The Resistance of Human APOBEC3H to HIV-1 NL4-3 Molecular Clone Is Determined by a Single Amino Acid in Vif

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Abstract

Some human APOBEC3 family cytidine deaminases have antiviral activity against HIV-1 and other retroviruses. The single deaminase domain APOBEC3H (A3H) enzyme is highly polymorphic and multiple A3H haplotypes have been identified. A3H haplotype II (A3H-haplI) possesses the strongest activity against HIV-1. There remains, however, uncertainty regarding the extent to which A3H-hapII is sensitive to HIV-1 Vif mediated degradation. We tested, therefore, the two different reference Vif proteins widely used in previous studies. We show that A3H-haplI is resistant to NL4-3 Vif while it is efficiently degraded by LAI Vif. Co-immunoprecipitation assays demonstrate that LAI Vif, but not NL4-3 Vif associates with A3H-hapII. Chimeras between NL4-3 and LAI Vif identify the amino acid responsible for the differential degradation activity: A histidine at position 48 in Vif confers activity against A3H-haplI, while an asparagine abolishes its anti-A3H activity. Furthermore, the amino acid identity at position 48 only affects the degradation of A3H-hapII, whereas recognition of and activity against human A3D, A3F and A3G are only minimally affected. NL4-3 encoding 48H replicates better than NL4-3 WT (48N) in T cell-lines stably expressing A3H haplI, whereas there is no fitness difference in the absence of APOBEC3. These studies provide an explanation for the conflicting reports regarding A3H resistance to Vif mediated degradation.

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Introduction

The human APOBEC3 family consists of seven deaminase proteins (A3A to A3H) that can restrict HIV-1 in cell culture [1,2]. They exert their activity by deaminating single stranded viral cDNA during reverse transcription resulting in numerous G-to-A mutations in the provirus [3,4,5]. HIV-1 Vif can counteract the restrictive activity of several APOBEC3 proteins by mediating their proteasomal degradation [6,7].

APOBEC3H (A3H), one of the single deaminase domain enzymes, was initially found to lack anti-HIV-1 activity due to its low protein stability [8,9,10]. However, several studies showed in 2008 that, in addition to the unstable reference wild-type A3H protein (WT, A3H-haplI), multiple other A3H haplotypes exist [11,12,13]. In contrast to A3H-haplI, A3H-haplII, hapIII, hapIV and hapV are stably expressed and can potently restrict HIV-1. A3H-haplII (RDD) differs at three amino acid positions (G105R, K121D and E178D) from A3H-haplI (GKE), but only the arginine (R) at position 105 is responsible for the increased protein stability [11,12]. The allelic frequency of the active A3H-haplII is high in African and low in European and Asian populations [11,14].

While there is good agreement on the potent antiviral activity of A3H-haplII, there remains uncertainty regarding its susceptibility to HIV-1 mediated proteosomal degradation. Indeed, multiple studies looking at the sensitivity of A3H-haplII to HIV-1 Vif revealed discrepant results; In some reports A3H-haplII expression was unaffected by HIV-1 Vif co-expression and no difference in restriction was observed between HIV-1 WT and HIV-1 lacking a functional Vif [HIV-1 ΔVif] when produced in the presence of A3H-haplII [12,14,15,16,17]. Other studies showed, however, efficient degradation of A3H-haplII by Vif and, consequently, infectivity of HIV-1 ΔVif but not of HIV-1 WT was reduced in the presence of A3H-haplII [11,17,18,19,20]. Interestingly, the studies that observed sensitivity of A3H-haplII to HIV-1 Vif also found that it was solely determined by the nature of the amino acid located at A3H position 121 [18,20]. Replacing the aspartic acid (D) in A3H-haplII with a lysine (K, RDD to RKD) at this position resulted in a Vif-resistant protein that would restrict HIV-1 in a Vif-independent manner [10,20].

The commonly used HIV molecular clones, NL4-3 and LAI, differ in their Vif coding region at multiple positions. We hypothesized that these Vif differences affect the resistance to A3H-haplII. Most of the studies that reported A3H-haplII being sensitive to Vif used the HIV-1 LAI clone [11,18,19], whereas the studies that observed A3H-haplII resisting Vif degradation used HIV-1 NL4-3 [12,14,15,16]. Only one study made a direct
counteracted A3H-hapII. The infectivity data is well supported length HIV-1 both counteracted A3G, but only LAI Vif
Vif also counteracts A3G more efficiently than NL4-3 Vif. These
WT was more sensitive to A3G than LAI WT, suggesting that LAI
type viruses counteracted A3G efficiently. Interestingly, NL4-3
Deficient HIV-1 NL4-3 and LAI
A3H Sensitivity to NL4-3 and LAI Vif
The aim of this study was to clarify the discrepancies regarding
A3H resistance to HIV-1 Vif. We systematically assessed the
properties of NL4-3 and LAI Vif and show that A3H-hapII is
efficiently counteracted by LAI Vif but not to NL4-3 Vif. This
difference in A3H recognition can be attributed to a single amino
cid difference at position 48. Exchanging the respective
amino acids at position 48 between NL4-3 and LAI Vif reversed
the degradation phenotype against A3H-hapII. Furthermore, we
show using single cycle and multiple round infections that Vif
position 48 is highly specific for A3H-hapII degradation as the
neutralization of other APOBEC3 members was unaffected.

Results
A3H Sensitivity to NL4-3 and LAI Vif
To test whether the difference in Vif sensitivity of A3H-hapII is
cased by the different Vif variants, we first determined the
iciency of Vif-mediated A3H-hapII degradation in the producer
cell. We co-transfected increasing amounts of NL4-3 or LAI Vif
pCRV1 expression plasmids with A3H-hapII or A3G expression
plasmids. A3H and A3G protein levels were analyzed two days
after transfection by western blot (Figure 1A) and the intensities of
the unsaturated A3H signals were quantified (Figure 1B). NL4-3
and LAI Vif showed different effects on A3H expression levels
despite being expressed to similar levels (Figure 1A). NL4-3 Vif,
even at high expression levels, only modestly degraded A3H-
 hapII, whereas small quantities of LAI Vif were sufficient for
efficient A3H-hapII degradation (Figure 1B). Both Vif variants
degraded A3G with similar efficiency indicating that the two Vif
variants are functionally comparable with respect to A3G but
specifically differ in their efficiency to degrade A3H-hapII
(Figure 1A and 1B).

A3H-hapII and A3G Restriction of Vif Proficient and Vif
Deficient HIV-1 NL4-3 and LAI
Next, we tested whether Vif expressed in cis from their respective
full-length HIV-1 molecular clone would counteract A3H-hapII
restriction. Increasing amounts of A3H-hapII and A3G were
transfected with full-length NL4-3, LAI or their corresponding Vif
deleted counterparts (ΔVif). Infectivity was measured by infecting
TZM-bl reporter cells [21]. Figure 2 shows that NL4-3 WT and
NL4-3 ΔVif were restricted to similar levels by A3H-hapII,
whereas LAI WT was resistant to A3H-hapII restriction. In good
agreement with numerous reports [reviewed in [22]], A3G
potently restricted NL4-3 ΔVif and LAI ΔVif, but the two wild-
type viruses counteracted A3G efficiently. Interestingly, NL4-3
WT was more sensitive to A3G than LAI WT, suggesting that LAI
Vif also counteracts A3G more efficiently than NL4-3 Vif. These
results show that NL4-3 Vif and LAI Vif expressed from full-
length HIV-1 both counteracted A3G, but only LAI Vif
counteracted A3H-hapII. The infectivity data is well supported
by the A3H-hapII and A3G Vif degradation results (Figure 1).
Taken together, A3H-hapII is counteracted by LAI Vif, but not by
NL4-3 Vif.

Contribution of the Three A3H-hapII Single Nucleotide
Polymorphisms to Vif Resistance
In order to determine the putative contribution of A3H
haplotypes to the observed Vif phenotype, we analyzed the
impact of the three amino acids differing between A3H-hapI
(GKE) and A3H-hapII (RDD) on the restriction of WT NL4-3 and
LAI and their Vif deleted counterparts. Previous studies
showed that the G105R change promotes protein stability and
antiviral activity [11,12], whereas K121D renders A3H-hapII
sensitive to Vif degradation [18]. Additional A3H haplotypes
encoding other combinations of these three amino acids (e.g.
hapV-RDE, hapVI-GKD and hapVII-RKE) were recently
described [14]. A panel of A3H mutants comprising all the
possible combinations of the three residue changes were tested for
antiviral activity and Vif sensitivity [Figure 3A, [12]]. We also
included the artificial A3H-hapII RED variant, which was shown
to be sensitive to NL4-3 Vif [20]. We analyzed the effect of these
A3H variants on infectivity of NL4-3 and LAI molecular clones
with and without Vif. All A3H variants encoding 105G, including
A3H-hapI, failed to restrict HIV-1 (A3H-GKE, GDE, GDK, and
GDD, Figure 3B). The 105R encoding variants (RKE, RDD,
R KD and RDE) restricted NL4-3 WT, NL4-3 ΔVif and LAI ΔVif
irrespective of the amino acid at position 121 (Figure 3B). LAI Vif
could only efficiently counteract the restriction of the 121D
variants (A3H-RDD and RDE), but not that of A3H-hapII encoding
121K (A3H-RKE and RKD, Figure 3B). The A3H RED variant
was also sensitive to LAI Vif but, more importantly, also showed some sensitivity to NL4-3 Vif, indicating that the
glutamic acid (E) at position 121 renders A3H sensitive to both
LAI and NL4-3 Vif (Figure 3B). Western blot analysis of A3H
expression in 293T cells showed that all four 105G carrying A3H
variants (GKE, GDE, GDK and GDD) were poorly expressed and
failed to restrict HIV lacking Vif (Figure 3B and 3C). A3H RKE
and RKD variants were resistant to NL4-3 and LAI Vif-mediated
degradation. A3H RDD and A3H RDE were both exclusively
degraded by LAI Vif, but not by NL4-3 Vif. The artificial A3H
RED variant was degraded by both NL4-3 and LAI Vifs.
Combined, the A3H degradation results in Figure 3C are in
excellent agreement with the restriction patterns depicted in
Figure 3B. Taken together, these data confirm that the amino
acid at position 121 determines the sensitivity of A3H to degradation
by specific Vif variants: An aspartic acid (A3H 121D) mediates A3H
sensitivity to LAI Vif [10], a glutamic acid (A3H 121E) renders
A3H sensitive to both LAI and NL4-3 Vif and a lysine (121K)
results in resistance of A3H to both Vif alleles.

Association of A3H-hapII with LAI Vif, but not with NL4-3
Vif
The observation that A3H-hapII is sensitive to LAI Vif but not
NL4-3 Vif could be explained by a difference in Vif binding to
A3H-hapII. We studied the interaction of A3H-hapII with both
Vif variants by co-immunoprecipitation. HA-tagged A3H-hapII
was co-transfected with empty pCRV1 plasmids or pCRV1
expressing NL4-3 and LAI Vif in 293T cells. To block A3H
degradation by LAI Vif, the proteasome inhibitor, clasto-
Lactacystin β-lactone, was added to the transfected HEK 293T
cells 24 hours prior to cell lysis. Cleared lysates were incubated
with α-HA coated beads and extensively washed with lysis buffer
cut. Western blot analysis of the cell lysates showed that
Vifs and A3H were equally expressed and that A3H-hapII was not degraded by LAI Vif in the presence of proteasome inhibitor (Figure 4). LAI Vif co-precipitated efficiently with A3H-hapII, whereas only very little NL4-3 Vif was bound (Figure 4). Together, this indicates that LAI Vif, but not NL4-3 Vif, can efficiently associate with A3H-hapII. This ability of LAI Vif correlates with its efficiency to degrade and counteract the antiviral activity of A3H-hapII.

Vif Position 48 Determines A3H hapII Degradation and Rescue of Infectivity

NL4-3 and LAI Vifs only differ at nine positions in the N-terminal region (see Figure 5A), which is the portion of Vif critical
for interaction with several APOBEC3 proteins. In order to pinpoint the differential anti-A3H activity at a single residue level, we generated a set of Vif chimeras in which LAI Vif amino acids were introduced into NL4-3 Vif (Figure 5A). The A3H-hapII degradation efficiency in the presence of the WT NL4-3, WT LAI and the chimeras was analyzed by western blot. The introduction of either LAI amino acids RS or VGRG into NL4-3 Vif did not result in an increase in activity, indicating that these regions are dispensable for specific A3H-hapII recognition (Figure 5B). However, a NL4-3 Vif variant with amino acids PHR efficiently degraded A3H-hapII. Additional single amino acid changes within the PHR stretch revealed that the introduction of a histidine at position 48 was sufficient to fully confer activity towards A3H-hapII. Indeed, the reverse change in LAI Vif, H48N, resulted in a complete loss of A3H-hapII degradation activity (Figure 5B). We next replaced the Vif position 48 in the full-length molecular clones NL4-3 and LAI and tested their infectivity levels in the presence of A3H-hapII (Figure 5C). Both NL4-3 WT and NL4-3 ΔVif were restricted, but the restriction was clearly relieved for NL4-3 N48H. Conversely, LAI WT displayed high infectivity levels, but the H48N change efficiently restricted LAI H48N, similar to LAI ΔVif. In summary, the difference in counteracting A3H-hapII between NL4-3 and LAI Vif can be attributed to a single amino acid at position 48.

Vif Position 48 is Specific for A3H-hapII Recognition

To test whether Vif position 48 affected the neutralization of other APOBEC3 proteins in addition to A3H-hapII, we analyzed the infectivity levels of WT NL4-3 and LAI as well as the corresponding Vif deleted and 48 substituted molecular clones in
the presence of all seven human APOBEC3 members. NL4-3 ΔVif and LAI ΔVif were both restricted by A3B, A3D, A3F, A3G and A3H hapII to varying degrees (Figure 6). The restriction by A3D, A3F and A3G was relieved by all the Vif variants. Both LAI and NL4-3 48H Vif efficiently counteracted A3H-hapII, whereas NL4-3 and LAI 48N showed low infectivity in the presence of A3H-hapII. In these single cycle infectivity experiments, the Vif 48 mutants in both NL4-3 and LAI molecular backgrounds counteracted A3D, A3F and A3G as efficiently as the parental clones indicating that the Vif substitution at position 48 specifically affects A3H recognition without impacting other APOBEC3 proteins.

Figure 4. Association of A3H-hapII with LAI Vif, but not with NL4-3 Vif. 5 μg HA-tagged A3H-hapII was co-transfected with 1 μg of the indicated Vif expression plasmids in 293T cells in 10-cm dishes. Cells were treated with clasto-Lactacystin β-lactone (10 μM) for ten hours and cells were lysed in mild lysis buffer 2 days post transfection. Lysates were cleared and incubated with anti-HA tagged beads (Sigma) for one hour at 4°C. Beads were extensively washed with lysis buffer and proteins were eluted by boiling in sample loading buffer. Proteins were analyzed by western blot.
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Figure 5. Vif 48H is required for A3H hapII degradation and rescue of infectivity. (A) Schematic overview of the first 61 amino acids of NL4-3 and LAI Vif. Three regions that are different between NL4-3 and LAI are indicated by black boxes. (B) 50 ng NL4-3 or LAI or mutant Vif expression plasmids was co-transfected with 100 ng of FLAG-tagged A3H-hapII in 293T cells. Two days post transfection cells were lysed and analyzed by western blot. (C) A3H-hapII restriction of Vif proficient mutant Vif and Vif deficient HIV-1 NL4-3 and LAI. 50 ng A3H-hapII was co-transfected with the different indicated HIV molecular clones (500 ng) in 293T cells. Two days post infection cleared supernatants were used to infect TZM-bl reporter cells and β-Galactosidase was measured two days later. One representative experiment consisting of triplicate transfections is shown. Error bars represent standard deviations from triplicate transfections.
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observed with the empty vector and A3G expressing cells, indicating that N48H also efficiently counteracts A3H in the setting of spreading infections. Of note, both viruses replicated with similar efficiencies on cells expressing the control empty vector (Figure 7B). Interestingly, the replication of NL4-3 WT was slightly improved compared to the N48H mutant on A3G expressing SupT1 cells, which may indicate that a Vif encoding 48N is somewhat better adapted to counteract A3G (Figure 7B). Taken together, the data indicate that Vif position 48 is specific for counteracting A3H in both single cycle as well as multiple round infection models.

Discussion

Over the past years, a series of publications established that some human A3H proteins (e.g., A3H hapII) exert potent activity against HIV-1, however, conflicting data regarding the sensitivity of A3H-hapII to HIV-1 Vif remain. Here we show that A3H-hapII is resistant to NL4-3 Vif but sensitive to LAI Vif, which reconciles the published findings based on which Vif was used in the respective studies. The difference in Vif activity against A3H-hapII is likely caused by its ability to interact with A3H-hapII as shown by co-immunoprecipitation (Figure 4). We pinpointed the difference in Vif activity against A3H-hapII to a critical Vif residue at position 48 (Figure 5). Our group recently reported the importance of this specific Vif residue in combination with Vif position 39 (especially for HIV-1 subtype F Vifs) to efficiently degrade A3H-hapII [17]. NL4-3 and LAI both already contain the active phenylalanine at position 39, making the amino acid identity at position 48 the sole determinant for A3H-hapII neutralization in the context of these two commonly used subtype B molecular clones.

In addition, we show that the A3H RED variant is sensitive to both LAI and NL4-3 Vif [20], pointing to D121E as a molecular determinant for A3H sensitivity (Figure 3). Although this variant has not been found in cellular transcripts in the human populations, chimpanzees and the reconstructed human ancestral A3H encode a glutamic Acid (E) at position 121 [11]. We speculate that the E121D change in A3H-hapII in modern humans may be beneficial, as it would protect from a broader spectrum of HIV-1 strains similar to NL4-3.

Two A3H variants encoding RKE and RKD showed a strong restriction of HIV-1 and were resistant to both Vifs tested (Figure 3B). Interestingly, the A3H RKE genotype variant was detected in at least one Caucasian individual [14], suggesting that some humans may encode A3H variants that are both active against and resistant to HIV-1.

A recent study showed that the A3H-hapII genotype correlates with decreased plasma viremia in HIV-1 infected individuals indicating a role of A3H in HIV-1 disease progression [23]. In addition, a study that included Vifs from HIV-1 infected individuals with different A3H haplotypes indicated that a HIV-1 infected patient homozygous for A3H-hapII harbored Vif variants that were more efficient in counteracting A3H-hapII compared to patients heterozygous or homozygous for the inactive A3H-hapI [18]. These observations point to the possibility that an HIV patient’s Vif adapts to degrading active A3H-hapII, but not inactive A3H proteins since the later variants fail to exert selection pressure since their neutralization is not required for efficient replication. This notion is further supported by our previous study, which showed that a large panel of Vifs alleles derived from different HIV-1 subtypes degraded A3G and A3F efficiently, whereas only half of these Vifs variants could counteract A3H-hapII [17]. Vif protein sequence analysis of 1,286 HIV-1 subtype B Vifs (HIV Los Alamos database, premade Vif protein alignment) shows that position 48 either encodes a histidine (H, 71.5%) or an asparagine (N, 28.3%). Thus, while the majority of subtype B Vifs likely display activity against A3H hapII there remains a
substantial number of subtype B Vif variants that lacks specific activity to degrade A3H.

The amino acid identity at Vif position 48 specifically affects A3H degradation, whereas A3D, A3F and A3G degradation in single cycle infectivity assays remained unchanged (Figure 6A and 6B). Testing the specificity of NL4-3 Vif 48N and 48H in multiple round infection experiments in T cell lines showed that replication is identical in the absence of APOBEC3 expression (Figure 6A). A sizable, 10-fold reduction in replication is observed in SupT1 cells expressing A3H hapII (Figure 6C), underscoring that the results observed in single cycle infectivity assays are well recapitulated in spreading infections. Interestingly, we observed a small replication delay for NL4-3 48H on cells expressing A3G (Figure 6B), which may indicate that viruses that are better adapted to counteracting A3H-hapII may have lost some activity against A3G.

Collectively, these data suggest a scenario in which the role of A3H on HIV-1 disease progression is both affected by the patients’ own A3H repertoire, but also by the ability of the HIV-1 Vif protein to counteract the respective A3H variants. Future studies are required to elucidate the role of A3H on HIV-1 replication in the context of different subtypes as well as on HIV-1 disease and AIDS progression.

Materials and Methods

Plasmids

The replication-competent molecular clones NL4-3 [24] and NL4-3 ΔVif [25] and LAI [26] were provided by the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health NIH Reagent Program [25].

The LAI ΔVif molecular clone was constructed by deleting the NdeI-StuI fragment resulting in the deletion of 284 nucleotides in the vif open reading frame. NL4-3 N48H and LAI H48N were constructed using standard overlap PCR mutagenesis.

The mammalian expression plasmids pTR600 containing amino-terminally FLAG-tagged A3H hapl-GKE, A3H hapII-RDD, the six site-directed A3H mutants and A3G were described previously [12]. The A3H RED and Vif mutants were constructed using standard overlap PCR mutagenesis, as previously described, using A3H hapII-RDD and NL4-3 and LAI Vifs as template, respectively [12]. All primer sequences are available upon request.

NL4–3 Vif and LAI Vif proteins were expressed using pCRV1 as described previously [27].

Culture of Cell Lines

HEK 293T and TZM-bl reporter cells were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin. TZM-bl cells were provided by the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health NIH Reagent Program [28].

SupT1 T-cells expressing empty vector, 3xHA tagged A3G and untagged A3H hapII were kindly provided by Dr. R. Harris [16]. SupT1 cells were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin-streptomycin and 0.5 mg/ml G418 (Mediatech).

Vif-mediated A3H and A3G Degradation

The FLAG-tagged A3H-hapII and A3G expression vectors (100 ng) were co-transfected with increasing amounts of NL4-3 or LAI Vif pCRV1 expression plasmids (0, 2.5, 5, 10, 25 and 50 ng) and pCRV1 empty plasmid (total amount of pCRV1 50 ng). The transfections were performed in a 24-well format using 4 µg/ml polyethylenimine (PEI; Polysciences, Inc.). Transfected cells were lysed two days post-transfection in 1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 5 mM EDTA. Five µl of 4x lithium dodecyl sulfate (LDS) sample buffer (NuPAGE; Invitrogen) and 2 µl of sample reducing agent (NuPAGE; Invitrogen) were added to 13 µl of the lysate and heated for an additional 10 min at 70°C. Proteins were separated on 10% SDS-polyacrylamide gels (Invitrogen), transferred onto polyvinylidene difluoride (PVDF) membranes (Pierce), and probed with anti-FLAG M2 monoclonal antibody (Sigma), rabbit
polyclonal Vif antiserum (AIDS reagent, catalog number 2221) [29] and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Sigma) or anti-Tubulin (Sigma) to ensure equal protein loading. Membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma), developed with SuperSignal West Pico (Pierce), and detected by using the Fujifilm Intelligent Lightbox LAS-3000 instrument and Image Reader LAS-3000 software. For quantification, non-saturated signals were background subtracted and FLAG signals without Vif were set at 100%.

**Assessment of Viral Infectivity using Single Cycle Infectivity Assays**

The FLAG-tagged A3H-hapII and A3G expression vectors (0, 25, 50 and 100 ng) were co-transfected with the different HIV molecular clones, NL4-3 WT, NL4-3 ΔVif, LAI and LAI ΔVif (500 ng) in 293T cells. For the A3H variants 20 ng was co-transfected with the different HIV molecular clones (500 ng). The transfected SuperSignal was performed in a 24-well format using 1 μg/ml polyethyleneimine (PEI; Polysciences, Inc.). The culture medium was replenished after 24 h and the supernatants were harvested 48 h after transfection, clarified by centrifugation, and used to infect TZM-bl reporter cells. TZM-bl cells were infected in triplicate with 20 μl of cell-free viral supernatants in 96-well plates. Beta-galactosidase activity was quantified 48 h after infection by using chemiluminescent substrate (Tropix; Perkin-Elmer), as previously described [12]. The data from three independent transfections were used to calculate average values and standard deviations.

**Assessment of Viral Replication in Spreading Infection Experiments**

Viral stocks were generated by transfecting HEK 293T cells with 500 ng of NL4-3 WT and NL4-3 ΔN5H molecular clones in 24-well plates using 4 μg/ml polyethyleneimine (PEI; Polysciences, Inc.). Supernatants were harvested 2 days post transfection, clarified by centrifugation, filtered and aliquots stored at −80°C until further use. Infectivity titers of the viral stocks were determined by infecting TZM-bl reporter cells in triplicate with serial dilutions of each virus as previously described [30].

1.5 × 10⁶ cells of each SupT1 cell line (empty, A3G, A3H) were infected (MOI 0.5) in a 24-well format (1.5 ml) and washed with PBS 10 hours post infection. Every day over a two week period, culture supernatants (200 μl) were collected, clarified and stored at −80°C. Cultures were supplemented with fresh media each day (250 μl). At the end of the infection, the cryo-preserved culture supernatants were used to infected TZM-bl cells in triplicate (10 μl, 96-well plates). Beta-galactosidase activity was quantified 48 h after infection by using chemiluminescent substrate (Tropix; Perkin-Elmer), as previously described [12]. Two independent infections of SupT1 cell lines were performed.

**Co-immuno-precipitation**

5 μg of 3xHA-tagged A3H-hapII in PTR600 or empty pTR600 were co-transfected with 1 μg of pCRV1-NL4-3 Vif, pCRV1-LAI Vif or empty pCRV1 in 293T cells in 10-cm dishes. Cells were treated with elaste-Lactacystin β-lactone (10 μM, Sigma) for 10 hours and 40 hours post transfection the cells were washed with PBS and lysed in mild lysis buffer (1% Triton X-100 in PBS supplemented with EDTA-free protease inhibitor cocktail, Roche). Lysates were cleared by centrifugation at 8,000 g for 10 minutes and incubated with anti-HA tagged beads (Sigma) for one hour at 4°C. Supernatants were discarded and the beads were washed by incubating with mild lysis buffer for another hour at 4°C. Subsequently, beads were washed 4 times with mild lysis buffer and proteins were eluted by boiling in LDS loading buffer (Sigma). Proteins were analyzed by western blot using anti-HA monoclonal antibody (Sigma), rabbit polyclonal Vif antiserum (AIDS reagent, catalog number 2221) [29] and anti-GAPDH (Sigma).

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**Author Contributions**

Conceived and designed the experiments: MO. Performed the experiments: MO ML MB. Analyzed the data: MO VS. Wrote the paper: MO VS.

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