A BIOLOGICAL "ARMS RACE"

APOBEC3F Diversity and its Influence on Lentiviral Resistance

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RESTRICTION FACTORS ARE IMPLICATED IN LONG-TERM EVOLUTIONARY "ARMS RACES," IN WHICH VIRAL ANTAGONISTS DRIVE THE EVOLUTION OF HOST PROTEINS, AND VICE VERSA. CONSEQUENTLY, RESTRICTION FACTORS ARE REMARKABLY VARIABLE, DISPLAYING POLYMORPHISM WITHIN SPECIES AND DIVERGENCE BETWEEN SPECIES AS A RESULT OF POSITIVE SELECTION. THIS PAPER INVESTIGATES DIVERSITY IN THE APOBEC3F (A3F) RESTRICTION FACTORS OF OLD WORLD PRIMATES IN ORDER TO DETERMINE WHETHER THEY DISPLAY EVIDENCE OF INVOLVEMENT IN AN EVOLUTIONARY "ARMS RACE." WE SPECULATED THAT GENETIC VARIABILITY IN A3F COULD REFLECT EVOLUTIONARY CONFLICT WITH THE VIF PROTEINS OF PRIMATE LENTIVIRUSES, WHICH ARE KNOWN TO ENHANCE VIRAL REPLICATION BY BINDING AND DEGRADING HOST A3 PROTEINS. A3FS OF SEVERAL OLD WORLD PRIMATE SPECIES WERE GENOTYPED, AND THE SEQUENCES REVEALED BOTH INTRA-SPECIES DIVERSITY AND INTER-SPECIES DIVERGENCE. REPRESENTATIVE RHESUS MACAQUE (MACACA MULATTA) SEQUENCES WERE CLONED AND TESTED FOR SENSITIVITY TO VIFS FROM VARIOUS SIMIAN IMMUNODEFICIENCY VIRUSES (SIVS). EVOLUTION OF A3F IN THE RHESUS LINEAGE IS NOT DUE TO SELECTION BY SIVS, BUT MAY REFLECT ANTAGONISM BY ANOTHER RETROVIRUS.

INTRODUCTION

I. Restriction factors: barriers to cross species transmission

Acquired Immune Deficiency Syndrome (AIDS) was first identified as a disease in 1981, as increasing numbers of homosexual men were dying of opportunistic infections after becoming immunocompromised.[1] The causative agent was soon discovered to be a lentivirus, classified as Human Immunodeficiency Virus Type 1 (HIV-1).[2,3] HIV-1 and its close relative HIV Type 2 (HIV-2) arose in human populations as a result of cross-species transmission events from natural simian hosts of the Simian Immunodeficiency Viruses (SIVs). The closest simian relative of HIV-1 is the strain naturally infecting chimpanzees (SIVcpz),[4] while the closest simian relative of HIV-2 is the strain naturally infecting sooty mangabeys (SIVsm).[5] Over 40 species-specific SIVs have since been identified in non-human primate species. A fraction of these infections result in immunodeficiency in the host species, while most are nonpathogenic in their natural hosts.[6]

The SIV of a given primate species cannot readily infect a different species without undergoing numerous adaptations. Part of this adaptation process involves evading host antiviral proteins known as restriction factors. Restriction factors are host proteins that utilize numerous mechanisms to interfere with viral infections as part of the innate immune response. Some interact directly with viral factors, while others make the cellular environment unsuitable for sustained viral replication.[7] These defensive genes are subject to relatively rapid evolution under the selective pressure of viral counter-restriction mechanisms, and variants that confer greater fitness to the host during a viral outbreak will become fixed in a population much faster than would normally be expected as a result of genetic drift alone. The virus, in turn, can adapt to infect the host carrying these more fit alleles, creating a new selective pressure on the host. Thus, over evolutionary time, a comparatively large number of non-synonymous, or protein-altering, mutations will be evident in genes that have participated in these virus-host "arms races."[8]

Genes can be examined for evidence of positive selection by comparing the rate of fixation of non-synonymous mutations (N) to the rate of fixation of synonymous mutations (S). dN/dS analysis demonstrates whether fixation of non-synonymous changes is occurring faster than expected under neutral selection alone. A dN/dS value greater than one indicates that a particular site is evolving under positive selection, while a dN/dS value less than one indicates that a site is evolving under purifying selection.[9]

This idea of a virus-host "arms race" is a fairly simplified model. RNA viruses can diversify their genome at a much higher rate than their hosts can. Hosts keep up through diversity of antiviral mechanisms rather than rapid counterrevolution. A viral variant that efficiently evades one host restriction mechanism will likely become more susceptible to another.[10] However, over evolutionary time and as a result of many interactions with diverse viruses, host restriction factors bear signatures of positive selection.

Another defining factor of restriction factors is their relationships with viral antagonist proteins. In many cases, viruses have evolved specific proteins to evade the mechanism of a particular restriction factor. This virus-host relationship is highly species-specific because of the rapid adaptation process. As a general rule, any given virus will have accessory proteins that are most effective at neutralizing the restriction mechanisms of its natural host, but may not be effective against the homologous restriction mechanisms of other organisms.[11]

A variety of restriction factors have been implicated in differential host resistance to primate lentiviruses, like HIV and the SIVs. Lentiviruses are part of the retrovirus family, and their life cycle is well understood. In order to infect a new target cell, the virion will first bind to a CD4 receptor and co-receptors on the cell surface. The virion membrane fuses with the cell membrane and the capsid is released into the cytoplasm. The viral RNA within the capsid then undergoes reverse transcription in the cytoplasm to form DNA that is transported into the nucleus and integrated into the host genome. The viral DNA is transcribed and translated using host machinery, and then viral RNA and proteins are localized to the cell membrane to form new virions. The restriction factors of primate lentiviruses have evolved to interfere with nearly every aspect of this life cycle, and thus act as barriers to cross-species transmission.

II. The APOBEC3 subfamily: a cluster of potent lentiviral restriction factors

The Apolipoprotein B mRNA-editing catalytic polypetide-3 (APOBEC3) subfamily of cytidine deaminases represents one such group of restriction factors. Primates encode seven APOBEC3 (A3) genes that form a cluster on chromosome 22 in humans and chromosome 10 in rhesus macaque monkeys: A3A, A3B, A3C, A3D, A3F, A3G, and

A3H. A3 proteins are most highly expressed in HIV-1 target cell types such as T-cells, macrophages, and dendritic cells, and they are active in restricting retroviruses as well as some other viruses that do not replicate via reverse transcription.[12,13] Human A3D, A3F, A3G, and A3H are known to be involved with HIV restriction, and each of these proteins has a functionally conserved homolog in rhesus macaques.[14]

The structure of A3 proteins is characterized by a cytidine deaminase domain consisting of six α-helices coordinated around five β-strands. This domain sits between a short N-terminal α-helical domain and a short C-terminal peptide.[15] The deaminase domain contains a conserved zinc-binding catalytic HXEX23-28PCX2-4C motif that coordinates deaminase activity. A3A, A3C, and A3H contain a single deaminase domain, while A3B, A3D, A3F, and A3G contain two deaminase domains. In the four A3s with two deaminase domains, only the C-terminal domain (CTD) is catalytically active.[16,17] A3G and A3F have the greatest sequence similarity of the seven A3s, and they share many structural features that have been elucidated from the crystal structures of their CTDs. The basic structure is maintained between the proteins, but differences paralleling the divergence in primary structure are evident.[18]

A3G is the most widely studied of the A3 subfamily, as it is believed to play the largest role in HIV-1 restriction.[19] However, A3D, A3F, and A3H have also been implicated in viral restriction. A3s act by inducing cytidine-to-uracil mutations in the viral negative sense cDNA produced during reverse transcription, consistent with the proteins' known cytidine deaminase function.[20] Studies have shown A3G to be the more potent editor in humans, producing about 10 times more mutations in HIV-1 reverse transcripts than A3F in cell culture.[21] Yet, A3F is stably co-expressed with A3G in human cells and is also packaged into HIV-1 virions to inhibit infectivity.[22] A3G preferentially deaminates cytidines on the 3' end of 5'-CCC-3' or 5'-CC-3' sequences, while A3F and other A3 proteins preferentially deaminate the 3' cytidine in 5'-TC-3' sequences.[23]

III. Interaction between APOBEC3 and Vif

Soon after the discovery of HIV-1, the viral protein termed viral infectivity factor (Vif) was found to be necessary for successful infection.[24] However, it was not necessary in every cell type; HIV-1Δvif could infect certain permissive cell lines, while infection was unsuccessful in others.[25] It was only later determined that A3 proteins were responsible for imparting resistance to Vif-deficient virus in these non-permissive cell types.[26] Vif has two domains, one crucial for binding A3 proteins and one that contains a highly conserved sequence required for mediating degradation.[27] Vif acts by targeting cytoplasmic A3 proteins for proteosomal degradation (Figure 1).[28] The interaction between A3s and Vif is a crucial determinant of the species specificity of a virus, and it is likely that a Vif from a given viral strain has evolved precisely to counteract the A3 proteins of its host species.[29,30] The critical sites involved in A3-Vif interactions have largely been elucidated via mutagenesis studies, and as little as a single amino acid can be responsible for conferring the ability of A3 proteins to resist degradation by Vif. In A3G, the Vif binding site has been localized to the N-terminal deaminase domain (NTD). It was found that the Vif of an SIV strain isolated from African green monkeys (SIVagm) was inactive against human A3G (hA3G) but active against African green monkey A3G (agmA3G). This discrepancy was mapped to amino acid 128, an aspartic acid in hA3G and a lysine in agmA3G.[31] This interface was expanded upon to include the proline at position 129 and the aspartic acid at position 130. Together, these three residues create a crucial negatively charged binding surface for HIV-1 Vif in hA3G.[32] Contrary to A3G, the HIV-1 Vif binding site in A3F is localized at the CTD. The region comprising amino acids 289EFLARH294 in hA3F and agmA3F has been shown to be a critical region for HIV-1 Vif interaction.[33] Another study suggested that amino acid 324 is a critical residue that confers hA3F susceptibility to HIV-1 Vif and rhesus macaque A3F (rhA3F) resistance to HIV-1 Vif.[34] More recently, the negatively charged surface of α-helices 3 and 4 in hA3F has been strongly implicated in HIV-1 Vif binding.[35] In HIV-1 Vif,

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highly conserved N-terminal motifs are involved with A3G and A3F binding.[36,37]

IV. Objective

While much attention has been given to the primary structures of A3s in terms of their effect on cross-species transmission of primate lentiviruses, examination of the diversity in these genes has been largely unconsidered. The most widely utilized primate model for investigating lentiviral infection and disease is the SIV infecting rhesus macaques (SIVmac), with rhesus macaques of Indian origin being the best characterized species.[38] Rhesus macaques are not natural hosts of SIV in the wild; rather, SIVmac emerged from cross-species transmission events in US primate centers and likely originated from infected sooty mangabeys.[39,40] The adaptations allowing the virus to stably infect rhesus macaques likely occurred as a result of serial passages of the virus through multiple animals.[41] Unlike SIVsm in its natural host, SIVmac is pathogenic in rhesus macaques, resulting in an AIDS-like disease. Given the well-established history of its emergence, SIVmac is an excellent model for studying the adaptive processes that viruses must undergo in order to successfully infect a new host species.

Recent work has demonstrated that the rhesus macaque A3G gene (rhA3G) is polymorphic at an N-terminal site, where three sequence variations are apparent. The variants displayed differential susceptibilities to SIVsm Vif-mediated degradation in cell culture, but all three could be degraded by SIVmac Vif.[42] Thus, it is likely that SIVmac Vif had to adapt to counteract the SIVsm Vif-resistant rhesus macaque A3G allele in order for pathogenic SIVmac to emerge. It is also known that A3G has evolved under strong positive selection, and that Vif is not wholly responsible for this phenomenon.[43]

The present study is intended to further characterize the degree of variation in other A3 family members, more specifically A3F. Old World primate A3Fs were examined for the genetic signatures of a rapidly adapting restriction factor. In a manner analogous to A3G, polymorphic sites in A3F may indicate potential determinants of the ability of SIVmac to infect its novel host. Polymorphic sites were investigated for their potential influence on the ability of rhA3F to resist degradation by Vifs derived from various SIV strains. Examination of allelic variance in A3F provides valuable insight into the barriers to cross-species transmission of lentiviruses, and offers further support to the idea of an evolutionary "arms race" between restriction factors and their viral antagonists.

MATERIALS AND METHODS

Cell Lines

HEK293T cells were maintained in Dulbecco's modified Eagles medium (DMEM) containing 10% FBS, 1% L-glutamine, 1% Penicillin Streptomycin mixture, and 2.5% HEPES.

Reverse Transcriptase PCR (RT-PCR) and Cloning

RT-PCR reactions were performed on RNA samples (extracted previously at the New England Primate Research Center) using the Invitrogen (Carlsbad, CA) SuperScript® III One-Step RT-PCR System or the QIAGEN (Hilden, Germany) OneStep RT-PCR Kit according their associated protocols. Gateway cloning forward and reverse primers were used (Table 1). Products were identified via gel electrophoresis and purified using QIAGEN QIAquick Gel Extraction Kit according to the protocols specified in the kit. Purified DNA samples were cloned using the Invitrogen Gateway® Cloning system and its associated protocols. Purified PCR samples were cloned into Invitrogen pENTR®/D-TOPO plasmids. The clones were transformed into Invitrogen One Shot® Stbl3™ chemically competent E.coli and transformants were selected for via kanamycin selective plating. Plasmid DNA was isolated using the QIAGEN QIAprep Spin Miniprep Kit and its associated protocols, and then sent to Eton Bioscience (Charlestown, MA) for sequencing to confirm the identity of A3F sequence. Selected clones were then subcloned into Invitrogen pcDNA3.2™/V5-DEST plasmids (Figure 4). The clones were transformed into Invitrogen One Shot® Stbl3™ competent E.coli and transformants were selected for via ampicillin selective plating. Plasmid DNA was isolated as before, and clones were sequenced again to confirm A3F insertion.

dN/dS Analysis

A3F sequences from rhesus macaques and other primates obtained from our own clones and from the NCBI database (see Appendix for sequences) were trimmed and aligned using the Multiple Align function in Geneious 6.1.8 software (created by Biomatters, available at http://www.geneious.com). The trimmed sequences were edited such that extraneous sequence before or after the A3F open reading frame was deleted. The sequences were up-

loaded to Datamonkey, a public server for comparative analysis of sequence alignments.[44,45,46] All three available models for dN/dS analysis (SLAC, FEL, REL) were run for the relatively small data set of 15 sequences. As suggested by prior research, the SLAC and FEL models were run with P values of 0.25, while the REL model was run with the Datamonkey-suggested Bayes factor of 50.[47]

Genotyping

PCR was performed on rhesus macaque genomic DNA samples obtained from multiple US primate research centers using Invitrogen Platinum® PCR SuperMix and its associated protocols. The primers used were genotyping forward and reverse (Table 1). The PCR products were visualized via gel electrophoresis, purified using the QIAGEN QIAquick Gel Extraction Kit according to its associated protocols, and sent to Eton Bioscience for sequencing. The sequence chromatograms were aligned using Geneious 6.1.8 software and examined for the presence of the SKEH/FQQY variants.

Mutagenesis

Around-the-horn PCR was used to mutate amino acids 60, 61, 63, and 64 from the FQQY variant to the SKEH variant (or vice versa) in select rhA3F clones. PCR was performed using Thermo Scientific (Waltham, MA) Phusion Flash High Fidelity PCR Master Mix and its associated protocols, and the patch mutant forward and reverse primers were used. PCR products were digested with 10U of DpnI (New England Biolabs; Ipswich, MA) and purified using the QIAGEN QIAquick PCR Purification Kit and its associated protocols. Ligation was performed using the Promega (Madison, WI) LigaFast™ Rapid Ligation System and its associated protocols. The clones were transformed into Invitrogen One Shot® Stbl3™ competent E.coli, and transformants were selected for via ampicillin selective plating. Plasmid DNA was isolated using the QIAGEN QIAprep Spin Miniprep Kit and its associated protocols, and then sent to Eton Bioscience for sequencing to confirm successful mutagenesis of A3F.

Immunoblotting

Plasmids to be used in cell culture were prepared using the Invitrogen PureLink® HiPure Plasmid Maxiprep Kit and its associated protocols. HEK293T cells were seeded in 6-well plates at a density of 800,000-1,000,000 cells/well or 12-well plates at a density of 200,000-400,000 cells/well 24-48 hours prior to transfection. Cells were transfected with 1-3ug total DNA using GenJet reagent (SignaGen Laboratories; Gaithersburg, MD) and its associated protocols. Cells were harvested 48 hours post-transfection using 100-200uL IP Lysis Buffer. Samples were added to 2x Laemmli Buffer and boiled for 10 minutes. Proteins were separated using SDS/PAGE and then transferred to PVDF or nitrocellulose membranes via Bio-Rad (Hercules, CA) Mini Trans-Blot Electrophoretic Transfer Cell and its associated protocols. Membranes were blocked overnight in 1.25% milk, washed in 1x PBS-0.1% Tween-20 solution, and incubated for 1 hour in anti-V5-HRP (Novex; Carlsbad, CA) to probe for A3 protein or anti-β-actin-HRP antibody (Abcam; Cambridge, MA) to probe for β-actin. Alternatively, a primary mouse monoclonal anti-β-actin antibody (Sigma-Aldrich; St. Louis, MO) and secondary anti-mouse-HRP antibody (Thermo Scientific) were used to probe for β-actin. All antibodies were diluted in 1.25% milk according to manufacturer's specifications. After washing again, blots were developed using either Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences; Little Chalfont, Buckinghamshire, United Kingdom), or Thermo Scientific SuperSignal™ West Femto Maximum Sensitivity Substrate and then visualized using Bio-Rad ChemiDoc MP gel imaging system.

RESULTS

I. Investigating diversity and positive selection in Old World primate A3F

In order to determine whether primate A3F displays the hallmarks of a restriction factor that has been antagonized over time, rhesus macaque (Macaca mulatta) A3F sequences were first investigated for evidence of polymorphism. To examine polymorphism within species, RT-PCR was performed on RNA samples obtained from 10 different rhesus macaques. The resulting DNA was cloned and the sequences were examined for both silent and non-synonymous polymorphisms. The data showed that A3F is a highly polymorphic gene in rhesus macaques, and that many of the single nucleotide polymorphisms result in amino acid changes (Figure 2).

To examine inter-species diversity, RT-PCR was also performed on multiple RNA samples from sooty mangabeys (Cercocebus atys), crab-eating macaques (Macaca fascicularis), and pigtail macaques (Macaca nemestrina). A3F coding sequences from these related primates showed divergence from each other and from rhesus macaques,

demonstrating that there are fixed differences between A3F genes in Old World primates (Figure 3).

After establishing that A3F is a polymorphic gene in rhesus macaques and that fixed differences exist between A3Fs of related Old World primates, a dN/dS analysis was performed in order to determine whether any codons have evolved under positive selection. The species that were included in this analysis were rhesus macaques, crab-eating macaques, pigtail macaques, sooty mangabeys, African green monkeys (Chlorocebus sabaeus), humans (Homo sapiens), chimpanzees (Pan troglodytes), bonobos (Pan paniscus), and drills (Mandrillus leucophaeus). These sequences were uploaded to Datamonkey, a public server for comparative analysis of sequence alignments (See dN/dS section in Materials and Methods). 11 codons were found to be evolving under positive selection. The sites were mapped relative to the deaminase domains and catalytic motifs in A3F (Figure 4), and seven sites were found to overlap with those previously identified to be polymorphic in rhesus macaques.

II. A closer look at a polymorphic and positively selected site in rhesus macaques

Four codons at positions 60, 61, 63, and 64 in rhesus macaques showed evidence of both intra-species variability and positive selection. In the rhA3F clones, these sites were linked and appeared as either 60FQPQY64 (henceforth referred to as FQQY) or 60SKPEH64 (henceforth referred to as SKEH). 20 of 35 clones showed the FQQY sequence, while 15 clones showed the SKEH sequence. The patch change from FQQY to SKEH is fairly significant at the amino acid level: bulky hydrophobic phenylalanine is exchanged for a small polar serine, polar uncharged glutamine is exchanged for a positively charged lysine and then a negatively charged glutamic acid, and hydrophobic tyrosine is exchanged for a positively charged histidine. Thus, the SKEH variant has three charged amino acids, whereas the FQQY variant is a neutral surface.

A broader genomic survey was done in order to better understand the level of diversity in the patch. Targeted PCR was used to amplify an approximately 680 base pair (bp) stretch of A3F from genomic DNA samples representing 44 different rhesus macaques. 15 animals appeared to be heterozygous FQQY/SKEH, 17 animals appeared to be homozygous FQQY/FQQY, and 12 animals appeared to be homozygous SKEH/SKEH. This frequency distribution correlated to the results from the cloned sequences. Thus, it is reasonable to conclude that this patch has maintained a high level of diversity within rhesus macaques, and that this diversity is not unique to a particular population of animals or the result of a small data set.

III. The functional significance of diversity

After demonstrating that there is a high level of diversity in primate A3F and that some sites in the gene have evolved under positive selection, experiments were done to determine whether the different A3F alleles displayed differential susceptibilities to Vifs of various SIV strains. First, A3F clones from rhesus macaques, crab-eating macaques, sooty mangabeys, and pigtail macaques were tested for expression in HEK293T cells. Of the 17 clones tested, 11 were found to express, as visualized via Western blot (Figure 5). They expressed at variable levels, and only a subset of clones that represented diverse sequences and were well-expressed were chosen for further examination.

In addition to these clones representing naturally occurring rhA3Fs, three point mutants were made by exchanging the FQQY and SKEH patches by site-directed mutagenesis. The resulting clones contained identical sequence to their parent rhA3F clone except for the four nucleotides that are responsible for the amino acid changes at this patch. These point mutants were also tested for expression in HEK293T cells, and two of the three clones tested were found to be highly expressed as detected via Western blot (Figure 5).

In order to determine whether the rhA3F variation confers differential resistance to Vif-mediated degradation, HEK293T cells were simultaneously transfected with A3F expression constructs and Vif expression constructs containing the Vif coding sequences of different SIV strains. The expression of A3F in the presence of these Vif challenges was then detected via Western blot and compared between the different clones as an indication of susceptibility to Vif-mediated degradation. Two rhA3G expression constructs were also included as controls because their phenotypes against these Vifs have been published previously.[48] A representative blot is shown in Figure 6, and it reveals three interesting observations. First, multiple rhA3Fs displayed resistance to the Vif derived from rhesus macaque-adapted SIVmac239. Second, nearly all of the rhA3Fs were degraded by a SIVsm Vif and by Vif from the stump-tailed macaque SIV strain (SIVstm). These are

somewhat surprising results, considering that the A3F-Vif interaction tends to be species-specific. Viral antagonist proteins are generally well-adapted to the restriction factors of their hosts, so one would expect Vif from rhesus macaque-adapted SIVmac to degrade the rhA3Fs and Vifs from related SIVs to not be as effective. However, this was not the case; SIVmac Vif did not degrade rhA3Fs, while Vifs from related SIVsm and SIVstm did. Finally, the FQQY/SKEH patch genotype did not affect sensitivity to any of the Vifs tested.

"The interaction between A3s and Vif is a crucial determinant of the species specificity of a virus, and it is likely that a Vif from a given viral strain has evolved precisely to counteract the A3 proteins of its host species."

DISCUSSION

Investigations into the diversity of primate A3F revealed that the gene displays the hallmarks of a restriction factor that has been subject to positive selective pressure by viral antagonists over evolutionary time. A3F is a highly polymorphic gene within rhesus macaques, and many single-nucleotide polymorphisms lead to significant amino acid changes. Charged residues are often implicated in protein-protein interactions, and therefore in the context of a restriction factor. Polymorphisms that change the charge of the amino acid may be more likely to be involved in an evolutionary "arms race" than more functionally conservative polymorphisms.

Examination of the inter-species variation via dN/dS analysis indicated that multiple sites within the gene have been subject to positive selection, and some of these sites overlap with the polymorphic sites found in rhesus macaques. It is important to note that the input for dN/dS analysis had 15 sequences representing 10 different primate species. This does not represent the full range of primate A3Fs, and the use of multiple A3F variants of certain species may have artificially influenced the results. However, eliminating variable alleles of a given species also would also have had the potential of producing artificially conservative results. Comparing results of multiple analyses that differ in their level of conservativeness helped to address this issue, and 11 sites in primate A3F were shown to be under the influence of positive selection. This is potentially only a subset of sites that are subject to positive selection, as the different analysis models identified as many as 25 positively selected sites.

An interesting patch of four linked amino acids was identified at codons 60, 61, 63, and 64, where two sequences were found at similar frequencies in rhA3F clones. dN/dS analysis suggested that these codons have been subject to positive selective pressure. The FQQY allele appeared at a slightly higher frequency than the SKEH allele in a broader genomic analysis of the region in rhesus macaques; however, the less common SKEH haplotype did appear across multiple animals and clones, unlike many other polymorphic sites where the less common allele appeared at a significantly lower frequency and sometimes only in one animal. The changes at the amino acid level in this patch are rather significant, as three charged amino acids are introduced in the SKEH haplotype while the patch is neutral in the FQQY haplotype. This patch was also notable because of its location in the structure of A3F. It borders the N-terminal catalytic motif, and is adjacent to a known determinant of strain-specific resistance to SIV-Vif mediated degradation in A3G. Polymorphism at residue 59 in rhA3G confers variable susceptibility to degradation by SIVmac Vif.[49] Together, these observations suggest that this patch may play a key role in an interaction with a viral protein, particularly with SIV Vif. However, functional tests did not suggest that variation at this site plays a role in determining susceptibility to Vif, as rhA3F clones that contained variable patch alleles did not display differential resistance to Vifs from various SIV strains.

Despite this patch's proximity to residue 59, it is not necessarily surprising that it does not play a role in rhesus A3F interaction with Vif. It is known that HIV-1 Vif interacts with the CTD in A3F, unlike in A3G where it interacts with the NTD. While this interaction has not been precisely

mapped with SIV Vifs, it is reasonable to believe that they will behave analogously to HIV-1 Vif and interact at the CTD in A3F. Therefore, this NTD patch would not be expected to play a direct role in interactions with Vif.

Functional tests did reveal multiple surprising phenotypes relating to the SIV Vifs. The rhA3F clones tested displayed resistance to degradation by SIVmac Vif. SIVmac239 is a cloned viral isolate that is well adapted to its rhesus macaque host. Thus, one would expect the Vif derived from this macaque-adapted virus to degrade the rhA3F clones, yet this was not observed. rhA3F function was further tested against a more diverse panel of macaque-adapted Vifs (data not shown), and similarly to the SIVmac239 isolate, the rhA3F alleles were resistant to degradation. Thus, this phenotype does not appear to be a specific phenomenon of the SIVmac239 isolate, but rather a more general property of rhA3F. It is important to note that the rhA3G controls confirm that this SIVmac239 Vif clone is active and behaves as expected with these rhA3G alleles. It is possible to speculate that SIVmac239 evolved to effectively counteract rhA3G, the more potent deaminase, but in doing so it traded off the ability to neutralize rhA3F. This proposal is supported by the fact that the more ancestral SIVsmE041 Vif completely degraded the rhA3F clones tested, with the exception of rhA3F-WI, which showed an intermediate phenotype.

A3F is known to restrict viruses other than lentiviruses, so it is reasonable to believe that a different retrovirus may be responsible for driving this high level of diversity in rhesus macaque A3F. A potential candidate is simian foamy virus (SFV), a member of the Spumavirus genus of retroviruses. FVs are distinct from other retroviruses in many aspects, including their gene expression, protein processing, and replication. They are found in non-human primates, cats, cows, and horses, as well as humans who have been infected by non-human primates. They cause a persistent infection, but there are no known pathologies in natural or human hosts.[50,51] It has been suggested that SFVs have co-evolved with their Old World primate hosts for over 30 million years, marking them as the oldest known RNA viruses in vertebrates.[52] A3 proteins have been implicated in restriction of FVs, and the FV accessory protein Bet has been found to counteract A3 restriction mechanisms analogously to HIV-1 Vif.[53,54] The relatively old age of FVs as compared to SIVs, their ubiquitous presence in Old World primates, and the existence of an accessory protein that may have anti-A3 properties together suggest that SFVs may have acted as a selective pressure on A3F.

This investigation offered valuable insight into the genetic diversity of A3F in Old World primates. The presence of unusually high intra- and inter-species variability indicates that A3F has been continuously exposed to positive selective pressure. While SIV may not necessarily be the virus responsible for driving the polymorphism in rhesus macaque A3F, understanding the restriction factor's genetic variability opens a line of inquiry into other potential selective pressures. It is also important to note that only one linked polymorphic patch was investigated; there are several other sites that may play a role in interactions with Vif. Further mutagenesis studies will reveal whether any of these sites play a role in SIV restriction, or whether it truly is another virus altogether that has provided the selective pressure on rhA3F. In addition, the sooty mangabey, crab-eating macaque, and pigtail macaque A3F clones have not been fully characterized in terms of their susceptibility to various Vifs. This information will provide a more complete picture of the role that Vif has played in driving A3F evolution, and further elucidate mechanisms of cross-species transmission of primate lentiviruses.

ENDNOTES

1. Sharp and Hahn 2008.
2. Barre-Sinoussi et al., 1983.
3. Gallo et al., 1984.
4. Huet et al., 1990.
5. Hirsch et al., 1989.
6. VandeWoude and Apetrei 2006.
7. McCarthy and Johnson 2014.
8. Emerman and Malik 2010.
9. Hurst 2002.
10. Meyerson and Sawyer 2011.
11. Harris et al., 2012.
12. Koning et al., 2009.
13. Desimmie et al., 2014.
14. Harris et al., 2012.
15. Jarmuz et al., 2002.
16. Ibid..
17. Desimmie et al., 2014.
18. Bohn et al., 2013.
19. Desimmie et al., 2014.
20. Mangeat et al., 2003.
21. Bishop et al., 2004.
22. Wiegand et al., 2004.
23. Harris and Liddament 2004.
24. Sodroski et al., 1986.
25. Gabuzda et al., 1992.
26. Sheehy et al., 2002.
27. Marin et al., 2003.
28. Sheehy et al., 2003.
29. Simon et al., 1998.
30. Holmes et al., 2007.
31. Bogerd et al., 2004.
32. Huthoff and Malim 2007.
33. Smith and Pathak 2010.
34. Albin et al., 2010.
35. Land et al., 2014.
36. He et al., 2008.
37. Russell and Pathak 2007.
38. Hatziioannou and Evans 2012.
39. Apetrei et al., 2005.
40. Mansfield et al., 1995.
41. Marx et al., 2004.
42. Krupp et al., 2013.
43. Sawyer et al., 2004.
44. Delport et al., 2010.
45. Kosakovsky Pond and Frost 2005a.
46. Kosakovsky Pond et al., 2005.
47. Kosakovsky Pond and Frost 2005b.
48. Krupp et al., 2013.
49. Ibid..
50. Murray and Linial 2006.
51. Meiering and Linial 2001.
52. Switzer et al., 2004.
53. Russell et al., 2005.
54. Lochelt et al., 2005.

REFERENCES

Albin, J. S., R. S. LaRue, J. A. Weaver, W. L. Brown, K. Shindo, E. Harjes, H. Matsuo, and R. S. Harris. 2010. "A Single Amino Acid in Human APOBEC3F Alters Susceptibility to HIV-1 Vif." *The Journal of Biological Chemistry* 285 (52): 40785-40792. doi: 10.1074/jbc.M110.173161.

Alce, T. M. and W. Popik. 2004. "APOBEC3G is Incorporated into Virus-Like Particles by a Direct Interaction with HIV-1 Gag Nucleocapsid Protein." *The Journal of Biological Chemistry* 279 (33): 34083-34086. doi: 10.1074/jbc.C40023520.

Apetrei, C., A. Kaur, N. W. Lerche, M. Metzger, I. Pandrea, J. Hardcastle, S. Falkenstein, et al. 2005. "Molecular Epidemiology of Simian Immunodeficiency Virus SIVsm in U.S. Primate Centers Unravels the Origin of SIVmac and SIVstm." *Journal of Virology* 79 (14): 8991-9005. doi: 10.1128/JVI.79.14.8991-9005.2005.

Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, et al. 1983. "Isolation of a T-Lymphotropic Retrovirus from a Patient at Risk for Acquired Immune Deficiency Syndrome (AIDS)." *Science (New York, N.Y.)* 220 (4599): 868-871.

Bishop, K. N., R. K. Holmes, A. M. Sheehy, N. O. Davidson, S. J. Cho, and M. H. Malim. 2004. "Cytidine Deamination of Retroviral DNA by Diverse APOBEC Proteins." *Current Biology: CB* 14 (15): 1392-1396. doi: 10.1016/j.cub.2004.06.057.

Bogerd, H. P., B. P. Doehle, H. L. Wiegand, and B. R. Cullen. 2004. "A Single Amino Acid Difference in the Host APOBEC3G Protein Controls the Primate Species Specificity of HIV Type 1 Virion Infectivity Factor." *Proceedings of the National Academy of Sciences of the United States of America* 101 (11): 3770-3774. doi: 10.1073/pnas.0307713101.

Bohn, M. F., S. M. Shandilya, J. S. Albin, T. Kouno, B. D. Anderson, R. M. McDougle, M. A. Carpenter, et al. 2013. "Crystal Structure of the DNA Cytosine Deaminase APOBEC3F: The Catalytically Active and HIV-1 Vif-Binding Domain." *Structure (London, England : 1993)* 21 (6): 1042-1050. doi: 10.1016/j.str.2013.04.010.

Delport, W., A. F. Poon, S. D. Frost, and S. L. Kosakovsky Pond. 2010. "Datamonkey 2010: A Suite of Phylogenetic Analysis Tools for Evolutionary Biology." *Bioinformatics*

(Oxford, England) 26 (19): 2455-2457. doi: 10.1093/bioinformatics/btq429.

Desimmie, B. A., K. A. Delviks-Frankenberrry, R. C. Burdick, D. Qi, T. Izumi, and V. K. Pathak. 2014. "Multiple APOBEC3 Restriction Factors for HIV-1 and One Vif to Rule them all." *Journal of Molecular Biology* 426 (6): 1220-1245. doi: 10.1016/j.jmb.2013.10.033.

Emerman, M. and H. S. Malik. 2010. "Paleovirology--Modern Consequences of Ancient Viruses." *PLoS Biology* 8 (2): e1000301. doi: 10.1371/journal.pbio.1000301.

Gabuzda, D. H., K. Lawrence, E. Langhoff, E. Terwilliger, T. Dorfman, W. A. Haseltine, and J. Sodroski. 1992. "Role of Vif in Replication of Human Immunodeficiency Virus Type 1 in CD4+ T Lymphocytes." *Journal of Virology* 66 (11): 6489-6495. doi: Role of Vif in Replication of Human Immunodeficiency Virus Type 1 in CD4+ T Lymphocytes.

Gallo, R. C., S. Z. Salahuddin, M. Popovic, G. M. Shearer, M. Kaplan, B. F. Haynes, T. J. Palker, R. Redfield, J. Oleske, and B. Safai. 1984. "Frequent Detection and Isolation of Cytopathic Retroviruses (HTLV-III) from Patients with AIDS and at Risk for AIDS." *Science (New York, N.Y.)* 224 (4648): 500-503.

Harris, R. S., J. F. Hultquist, and D. T. Evans. 2012. "The Restriction Factors of Human Immunodeficiency Virus." *The Journal of Biological Chemistry* 287 (49): 40875-40883. doi: 10.1074/jbc.R112.416925.

Hatziioannou, T. and D. T. Evans. 2012. "Animal Models for HIV/AIDS Research." *Nature Reviews.Microbiology* 10 (12): 852-867. doi: 10.1038/nrmicro2911.

He, Z., W. Zhang, G. Chen, R. Xu, and X. F. Yu. 2008. "Characterization of Conserved Motifs in HIV-1 Vif Required for APOBEC3G and APOBEC3F Interaction." *Journal of Molecular Biology* 381 (4): 1000-1011. doi: 10.1016/j.jmb.2008.06.061.

Hirsch, V. M., R. A. Olmsted, M. Murphey-Corb, R. H. Purcell, and P. R. Johnson. 1989. "An African Primate Lentivirus (SIVsm) Closely Related to HIV-2." *Nature* 339 (6223): 389-392.

Holmes, R. K., F. A. Koning, K. N. Bishop, and M. H. Malim. 2007. "APOBEC3F can Inhibit the Accumulation of HIV-1 Reverse Transcription Products in the Absence of Hypermutation. Comparisons with APOBEC3G." *The Journal of Biological Chemistry* 282 (4): 2587-2595.

Huet, T., R. Cheynier, A. Meyerhans, G. Roelants, and S. Wain-Hobson. 1990. "Genetic Organization of a Chimpanzee Lentivirus Related to HIV-1." *Nature* 345 (6273): 356-359.

Hurst, L. D. 2002. "The Ka/Ks Ratio: Diagnosing the Form of Sequence Evolution." *Trends in Genetics : TIG* 18 (9): 486.

Huthoff, H. and M. H. Malim. 2007. "Identification of Amino Acid Residues in APOBEC3G Required for Regulation by Human Immunodeficiency Virus Type 1 Vif and Virion Encapsidation." *Journal of Virology* 81 (8): 3807-3815. doi: 10.1128/JVI.02795-06.

Jarmuz, A., A. Chester, J. Bayliss, J. Gisbourne, I. Dunham, J. Scott, and N. Navaratnam. 2002. "An Anthropoid-Specific Locus of Orphan C to U RNA-Editing Enzymes on Chromosome 22." *Genomics* 79 (3): 285-296. doi: 10.1006/geno.2002.6718.

Koning, F. A., E. N. Newman, E. Y. Kim, K. J. Kunstman, S. M. Wolinsky, and M. H. Malim. 2009. "Defining APOBEC3 Expression Patterns in Human Tissues and Hematopoietic Cell Subsets." *Journal of Virology* 83 (18): 9474-9485. doi: 10.1128/JVI.01089-09.

Kosakovsky Pond, S. L. and S. D. Frost. 2005. "Datamonkey: Rapid Detection of Selective Pressure on Individual Sites of Codon Alignments." *Bioinformatics (Oxford, England)* 21 (10): 2531-2533. doi: 10.1093/bioinformatics/bti320.

Kosakovsky Pond, S. L. and S. D. Frost. 2005. "Not so Different After all: A Comparison of Methods for Detecting Amino Acid Sites Under Selection." *Molecular Biology and Evolution* 22 (5): 1208-1222. doi: 10.1093/molbev/msi105.

Kosakovsky Pond, S. L., S. D. Frost, and S. V. Muse. 2005. "HyPhy: Hypothesis Testing using Phylogenies." *Bioinformatics (Oxford, England)* 21 (5): 676-679. doi: 10.1093/bioinformatics/bti079.

Krupp, A., K. R. McCarthy, M. Ooms, M. Letko, J. S. Morgan, V. Simon, and W. E. Johnson. 2013. "APOBEC3G Poly-

morphism as a Selective Barrier to Cross-Species Transmission and Emergence of Pathogenic SIV and AIDS in a Primate Host." *PLoS Pathogens* 9 (10): e1003641. doi:10.1371/journal.ppat.1003641.

Land, A. M., N. M. Shaban, L. Evans, J. F. Hultquist, J. S. Albin, and R. S. Harris. 2014. "APOBEC3F Determinants of HIV-1 Vif Sensitivity." *Journal of Virology* 88 (21): 12923-12927. doi:10.1128/JVI.02362-14.

Lochelt, M., F. Romen, P. Bastone, H. Muckenfuss, N. Kirchner, Y. B. Kim, U. Truyen, et al. 2005. "The Antiretroviral Activity of APOBEC3 is Inhibited by the Foamy Virus Accessory Bet Protein." *Proceedings of the National Academy of Sciences of the United States of America* 102 (22): 7982-7987. doi: 10.1073/pnas.0501445102.

Mangeat, B., P. Turelli, G. Caron, M. Friedli, L. Perrin, and D. Trono. 2003. "Broad Antiretroviral Defence by Human APOBEC3G through Lethal Editing of Nascent Reverse Transcripts." *Nature* 424 (6944): 99-103. doi: 10.1038/nature01709.

Mansfield, K. G., N. W. Lerch, M. B. Gardner, and A. A. Lackner. 1995. "Origins of Simian Immunodeficiency Virus Infection in Macaques at the New England Regional Primate Research Center." *Journal of Medical Primatology* 24 (3): 116-122.

Marin, M., K. M. Rose, S. L. Kozak, and D. Kabat. 2003. "HIV-1 Vif Protein Binds the Editing Enzyme APOBEC3G and Induces its Degradation." *Nature Medicine* 9 (11): 1398-1403. doi: 10.1038/nm946.

Marx, P. A., C. Apetrei, and E. Drucker. 2004. "AIDS as a Zoonosis? Confusion Over the Origin of the Virus and the Origin of the Epidemics." *Journal of Medical Primatology* 33 (5-6): 220-226. doi: 10.1111/j.1600-0684.2004.00078.x.

McCarthy, K. R. and W. E. Johnson. 2014. "Plastic Proteins and Monkey Blocks: How Lentiviruses Evolved to Replicate in the Presence of Primate Restriction Factors." *PLoS Pathogens* 10 (4): e1004017. doi:10.1371/journal. ppat.1004017.

Meiering, C. D. and M. L. Linial. 2001. "Historical Perspective of Foamy Virus Epidemiology and Infection." *Clinical Microbiology Reviews* 14 (1): 165-176.

Meyerson, N. R. and S. L. Sawyer. 2011. "Two-Stepping through Time: Mammals and Viruses." *Trends in Microbiology* 19 (6): 286-294. doi: 10.1016/j.tim.2011.03.006.

Murray, S. M. and M. L. Linial. 2006. "Foamy Virus Infection in Primates." *Journal of Medical Primatology* 35 (4-5): 225-235. doi: 10.1111/j.1600-0684.2006.00171.x.

Russell, R. A. and V. K. Pathak. 2007. "Identification of Two Distinct Human Immunodeficiency Virus Type 1 Vif Determinants Critical for Interactions with Human APOBEC3G and APOBEC3F." *Journal of Virology* 81 (15): 8201-8210. doi: 10.1128/JVI.00395-07.

Russell, R. A., H. L. Wiegand, M. D. Moore, A. Schafer, M. O. McClure, and B. R. Cullen. 2005. "Foamy Virus Bet Proteins Function as Novel Inhibitors of the APOBEC3 Family of Innate Antiretroviral Defense Factors." *Journal of Virology* 79 (14): 8724-8731. doi: 10.1128/JVI.79.14.8724-8731.2005

Sawyer, S. L., M. Emerman, and H. S. Malik. 2004. "Ancient Adaptive Evolution of the Primate Antiviral DNA-Editing Enzyme APOBEC3G." *PLoS Biology* 2 (9): E275. doi: 10.1371/journal.pbio.0020275.

Sharp, P. M. and B. H. Hahn. 2011. "Origins of HIV and the AIDS Pandemic." *Cold Spring Harbor Perspectives in Medicine* 1 (1): a006841. doi: 10.1101/cshperspect.a006841.

Sheehy, A. M., N. C. Gaddis, J. D. Choi, and M. H. Malim. 2002. "Isolation of a Human Gene that Inhibits HIV-1 Infection and is Suppressed by the Viral Vif Protein." *Nature* 418 (6898): 646-650. doi: 10.1038/nature00969.

Sheehy, A. M., N. C. Gaddis, and M. H. Malim. 2003. "The Antiretroviral Enzyme APOBEC3G is Degraded by the Proteasome in Response to HIV-1 Vif." *Nature Medicine* 9 (11): 1404-1407. doi: 10.1038/nm945.

Simon, J. H., D. L. Miller, R. A. Fouchier, M. A. Soares, K. W. Peden, and M. H. Malim. 1998. "The Regulation of Primate Immunodeficiency Virus Infectivity by Vif is Cell Species Restricted: A Role for Vif in Determining Virus Host Range and Cross-Species Transmission." *The EMBO Journal* 17 (5): 1259-1267.

Smith, J. L. and V. K. Pathak. 2010. "Identification of Specific Determinants of Human APOBEC3F, APOBEC3C,

and APOBEC3DE and African Green Monkey APOBEC3F that Interact with HIV-1 Vif." *Journal of Virology* 84 (24): 12599-12608. doi: 10.1128/JVI.01437-10.

Sodroski, J., W. C. Goh, C. Rosen, A. Tartar, D. Portetelle, A. Burny, and W. Haseltine. 1986. "Replicative and Cytopathic Potential of HTLV-III/LAV with Sor Gene Deletions." *Science (New York, N.Y.)* 231 (4745): 1549-1553.

Switzer, W. M., M. Salemi, V. Shanmugam, F. Gao, M. E. Cong, C. Kuiken, V. Bhullar, et al. 2005. "Ancient Co-Speciation of Simian Foamy Viruses and Primates." *Nature* 434 (7031): 376-380. doi: 10.1038/nature03341.

VandeWoude, S. and C. Apetrei. 2006. "Going Wild: Lessons from Naturally Occurring T-Lymphotropic Lentiviruses." *Clinical Microbiology Reviews* 19 (4): 728-762.

Wiegand, H. L., B. P. Doehle, H. P. Bogerd, and B. R. Cullen. 2004. "A Second Human Antiretroviral Factor, APOBEC3F, is Suppressed by the HIV-1 and HIV-2 Vif Proteins." *The EMBO Journal* 23 (12): 2451-2458.

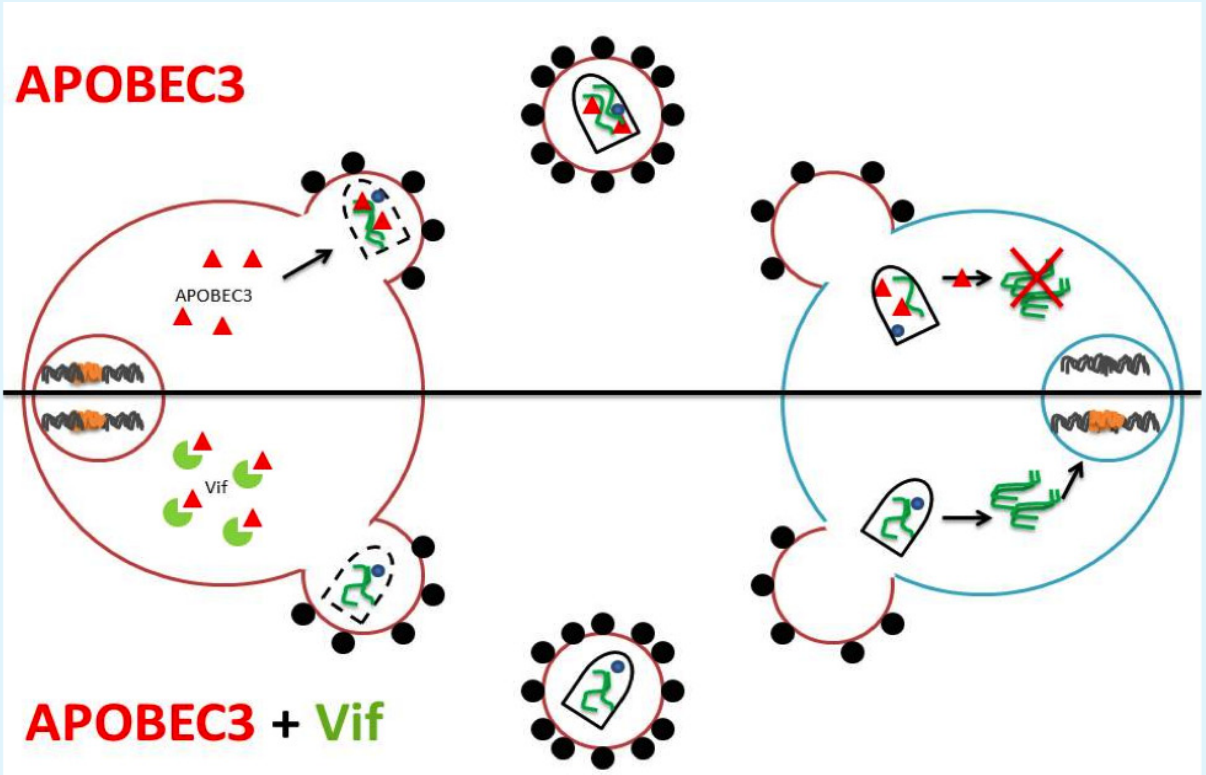

FIGURE 1: A3 PROTEINS INTERFERE WITH LENTIVIRAL REPLICATION IN THE ABSENCE OF VIF. THE TOP HALF OF THE FIGURE EXEMPLIFIES A CELL INFECTED WITH A VIRUS THAT DOES NOT ENCODE VIF. HOST A3 PROTEINS (TRIANGLES) INCORPORATE INTO A BUDDING VIRION, AND THEN INTERFERE WITH THE PROCESS OF REVERSE TRANSCRIPTION IN THE TARGET CELL TO PREVENT FURTHER VIRAL REPLICATION. THE BOTTOM HALF OF THE FIGURE EXEMPLIFIES A CELL INFECTED WITH A VIRUS THAT ENCODES VIF. VIF (¾ CIRCLES) PREVENTS A3 PROTEINS FROM INCORPORATING INTO THE BUDDING VIRION BY TARGETING THEM FOR PROTEOSOMAL DEGRADATION, ALLOWING REPLICATION TO CONTINUE UNHINDERED IN THE TARGET CELL.

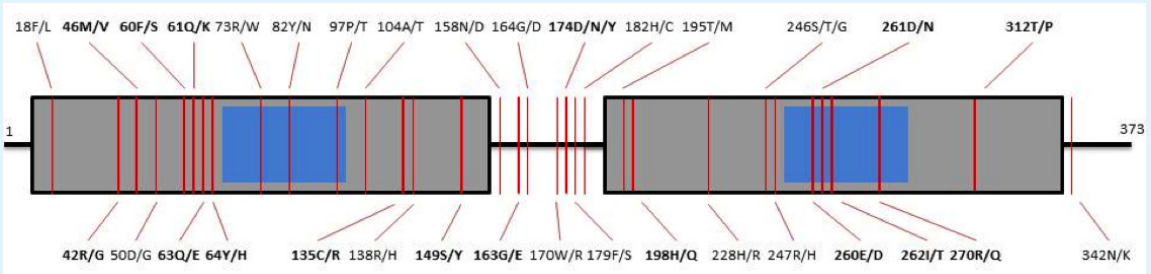

FIGURE 2: DISTRIBUTION OF POLYMORPHIC SITES IN RHESUS MACAQUE A3F. THE DARK GRAY BOXES REPRESENT THE CYTIDINE DEAMINASE DOMAINS IN A3F, AND THE BLUE SHADING INDICATES THE CATALYTIC MOTIF. THE LINES SHOW THE APPROXIMATE LOCATION OF SITES FOUND TO CONTAIN NON-SYNONYMOUS POLYMORPHISMS IN TWO OR MORE RHESUS MACAQUE CLONES. THE CODON NUMBERS AND CODED AMINO ACIDS ARE INDICATED ABOVE OR BELOW THE IMAGE. ONLY POLYMORPHISMS THAT WERE SEEN IN AT LEAST TWO CLONES ARE INCLUDED. BOLDED LINES AND CODON NUMBERS INDICATE THAT THE POLYMORPHISM WAS FOUND IN MULTIPLE ANIMALS.

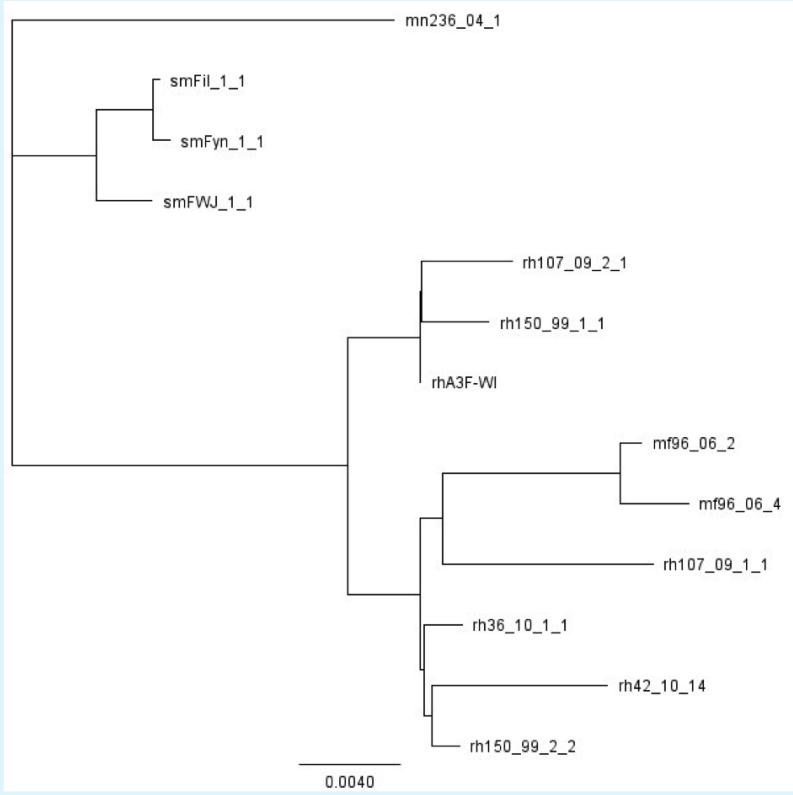

FIGURE 3: INTER-SPECIES DIVERSITY OF A3F IN OLD WORLD PRIMATES. THE PHYLOGENETIC TREE SHOWS THE DEGREE OF DIVERGENCE BETWEEN RHESUS MACAQUE A3F CLONES (RH), SOOTY MANGABEY A3F CLONES (SM), CRAB-EATING MACAQUE A3F CLONES (MF), AND PIGTAIL MACAQUE A3F CLONES (MN). THE SPECIES TEND TO GROUP TOGETHER INTO DISTINCT LINEAGES, AND DIVERSITY WITHIN SPECIES IS EVIDENT WITHIN THESE LINEAGES.

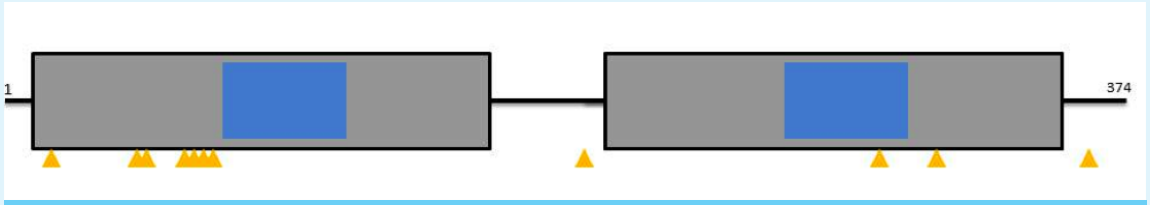

FIGURE 4: DISTRIBUTION OF POSITIVELY SELECTED SITES IN PRIMATE A3F. THE GREY BOXES REPRESENT THE CYTIDINE DEAMINASE DOMAINS IN A3F, AND THE SHADED AREAS INDICATES THE CATALYTIC MOTIFS. THE TRIANGLES SHOW THE APPROXIMATE LOCATIONS OF POSITIVELY SELECTED CODONS IDENTIFIED VIA DN/DS ANALYSIS. THESE SITES ARE DISTRIBUTED FAIRLY EVENLY THROUGHOUT THE A3F GENE.

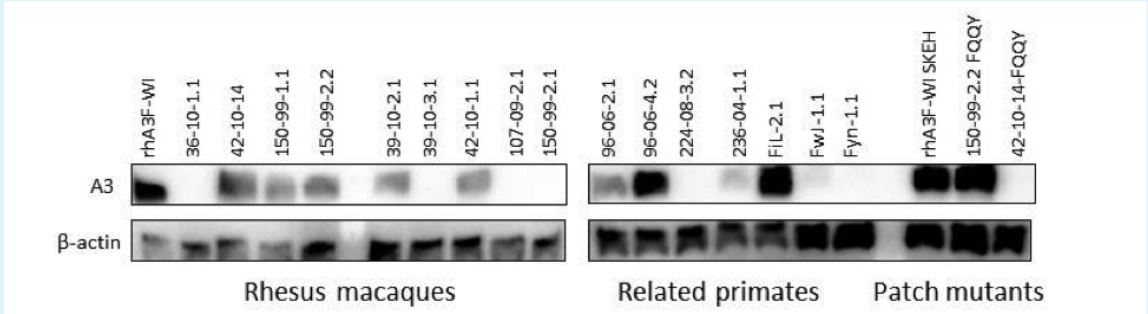

FIGURE 5: A3F ALLELES SHOW VARIABLE LEVELS OF EXPRESSION IN CELL CULTURE. A3F EXPRESSION CONSTRUCTS WITH CODING SEQUENCE ORIGINATING FROM RHESUS MACAQUES (LANES 1-10), CRAB-EATING MACAQUES (LANES 11-12), PIGTAIL MACAQUES (LANES 13-14), OR SOOTY MANGABEYS (LANES 15-17), ALONG WITH THE RHESUS MACAQUE PATCH MUTANTS (LANES 18-20) WERE TRANSFECTED IN HEK293T CELLS. CELLS WERE HARVESTED AFTER 48 HOURS, AND PROTEINS WERE SEPARATED VIA SDS/PAGE AND VISUALIZED VIA WESTERN BLOT (SEE IMMUNOBLOTTING SECTION IN MATERIALS AND METHODS).

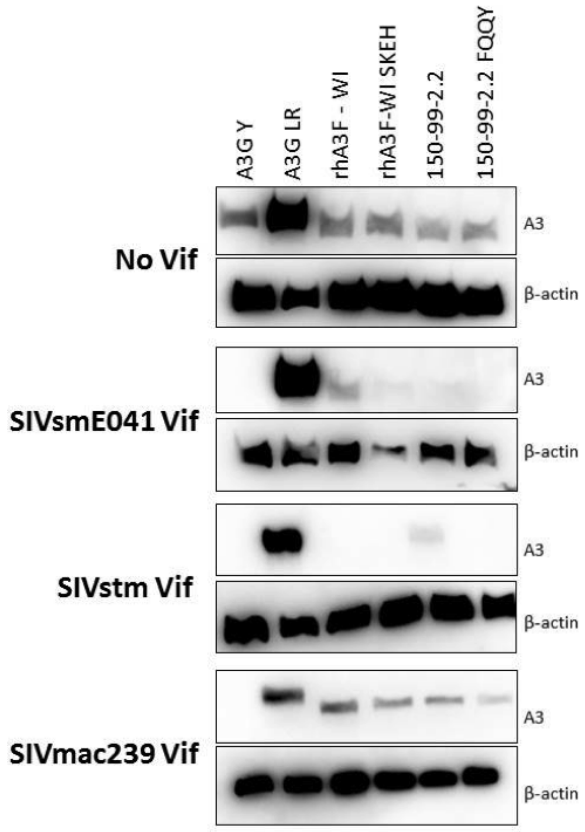

FIGURE 6: WESTERN BLOT SHOWING RHA3F SUSCEPTIBILITY TO SIV VIF-MEDIATED DEGRADATION. A3 AND VIF EXPRESSION CONSTRUCTS WERE CO-TRANSFECTED INTO HEK293T CELLS. CELLS WERE HARVESTED 48 HOURS POST-TRANSFECTION AND PROTEINS WERE SEPARATED VIA SDS/PAGE AND VISUALIZED VIA WESTERN BLOT (SEE IMMUNOBLOTTING SECTION IN MATERIALS AND METHODS). THE A3GS WERE INCLUDED AS CONTROLS FOR VIF EFFECTIVENESS, AS THEIR SUSCEPTIBILITIES TO THESE VIFS HAVE BEEN PREVIOUSLY INVESTIGATED (KRUPP ET AL. 2013). THE RHA3FS ARE SUSCEPTIBLE TO SIVSME041 VIF AND SIVSTM VIF, BUT ARE RESISTANT TO SIVMAC239 VIF. THE FQQY/SKEH PATCH DOES NOT AFFECT SUSCEPTIBILITY TO THE VIFS TESTED.