DNA repair pathways including the non-homologous end joining (NHEJ) repair pathway. However, whether or not the NHEJ machinery is involved is controversial, and identification of the specific enzymes conducting this gap repair step is anticipated in future studies (Turlure et al., 2004; Smith and Daniel, 2006; Yoder et al., 2006).

HOST PROTEINS ASSOCIATED WITH THE RETROVIRAL INTEGRATION COMPLEX

The PIC, the key nucleoprotein complex responsible for the integration, is formed in the cytoplasm following the reverse transcription of viral DNA from the RNA genome (**Figure 2**). Although the full composition of this nucleoprotein complex has yet to be determined, most of the viral protein components of the PIC are thought to be derived from the core of the infecting virion, which includes not only IN but also reverse transcriptase (RT), matrix (MA), capsid (CA), and some HIV-1 accessory proteins (Suzuki and Craigie, 2007). With respect to the cellular components of the PIC, significant efforts have been made to identify them through surveys of IN-interacting proteins using yeast two-hybrid screenings and/or co-immunoprecipitation analysis (**Figure 2**; Turlure et al., 2004).

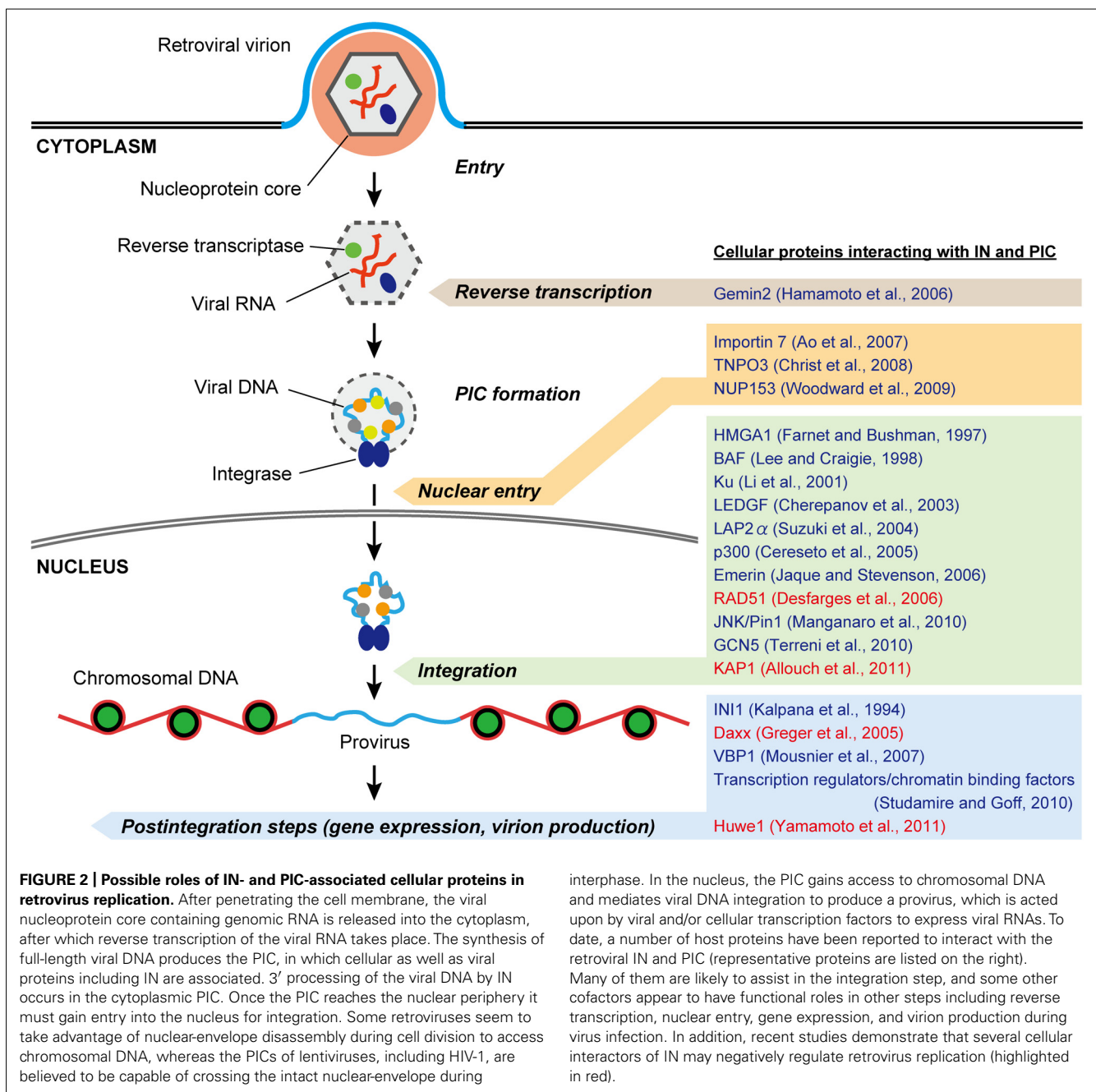
Integrase interactor 1 (INI1), also known as hSNF5, was the first binding partner of HIV-1 IN identified by yeast two-hybrid assay (Kalpana et al., 1994). This nuclear protein is the human homolog of the yeast SNF5 protein, a transcription activator and part of the SWI/SNF complex in mammalian systems (Wang et al., 1996). Interaction with the HIV-1 IN requires a direct imperfect repeat region of INI1 termed Rpt1 (Yung et al., 2001). The function of INI1 in HIV-1 integration was demonstrated in an *in vitro* integration assay where it stimulated the strand transfer activity of recombinant IN (Kalpana et al., 1994). However, the role of INI1 during virus replication is still a matter of debate. Co-transfection of the minimal IN-binding domain of INI1, including the Rpt1 region, with HIV-1 proviral DNA in HEK293 cells resulted in a nearly 10,000-fold reduction in virus production from the transfected cells (Yung et al., 2001). Furthermore, subsequent studies showed that INI1 is specifically incorporated into HIV-1 virions during virus production (Yung et al., 2004), suggesting a possible role of INI1 in the late stage of HIV-1 replication rather than in the integration step. Interestingly, it was revealed that IN and INI1 selectively recruit the components of the Sin3a-HDAC1 complex into HIV-1 virions, whereafter it is required for the reverse transcription step following the infection of subsequent target cells (Sorin et al., 2009). Thus, INI1 does not seem to

be involved in the integration step in spite of its interaction with IN.

Lens epithelium-derived growth factor (LEDGF) is the first cellular protein to have been demonstrated as a *bona fide* cofactor for HIV-1 integration (Engelman and Cherepanov, 2008). LEDGF is a transcriptional regulatory protein with a molecular mass of 76 kDa from the hepatoma-derived growth factor (HDGF)-related protein (HRP) family, and was found to be strongly associated with chromatin throughout the cell cycle. This protein was identified as an interaction partner of HIV-1 IN through a co-immunoprecipitation study in human cells (Cherepanov et al., 2003) and has been shown to stimulate *in vitro* integration activity of IN (Cherepanov et al., 2004). LEDGF comprises several functional domains that have been implicated in the integration process. The N-terminal of LEDGF contains the PWWP (proline-tryptophan-tryptophan-proline) domain that is highly conserved amongst the members of the HRP family and was revealed to be crucial for protein-protein interaction and/or DNA-binding. Furthermore, the N-terminal also contains a putative nuclear localization signal (NLS) and dual copies of the AT-hook DNA-binding motif (Engelman and Cherepanov, 2008). In addition, a limited proteolysis analysis of recombinant LEDGF has shown that an evolutionally conserved domain, termed the integrase-binding domain (IBD), is included in its C-terminal and as its name suggests, this domain was found to be crucial for the specificity of the interaction with HIV-1 IN (Engelman and Cherepanov, 2008).

LEDGF is likely to play a critical but not strictly essential role in HIV-1 integration and replication. A study performed using intensified RNA interference demonstrated a significant reduction of HIV-1 replication in human CD4⁺ T cells in which there was a complete knockdown of endogenous LEDGF (Llano et al., 2006). Moreover, a knockout study done in mouse embryonic fibroblasts (MEFs) cell lines reported a reduction of 90% in HIV-1 infectivity upon the depletion of LEDGF/p75 and that this reduction was ablated upon re-expression of LEDGF (Shun et al., 2007). In these studies, it was found that the block in HIV-1 infection occurs specifically at the integration step, and that both the PWWP and IBD domains are of critical importance for HIV-1 integration and replication (Llano et al., 2006; Shun et al., 2007). Based on these observations, a simple mechanism model is proposed for LEDGF's function in HIV-1 integration: LEDGF serves as a molecular adaptor that tethers HIV-1 IN to the target DNA (i.e., chromatin). Since LEDGF is a transcriptional coactivator, this tethering activity is thought to be responsible for targeting the integration site of HIV-1 into transcriptionally active regions (Engelman and Cherepanov, 2008).

After the PIC is formed in cytoplasm, it needs to be shuttled into the nucleus to allow the integration process to take place. One of the striking features of HIV-1 is its ability to get across the intact nuclear envelope easily even in non-dividing cells. This phenomenon is believed to be strongly associated with the karyophilic properties of the HIV-1 PIC (Suzuki and Craigie, 2007). Although the main component causing the PICs to be actively imported into the nucleus remains undetermined, some host-encoded proteins that interact with IN have been reported to facilitate the nuclear import of HIV-1 PICs. To date, importin 7 and TNPO3, have been



reported as IN-interacting importins that direct the HIV-1 PICs to the nucleus (Fassati et al., 2003; Ao et al., 2007; Christ et al., 2008). Both proteins are members of the importin β family, which act as import receptors for cargo molecules through association with nucleoporin (NUP) of the nuclear pore complex (Suzuki and Craigie, 2007). While a confocal microscopy analysis of digitonin-permeabilized human cells showed the involvement of importin 7 in the nuclear accumulation of HIV-1 integration complexes, the functional role of this protein in virus replication is still unresolved (Fassati et al., 2003; Zaitseva et al., 2009). TNPO3, an HIV-1 IN-binding protein identified by yeast two-hybrid screening, was found to promote viral nuclear import as well (Christ et al., 2008).

Although subsequent studies have confirmed the requirement of TNPO3 for efficient replication of HIV-1, the TNPO3–CA interaction, rather than the TNPO3–IN interaction, is likely to be required for the nuclear import of PIC in infected cells (Krishnan et al., 2010; De Iaco and Luban, 2011). By the same token, another cellular protein regulating nucleocytoplasmic trafficking, NUP153, was also shown to interact with HIV-1 IN by a pull-down assay (Woodward et al., 2009), but again, viral CA appears to be the viral determinant for the NUP153-mediated nuclear entry of the PIC (Matreyek and Engelman, 2011).

Intriguingly, a certain host protein called Gemin2 may be of importance to the reverse transcription process of HIV-1 via

its physical association with IN. Gemin2 is a component of the survival of motor neurons (SMN) complex that is essential for the biogenesis of spliceosomal small nuclear ribonucleoproteins (snRNPs) and was identified as a novel interactor of HIV-1 IN by a yeast two-hybrid screening (Hamamoto et al., 2006). Although this cellular protein is found in the HIV-1 PIC, depletion of endogenous Gemin2 by siRNA in HIV-1-infected primary monocyte-derived macrophages resulted in impairment of the early reverse transcription step rather than the integration step (Hamamoto et al., 2006). Furthermore, a recent study showed that viral DNA synthesis by HIV-1 RT *in vitro* is enhanced by the concerted action of Gemin2 and IN (Nishitsuji et al., 2009). This augmentation of RT activity by an IN interaction appears to be reasonable because functional interaction between HIV-1 RT and IN has been revealed (Wilkinson et al., 2009). Hence, it would be assumed that Gemin2 serves as cofactor that stimulates and/or stabilizes the formation of the reverse transcription complex to initiate DNA synthesis through its interaction with IN (Masuda, 2011).

Several cellular proteins have been also identified as components of HIV-1 and Moloney murine leukemia virus (MoMLV) PIC by the *in vitro* reconstitution analysis and immunoprecipitation assays of PICs isolated from virus-infected cells. These include HMG proteins, barrier-to-autointegration factor (BAF, discussed below), Ku, and LEM proteins (Figure 2). Although there are some controversies as to whether these PIC-associated factors are indispensable to retrovirus infection, they may be indirectly involved in the integration process by stabilizing the nucleoprotein complex, promoting the nuclear retention of the PIC, or protecting host cells from viral DNA termini-induced apoptosis (Turlure et al., 2004; Van Maele et al., 2006; Suzuki and Craigie, 2007). Besides the host proteins listed above, more extensive discussions about the other cellular factors interacting with the IN/PIC and their roles in retrovirus replication are presented in previous comprehensive reviews (Turlure et al., 2004; Al-Mawsawi and Neamati, 2007; Goff, 2007; Studamire and Goff, 2010).

POTENTIAL CELLULAR INHIBITORS AGAINST INTEGRATION

In addition to the cellular enhancers for retroviral integration, the existence of potential antiviral proteins controlling the integration activity of IN and PIC has been revealed by several recent studies. Understanding the molecular details by which these factors hamper the integration reaction may potentially lead to future therapeutic inventions against retroviral infectious diseases. Recent findings on the potential restriction factors for integration process are summarized in this section, and their molecular inhibitory mechanisms are discussed.

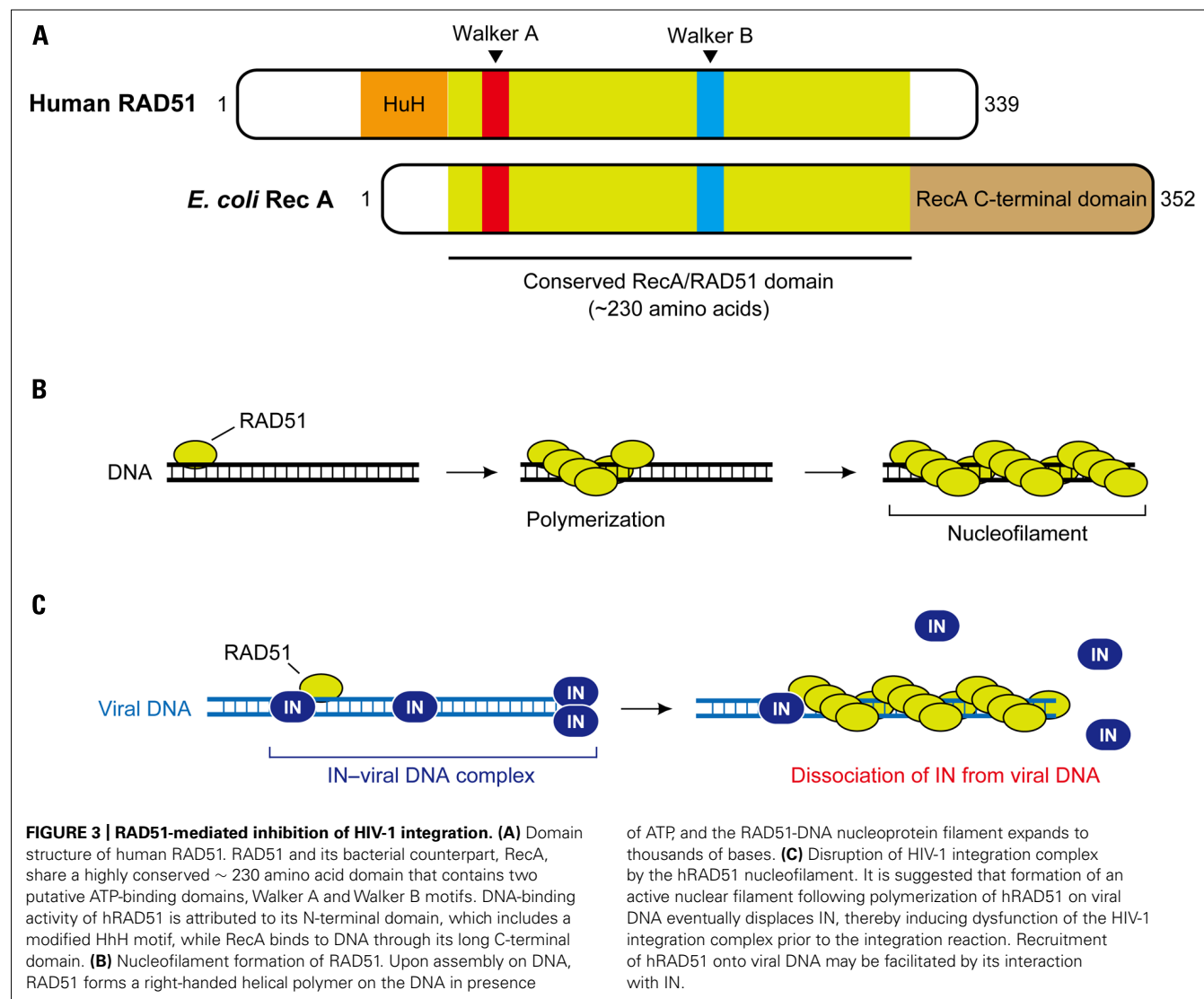
RAD51

Although retroviral integration is completed by the gap repair step, which is believed to be mediated by the cellular DNA repair enzymes (Daniel et al., 1999; Lewinski and Bushman, 2005), one of the homologous recombination (HR) proteins, RAD51, has been shown to suppress HIV-1 integration (Desfarges et al., 2006; Cosnefroy et al., 2012). The downregulation of integration by RAD51 was first observed in a unique integration assay using a budding yeast strain in which DNA substrates containing zeocin-resistance

gene flanked by HIV-1 U3 and U5 LTR ends can be integrated into genomic DNA by HIV-1 IN expressed by the yeast (Desfarges et al., 2006). When a haploid yeast strain deficient for the RAD51 encoding gene was examined using the integration assay, higher numbers of zeocin-resistant clones were obtained, indicating an increase in integration events in the yeast cells lacking RAD51. This result suggests that RAD51 negatively regulates the HIV-1 IN-catalyzed integration reaction in yeast. In accordance with the yeast integration assay, additional *in vitro* analyses using purified recombinant proteins showed that human RAD51 (hRAD51) inhibited integration by IN. Interestingly, HIV-1 IN binds hRAD51 *in vitro*, suggesting that the inhibition of the integration reaction may be mediated through the physical interaction between IN and RAD51 (Desfarges et al., 2006).

Human RAD51 belongs to the RAD52 epistasis group, and members of the RAD52 epistasis group are highly conserved in yeast and humans, which mediate mitotic HR events as well as chromosome segregation during meiosis (San Filippo et al., 2008). RAD51 is also an ortholog of the *E. coli* RecA (Figure 3A). The RecA family proteins possess a conserved, ~230 amino acid core domain that is involved in the binding and hydrolysis of ATP through Walker A and B motifs, with the human RAD51 also bearing a long N-terminal and a short CTDs (Figure 3A; Lin et al., 2006; Wang et al., 2008). The RAD51 protein exists as a homo-oligomer in solution, and binds to single-stranded (or double-stranded) DNA to form a right-handed helical presynaptic nucleoprotein. In the presence of ATP, RAD51 can polymerize on the DNA to form a nucleofilament that expands for thousands of bases (or base pairs, Figure 3B). This nucleoprotein filament serves as the catalytic center for DNA strand-exchange reactions during HR (San Filippo et al., 2008).

The formation of the nucleofilament is also likely to be important for the downregulation of HIV-1 IN activity by RAD51. In a recent report, Cosnefroy et al. (2012) set up an *in vitro* integration assay for IN in which active nucleofilament formation of RAD51 on substrate DNA was allowed to take place. They revealed that strong inhibition of HIV-1 IN activity was only observed with wild-type hRAD51 in the presence of ATP, but not in the reactions without ATP or with an hRAD51 mutant containing a single amino acid substitution in the Walker A motif (K133A) that ablated hRAD51-ATP-binding and therefore prevented hRAD51 polymerization (Cosnefroy et al., 2012). An additional *in vitro* experiment using a substrate DNA containing LTR sequences complexed with HIV-1 IN showed that even after the IN-viral DNA complexes were formed, subsequent incubation with hRAD51-induced dissociation of IN from its substrate viral DNA. The dissociation effect was not observed with hRAD51 in the absence of ATP or with the K133A mutant (Cosnefroy et al., 2012). Given that RAD51 can polymerize on DNA in an ATP-dependent manner, these observations suggest that the formation of the nucleofilament on viral DNA is responsible for the RAD51-induced restriction of HIV-1 integration via displacement of IN (Figure 3C). Furthermore, when an allosteric effector of RAD51, RS-1 (RAD51-stimulatory compound 1), which promotes formation of the active RAD51 nucleofilament, was used, HIV-1 integration and replication was significantly impaired in CD4⁺ cells. This was accompanied in an increase in 2-LTR circles,

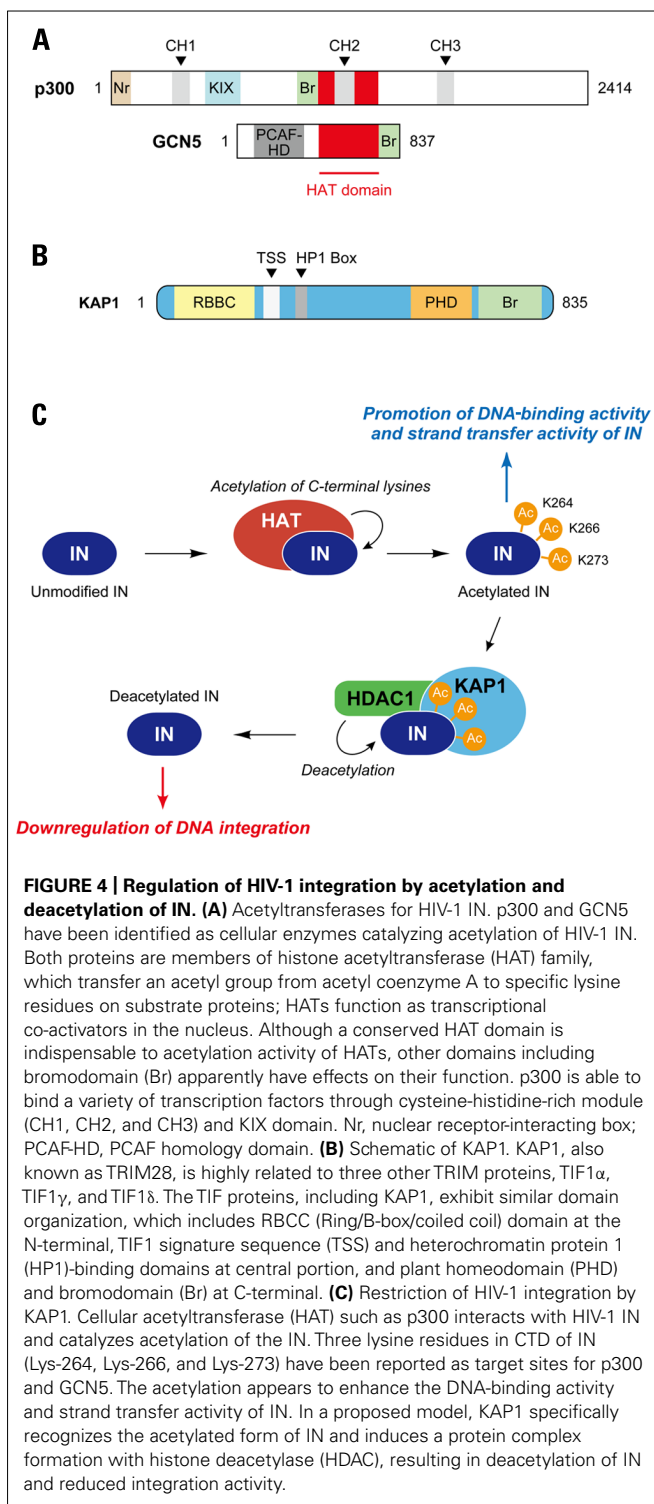


which is a byproduct from the failure to integrate (Cosnefroy et al., 2012). This *in vivo* experiment supports the proposed model in which hRAD51 recruited by HIV-1 IN is able to release the IN from viral DNA via formation of the nucleofilament, resulting in dysfunction of the PIC (Figure 3C). Although it is still unclear whether the hRAD51 in a natural setting could restrict HIV-1 integration, this study provides the possibility of using a specific stimulator of RAD51 such as RS-1 for a new antiviral approach against HIV infection.

KAP1

Posttranslational modifications (PTMs) including phosphorylation, ubiquitination, and acetylation is the chemical alternation of a protein's primary structure after its translation, a process which is well known to have a great impact on various protein functions such as enzymatic activity, protein-protein interaction, and subcellular localization (Walsh et al., 2005). Although the association between PTMs and the retroviral integration process has not been fully addressed, it was demonstrated that the enzymatic

activity of HIV-1 IN is positively regulated by histone acetyl transferases (HATs; Cereseto et al., 2005; Terreni et al., 2010). HATs are the cellular enzymes that transfer an acetyl group from acetyl-coenzyme A (acetyl-CoA) to the ϵ -amino group of certain basic lysine residues of histone's N-terminal, which modifies the accessibility of DNA to other proteins (Roth et al., 2001). p300, the first HAT protein that was reported to catalyze the acetylation of HIV-1 IN, is a ~300 kDa nuclear phosphoprotein originally isolated as an interaction partner of adenovirus E1A (Stern and Berger, 2000). This HAT is also a paralog of the CREB-binding protein (CBP), and the p300/CBP family proteins contain several identical domains necessary for their function as HATs, which include three cysteine-histidine rich domains (CH1, CH2, CH3), a KIX domain, a bromodomain, and a HAT domain. (Figure 4A). p300 directly binds to and acetylates three specific lysine residues, Lys-264, Lys-266, and Lys-273, of the C-terminal region of HIV-1 IN (Figure 4C), which is likely to enhance the binding affinity to LTR DNA as well as strand transfer activity of IN *in vitro* (Cereseto et al., 2005). Importantly, an HIV-1 strain



that contained arginine substitutions at the critical C-terminal lysine residues on IN exhibited a significant replication defect in CD4⁺ cells, and the replication defect occurred at the integration step, indicating the importance of C-terminal acetylation of IN in HIV-1 integration (Cereseto et al., 2005). Another HAT, GCN5 (Figure 4A), was also found to mediate the acetylation

of HIV-1 IN at the same C-terminal lysines, and HIV-1 integration was shown to be impaired in GCN5 knockdown cells (Terreni et al., 2010).

In the case of histones, acetylation of the internal lysines affects several aspects of nucleosome function and generates new protein-protein interactions. However, it is a reversible process, and transcriptional activation of the nucleosome is repressed by deacetylation mechanisms, which are mediated by histone deacetylases (HDAC; Shahbazian and Grunstein, 2007). It is likely that deacetylation of HIV-1 IN also takes place. To find a cellular partner that selectively recognizes the acetylated IN, Cereseto and colleagues developed a unique yeast two-hybrid screening assay, in which acetylated HIV-1 IN was constitutively expressed as the bait by fusion of the p300 HAT domain to IN (Allouch et al., 2011). From the screening of a human lymphocyte cDNA library, they identified that a cellular protein, KAP1, was able to interact with acetylated IN (Figure 4B; Allouch et al., 2011). KAP1 was first identified as a transcriptional corepressor that is recruited to its target genes via interaction with the Kruppel-associated box-domain-containing (KRAB) zinc-finger DNA proteins (Friedman et al., 1996; Peng et al., 2000). Interestingly, KAP1 is a member of the human TRIM family, and hence contains a RBCC (Ring [really interesting new gene] finger, two B-box zinc fingers and a coiled coil) domain at the N-terminal, which is a characteristic feature of the TRIM family protein domains (Figure 4B; Iyengar and Farnham, 2011). Additionally, KAP1, also called TRIM28 and transcription intermediary factor (TIF) 1 β , is composed of a plant homeodomain (PHD) and bromodomain at the C-terminus, and contains a central TIF1 signature sequence (TSS) domain and a heterochromatin protein 1 (HP1)-binding domain, which are found in other TIF1 subfamily members (Iyengar and Farnham, 2011). In accordance with the yeast two-hybrid screen, preferential binding of KAP1 to acetylated HIV-1 IN was confirmed by *in vitro* and *in vivo* analysis. Importantly, knockdown of KAP1 increased the level of integration in HIV-1-infected cells, which was accompanied by a decrease in 2-LTR circles (Allouch et al., 2011). When the infection experiments were performed with HIV-1 carrying mutations in the C-terminal lysines (Lys-264, Lys-266, Lys-273) of IN, knockdown of KAP1 did not significantly increase the levels of integrated DNA, indicating that KAP1-mediated inhibition was related to the C-terminal acetylation of IN. Consistent with this, the level of acetylated IN was shown to be decreased by expression of KAP1 in cells (Allouch et al., 2011). KAP1 has been reported to form complexes with HDAC to regulate transcription at specific target sites on the genome via modification of histone structure (Iyengar and Farnham, 2011). Together with the observation that KAP1 has a higher affinity to acetylated IN, the evidence raises a possibility that KAP1 may serve as a scaffolding mediator that recruits HDAC to acetylated HIV-1 IN (Figure 4C). Indeed, HIV-1 IN formed a complex with KAP1 and HDAC1, and HIV-1 integration was enhanced by treatment with the HDAC inhibitors (Allouch et al., 2011). These evidences therefore support the proposed model that KAP1 recruits the HDAC to acetylated IN allowing for the HDAC1-mediated deacetylation of IN to take place, which results in the subsequent reduction of integration efficiency in HIV-1 infection (Figure 4C).

A different inhibitory mechanism of KAP1 has been also demonstrated in other retroviruses and endogenous retroviruses, which involves the transcriptional repression of the provirus in embryonic carcinoma and embryonic stem cells (Wolf and Goff, 2007, 2009; Wolf et al., 2008; Matsui et al., 2010; Rowe et al., 2010). The KAP1-mediated inhibition of viral transcription appeared to be largely attributable to the recruitment of KAP1 to certain types of primer binding sites (PBS) near the 5' end of the provirus containing PBS sequences complementary to tRNA^{Pro} (MoMLV and human T-lymphotropic virus type I [HTLV-1]) and tRNA^{Lys-1,2} (spumavirus and Mason–Pfeizer monkey virus; Wolf et al., 2008; Wolf and Goff, 2009). In contrast, HIV-1 is not susceptible to the transcriptional silencing induced by KAP1 as its PBS is complementary to neither tRNA^{Pro} nor tRNA^{Lys-1,2} (Wolf et al., 2008). Since the study by Cereset and colleagues showed that MoMLV integration was not affected by depletion of KAP1 (Allouch et al., 2011), the KAP1-mediated retroviral integration block should only occur in HIV-1 replication due to the specific interaction between KAP1 and HIV-1 IN. Therefore, it can be concluded that KAP1 is an intrinsic restriction factor against a broad range of retroviruses, which inhibits either the integration or post-integration step. However, despite the ubiquitous expression of KAP1 in many cell types, substantial levels of HIV-1 integration can still be detected, indicating KAP1 may not be a very powerful integration inhibitor. Yet, it would shed light on a potentially novel KAP1-based strategy for the treatment of retrovirus infections.

REGULATION OF INTEGRASE STABILITY BY PHOSPHORYLATION: IMPLICATION OF UNKNOWN CELLULAR INHIBITORY FACTOR INVOLVED IN DEGRADATION OF UNPHOSPHORYLATED INTEGRASE

Phosphorylation is another type of PTM that is also likely to influence in HIV-1 integration. Recently, phosphorylation of HIV-1 IN by c-Jun NH₂-terminal kinase (JNK) was reported (Manganaro et al., 2010). JNK is one of the major groups of mitogen-activated protein kinases (MAPKs), a family of well-studied serine/threonine kinases involved in signal transduction from extracellular stimuli such as growth factors, cytokines, infection, and stress (Raman et al., 2007; Cargnello and Roux, 2011). JNK consists of three variants, JNK1, JNK2, and JNK3, which are generated through alternative splicing but exhibit distinct expressions and functions (Sabapathy et al., 2004). The MAPK signaling pathway can also be activated by the interaction of T cell receptors with antigen-major histocompatibility complex (MHC) class II molecules, resulting in T cell activation (Weiss and Littman, 1994). In HIV infection, the requirement for the activation states of CD4⁺ T cells for efficient initiation and maintenance of replication has been well established (Stevenson et al., 1990; Pope and Haase, 2003; Stevenson, 2003; Chiu et al., 2005). In particular, it has been demonstrated that HIV-1 infection is restricted at the post-entry stages in resting T cells, following reverse transcription and prior to integration (Bukrinsky et al., 1992; Pierson et al., 2002; Coiras et al., 2009).

Manganaro et al. (2010) reported that although expression levels of JNK was low in the resting CD4⁺ T cells, it became activated upon stimulation, and that even in activated CD4⁺ T cells, HIV-1

infection was impaired by treatment with a specific JNK inhibitor, SP600125. Furthermore, decreased amounts of integrated DNA was detected in the SP600125-treated activated CD4⁺ T cells, indicating JNK contributes to the efficient infection and integration of HIV-1. Importantly, IN was found to be phosphorylated, and a conserved serine residue in the CCD, Ser-57, was identified as the target site of phosphorylation. Since the phosphorylated IN could not be detected in cells treated with SP600125, JNK appeared to be the kinase responsible for the phosphorylation of IN (Manganaro et al., 2010).

Next question to be asked was how the IN phosphorylation augmented the efficiency of HIV-1 integration. *In vitro* integration activity assays using recombinant IN showed that an alanine substitution at the Ser-57 residue did not affect the strand transfer activity of HIV-1 IN, suggesting that this serine residue itself is not involved in the enzymatic activity of IN (Manganaro et al., 2010). Meanwhile, Manganaro et al. (2010) found that the Ser-57 and a flanking Pro-58 are conserved amongst most of the HIV-1 clades and subtypes, and that the phosphorylated of the serine/proline motif can potentially be recognized by peptidyl-prolyl *cis-trans* isomerase (PPIase), Pin1 (Lu and Zhou, 2007). Pin1 is a regulator of phosphorylation signaling which specifically recognizes phosphorylated serine or threonine residues preceding a proline. Pin1 then catalyzes the structural rearrangement of a target molecule through *cis-trans* isomerization of its specific proline (Lu and Zhou, 2007). Pin1 binds wild-type HIV-1 IN, but not the IN mutant with a single amino acid mutant on the phosphorylation site (S57A). As expected, treatment of IN-expressing cells with a Pin1 inhibitor, Pib, decreased the stability of HIV-1 IN. Moreover, *in vitro* incubation of recombinant IN with Pin1 increased IN resistance against a protease, subtilisin, suggesting that Pin1 induced a conformational change in phosphorylated IN, thereby rendering it less sensitive to protein degradation (Manganaro et al., 2010). Supporting this notion, treatment of cells with a Pin1 inhibitor or the depletion of endogenous Pin1 by siRNA reduced the level of integrated DNA in HIV-1-infected cells without any impairment of the reverse transcription step (Manganaro et al., 2010). More important, whereas HIV-1 carrying the S57A IN mutant exhibited decreased integration activity, treatment with the proteasome inhibitor MG132 restored integration activity (Manganaro et al., 2010), demonstrating that JNK-mediated phosphorylation of HIV-1 IN at Ser-57 and subsequent prolyl *cis-trans* isomerization by Pin1 increased the stability of IN and thereby enhanced integration efficiency in infected CD4⁺ T cells. Taken together, resting CD4⁺ T cells could have lower expression/activation levels of JNK, leading to decreased IN phosphorylation and consequently decreased IN stability, resulting in inefficient HIV-1 replication. Also, one could speculate that extracellular stimulations, including cytokines and chemokines, may enhance integration in resting T cells via activation of the JNK pathway (Cameron et al., 2010; Guo et al., 2011).

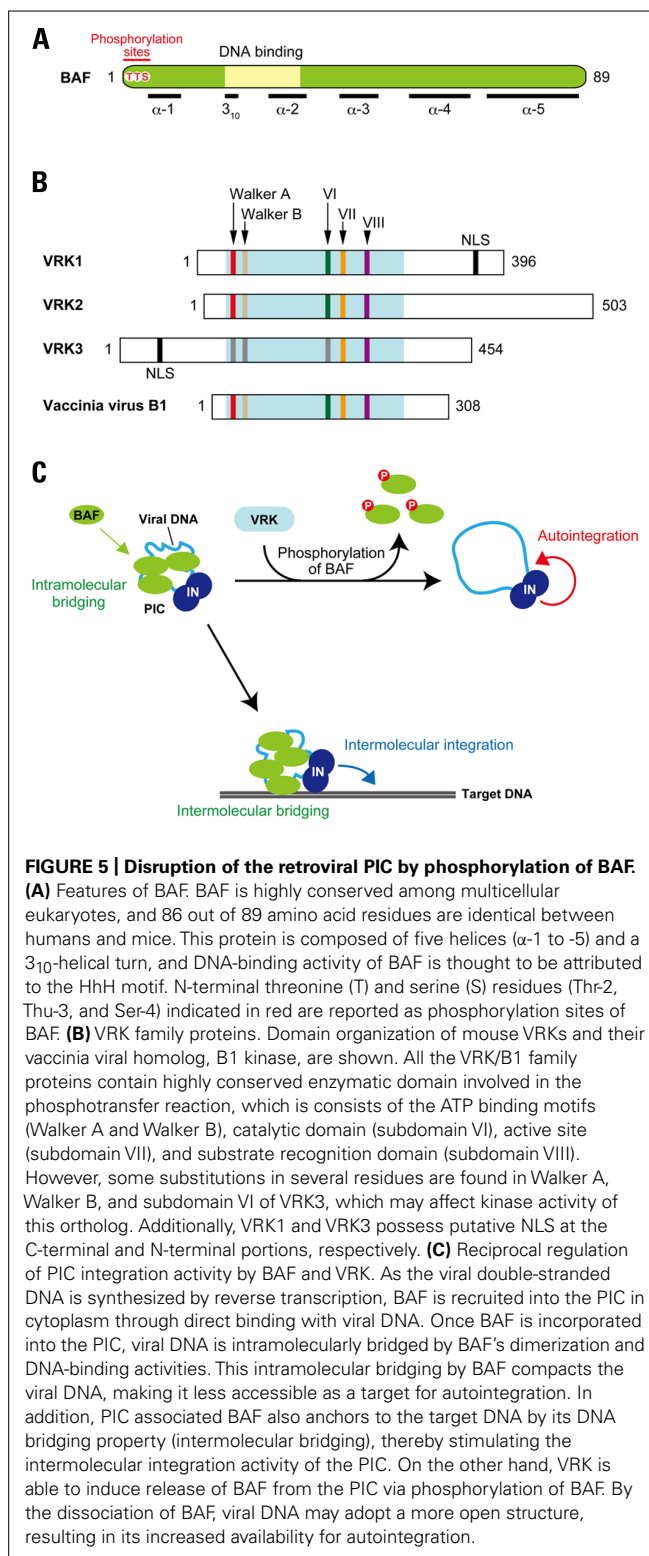
Of particular interest is that the study by Manganaro et al. (2010) also implies yet-to-be identified host factor(s) participating the degradation of IN, particularly unphosphorylated form of IN, in the resting T cells. HIV-1 IN is well known to undergo proteasome-mediated degradation via ubiquitination (Devroe et al., 2003). A previous study has reported that the von

Hippel–Lindau binding protein 1 (VBP1) and cullin2-based von Hippel–Lindau (Cul2/VHL) ubiquitin ligase cooperate to mediate the polyubiquitination and subsequent proteasomal degradation of IN, but this event was likely to occur at a post-integration step and also to promote gene expression from the integrated provirus in the nucleus (Mousnier et al., 2007). Therefore, further work will be required to identify novel ubiquitin–proteasome proteolytic pathways that drive the cytoplasmic degradation of HIV-1 IN in a manner that restricts integration.

VACCINIA-RELATED KINASES

Integration reactions mediated by PICs can be categorized into two pathways: (i) intermolecular integration into a target DNA and (ii) intramolecular integration into the viral DNA, which is also called autointegration (Lee and Craigie, 1994). In order to establish productive infection, the retrovirus (or retroviral PIC) needs to avoid the suicidal autointegration pathway. Hence, a strong preference for intermolecular integration is one of the key features of the retroviral PIC (Lee and Craigie, 1994). BAF was identified as a host factor responsible for blocking the autointegration of MoMLV PICs (Lee and Craigie, 1998). BAF is a small dimeric protein (Margalit et al., 2007) and shown as a cellular component of HIV-1 and MoMLV PICs (Suzuki and Craigie, 2002; Lin and Engelman, 2003). Crystal structure analysis has revealed that BAF binds double-stranded DNA through its helix-hairpin-helix (HhH) motif (Bradley et al., 2005; **Figure 5A**). Importantly, the dimerization of BAF appears to cross-bridge DNA, thereby preventing the autointegration of retroviral PICs by compacting viral DNA into rigid structure (Lee and Craigie, 1998; Suzuki and Craigie, 2002; Bradley et al., 2005).

Barrier-to-autointegration factor is involved in the organization of nuclear architecture through interaction with lamin A and lamina-associated polypeptide 2-emerin-MAN1 (LEM) family proteins, and therefore predominantly localizes in the nucleus during interphase (Margalit et al., 2007). On the other hand, in the mitotic phase, BAF is diffusely localized and subsequently assembles on the chromatin core region at anaphase to form a scaffold for the gathering of lamin A and LEM proteins to reconstitute the nuclear envelope (Segura-Totten and Wilson, 2004; Margalit et al., 2007). Intriguingly, these cell cycle-dependent actions of BAF appear to be regulated by the phosphorylation (Margalit et al., 2007). So far, vaccinia-related kinases (VRKs) have been identified as the cellular kinases that participate in the BAF phosphorylation (**Figure 5B**; Nichols et al., 2006). VRKs are a family of serine/threonine kinases, and three alternative splicing isoforms, VRK1, VRK2, and VRK3, are found in human and murine cells (Nichols et al., 2006; Klerkx et al., 2009). They share several domains comprised of ATP-binding motifs (Walker A and Walker B), catalytic domains (subdomain VI), active sites (subdomain VII), and substrate recognition domains (subdomain VIII; **Figure 5B**; Boyle and Traktman, 2004). VRK was first identified as cellular homolog of the poxvirus (vaccinia virus) B1 kinase that plays an essential role in viral DNA replication in the early stage of vaccinia virus infections (**Figure 5B**; Nichols et al., 2006). Importantly, VRK-mediated phosphorylation of BAF causes the loss of its DNA-binding ability (Nichols et al., 2006). Depletion of VRK1 in *Caenorhabditis elegans* results in several mitotic



defects and BAF delocalization (Gorjanacz et al., 2007), suggesting that the release of BAF from chromatin during mitosis is regulated by the VRK family proteins in a phosphorylation-dependent manner.

Since the DNA-binding property of BAF is crucial to the authentic integration activity of the retroviral PIC, down-modulation of the BAF activity by phosphorylation may disrupt the integration function of the PIC through dissociation of BAF from viral DNA. Indeed, our recent study revealed that BAF could be removed from the PIC following treatment with recombinant VRK1 *in vitro* (Suzuki et al., 2010). More important, the intermolecular integration activity of the PIC assay was strongly inhibited by the VRK1 treatment, and this was accompanied by an increase in autointegration activity (Lee and Craigie, 1994; Suzuki et al., 2010). Little inhibition of the PIC activity was observed in following treatment with mutant VRK1 harboring alanine substitutions in the catalytic subdomain VI, and also in PICs treated with wild-type VRK1 in the presence of a well-known kinase inhibitor, staurosporine, indicating that disruption of PIC function is attributed to the kinase activity of VRK1 (Suzuki et al., 2010). Based on these findings, a potential inhibitory mechanism by which VRK1 restricts PIC integration activity can be proposed: phosphorylation by VRK1 dissociates BAF from the PIC, resulting in destructive autointegration of viral DNA (Figure 5C).

When other members of the VRK family of proteins were assessed for their ability to inhibit the intermolecular integration activity of PICs, VRK2, but not VRK3, was found to abrogate the function of PICs in a similar manner with VRK1 (Suzuki et al., 2010). This raised the question of why VRK3 was unable to inhibit PIC activity. Phosphorylation of BAF exhibits biphasic phases (hypophosphorylation and hyperphosphorylation), with the phosphorylation taking place on the Thr-2, Thr-3, and Ser-4 located on the N-terminus of BAF (Figure 5A). VRK1 and VRK2 are able to catalyze the hyperphosphorylation (Nichols et al., 2006). However, the BAF product phosphorylated by VRK3 is the hypophosphorylated form, and an *in vitro* kinase assay

using BAF mutants, in which Thr-2, Thr-3, and/or Ser-4 had been substituted with alanine, revealed that Ser-4 is the sole site for the VRK3-mediated hypophosphorylation (Suzuki et al., 2010). Ser-4 has been also indicated as initial phosphorylation site by VRK1 and VRK2 (Nichols et al., 2006) and our preliminary results of an *in vitro* kinase assay using BAF mutants showed that subsequent phosphorylation likely takes place on Thr-3 (Suzuki and Suzuki, unpublished data). These results suggest that Ser-4 is the primary target of all VRK family proteins, but that Thr-3 is an additional preferred target of VRK1 and VRK2. The hyperphosphorylation of BAF at both the N-terminal Thr-3 and Ser-4 should therefore be a prerequisite for the functional disruption of PIC function. Although the findings of our *in vitro* studies propose that VRK is a cellular kinase harboring potentially restrictive activity against retroviral integration, delineating the precise role of the VRK family of proteins in retrovirus infection and integration *in vivo* requires further experimentation.

CONCLUSION

As discussed in this review, the processes leading up to integration are assisted by a number of host proteins. Furthermore, it has become evident that there are some cellular proteins that act as potential restriction factors. Research into intracellular effectors and inhibitors of retroviral integration therefore provides an important insight for the development of novel classes of anti-HIV drugs that can act synergistically with existing drugs targeting the active sites of viral enzymes or the binding pockets of cellular receptors.

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Role of nucleocytoplasmic RNA transport during the life cycle of retroviruses

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Retroviruses have evolved mechanisms for transporting their intron-containing RNAs (including genomic and messenger RNAs, which encode virion components) from the nucleus to the cytoplasm of the infected cell. Human retroviruses, such as human immunodeficiency virus (HIV) and human T cell leukemia virus type 1 (HTLV-1), encode the regulatory proteins Rev and Rex, which form a bridge between the viral RNA and the export receptor CRM1. Recent studies show that these transport systems are not only involved in RNA export, but also in the encapsidation of genomic RNA; furthermore, they influence subsequent events in the cytoplasm, including the translation of the cognate mRNA, transport of Gag proteins to the plasma membrane, and the formation of virus particles. Moreover, the mode of interaction between the viral and cellular RNA transport machinery underlies the species-specific propagation of HIV-1 and HTLV-1, forming the basis for constructing animal models of infection. This review article discusses recent progress regarding these issues.

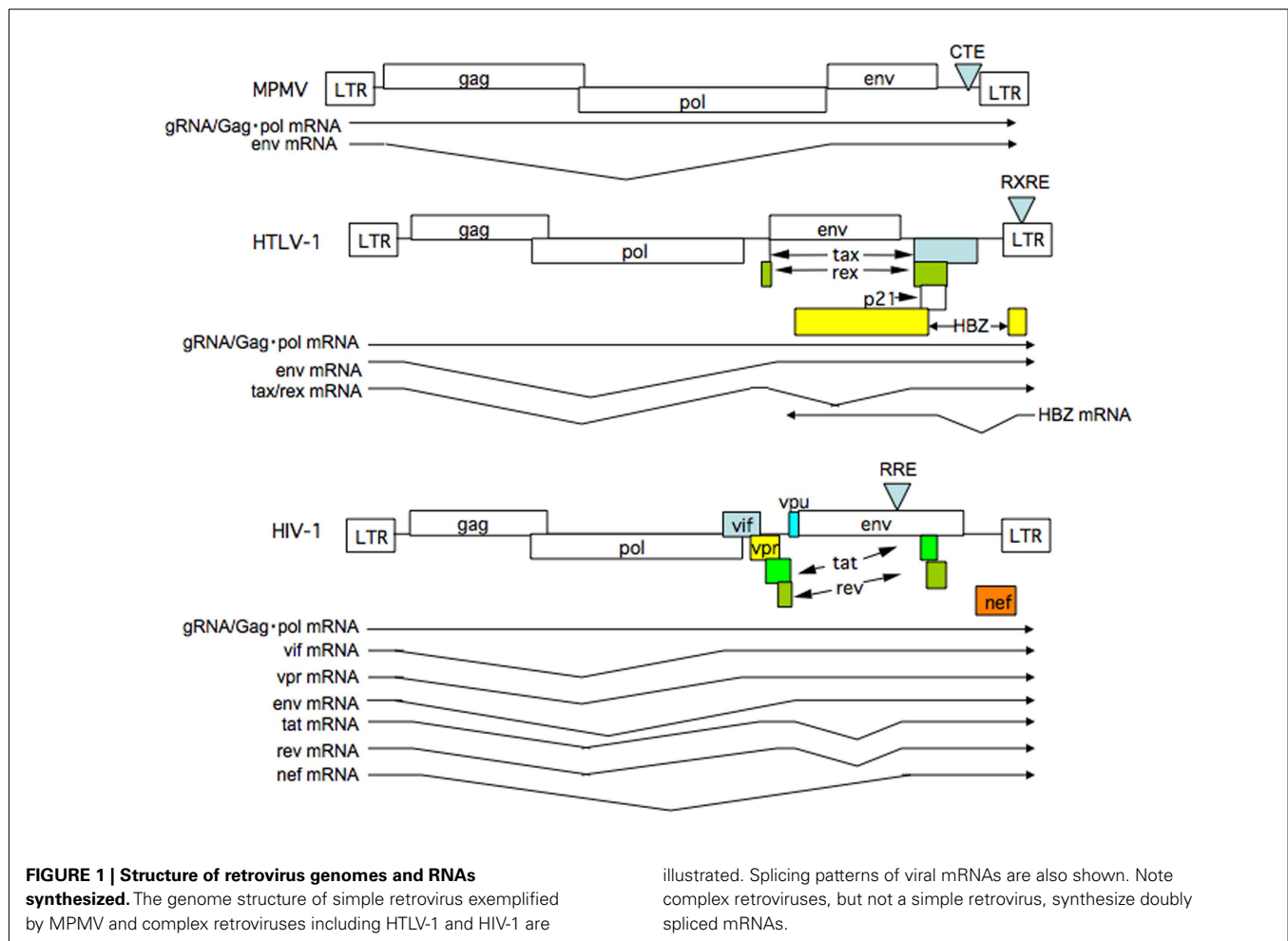
Keywords: RNA export, HTLV-1, HIV-1, infection rat models for, CRM1, simple retrovirus, species-specific barrier, Rex/Rev

Almost all eukaryote cellular messenger RNAs (mRNAs) undergo splicing to remove introns, and only fully spliced mRNAs are able to reach the cytoplasm (Legrain and Rosbash, 1989; Fischer et al., 1994). The nuclear retention of unspliced, or incompletely spliced, mRNAs is strictly regulated because incompletely spliced mRNAs yield proteins that are non-functional, or even deleterious to the cell. However, the expression of retroviral genes is a notable exception. Retroviruses are subdivided into two categories, simple and complex, according to their genome structure (Goff, 2001). Simple retroviruses encode mainly structural genes, whereas complex retroviruses harbor several accessory genes that play important roles in their replication and pathogenesis. All retroviruses share a common genome structure comprising long terminal repeat (LTR) sequences at either end and centrally located gag, pol, and env genes, which encode the component proteins of the virions (Figure 1). All except foamy viruses (Yu et al., 1996) utilize the 5'-LTR as the sole promoter, resulting in the synthesis of a single full-length viral RNA as the primary transcript. Since translation preferentially starts at the first AUG codon (methionine) in eukaryotes (Kozak, 1992), viruses must position the initiation codons for all their genes (which are located within the primary transcript) close enough to the 5'-cap to ensure appropriate and efficient translation. Retroviruses utilize alternative splicing of the full-length transcript to produce the appropriate mRNAs. This means that the introns of the full-length primary transcript encode the gag gene, for instance. The mRNA must then be exported and translated in the cytoplasm. In addition, viral genomic RNAs (gRNAs), which are apparently indistinguishable to gag mRNA, must be exported to the cytoplasm for viral replication. Therefore, retroviruses have evolved the means to circumvent cellular restrictions on the export of their intron-containing RNAs. Studies

regarding this issue have not only shed light on the mechanisms underlying viral replication, but have also led to the discovery of the nucleocytoplasmic transport systems in higher eukaryotes. Moreover, recent findings indicate that the correct mode of nucleocytoplasmic transport of viral RNA and proteins forms the basis of subsequent cytoplasmic events involved in efficient viral replication and, in some cases, forms the basis for species-specific propagation.

TRANSPORT OF HTLV-1 RNA

Human T cell leukemia virus type 1 (HTLV-1) is the causative agent of human adult T cell leukemia (ATL), a chronic progressive neurological disorder termed HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP; Hinuma et al., 1981; Kalyanaraman et al., 1982; Osame et al., 1986; Jacobson et al., 1988), and other diseases. HTLV-1 encodes an unspliced, intron-containing 9 kb RNA, which encodes Gag and Gag-Pol, a singly spliced 4 kb mRNA encoding Env, and multiply spliced, intron-free 2 kb mRNAs encoding Tax and Rex (Green and Chen, 2001). Recently, the HTLV-1 leucine zipper factor (HBZ) gene was identified, which is encoded by a complementary strand of the HTLV-1 genome, driven by a promoter residing in the 3' LTR (Figure 1). Tax and HBZ are implicated in oncogenesis (Satou et al., 2006; Matsuoka and Jeang, 2011). Historically, Rex was the first protein to be identified as a factor that induces the cytoplasmic expression of intron-containing 9 and 4 kb RNAs (Inoue et al., 1986; Hidaka et al., 1988). Rex binds directly to viral RNAs containing Rex response element (RXRE) located in the 3' LTR. RXRE, which consists of a highly ordered stem-and-loop structure, functions as a Rex binding site (Ballaun et al., 1991; Bogerd et al., 1991). Since all HTLV-1 RNAs contain RXRE, Rex also enhances transport of

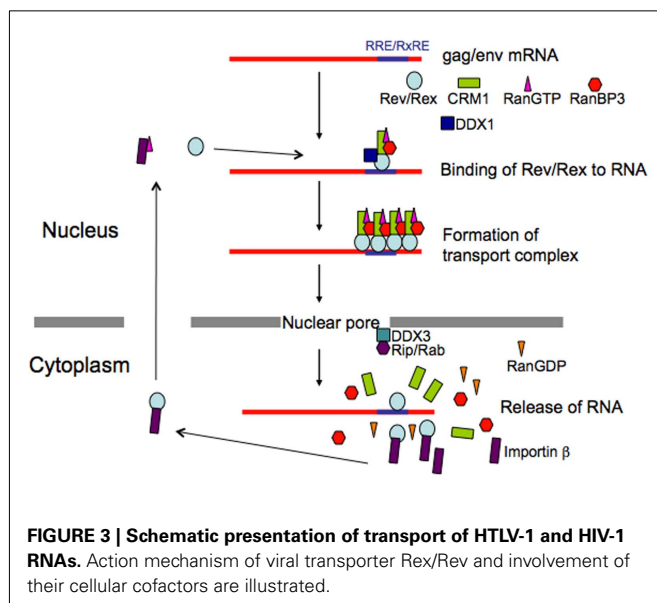
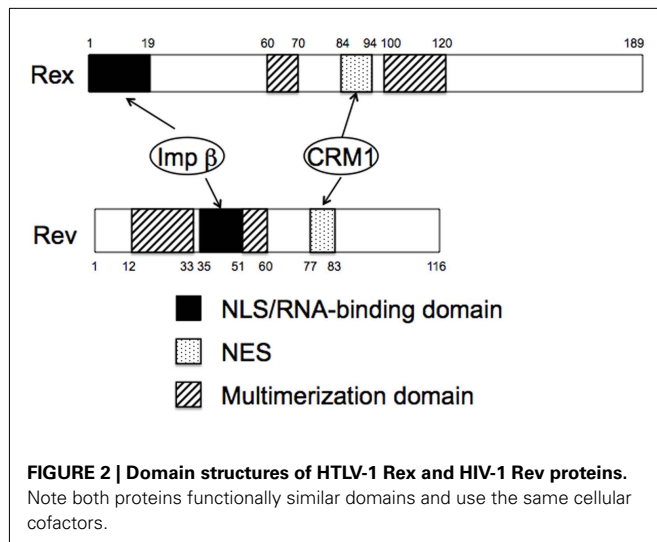


fully spliced 2 kb mRNA, although this RNA can be transported via the normal route for cellular mRNAs that do not require Rex (Bai et al., 2012). The sequence elements that retain HTLV-1 unspliced RNAs in the nucleus have been identified in the pol-env and LTR regions of the HTLV-1 genome (Saiga et al., 1997; King et al., 1998).

An important property of Rex, which is essential for its role, is its ability to dynamically shuttle between the cytoplasm and nucleus, although Rex is predominantly localized in the nucleus and nucleolus (Bogerd et al., 1996; Kim et al., 1996; Siomi et al., 1988). Rex is synthesized in the cytoplasm and then imported into the nucleus. In the nucleus, Rex directly binds to RXRE, after which it escorts the viral RNAs into the cytoplasm (Bogerd et al., 1991). Finally, Rex is released from its cognate RNAs for recycling. Extensive mutational analyses show that Rex contains functional domains that are essential for its function (Figure 2). The basic domain is rich in arginine residues and maps between amino acids (aa) 1–19 in the 189 aa Rex protein. This domain serves as a nuclear/nucleolar localization signal (NLS), and foreign proteins containing these residues are localized to the nucleus/nucleolus (Siomi et al., 1988). Importantly, the same region mediates binding to RXRE, thereby determining its specific target RNA (Bogerd et al., 1991). The second essential domain, which maps between aa 84 and 94 of Rex, is a leucine-rich activation domain that functions as a nuclear export

signal (NES; Bogerd et al., 1996). The third important property of Rex is multimerization. The domain responsible for this was first ascribed to a 60–70 aa region of Rex (Weichselbraun et al., 1992); however, subsequent *in vivo* two-hybrid assays revealed that a 100–120 aa region was also involved in multimerization (Heger et al., 1998). Multimerization of Rex on its cognate RNA is generally thought to be critical for its ability to export viral RNAs, although it is not required for export of the Rex protein itself (Heger et al., 1998). Although shuttling proteins have now been identified in a wide range of other viral and cellular proteins, multimerization may distinguish Rex from other shuttling proteins that are not involved in RNA export since their multimerization is not necessarily required for their functioning (Weichselbraun et al., 1992; Bogerd and Greene, 1993; Heger et al., 1998).

A detailed understanding of Rex shuttling was obtained from studies that identified the cellular factors binding to the functional domains, and the cellular machinery involved in protein transport into and out of the nucleus (Figure 3). Transport of all macromolecules occurs via nuclear pore complexes (NPCs), which comprise at least 50 different proteins, termed nucleoporins (Doye and Hurt, 1997). The import of proteins containing an NLS is mediated by the importin family of import receptors, which have affinity for nucleoporins, and the GTP-bound status of Ran (RanGTP; Mattaj



and Englmeier, 1998). Rex directly binds to importin β through its NLS (Palmeri and Malim, 1999). Translocation through the pore is thought to be facilitated by sequential direct interactions between importin β and various nucleoporins. Ran is a small GTPase that can exist in either the GTP or GDP-bound state. RanGTP reflects the nuclear localization of chromatin binding protein, RCC1, which specifically catalyzes the exchange of guanine nucleotides on Ran, and RanGDP, the predominant form of Ran in the cytoplasm, reflects the cytoplasmic location of a GTPase-activating protein, RanGAP. One role of RanGTP is to promote cargo release in the nucleus by dissociating the imported receptor–cargo complexes; RanGDP has no affinity for importin β (Mattaj and Englmeier, 1998). Since the NLS of Rex overlaps with its RNA binding domains, binding to importin β in the cytoplasm may facilitate the release of viral RNAs from Rex (Bogerd et al., 1991; Siomi et al., 1988; Palmeri and Malim, 1999).

The cellular cofactor that interacts with the NES of Rex is human (h)CRM1. CRM1 was originally identified as binding the NES of the human immunodeficiency virus (HIV)-1 Rev protein. Sequence similarity to importin β suggested that hCRM1 was an export receptor (Fornerod et al., 1997a; Fukuda et al., 1997). Subsequently, hCRM1 was shown to form a specific complex with NES; however, unlike the Rex–importin β complex, this only occurred in the presence of RanGTP (Fornerod et al., 1997a). The association between Rex and hCRM1 was confirmed *in vivo* using a two-hybrid assay (Hakata et al., 1998). Moreover, CRM1 directly binds to Ran-binding protein 3 (RanBP3), which binds to RCC1 in a Ran-dependent manner and increases the nucleotide exchange activity of RCC1, resulting in high local concentrations of RanGTP. Consequently, RanBP3 acts as a scaffold protein through which the components of the export complex are concentrated around the RCC1 site, thereby promoting complex assembly. RanBP3 continues to interact with hCRM1 to stabilize the hCRM1–substrate–RanGTP complex, thereby forming a quaternary complex (Englmeier et al., 2001; Nemergut et al., 2002). Since CRM1 associates with various nucleoporins (Fornerod et al., 1997b), the Rex–hCRM1–RanGTP–RanBP3 complex is translocated to the cytoplasm across the NPC; this occurs via possible hydrophobic interactions between hCRM1 and nucleoporins. Following this, the RanGTP within the complex is converted to RanGDP by RanGAP and RanBP1 (and probably RanBP2), leading to the dissociation of the complex and release of the substrate into the cytoplasm (Askjaer et al., 1999; Kehlenbach et al., 1999).

An important question is whether cellular cofactors, such as hCRM1, are involved in the multimerization of Rex proteins (Hakata et al., 1998). Therefore, we examined the multimerization of Rex proteins using a two-hybrid assay. First, we found that a Rex mutant, RexM90 (which harbors a mutation in the NES), lost the ability to bind to hCRM1 and to form multimers. Second, we found that the RNA transport activity of RexM64, which harbors a mutation in the multimerization domain, was partially restored by the overexpression of hCRM1, and this coincided with the partial restoration of multimerization. Third, a dominant-negative (DN) mutant of Rex, termed TAGRexM64, which sequesters Rex cofactors (Katahira et al., 1995), abrogated the multimerization and activity of the wild-type Rex protein. Both functions were simultaneously restored by the overexpression of hCRM1. Taken together, these results suggest that the multimerization of Rex *in vivo* requires both the intrinsic ability of Rex to form multimers, and the hCRM1 protein. Moreover, these results also suggest that the multimerization of Rex is not a prerequisite for interaction with hCRM1 but, rather, that Rex initially interacts with hCRM1, leading to the multimerization of Rex proteins in a step that is favored by its intrinsic capacity to form oligomers on RXRE. This, in turn, would favor a structure that facilitates interactions with other hCRM1 molecules on the RNA (Hakata et al., 1998). Comparison of human and rat CRM1 (rCRM1) mapped the CRM1 domain that induces Rex multimerization to the central region, particularly a region containing two residues (aa 411 and 414; Hakata et al., 2003); this domain is different from the region spanning aa 566–720 that is involved in binding to the NES (Ossareh-Nazari and Dargemont, 1999). These data suggest that CRM1 not only functions as an export receptor

but also participates in the formation of the RNA export complex through a high-order interaction with Rex. Curiously, the CRM1 region responsible for the interaction with RanBP3, which comprises four residues (aa 411, 414, 478, and 484), and the region responsible for Rex multimerization form an overlapping domain (Hakata et al., 2003).

RNA EXPORT STEP AS A MAJOR SPECIES-SPECIFIC BARRIER FOR HTLV-1

Appropriate animal models of HTLV-1 infection would allow us to analyze the pathogenesis and oncogenesis of HTLV-1-associated diseases, which could lead to the development of therapeutic and preventative measures. HTLV-1 is able to infect experimental animals such as monkeys, rabbits, and rats (Akagi et al., 1985; Nakamura et al., 1987; Oka et al., 1992). The utility of monkey and rabbit models is limited, however, because of the difficulties in breeding a large number of these animals and the lack of inbred strains. Consequently, it would be more convenient to use small animal models. In particular, rat models have been extensively developed to study HTLV-1-associated diseases (Ishiguro et al., 1992; Ohashi et al., 2000). However, although the current rat models have certainly allowed us to understand HTLV-1-associated diseases better, they are still incomplete because of the poor replication of HTLV-1 in rats.

Human T cell leukemia virus type 1 can infect a number of rat cell types, which indicates that rat cells possess the receptors for viral attachment and penetration (Li et al., 1996; Sutton and Littman, 1996). Thus, the blockade of viral propagation within the rat cells must occur during subsequent steps. Identification of the blocking step and the responsible host factor(s) could lead to the construction of transgenic rats that express the critical human factor(s), which are highly susceptible to HTLV-1 infection.

The first hint of a blocking step came in a report showing that the viral mRNAs encoding Gag and Env proteins are produced only at low levels in rat cells despite the fact that the mRNA for Tax/Rex protein is abundant (Koya et al., 1999). This observation led us to hypothesize that Rex may function poorly in rat cells. Indeed, we found that the activity of Rex in rat cells is quite low compared with that in human cells. As the functions of Rex depend largely on the CRM1 protein, we examined whether rCRM1 can act as a cofactor for Rex activity as it does in human cells, although CRM1 is highly conserved from yeast to mammals, and hCRM1 can function in yeast cells as an export receptor. Actually, only 24 out of 1027 amino acids are different between rat and human CRM1 (Hakata et al., 2003). Also, we found that both rCRM1 and hCRM1 could bind to and export the Rex protein to the cytoplasm with similar efficiency. However, unlike hCRM1, rCRM1 could not efficiently support Rex function because of its poor ability to induce the Rex–Rex interaction required for RNA export into the cytoplasm (Figure 4). These results underline the absolute requirement of hCRM1 for Rex multimerization. It was also concluded that the poor ability of rCRM1 to act as a cofactor for Rex is responsible for the poor replication of HTLV-1 in rats (Hakata et al., 2001). This notion was further supported by a study showing that a HTLV-1-transformed rat CD4⁺ T cell line, which was a poor producer of HTLV-1, efficiently synthesized functional Env and Gag proteins, which were then fully processed, and produced infectious HTLV-1

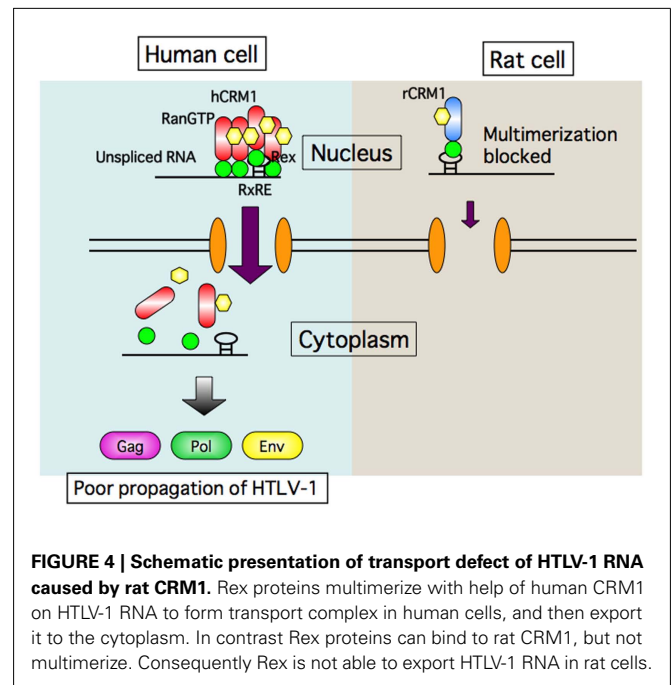


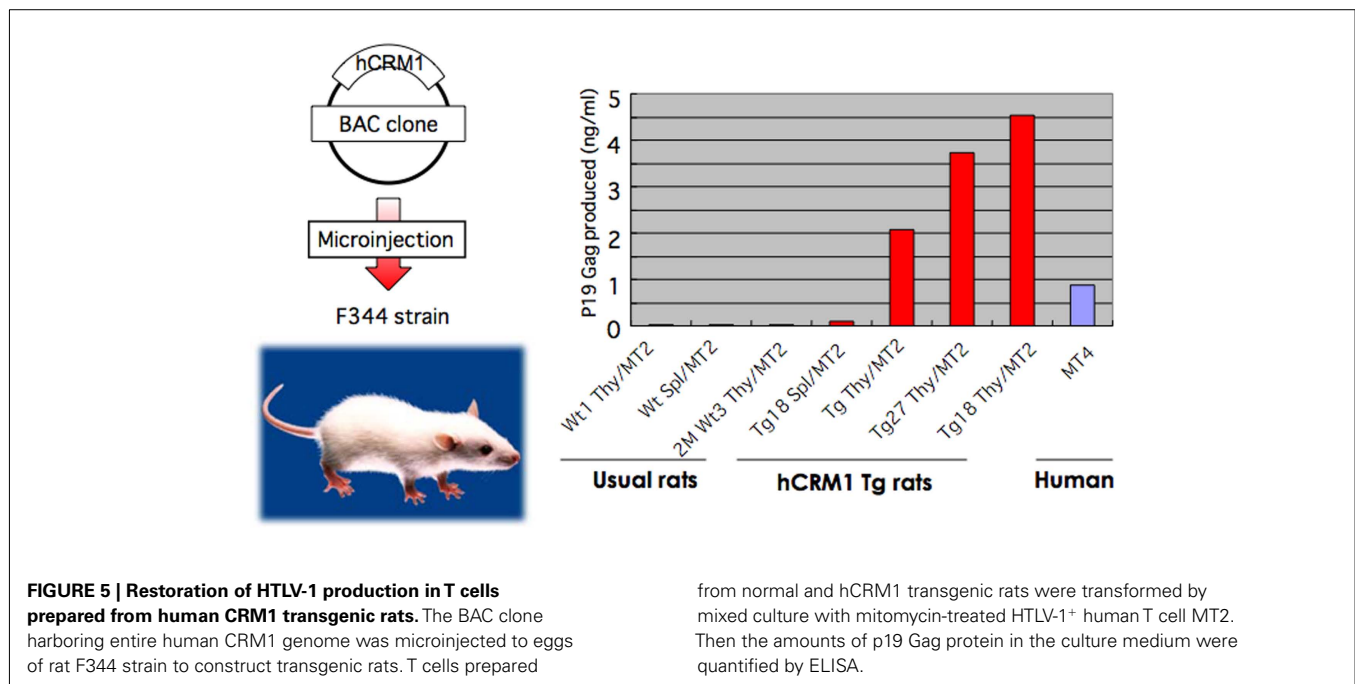
FIGURE 4 | Schematic presentation of transport defect of HTLV-1 RNA caused by rat CRM1. Rex proteins multimerize with help of human CRM1 on HTLV-1 RNA to form transport complex in human cells, and then export it to the cytoplasm. In contrast Rex proteins can bind to rat CRM1, but not multimerize. Consequently Rex is not able to export HTLV-1 RNA in rat cells.

progeny viruses at the level comparable to HTLV-1-transformed human T cells when it was transduced with a retroviral vector expressing hCRM1 at physiological levels (Zhang et al., 2006).

Transgenic (Tg) rats were constructed using an artificial bacterial chromosome clone containing the entire regulatory and coding regions of the CRM1 gene (Figure 5), since the expression of the CRM1 gene is elaborately regulated by a protein kinase C pathway during lymphocyte activation, initially in a post-transcriptional and subsequently in a transcriptional manner. Consequently, Tg rats expressed hCRM1 protein in a manner similar to that of intrinsic rCRM1 in various organs. HTLV-1-infected T cell lines derived from these Tg rats produced 100- to 10,000-fold more HTLV-1 than T cells from wild-type rats, and the absolute levels of HTLV-1 were similar to those produced by human T cells. We also observed increased dissemination of HTLV-1 into the thymuses of Tg rats after intraperitoneal inoculation, although the proviral loads were low in both wild-type and Tg rats (Takayanagi et al., 2007). Furthermore, rat regulatory T (Treg) cells were preferentially transformed by HTLV-1 (Takayanagi et al., 2009), as is the case for human Treg cells (Chen et al., 2006). Therefore, the Tg rat model forms the basis for an improved animal model for HTLV-1 infection.

RNA TRANSPORT OF HIV

Human immunodeficiency virus-1 belongs to the subfamily of complex retroviruses and, like HTLV-1, also encodes accessory genes besides gag and env; however, HIV-1 shows more complex patterns of mRNA expression. HIV-1 produces one unspliced 9 kb RNA encoding Gag and Gag–Pol, five singly spliced 4 kb mRNAs encoding Vif, Vpr, Vpu, and Env, and 16 multiply spliced 2 kb mRNAs encoding Tat, Rev, and Nef (Figure 1; Freed and Martin, 2001). As is the case for HTLV-1 Rex, the 9- and 4-kb viral RNAs of HIV-1 require the viral protein Rev for export (Cullen,



1991). However, expression of 2 kb RNAs is not augmented by Rev, since the Rev response element (RRE), an approximately 300 nucleotide region with a highly ordered stem-and-loop structure that functions as a Rev binding site, is located in the env gene rather than the 3' LTR (Malim et al., 1990; Mann et al., 1994). Consequently, during the early phase of infection when Rev expression is low, proteins encoded by the 2-kb class of mRNAs are expressed. During the late phase, when Rev is expressed in sufficient quantities, the remaining viral proteins are produced (Kim et al., 1989), including the virion constituents and infectivity enhancing and virion-releasing factors, all of which work toward the efficient production and dissemination of progeny virus.

Rev is a functional homolog of Rex, although Rev and Rex do not show any homology at the amino acid level. However, they do have similar domain structures and function in a similar manner (Figures 2 and 3), as exemplified by the fact that Rex can replace Rev (Ahmed et al., 1990). The basic domain rich in arginine residues, which maps between aa 35 and 50 in the 116 aa Rev protein, serves as an NLS, which recruits importin β and binds to the RRE (Daly et al., 1989; Malim et al., 1991; Tiley et al., 1992; Madore et al., 1994). Replacing the amino-terminal domain of the Rex protein with the RNA binding domain of the Rev protein yields a chimeric protein that can specifically interact with the RRE (Kubota et al., 1991). A leucine-rich activation domain, which maps between aa 73 and 83, acts as an NES that associates with CRM1 in the presence of RanGTP (Fisher et al., 1995; Wen et al., 1995). *In vivo* randomization selection experiments identified the consensus sequence, which contains relatively evenly spaced bulky hydrophobic residues (often leucine) that are crucial for NES function (Bogerd et al., 1996; Kim et al., 1996). *In vitro* studies of the multimerization of recombinant Rev proteins bound to the RRE-containing RNA defined two essential domains: one between aa 12 and 33 and one between aa 46 and 60 (Malim et al., 1991).

The C-terminal region of Rev is highly conserved between HIV-1 strains and supports efficient multimerization and subsequent Rev function, probably by stabilizing its conformation. In contrast to Rex, Rev is able to dimerize without CRM1 molecules *in vitro*, although *in vivo* two-hybrid assays suggest that CRM1 improves dimerization (Chaitanya and Belasco, 2001; Hakata et al., 2002; Matthew et al., 2008). Early studies suggested that Rev binds initially as a monomer to a single high-affinity Rev binding site within the RRE (Battiste et al., 1996), but recent studies on the tertiary structure of Rev indicate that the dimerization of Rev induces conformational changes in the position of the arginine-rich domain to facilitate RNA binding (Daugherty et al., 2010). RNA bound to the RRE augments further Rev multimerization, recruiting up to eight Rev molecules on the viral RNA, which subsequently recruit several hCRM1 molecules (Mann et al., 1994; Chaitanya and Belasco, 2001). Thus, multimerization, RNA binding to Rev, and Rev-CRM1 interactions are highly cooperative processes. The process is further accelerated by the DEAD (D-E-A-D = Asp-Glu-Ala-Asp) box helicase, DDX1, which is an RNA-dependent ATPase that binds directly to Rev. Knockdown of DDX1 greatly reduces Rev function, supporting the important role of DDX1 (Fang et al., 2004; Robertson-Anderson et al., 2011; Edgcomb et al., 2012). After the transport complex is formed, which comprises HIV-1 RNA, Rev, CRM1, and other cellular components, it moves to the nuclear pores, possibly via the actin filament network (Kimura et al., 2000). Multiple CRM1 molecules, which have affinity for nucleoporins, may be required to pass through the nuclear pore because the transport complex could be longer than the depth of the nuclear pore; thus, the rear portion of the complex is still within the nucleus when the front portion reaches the cytoplasm. CRM1 molecules associated with Rev within the front portion of the ribonucleoprotein complex (in the cytoplasm) will dissociate due to the lack of RanGTP (Fornerod et al., 1997a). Thus, additional

CRM1 molecules may be required to associate with the rear part of the RNA complex to enable passage of the whole RNA complex out of the nucleus. Another DEAD box protein, DDX3, which is located outside of the nuclear pore, associates with CRM1 directly and facilitates the passage of the complex through the nuclear pore and the release of RNA into the cytoplasm by inducing conformational changes in the ribonucleoprotein complex (Yedavalli et al., 2004). Additionally, Rip (also named Rab), which indirectly binds to Rev and has affinity for nuclear pores, is thought to function in releasing the viral RNA within the cytoplasm (Sanchez-Velar et al., 2004; Yu et al., 2005).

Suboptimal viral RNA splicing is also important for the function of Rev. Both inefficient branch point region and polypyrimidine tract are thought to be responsible for the low splicing efficiency of the HIV-1 *tat/rev* intron, which comprises most of the HIV-1 *env*-coding sequences (Staffa and Cochrane, 1994). Two additional elements, an exon-splicing enhancer (ESE) and an exon-splicing silencer (ESS), both of which modulate the overall efficiency of the 3' *tat-rev* splice site, were identified within the 3' terminal exon (Staffa and Cochrane, 1995). It has been proposed that inefficient splicing causes the retention of unspliced, or partially spliced, mRNAs since incomplete spliceosome formation at the splice sites inhibits export (Chang and Sharp, 1989).

Secondary sequence elements, which retain unspliced HIV-1 RNAs within the nucleus, are termed *cis*-acting repressive sequences (CRSs) or instability sequences (INSS; Cochrane et al., 1991; Maldarelli et al., 1991; Schneider et al., 1997). When appended in *cis* to heterologous reporter genes, these elements inhibit their expression. *In situ* hybridization studies demonstrated discrete localization of unspliced *gag* and *env* RNAs. In contrast to fully spliced mRNA, which is dispersed throughout both the nucleus and cytoplasm, the location of unspliced *gag* and *env* RNAs does not coincide with splicing factor SC35-containing nuclear speckles. Deletion of the intron sequence containing the CRS resulted in a shift from discrete regional localization within the nucleus to a diffuse localization throughout the cell. On the other hand, mutations that affect splicing efficiency do not alter the sequestration of unspliced RNAs (Seguin et al., 1998). Based on these results, one may hypothesize that CRSs (in conjunction with other elements that cause suboptimal splicing) cause significant amounts of full-length primary RNA, or partially spliced RNA, to accumulate. This RNA is inaccessible to subsequent splicing due to its localization in discrete regions within the nucleus. This sequestration of intron-containing RNAs may be a prerequisite for subsequent export by Rev. Indeed, addition of the RRE to β -globin mRNA, which contains efficient splice sites, does not make it responsive to Rev unless the splice sites are replaced by more inefficient ones, or a CRS is introduced into the mRNA. The finding that hnRNP A1 and a 50-kDa protein associate with the CRS suggests a connection between the CRS and RNA splicing, since hnRNP A1 controls the selection of splice sites (Najera et al., 1999).

RNA EXPORT AS A SPECIES-SPECIFIC BARRIER FOR HIV-1

Current animal models of HIV-1 disease use non-human primates (Veazey et al., 2005; Koff et al., 2006) or severe combined immunodeficiency (SCID) mice transplanted with human hemopoietic

stem cells and fetal tissues (Shultz et al., 2007; Watanabe et al., 2007). These models have made significant contributions to our understanding of lentiviral pathogenesis and have assisted in the development of several therapeutic strategies. However, they also have significant shortcomings, such as limited availability and high cost (for non-human primates), they are permissive only for related retroviruses, and they often show aberrant immune responses. Therefore, new animal models are needed. Rodent models susceptible to infection by human viruses would be ideal, since the inbred strains are well characterized and can be genetically manipulated.

Studies on rodent cell-specific defects in the HIV-1 life cycle have facilitated the identification and characterization of host cell gene products that are essential for viral replication, and these may provide a molecular basis for generating fully permissive small animal models. The major block to HIV-1 replication is at the level of cell entry. It is thought that this hurdle may be overcome by introducing human CD4 and CCR5 into the animal cells, which would serve as receptors for HIV-1. However, Tg mice and rats expressing human CD4 and CCR5 do not appear to support HIV-1 replication (Browning et al., 1997; Keppler et al., 2002; Goffinet et al., 2007), suggesting the presence of additional blocks post-entry.

Studies of the viral transcription activator, Tat, indicate the existence of a profound block at the transcription stage in murine cells (Bieniasz et al., 1998; Garber et al., 1998) and in rat T cells but, curiously, not in rat macrophages or fibroblasts (Okada et al., 2009). Expression of the human CyclinT1 (hCycT1), a cellular cofactor for Tat, in mouse and rat T cells restored viral transcriptional activity (Okada et al., 2009). A single amino acid difference between human and mouse CyclinT1 (mCycT1), which has a tyrosine at residue 261 in place of the cysteine in hCycT1, caused almost complete loss of Tat cofactor activity (Bieniasz et al., 1998; Garber et al., 1998).

In contrast to the definitive results obtained for Rex in rat cells, the existence of a profound block in Rev function in rodent cells remains controversial, although a reduced level of the HIV-1 9 kb transcript has been reported (Bieniasz and Cullen, 2000; Keppler et al., 2001). Some studies have reported impaired Rev activity (Trono and Baltimore, 1990; Marques et al., 2003), whereas others have ascribed the reduced transcript levels to over splicing or the reduced stability of unspliced transcripts in murine cells compared with human cells (Malim and Cullen, 1991); a problem corrected by the expression of the human p32 protein (Zheng et al., 2003). However, Malim et al found that the Gag protein is still efficiently synthesized in murine cells, indicating that Rev is functional, although it is not transported to the plasma membrane, resulting in defective virion formation. When Gag mRNA is transported via a different route using the cellular transporter, Tap, instead of the Rev-CRM1 system, Gag protein was synthesized and transported to the surface membrane, leading to efficient virus production (Swanson et al., 2004). This indicates that the RNA export step is a one of the species-specific barriers to HIV-1 propagation, and that the mode of mRNA nucleocytoplasmic transport affects subsequent processes occurring in the cytoplasm.

The situation in rat cells is somewhat different to that in murine cells. Even when HIV-1 genes are expressed from transfected plasmids (which circumvents the block at the entry step), virus

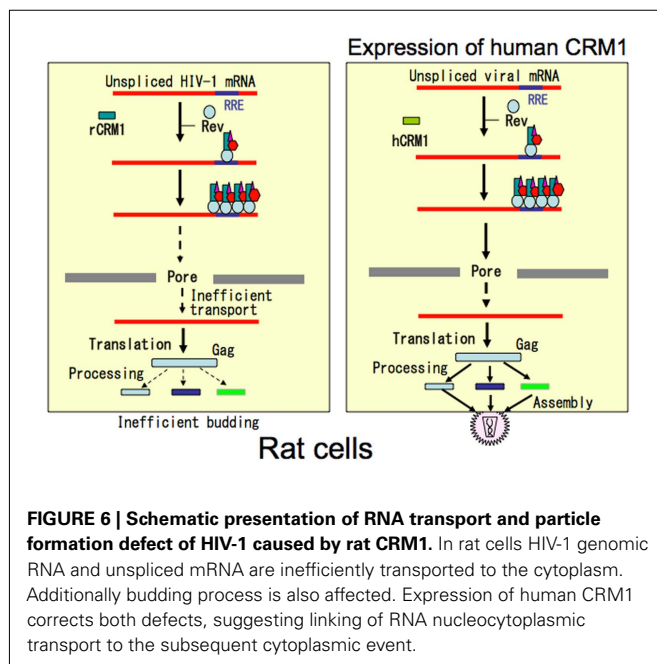
production in rat cells is still poor. However, it increases markedly in rat macrophages and fibroblasts expressing hCRM1 (but only marginally in rat T cells), which is in sharp contrast to cells transfected with hCycT1 (Okada et al., 2009). Further analyses revealed that hCRM1 enhanced Gag mRNA export a couple of times at most in rat fibroblasts. The amount of Gag protein within the cells increased two to three times, which paralleled the increase in Gag mRNA in the cytoplasm, although the trafficking of Gag to the plasma membrane did not increase. However, the number of viral particles shed into the culture medium increased 20- to 50-fold, accompanied by the increased processing of the p55Gag precursor to fully matured Gag proteins. Even a protease-deficient viral construct that caused accumulation of the p55 Gag precursor in the cytoplasm facilitated the release of viral-like particles in the presence of hCRM1, suggesting increased budding at the plasma membrane. The N-terminal half of hCRM1 was found to be responsible for this as the rCRM1–Rev interaction was much less efficient. These facts suggest that incomplete formation of transport complexes in the nucleus may affect the mechanisms underlying particle formation (Figure 6; Nagai-Fukataki et al., 2011). Indeed, the interaction between Rev and the RRE is essential for proper encapsidation of gRNA into virus particles (Blissenbach et al., 2010; Cockrell et al., 2011). Moreover, HIV-1 Gag proteins gain access to the nucleus because they have NLSs (Bukrinsky et al., 1993) and NESs that associate with CRM1 (Dupont et al., 1999). Thus, it would be interesting to examine the role of intra-nuclear Gag proteins in virus morphogenesis. More studies are required to conclude since contradictory results that NLS defective mutant Gag proteins still support viral-like particle formation has been also reported (Grewe et al., 2012).

RNA EXPORT OF SIMPLE RETROVIRUSES

Although simple retroviruses, exemplified by the Mason-Pfizer monkey virus (MPMV), encode only the gag, pol, and env genes

located centrally between the two LTRs (Figure 1; Goff, 2001), they also require the means to circumvent cellular restrictions, since the gag coding region is located within the intron of the pre-mRNA encoding the envelope proteins. To express the intron-containing gag mRNA and gRNA, inefficient splicing is necessary (as in the case of HIV-1), which is demonstrated by the finding that the modification of their splice sites increases their efficiency, resulting in the over splicing of retroviral RNAs, which renders the virus replication incompetent (Katz and Skalka, 1990). However, inefficient splicing is not sufficient; a sequence known as the constitutive transport element (CTE; first identified in the 3'-untranslation region of MPMV by Bray et al., 1994) is also required for efficient export. Since none of the simple retroviruses encode accessory proteins such as Rex and Rev, CTE was thought to recruit cellular proteins. Subsequently, a cellular protein called transporter associated with antigen processing (TAP) was identified, which binds CTE directly, and TAP sequence similarity to yeast Mex67 (which is involved in mRNA transport) indicates that CTE accesses the cellular mRNA export pathway (Grüter et al., 1998; Katahira et al., 1999). The fact that the RNA export pathway involving CTE is independent from those involving RRE/Rev or RXRE/Rex was illustrated by its sensitivity to inhibition by a DN mutant, TAGRexM64, which inhibits Rev/Rex function by sequestering the cellular cofactor, CRM1 (Katahira et al., 1995; Kiyokawa et al., 1997). The ribonucleoprotein complex containing simple retroviral RNA must also undergo a conformational change during export, which is mediated by RNA helicase A (Tang et al., 1997). Release of viral RNA into the cytoplasm is mediated by another RNA helicase, Dbp5, which localizes outside the nuclear pores (LeBlanc et al., 2007).

An intriguing question is: are gRNA and unspliced gag mRNA exported via different pathways that determine whether they are encapsidated or translated, respectively? Rous sarcoma virus (RSV), a simple avian retrovirus, contains direct repeat (DR) elements (Ogert et al., 1996) that mediate the cytoplasmic accumulation of unspliced viral RNA in a manner similar to that of MPMV CTE, since DN TAP and Dbp5 mutants abrogate DR-dependent RNA expression (LeBlanc et al., 2007). On the other hand, RSV Gag is reported to gain access to the nucleus via its NLS, which interacts with importin α/β , transportin SR, and importin 11 (Butterfield-Gerson et al., 2006; Gudleski et al., 2010), and is exported via its NES, which is associated with CRM1 (Scheifele et al., 2002, 2005). Gag binds to gRNA at the packaging element, ψ , and another unidentified site (D'Souza and Summers, 2005), and then forms multimers on gRNA to assemble viral core particles (Scheifele et al., 2007). Thus, it is conceivable that Gag proteins drive the transport of viral particles formed in the nucleus. This notion is supported by the following observations: First, transient nuclear trafficking of Gag is required for efficient gRNA encapsidation (Garbitt-Hirst et al., 2009), suggesting that Gag may initially bind to gRNA in the nucleus. Second, formation of Gag:gRNA prevents the association between Gag and importins, while the binding of Gag to gRNA instead enhances its association with CRM1, consequently promoting the export of the Gag–gRNA complex to the cytoplasm (Gudleski et al., 2010; Parent, 2011). These facts suggest the intriguing possibility that gag mRNA is transported through the



TAP pathway to be translated, whereas gRNA (which appears to be indistinguishable from gag mRNA), is exported by the Gag–CRM1 pathway for encapsidation.

Detailed analyses on mouse mammary tumor virus (MMTV) and human endogenous retrovirus K (HERV-K) that had been initially classified as simple retroviruses revealed doubly spliced mRNAs as the case of complex retroviruses. Moreover, such mRNAs have been found to encode Rev like proteins such as Rem in MMTV (Indik et al., 2005; Mertz et al., 2005) and K-Rev in HERV-K (Yang et al., 1999) that export their cognate viral RNAs through CRM1 pathway. The results suggest that these viruses belong to complex retrovirus family. The presence of K-Rev in HERV-K that integrated in primate genome ≥ 30 million years ago may evoke the possibility of evolutionary relation in the acquisition of Rev by exogenous lentiviruses (Yang et al., 1999).

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Animal models on HTLV-1 and related viruses: what did we learn?

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Retroviruses are associated with a wide variety of diseases, including immunological, neurological disorders, and different forms of cancer. Among retroviruses, Oncovirinae regroup according to their genetic structure and sequence, several related viruses such as human T-cell lymphotropic viruses types 1 and 2 (HTLV-1 and HTLV-2), simian T cell lymphotropic viruses types 1 and 2 (STLV-1 and STLV-2), and bovine leukemia virus (BLV). As in many diseases, animal models provide a useful tool for the studies of pathogenesis, treatment, and prevention. In the current review, an overview on different animal models used in the study of these viruses will be provided. A specific attention will be given to the HTLV-1 virus which is the causative agent of adult T-cell leukemia/lymphoma (ATL) but also of a number of inflammatory diseases regrouping the HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP), infective dermatitis and some lung inflammatory diseases. Among these models, rabbits, monkeys but also rats provide an excellent *in vivo* tool for early HTLV-1 viral infection and transmission as well as the induced host immune response against the virus. But ideally, mice remain the most efficient method of studying human afflictions. Genetically altered mice including both transgenic and knockout mice, offer important models to test the role of specific viral and host genes in the development of HTLV-1-associated leukemia. The development of different strains of immunodeficient mice strains (SCID, NOD, and NOG SCID mice) provide a useful and rapid tool of humanized and xenografted mice models, to test new drugs and targeted therapy against HTLV-1-associated leukemia, to identify leukemia stem cells candidates but also to study the innate immunity mediated by the virus. All together, these animal models have revolutionized the biology of retroviruses, their manipulation of host genes and more importantly the potential ways to either prevent their infection or to treat their associated diseases.

Keywords: HTLV-1, BLV, STLV-1, animal models, ATL

INTRODUCTION

The family Retroviridae is composed of numerous non-icosahedral, enveloped viruses that possess two copies of a single-stranded RNA genome. The Retroviridae have two defining hallmarks of replication: the reverse transcription of the genomic RNA into a linear double-stranded DNA copy and the subsequent covalent integration of this DNA into the host genome. Among retroviruses, the Oncovirus family regroups many viruses having a clinical, economical, and veterinary significance. Human T-cell lymphotropic virus (HTLV)-1 belongs to the Delta-type retroviruses, which also include HTLV-2, -3, and -4, simian T-cell leukemia viruses STLV-1, -2, -3, -4, and -5, and bovine leukemia virus (BLV; **Table 1**). Animal models provide an excellent tool to understand the biology of oncoviruses related diseases, and to develop vaccines or targeted therapies. These animal models vary from naturally infected hosts to established or engineered animal models that mimic the related disease in patients (**Table 1**).

NATURALLY INFECTED HOSTS

BOVINE LEUKEMIA VIRUS

The symptoms of BLV were first discovered in 1871 when Leisering reported the occurrence in cattle of a disease called “leukosis” leading to splenomegaly associated with yellowish nodules in spleens of infected cows (Leisering, 1871). This spleen disruption is consecutive to tumor formation and is the most spectacular clinical manifestation in BLV infected cattle. Tumors result from accumulation of transformed B cells in the spleen as well as diverse organ infiltration of the liver, heart, eye, skin, lung, and lymph nodes (reviewed in Burny et al., 1987; Olson and Miller, 1987; Willems et al., 1999; Meas et al., 2002). This fatal lymphoma or lymphosarcoma occurs in <5–10% of infected animals, predominantly adult cattle older than 4–5 years (Ferrer, 1980; Burny et al., 1985, 1988) whereas the great majority of infected animals (around 70%) remain asymptomatic carriers of the virus. These animals can only be identified by the presence of anti-BLV antibodies

Table 1 | BLV and PTLV: related hosts, diseases, and animal models.

Virus	Host	Disease	Animal models
Bovine leukemia virus (BLV)	Cattle	Leukosis	- Sheep - Rats - Rabbits
Human T-cell leukemia virus (HTLV-1)	Humans	- Adult T cell leukemia/lymphoma (ATL) - HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) - Infective dermatitis - Ocular lesions - Inflammatory arthropathy and polymyositis	- Primates - Rabbits - Rats - Mice models (xenografts, humanized, transgenics).
Simian T-cell leukemia virus (STLV)	Non-human primates	Unknown	Non-human primates

and/or of proviral DNA (Kettmann et al., 1976; Burny et al., 1988; Kettmann and Burny, 1994). In these settings, <1 % of peripheral blood cells in animals are found to be infected by the virus (reviewed in Gillet et al., 2007). BLV can be transmitted through the milk horizontally (Ferrer and Piper, 1978). Nowadays, BLV causes major economical losses in cattle production and export (Trono et al., 2001; Motton and Buehring, 2003; Ott et al., 2003; Rhodes et al., 2003).

PRIMATE T-CELL LYMPHOTROPIC VIRUSES: HUMAN T-CELL LEUKEMIA VIRUS AND SIMIAN T-CELL LEUKEMIA VIRUS

The primate T-cell lymphotropic viruses (PTLV) regroup the HTLVs (HTLV-1, -2, -3, and -4) as well as their related simian counterparts STLV-1, -2, and -3 (Lairmore and Franchini, 2007). Two additional STLV (-4 and -5) belong also to PTLVs but have no human counterparts discovered to date. While PTLV-1 and PTLV-2 strains have been extensively studied since the 1980s, studies on PTLV-3 are more recent and have increased in number since the discovery of HTLV-3 in 2005 (Calattini et al., 2005; Wolfe et al., 2005). HTLV-4, the fourth human HTLV retrovirus, was also discovered in 2005, but a simian counterpart of this virus has not been identified to date (Wolfe et al., 2005; Sintasath et al., 2009).

Simian T-cell leukemia viruses

The high percentage of homologies between HTLV and STLV strains, led to the demonstration that most HTLV subtypes arose from interspecies transmission between monkeys and humans. STLVs have been documented in more than 30 non-human primate (NHP) species from sub-Saharan Africa and Asia (Locatelli and Peeters, 2012). STLV-1 has been documented in captive but wild-caught chimpanzees and gorillas from west Central Africa (Gessain and Mahieux, 2000; Nerrienet et al., 2004). STLV-2 has only been documented in bonobos, an ape species endemic to Democratic Republic of Congo (Van Brussel et al., 1998). The first strain of STLV-3 was isolated in 1994, after the long-term co-culture of human cord blood lymphocytes with the peripheral blood mononuclear cells (PBMCs), obtained from an Eritrean sacred baboon that had been kept in captivity in a research laboratory in Leuven, Belgium (Goubau et al., 1994). Sequence

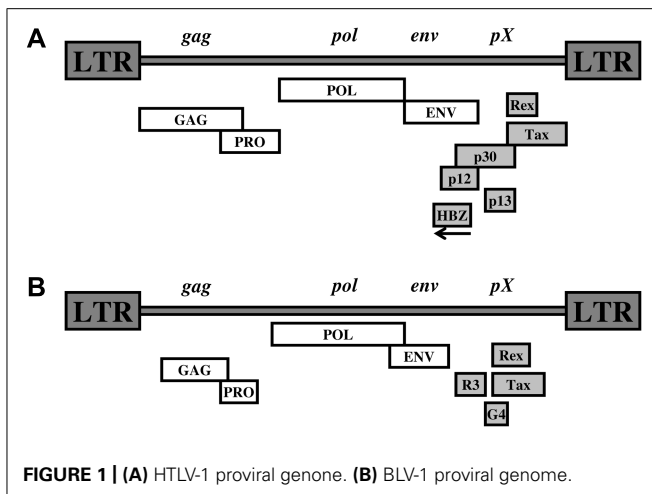
comparisons of STLV-3 full-length proviruses pointed out that these strains are highly divergent from HTLV-1, HTLV-2, or STLV-2 prototype sequences (around 40% nucleotide divergence; Meertens et al., 2002, 2003; Meertens and Gessain, 2003).

Human T-cell leukemia viruses

HTLV-1 is the first human retrovirus discovered, and is the etiological agent of two distinct diseases: adult T-cell leukemia/lymphoma (ATL; Poiesz et al., 1980; Hinuma et al., 1981, 1982; Yoshida et al., 1982) and tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM). ATL is an aggressive malignancy of mature activated CD4⁺ T cells, characterized by frequent visceral involvement, malignant hypercalcemia and opportunistic infections secondary to T cell immunosuppression. TSP/HAM is a slowly progressive neurodegenerative disorder in which lesions in the central nervous system (CNS) cause progressive weakness, stiffness, and a lower limb spastic paraparesis leading to the paralysis of the legs (Gessain et al., 1985; Rodgers-Johnson et al., 1985; Osame, 1986).

HTLV-1 infects approximately 20 million individuals worldwide (Matsuoka, 2003). Endemic areas include Japan, the Caribbean, inter-tropical Africa, Brazil, Eastern Europe, and the Middle East (Kaplan and Khabbaz, 1993; Abbaszadegan et al., 2003; Nagai and Osame, 2003). ATL develops in a small percentage (4%) of HTLV-1-infected individuals after a long period of clinical latency (20–40 years following viral infection; Hermine et al., 1998; Bazarbachi and Hermine, 2001; Bazarbachi et al., 2004). Yet, ATL is characterized by the monoclonal integration of HTLV-1 provirus in the tumor cells (Yoshida et al., 1984). Typical ATL cells are characterized by unusual morphology with lobulated nucleus, known as “flower cells” (Shimoyama et al., 1983). These malignant lymphocytes are activated CD4⁺ T cells with increased expression of the alpha chain of the interleukin (IL)-2 receptor (Waldmann et al., 1985; Okayama et al., 1997).

In addition to the classical structural genes required for retroviral replication, the HTLV-1 genome encodes a series of accessory and regulatory proteins (Figure 1A) such as the viral transcriptional activator Tax (Slamon et al., 1984) and the HTLV-1 bZIP factor gene (HBZ), a recently discovered unique viral protein



encoded from the 3' LTR in the complementary strand of the proviral genome (Gaudray et al., 2002). Both Tax and HBZ were shown to be linked to HTLV-1 pathogenesis (Boxus and Willems, 2009; Kannian and Green, 2010).

The diversity in prognosis and clinical features of patients with ATL led to the Shimoyama classification of this disease into four clinical subtypes: acute, lymphoma, chronic, and smoldering forms (Shimoyama, 1991). This classification is largely based on the extent of systemic leukemia, hypercalcemia, and organ involvement. The chronic and smoldering subtypes are considered indolent, but eventually have poor long-term survival. The acute and lymphoma forms generally have a worse prognosis mainly due to their resistance to the conventional chemotherapies, a large tumor burden, hypercalcemia, and/or frequent infectious complications as a result of a profound T-cell immunodeficiency (Shimoyama, 1991; Hermine et al., 1998; Bazarbachi and Hermine, 2001; Bazarbachi et al., 2004).

HTLV-1 is transmitted by three routes: (1) vertical (mother-to-child through breast-feeding), (2) horizontal (sexual), and (3) parenteral (blood transfusion or intravenous drug abuse) routes. Via any of the routes, infected cells are essential for transmission of HTLV-1, which has been demonstrated by the absence of seroconverters among recipients of fresh frozen plasma transfusions (Okochi et al., 1984).

A genetically related virus, HTLV-2, has been identified and isolated (Kalyanaraman et al., 1982). However, there has been no demonstration of a definitive etiological role for HTLV-2 in a human disease to date. HTLV-2 was originally identified from a patient with a variant form of hairy T-cell leukemia (Kalyanaraman et al., 1982) and is so far loosely correlated with TSP/HAM (Murphy et al., 1997a) or other opportunistic infections attributable to immunocompromised patients (Murphy et al., 1997b).

In 2005, the discovery of HTLV-3, a third HTLV type, was reported in two Cameroonian asymptomatic individuals living in the rainforest area of the southern part of the country (Calattini et al., 2005; Wolfe et al., 2005; Mahieux and Gessain, 2011). The fourth HTLV type (HTLV-4) was also reported in 2005 and consists only, so far, of a unique human strain, whose provirus was also

found in the blood of a hunter living in Cameroon (Calattini et al., 2005). However, association of HTLV-3 and -4 with any human disease remains unconfirmed due to the limited number of cases in which these viruses have been identified (Thomas et al., 2010; Zheng, 2010; Mahieux and Gessain, 2011; Welsh, 2011).

EXPERIMENTAL ANIMAL MODELS

ANIMAL MODELS FOR BLV

Sheep are excellent models to study and follow BLV infection

Large animals often provide a more relevant model of human cancer as compared to mice, since disease chronology and relevant physiology are more accurately replicated. BLV transmission has been reported in rabbits (Wyatt et al., 1989; Onuma et al., 1990), rats (Altanerova et al., 1989; Boris-Lawrie et al., 1997), chicken (Altanerova et al., 1990), pigs (Mammerickx et al., 1981), and goats (Olson et al., 1981). However, the most consistent model to study BLV infection is the sheep (Djilali et al., 1987; Djilali and Parodi, 1989; Zhao et al., 2005).

Although BLV-associated ovine leukemia is a B-cell malignancy, it shares many similarities with ATL and has been extensively studied as a model for unraveling leukemogenic mechanisms (Willems et al., 2000; Gillet et al., 2007; Merimi et al., 2009). Since the complete onset of the disease occurs in a relatively short period of time (18 months average), this model was used for studying anti-leukemic immune responses prior to tumor onset. In addition, this model has been extensively used to develop potential treatment or vaccine against BLV infection in cattle as well as to correlate these approaches with HTLV-1-associated ATL.

As for the other complex retroviruses, in addition to the Gag, Pol, and Env structural and enzymatic proteins, the genome of BLV encodes essential regulatory and accessory proteins such as Tax and Rex (Figure 1B).

Malignant progression following BLV infection in sheep is dependent on the viral Tax oncoprotein (Yoshida et al., 1982; Burny et al., 1987; Willems et al., 1990; Schwartz and Levy, 1994; Marriott et al., 2002; Szynal et al., 2003; Jeang et al., 2004; Yoshida, 2005; Klener et al., 2006; Matsuoka and Jeang, 2007). Van den Broeke et al. (2010) developed a retrovirus transduction system to generate autologous B-cell lines expressing Tax. This group demonstrated that the induction of a Tax-specific cytotoxic response by DNA immunization or viral infection of naïve animals was not predictive of disease outcome and did not prevent tumor development. On the other hand, Florins et al. (2009) demonstrated that the integrity of the spleen is required to control pathogenesis because asplenia decreased the efficiency of the immune response and induced an imbalance in cell dynamics resulting in accelerated onset of leukemia.

Bovine leukemia virus-infected sheep were also used to provide insights on the molecular genetic and epigenetic modulation of viral expression. On the genetic level, infectious proviruses were cloned and injected into sheep or calves to study the viral genetic determinants required for infection and pathogenesis (Rovnak et al., 1993; Adam et al., 1994; Bartoe et al., 2000; Tana et al., 2001). One proviral clone (clone 344) leads to tumor or leukemia after a mean latency period of 33 months (Lefebvre et al., 2002). This clone has been used to construct a series of derivative proviruses harboring mutations or deletions in different parts of the genome

including *gag*, *pol*, or *env* genes whose deletions destroy infectivity *in vivo* (Onuma et al., 1987; Adam et al., 1994). The deletion of the region which expands from the end of the *env* gene to the splice acceptor site of the *tax/rex* mRNA does not impair infectivity (Adam et al., 1994). Since these sequences correspond respectively to the third and second exons of the R3 and G4 mRNAs, it appears that these genes are not essential for infectivity *in vivo*. When p12I and p13II/p30II orthologs of R3 and G4 were deleted, similar results were obtained (Collins et al., 1998; Van den Broeke et al., 2001; Silverman et al., 2004). Importantly, the R3/G4 deletion greatly interferes with the efficiency of BLV propagation and restricts pathogenesis (Powers et al., 1991; Lefebvre et al., 2002). However, one out of 20 sheep infected with a R3/G4 mutant developed a lymphoma after 7.5 years of latency, demonstrating that the deleted sequences are not strictly required for pathogenesis (Florins et al., 2007). Among other isolates, clone 395 is deficient for infectivity *in vivo*, due to the presence of a mutation at codon 303 of the Tax protein (Adam et al., 1994; Tajima et al., 1998; Twizere et al., 2003). This result illustrates that Tax transactivation activity is required for viral infectivity *in vivo*. In contrast, a provirus (Tax106+293) harboring mutated phosphorylation sites remains infectious and propagates at wild-type levels in sheep. In addition, the Tax106+293 mutant is pathogenic despite a loss in its ability to transform primary cells *in vitro* (Szynal et al., 2003). The BLV transcriptional promoter located in the 5' LTR contains suboptimal binding sequences for the CREB transcription factor. Remarkably, the cyclic-AMP responsive site (CRE) consensus "TGACGTCA" is never strictly conserved. When a perfect CRE sequence is restored, the promoter's activity increases. However, the proviral loads are drastically reduced in sheep infected with a virus harboring this type of change (Calomme et al., 2004).

On the epigenetics level, a subtle equilibrium between the virus, which attempts to replicate, and the immune response, which seeks to exert tight control of the pathogen appeared to be tightly regulated by histone acetylation and DNA hypermethylation. In BLV infected cells, the virus is stably integrated apparently in a transcriptionally silent state (Kettmann et al., 1980; Gupta and Ferrer, 1982; Kashmiri et al., 1985; Van den Broeke et al., 1988; Lagarias and Radke, 1989; Kerkhofs et al., 1996; Merimi et al., 2007a,b). Two epigenetic mechanisms, histone acetylation and DNA hypermethylation, correlate with BLV transcriptional repression (Merezak et al., 2002; Tajima et al., 2003; Calomme et al., 2004; Nguyễn et al., 2004; Achachi et al., 2005; Pierard et al., 2010). A key observation in the BLV sheep model was a paradoxical decrease in proviral loads when increasing the BLV promoter efficiency (Merezak et al., 2001). This process was highly modulated by epigenetic modifications on the promoter sequence. In this context, a therapeutic approach based on the modulation of host epigenetic mechanisms was proposed to treat BLV infection and disease (Grange et al., 2000; Novakovic et al., 2004; Achachi et al., 2005). Different histone deacetylase (HDAC) inhibitors including valproate (VPA), trichostatin A (TSA), and trapoxin (TPX) efficiently enhanced viral transcription directed by the BLV promoter *in vitro* (Merezak et al., 2002; Achachi et al., 2005). HDAC inhibitors also increased viral expression during *ex vivo* short-term culture of PBMCs from BLV-infected sheep and cattle (Merezak et al., 2002; Achachi et al., 2005). VPA-induced hyperacetylation of histone H3 (Bouzar et al.,

2009) and in the absence of any other cytotoxic drug, VPA-induced tumor regression in BLV-infected sheep. However, this therapy was inefficient for preventing primary infection or reducing proviral load in asymptomatic sheep (Achachi et al., 2005).

The BLV-infected sheep model was also used to unravel the relative importance of cell proliferation versus apoptosis during the process of leukemogenesis associated with infection by complex oncoviruses. Debacq et al. (2002) measured the rates of cell proliferation and death in the BLV-ovine system, by using the i.v. injection of 5-bromodeoxyuridine into BLV-infected sheep. Their results showed that the increase in the number of B cells during BLV-induced lymphocytosis results from an increased cell proliferation rather than a reduced cell death.

Rats and rabbits provide a tool for BLV vaccination

Ideally, the optimal vaccine shall contain a large number of viral factors permanently stimulating the immune response. Attenuated derivatives of BLV proviruses meet these requirements (Willems et al., 1993, 1997, 2000; Boris-Lawrie et al., 1997; Kucerova et al., 1999; Kerkhofs et al., 2000; Altanerova et al., 2004; Debacq et al., 2004; Florins et al., 2007). Replication-competent BLV proviruses lacking accessory genes and *cis*-acting LTR sequences were designed and evaluated in rats and rabbits. A first generation of these genetically simpler viruses was constructed by co-injection of independent vectors encoding *gag-pol* and *env* genes. These constructs were devoid of *tax*, *rex*, *R3*, and *G4* and contained promoter *cis*-acting regulatory sequences of spleen necrosis virus (SNV). These BLV simpler hybrid derivatives were infectious and induced specific antibodies in a rat model (Boris-Lawrie et al., 1997). A second type of virus contained *gag*, *pol*, and *env* genes in a single genome under the control of SNV regulatory sequences. This viral vector was competent for replication and induced antibody responses against *gag* and *env* structural proteins in rats and rabbits (Boris-Lawrie et al., 1997; Kucerova et al., 1999; Altanerova et al., 2004). This viral vector induced protection against viral challenge in a rabbit model and decreased the proviral load (Altanerova et al., 2004).

ANIMAL MODELS FOR PTLV

Non-human primates are both natural hosts and experimental models for STLV infections

Various species of NHP serve as the natural hosts for at least six exogenous retroviruses, including gibbon ape leukemia virus (GaLV), simian sarcoma virus, simian immunodeficiency virus (SIV), STLV, simian type D retrovirus (SRV), and simian foamy virus (SFV; Lowenstine and Lerche, 1988). Asian monkeys of the genus *Macaca*, are natural hosts for three of these viruses (SRV, SFV, STLV; Lowenstine et al., 1986; Daniel et al., 1988). These Macaques were widely used in diverse studies including vaccination and toxicology against retroviruses.

Using the primate animal model, Dezzutti et al. (1987) challenged vaccinated pig-tailed macaques with the HTLV-1 subunit vaccine by an STLV-1-infected cell line. An antibody response developed to HTLV-1 and STLV-1 viral proteins recognizing both *gag* and *env* proteins. Importantly, mononuclear cells from immunized monkeys produced a greater cytotoxic activity demonstrating that the HTLV-1 subunit vaccine was

successful in protecting the pig-tailed macaques from the STLV-1 infection.

The primate animal model was also used in order to investigate the mode of transmission of HTLV-1 by studying the transmission of its related STLV-1. This study consisted of breeding seronegative macaques females with seropositive males and showed that sexual contact is important in the transmission of STLV-1, but it may not be an efficient mode of viral infection (Lazo et al., 1994).

On the molecular level, since the expression of the HTLV-1 provirus is epigenetically regulated, and since the low level of viral expression is associated with proviral chromatin deacetylation and condensation (Ego et al., 2002; Lu et al., 2004), an STLV-1 model was of great importance to study these mechanisms *in vivo*. Indeed, approximately 3% of all HTLV-1 infected persons will develop TSP/HAM against which there is currently no efficient treatment. Differences between the immune systems of rodents and humans cannot be ignored, particularly in models of TSP/HAM, because immune-mediated mechanisms appear to contribute to its development (Moore et al., 1989; Levin et al., 1997). As a correlation exists between the proviral loads (PVL) and the clinical status of the carrier, it is thought that diminishing the PVL could prevent later occurrence of the disease. In order to decrease the PVL, the STLV-1 model consisted of baboons (*Papio papio*) that are naturally infected with this virus. Baboons constitute an interesting, but little-used, model of asymptomatic HTLV-1 infection (Wolfe et al., 2005). Indeed, their immune system is very similar to the humans and the animals are naturally infected with STLV-1, and some develop STLV-1-associated diseases, such as ATL (Allan et al., 2001). Afonso et al. (2010) conducted a study combining VPA and zidovudine (AZT) in a series of baboons. They showed that the VPA/AZT combination induced a strong decrease in the PVL, which correlated with an increase in the STLV-1-specific cytotoxic T-cell population.

Non-human primates were also used to test the hypothesis that coinfection with human immunodeficiency virus (HIV) and HTLV-1 or -2 accelerates progression to AIDS. Fultz et al. (1999) inoculated pig-tailed macaques with the simian counterparts, SIV and STLV. During 2 years of follow-up of singly and dually infected macaques, no differences in SIV burdens, onset of disease, or survival were detected. However, in the first coinfecting macaque that died of AIDS (1 year after infection), >50% of CD4⁺ and CD8⁺ lymphocytes expressed CD25. On the basis of the low incidence of HTLV-1- and STLV-1-associated disease during natural infections, this early evidence of neoplastic disease was unexpected. In the same direction, Gordon et al. (2010) generated a coinfection animal model to investigate the effect of HTLV-2 on T-cell response and its impact on SIV. They found that inoculation of irradiated HTLV-2 cells in macaques elicited humoral and T-cell responses to HTLV-2 at both systemic and mucosal sites. Their data provided insights on the potential development of an attenuated HTLV-2-based vector vaccine for HIV-1.

Rabbits are excellent models to study the immunological response against HTLV-1

Haynes et al. (2010a) established a rabbit HTLV-1 infection model to study early spatial and temporal events of the viral infection. Twelve-week-old rabbits were injected intravenously with

cell-associated HTLV-1. Blood and tissues were collected at defined intervals throughout the study to test the early spread of the infection. Antibody and hematologic responses were monitored throughout the infection. This group showed that intravenous infection with cell-associated HTLV-1 targets lymphocytes located in both primary lymphoid and gut-associated lymphoid compartments. A transient lymphocytosis that correlated with peak virus detection parameters was observed by 1 week postinfection, before returning to baseline levels, suggesting that HTLV-1 promotes lymphocyte proliferation preceding early viral spread in lymphoid compartments to establish and maintain persistent infection (Haynes et al., 2010a). Moreover, Haines et al. developed an oral model of HTLV-1 transmission in rabbits to allow testing of the mucosal microenvironment during the early stages of orally acquired HTLV-1 (Martin et al., 2011). Valeri et al. (2010) suggested that infection of dendritic cells might be required for the establishment and maintenance of HTLV-1 infection in primate species. This conclusion was reached after their interesting finding established after the ablation of p12, p30, and HBZ proteins. In fact, none of these proteins, when ablated, could affect viral infectivity in rabbits. Interestingly, in rabbits, only the absence of HBZ is associated with a consistent reduction in virus levels. In contrast, in macaques, the absence of HBZ or p30 was associated with reversion of the mutant virus to the wild-type genotype. The macaques exposed to the p12 knockout remained seronegative. Interestingly, p12 and p30 mutants were severely impaired in their ability to replicate in human dendritic cells (Valeri et al., 2010). Furthermore, since HTLV-1-infected patients treated with immunosuppressive drugs, typically for organ or bone marrow transplantation procedures, often exhibit an accelerated or altered course for the development of HTLV-1-associated diseases (Gout et al., 1990; Brezin et al., 1995; Tsukasaki et al., 1999; Tajima and Aida, 2000), the rabbit model was used to evaluate the effects of immune suppression on the early spread of HTLV-1 infection upon treatment with cyclosporine A. Haynes et al. (2010b) concluded that immunologic control during early virus exposure determines subsequent HTLV-1 spread and has important implications for therapeutic intervention strategies and the development of HTLV-1-associated diseases.

Rats provided new insights on the relationship of regulatory T cells and ATL

The relationship of ATL cells with regulatory T cells (Treg) was intensively studied in order to explain the reasons behind the immunodeficiency in ATL patients. Some ATL cells and HTLV-1-infected human cells express Foxp3 and related molecules, such as CTLA-4 and GITR (Karube et al., 2004; Kohno et al., 2005; Matsubara et al., 2005; Roncador et al., 2005). To analyze the contribution of Foxp3 and Treg associated molecules to the development of ATL in more detail, various rat models for HTLV-1 infection including inbred and immunocompromised rats were generated (Ohashi et al., 1999; Nomura et al., 2004). Shinagawa et al. constructed a transgenic rat expressing human CRM1 (hCRM1), a cellular cofactor of Rex, and demonstrated that T cells derived from transgenic rats allowed production of HTLV-1 as efficiently as human T cells (Hakata et al., 2001; Takayanagi et al., 2007; Martin et al., 2011). Their results suggest the presence of

inhibitor(s) during the entry process in rat dendritic cells (Martin et al., 2011).

Mouse models: a breakthrough in HTLV studies

Small animal models are the most efficient method of studying human afflictions. This is particularly evident in the study of the human retroviruses, especially HTLV-1. Indeed, although simian models were very useful to elucidate the mechanisms of early infection and cell-to-cell transmission and to study antiviral immunological responses and potential vaccine development, they remain expensive and difficult to maintain. Mice provide a cost-effective and highly reproducible model to study factors related to ATL development and the preclinical efficacy of potential therapies. Transgenic mice have provided important insight into viral genes responsible for lymphocyte transformation. Expansion of various strains of immunodeficient mice has accelerated the testing of drugs and targeted therapy against ATL.

XENOGRAFT MICE MODELS

DEVELOPMENT OF IMMUNOCOMPROMIZED MICE STRAINS

Over the past two decades, the construction of humanized animal models through the transplantation and engraftment of human tissues or progenitor cells into immunocompromised mouse strains has allowed the development of a reconstituted human tissue scaffold in a small animal system. The first humanized mouse model was developed in 1983 through the discovery of the *scid* mutation in CB-17 *scid/scid* (SCID) mice (Bosma et al., 1983). This mouse contains a spontaneous non-sense mutation in the gene for the protein kinase DNA activated catalytic polypeptide (Pkrdc). The Pkrdc enzyme is necessary for the efficient recombination of the B- and T-cell receptors. Without this enzyme, mature B and T cells do not develop. The SCID mouse retains normal macrophage, antigen-presenting cell, and natural killer (NK) cell functions (Bosma et al., 1983). SCID mice are used extensively in human stem cell and tumor cell engraftment studies. This mouse model resulted in animals demonstrating improved engraftment efficiency and infectivity. The SCID/beige mouse (CB17.B6-Prkdcscid/Lystbg) is a double mutant mouse in which the SCID mutation is retained, but these mice have an additional beige mutation in the *Lyst* gene that results in altered lysosomal trafficking. These mice have defective B- and T-cell function, NK cell activity, and granulocyte properties.

Engraftment efficiency was further improved through the integration of the non-obese diabetic (NOD) mutation leading to the creation of NOD/SCID, a good model used to study the development of autoimmune-mediated insulin-dependent diabetes mellitus. The resultant NOD/SCID mice lack functional B and T cells, have low NK cell activity, lack complement activity, and have impaired macrophage and antigen-presenting cell function. Other immunodeficient models were also created including NOD/SCID β 2-microglobulin^{null} animals. These later were produced with development of the NOD/SCID mouse containing a targeted mutation in the β -2 microglobulin gene, encoding a protein necessary for the presentation of antigens *via* major histocompatibility class I. These mice lack all the immune functions that their less immunodeficient NOD/SCID predecessors also lack but have more complete elimination of NK-cell function.

Further efforts at minimizing the immune response resulted in the generation of NOG (NOD/Shi-*scid* IL2 γ ^{-/-}) mice. These mice are homozygous for the SCID mutation and a targeted disruption of the IL-2 γ gene mutation. The γ chain is common to the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. NOG mice are easily transplanted with human cells that would not normally transplant with the same efficiency in the more immunocompetent mouse models. NOG mice lack B- and T-cell development as well as NK-cell function and have a severe reduction in interferon (IFN)- γ production from dendritic cells. In order to further reduce the innate murine immune system, the Rag2^{-/-} γ c^{-/-} model was generated and constituted an important advancement for the engraftment of human CD34⁺ hematopoietic stem cells. These mutant mice were created by crossing homozygous recombinase activating gene 2 (Rag2) knockout mice with homozygous common cytokine receptor γ chain (γ c) knockouts. The Rag2 mutation results in the lack of maturation of thymus derived T cells and peripheral B cells whereas the γ c mutation results in the lack of the functional subunit of the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors, preventing the development of lymphocytes and NK cells. The Rag2 knockout is not a leaky mutation: it does not result in spontaneously forming tumors, and does not confer radiation-sensitivity to the mice as the SCID mutation does. Therefore, the Rag2^{-/-} γ c^{-/-} mouse may be an ideal scaffold for repopulation of the animal with human hematopoietic cells (Greiner et al., 1995; Hesselton et al., 1995; Christianson et al., 1997; Ito et al., 2002; Ishikawa et al., 2005; Shultz et al., 2005; Ito et al., 2008; Pearson et al., 2008). Together, these animal models have revolutionized the investigation of retroviral infections *in vivo*.

XENOGRAFTS OF HTLV-1 TRANSFORMED OR ATL CELLS IN IMMUNE-COMPROMIZED MICE

Xenografts of ATL cells or cell lines into immunodeficient mice replicate features of ATL and provide systems to test therapies (Zimmerman et al., 2010). Early attempts to establish an HTLV-1 infection *in vivo* involved inoculation of the CB17-*scid* mice with peripheral blood lymphocytes or PBMCs from HTLV-1 healthy carriers. These experiments were promising although limited in success due to engraftment inefficiencies and poor detection of viral integration (Feuer et al., 1993; Kondo et al., 1993). Feuer et al. (1996) used the SCID mouse model to compare the engraftment achieved with either HTLV-1-infected human hematopoietic progenitor CD34⁺ cells or *in vitro* HTLV-1 transformed cell lines SLB-1 and MT-2. This group showed that not only human hematopoietic progenitor cells could be infected *via* co-culture with cell lines transformed with HTLV-1 and HTLV-2, but that upon inoculation into immunocompromised mice, infection could be detected in biopsies from the thymus or the liver. When the same model was challenged using only the transformed cell lines SLB-1 and MT-2, infection could be also be detected in biopsies from the same organs, although levels were not as impressive as those achieved with the hematopoietic progenitor cells. These results pointed to a role for hematopoietic cells in HTLV-1 infection (Feuer et al., 1996).

To improve the efficacy of engraftment, Liu et al. (2002) used different HTLV-1 infected cell lines (RV-ATL cells) derived from a

patient sample. This group noted that a higher level of engraftment could be achieved through the use of an HTLV-1 transformed cell line as opposed to cell lines that were immortalized through transfection, which did not produce lymphomas in NOD/SCID animals. These cells were reported to establish tumors readily, but must be propagated through mice because they did not remain viable in cell culture (Liu et al., 2002). This RV-ATL cell line was reported to engraft in approximately 75% of the SCID/beige mice, whereas transformed cells (HT-1-RV, SLB-1, MT-2, ACH, and ACH.p12) were unable to establish engraftment (Liu et al., 2002). These results illustrate the significant difference between ATL cell lines derived from patients versus those transformed *ex vivo* by HTLV-1. Furthermore, using C3H/HEJ model inoculated with MT-2 cells, Tanaka et al. (2001) demonstrated integration of the virus and concentration of infected cells in lymphoid tissue.

Kawano et al. (2005) developed a novel xenogeneic engraftment model in which primary ATL cells are transplanted intravenously into neonatal NOG SCID mice. Engrafted ATL cells were dually positive for human CD4 and CD25, and displayed patterns of HTLV-1 integration identical to those of donors. Engrafted mice showed monoclonal or polyclonal proliferation of ATL cells in blood and lymph nodes, evidenced by clinical features specific to each subtype of transplanted ATL.

TARGETING THE NF- κ B PATHWAY IN XENOGRAFT MODELS

Nuclear factor- κ B (NF- κ B) is a transcription factor constitutively activated in HTLV-1 infected and ATL cells (reviewed in Kfoury et al., 2005). NF- κ B regulates the expression of a wide variety of genes implicated in proliferation, angiogenesis, invasion, and metastasis. Importantly, HTLV-1-induced transformation is dependent on the NF- κ B activation, which makes this pathway an ideal target for therapeutic attack.

In tissue culture and mouse models, non-specific inhibitors of the NF- κ B pathway such as sodium salicylate or cyclopentenone prostaglandins can increase the sensitivity of Tax-tumor cells to apoptosis and repress NF- κ B-inducible cytokines IL-6, IL-10, IL-15, and IFN- γ (Portis et al., 2001). The proteasome inhibitor bortezomib is another non-specific inhibitor of the NF- κ B pathway that is capable of inhibiting proliferation of ATL cells *ex vivo* and sensitizing them to apoptosis (Nasr et al., 2004; Mitra-Kaushik et al., 2004). Bortezomib inhibits the degradation of the NF- κ B inhibitor I κ B α , resulting in reversal of NF- κ B activation. Hence, bortezomib treatment slowed tumor growth in an allograft model of ATL by increasing apoptosis, but toxicity constraints limited its efficacy (Mitra-Kaushik et al., 2004). Similarly, when bortezomib was administered into SCID mice bearing tumors, it suppressed tumor growth *in vivo*; confirming that bortezomib was effective against ATL cells *in vivo* (Satou et al., 2004).

Another inhibitor of NF- κ B DNA binding activity, BAY 11-7082, was also shown to induce tumor regression in ATL transplanted NOG mice (Dewan et al., 2003). Similarly, Ohsugi et al. (2006, 2007) explored the use of the NF- κ B inhibitor dehydromethylepoxyquinomicin (DHMEQ) as a therapeutic agent. They established a model for infection in the NOD/SCID β 2-microglobulin null mice by sublethally irradiating 7–10-week-old animals and injecting them with transformed HTLV-1 cell lines

the following day. Treatment with DHMEQ showed increased survival and growth inhibition of ATL cells in animals that had been infected through inoculation with HTLV-1 producing cell lines.

Finally, under a similar aim in targeting and understanding the NF- κ B involvement *in vivo*, Nitta et al. (2008) utilized a mouse model with a defect in NF- κ B inducing kinase (NIK) gene resulting in a phenotype of alymphoplasia (*aly/aly*). These investigators used this model to evaluate the importance of NIK for the establishment of HTLV-1 infection and associated pathology. *Aly/aly* mice were compared with C57BL/6J and BALB/c mice. All animals were inoculated intra-peritoneally with MT-2 cells, and PCR was used to evaluate PVL. *Aly/aly* animals demonstrated dramatically lower PVLs, suggesting that NIK plays an essential role in HTLV-1 infection and could serve as a potential target for therapeutic intervention (Nitta et al., 2008). Altogether, these results confirm the importance of NF- κ B activation in ATL development and demonstrate that NF- κ B inhibition can slow ATL growth, but is not sufficient for ATL eradication.

TESTING MONOCLONAL ANTIBODIES IN XENOGRAFT MICE MODELS

The expression of markers on the cell surface of ATL cells implanted in mice has made them an excellent target for pre-clinical trials with monoclonal antibodies. Monoclonal antibodies directed against IL-2R α (Phillips et al., 2000), CD25, CD52, and CD2 (Zhang et al., 2003a,b, 2005) were tested. Phillips et al. (2000) established a NOD/SCID animal model by introducing cells from an ATL patient (MET-1), which are activated T cells that express CD2, CD3, CD4, CD25, CD122, and CD52, into the mice. The disease progressed to death in this animal model after approximately 4–6 weeks. When they treated the animals with humanized anti-Tac (HAT), murine anti-Tac (MAT), and 7G7/B6, all of which are directed to CD25 (IL-2R α), they noticed that all of the treatments significantly delayed the progression of the leukemia and prolonged the survival of the tumor-bearing mice. Moreover, and using the same animal model, Zhang et al. (2005) investigated the therapeutic efficacy of flavopiridol, an inhibitor of cyclin-dependent kinases, alone and in combination with HAT. They obtained a prolonged survival and a dramatically enhanced antitumor effect, with the combination therapy. Zhang et al. (2005) evaluated the efficacy of Campath-1H (alemtuzumab; a humanized monoclonal antibody directed to CD52), alone and in combination with HAT or with MEDI-507 directed to CD2. They noticed that the survival of the group receiving the Campath-1H was significantly longer than that of the group receiving the HAT. Furthermore, the main tumor killing mechanism with Campath-1H *in vivo* involves FcR γ containing receptors (e.g., FcR γ III) on polymorphonuclear leukocytes and macrophages that mediate antibody-dependent cellular cytotoxicity and/or trigger cross-linking induced apoptosis (Zhang et al., 2003a,b). The outcome of these mouse studies may be predictive of successful therapy for human patients, because a complete response has been reported in an ATL patient treated with alemtuzumab (anti-CD52; Mone et al., 2005). Maeda et al. (2010) investigated the effect of CD30-mediated therapy on ATL by using SGN-30, a chimeric anti-CD30 mAb, and

SGN-35, a monomethyl auristatin E-conjugated anti-CD30 mAb, *in vitro* and *in vivo*. They used NOD/SCID mice subcutaneously engrafted with HTLV-1-infected cell lines. Both mAbs significantly inhibited the growth of HTLV-1-infected cell tumors in NOD/SCID xenograft models, suggesting that CD30-mediated therapy with SGN-30 or SGN-35 would be useful for patients with ATL.

HUMANIZED MICE MODELS

Miyazato et al. (2006) took the next step in 2006 when they designed an experiment utilizing the NOG mouse model. Their investigation involved inoculation with human PBMCs in order to establish a humanized system, followed by inoculation with the MT-2 cell line to allow for the required cell-to-cell transmission essential for HTLV-1 infection. Important findings included the detection of an increased PVL in both CD4⁺ and CD8⁺ T cells. Additionally, they were able to demonstrate that prophylaxis with the reverse transcriptase inhibitors tenofovir and azidothymidine (AZT) was successful in preventing new HTLV-1 infection in these animals. Takajo et al. (2007) were able to achieve similar results in 2007 when they also established HTLV-1 infection in NOG mice through the inoculation of PBMCs from HTLV-1-infected individuals. Although the approach was different, they confirmed that these animals could harbor HTLV-1 infection and they demonstrated the presence of detectable viral integration.

Another attempt to explore treatment options included a novel approach to detect tumor growth. Shu et al. (2007) established a bioluminescent mouse model in the older CB17-*scid* model by infecting the animals with the ATL cell line, RV-ATL, and a lentivirus harboring the luciferase gene. These investigators were able to non-invasively measure the tumor growth and expansion that occurred in the recipient mice. Additionally, they tested a bisphosphonate, zoledronic acid, and the proteasome inhibitor bortezomib. Both compounds demonstrated some level of success in reducing the development of tumors, as well as levels of parathyroid hormone related protein (PTHrP) and macrophage inflammatory protein-1 α (MIP-1 α) which are both indicators of malignant hypercalcemia, a complication observed in 60% of acute ATL patients (Shu et al., 2007).

Chen et al. (2009) utilized the NOD/SCID mouse inoculated with an ATL cell line, MET-1, in their investigation of the use of a HDAC inhibitor, depsipeptide, along with daclizumab as a therapeutic option in the murine HTLV-1 infection model. They demonstrated that both depsipeptide and daclizumab alone and when used in combination were able to increase the survival of the animals.

ROLE OF HEMATOPOIETIC STEM CELLS IN HTLV-1 HUMANIZED MICE MODELS

In order to identify the molecular and cellular events that control the initiation and progression of ATL and potential therapeutic targets to block tumor development, Banerjee et al. (2010) generated an HTLV-1-infected humanized (HU-NOD/SCID) mouse model. This model was obtained by inoculation of NOD/SCID mice with CD34⁺ hematopoietic progenitor and stem cells (CD34⁺ HP/HSCs) infected *ex vivo* with HTLV-1.

HTLV-1-HU-NOD/SCID mice exclusively developed CD4⁺ T-cell lymphomas with characteristics similar to ATL. Importantly, an increased proliferation of infected human stem cells (CD34⁺CD38⁻) in the bone marrow was observed in mice developing malignancies. Furthermore, CD34⁺ HP/HSCs purified from the PBMCs of an HTLV-1-infected patient revealed proviral integrations suggesting viral infection of human bone marrow-derived stem cells. NOD/SCID mice reconstituted with CD34⁺ HP/HSCs transduced with a lentivirus vector expressing the HTLV-1 oncoprotein Tax also developed CD4⁺ lymphomas. The recapitulation of a CD4⁺ T-cell lymphoma in HU-NOD/SCID mice suggests that HSCs provide a viral reservoir *in vivo* and act as cellular targets for cell transformation in humans.

Tezuka et al. reported the development of ATL-like disease in humanized mice (huNOG) by the intra-bone marrow transplantation of NOG-SCID mouse with CD133⁺ hematopoietic stem cells purified from human cord blood infected with HTLV-1 (Martin et al., 2011). Inverse PCR analysis of provirus integration sites revealed oligoclonal expansion of infected T cells in CD4⁺/CD25⁺ T cells similar to HTLV-1-infected humans. Villaudy et al. (2011) reported that HTLV-1 induces alterations of the thymus of Rag2⁻/IL-2R γ c⁻ mice leading to expanded populations of mature CD4⁺/CD25⁺ T cells and other pathological features such as splenomegaly and lymphomas as compared to mock-infected mice. This unique model system was then used to test anti-cancer drugs, further illustrating the usefulness of the model (Martin et al., 2011; Villaudy et al., 2011).

ROLE OF THE IMMUNE SYSTEM IN HTLV-1 INFECTION AND ATL DEVELOPMENT

In order to understand the immune response against HTLV-1 in infected patients, xenografted mouse models were of great importance. Stewart et al. (1996) demonstrated that SCID mice NK cells mediated specific lysis of HTLV-1-expressing cell lines, suggesting that the absence of HTLV-1 expression in patient-derived ATL lines allows these cells to evade immune surveillance. Whole-body irradiation or administration of antibodies to abrogate NK-cell function proved necessary to establish engraftment of non-leukemic cell lines such as SLB-1 cells (Feuer et al., 1995; Uchiyama, 1996; Liu et al., 2002). MT-2 cells developed tumors at the site of injection in SCID mice treated with anti-asialo GM-1 antibody, which functionally inactivates NK cells (Ishihara et al., 1992). Indeed, invariant NK T cells (iNKT) are inversely correlated to PVLs in ATL patients (Azakami et al., 2009).

TRANSGENIC MICE MODELS OF HTLV-1

Transgenic animal technology has been useful for the direct demonstration of the tumorigenic potential of oncogenes *in vivo*. Over the recent years, a wide variety of oncogenes and proto-oncogenes from viral and cellular sources have been inserted into the germline of mice with subsequent development of neoplasia. These models continue to provide new insights into the molecular mechanisms of HTLV-1-associated transformation. None of the transgenic mice models fully recapitulate HTLV-1-associated disease, but many have been useful to investigate Tax-mediated disruption of lymphocyte function or provide evidence that Tax is an oncoprotein.

THE HTLV-1 ONCOPROTEIN Tax

In addition to its effects on the transactivation of the viral LTR, Tax has pleiotropic cellular functions (Franchini, 1995; Gatza et al., 2003; Matsuoka, 2003). It stimulates the transcription of several cellular genes through activation of critical transcription factors such as NF- κ B (Sun et al., 1994; Good and Sun, 1996; Uhlik et al., 1998; Xiao et al., 2001); cyclic AMP response element-binding protein (CREB; Zhao and Giam, 1992; Suzuki et al., 1993), serum responsive factor (SRF; Fujii et al., 1992), and activated protein 1 (AP-1; Fujii et al., 2000). Tax also represses the expression of cellular genes such as DNA polymerase β (Jeang et al., 1990), cyclin A (Kibler and Jeang, 2001), and transforming growth factor β (Arnulf et al., 2002). Moreover, Tax is involved in the regulation of apoptosis through the activation of apoptosis-suppressing genes such as Bcl-XL (Nicot et al., 2000) and repression of apoptosis-inducing genes such as Bax (Brauweiler et al., 1997). Tax inhibits tumor suppressor proteins such as p53 and p16, interferes with cell cycle checkpoint control and enhances the accumulation of mutations in HTLV-1-infected cells through the repression of DNA repair (Suzuki and Yoshida, 1997; Pise-Masison et al., 1998). Tax also influences the microenvironment: it induces angiogenesis and gap junction mediated communication between infected cells and endothelial cells, hence contributing to the extravasation and invasiveness of ATL cells (El-Sabban et al., 2002; Bazarbachi et al., 2004). Recent observations suggested that Tax also modulates the micro-RNA environment thereby adding another level of complexity to its cellular functions (Jeang, 2010). Indeed, a recent study (Yamagishi et al., 2012) has shown that Mi-RNA31 negatively down-regulates the non-canonical NF- κ B pathway by targeting NIK. Aberrant up-regulation of polycomb proteins contribute to miR-31 down-regulation epigenetically leading to activation of NF- κ B and apoptosis resistance in ATL cells (Yamagishi et al., 2012). Furthermore, more than 20 cellular proteins have been reported to interact with Tax, including a number of cytoplasmic proteins, such as MEKK1, MAD1, CBP, RelA, and I κ B kinase subunits, as well as other nuclear proteins that are not found in Tax Speckled Structures (TSS), including p16^{INK4a} and p15^{INK4b} (Jin et al., 1998; Yin et al., 1998; Xiao et al., 2000; Azran et al., 2005). Interactions of Tax with these proteins have profound effects on normal host cell processes and in many cases have been shown to be essential for or to enhance cellular transformation.

Among the properties of Tax, activation of the NF- κ B pathway plays a crucial role in the proliferation and transformation of HTLV-1-infected T cells (Yamaoka et al., 1996; Robek and Ratner, 1999). In unstimulated cells, NF- κ B is found in an inactive cytosolic complex, associated with I κ B. Upon cell stimulation, the I κ B proteins are phosphorylated by the I κ B kinase (IKK) complex, then ubiquitinated and subsequently degraded by the proteasome. Consequently, RelA-containing NF- κ B proteins translocate to the nucleus, bind specific promoters, and activate NF- κ B-dependent gene transcription (Li and Gaynor, 2000). Tax acts at multiple levels to initiate and maintain a permanent NF- κ B activation (reviewed in Geleziunas et al., 1998; Sun et al., 2000; Jeang et al., 2004). A critical step is the recruitment of Tax to the IKK- γ regulatory component of the IKK complex (Yamaoka et al., 1998). Tax/IKK γ association leads to activation of the IKK α and IKK β kinases resulting in I κ B phosphorylation, ubiquitylation,

and proteasomal degradation. The precise subcellular localization where these events occur and their molecular requirements remain largely unknown. We and others recently demonstrated that NF- κ B activation is dependent on Tax post-translational modifications, namely ubiquitylation and sumoylation (Lamsoul et al., 2005; Nasr et al., 2006; Kfoury et al., 2008, 2011, 2012), which result in the activation of the IKK complex, phosphorylation of the NF- κ B inhibitor I κ B, ultimately resulting in the nuclear translocation of the active NF- κ B subunits and activation of NF- κ B-dependent genes (reviewed in Kfoury et al., 2005, 2012). In addition, Peloponese et al. (2004) reported that ubiquitin addition modifies Tax in a proteasome-independent manner from an active to a less-active transcriptional form. Finally, Tax subcellular distribution and its interaction with cellular proteins respond dynamically to cellular stress (Gatza and Marriott, 2006).

Altogether, these multiple activities of Tax cooperate to promote infected T-cell proliferation, generate cellular defects and lead to subsequent transformation. This oncogenic potential of Tax was demonstrated through its ability to transform a rat fibroblast cell line (Matsumoto et al., 1997) and immortalize primary T cells *in vitro* (Grassmann et al., 1992).

Tax TRANSGENIC MICE MODELS

Tax in transgenic mice models is sufficient to cause oncogenesis. However, leukemia and lymphoma are rare. The first transgenic mice expressing the HTLV-1 tax gene (originally called HTLV-1 tat when mice were produced) under the control of the LTR promoter [Tg (HIV-tat) 6-2Gja] developed multicentric mesenchymal tumors of the nose, ear, mouth, tail, and foot (Nerenberg et al., 1987). These tumors were characterized by a typical spindle cell component with infiltration of granulocytes. Although this transgenic system is not appropriate for the study of ATL, it proved that the tax gene encodes an oncoprotein. In addition to the mesenchymal tumors described above, these transgenic mice developed a disease characterized by degeneration of oxidative muscle fibers. As such, this transgenic mouse helped to understand some aspects of HTLV-1-associated myopathies (Nerenberg and Wiley, 1989). LTR-tax mice showed skeletal abnormalities. Bones were grossly thicker and more fragile, whereas histologically they exhibited high bone turnover characterized by increases in osteoclasts and osteoblasts (Ruddle et al., 1993). Combining the LTR-tax mice with LTR- β gal (β galactosidase) mice generated a bitransgenic mouse in which the transactivator protein acts on the LTR to increase expression of β gal. The enzyme was detected in bone, muscle, cartilage, exocrine glands, and mesenchymal tumors (Benvenisty et al., 1992). Recently, Swaims et al. (2010) focused on the role of HTLV-1 expression in chronically infected CD4⁺ T cells using LTR-Tax transgenic mice. In this system, immune activated Tax-expressing CD4⁺ T cells express characteristics of several different CD4⁺ T cell subtypes, suggesting that HTLV-1 Tax induces changes in the normal pattern of CD4⁺ subtype specification (Kress et al., 2011).

Several transgenic C57/CBA mouse strains were generated with tax gene under the regulatory control of CD-3 ϵ promoter enhancer sequence designed to target expression to leukocytes. These mice developed mesenchymal tumors at wound sites as well as mammary and salivary adenomas (Hall et al., 1998). Another

model of Tax transgenics was developed in C57BL/6TgN mice (huGMZBTax) under the control of the granzyme B promoter. In this model, Tax expression was restricted to CD4⁺, CD8⁺, NK cells, and lymphokine-activated killer cells (Grossman et al., 1995). These mice exhibit a large granular lymphocytic (LGL) leukemia and neutrophilic dominated inflammation at sites of trauma admixed with LGL leukemic cells. These mice also developed splenomegaly, lymphadenopathy, and masses on the ears, legs, and tail (Grossman et al., 1995). As in ATL patients, these mice had malignant hypercalcemia and osteolytic bone lesions associated with metastasis (Gao et al., 2005). This model was also used to study the contribution of p53 inactivation to Tax-mediated tumorigenesis (Portis et al., 2001). Primary Tax-induced tumors and tumor-derived cell lines exhibited functional inactivation of the p53 apoptotic pathway and were resistant to an apoptosis-inducing stimulus. In contrast, p53 mutations in tumors were found to be associated with secondary organ infiltration. Furthermore, mating Tax transgenic mice with p53-deficient mice demonstrated minimal acceleration in initial tumor formation, but significantly accelerated disease progression and death in mice heterozygous for p53 suggesting that functional inactivation of p53 by HTLV-1 Tax, is not critical for initial tumor formation, but contributes to late-stage tumor progression (Portis et al., 2001). Using the same transgenic model, Rauch et al. (2009) reported that Tax expression in IL-15 knockout mice led to the development of larger and more aggressive tumors, suggesting caution against IL-15 blockade as an ATL therapy (Martin et al., 2011).

An advantageous model to study the role of inflammation and its relationship with tumor development was obtained when the C57BL/6TgN (huGMZBTax) were crossed with an IFN- γ knockout strain. The obtained mouse model showed an enhanced rate of lesion development (Mittra-Kaushik et al., 2004). More exploitations were done on imaging tumor engraftment *in vivo*. Indeed, Rauch et al. (2009) evaluated Tax-mediated activation of luciferase in LTR-luciferase (LTR-LUC) mice [C57BL/6TgN(LtrLuc)] refining the C57BL/6TgN mouse model. They reported that microscopic intraepithelial lesions precede the onset of peripheral subcutaneous tumors and that Tax is sufficient for inducing tumors. These results suggest that the viral oncoprotein activates lymphocytes to cause NK/T-cell recruitment, activation, and subsequent transformation.

In order to more specifically target Tax expression to leukocytes, bitransgenic doxycycline inducible mice [Tg (EmuSR-tTa) 83Bop] were generated to specifically control expression of wild-type or selected tax mutants in the lymphocyte compartment. This model showed skin manifestations as in ATL patients with a fatal dermatologic disease characterized by infiltration of Tax-positive T cells into the dermis and epidermis. Addition of doxycycline (suppression of Tax expression) resulted in the resolution of lesions (Kwon et al., 2005).

Hasegawa et al. (2006) generated tax transgenic mice in which the transgene expression was restricted to the thymus by the Lck promoter [C57BL/6-Tg (Lck-HTLV-1 Tax)]. This mouse model was a further confirmation that Tax alone can induce leukemia and hence represents the powerful oncogene of HTLV-1 virus. Indeed, HTLV-1 Tax transgenic mice were generated using the *lck* proximal promoter to restrict transgene expression to developing

thymocytes. Following prolonged latency periods (around 18 months), animals developed diffuse large cell lymphomas and leukemia with clinical, pathological and immunological features characteristic of acute ATL with characteristic flower cells, and extensive lymphomatous infiltration of the spleen, lymph nodes, bone marrow, liver, kidney, and lung by malignant T lymphocytes highly expressing CD25 (Hasegawa et al., 2006). As in ATL patients, mice showed marked leukocytosis, hypercalcemia, and high level of LDH and constitutive activation of the NF- κ B pathway (Hasegawa et al., 2006; El Hajj et al., 2010).

IDENTIFICATION OF ATL STEM CELLS IN MURINE ATL DERIVED FROM Tax TRANSGENICS

Transferring murine ATL splenic cells derived from Tax transgenics (Hasegawa et al., 2006) to NOD/SCID mice allowed the identification of the first candidate ATL stem cells in a side population (0.06%), which overlapped with a minor population of CD38⁻/CD71⁻/CD117⁺ cells (0.03%). In addition, lymphoma and ATL stem cells could also be demonstrated in the bone marrow and in both osteoblastic and vascular niches. In these ATL stem cells, *Tax*, *Notch1*, and *Bmi1* expression was down-regulated, suggesting that they were derived from Pro-T cells or early hematopoietic progenitor cells (Yamazaki et al., 2009). Using the same Tax transgenic model, Kawaguchi et al. (2009) demonstrated that AMD3100, a CXCR4 antagonist, inhibited infiltration of lymphomatous cells into liver and lung tissues *in vivo*. Their results demonstrated the involvement of the stromal cell-derived factor-1 α (SDF-1 α) and its receptor CXCR4 interaction as one mechanism of leukemic cell migration and this may provide a novel target as part of combination therapy for ATL. The same tax transgenic mouse model generated by Hasegawa et al. (2006) was used in an interesting study using a bioinformatics approach where Suzuki et al. (1993) could identify in a comparative proteomic analysis, proteins differentially expressed in Tax induced lymphoma (Martin et al., 2011). Strikingly, among the more than 700 proteins detected, levels of 53 proteins were increased in stem cells, including one membrane protein, which might potentially serve as a new target of antibody based therapy (Martin et al., 2011).

Tax TRANSGENICS AS A PLATFORM TO TEST TARGETED THERAPY OF ATL

In addition to the xenograft humanized mouse models, transgenic mice provided insights to clinicians before the development of phase I clinical trials. They were used in many translational studies to examine the effect of different targeted therapies of ATL (summarized in Zimmerman et al., 2010).

Adult T-cell leukemia/lymphoma is resistant to chemotherapy and carries a very poor prognosis (reviewed in Shimoyama, 1991; Bazarbachi et al., 2004). Multiple small studies using AZT and IFN showed response in ATL patients. A worldwide meta-analysis recently showed that antiviral therapy significantly increased 5-year survival of ATL patients from 20 to 50% (Bazarbachi et al., 2010). Unfortunately, most patients eventually relapse, which underlines the need for new therapeutic approaches. Using an *in vitro* model of ATL derived cell lines and freshly isolated ATL leukemic cells, Bazarbachi et al. (1999) showed that arsenic

trioxide synergizes with IFN to selectively induce G1 arrest and apoptosis in ATL cells. This combination yielded promising clinical results in relapsed/refractory ATL patients (Hermine et al., 2004). Critically, this drastic phenotype was associated to rapid proteasome-mediated Tax degradation upon exposure to the drug combination (El-Sabban et al., 2000; Nasr et al., 2003). Promising results were recently obtained in *de novo* ATL patients treated with arsenic trioxide, IFN, and AZT, with 100% response rate including 70% complete remission rate (Kchour et al., 2009).

El Hajj et al. (2010) recently reported that the combination of arsenic trioxide and IFN cures Tax-driven murine ATLs through selective targeting of leukemia initiating cell (LIC) activity. We used a transplantation model of murine ATL by transferring murine ATL splenic cells derived from Tax transgenics (Hasegawa et al., 2006) to NOD/SCID mice. These mice develop ATL-like disease manifested by diffuse large cell lymphomas and leukemia with clinical, pathological, and immunological features characteristic of acute ATL with typical flower cells, and extensive lymphomatous infiltration of the spleen, lymph nodes, bone marrow, liver, kidney, and lung by malignant T lymphocytes highly expressing CD25 (Hasegawa et al., 2006). As in ATL patients, mice showed marked leukocytosis, hypercalcemia, and high level of LDH and constitutive activation of the NF- κ B pathway (Hasegawa et al., 2006; El Hajj et al., 2010). Unexpectedly, ATL cells did not respond to arsenic and IFN by apoptosis and/or cell cycle arrest *in vivo* and therefore this regimen does not induce rapid tumor regression or massive cell death in treated animals. Importantly, on the other hand, this combination therapy used in primary mice immediately reduces leukemia transplantation into untreated secondary recipients and totally abrogates leukemia transplantation into untreated tertiary recipients. In other words, the primary tumor continues to grow and only exhausts much later, due to the specific targeting of LIC activity. Adding the proteasome inhibitor bortezomib essentially blocks the degradation of Tax triggered by the arsenic/IFN combination, and eliminates the enhancement of survival in secondary and tertiary recipients. This reversal of ATL LIC eradication by proteasome inhibition is a significant indication that ATL cells are addicted to continuous Tax expression for their LIC activity (stemness) but not for their short-term tumor growth.

Since the action of the arsenic/IFN combination is very specific to both HTLV-1-infected cells and Tax-driven murine leukemia, it is most likely that therapy-induced loss of the driving oncogene underlies responsiveness to therapy.

HBZ TRANSGENICS DEVELOP TUMORS BUT NOT ATL

Despite the fact that the expression of Tax is frequently disrupted in ATL (Matsuoka and Jeang, 2007), the HTLV-1 bZIP factor (HBZ) gene, which is encoded by the minus strand of the HTLV-1 genome (Larocca et al., 1989; Gaudray et al., 2002), is transcribed in all ATL cases (Satou et al., 2006). The HBZ gene product promotes the proliferation of ATL cells (Satou et al., 2006; Arnold et al., 2008). Furthermore, HBZ mRNA expression in HAM/TSP patients was well correlated with disease severity (Saito et al., 2009). Satou et al. (2011) generated transgenic mice containing the HBZ gene under control of a murine CD4-specific

promoter/enhancer/silencer (HBZ-Tg mice). HBZ-Tg mice spontaneously developed systemic dermatitis, alveolitis, and lymphoma as they aged and expressed the HBZ gene in all murine CD4⁺ cells. This group demonstrated that transgenic expression of HBZ in CD4⁺ T cells induced T-cell lymphomas and systemic inflammation in mice. Importantly, whereas human ATL cells and murine ATL cells derived from tax transgenics displayed constitutive activation of the NF- κ B pathway, NF- κ B was not activated in T-cell lymphomas observed in HBZ transgenics, hence demonstrating that HBZ alone cannot maintain the ATL phenotype *in vivo*.

Since the immune system plays a major role in HAM/TSP progression and since HBZ mRNA expression was correlated with disease severity (Saito et al., 2009), Satou et al. (2011) used the HBZ transgenic mouse model to study the CD4⁺Foxp3⁺ Treg population specifically that ATL cells were shown to functionally and phenotypically resemble Foxp3⁺CD25⁺CD4⁺ Treg cells, which control immune responses against self- and non-self-antigen (Sakaguchi et al., 2008). In HBZ-transgenic mice, CD4⁺Foxp3⁺ Treg cells and effector/memory CD4⁺ T cells increased *in vivo*. As a mechanism of increased Treg cells, HBZ expression directly induced Foxp3 gene transcription in T cells. However, the increased CD4⁺Foxp3⁺ Treg cells in HBZ transgenic mice were functionally impaired while their proliferation was enhanced (Satou et al., 2011). To further investigate the expression of Foxp3, Taguchi et al. used the HBZ transgenics to investigate the production of cytokines and they provided data to support the concept that altered Foxp3 expression in iTreg cells might result in systemic inflammation (Matsuoka and Green, 2009; Martin et al., 2011).

CONCLUSION

Collectively, advances in the development of animal models have extended opportunities to better understand the biology of HTLV-1-related diseases. Rising from naturally infected to genetically engineered models, these animals provided new insights on the biology of HTLV-1 and related viruses. They allowed a better understanding of the multiple genetic, epigenetic, and cellular aberrations that occur during the progression of leukemia in humans, cattle, and monkeys. They highlighted the immune response against these viruses providing new insights on the potential development of vaccines. Humanized mice models using immunocompromised animals pointed to the importance of hematopoietic stem cells in HTLV-1 infection and ATL development. Genetically engineered mice demonstrated that Tax triggers ATL development, allowed the identification of potential ATL stem cells and the preclinical development of targeted therapies such as monoclonal antibodies or the combination of arsenic trioxide and IFN- α .

Using the translation of knowledge from the laboratory bench to appropriate animal models and subsequently to patients provides hope for the prevention, and/or development of targeted and efficacious treatments against a highly refractory neoplasm such as ATL. For the first time, a proposed drug regimen showed an efficient and specific targeting of the leukemia initiating cells and was translated into an excellent responsiveness of human patients yielding to improved survival and potential cure of ATL.

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The role of genes domesticated from LTR retrotransposons and retroviruses in mammals

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The acquisition of multiple genes from long terminal repeat (LTR) retrotransposons occurred in mammals. Genes belonging to a sushi-ichi-related retrotransposon homologs (*SIRH*) family emerged around the time of the establishment of two viviparous mammalian groups, marsupials and eutherians. These genes encode proteins that are homologous to a retrotransposon Gag capsid protein and sometimes also have a Pol-like region. We previously demonstrated that *PEG10* (*SIRH1*) and *PEG11/RTL1* (*SIRH2*) play essential but different roles in placental development. *PEG10* is conserved in both the marsupials and the eutherians, while *PEG11/RTL1* is a eutherian-specific gene, suggesting that these two domesticated genes were deeply involved in the evolution of mammals via the establishment of the viviparous reproduction system. In this review, we introduce the roles of *PEG10* and *PEG11/RTL1* in mammalian development and evolution, and summarize the other genes domesticated from LTR retrotransposons and endogenous retroviruses (ERVs) in mammals. We also point out the importance of DNA methylation in inactivating and neutralizing the integrated retrotransposons and ERVs in the process of domestication.

Keywords: domesticated genes, LTR retrotransposons and ERVs, mammals, development and evolution

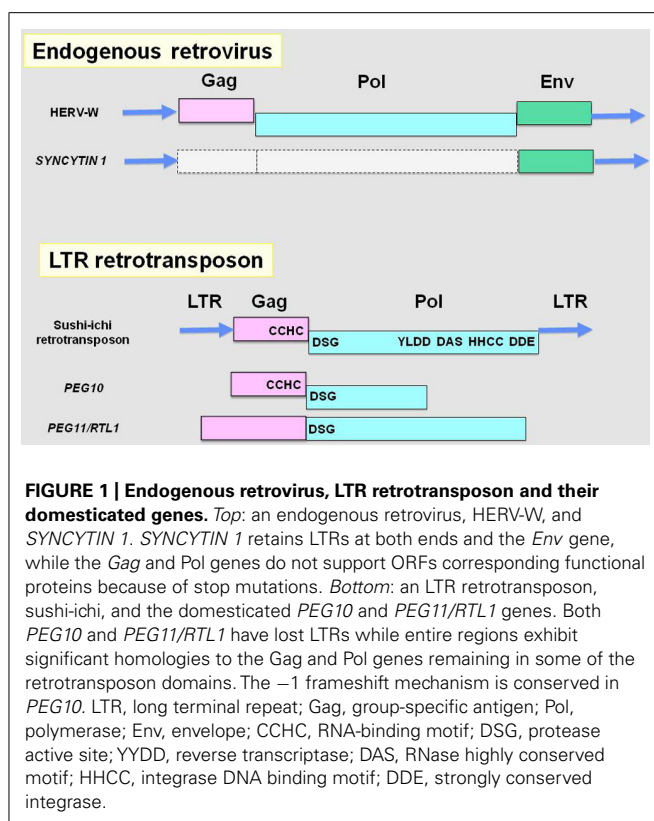
INTRODUCTION

Domestication (exaptation, co-option) is an extended mode of restricting the retrotransposons, endogenous retroviruses (ERVs), and DNA transposons that are integrated into host genomes. It has been proposed that host organisms make use of such transposable DNA elements as a genetic resource of genes for novel purposes (Brosius and Gould, 1992; Smit, 1999). Telomerase, which maintains the telomere end repeats in chromosomes in eukaryotes, and two recombination activating genes (*RAG1* and *RAG2*) that are essential for producing the vast diversity of immunoglobulin types by V(D)J recombination in vertebrates, are good examples. The former was derived from a reverse transcriptase of a long terminal repeat (LTR) retrotransposon or retrovirus (Nakamura and Cech, 1998) and the latter from a transposase of a DNA transposon (Agrawal et al., 1998; Hiom et al., 1998). Mammalian centromere-associated protein B (CENP-B) facilitates centromere formation and is a DNA-binding protein derived from a transposase of the *pogo*-like DNA transposon family (Tudor et al., 1992; Casola et al., 2008). Although it bears considerable similarity to three fission yeast proteins, ARS-binding protein (Abp1), CENP-B homologs 1 and 2 (Cbh1 and Cbh2), which also exhibit centromere binding, it was recently reported that the origin of mammalian CENP-B is different from that of the three fission yeast proteins. That is, they are all derived from distinct *pogo*-like DNA transposons, indicating that convergent domestication occurred in the mammalian and fission yeast lineages. In plants, the Arabidopsis *far-red elongated hypocotyls 3* (*FHY3*) and *far-red-impaired response* (*FAR1*) genes are derived from an ancient Mutator-like transposase, a kind of DNA transposons. They encode transcription factors essential for the light response via phytochrome A signaling (Lisch et al., 2001;

Hudson et al., 2003; Lin et al., 2007). From these data, it is clear that the domestication of transposable elements had a profound effect on quite a large numbers of animals and plants during the course of biological evolution, even though only few cases are currently known.

The retrotransposons, ERVs, and their remnant DNA sequences occupy approximately 40% of the mammalian genome and they have long been thought to be either “selfish” genes or useless “junk.” Is it thus the case that domestication events are very rare in mammals? Are there any domesticated genes which are present in a mammalian-, therian-, and eutherian-specific manner? Alternatively, are there domesticated genes which have been conserved in a more restricted manner, i.e., as species- and strain-specific genes? If so, it would be highly probable that they have contributed to mammalian evolution in various ways and to different degrees.

The recent availability of mammalian genome sequence information enabled us to identify dozens of novel domesticated genes from LTR retrotransposons/ERVs. In 2000, human *SYNCYTIN* (*ERVWE1*) was identified as the first candidate domesticated gene derived from ERVs in mammals (Blond et al., 2000; Mi et al., 2000). As shown in **Figure 1**, it derives from an *envelope* (*Env*) gene of a human-specific endogenous retrovirus, HERV-W, and was suggested by *in vitro* study to mediate placental cytotrophoblast fusion so as to produce syncytiotrophoblast cells in human placental morphogenesis. Interestingly, humans have two *SYNCYTIN* genes, but they are primate-specific genes (Blaise et al., 2003). Similar genes (also called *Syncytin*) were also discovered in several mammalian lineages that were independently acquired from *Env* genes from different ERVs (Dupressoir et al., 2005; Heidmann et al., 2009).



Finally, mouse *SyncytinA* and *B* have been to be essential placental genes using knockout mice (Dupressoir et al., 2009, 2011).

In 2001, the first and second candidate domesticated genes from a sushi-ichi-related LTR retrotransposon were identified as *paternally expressed 10* (*PEG10*; Ono et al., 2001) and *paternally expressed 11/retrotransposon-like 1* (*PEG11/RTL1*; Charlier et al., 2001). They encode proteins homologous to a retrotransposon *Gag* and a *Pol* protein, respectively (Figure 1). Combined with definitive genetic studies using knockout mice, *PEG10* and *PEG11/RTL1* have been shown to be essential for mammalian development via placenta formation and the subsequent maintenance of its placental function, respectively (Ono et al., 2006; Sekita et al., 2008). As *PEG10* is conserved in all the eutherian and marsupial species, it is a therian-specific gene (Suzuki et al., 2007), while *PEG11/RTL1* is eutherian-specific (Edwards et al., 2008). All these findings demonstrated that these two domesticated genes are essential in the current mammalian developmental system and indicate that they have been critically involved in the establishment and diversification of viviparous mammals. In other words, these domesticated genes could be major players in the macroevolution of mammals (Kaneko-Ishino and Ishino, 2010).

The concept of macroevolution by such domesticated genes from the LTR retrotransposons/ERVs and the DNA transposons, as well as rewiring gene regulatory networks by non-LTR retrotransposons (Kuwabara et al., 2009; Lynch et al., 2011; Schmidt et al., 2012) is a subject of interest not only to biologists, but also to those in the general public who are interested in biological

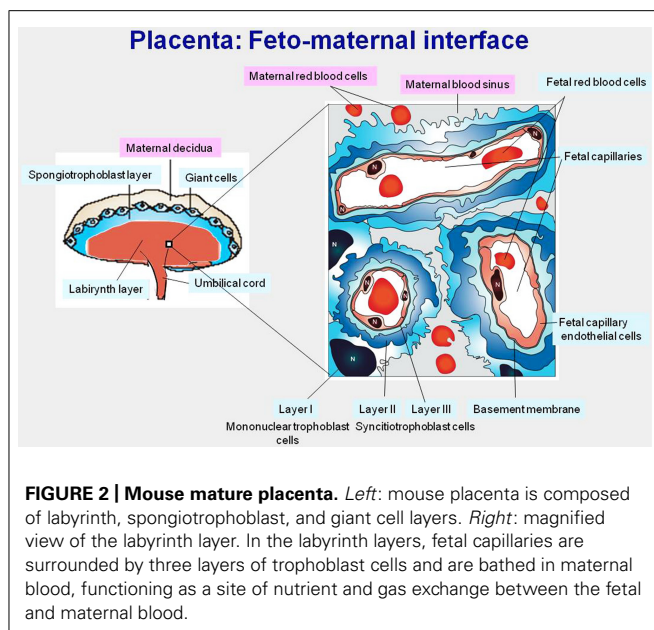
evolution and the origin of human beings. It is of special interest because it implies the existence of a unique long-term relationship between the transposable elements and the emergence of mammals.

In this review, we introduce the essential role played by *PEG10* and *PEG11/RTL1* in mammalian development via placenta formation, and summarize the current understanding of domesticated genes from the LTR retrotransposons/ERVs, especially those in the mammalian lineages. We also discuss the critically important role of DNA methylation in the process of retrotransposon domestication.

PEG10 AND PEG11/RTL1 IN MAMMALIAN DEVELOPMENT AND EVOLUTION

PEG10 and *PEG11/RTL1* were identified as paternally expressed genes in the course of an investigation on genomic imprinting (Charlier et al., 2001; Ono et al., 2001). Genomic imprinting is a mammalian-specific epigenetic mechanism regulating the parent-of-origin expression of a subset of specific genes. For these imprinted genes, the two parental alleles are not equivalent: some of the genes are transcribed only from maternally transmitted alleles (maternally expressed genes, *MEGs*) and the others are transcribed only from paternally transmitted alleles (paternally expressed genes, *PEGs*; Kaneko-Ishino et al., 2006). Then, genomic imprinting plays an essential role in mammalian development, growth, and behavior via the activity of certain critically important imprinted genes. In mice, there are more than 10 imprinted regions which have been identified, consisting of both *PEGs* and *MEGs*. Among them, a proximal region of chromosome 6 is known to cause early embryonic lethality upon maternal duplication, while maternal duplication of a distal region of chromosome 12 causes late embryonic/neonatal lethality associated with growth retardation (Cattanach and Beechey, 1990; see also Genomic imprinting map: http://www.har.mgu.ac.uk/research/genomic_imprinting/). Mouse *Peg10* and *Peg11/Rtl1* are the major genes responsible for the lethal phenotypes observed in these imprinted regions, respectively (Ono et al., 2006; Sekita et al., 2008). Using knockout mice, we demonstrated that *Peg10* and *Peg11/Rtl1* play essential roles in early placenta formation and maintenance of the placenta in the mid-to-late stages of gestation, respectively. No labyrinth or spongiotrophoblast formation was observed in the placenta of *Peg10* knockout mice. The labyrinth layer is a central part of the mouse placenta in which fetomaternal interactions take place. A large portion of the fetal capillaries exist in the labyrinth layer and allow an exchange of nutrients and gases between maternal and fetal blood cells (Figure 2). Mouse embryos require nutrient supply from the placenta starting on day 9.5 of gestation, therefore, *Peg10* KO embryos do not survive beyond this stage.

PEG10 encodes two open reading frames exhibiting the highest homology to the *Gag* and *Pol* proteins of the sushi-ichi retrotransposon, respectively, and produce two types of proteins, one derived from ORF1 and the other from both ORF1 and 2 (Ono et al., 2001; Volff et al., 2001; Figure 1). The *PEG10* protein retains a CCHC RNA-binding motif in the *Gag* protein and there is a DSG protease domain in the *Pol* protein. The -1 frameshift mechanism which produces a *Gag*-*Pol* fusion



protein that is unique to LTR retrotransposons and ERVs is conserved in *PEG10*, providing strong evidence for its origin from an LTR retrotransposon (Ono et al., 2001; Shigemoto et al., 2001; Manktelow et al., 2005). The biochemical function of the *PEG10* protein has yet to be elucidated. However, it was reported that *PEG10* is highly expressed in a great majority of hepatocellular carcinomas and confers oncogenic activity. Furthermore, the *PEG10* protein is reportedly associated with a member of the “seven in absentia homolog” family (*SIAH1* protein) that acts as a mediator of apoptosis. Overexpression of *PEG10* decreased the cell death mediated by *SIAH1*, suggesting that *PEG10* has a growth promoting function related to apoptosis in somatic cells (Okabe et al., 2003).

The genomic record shows that *PEG10* is conserved in the eutherian and marsupial mammals among the vertebrates (Suzuki et al., 2007). As the placenta is an organ unique to the viviparous reproduction system in these two mammalian groups, it is clearly evident that this gene domesticated from the LTR retrotransposon contributed to the establishment of the current developmental systems of viviparous mammalian groups as a positively selected gene (Suzuki et al., 2007; Kaneko-Ishino and Ishino, 2010). Thus, *PEG10* is a very good example of Darwinian evolution and natural selection at work in a macroevolutionary process beyond the individual species which led to the establishment of a subclass of mammals, the therians (Figure 3).

The *PEG11/RTL1* protein also possesses homology to both the Gag and Pol proteins, including the DSG protease domain in the latter, although no frameshift is required in this case (Charlier et al., 2001). The amino acid sequence homology between the *PEG10* and *PEG11/RTL1* proteins is approximately 20–30%, indicating their different functions. Mouse *Peg11/Rtl1* knockout clearly showed that *Peg11/Rtl1* has both a different role from *Peg10* and is essential for the maintenance of placental function in the mid-to-late fetal stages (Sekita et al., 2008). *Peg11/Rtl1* is expressed in endothelial cells (of extraembryonic mesoderm

lineage) of the fetal capillaries in the labyrinth layer, in contrast with *Peg10*, which is expressed in the labyrinth and spongiotrophoblast cells (of extraembryonic endoderm lineage; Figure 2). As mentioned above, the fetal capillary is the place where fetomaternal interaction occurs. The loss of *Peg11/Rtl1* causes clogging in many of the fetal capillaries in the labyrinth layer because of the phagocytosis of endothelial cells carried out by the surrounding trophoblast cells. The *Peg11/Rtl1* protein may protect endothelial cells against placental trophoblast cells, which have a highly invasive and hence dangerous nature, although its biochemical function awaits demonstration. It should be noted that the loss and overexpression of *PEG11/RTL1* are thought to attribute to the etiology of two different human imprinted diseases, maternal and paternal disomies of human chromosome 14 (matUPD14 and patUPD14), where *PEG11/RTL1* is located, respectively (Kagami et al., 2008). In these cases, *PEG11/RTL1* plays a major role, not only in placental function, but also in fetal and postnatal growth.

PEG11/RTL1 is conserved in all eutherian mammals but is absent from marsupial mammals, and is, therefore, a eutherian-specific gene (Edwards et al., 2008). Marsupials use a choriovitelline placenta (yolk sac placenta), which is different from the eutherian chorioallantoic placenta and give birth to their young after a very short gestation period compared to the eutherians (Renfree, 2010). *PEG11/RTL1* function is necessary for the latter to complete their longer gestational period. Therefore, it is probable that *PEG11/RTL1* has a role in the reproduction system of eutherians, which have the chorioallantoic placenta and that it thus contributed to the diversification of these two viviparous mammalian groups. We can say that *PEG11/RTL1* provides another good example of macroevolution in mammals (i.e., the establishment of an infraclass of mammals, the eutherians) by domesticated genes from LTR retrotransposons (Kaneko-Ishino and Ishino, 2010; Figure 3).

OTHER *SIRH* FAMILY GENES DERIVING FROM THE *SUSHI-ICHI*-RELATED RETROTRANSPOSON

PEG10 and *PEG11/RTL1* belong to a sushi-ichi-related retrotransposon homolog (*SIRH*) family consisting of 12 genes, including these two genes as *SIRH1* and *SIRH2*, respectively (Figure 2; Ono et al., 2006). It is also called the mammalian-specific retrotransposon transcripts (*MART*; Brandt et al., 2005a,b) or *SUSHI* family (Youngson et al., 2005). The *SIRH1–11* genes are conserved in the eutherian species but no marsupial orthologs have been found, yet nevertheless, *SIRH12* is derived from a marsupial-specific insertion event (Ono et al., 2011; Figure 3). Among the *SIRH* family genes, *PEG10* (*SIRH1*), *PEG11/RTL1* (*SIRH2*), and *SIRH9* share homology to both the Gag and Pol proteins, while all the others bear homology only to the Gag protein, but encode proteins of more than 100 amino acid sequences (Brandt et al., 2005a,b; Youngson et al., 2005; Campillos et al., 2006; Ono et al., 2006).

SIRH12 is present in the tammar wallaby, an Australian marsupial species, but its amino acid sequence is degenerated in the gray short-tailed opossum, a South American marsupial species, suggesting that it is only functional in the former (Ono et al., 2011). No ortholog has been reported in any eutherian species

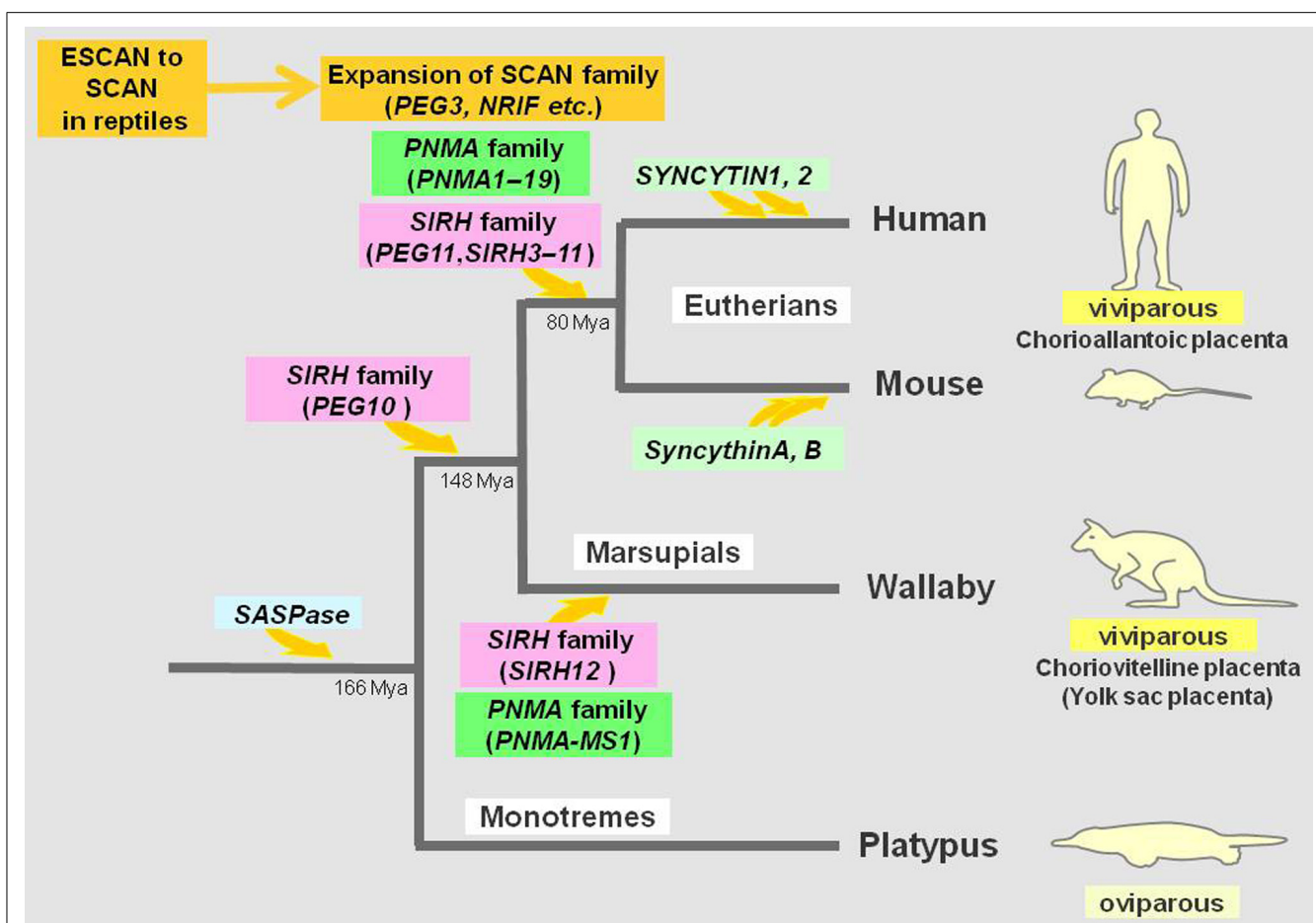


FIGURE 3 | Domestication from LTR retrotransposons and ERVs in mammals. The acquisition of *SASPase* occurred in a common mammalian ancestor. *PEG10* was domesticated in a common therian ancestor while *PEG11/RTL1*, *SIRH3-11*, and *PNMA1-19* were domesticated in a common eutherian ancestor with subsequent loss of some of the *PNMA* genes in rodents. The *ESCAN* domain was domesticated in lower vertebrates and its transition to the *SCAN* domain took place by combining with the zinc finger and/or *KRAB* motifs which had already occurred in

certain reptiles. In any event, the expansion of *SCAN* family is obvious in the eutherians. *SIRH12* and *PNMA-MS1* are derived from marsupial-specific domestication events. The *SYNCYTIN*s were independently recruited in several different mammalian lineages. The eutherians and marsupials are viviparous, having chorioallantoic and choriovitelline placentas (yolk sac placentas), respectively. Both *PEG10* and *PEG11/RTL1* are essential for the proper formation of efficient chorioallantoic placentas.

in the corresponding region between the *ectodermal-neural cortex* (*ENC1*) and *rho-guanine nucleotide exchange factor* (*RGNEF*) genes where wallaby *SIRH12* and opossum pseudo *SIRH12* are located in the marsupial genome. These findings demonstrate that the *SIRH* family of genes appeared mainly around the establishment of the therian mammals, one (*PEG10*) before and all the others after the split of the marsupials and eutherians (Figure 3). Consequently, the eutherians and the marsupials have a different set of *SIRH* family genes except for *PEG10*. It is likely that *SIRH3-11* and *SIRH12* also have roles in the eutherian and marsupial developmental and reproductive systems as well as *PEG10* and *PEG11*. As they are expressed in the brain, ovary, and testis as well as the placenta, they may be related to ovulation, gestation, delivery, and/or maternal nursing behaviors, including lactation, as well as placenta formation. Their respective functions are now under investigation using knockout mice.

PNMA-FAMILY GENES FROM THE Gypsy12_DR-RELATED LTR RETROTRANSPOSON

A paraneoplastic Ma antigen (*PNMA*) family is another large family consisting of eutherian- and marsupial-specific genes (Schüller et al., 2005; Iwasaki et al., in preparation; Figure 3). *PNMA*-family genes (*PNMA1-3*) were first identified as genes encoding neuronal auto-antigens using sera from human patients with paraneoplastic neurological syndromes (Voltz et al., 1999; Rosenfeld et al., 2001). By comprehensive search of a protein database, Schüller et al. (2005) identified additional human *PNMA* genes, *MOAP1/PNMA4*, *PNMA5*, and *PNMA6*, among which *PNMA6* has no mouse ortholog. Campillos et al. (2006) performed a genome-wide search for *PNMA* genes and identified a total 15 genes and 1 pseudogene in humans. They also showed that all of the *PNMA* genes were related to a Gypsy12_DR-related Gag protein group of the Ty3/Gypsy LTR retrotransposons isolated from zebrafish (*Danio rerio*) and that there was no Gypsy12_DR-derived

sequences in birds. Recently, Iwasaki and colleagues identified novel *PNMA* genes by a re-examination of the entire mouse and human entire genome sequences and the *PNMA*-family genes found thus far number 15 and 19 in mice and humans, respectively; all of these genes have Gag-like proteins, but none are homologous to the Pol protein. The difference in number may be due to the rodent-specific deletion of the *PNMA6A–6D* genes on X chromosome (Schüller et al., 2005; Iwasaki et al., in preparation).

No knockout mouse studies on *PNMA* genes have been reported, but there are reports indicating that these genes are involved in important biological pathways and related to human diseases. *PNMA4*-deficient cells exhibit aggressive anchorage-independent growth, suggesting that *PNMA4* has an important role in regulating apoptosis signaling in a strict temporal manner in mammalian cells, because the *PNMA4*/MOAP-1 protein is short-lived and constitutively degraded by the ubiquitin–proteasome system (Lee et al., 2009).

Cho et al. (2008a, 2011) reported *PNMA10* to be a candidate gene for X-linked mental retardation (*XLMR*) in humans. In mice, *Pnma10/Zcchc12* is expressed in the embryonic ventral forebrain in a cholinergic–neuron-specific manner (Cho et al., 2011), and is known to act as a transcriptional co-activator for bone morphogenic protein (BMP) signaling by binding to the SMAD family of proteins (Cho et al., 2008b). Therefore, it is likely that *PNMA10* is related to the evolution of brain function in mammals.

Recently, *Pnma14/CCDC8* was suggested to be one of the genes responsible for 3-M syndrome (Hanson et al., 2011a,b). 3-M syndrome is an autosomal-recessive disease characterized by severe postnatal growth restriction, leading to a significantly diminished stature. *CULLIN7 (CUL7)* and *Obscure-like 1 (OBSL1)* are both related to the transcription of *insulin-like growth factor-binding protein (IGFBP)* genes and have been identified as two of the genes involved in 3-M syndrome (Huber et al., 2005; Hanson et al., 2009). Importantly, the *OBSL1* protein interacts with both the *PNMA14/CCDC8* and *CUL7* proteins, indicating that this protein complex is responsible for the growth retardation observed in 3-M patients. These findings suggest that *PNMA* genes play an important role in the eutherian development and growth that are impacted by human diseases.

We have recently identified two marsupial-specific *PNMA* genes, *PNMA-MS1* and *-MS2* (Iwasaki et al., in preparation; **Figure 3**). *PNMA-MS1* exists in the same genome location in both the Australian (tammar wallaby) and South American (gray short-tailed opossum) marsupial species, but no orthologs exist in the eutherians, suggesting that they are derived from a marsupial-specific domestication event similar to that of *SIRH12*. *PNMA-MS2* was only found in the opossum because there is a gap in the corresponding region of the wallaby genome sequence. However, it is clear that no ortholog exists in any eutherian species. Thus, it is also clear that *PNMA*-family genes were independently domesticated in the eutherian and the marsupial lineages, and may have certain eutherian- and marsupial-specific functions, respectively.

THE RETROVIRAL-LIKE ASPARTIC PROTEASE *SASPase* IS CONSERVED IN MAMMALS

Skin aspartic protease (*SASPase*), which is known a retroviral-like aspartic protease (Bernard et al., 2005), plays a key role in

determining the texture of skin by modulating the degree of hydration via the processing of profilaggrin (Matsui et al., 2006, 2011; Barker et al., 2007). *SASPase* is a single gene conserved in the eutherians, marsupials, and presumably the monotremes (Matsui, personal communication), and thus is a mammalian-specific gene (**Figure 3**). The profilaggrin protein comprises a tandem array of filaggrin monomers and the *SASPase* is its specific protease which produces the filaggrin monomer (Matsui et al., 2011). The *Filaggrin* gene has recently been identified to be etiologically responsible for atopic dermatitis (Barker et al., 2007). Interestingly, both *SASPase* and profilaggrin are unique to mammals and expressed exclusively in the stratified epithelia in skin. Therefore, it is highly likely that they contributed to the establishment of the mammalian-specific skin barrier system. Aberrant *SASPase* expression in transgenic mice reportedly leads to impaired skin regeneration and skin remodeling after cutaneous injury or chemically induced hyperplasia (Hildenbrand et al., 2010), and *SASPase*-deficient mice exhibit fine wrinkles on the sides of the adult body (Matsui et al., 2006).

SCAN-FAMILY GENES RAPIDLY EXPANDED IN THE COURSE OF EUTHERIAN EVOLUTION

The SCAN-family is not a mammalian-specific gene family because its ancestral form exists in non-mammalian vertebrates, but nevertheless, an enormous expansion occurred in the eutherian species (**Figure 3**). The SCAN motif consists of only a C-terminal portion of the Gag capsid (CA) protein and, in mammals, it always accompanied by multiple C2H2 zinc finger motifs and/or Krüppel-associated box (KRAB) domains neither of which is of retrotransposon origin. It is suggested that the former part was already domesticated at or near the root of the tetrapod animal branch from a full-length CA gene derived from a Gmr1-like retrotransposon. This is called the extended SCAN (ESCAN) domain and that either it or its truncated SCAN motif combined with the zinc finger and/or KRAB motifs in the Anolis lizard (Emerson and Thomas, 2011). Approximately, 60 and 40 genes are known in humans and mice, respectively, and some of them are involved in development and differentiation as transcription factors, such as *ZNF202*, *ZNF197*, *ZNF444*, *ZNF274* (*neurotrophin receptor interacting factor*, *NRIF*), *Zfp496* (*NSD1-interacting zinc finger protein 1*, *Nizp1*) and *Zfp263* (*NT2*; Edelstein and Collins, 2005).

Therefore, it is highly likely that some of the SCAN-family genes are related to certain eutherian-specific functions. One example is *paternally expressed gene 3 (PEG3)* (Kuroiwa et al., 1996) that was reported to be essential for maternal nursing behavior as well as promoting embryonic growth (Li et al., 1999). The *PEG3* protein has very unique structural features among C2H2 zinc finger proteins, such as amino acid sequences for 11 C2H2 zinc finger motifs and a wider spacing of these motifs. The C2H2 zinc finger proteins comprise the largest class of eukaryotic transcription factors, yet no other C2H2 zinc finger proteins have such features (Kuroiwa et al., 1996). *PEG3* is widely expressed during fetal development of mice, and strongly in adult neurons and skeletal muscle. The *Peg3* KO offspring are approximately 20% smaller at birth, with markedly reduced nursing behavior and a reduced number of oxytocin-positive neurons in the hypothalamus of *Peg3* KO females (Li et al., 1999). Human *PEG3* has

tumor-suppressing activity in glioma cell lines by its capacity to inhibit Wnt signaling, and the loss of its expression is reportedly observed in gliomas (Kohda et al., 2001; Maekawa et al., 2004; Jiang et al., 2010).

INDEPENDENT DOMESTICATION EVENTS OF THE *SYNCYTIN*s IN DIFFERENT LINEAGES IN EUTHERIANS

As mentioned in Section “Introduction,” *SYNCYTIN* was first discovered in humans (Blond et al., 2000; Mi et al., 2000). Although there are many *Env*-related DNA sequences in the human genome, only two exhibit fusogenic activity in cell fusion assays and now these are called *SYNCYTIN1* and 2 (Blaise et al., 2003). They are derived from different human-specific ERVs, HERV-W, and HERV-FRD, and became integrated into a primate lineage 25 and >40 MYA, respectively (Figure 3). Recent studies demonstrated that similar genes exist in an order- or family-specific manner in several mammalian lineages, i.e., producing syncytiotrophoblast cells by cell fusion in the placenta. Mice also have two *Syncytin* genes, *SyncytinA* and *B*, derived from Muridae family-specific integrations of HERV-F/H-related ERV(s) approximately 20 MYA (Dupressoir et al., 2005; Figure 3), and rabbits (*Oryctolagus cuniculus*) have another *SYNCYTIN-Ory1* from Leporidae family-specific integration of a different type-D retrovirus 12–30 MYA (Heidmann et al., 2009). Therefore, at least three independent domestication events have been confirmed in the eutherians, indicating that domestication from ERVs which were actively functioning during the time of mammalian radiation.

SyncytinA knockout mice exhibit mid-fetal lethality because of the structural abnormality of the placenta (Dupressoir et al., 2009), and double knockout of both *SyncytinA* and *B* causes an even more severe phenotype, early embryonic lethality (Dupressoir et al., 2011). Among the eutherians, placental morphology and functions are quite substantially diverged. Therefore, it is very interesting that the *SYNCYTIN*s from the ERVs appear to have important roles in the placenta that they play in an order- or family-specific manner, while *PEG10* and *PEG11/RTL1* from the LTR retrotransposons are conserved in the therians and eutherians, respectively, and presumably have contributed to the establishment of the basic structure of viviparous reproductive systems in the current eutherian species.

RESISTANCE TO VIRAL INFECTION BY DOMESTICATED VIRAL GENES

ERVs have long been thought to confer resistance to infection by exogenous retroviruses. Well-known examples are *Friend virus susceptibility 1* and 4 (*Fv1* and *Fv4*) and *resistance to mink cell focus-forming (MCF) virus (Rmcf)* genes, which exhibit resistance to murine leukemia viruses (MuLVs) in mice (Pincus et al., 1971; Suzuki, 1975; Hartley et al., 1983). *Fv1* is derived from the Gag region of an ancient MERV-L element (Best et al., 1996; B  nit et al., 1997), whereas *Fv4* and *Rmcf* consist of intact *Env* genes, the expression of which prevents infection via receptor interference (Ikeda et al., 1985; Lyu and Kozak, 1996; Taylor et al., 2001; Jung et al., 2002). Endogenous betaretroviruses (enJSRVs) in sheep are another example (Dunlap et al., 2005). The ovine genome possesses approximately 20 copies of enJSRVs that are highly related

to two exogenous oncogenic viruses, Jaagsiekte sheep retrovirus (JSRV) and enzootic nasal tumor virus. It has been proposed that the enJSRVs *Env* genes are beneficial to the host and help protect of the uterus from viral infection and act as regulators of placental morphogenesis and function. They exist as species- or strain-specific genes, meaning that they are derived from recent domestication events compared to the *SIRH*-, *PNMA*-, and *SCAN*-family genes as well as the *SASPase* gene.

Therefore, it is clear that the domestication from LTR retrotransposons and ERVs has a very long history, dating from around the time of the establishment of vertebrates, on through the establishment and diversification of mammals and ultimately to the radiation of each mammalian species. Koala retrovirus (KoRV) has recently been reported to cause leukemia, lymphoma, and immunosuppression in the Australian Koala population (Tarlington et al., 2006, 2008; Stoye, 2006). Interestingly, KoRV is currently undergoing endogenization and it is likely that it entered the koala genome within the last 200 years. Therefore, retrotransposon endogenization may be a fairly ordinary process in the long course of evolution, and novel genes may continue to appear by this mechanism in the future.

GENE DUPLICATION OF DOMESTICATED GENES

Although dozens of domesticated genes have been found in mammals, this does not necessarily mean that independent domestication events have happened as often as the number of domesticated genes. Certain domesticated genes have apparently been produced by the gene duplication of an originally domesticated gene, such as in the *SCAN* family of genes. The *SCAN* domain was domesticated long before the emergence of mammals in the lower vertebrates (*ESCAN*) and then a new combination of this domain and zinc finger and/or KRAB motifs produced the *SCAN*-family gene prototype in a reptile, and its expansion occurred during radiation of the eutherians (Emerson and Thomas, 2011; Figure 3).

SIRH4, 5, and 6 as well as *PNMA6A*, 6B, 6C, and 6D, are other clear examples of gene duplication. The domestication of the original gene must have occurred in the ancestral eutherian mammals, but these clusters were produced by gene duplication because they encode very nearly the same coding frames. It is interesting to elucidate whether they are in the process of diversifying into genes with different functions or there is some as yet unknown reason for them to multiply and increase their copy numbers in this way. Nevertheless, as discussed above, at least two independent domestication events occurred in the eutherians and the marsupials in the *SIRH* and *PNMA* families, and at least four independent domestications have been confirmed in three different eutherian lineages in the case of the *SYNCYTIN*s (Figure 3).

THE ESSENTIAL ROLE OF DNA METHYLATION IN THE DOMESTICATION PROCESS

Retrotransposons are potentially harmful to host organisms because their integration not only causes genetic diseases by disrupting essential genes, but also induces chromosomal deletion as well as recombination by DNA homologous recombination between the two of them. Their integration could also disturb transcription of neighboring genes. Thus, host organisms must

prevent any further propagation that would result in an accumulation of new insertion events by regulating their transcription. How are they critically silenced and yet stably inherited from generation to generation in a manner similar to endogenous DNA sequences in the host genome?

Mammals have adopted certain defense mechanisms against them, such as DNA methylation and histone modifications (Rowe and Trono, 2011). The integrated retrotransposons are usually heavily DNA methylated and transcriptionally silenced in almost all somatic cells. They have the character of being neutral genes in the mammalian genome. According to the neutral theory of molecular evolution proposed by Kimura (1968, 1983), such neutral mutations are fixed in a population by the mechanism of random drift. Ohta (2002) proposed in her “nearly neutral theory” as an extension of neutral evolution that less harmful mutations could become fixed in a population if the population size were sufficiently small). We previously proposed the hypothesis that in the course of retrotransposon domestication the neutral or nearly neutral evolution preceded Darwinian evolution and helped supply novel genes for novel purposes from the integrated retrotransposons (Kaneko-Ishino et al., 2006; Kaneko-Ishino and Ishino, 2010). In brief, we assume that either neutral or nearly neutral evolution played essential background roles by both inactivating and neutralizing integrated retrotransposons. Subsequently, their gradual conversion from silenced harmful genes to slightly advantageous genes took place as the result of multiple mutations. A loss of such silencing, at least in a subset of tissues, was ultimately required for the “new gene” to have a certain function. Darwinian forces then came into play, and by natural selection certain of these genes became more usefully functional and thus advantageous for the host organisms. It should be noted that in extraembryonic organs, such as the yolk sac and placenta in mammals, the DNA methylation levels are lower than those in other embryonic and adult tissues. Therefore, a leaky expression of retrotransposons and retroviruses constantly occurs. In this situation, the integrated retrotransposons and their subsequent mutated forms would be less harmful. However, in the case of advantageous mutations, a swift transition from the state of nearly neutral evolution to that of Darwinian evolution would take place. In this regard, the extraembryonic tissues might have been a site of retrotransposon domestication during the course of mammalian evolution, which is consistent with the fact that the domesticated *PEG10*, *PEG11/RTL1*, and *SYNCYTIN* genes play essential roles in the placenta (Kaneko-Ishino and Ishino, 2010).

In this hypothetical scenario, various epigenetic mechanisms, such as DNA methylation and/or histone modification, might have played a critical role. In mammals, DNMT1 is the essential maintenance DNA methyltransferase and the loss of its activity causes early embryonic lethality associated with overexpression of IAP retrovirus (Li et al., 1992; Walsh et al., 1998). The two *de novo* DNA methyltransferase DNMT3A and DNMT3B are also essential for mammalian development, and the loss of their activities causes lethality in the postnatal and embryonic period in mice, respectively (Okano et al., 1999). Overexpression of IAP retrovirus was also observed in *Dnmt3a* and *3b* double knockout mice, although to a lesser degree than *Dnmt1* KO mice (Okano et al., 1999). DNMT3L does not have DNA

methyltransferase activity itself, but has an essential function of producing a different DNA methylation status in female- and male-derived genomic DNA in the process of establishing the genomic imprinting memories associated with *DNMT3A* (Bourc’his et al., 2001; Hata et al., 2002). It is known that this complex is also essential for retrotransposon methylation in the paternal germ line (Bourc’his et al., 2001; Bourc’his and Bestor, 2004). The coincident emergence of *DNMT3L* in the therian mammals is highly suggestive, both for the origin of the genomic imprinting mechanism as well as the abundance of LTR retrotransposons/ERVs, each of which is specific to the therian genome (Yokomine et al., 2006). It should be noted that H3K9 methyltransferase ERG-associated protein with SET domain (ESET, also called SETDB1) coupled with KRAB-associated protein 1 (KAP1, also called TRIM28) and zinc finger protein ZFP806 is required for H3K9 trimethylation as well as the repression of the retrotransposons and ERVs in undifferentiated mouse ES cells (Wolf and Goff, 2007, 2009; Matsui et al., 2010). Such a DNA methylation-independent pathway may be necessary, because DNA methylation is dynamically reprogrammed during the early embryonic period in mammals.

Finally, we would like to consider how the mammalian viviparous reproductive system originally started using the retrotransposon-derived *PEG10* gene. If this new reproductive system first happened in a single individual, was it possible for such an individual to survive and propagate his or her offspring? It is worth mentioning that the nearly neutral theory of molecular evolution can also explain how new species originated not from a single individual, but rather from a population subset (Kimura, 1983). Preadaptive mutations were already distributed in a neutral manner. Adaptive functions emerged under the selective pressures of a new environment. This suggests the neutral evolution process could also play a role as an “evolutionary capacitor,” as predicted in the case of heat shock protein (Hsp) 90, where genotypic variations in other genes are masked and therefore are accumulated without causing any evident phenotypic changes in the chaperone activity of Hsp90 *per se* (Rutherford and Lindquist, 1998; Bergman and Siegel, 2003). However, this original scenario has recently come under challenge because Hsp90 also acts as a suppressor of retrotransposons and its mutation induces retrotransposon transposition, thus causing a number of secondary mutations (Specchia et al., 2010; Gangaraju et al., 2011).

DNA methylation is commonly observed in a wide range of organisms, from bacteria to plants and animals, although certain model organisms do in fact lack this feature. We propose that changes in DNA methylation in genome regulation systems gave rise to the great diversity of the organisms across the earth. In particular, as mammals developed their particularly specialized DNA methylation system, mammalian evolution was advanced by a series of retrotransposon domestication events. Retrotransposons serve as a double-edged sword in development and evolution, i.e., either harmful or beneficial depending on which time scale is used. The domestication of retrotransposons seems likely to be a very rare event, but once it has taken place, its impact is profound, which is especially the case in mammalian evolution. That may provide the *raison d’être* for the LTR retrotransposons in the mammalian genome.

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Modes of retrotransposition of long interspersed element-1 by environmental factors

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Approximately 42% of the human genome is composed of endogenous retroelements, and the major retroelement component, long interspersed element-1 (L1), comprises ~17% of the total genome. A single human cell has more than 5×10^5 copies of L1, 80~100 copies of which are competent for retrotransposition (RTP). Notably, L1 can induce RTP of other retroelements, such as *Alu* and *SVA*, and is believed to function as a driving force of evolution. Although L1-RTP during early embryogenesis has been highlighted in the literature, recent observations revealed that L1-RTP also occurs in somatic cells. However, little is known about how environmental factors induce L1-RTP. Here, we summarize our current understanding of the mechanism of L1-RTP in somatic cells. We have focused on the mode of L1-RTP that is dependent on the basic helix-loop-helix/per-arnt-sim (bHLH/PAS) family of transcription factors. Along with the proposed function of bHLH/PAS proteins in environmental adaptation, we discuss the functional linking of L1-RTP and bHLH/PAS proteins for environmental adaptation and evolution.

Keywords: LINE-1, environmental factors, retrotransposition, bHLH/PAS family

INTRODUCTION

Approximately half of the human genome (~45%) is composed of transposable elements, most of which are endogenous retroelements (~42%; Lander et al., 2001; Bannert and Kurth, 2004). Notably, humans have more endogenous retroelements than do mice (~37%) or fruit flies (~3.6%), implying a possible role for endogenous retroelements in evolution (Kazazian, 2004). In humans, ~8% of the genome comprises human endogenous retroviruses (HERV) that have gene structures similar to the long-terminal repeats (LTRs) of lentiviruses (Bannert and Kurth, 2004). By contrast, ~34% of retroelements are of non-LTR types, half of which are long interspersed element-1 (L1). A single human cell contains more than 5×10^5 copies of L1, 80~100 copies of which are competent for genome shuffling by retrotransposition (RTP; Brouha et al., 2003). Interestingly, ~10% of such RTP-competent L1 sequences are “hot” and account for more than 80% of the total RTP activity (Brouha et al., 2003).

L1 is actively expressed in embryonic stem cells (Georgiou et al., 2009), and L1-RTP occurs in oocytes and during early embryonic development (van den Hurk et al., 2007; Kano et al., 2009). L1-RTP is also coupled with neuronal cell differentiation (Muotri et al., 2005), supporting the hypothetical role of L1-RTP in the plasticity of nerve cells. Moreover, L1-RTP was shown to be a critical event at an early stage of cell division in the fertilized egg, although its exact role remains elusive (Vitullo et al., 2012). While these lines of evidence strongly suggest that L1-RTP is pivotal for early embryogenesis, recent observations indicate that L1-RTP also occurs in somatic cells. Interestingly, some studies suggest that the L1 copy number is increased in the human brain (Muotri et al.,

2005; Baillie et al., 2011), and greater L1 activity was detected in patients with defective ataxia telangiectasia mutated (*ATM*) genes (Coufal et al., 2011). Moreover, new somatic inserts were identified in the tumors, suggesting increased activity therein (Iskowitz et al., 2010; Ting et al., 2011). These observations support the idea that somatic cells possess the machinery that is involved in the induction of L1-RTP, but little is known about the cellular factors required for L1-RTP. Recently, we found that L1-RTP was induced by environmental compounds that included carcinogens (Okudaira et al., 2010, 2011; Ishizaka et al., 2012). L1-RTP induction by these compounds was dependent on members of the basic helix-loop-helix/per-arnt-sim (bHLH/PAS) protein family, which has been proposed to be associated with the environmental adaptation of living organisms (Beischlag et al., 2008; McIntosh et al., 2010). Here, we provide an overview of our current understanding of the mechanism of L1-RTP coupled with bHLH/PAS proteins, and discuss the role of L1-RTP in relation to environmental adaptation and evolution.

L1 AND THE ROLES OF L1-ENCODED PROTEINS

L1 is ~6 kb in length and is composed of a 5'-untranslated region (UTR), two non-overlapping open reading frames (ORFs), and a 3' UTR with a poly(A) tail (Goodier and Kazazian, 2008). L1 has evolved along a single, unbroken lineage for the past 40 million years (myrs) in primates, and five subfamilies of L1 (L1PA1-5) have developed within the past 25 myrs in hominoid primates (Lee et al., 2007). Interestingly, species-specific L1 subfamilies emerged in *Homo sapiens* and *Pan troglodytes* after their divergence 6 myrs ago. In humans, active L1 (Ta-1) arose from the Ta-0 subfamily

~4 myrs ago, and expanded as a dominant subfamily thereafter. Notably, it has also been shown that ~69% of L1-inserted loci are polymorphic for the presence or absence of the Ta-1 insert, and that ~90% of such loci possess Ta-1d inserts, the youngest subset of Ta-1, which arose ~1.4 myrs ago (Boissinot et al., 2000). Of note, Ta-1d accounts for approximately two-third of the Ta-1 subfamily, indicating that it has been selectively expanded during the evolution of *H. sapiens*. Intriguingly, humans have higher numbers of RTP-competent L1 sequences (80~100 copies) than do chimpanzees (~5 copies), although there are similar numbers of species-specific inserts (1200–2000 copies) in humans and chimpanzees (Lee et al., 2007).

L1 encodes two proteins: ORF1 and ORF2 (Goodier and Kazazian, 2008). ORF1 is a 40-kDa protein that acts on L1-mRNA *in cis* (Wei et al., 2001) and functions as its chaperone (Martin et al., 2005). ORF2 is a protein of about 150 kDa with both reverse transcription (RT; Mathias et al., 1991) and endonuclease (Feng et al., 1996) activities. ORF2 recognizes 5'-TTAAAA hexanucleotides in the genome and induces a nick between 3'-AA and TTTT in the complementary strand (Jurka, 1997; Gilbert et al., 2005). It has been proposed that first-strand DNA is synthesized by target site-primed RT, in which the poly(A) tail of L1 mRNA anneals to the poly-T stretch of nicked genomic DNA (Kazazian, 2004; Babushok and Kazazian, 2007; Goodier and Kazazian, 2008). ORF1 and ORF2 complete the entire process of L1-RTP and also transpose other elements, such as *Alu* and *SVA* (SINE-VNTR-Alus: short interspersed element, SINE; variable number of tandem repeats, VNTR; and *Alu*; Dewannieux et al., 2003; Wallace et al., 2008; Hancks and Kazazian, 2012; Raiz et al., 2012), indicating that L1 functions as a driving force of genome shuffling.

L1-RTP IN SOMATIC CELLS IS INDUCED BY ENVIRONMENTAL FACTORS REPORTED INDUCERS

The environmental triggers, gamma irradiation (Farkash et al., 2006), and heavy metals (El-Sawy et al., 2005; Kale et al., 2005), such as mercury, cadmium, and nickel, have been shown to induce L1-RTP. Although it was proposed that L1-RTP induced by nickel occurs at the post-transcriptional level (El-Sawy et al., 2005), the precise mode of induction of L1-RTP remains to be clarified. Stribinskis and Ramos (2006) also reported that benzo[a]pyrene (B[a]P) induced L1-RTP. They found that B[a]P-induced L1-RTP depended on the aryl hydrocarbon receptor (AhR). Interestingly, however, it was shown that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin), a well-known ligand of AhR (Beischlag et al., 2008; McIntosh et al., 2010), did not induce L1-RTP. In a proposed mechanism, ligand-bound AhR activates expression of metabolic enzymes encoded by cytochrome P450 (*CYP*) genes, which in turn convert B[a]P into a genotoxic compound that induces L1-RTP (Stribinskis and Ramos, 2006). By contrast, TCDD is not a genotoxic compound, and does not induce L1-RTP.

NEWLY IDENTIFIED ENVIRONMENTAL FACTORS AND NOVEL MODES OF L1-RTP

We recently discovered that 6-formylindolo[3,2-*b*]carbazole (FICZ), a tryptophan photoproduct and a putative physiological AhR ligand (Wincent et al., 2009), induces L1-RTP (Okudaira

et al., 2010). Experiments using short-interfering RNA (siRNA) along with an AhR inhibitor revealed that the L1-RTP induced by FICZ was not dependent on AhR, but required AhR nuclear translocator-1 (ARNT1; Hoffman et al., 1991). Chromatin recruitment of ligand-bound AhR is dependent on the ARNT1 nuclear localization signal (Eguchi et al., 1997). Moreover, it was also shown that addition of FICZ initiated the molecular interaction between ARNT1 and ORF1, and promoted the recruitment of ORF1 to the chromatin-rich fraction (Okudaira et al., 2010). Given that there have been no reports indicating that ARNT1 by itself functions as a receptor for environmental compounds, it is likely that a novel cellular factor that functions as an FICZ receptor cooperates with ARNT1 in the induction of L1-RTP.

Further studies demonstrated that dimethylbenzoanthracene (DMBA), B[a]P, and 3-methylcholoranthrene (3-MC) induced L1-RTP (Okudaira et al., 2011; Ishizaka et al., 2012). Interestingly, the results of siRNA-based experiments revealed that the induction of L1-RTP by these carcinogens depended on AhR. Moreover, the induction of L1-RTP by DMBA required ARNT1, whereas L1-RTP induced by B[a]P and 3-MC did not. Notably, *ARNT1* siRNA blocked mRNA expression of the *CYP* gene *CYP1A1*. Additionally, it has been ascertained that the expression of *CYP1A1* mRNA depends on a heterodimer of AhR and ARNT1 (AHRC, AhR complex). These observations indicate that *ARNT1* siRNA efficiently attenuated the biological function of the AHRC, confirming that the induction of L1-RTP by B[a]P and 3-MC was independent of ARNT1. Moreover, the expression of *CYP1A1* mRNA in response to environmental factors requires the chromatin recruitment of ligand-bound AhR, which is dependent on the ARNT1 nuclear localization signal (Eguchi et al., 1997), lending support to the notion that a cellular factor, the function of which was similar to that of ARNT1, could contribute to chromatin recruitment of ORF1. Since AhR forms a complex with estrogen receptor α (ER α ; Ohtake et al., 2003), we examined whether ER α was involved in induction of L1-RTP. Notably, experiments using ER α siRNA indicated that L1-RTP induced by both B[a]P and 3-MC depended on ER α (Ishizaka et al., 2012).

It has been shown that B[a]P increased the expression of L1 mRNA (Stribinskis and Ramos, 2006), whereas nickel chloride did not (El-Sawy et al., 2005). We also observed no apparent increase in L1 transcripts with FICZ (Okudaira et al., 2010). These observations suggest that the modes of L1-RTP triggered by environmental compounds are regulated differently at the transcriptional and post-transcriptional levels. It is conceivable that the cellular machinery that functions at the post-transcriptional level is regulated differently depending on the compound (Table 1). Although further study is required, the data support the idea that genotoxic carcinogens induce L1-RTP in an AhR-dependent manner. Moreover, the modes of L1-RTP induced by environmental compounds were different, suggesting that the integration sites of L1 differ depending on the trigger. We will discuss this possibility further later (Figure 1).

MITOGEN-ACTIVATED PROTEIN KINASES ARE REQUIRED FOR RTP INDUCED BY ENVIRONMENTAL COMPOUNDS

In mammalian cells, six groups of MAPKs – extracellular signal-regulated protein kinase1/2 (ERK1/2), ERK5, JNK, p38, ERK3/4,

Table 1 | Summary of cellular factors required for L1-RTP by environmental compounds.

Cellular factors	Inducers			
	FICZ	B[a]P	3-MC	DMBA
AhR	–	○	○	○
ARNT1	○	–	–	○
ER α	N.T.	○	○	–
MAPKs				
SB202190	○	○	○	–
SP600125	○	○	–	–

○, dependent; –, independent; N.T., not tested.

The induction of L1-RTP was examined by a PCR-based assay.

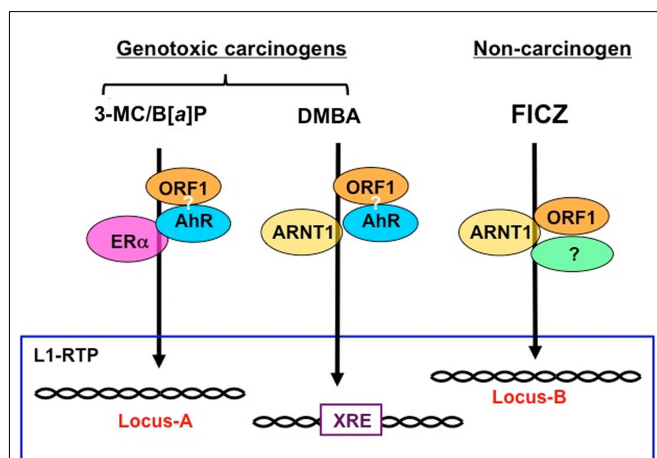


FIGURE 1 | Schematic modes of L1-RTP triggered by environmental compounds. Given that ORF1, which associates with retroelements, is recruited to the chromatin, regions of L1-RTP are likely determined by the binding partners of ORF1. As shown in **Table 1**, the induction of L1-RTP by environmental compounds depended on different sets of bHLH/PAS proteins. The induction of L1-RTP by DMBA depended on AhR and ARNT1, whereas that by 3-MC and B[a]P required AhR. Interestingly, L1-RTP by 3-MC and B[a]P did not require ARNT1, but depended on ER α . By contrast, FICZ-induced L1-RTP depending on ARNT1, whereas it did not require AhR. It is plausible that the regions where these compounds insert L1 are different: DMBA induces L1-RTP in the vicinity of XRE, whereas 3-MC and B[a]P induce L1-RTP in the region determined by AhR and ER α . The locus of L1-RTP by FICZ would differ from those determined by these genotoxic carcinogens. To prove this, it is necessary to identify the association of ORF1 and AhR and genome regions where L1 is inserted in response to each environmental compound.

and ERK7/8 – have been identified and shown to be activated by intracellular and extracellular stimuli (Cargnello and Roux, 2011). Among these MAPKs, the cellular signal cascades activated by ERK1/2, p38, and JNK have been well characterized because of the availability of inhibitors. PD98059, SB202190, and SP600125 are inhibitors of ERK1/2, p38, and JNK, respectively. To examine the involvement of MAPKs in the induction of L1-RTP, the effects of these inhibitors on the induction of L1-RTP

have been analyzed. SB202190 blocked FICZ-induced L1-RTP, and siRNA specific for cyclic-AMP responsive element binding protein (*CREB*) mRNA efficiently attenuated FICZ-induced L1-RTP (Okudaira et al., 2010). Additionally, FICZ-induced phosphorylation of CREB in an ARNT1-dependent manner, and SB202190 blocked the FICZ-induced phosphorylation of CREB and chromatin recruitment of ORF1. These observations verified that p38 is required for the induction of L1-RTP by FICZ at the step of chromatin recruitment of ORF1. Further analysis on the mode of L1-RTP induced by 3-MC and B[a]P revealed that the L1-RTP induced by 3-MC was coupled to CREB phosphorylation, and that both *CREB* siRNA and SB202190 abrogated L1-RTP induction by the compound. The effects of MAPK inhibitors are summarized in **Table 1**. The next challenge is to determine how MAPKs and the phosphorylation of MAPK substrates are involved in the induction of L1-RTP. One plausible role of these molecules is driving the chromatin recruitment of ORF1 or retroelements in response to environmental compounds (Chiu and Greene, 2008). Several studies suggest that retroelements, such as Alu, are present in a cytoplasmic high-molecular weight complex due to the function of APOBEC3 proteins, an innate restriction molecule (Chiu and Greene, 2008).

FUNCTIONAL INTERACTION OF bHLH/PAS PROTEINS AND L1-RTP

BIOLOGY OF bHLH/PAS PROTEINS

The bHLH/PAS family is composed of numerous transcriptional factors with PAS domains consisting of approximately 275 amino acids. Family members have sequence homology to the clock gene *period* (*per*) from *Drosophila melanogaster*, which is involved in the control of circadian rhythms; the *arnt* gene in mammals, which is required for signaling pathways activated in response to dioxin or polyaromatic hydrocarbons; and the *single-minded* (*sim*) gene, a neurodevelopmental regulator in flies (Beischlag et al., 2008; McIntosh et al., 2010). It is interesting to note that the bHLH/PAS family is involved in these three apparently different biological responses. The PAS domain has two sub-domains, PAS-A and PAS-B, each of which comprises approximately 70 amino acids. The PAS-A domain functions as a binding site for other PAS-A domain-containing proteins (McIntosh et al., 2010). In contrast, the PAS-B domain is involved in interactions with other classes of proteins, such as heat shock protein 90 and small molecules. One of the best-characterized functions of bHLH/PAS proteins is in the response to environmental pollutants, such as polyaromatic hydrocarbons (PAH; Beischlag et al., 2008). When PAH binds to the PAS-B domain of AhR, a heterodimeric complex of ligand-bound AhR and ARNT1 (AHRC) is formed. AHRC is then recruited to chromatin, where it recognizes a xenobiotic responsive element (XRE; Probst et al., 1993) and activates the expression of genes encoding xenobiotic metabolic enzymes (Beischlag et al., 2008). Interestingly, the AHRC also activates expression of AhR repressor (AhRR), which has both bHLH and PAS-A domains, but not a PAS-B domain. It has been proposed that the expression of AhRR down-regulates the activity of the AHRC by a feedback mechanism (Mimura et al., 1997).

CHROMATIN RECRUITMENT OF ORF1 IS DEPENDENT ON bHLH/PAS PROTEINS

Experiments involving the forced expression of a plasmid DNA encoding *ORF1* cDNA revealed that ORF1 is present in cytoplasmic stress granules in ribonucleoprotein complexes (Hohjoh and Singer, 1996; Goodier et al., 2007). Given that ORF1 acts *in cis* on L1-mRNA, it is conceivable that ORF1 is recruited from the cytoplasm to chromatin. Consistently, we observed that FICZ-induced enrichment of ORF1 in the chromatin-rich fraction and also triggered the physical association of ORF1 and ARNT1 (Okudaira et al., 2010). Additional experiments revealed that carcinogen-induced L1-RTP was dependent on AhR (Stribinskis and Ramos, 2006; Okudaira et al., 2011). These observations led us to hypothesize that ORF1 is functionally associated with bHLH/PAS proteins, and that both bHLH/PAS proteins and MAPK are involved in the chromatin recruitment of ORF1.

The importance of bHLH/PAS proteins in L1-RTP suggests that L1-RTP in the genome induced by environmental compounds may be directed. As summarized in **Table 1**, environmental carcinogens induce L1-RTP in an AhR-dependent manner. By contrast, FICZ-induced L1-RTP was not dependent on AhR. Instead, it requires ARNT1 and an additional cellular factor. Interestingly, the dependence of carcinogen-induced L1-RTP on ARNT1 differed among compounds: L1-RTP induced by DMBA required ARNT1, whereas that induced by 3-MC and B[a]P depended on AhR and ER α . These observations suggest that DMBA induces L1-RTP in the vicinity of XRE, which the AHRC targets for induction of mRNA expression of genes, such as *CYP* genes. In contrast, 3-MC and B[a]P would induce L1-RTP in the genome region determined by AhR and ER α . Moreover, the region in which FICZ-induced L1-RTP occurs differs from that in which carcinogen-induced L1-RTP occurs (**Figure 1**). It is important to analyze the regions of the newly integrated L1 to obtain novel information on the roles of L1-RTP in carcinogen-induced genetic alternations.

L1-RTP IS LINKED TO THE DEVELOPMENT OF VARIOUS DISEASES

GENETIC ERRORS

L1-RTP accidentally disrupts structures of functional genes and gives rise to inborn errors (Goodier and Kazazian, 2008). Since the discovery of aberrant insertions of L1 in the gene encoding factor VIII in 2 of 240 sporadic hemophilia A patients (Kazazian et al., 1988), at least 20 genetic disorders have been identified as resulting from L1 insertional mutagenesis (Hancks and Kazazian, 2012). Including genetic alterations by *Alu* and SVA, 96 genetic disorders have been so far identified (Hancks and Kazazian, 2012). In addition, abnormal L1 insertion was detected in a patient with branchio-oto-renal syndrome, in which L1 disrupted the locus responsible for the disease (Morisada et al., 2010). Of note, a recent study of 18 unrelated patients with neurofibromatosis type 1 (*NF1*) identified insertions of 14 *Alu*, three L1, and one poly(T) stretch within the *NF1* gene, indicating the retrotransposon insertions account for ~0.4% of all *NF1* mutations (Wimmer et al., 2011). Importantly, inserted L1, which was responsible for *NF1* disruption, was Ta-1, which is the youngest subfamily of human L1 (Boissinot et al., 2000;

Lee et al., 2007; Wimmer et al., 2011). Moreover, six different insertions were identified within the 1.5-kb region between exons 21 and 23. As the *NF1* gene is 280 kb in length, it was proposed that the insertions of endogenous retroelements into the *NF1* gene occurred non-randomly (Wimmer et al., 2011).

TUMORIGENESIS

During early studies of the alterations of cancer-related genes, aberrant L1 insertions in the *c-myc* and *APC* genes were found in breast and colon carcinoma, respectively (Morse et al., 1988; Miki et al., 1992). It was recently proposed that L1 functions as a natural mutagen for genetic alterations, and L1-RTP has been detected in lung carcinomas and pancreatic adenocarcinomas (Iskow et al., 2010; Ting et al., 2011). Interestingly, L1-RTP is associated with the expression of major satellite repeats and malignant tumor phenotypes (Ting et al., 2011). L1-RTP induces a variety of genetic alternations, including gene deletions, inversions, and inter/intra-chromosome translocations (Gilbert et al., 2002, 2005; Symer et al., 2002). Moreover, ORF2 induces DNA damage (Gasior et al., 2006). In addition to these direct effects of L1, the human genome is susceptible to genetic alterations by *Alu*–*Alu* non-allelic homologous recombination (NAHR; Belancio et al., 2010; Konkel and Batzer, 2010). For example, it was shown that *Alu*–*Alu* NAHR induced structural alterations in the tumor suppressor gene *BRCA1*, and the *MLL-1* and *Myb* genes, in cancers cells (Strout et al., 1998; Mazoyer, 2005; O'Neil et al., 2007). Notably, the frequency of deletion via *Alu*–*Alu* NAHR under conditions of p53 deficiency was 40–300 times higher than that observed in the presence of wild-type p53 (Gebow et al., 2000), suggesting the importance of *Alu*–*Alu* NAHR during multistep carcinogenesis (Hanel and Moll, 2012). Environmental stimuli possibly function as risk factors for carcinogenesis by modulating genetic alternations via L1-RTP.

Although there is no model system to evaluate the association of L1-RTP with tumorigenesis, we recently showed that L1-RTP was common in skin tumors induced by DMBA and 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Balmain et al., 1984; Okudaira et al., 2011). Of 15 skin tumors, 13 were positive for L1-RTP. It has been proposed that DMBA induces *H-ras* activation (Nelson et al., 1992), whereas TPA promotes the growth of transformed cells in a manner depending on TPA susceptibility locus (*Psl*) that is located on chromosome 9 (Angel et al., 1997). Recently, glutathione S-transferase $\alpha 4$ was identified as a candidate *Psl* gene (Abel et al., 2010). Interestingly, it was recently reported that activation of the *H-ras* gene in normal cells activates an ATM-dependent growth-arrest signal (Bartkova et al., 2006; Di Micco et al., 2006). Since ATM-dependent signaling causes senescence, an additional genetic alteration is required for tumor development; otherwise, tumor growth would be prevented. It remained elusive to clarify how TPA-induced L1-RTP is linked with the functional modification of the *Psl* gene product and attenuation of the ATM-dependent growth-arrest signal. Although it remains unclear whether L1-RTP is really involved in tumorigenesis, DMBA/TPA-induced skin carcinogenesis would be a good animal model for clarification of the role of L1-RTP in tumor development.

AUTOIMMUNE DISEASES

Intriguingly, a positive link between L1-RTP and the development of autoimmune diseases was proposed recently (Crow, 2010). Originally, heterozygous mutations of *Trex1*, a gene that encodes the 3' repair exonuclease 1, were detected in familial chilblain lupus and Aicardi–Goutières syndrome (Rice et al., 2007). Interestingly, *Trex1*-deficient mice provided evidence that *Trex1* functions as a negative regulator of the interferon-regulatory DNA response (Stetson et al., 2008). Without *Trex1* function, mice die due to severe autoimmunity. However, such lethal effects of *Trex1* deficiency were attenuated in mice with *IRF3*, *IFN α 1*, and *RAG2* gene deficiencies (Stetson et al., 2008). Moreover, single-stranded DNA derived from endogenous retroelements accumulated in *Trex1*-deficient cells, and *Trex1* overexpression blocked RTP of L1 (Stetson et al., 2008). Consistent with these experimental data, *Trex1* activity was reduced in the synovial fibroblasts of rheumatoid arthritis patients (Neidhart et al., 2010). These observations suggest that RTP-induced production of DNA derived from endogenous retroelements is a potential molecular mechanism for the development of autoimmune disorders.

FUTURE PERSPECTIVES

L1-RTP depends on three steps: transcription, RT, and integration. It is known that the expression of L1 is regulated by the methylation of CpG islands in the 5' UTR region (Hata and Sakaki, 1997; Woodcock et al., 1997; Muotri et al., 2010). Moreover, the 5' UTR of L1 contains a ubiquitously active antisense promoter that encodes siRNAs that effectively suppress L1-RTP (Yang and Kazazian, 2006). Although these reports indicate that L1 activity is regulated at the transcriptional level, our observations and those of others indicate that L1-RTP is also regulated post-transcriptionally. Environmental compounds induce the chromatin recruitment of ORF1 via bHLH/PAS proteins, suggesting that the functional coupling of L1-RTP and bHLH/PAS proteins is another pivotal step in the regulation of L1-RTP.

Since bHLH/PAS proteins are transcription factors that strictly recognize *cis* elements, it is plausible that the induction of L1-RTP in the genome depends on the selection of bHLH/PAS proteins by the individual compounds. DMBA-induced L1-RTP depends on both AhR and ARNT1, and it is likely that L1-RTP is induced in the vicinity of the XRE (Figure 1). Further study is required to identify a target locus for the induction of L1-RTP, which would provide novel information regarding the biological relevance of L1-RTP in somatic cells.

It is interesting to note that *Candida albicans* possesses a non-LTR-type retroelement with a structure similar to that of human L1 (Dong et al., 2009). This was shown to be functional in *Saccharomyces cerevisiae*, indicating that an endogenous retroelement similar to human L1 is functional in lower eukaryotes. Conversely, AhR homologs are present in the genomes of lower eukaryotes such as *D. melanogaster* and *Caenorhabditis elegans* (Hahn, 2002). Combined with our observation that L1-RTP and bHLH/PAS are linked functionally, these results suggest that genome shuffling by bHLH/PAS-dependent L1-RTP may facilitate adaptation to environmental changes. It is tempting to speculate that bHLH/PAS molecules recognize environmental

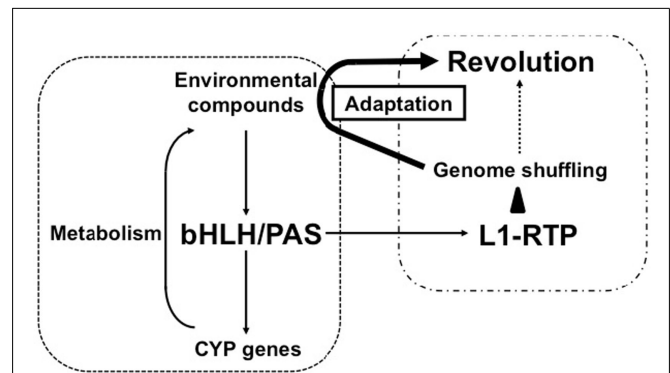


FIGURE 2 | Possible functional link between bHLH/PAS proteins and L1-RTP in environmental adaptation and evolution. As a

well-characterized biological function, bHLH/PAS proteins of AhR and ARNT1 induce gene expression of *CYP* genes in response to various compounds. The induced *CYP* proteins metabolize the environmental compounds and detoxify them. By contrast, L1-RTP induces genome shuffling, and random mutagenesis increases the chance of the emergence of living organisms that possess novel characteristics. As shown here, bHLH/PAS proteins are involved in the induction of L1-RTP, indicating the functional link between these two well-conserved cellular activities. The novel properties can overcome the disadvantageous effects of environmental compounds, enabling living organisms to survive under the selective pressure in the altered environment and contributing to evolution.

pollutants and promote genome shuffling by RTP to generate novel cellular properties that can overcome changes in the environment (Figure 2). Such property of L1 might contribute to revolution.

After the divergence of humans and chimpanzees about 6 myrs ago, both L1s continued to propagate, and Ta-1d emerged as the major subfamily in *H. sapiens* (Boissinot et al., 2000). Together with our observation that L1-RTP is induced in somatic cells by environmental factors, this affords the opportunity to speculate about novel aspects of L1 biology. Since human- and chimpanzee-specific L1 copy numbers are similar (Lee et al., 2007), it is likely that L1-RTP in the germ cells of both species is strictly regulated. However, the observation that *H. sapiens* has more copies of active L1 than do chimpanzees suggests that “hot” L1 offers an unidentified advantage to human activity. Interestingly, L1-RTP was induced in the dentate gyrus of the hippocampus when mice performed voluntary exercise (Muotri et al., 2009), and some data suggest that L1 copy numbers are increased in the human hippocampus (Coufal et al., 2011). One plausible direction for future research on L1 would be to focus on its role in the central nervous system (Hancks and Kazazian, 2012).

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Retroelements versus APOBEC3 family members: No great escape from the magnificent seven

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Retroelements comprise a large and successful family of transposable genetic elements that, through intensive infiltration, have shaped the genomes of humans and other mammals over millions of years. In fact, retrotransposons now account for approximately 45% of the human genome. Because of their genomic mobility called retrotransposition, some retroelements can cause genetic diseases; such retrotransposition events occur not only in germ cells but also in somatic cells, posing a threat to genomic stability throughout all cellular populations. In response, mammals have developed intrinsic immunity mechanisms that provide resistance against the deleterious effects of retrotransposition. Among these, seven members of the APOBEC3 (A3) family of cytidine deaminases serve as highly active, intrinsic, antiretroviral host factors. Certain A3 proteins effectively counteract infections of retroviruses such as HIV-1, as well as those of other virus families, while also blocking the transposition of retroelements. Based on their preferential expression in the germ cells, in which retrotransposons may be active, it is likely that A3 proteins were acquired through mammalian evolution primarily to inhibit retrotransposition and thereby maintain genomic stability in these cells. This review summarizes the recent advances in our understanding of the interplay between the retroelements currently active in the human genome and the anti-retroelement A3 proteins.

Keywords: retroelements, retrotransposition, LINE-1, Alu, APOBEC3, HIV-1, Vif, restriction factors

INTRODUCTION

The evolution of vertebrate genomes has been driven in part by the long history of their interaction with genetic transposable elements. These so-called retrotransposons, which replicate via RNA intermediates, can be divided into two groups depending on the presence or absence of long terminal repeats (LTRs). LTR retrotransposons are endogenous retroviruses that constitute nearly 10% of murine and human genomes, but they have been rendered mostly inactive due to the accumulation of mutations, although some murine intracisternal A-particles (IAP) and MusD sequences remain viable (Dewannieux et al., 2004; Ribet et al., 2004). Non-LTR retrotransposons comprise the majority of transposable elements; in fact, collectively, they account for more than one third of the human genome. They can be further subdivided into three types; long interspersed elements (LINEs), short interspersed elements (SINEs), and the composite hominid-specific retrotransposons, each of which contain the only transposable elements currently active in the human genome, i.e., LINE-1, Alu, and SINE-VNTR-Alu (SVA), respectively (Deininger and Batzer, 2002; Ostertag et al., 2003).

Retrotransposition, discussed in greater detail below, involves the reverse transcription of an RNA intermediate with subsequent genomic integration in a process driven by retrotransposon-encoded RNA-dependent DNA polymerase and endonuclease. The integration of these elements may have harmful consequences for the host, compromising genomic stability via insertions, deletions, and DNA rearrangements and thereby posing

a threat to human health, as described in several reports of retrotransposition-induced genetic disorders (Kazazian et al., 1988; Wallace et al., 1991; Kobayashi et al., 1998). In response, eukaryotic organisms have evolved mechanisms to restrict uncontrolled retrotransposition. Anti-retroelement strategies include transcriptional silencing through DNA methylation (Walsh et al., 1998; Bourc'h and Bestor, 2004; Burden et al., 2005), post-transcriptional silencing via RNA interference (Soifer et al., 2005; Yang and Kazazian, 2006), and some cellular factors inhibiting retrotransposition at the post-translational level. Of these cellular factors, seven members of the apolipoprotein B mRNA-editing catalytic polypeptide-like 3 (APOBEC3; referred to hereafter as the A3) family of cytidine deaminases have been shown to act as potent inhibitors of a wide range of both exogenous retroviruses and endogenous retroelements (Sheehy et al., 2002; Esnault et al., 2005; Chen et al., 2006; Kinomoto et al., 2007). In this review, we focus on active endogenous retroelements, their deleterious effects on the human genome, and the anti-retroelement activity of A3 proteins.

RETROTRANSPOSONS: AN OVERVIEW

Unlike the murine LTR retrotransposons IAP and MusD, human versions, such as human endogenous retroviruses (HERV), have been mostly fossilized, and even those that are not are non-transposable. In contrast, many copies of human non-LTR retrotransposons can replicate through an RNA/protein complex intermediate and integrate into the host genome at a new site.

The LINE retrotransposons, typified by LINE-1 (L1), account for approximately 17% of the human genome, corresponding to >500,000 copies (of which 100 copies are retrotransposition-competent). L1 retrotransposons are 6 kb in length and contain a 5' untranslated region (UTR) that harbors a Pol II promoter; two ORFs necessary for their own replication; and a 3' UTR containing a polyadenylation signal, followed by a poly(A) tail (**Figure 1A**, top). Briefly, L1 elements are first transcribed by RNA-polymerase II using a promoter located at the L1 5' region (Ostertag and Kazazian, 2001). ORF1, encoding an RNA-binding protein, and ORF2, encoding a protein with reverse transcriptase and endonuclease activity, are then translated in the cytoplasm. The resulting proteins associate with L1 RNA to form a ribonucleoprotein (RNP) complex (Martin, 1991; Hohjoh and Singer, 1996; **Figure 1B**) that is transported back into the nucleus, where L1 is integrated into the host genome through a target-primed reverse transcription (Cost et al., 2002).

The human genome also contains more than 1 million copies of Alu elements; these are the most common SINE retrotransposons, representing 11% of our genome. The typical Alu element is approximately 300 bp in length and is formed by the fusion of two 7SL-RNA gene-derived monomers separated by an A-rich linker, followed by a poly(A) tail (Kriegs et al., 2007; **Figure 1A**, middle). Likewise, there are ~2700 copies of the composite SVA elements in the human genome. SVAs, which are approximately 2 kb long, are composed of CCCTCT hexameric repeats that are followed by an inverted Alu-like region, a region containing a variable number of tandem repeats (VNTRs), and a partial HERV-K *env*-LTR sequence termed SINE-R that ends with a polyadenylation signal, followed by a poly(A) tail (Ostertag et al., 2003; **Figure 1A**, bottom). Unlike L1, Alu and SVA elements are non-autonomous since they do not encode functional reverse transcriptase or endonuclease; instead, they use the enzymatic machinery of L1 for retrotransposition. Once Alu and SVA elements have been transcribed and exported to the cytoplasm, they hijack the L1-encoded enzymes in the vicinity of the ribosomes through mechanisms that are as-yet unclear (**Figure 1C**; Dewannieux et al., 2003; Ostertag et al., 2003).

RETROTRANSPOSONS IN HUMAN DISEASES

Approximately 100 examples of disease-causing retrotransposon insertions are currently reported in the literature. It is estimated that *de novo* insertions of L1, Alu, and SVA elements are responsible for approximately 0.3% of all disease-causing human mutations, corresponding to event rates of 1:100, 1:20, and 1:900 births, respectively (Cordaux and Batzer, 2009). L1-induced genetic diseases include the following: Duchenne muscular dystrophy and X-linked dilated cardiomyopathy, resulting from insertions in the dystrophin gene (Narita et al., 1993; Yoshida et al., 1998); progressive chorioretinal degeneration, caused by the CHM gene disruption (van den Hurk et al., 2003); hemophilia A and B, due to insertions in the factor VIII and IX genes, respectively (Kazazian et al., 1988; Li et al., 2001; Mukherjee et al., 2004); and chronic granulomatous disease, the result of a mutation arising from an insertion in the CYBB

gene (Meischl et al., 2000). Genetic diseases linked to Alu integration events include neurofibromatosis via an insertion in the NF1 gene (Wallace et al., 1991; Wimmer et al., 2011); Apert syndrome, a severe autosomal dominant disorder, due to integration of the element into the fibroblast growth-factor receptor 2 (FGFR2) gene (Oldridge et al., 1999); and progressive renal failure (Dent's disease) due to disruption of the renal chloride channel (CLCN5) gene (Claverie-Martin et al., 2005). The involvement of SVA retrotransposition in human diseases has also been documented; namely, an insertion in the ARH gene leads to autosomal recessive hypercholesterolemia (Wilund et al., 2002); disruption of the BTK gene causes X-linked agammaglobulinemia (XLA; Rohrer et al., 1999); and disruption of the fukutin gene results in Fukuyama-type congenital muscular dystrophy (Kobayashi et al., 1998). Importantly, ongoing retrotransposon insertions seem to occur not only in germ cells and early embryos but also in brain tissues (Coufal et al., 2009; Baillie et al., 2011), somatic cells *in vitro* (Kubo et al., 2006; Rangwala et al., 2009), and somatic malignant tissues (Economou-Pachnis and Tschlis, 1985; Morse et al., 1988; Miki et al., 1992). Several reports have also shown retrotransposon-induced recombination in certain types of cancer (Schichman et al., 1994; Jeffs et al., 1998).

CELLULAR MECHANISMS LIMITING THE ACTIVITY OF RETROELEMENTS AND RETROVIRUSES

As noted above, since unrestricted retrotransposition would result in genome instability, eukaryotic organisms have developed several strategies to restrict these mobile elements. Firstly, retrotransposition can be regulated at the transcriptional level through several transcription factors. For example, L1 transcription is positively regulated by SOX11 (Tchenio et al., 2000), RUNX3 (Yang et al., 2003) and YY1 (Athaniar et al., 2004), and negatively regulated by SRY (Tchenio et al., 2000) and SOX2 (Muotri et al., 2005). DNA methylation by the methyl-CpG-binding protein MeCP2 results in the repression of L1 transcription in neurons (Walsh et al., 1998; Burden et al., 2005; Muotri et al., 2010). Secondly, retrotransposable elements are also susceptible to post-transcriptional regulation. For instance, endogenously encoded small interfering RNAs have been shown to reduce L1 retrotransposition *in vitro* (Soifer et al., 2005; Yang and Kazazian, 2006). Additionally, L1 transcripts that contain multiple polyadenylation signals lead to premature polyadenylation, resulting in the attenuation of L1 activity via truncation of its full-length transcripts (Perepelitsa-Belancio and Deininger, 2003). Thirdly, some cellular factors regulate retrotransposition at the post-translational level. In mice, the 3'-5' exonuclease Trex1 digests retroelement-derived DNA to suppress the autoimmune response (Stetson et al., 2008). Consistent with this, mutations in human Trex1 cause autoimmune diseases like familial chilblain lupus and Aicardi-Goutieres syndrome (Crow et al., 2006). Likewise, HIV-1 restriction factors such as the cytidine deaminases, the focus of this review, can inhibit L1 and Alu retrotransposition through a mechanism that is still unknown.

In humans, the cellular cytidine deaminase family comprises several members, including activation-induced cytidine

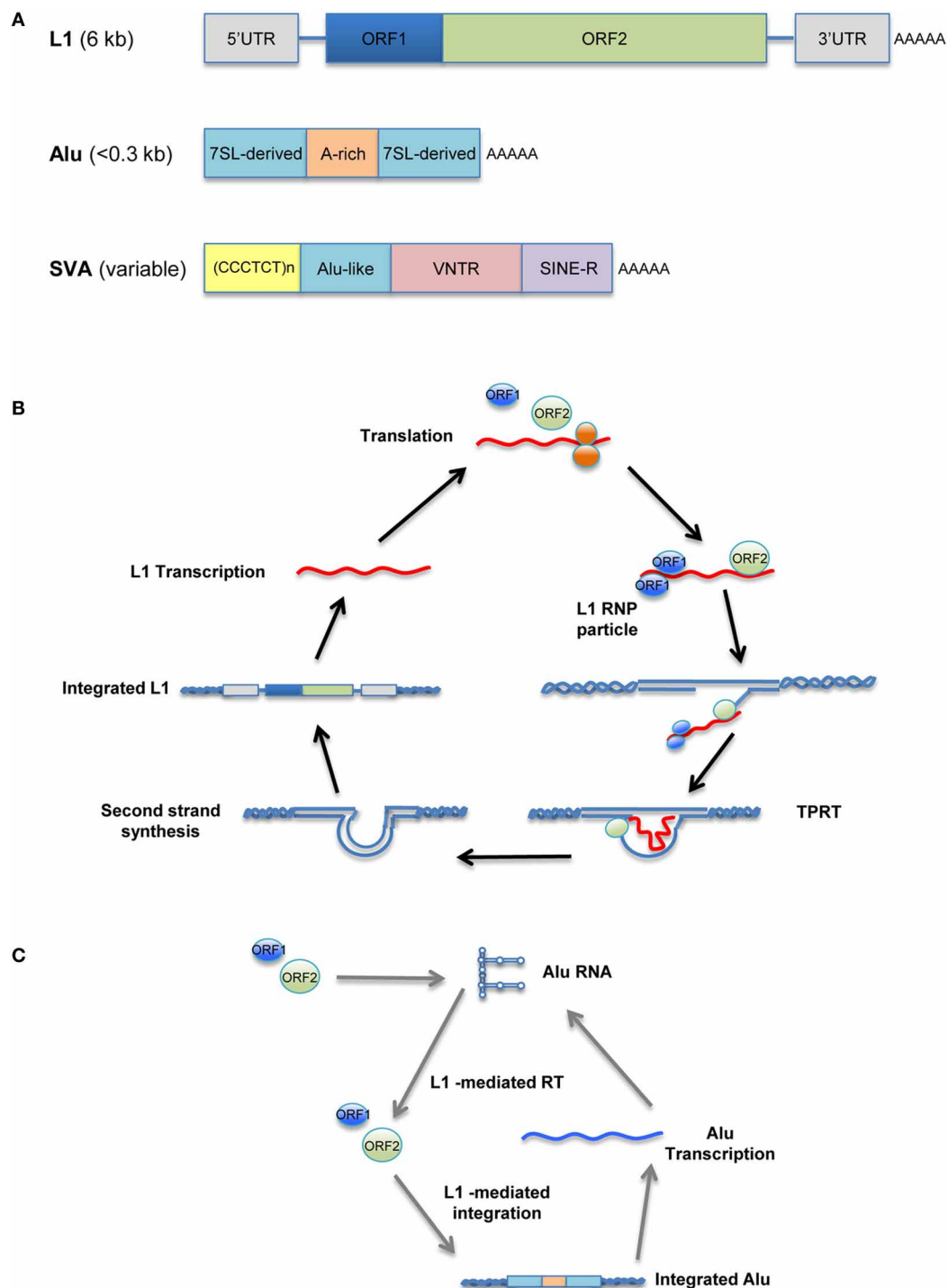


FIGURE 1 | Retrotransposition cycle. Schematic representation of active human retrotransposons. **(A)** Top: L1 genomic organization, from the left: 5'UTR, untranslated region; ORF-1, encoding an RNA-binding protein; linker region; ORF-2, encoding reverse transcriptase and endonuclease; 3'UTR; AAA, poly(A) tail. Middle: Alu organization, from the left: 7SL-derived monomer; A-rich linker, A₅TACA₆; 7SL-derived monomer; AAA, poly(A) tail. Bottom: SVA organization, from the left: (CCCTCT)_n, hexamer repeat; inverted Alu-like sequence; VNTR, variable number of tandem repeats; SINE-R, HERV-K-derived sequence; AAA, poly(A) tail. **(B)** Retrotransposition cycle: L1 elements are transcribed by

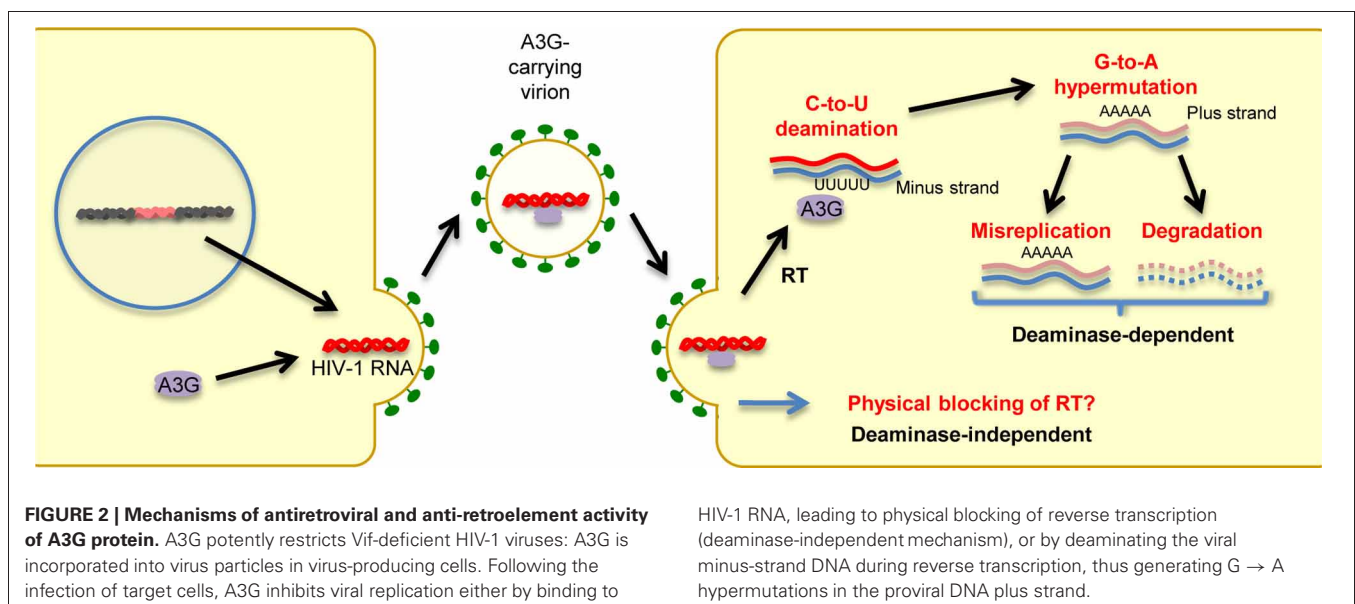
RNA-polymerase II from an L1 promoter sequence. The L1 mRNA template is exported to the cytoplasm and translated. Retrotransposon-encoded proteins actively bind the L1 RNA transcript, forming a ribonucleoprotein particle (RNP) that is imported back into the nucleus. There, the L1-encoded endonuclease nicks an L1 target sequence (5'-TTTT/AA-3') and the 3'-OH generated is used as a primer for target-primed reverse transcription (TPRT) by the L1-encoded reverse transcriptase, resulting in *de novo* integration into the host genome. **(C)** Alu as well as SVA elements are transcribed and hijack the L1-encoded enzymatic machinery to complete their respective retrotransposition cycles.

deaminase (AID), APOBEC1, APOBEC2, the A3 family, and APOBEC4 (Harris and Liddament, 2004; Conticello, 2008; Smith et al., 2012). APOBEC1 is the catalytic subunit of an RNA-editing complex that deaminates C⁶⁶⁶⁶ → U in the mRNA of the lipid-transport protein apolipoprotein B, thereby creating a premature stop codon that leads to a truncated protein in gastrointestinal tissues (Teng et al., 1993). APOBEC1 proteins from multiple small-animal species exhibit inhibitory activity against not only exogenous retroviruses (Ikeda et al., 2008) but also endogenous retroviruses, such as murine IAP and MusD sequences, as well as L1 elements (Ikeda et al., 2011). AID plays a role in the adaptive humoral immune system by inducing somatic hypermutations and class switch recombination, which allows affinity maturation and memory development; however, its precise mechanism of action remains to be determined (Honjo et al., 2005). As described in detail in a subsequent section, members of the A3 family are potent inhibitors of both exogenous retroviruses and endogenous retroelements. A3G, the most extensively studied member of the A3 family, was the first cytidine deaminase shown to restrict infection by Vif-deficient HIV-1 viruses. Briefly, as depicted in **Figure 2**, A3G is incorporated into budding virions and thus exerts its antiviral effect at the post-entry step in target cells, either by mediating extensive deamination of the minus-strand of viral DNA during reverse transcription, which results in G → A hypermutations in the proviral DNA plus strand (deaminase-dependent mechanism) (Harris et al., 2003; Mangeat et al., 2003; Zhang et al., 2003), or by binding to HIV-1 RNA, leading to physical impairment of reverse transcription (deaminase-independent mechanism; Newman et al., 2005; Bishop et al., 2006; Iwatani et al., 2007). Consequently, primate lentiviruses have evolved to counteract the antiretroviral activity of A3G by acquiring Vif. This accessory protein prevents A3G incorporation into virions through its proteasomal degradation (Marin et al., 2003; Sheehy et al., 2003; Stopak et al., 2003). We and others have shown that Vif proteins derived from different HIV-1 subtypes

differ in their potency of A3G inhibition, suggesting differential levels of viral fitness among clades (Iwabu et al., 2010; Binka et al., 2012). APOBEC2, a cardiac- and skeletal muscle-specific cytidine deaminase, is required for muscle development and early embryogenesis (Etard et al., 2010; Sato et al., 2010; Vonica et al., 2011). The physiological role of APOBEC4 remains to be determined.

DIFFERENTIAL ANTIVIRAL AND ANTI-RETROELEMENT ACTIVITIES OF A3 CYTIDINE DEAMINASES

Members of the A3 family contain either single (A3A, A3C, A3H) or double (A3B, A3DE, A3F, and A3G) cytidine deaminase domains (CDA). In A3G and A3F, the N-terminal CDA is responsible for RNA-dependent oligomerization, while the C-terminal CDA mainly mediates the deamination of single-stranded DNA (Hache et al., 2005; Newman et al., 2005). Some A3 family members strongly inhibit a wide range of exogenous retroviruses, as well as other viral pathogens, including herpesviruses, parvoviruses, papillomaviruses, and hepadnaviruses (Baumert et al., 2007; Vartanian et al., 2008; Narvaiza et al., 2009; Suspène et al., 2011b). The importance of A3 proteins *in vivo* has been demonstrated in murine studies in which mice lacking the A3 gene were shown to be more susceptible to viral infection than their wild-type counterparts (Okeoma et al., 2007, 2009; Takeda et al., 2008). A3 proteins also inhibit the mobilization of endogenous retroviruses, such as MusD, IAP, and the yeast LTR-retrotransposon Ty1 (Esnault et al., 2005; Schumacher et al., 2008), in addition to their inhibitory activity on L1 and Alu retrotransposition. The gene copy number of A3 family members is species-specific in mammals, in which except for primates, one, two, or three A3 proteins are encoded, whereas in humans and in non-human primates, seven A3 proteins have been recognized (A3A, A3B, A3C, A3DE, A3F, A3G, and A3H; Sawyer et al., 2004; OhAinle et al., 2006). Of note, expansion of the A3 gene cluster in primate genomes correlates with a



sharp reduction in retrotransposition activity, suggesting that these restriction factors have evolved to protect mammalian hosts from retroelements (Sawyer et al., 2004; Schumann, 2007). Antiretroviral and anti-retroelement potencies were shown to differ in the seven members of A3 family, independently of their subcellular localization (Kinomoto et al., 2007). However, the exact mechanism by which A3 proteins inhibit retrotransposition is unclear. The current findings on antiviral and anti-retroelement activities of A3 members are summarized below and in **Table 1**.

A3A

Human A3A (hA3A) lacks inhibitory activity against HIV-1 produced from 293T cells overexpressing this protein (Bishop et al., 2004; Bogerd et al., 2006a; Kinomoto et al., 2007; Hultquist et al., 2011), since it is not incorporated into virions (Goila-Gaur et al., 2007; Aguiar et al., 2008). In human monocytic cells as targets, however, hA3A blocks the early phase of HIV-1 infection (Peng et al., 2007; Koning et al., 2011) but is counteracted by the HIV-2/SIV (simian immunodeficiency virus) accessory protein Vpx (Berger et al., 2010, 2011). Also, hA3A can inhibit infections by adeno-associated virus (Chen et al., 2006; Narvaiza et al., 2009), human papillomavirus (HPV; Vartanian et al., 2008), porcine endogenous retrovirus (PERV; Dörrschuck et al., 2011), and human T-cell leukemia virus type 1 (HTLV-1; Ooms et al., 2012). Importantly, *in vitro* overexpression experiments have demonstrated that hA3A effectively inhibits the retrotranspositions of L1, Alu (Bogerd et al., 2006b; Chen et al., 2006; Muckenfuss et al., 2006; Kinomoto et al., 2007; Niewiadomska et al., 2007; Tan et al., 2009; Khatua et al., 2010; Ikeda et al., 2011), IAP, and MusD (Bogerd et al., 2006a; Chen et al., 2006; Ikeda et al., 2011) through a deaminase-independent mechanism. hA3A is intrinsically able to restrict infection of the genetically reconstituted HERV-K in a deaminase-dependent manner (Lee et al., 2008). In a recent report, hA3A was shown to induce somatic hypermutation in human mitochondrial and nuclear DNA; in the latter, this included genes associated with the development of cancer (Suspène et al., 2011a).

A3B

Human A3B (hA3B) is the sole member of the A3 family with an exclusive nuclear localization (Bogerd et al., 2006a; Muckenfuss et al., 2006; Stenglein and Harris, 2006; Kinomoto et al., 2007; Pak et al., 2011), sharing a common nuclear import mechanism with AID (Lackey et al., 2012). hA3B inhibits infections of HIV-1 and SIV, independently of the presence of Vif (Bishop et al., 2004; Yu et al., 2004; Doehle et al., 2005a; Hultquist et al., 2011). Since hA3B expression is extremely low in target CD4⁺ T-cells (Bishop et al., 2004; Doehle et al., 2005a; Koning et al., 2009; Refsland et al., 2010), Vif might have failed to evolve the mechanism to antagonize this antiretroviral factor. Also, hA3B restricts infections by murine leukemia virus (MLV; Doehle et al., 2005b; Kinomoto et al., 2007), PERV (Dörrschuck et al., 2011), HTLV-1 (Ooms et al., 2012), and Rous sarcoma virus (RSV; Wiegand and Cullen, 2007). Like hA3A, *in vitro* overexpression of hA3B inhibits the retrotranspositions of L1, Alu (Bogerd et al., 2006b; Muckenfuss et al., 2006; Stenglein

and Harris, 2006; Kinomoto et al., 2007; Niewiadomska et al., 2007; Khatua et al., 2010; Ikeda et al., 2011), IAP, and MusD (Bogerd et al., 2006a; Chen et al., 2006; Ikeda et al., 2011) in a deaminase-independent manner, while inhibiting the reconstituted HERV-K infection through a deaminase-dependent mechanism (Lee et al., 2008). In a recent study, endogenously expressed hA3B effectively restricted L1 retrotransposition in both transformed cells and human embryonic stem cells (Wissing et al., 2011).

A3C

Human A3C (hA3C) is abundantly expressed in numerous tissues and cell types (Jarmuz et al., 2002), and its expression is unresponsive to interferon- α (Koning et al., 2009). Although hA3C is efficiently incorporated into retroviral particles, it exhibits only partial antiviral activity against HIV-1, with or without Vif (Bishop et al., 2004; Yu et al., 2004; Bogerd et al., 2006a; Hultquist et al., 2011). By contrast, hA3C is able to efficiently block the replication of SIV, which also encapsidates this protein but is readily antagonized by SIV Vif (Yu et al., 2004). hA3C can inhibit infection of primate foamy virus (PFV), which carries an hA3 antagonistic Bet protein (Russell et al., 2005; Perković et al., 2009). The overexpression of hA3C results in a moderate inhibition of L1 and Alu retrotranspositions (Bogerd et al., 2006b; Chen et al., 2006; Muckenfuss et al., 2006; Kinomoto et al., 2007; Niewiadomska et al., 2007; Khatua et al., 2010) but effectively inhibits those of IAP, MusD, and Ty1 (Dutko et al., 2005; Chen et al., 2006). In a recent study, hA3C was shown to restrict infections by MLV (Langlois et al., 2005; Kinomoto et al., 2007), hepatitis B virus (HBV; Baumert et al., 2007; Köck and Blum, 2008), HPV (Vartanian et al., 2008), herpes simplex virus 1 and Epstein-Barr virus (Suspène et al., 2011b).

A3DE

Human A3DE (hA3DE) overexpression has moderate effects on L1 and Alu retrotransposition (Stenglein and Harris, 2006; Kinomoto et al., 2007; Niewiadomska et al., 2007; Tan et al., 2009; Duggal et al., 2011). Similarly, hA3DE exhibits low levels of anti-HIV-1 and anti-SIV activities, both of which are antagonized by the respective Vif proteins (Dang et al., 2006; Hultquist et al., 2011). The reduced activity is determined by a cysteine residue located at amino acid position 320 of hA3DE. Substitution with the corresponding tyrosine present in A3F resulted in a 20-fold increase of A3DE activity (Dang et al., 2011). Indeed, the chimpanzee version of A3DE, carrying a tyrosine residue at this position, shows much higher antiretroviral activity, while both human and chimpanzee A3DEs exhibit similar levels of inhibition against retroelements, suggesting that the host defense activity of A3DE against retroelements has been evolutionarily conserved (Duggal et al., 2011).

A3F/A3G

With regard to the antiretroviral potencies of human A3G (hA3G) and A3F (hA3F) proteins, overwhelming amount of information is well-summarized elsewhere (Harris and Liddament, 2004; Huthoff and Towers, 2008; Malim, 2009). Similar to hA3G, as introduced in the previous section, hA3F has been shown to

Table 1 | Antiviral and anti-retroelement spectrum of A3 family members.

A3 family	Exogenous		Endogenous	
	Non-retroviruses	Retroviruses	Non-human retroelements	Human retroelements
A3A (C/N)	AAV (Chen et al., 2006; Narvaiza et al., 2009)	HIV-1 (Peng et al., 2007; Koning et al., 2011; Schmitt et al., 2011)	IAP (Chen et al., 2006; Ikeda et al., 2011) MusD (Bogerd et al., 2006a; Chen et al., 2006)	L1 (Bogerd et al., 2006b; Chen et al., 2006; Muckenfuss et al., 2006; Kinomoto et al., 2007; Niewiadomska et al., 2007; Tan et al., 2009; Khatua et al., 2010; Ikeda et al., 2011)
	HPV (Vartanian et al., 2008)	SIV (Schmitt et al., 2011)	PERV (Dörrschuck et al., 2011)	Alu (Bogerd et al., 2006b; Muckenfuss et al., 2006; Niewiadomska et al., 2007; Tan et al., 2009; Khatua et al., 2010)
		HTLV-1 (Ooms et al., 2012)		Reconstituted HERV-K (Lee et al., 2008)
A3B (N)		HIV-1 (Bishop et al., 2004; Doehle et al., 2005a; Bogerd et al., 2006a; Kinomoto et al., 2007; Hultquist et al., 2011) SIV (Yu et al., 2004)	IAP (Bogerd et al., 2006a; Chen et al., 2006; Ikeda et al., 2011) MusD (Chen et al., 2006; Ikeda et al., 2011)	L1 (Bogerd et al., 2006b; Muckenfuss et al., 2006; Stenglein and Harris, 2006; Kinomoto et al., 2007; Niewiadomska et al., 2007; Khatua et al., 2010; Ikeda et al., 2011; Wissing et al., 2011)
		HTLV-1 (Ooms et al., 2012)	PERV (Dörrschuck et al., 2011)	Alu (Bogerd et al., 2006b; Muckenfuss et al., 2006; Stenglein and Harris, 2006; Niewiadomska et al., 2007)
		MLV (Doehle et al., 2005b; Kinomoto et al., 2007) RSV (Wiegand and Cullen, 2007)		Reconstituted HERV-K (Lee et al., 2008)
A3C (C/N)	HBV (Baumert et al., 2007; Köck and Blum, 2008) HPV (Vartanian et al., 2008)	HIV-1 (Bishop et al., 2004; Yu et al., 2004; Bogerd et al., 2006a; Hultquist et al., 2011)	IAP (Chen et al., 2006) MusD (Chen et al., 2006)	L1 (Bogerd et al., 2006b; Chen et al., 2006; Muckenfuss et al., 2006; Kinomoto et al., 2007; Niewiadomska et al., 2007; Khatua et al., 2010)
	HSV-1 (Suspène et al., 2011b)	SIV (Yu et al., 2004) PFV (Russell et al., 2005; Perković et al., 2009)	Ty1 (Dutko et al., 2005)	Alu (Bogerd et al., 2006b; Muckenfuss et al., 2006; Niewiadomska et al., 2007; Khatua et al., 2010)
	EBV (Suspène et al., 2011b)	MLV (Langlois et al., 2005; Kinomoto et al., 2007)		
A3DE (C)		HIV-1 (Dang et al., 2006; Hultquist et al., 2011)		L1 (Stenglein and Harris, 2006; Kinomoto et al., 2007; Niewiadomska et al., 2007; Duggal et al., 2011)
		SIV (Dang et al., 2006)		Alu (Tan et al., 2009)
A3F (C)		HIV-1 (Wiegand et al., 2004; Zheng et al., 2004; Bishop et al., 2006; Holmes et al., 2007; Yang et al., 2007)	IAP (Chen et al., 2006) MusD (Chen et al., 2006)	L1 (Turelli et al., 2004a; Muckenfuss et al., 2006; Stenglein and Harris, 2006; Kinomoto et al., 2007; Niewiadomska et al., 2007; Khatua et al., 2010)
		SIV (Bogerd et al., 2004; Mangeat et al., 2004; Schröfelbauer et al., 2004) XMRV (Paprotka et al., 2010)	Ty1 (Dutko et al., 2005; Schumacher et al., 2005) PERV (Dörrschuck et al., 2011)	Reconstituted HERV-K (Lee and Bieniasz, 2007; Lee et al., 2008)
		PFV (Russell et al., 2005; Delebecque et al., 2006) MLV (Langlois et al., 2005) RSV (Wiegand and Cullen, 2007) MPMV (Doehle et al., 2006)		

(Continued)

Table 1 | Continued

A3 family	Exogenous		Endogenous	
	Non-retroviruses	Retroviruses	Non-human retroelements	Human retroelements
A3G (C)	HBV (Turelli et al., 2004a; Köck and Blum, 2008)	HIV-1 (Harris et al., 2003; Mangeat et al., 2003; Zhang et al., 2003; Newman et al., 2005; Bishop et al., 2006)	IAP (Esnault et al., 2005; Ikeda et al., 2011)	L1 (Kinomoto et al., 2007; Niewiadomska et al., 2007; Khatua et al., 2010; Ikeda et al., 2011)
		SIV (Bogerd et al., 2004; Mangeat et al., 2004; Schröfelbauer et al., 2004)	MusD (Esnault et al., 2005; Chen et al., 2006; Schumacher et al., 2008; Ikeda et al., 2011)	Alu (Chiu et al., 2006; Hulme et al., 2007; Bulliard et al., 2009; Tan et al., 2009)
		MLV (Harris et al., 2003; Kobayashi et al., 2004; Doehle et al., 2005b; Langlois et al., 2005; Kinomoto et al., 2007)	Ty1 (Dutko et al., 2005; Schumacher et al., 2005)	Reconstituted HERV-K (Lee et al., 2008)
		XMRV (Groom et al., 2010; Paprotka et al., 2010)	PERV (Jónsson et al., 2007; Dörrschuck et al., 2011)	
		PFV (Russell et al., 2005; Delebecque et al., 2006)		
		MMTV (Okeoma et al., 2007)		
		EIAV (Bogerd et al., 2008)		
		HTLV-1 (Sasada et al., 2005; Fan et al., 2010)		
		RSV (Wiegand and Cullen, 2007)		
		MPMV (Doehle et al., 2006)		
A3H (C/N)	HPV (Vartanian et al., 2008)	HIV-1 (OhAinle et al., 2008; Harari et al., 2009; Li et al., 2010; Zhen et al., 2010; Wang et al., 2011)	PERV (Dörrschuck et al., 2011)	L1 (Kinomoto et al., 2007; OhAinle et al., 2008; Tan et al., 2009)
	HBV (Köck and Blum, 2008)	HTLV-1 (Ooms et al., 2012)		Alu (Tan et al., 2009)

(C), cytoplasmic localization; (N), nuclear localization; (C/N), diffuse cytoplasmic/nuclear localization; AAV, Adeno-associated virus; HPV, Human papillomavirus; HBV, Hepatitis B virus; HSV-1, Herpes simplex virus 1; EBV, Epstein-Barr virus; MLV, Murine leukemia virus; RSV, Rous sarcoma virus; PFV, Primate foamy virus; XMRV, Xenotropic murine leukemia virus-related virus; MPMV, Mason-Pfizer Monkey Virus; MMTV, Mouse mammary tumor virus; HTLV-1, Human T-cell leukemia virus type 1; EIAV, Equine infectious anemia virus; IAP, Intracisternal A particles; PERV, Porcine endogenous retrovirus; L1, Long interspersed element 1; HERV-K, Human endogenous retrovirus K.

potently restrict the replication of Vif-deficient HIV-1 viruses in target cells after its incorporation into budding virions through both deaminase-dependent and -independent mechanisms (Bishop et al., 2004; Wiegand et al., 2004; Zheng et al., 2004; Holmes et al., 2007; Yang et al., 2007). The *in vitro* overexpression of hA3G inhibits not only retroviral infections, such as those by HTLV-1 (Sasada et al., 2005; Fan et al., 2010), SIV (Bogerd et al., 2004; Mangeat et al., 2004; Schröfelbauer et al., 2004), PFV (Russell et al., 2005; Delebecque et al., 2006), equine infectious anemia virus (Bogerd et al., 2008), MLV (Harris et al., 2003; Kobayashi et al., 2004; Doehle et al., 2005b; Langlois et al., 2005; Kinomoto et al., 2007), Mason-Pfizer monkey virus (MPMV; Doehle et al., 2006), xenotropic murine leukemia virus-related virus (XMRV; Groom et al., 2010; Paprotka et al., 2010), PERV (Jónsson et al., 2007; Dörrschuck et al., 2011), and RSV (Wiegand and Cullen, 2007), but also retrotranspositions of

non-human LTR retroelements, such as IAP, MusD, and Ty1 (Dutko et al., 2005; Esnault et al., 2005; Chen et al., 2006; Schumacher et al., 2008; Ikeda et al., 2011). This cytidine deaminase is also effective against HBV (Turelli et al., 2004a; Köck and Blum, 2008). With regard to potential to inhibit L1 retrotransposition, conflicting results have been reported; some lines of evidence suggest that hA3G has anti-Alu activity (Chiu et al., 2006; Hulme et al., 2007; Bulliard et al., 2009; Tan et al., 2009) but little or no anti-L1 activity (Turelli et al., 2004b; Bogerd et al., 2006b; Muckenfuss et al., 2006; Stenglein and Harris, 2006). We and others, however, have shown that hA3G is also able to restrict L1 retrotransposition, albeit less potently than hA3A or hA3B, through deaminase-independent mechanisms (Kinomoto et al., 2007; Niewiadomska et al., 2007; Khatua et al., 2010; Ikeda et al., 2011). These discrepancies might be due to cell-type differences in hA3 protein expression levels, as

we have described previously (Kinomoto et al., 2007). A putative mechanism of L1 inhibition by hA3G is as follows: When L1 forms the RNP complex in the cytoplasm (**Figure 1B**, right half), cytoplasmic hA3G protein might be able to access the complex through the interaction with L1 RNA, and then enter the nucleus together with the complex. This could result in the effective inhibition of L1 reverse transcription, by physically blocking the access to the chromosomal DNA, or by impeding the movement of the reverse transcriptase on a template L1 RNA. In the case of the infection by reconstituted HERV-K, hA3G carries out deamination showing only marginal inhibition of infectivity (Lee et al., 2008). Among the viruses and retroelements described above, hA3F is known to inhibit infections of PFV (Russell et al., 2005; Delebecque et al., 2006), MLV (Langlois et al., 2005), XMRV (Paprotka et al., 2010), MPMV (Doehle et al., 2006), PERV (Dörrschuck et al., 2011), RSV (Wiegand and Cullen, 2007), and reconstituted HERV-K (Lee and Bieniasz, 2007; Lee et al., 2008), as well as the retrotransposons; L1 (Chen et al., 2006; Muckenfuss et al., 2006; Stenglein and Harris, 2006; Kinomoto et al., 2007; Niewiadomska et al., 2007; Khatua et al., 2010), Ty1 (Dutko et al., 2005; Schumacher et al., 2005), MusD and IAP (Chen et al., 2006).

A3H

Human A3H (hA3H) is the most distantly related of the hA3 members and is known for its functional polymorphisms. Currently, four major haplotypes (I–IV) have been identified in human populations, among which haplotype I has the highest allelic frequencies (OhAinle et al., 2008). Haplotypes I, III, and IV generate unstable proteins with very little, if any, antiretroviral and anti-retroelement activity. Haplotype II, however, expresses a stable protein with relatively high inhibitory activity on HIV-1 (OhAinle et al., 2008; Harari et al., 2009; Li et al., 2010; Zhen et al., 2010; Wang et al., 2011) and HTLV-1 (Ooms et al., 2012), and its overexpression effectively restricts L1 retrotransposition (OhAinle et al., 2008; Tan et al., 2009). These observations suggest that the relative lack of anti-retroviral and anti-retroelement potencies in hA3H is not due to insufficient enzymatic activity but to the instability of the protein. It should be noted that hA3H haplotype II is mainly localized to the cytoplasm, while

the haplotype I protein passively diffuses into the nucleus (Li and Emerman, 2011). The ability of hA3H to block infections of HPV (Vartanian et al., 2008), HBV (Köck and Blum, 2008), and PERV (Dörrschuck et al., 2011) has also been reported, although the responsible haplotypes have not been described.

CONCLUDING REMARKS

Retrotransposable elements have successfully proliferated over tens of millions of years of mammalian evolution, such that they now constitute 45% of the human genome. Retrotransposition spreads DNA fragments to different genomic sites and is thus considered to be one of the driving forces in genome evolution by contributing to the formation of new genes. On the other hand, the price to pay for such genomic innovation, in which retrotransposons integrate in their host genomes, is the potential disruption of essential genes, resulting in deleterious effects, some of which are clearly associated with genetic diseases and tumorigenesis. Consequently, to prevent uncontrolled retrotransposition, host organisms have evolved several defense mechanisms. Among these, the seven members of A3 family have the ability to restrict not only a broad range of exogenous retroviruses but also endogenous retroelements, as described herein. Interestingly, high-level A3 expression is seen in the testis and ovary and in embryonic stem cells (Jarmuz et al., 2002; Bogerd et al., 2006b; OhAinle et al., 2006), in which the retroelements are hypomethylated and therefore active (Bourc'his and Bestor, 2004; Dupressoir and Heidmann, 1996). These findings support the evolutionary acquisition of A3 proteins to protect these cells primarily from the genomic instability caused by the disruptive effect of endogenous retroelements. Further investigations of A3-mediated intrinsic immunity are likely to provide insights into the molecular mechanisms of the host defenses that do not allow retrotransposons to escape from the seven members of A3.

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Intrinsic immunity against retrotransposons by APOBEC cytidine deaminases

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Over 40% of the human genome is recognizable as having been derived from ancient retroelements, transported by an intracellular copy-and-paste process involving an RNA intermediate, with an additional few percent classified as DNA transposable elements. Endogenous retroviruses are long terminal repeat (LTR)-type retroelements that account for ~8% of human genomic DNA. Non-LTR members are present at extremely high copy numbers, with ~17% of the human genome consisting of long interspersed nuclear elements (LINEs). These LINEs modify vertebrate genomes not only through insertions, but also by the indirect replication of non-autonomous retrotransposons, such as short interspersed nuclear elements. As expected, vertebrate intrinsic immunity has evolved to support a balance between retroelement insertions that confer beneficial genetic diversity and those that cause deleterious gene disruptions. The mammalian cytidine deaminases encoded by the *APOBEC3* genes can restrict a broad number of exogenous pathogens, such as exogenous retroviruses, and the mobility of endogenous retroelements. Furthermore, APOBEC1 from a variety of mammalian species, which mediates the cytidine (C) to uridine (U) deamination of apolipoprotein B (apoB) mRNA, a protein involved in lipid transport, also plays a role in controlling mobile elements. These mammalian apoB mRNA-editing, catalytic polypeptide (APOBEC) cytidine deaminases, which can bind to single-stranded DNA (ssDNA) as well as RNA, are able to insert mutations into ssDNA and/or RNA as a result of their ability to deaminate C to U. While these APOBEC cytidine deaminases with DNA mutagenic activity can be deleterious to cells, their biological modifications, such as protein–protein interactions and subcellular localization, in addition to their ability to bind to RNA, appear to have conferred a role for APOBECs as a cellular defense system against retroviruses and retroelements. In support of this notion, the expansion of the single *APOBEC3* gene in mice to the seven *APOBEC3* genes found in primates apparently correlates with the significant enhancement of the restriction of endogenous retroelements seen in primates, including humans. This review discusses the current understanding of the mechanism of action of APOBEC cytidine deaminases and attempts to summarize their roles in controlling retrotransposons.

Keywords: APOBEC1, APOBEC3, AID, retrovirus, HIV-1, LINE-1, retroelements, endogenous retrovirus

INTRODUCTION

The ability of members of the apolipoprotein B (apoB) mRNA-editing, catalytic polypeptide (APOBEC) family to confer intrinsic immunity against mobile elements was initially recognized for human APOBEC3G, which can block the replication of a human immunodeficiency virus type 1 (HIV-1) mutant lacking the virus infectivity factor (*vif*) gene (Sheehy et al., 2002). APOBEC3 cytidine deaminases form one element of the cellular machinery that plays a role in the intrinsic restriction of two distinct classes of endogenous retroelements: non-long terminal repeat (non-LTR) retroelements, such as long interspersed nuclear elements (LINEs) and LTR retrotransposons (for reviews, see Holmes et al., 2007; Chiu and Greene, 2008; Koito and Ikeda, 2011, 2012). There is increasing evidence supporting the notion that the primary function of APOBEC3 cytidine deaminases could be to prevent the propagation of these intracellular mobile elements. Furthermore,

APOBEC1 from non-human mammals, such as rodents and rabbits, has prominent intrinsic immune functions, regulating retroelements including HIV-1 in addition to its integral roles in editing its primary substrate, apoB mRNA (Bishop et al., 2004; Ikeda et al., 2008, 2011; Petit et al., 2009).

Increasingly detailed sequence analyses have revealed that a large portion of the mammalian genome is composed of non-LTR retrotransposons, with LINE-1 (L1), the most common LINEs, contributing to >35% of the mass of the mammalian genomes (Lander et al., 2001; Waterston et al., 2002; Gibbs et al., 2004). Non-LTR retrotransposons, also called target-primed (TP) retrotransposons (Beauregard et al., 2008), predominantly undergo reverse transcription in the nucleus. These autonomous TP retrotransposons have modified host genomes not only by creating insertions, but also by their ability *in trans* to mediate the retrotransposition of cellular mRNAs to generate processed

pseudogenes (copies of genes that are no longer functional) and short interspersed nuclear elements (SINEs). These SINE retrotransposons further constitute one of the main components of the genomic repetitive fractions.

On the other hand, the replication cycle of LTR retrotransposons, also called extrachromosomally-primed (EP) retrotransposons (Beauregard et al., 2008), is different, in which reverse transcription with the formation of virus-like particles (VLPs) occurs exclusively in the cytoplasm of infected cells. LTR retrotransposons, also called endogenous retroviruses (ERVs), which are structurally similar to HIV-1 and other infectious retroviruses, entered the germ line as infectious retroviruses at several time points during the evolution of many organisms. These mobile elements have been inherited through successive generations in the classical Mendelian manner and have been accumulated by reinfection and/or retrotransposition throughout evolution in the host genomes. This review summarizes and discusses the advances in the general knowledge of the APOBEC family proteins as a cellular defense mechanism against endogenous invaders of the genome.

APOBEC FAMILY MEMBERS AS RESTRICTION FACTORS FOR NON-LTR RETROTRANSPOSONS

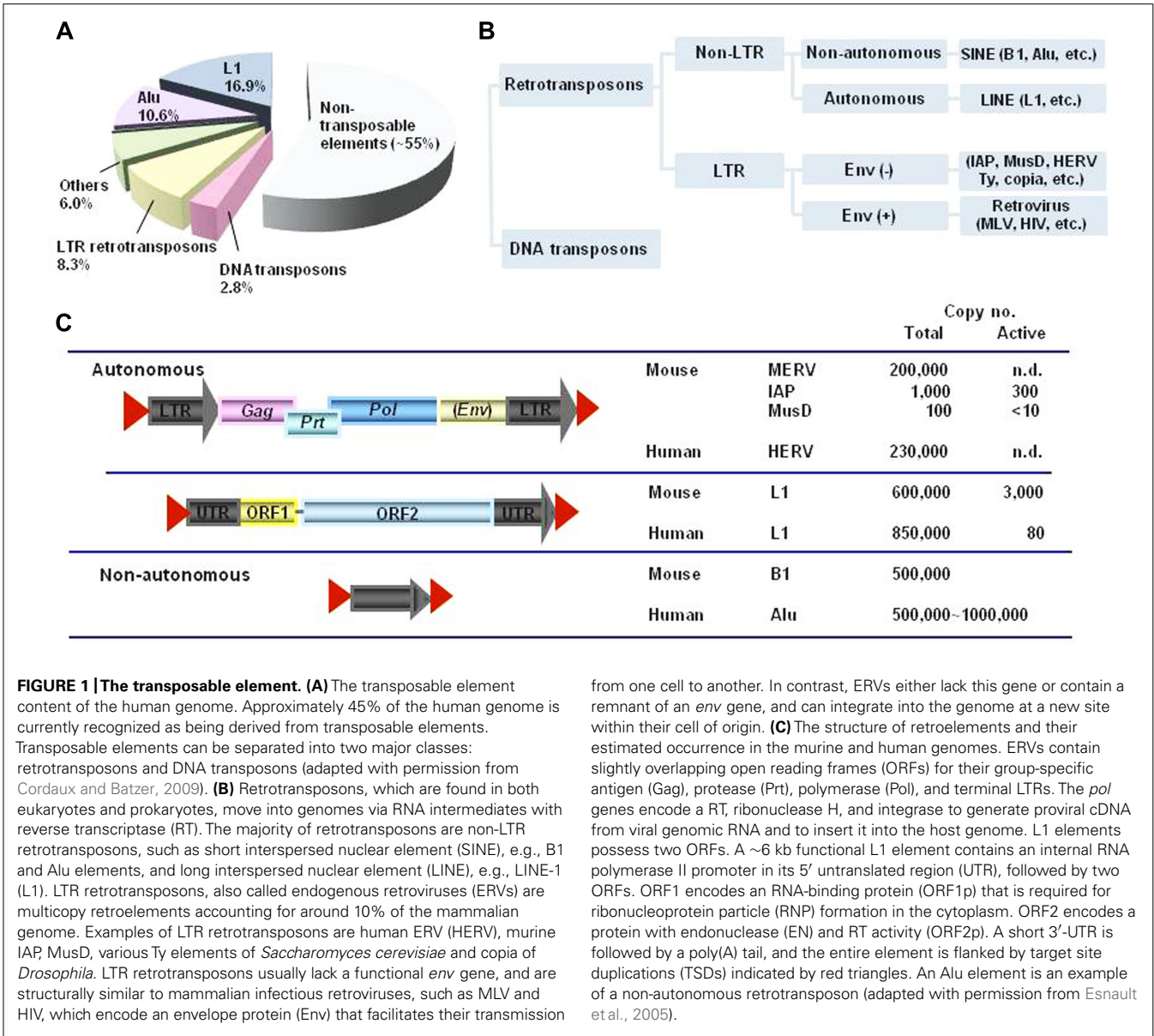
LINE-1 element is an autonomous retroelement, and comprises large fractions of the mammalian genomes (Figure 1). L1 is transcribed by RNA polymerase II to give a ~6-kb mRNA that encodes two open reading frames (ORF1p and ORF2p; Moran et al., 1996; Figure 2). ORF1p binds its own RNA to form a ribonucleoprotein (RNP) complex. In addition, ORF1p has a nucleic acid chaperon activity (Kolosha and Martin, 2003), which is also required for L1 retrotransposition (Martin et al., 2005). ORF2p has an endonuclease (EN) and reverse transcriptase (RT) domain, and forms a large RNP complex with the L1 RNA and ORF1p (Mathias et al., 1991; Feng et al., 1996; Kulpa and Moran, 2006). These structural alignments are well conserved in LINE-like elements from fish to mammals, although only mammals appear to limit L1 evolution to a single lineage (Furano et al., 2004). A comprehensive phylogenetic analysis based on the RT domain indicated that the LINES can be divided into 11 distinct clades, and that the entire group was likely present at the beginning of the evolution of eukaryotes (Malik et al., 1999). An L1 homolog from lower eukaryotes was demonstrated to be functional, indicating that L1s originated in the lower eukaryotes and expanded in many vertebrate species (Dong et al., 2009). These L1 retrotranspositions in various organisms have played, and continue to play, a significant role in shaping the host genomes through insertional mutagenesis, non-allelic recombination, and by mobilization *in trans* of non-L1 RNAs, such as SINEs (Bannert and Kurth, 2004; Kazazian, 2004; Cordaux and Batzer, 2009).

A family of host proteins that has been demonstrated to play a key role in the innate restriction of non-LTR retrotransposons is APOBEC. All members of human APOBEC3 family, APOBEC3A–APOBEC3H inhibit L1 to varying degrees (Kinomoto et al., 2007; Niewiadomska et al., 2007), with APOBEC3A and APOBEC3B being the most potent. Interestingly, the mechanisms underlying antiretroviral and anti-retrotransposon inhibition by the APOBEC family proteins appeared to differ, with the latter

being independent of enzymatic activity. Similar DNA editing-independent anti-L1 activity had been reported for activation induced deaminase (AID) and APOBEC1 proteins in multiple mammalian species (MacDuff et al., 2009; Ikeda et al., 2011). The replication cycle of non-LTR retrotransposon L1 differs from that of LTR retroelements, with reverse transcription occurring within the cytoplasm that results in the formation of identifiable VLPs (Figure 2). To date, the exact step of the L1 replication cycle targeted by the APOBEC and other DNA editing-independent L1 restriction machineries has yet to be determined.

It has been documented that human APOBEC3G interacts with cellular RNAs; mRNAs, tRNAs, and rRNAs, and almost 100 different cellular RNA binding proteins to assemble into high-molecular-mass (HMM) RNP complexes that are converted to a low-molecular-mass (LMM) form by RNase treatment (for a review, see Chiu and Greene, 2008), although intracellular HMM complex formation does not appear to be a common feature among APOBEC family proteins. APOBEC3A was reported to localize in both the nucleus and the cytoplasm and to become associated with HMM complexes in the presence of L1 (Niewiadomska et al., 2007). APOBEC1 proteins were also found to exist in an HMM form in both the presence and absence of L1 (Ikeda et al., 2011). Notably, and in sharp contrast to APOBEC3G, the distribution of APOBEC1s was not affected by RNase treatment, suggesting that this single-domain cytidine deaminase may interact differently and/or more strongly with cellular RNAs.

Additionally, a homogenous cytoplasmic distribution of APOBEC3 proteins, along with discrete cytoplasmic foci referred to as mRNA-processing bodies (P-bodies), which involved in host mRNA degradation, translational repression, and microRNA-mediated RNA-silencing machinery has been demonstrated (Kozak et al., 2006; Wichroski et al., 2006; Gallois-Montbrun et al., 2007). The accumulation of APOBEC3 proteins in P-bodies can be explained by several possible mechanisms. The simplest possibility is that the concentration of APOBEC3 proteins reflects their binding to a subset of endogenous RNAs, which are translationally repressed and accumulate in P-bodies. For example, APOBEC3 proteins might interact with transcripts from endogenous retroelements, and the transcripts might be expected to be translationally repressed by miRNAs, thereby accumulating in P-bodies (Klattenhoff and Theurkauf, 2008). However, it appears that the inhibitory activity of APOBEC3 proteins against L1 retrotransposition does not correlate with the intracellular HMM formation or P-body association (Niewiadomska et al., 2007). Interactions between APOBEC3s and Ago1 and Ago2, proteins associated with the RNA interference pathway, were demonstrated (Gallois-Montbrun et al., 2007). Further, APOBEC3 proteins appeared to play a role in preventing the decay of miRNA-targeted mRNA from P-bodies, thus allowing for translation of these mRNA (Huang et al., 2007). These observations suggest that the recruitment of APOBEC family proteins into cellular sites of RNA metabolism and RNA-silencing pathways may represent one mechanism for regulating its activity as an inhibitor of retroelement mobility, and as a possible regulator of cellular RNA function. P-body associated host factor Moloney leukemia virus 10 (MOV10), an RNA helicase that belongs to the DExD box superfamily, is demonstrated to regulate L1 mobilization (Arjan-Odedra



et al., 2012). In order to clarify the molecular mechanism through which L1 retrotransposition is inhibited, mainly in a deamination-independent manner, it is necessary to identify the exact step of L1 replication that is affected by these APOBEC proteins. Elucidation of this deamination-independent repressive activity of APOBECs on L1 retrotransposition may provide new insights into the consequences of deamination-independent HIV-1 inhibition by APOBEC3 proteins.

Despite the impact of L1 insertion on mammalian genome evolution, much of the L1 retrotransposition process, especially *in vivo*, remains unexplored. The majority of L1s are inactive due to the truncation, point mutations, and other rearrangements; however, it is estimated that the mouse and human genomes harbor 3,000 and ~100 copies of retrotransposition-competent L1 elements, respectively (Figure 1; Bannert and Kurth, 2004; Kazazian, 2004; Cordaux and Batzer, 2009). L1

retrotransposition has been demonstrated to result in the generation of novel polymorphisms in mammalian genomes, as well as a broad range of sporadic diseases in humans, including hemophilia A, Duchene muscular dystrophy, β -thalassemia, and colon cancer (Hancks and Kazazian, 2012). It was demonstrated that L1 RNA assembled into its RNP complex might be stable and could be carried over through fertilization using L1 transgenic rodent models, suggesting that the majority of *de novo* L1 retrotransposition usually occurs in early embryonic development (Kano et al., 2009). This scenario indicates that germ cells should have evolved several post-transcriptional defense mechanisms that strictly prevent the integration of transcribed L1 RNA into the genome. These defense mechanisms include post-transcriptional silencing via RNA interference (Yang and Kazazian, 2006), and APOBEC-mediated machinery may also contribute to the control of L1 retrotransposition in both early embryos and germ cells.

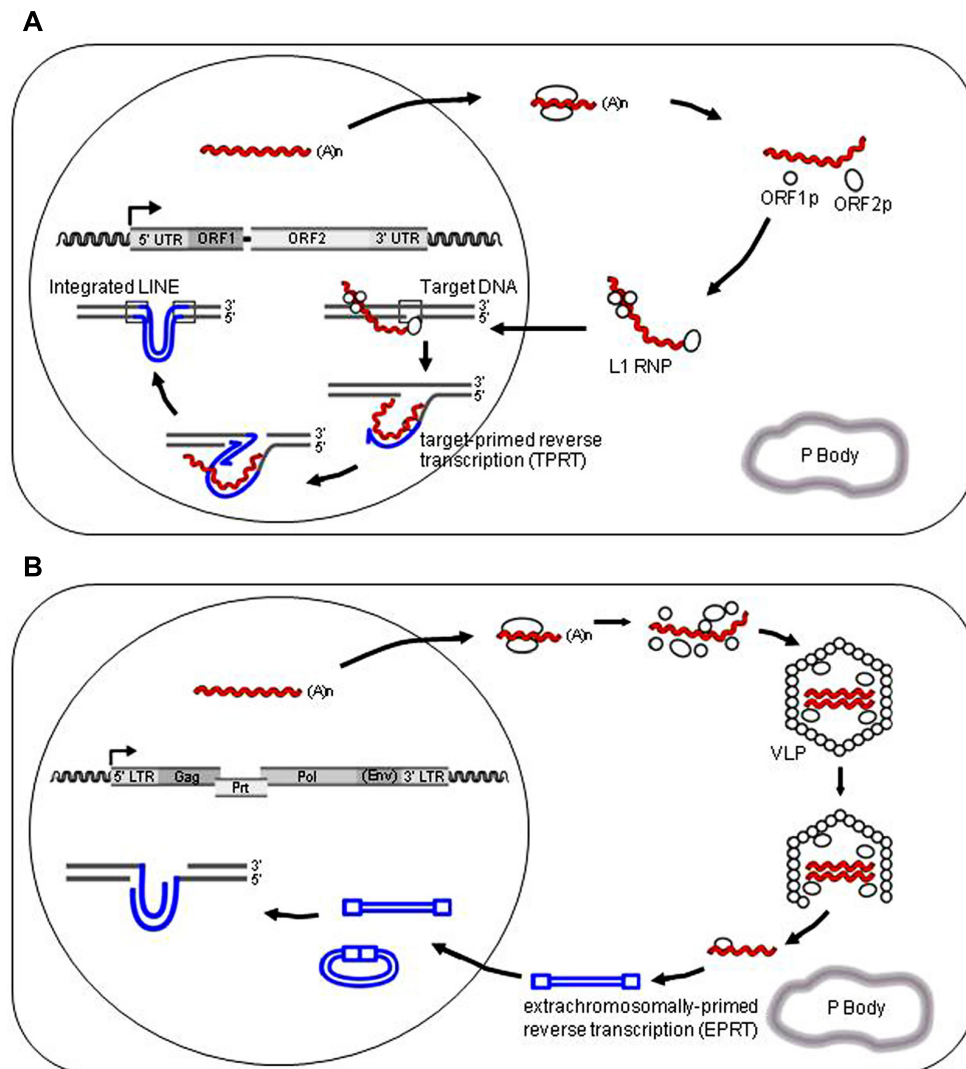


FIGURE 2 | Models for the retrotransposition cycle of retroelements.

(A) The retrotransposition pathway of L1 retroelements. A functional full-length L1 element contains an internal promoter in the 5' untranslated region (5' UTR) that initiates transcription. This is followed by two long open reading frames, ORF1 and ORF2, required for retrotransposition *in cis*. A short 3' UTR is followed immediately by a poly(A) tail, and the entire element is typically flanked by target site duplications. ORF1p and ORF2p preferentially associate with their own encoding RNAs ("cis preference") to form a RNP complex in the cytoplasm as a retrotransposition intermediate, and are critical for retrotransposition by a "copy and paste" mechanism. L1 DNA synthesis in the nucleus is based on "target-primed reverse transcription (TPRT)" in which ORF2p nicks the target chromosomal DNA, and then uses the resultant 3'-OH to prime the reverse transcription of L1 RNA as a template. Human APOBEC3 proteins have been documented to associate with stress granules, Staufen granules, or P-bodies (gray enclosure); however, it appears that the inhibitory activity of human

APOBEC3 proteins against L1 retrotransposition does not correlate with their P-body association. L1 genomic RNA and reverse-transcribed DNA are indicated by red and blue colors, respectively.

(B) The retrotransposition pathway of LTR-type retrotransposons (or endogenous retroviruses, ERVs), e.g., murine IAP, MusD, and yeast Ty1. The life cycle of these ERVs includes the formation of virus-like particles (VLPs) that remain intracellular. Reverse transcription of ERVs genomic RNA occurring in the cytoplasm is called "extrachromosomally-primed reverse transcription (EPRT)", and is a complicated, multistep process. Reverse-transcribed single-stranded DNA is thought to be sensitive to APOBEC-mediated deamination activity. The proviral copies that have escaped degradation can integrate, but exhibit G-to-A mutations that render them defective for subsequent rounds of retrotransposition. P-bodies can influence the life cycle of ERVs and HIV-1 in either a positive or negative manner. ERVs genomic RNA and reverse-transcribed DNA are indicated by red and blue colors, respectively.

In accord with this scenario, APOBEC3s mRNA is expressed in germ cells (Jarmuz et al., 2002; Mikl et al., 2005; Koning et al., 2009). Furthermore, APOBEC1 mRNA is expressed in germ cells in multiple mammalian species (Greeve et al., 1993; Ikeda et al., 2011), placing both APOBEC3 and APOBEC1 in a compartment

where endogenous retroelements may have the greatest impact *in vivo*.

Short interspersed nuclear elements were also demonstrated to be sensitive to the restriction activity by human APOBEC3 family members (Bogerd et al., 2006a; Hulme et al., 2007; Tan et al., 2009).

SINEs are transcribed by RNA polymerase III to give a ~300-nt non-coding RNA (Batzer and Deininger, 2002). Retrotransposition of non-autonomous retrotransposon SINEs depends on the L1 ORF2p with EN and RT activities (Dewannieux et al., 2003). It was reported that APOBEC3s do not require direct interactions with ORF1p with no known specific role in the L1 replication mechanism to inhibit the L1 retrotransposition (Lovsin and Peterlin, 2009). On the other hand, the interactions of ORF2p with APOBEC family proteins have not been addressed, thus far, since ORF2p within the cells is difficult to detect, even in the context of overexpression systems (Goodier et al., 2004). Therefore, the precise step(s) during which both LINE and SINE retrotransposition are affected by APOBEC family proteins are still unknown.

APOBEC1, as well as APOBEC3A and APOBEC3G, are able to inhibit nascent L1 DNA accumulation, suggesting that L1 reverse transcription, integration, and/or the intracellular movement of L1 RNPs are affected by these APOBEC enzymes (Kinomoto et al., 2007; Ikeda et al., 2011). The suppressive activity of these molecules against *de novo* L1 DNA synthesis occurred mainly in a deamination-independent manner, and was not affected by the subcellular localization of the proteins (Ikeda et al., 2011). It has not been described whether APOBECs interact with specific sequences in the Alu and L1 genomic RNA and/or L1-encoded ORF2p and/or host factor(s) that facilitate retrotransposition. These interactions might be able to interfere with the subsequent transport and/or nuclear import of cytoplasmic RNPs (for reviews, see Koito and Ikeda, 2011).

Although these genetic transposable elements have been mainly considered to be molecular fossils until recently, SINEs, which include murine B1 and human Alu elements, appear to play roles in the regulation of gene expression, the stress response, and proteome diversity (Chu et al., 1998; Ponicsan et al., 2010). This L1-mediated Alu retrotransposition has also been demonstrated to result in human diseases such as cancer (Dewannieux et al., 2003; Konkel and Batzer, 2010). Current studies are further emphasizing that SINEs insertions are involved in organizing and regulating intricate transcriptional pathways by dispersal of CCCTC-binding factor (CTCF), which acts as a master regulator of mammalian genomic boundaries that helps establish vertebrate insulators (Schmidt et al., 2012). Of note, increasingly detailed analyses of primate genomes informed that human genome contains threefold more abundant Alu sequences than that of the chimpanzee (Mikkelsen et al., 2005). Alu insertions appear to be particularly active in the human lineage after human–chimpanzee divergence, where they likely contribute to shaping some of the human-specific characteristics, such as brain size (Britten, 2010).

On the other hand, the L1 sequences of a transcript were demonstrated to possess a strong A-rich bias in the sense strand and serve as an evolutionary fine-tuner of the mammalian transcriptome by significantly decreasing RNA expression, and therefore protein expression (Han et al., 2004). Because L1 is also an abundant and broadly distributed mobile element, the inhibition of transcriptional elongation by L1 might profoundly affect the expression of endogenous human genes. Interestingly, recent studies further suggested that somatic genome mosaicism driven by L1 retrotransposition in the brain may influence brain activity (Bailly et al., 2011). The rapid expansion of non-LTR retrotransposons

is likely to have had a major impact on the landscape and plasticity of the host genome, and significantly increased the rate of mammalian evolution, especially that of primates. Current hypotheses predict that the rapid expansion of L1 and Alu elements exerted strong positive selective pressure that resulted in the rapid evolution of APOBEC3s in primates around 30–50 million years ago (Jarmuz et al., 2002; Zhang and Webb, 2004).

APOBEC FAMILY MEMBERS AS RESTRICTION FACTORS FOR LTR RETROTRANSPOSONS

Retroviruses that integrate into the germ line may be inherited vertically as ERVs, also known as LTR retrotransposons. Around 10% of the mammalian genomes is composed of these ERV elements (**Figure 1**), but the majority of them have been sufficiently degraded over time through mutations and deletions so that they are incapable of expressing infectious viruses (Bannert and Kurth, 2004; Kazazian, 2004; Jern and Coffin, 2008).

Following the discovery of the restriction activity of APOBEC3 proteins against HIV-1, similar DNA editing-dependent activities against murine ERVs, such as the intracisternal A particle (IAP) and MusD, were documented (Esnault et al., 2005, 2006; Bogerd et al., 2006b; Schumacher et al., 2008). The life cycle of IAP and MusD includes the formation of VLPs and reverse transcription in the cytoplasm of infected cells (**Figure 2**). IAP and MusD lack an extracellular phase and are not infectious, due to the absence of a functional *env* gene. The mouse genome contains numerous copies of IAP and MusD, of which about 300 IAP and 10 MusD copies are still active for autonomous intracellular retrotransposition (**Figure 1**; Dewannieux et al., 2004; Ribet et al., 2004). IAP and MusD mRNAs are expressed in germ cells, during early embryogenesis and in various tumor cells, and IAP VLPs are demonstrated to assemble and bud at the endoplasmic reticulum (ER) membrane (Heidmann and Heidmann, 1991; Dupressoir and Heidmann, 1996; Baust et al., 2003). In humans, IAP-like VLPs were detected in salivary tissues and in peripheral blood mononuclear cells, and appeared to be associated with Sjögren's syndrome and CD4⁺ T cell deficiencies, respectively (Garry et al., 1990; Gupta et al., 1992). IAP insertions can lead to mutations and contribute to pathological processes. Therefore, it is critical for host cells to maintain their retrotransposition at low levels in order to maintain genome stability.

The inhibitory activity of APOBEC3 proteins against IAP and MusD appeared to be based, at least in part, on cytidine deamination. Consistent with reports indicating that the inhibitory activity of APOBEC3 proteins against exogenous retrovirus, such as HIV-1, was mediated by their selective incorporation into retroviral particles through an RNA-dependent interaction with the Gag protein, direct interactions between APOBEC3 proteins and IAP Gag have been demonstrated (Bogerd et al., 2006b). The molecular mechanisms responsible for the editing of reverse-transcribed DNA from endogenous and exogenous retroviruses appeared to overlap (Esnault et al., 2005; Bogerd et al., 2006b). Further, a genetic analysis demonstrated that some endogenous murine leukemia viruses (MuLVs) in the C57BL/6J genome bear the signatures of mutations induced by the murine APOBEC3 protein (Jern et al., 2007), indicating that these ERVs (MERVs) have been in conflict with APOBEC3 during murine evolution. The APOBEC3 enzyme was

demonstrated to be dispensable for mouse development, survival and fertility (Mikl et al., 2005), although APOBEC3-knockout mice were more susceptible to Moloney MuLV (M-MuLV; Takeda et al., 2008; Low et al., 2009) and mouse mammary tumor virus (MMTV) replication (Okeoma et al., 2007). Although the murine APOBEC3 expressed in germ cells appears to be the likely mediator of the hypermutations observed in the MERVs, the participation of other cytidine deaminases in these modifications of the MERVs genome cannot be excluded at present. In addition, previous *ex vivo* studies on the effects of murine APOBEC3 on MuLV replication have been less clear (Doehle et al., 2005; Abudu et al., 2006; Zhang et al., 2008). MuLV is simple gammaretrovirus and does not encode any known *vif* analog. However, murine APOBEC3 does not induce obvious cytidine deamination when incorporated into MuLV virions. It is proposed that MuLV has evolved yet an unidentified mechanism for blocking the ability of APOBEC proteins to mediate deamination-dependent hypermutation (Browne and Littman, 2008; Rulli et al., 2008).

AID from multiple species, including lower vertebrates such as fish, and APOBEC1 proteins from several mammalian species were also found to possess the capacity to inhibit murine IAP and MusD elements (Esnault et al., 2006; MacDuff et al., 2009; Ikeda et al., 2011). These results raise the possibility that not only APOBEC3 proteins, but also AID and APOBEC1 cytidine deaminases participate in the intrinsic immunity of various vertebrates against the retrotransposition of endogenous and exogenous retroviruses. The catalytic activity of APOBEC1 appears to be critical for this repressive activity (Ikeda et al., 2008, 2011).

P-bodies appear to influence viral life cycles, including those of LTR retrotransposons within host cells, in either a positive or negative manner (reviewed in Beckham and Parker, 2008), although the underlying mechanism is not fully understood. Indeed, P-bodies play a major role during the replicative cycles of LTR retrotransposon Ty elements in yeast (Beliakova-Bethell et al., 2006). P-body is subcellular foci where Ty mRNA and proteins aggregate to facilitate their assembly and replication. On the other hand, the siRNA-mediated knockdown of RNA-induced silencing complex (RISC) and P-body-associated proteins was demonstrated to increase HIV-1 replication and IAP retrotransposition (Chable-Bessia et al., 2009; Nathans et al., 2009; Lu et al., 2011). P-body-associated host factor MOV10 is also demonstrated to inhibit IAP retrotransposition (Lu et al., 2012).

It was suggested that HIV-1 preferentially packages newly synthesized human APOBEC3G, rather than the RNA-bound APOBEC3G found in P-bodies or in the HMM complex (Soros et al., 2007; Ma et al., 2011). The efficiency of packaging into HIV-1 particles appears to correlate with the ability of APOBEC3G to binds to HIV-1 Gag nucleocapsid (NC) domain and to require bridging to heterologous single-stranded RNAs such as Pol II-transcribed poly(A)⁺ RNA and several Pol III-transcribed RNAs (Bogerd and Cullen, 2008). Among Pol III-transcribed short, non-coding RNAs, human 7SL RNA and Y RNAs were demonstrated to promote HIV-1 Gag NC binding by APOBEC3G, while some highly structured RNA molecules, such as the tRNA and rRNA, failed to rescue APOBEC3G:NC complex formation. This RNA bridging by APOBEC3G, not RNA binding by NC, appears to render APOBEC3G competent to associate with HIV-1 NC.

So far, no active ERVs have been isolated in the human genome (HERVs), despite evidence for recent (<200,000 years) amplification (Bannert and Kurth, 2004). However, although none of the HERVs are replication-competent due to their accumulation of mutations (deletions, termination, and frame shifts), more than 20 independent HERV families, which include proviruses that belong to beta-, gamma-, and spuma-retrovirus families, have been identified (Tristem, 2000). HERV families have been classified by the tRNA specificity of their primer binding site (PBS; Blomberg et al., 2009). Many HERV families have lost the ability to transfer, however, several HERV elements, e.g., HERV-K, HERV-H, HERV-W, and HERV-L, possess intact ORFs that encode structural genes and retain the capacity to be translated under certain conditions, including embryonic development and disease states (Kurth and Bannert, 2010).

The relationship between HERV elements and human diseases has been widely discussed following the detection of various HERV genome-derived mRNA, proteins, and even viral particles in patients with several diseases (Nelson et al., 1999; Mayer, 2001; Christensen, 2010). It has also been demonstrated that HERVs exhibit complex interactions with exogenous infectious viruses, such as HIV-1 and herpesviruses (Christensen, 2010). Of note, the most recently active HERVs, known as the HERV-K family with homology to MMTV (Mayer and Meese, 2005), which were reconstituted on the basis of ancient HERV-K sequences, could be restricted by APOBEC3 proteins in an *ex vivo* assay for their mobility (Lee and Bieniasz, 2007; Esnault et al., 2008; Lee et al., 2008). Moreover, the genetic analyses demonstrated that ancient HERV-K elements carry clear footprints of the deamination activity by human APOBEC3G, and to a lesser extent, APOBEC3F. The optimal sequence context of G-to-A mutations was consistent with human APOBEC3s-mediated editing (Armitage et al., 2008). This analysis provided the physiological relevance of the observed *ex vivo* assay. Primate APOBEC3s have been subjected to strong positive selection throughout primate evolution, and the rapid expansion of this gene family was suggested to occur in primates (Sawyer et al., 2004; Zhang and Webb, 2004).

It is still unclear whether human APOBEC3s have shaped the HIV-1 genome, because the results have been conflicting. Obviously, modern retroviruses such as HIV-1 were not a driving force that facilitated this rapid expansion of the *APOBEC3* locus on human chromosome 22q13 over millions of years of primate evolution, since HIV-1 has emerged and entered into the human population during the last 100 years (Korber et al., 2000; Keele et al., 2006; Worobey et al., 2008). In accord with this, it was currently demonstrated that the highly targeted motifs by human APOBEC3G and 3F (e.g., TGGG [the underlined G in the plus strand is deaminated to A]) have not been removed by selective pressure, suggesting the lack of an evolutionary footprint left by human APOBEC3s on the HIV-1 genome (Ebrahimi et al., 2011), although several studies have documented the possibility that evolutionary pressure from human APOBEC3s has shaped the HIV-1 genome (Yu et al., 2004; Armitage et al., 2008).

Based on these findings, it is reasonable to consider that the rapid evolution of APOBEC3s in primates can be attributed to the strong positive selective pressure from their targets, endogenous retrotransposons such as L1 and Alu elements, and that

their evolution has been further promoted by repeated retroviral infection, including HERVs. The *APOBEC3* locus appears to have undergone major expansion during the evolutionary radiation of primates (LaRue et al., 2008). In primate lineage, humans, chimpanzees, and rhesus macaques share similar *APOBEC3* locus architectures, with a seven-protein coding capacity of analogous domain organization (OhAinle et al., 2006; LaRue et al., 2008), indicating that rapid expansion of the *APOBEC3* locus started before the separation of hominoids from Old world monkeys such as rhesus macaques over 50 million years ago. These APOBEC3s in rhesus macaques are demonstrated to be packaged into and restrict HIV-1 and neutralized by the SIV mac239 Vif (Hultquist et al., 2011). This rapid expansion of the *APOBEC3* locus in primates may have caused a dramatic decline in the retrotransposon expansion activity in primates, since 35–50 million years ago (Lander et al., 2001). This scenario also raises the question why these unique rapid expansions of the *APOBEC3* locus have occurred only in primate lineages, since “interspersed repeats” (copies of transposable retroelements) appear to be characteristically abundant in mammalian genomes (Lander et al., 2001; Waterston et al., 2002; Gibbs et al., 2004). The activity of *APOBEC1* genes against retrotransposons may further expose evidence of a complex evolutionary history between APOBEC family and retrotransposons. The details of the expansion are not fully understood as the orthologs of many *APOBEC3* genes have not been sequenced in other placental mammals. It is tempting to speculate that the function of APOBEC family proteins, such as APOBEC1, in intrinsic

immunity has been taken over by expansion of APOBEC3s in primates, but they are conserved in the ancestor of placental mammals.

CONCLUSION

The spectrum of biological functions of the APOBEC family is expanding. Several members of this family play important roles in intrinsic immunity by regulating the spread of foreign and endogenous nucleic acids through non-editing and editing mechanisms. In doing so, they balance the beneficial and deleterious effects of retrotransposition on the host genome. While the restriction activity of the APOBEC family against retroviruses and retroelements is a fairly recent discovery, earlier studies of the zinc-dependent deaminase superfamily of both prokaryotes and eukaryotes that act on nucleosides and nucleotides have provided evidence of a complex evolutionary history. These research findings on the ancient origins of the APOBEC family, and its presence in widely divergent vertebrate lineages provide further insights into the co-evolution of the APOBEC family and retrotransposons.

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