Adaptive Evolution and Loss of Function of a Primate Intrinsic Immunity Gene

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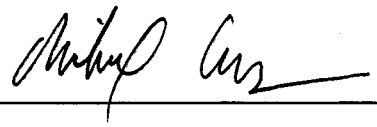

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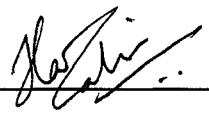

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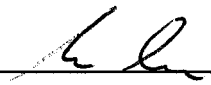

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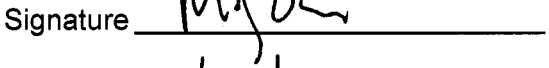
Signature 

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Abstract

Adaptive Evolution and Loss of Function of a Primate Intrinsic Immunity Gene

Molly OhAinle

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The evolution of retroviral elements and their hosts is inextricably tied together throughout the animal kingdom. Retroviral elements selfishly unleash an onslaught of tactics to replicate themselves within host cells while the host must fight back with an arsenal of weaponry that has been honed over hundreds of millions of years to protect host genomes from invasion by these selfish retroviral elements. Host and virus are perpetually locked in this evolutionary arms race that leaves distinct footprints on both genomes that can be "read" using molecular evolution techniques. Here I present an in depth study of one such host gene, APOBEC3H, that has dutifully served its primate hosts to limit retroviral replication for millions of years. Most humans, however, have recently lost the services of this retroviral defense gene, leaving these individuals more susceptible to several retroviral pathogens with large current-day impacts on human health and evolution, including HIV-1, LINE-1 and Alu elements.

TABLE OF CONTENTS

LIST OF FIGURES

Figure Number Page

LIST OF TABLES

PREFACE

"To lose an antiviral gene once may be regarded as misfortune; to lose it twice looks like carelessness."

-adapted from Oscar Wilde's *The Importance of Being Earnest*

The ancient conflict between pathogen and host genomes has been played out over millions of years. The threat imposed by retroviral elements has been broad in scope both in terms of diversity of invaders as well as the continued threat throughout evolution. Combating the threat posed by these retroviral elements is crucial as these invaders are capable of integrating into the host cell DNA, thereby jeopardizing the host's genomic integrity. Therefore, successful hosts have found ways to combat the challenge presented by retroviral invaders as a prerequisite to survival.

Recently, several genes and gene families, including the TRIM and APOBEC genes, have been described as frontline barriers to infection by retroelements in primates. Intrinsic immunity proteins are cell-autonomous and are thought to be key players in preventing the cross-species transmission of viruses as well as in controlling the replication of retrotransposons that have already invaded host genomes. The antagonistic interaction between host and virus results in genetic conflict. Such conflict has been proposed for the APOBEC3 genes found in primates and can be observed in the large expansion of this gene family in primates and the rapid evolution of several APOBEC3 family members that must keep pace with rapidly evolving pathogens.

In this dissertation, I describe my work aimed at uncovering the role of the primate APOBEC3H antiviral gene in the conflict between host and retrovirus.

Specifically, I examine the role of APOBEC3H in controlling and limiting retroviral replication.

In the first chapter I describe both the evolution of APOBEC3H as well as the first description of this protein as an antiretroviral gene. I find that although an APOBEC3H-like domain is conserved in eutherian mammals, this domain has never been duplicated. Further, this gene has evolved under positive selection throughout primate evolution consistent with its role as an antiviral gene. Through functional assays I show that this protein is capable of limiting HIV-1 replication, although this anti-HIV activity is primate-specific as is the case for other members of the APOBEC gene family. Specifically, the rhesus macaque homolog of APOBEC3H has potent anti-HIV activity while the human APOBEC3H homolog does not show any ability to affect HIV replication. I hypothesize that this is due to low steady-state expression of the human APOBEC3H protein relative to the macaque protein.

In the second chapter, I take a wider view of the evolution of the function of APOBEC3H throughout the primate lineage. Here I show that the function of APOBEC3H has changed dramatically over the last ~30 million years. Specifically, in addition to the human protein, the APOBEC3H protein from African green monkeys is also unstable and unable to inhibit retroviral replication. Other primate homologs show relatively high steady-state expression and varying abilities to inhibit the replication of several retroviral elements including HIV, SIV, MusD and LINE-1 elements. Further, I describe the truncation of the APOBEC3H coding sequence during the evolution of primates. This occurred when a nonsense mutation was fixed during the evolution of the human/chimpanzee ancestor. I find that this C-terminal domain (referred to as the "Tail") is necessary for the antiviral activity and cytoplasmic localization of the macaque APOBEC3H protein. It does not, however, contribute to the stability of APOBEC3H

proteins. Interestingly, I find that the chimpanzee APOBEC3H protein is capable of inhibiting a wide range of retroviral elements despite the fact that this protein lacks the Tail domain that is required for activity of the macaque homolog.

In the third chapter I examine the evolution of APOBEC3H specifically during the evolution of humans and chimpanzees since they last shared a common ancestor. While the chimpanzee APOBEC3H protein is stable and blocks retroviral replication, the human protein is unstable (due to a rapid turnover rate) and unable to mediate antiviral activities. I show that a reconstructed human/chimpanzee ancestral APOBEC3H protein is stable and functions as an antiviral, suggesting that loss of APOBEC3H stability and antiviral activity was specific to the human lineage. Further, a reconstructed APOBEC3H cDNA representing a Human Ancestor protein is also stable and has potent antiviral activity. Therefore, I propose that our recent human ancestors possessed an APOBEC3H allele that was stable and was capable of blocking the replication of a range of retroviral elements, including HIV and LINE-1 elements.

Finally, I map the loss of stability and antiviral activity to two, independent changes in human APOBEC3H alleles that are both polymorphic in the human population. A survey of APOBEC3H coding sequence from several different world populations reveals the presence of a haplotype without either destabilizing mutation that is present in the human population at a significant frequency. Therefore, functional APOBEC3H alleles do exist in the human population and may contribute to our different susceptibilities to retroviral elements.

ACKNOWLEDGEMENTS

This dissertation is the product of the thoughts and energy of much more than one person. I'd like to express my thanks and gratitude to several people who had a hand in this work.

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Finally, to Paul for loving what I do and always being there when I've needed encouragement. You had a huge hand in my success and I wouldn't be the scientist, much less the person I am today without you. So thank you.

DEDICATION

To my family, my husband and the one on the way

Chapter One:

INTRODUCTION

Retrotransposons, including retroviruses and retroviral elements, are distinguished from other virus families based on the fact that they reverse transcribe their positive-stranded, RNA genomes into DNA using a virally-encoded reverse transcriptase enzyme[1]. Some of these retroelements can replicate either exogenously (from cell-to-cell) or endogenously (within a single cell), while others replicate only endogenously. Further, they can be subdivided into either autonomously replicating (do not require help from other viral elements) or non-autonomously replicating (require machinery encoded by other viral elements) elements. I will describe the replication of each of these elements in order to show how they are inhibited by proteins of the mammalian intrinsic immune system. Specifically, this work explores the function of a member of the APOBEC3 family of proteins that are effectors of an antiviral defense that plays an important role in protecting primates and other mammals from a variety of genomic invasions [2].

RETROVIRUSES

LTR (Long Terminal Repeat)-containing retroviruses make up the *Retroviridae* family of viruses. Retroviruses can be divided into 2 subfamilies, the *Orthoretrovirinae* and the *Spumaretrovirinae*. Within the *Orthoretrovirinae* there are 6 genera: *Alpharetrovirus*, *Betaretrovirus*, *Gammaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus* and *Lentivirus*[3]. A single genus makes up the *Spumaretrovirinae*, *Spumavirus*. Viral genomes are 7 – 12kb in length, with two copies per virion. Retroviruses encode

three major proteins: *gag*, which encodes the viral structural proteins; *pol*, which encodes the enzymatic functions; and *env*, which encodes the viral envelope proteins (SU-surface and TM-transmembrane) required for receptor binding and fusion. Retroviruses can replicate either exogenously or endogenously, although the two are not mutually exclusive. The life cycle of retroviruses consists of: 1) entry and uncoating, 2) reverse transcription, 3) nuclear import and integration, 4) transcription and translation and, finally, 5) assembly, budding and virion maturation (Figure 1) [4-6] (the retroviral lifecycle has been reviewed in reference 2 and will be reviewed briefly here, with the exception of Reverse Transcription which will be covered in greater depth as details of reverse transcription are important for an understanding of APOBEC3 biology).

1: Entry and Uncoating

Receptor binding occurs via a specific interaction between the viral *env* glycoproteins and a target cell surface receptor. This interaction is thought to trigger a conformational change in the viral glycoproteins, resulting in the lipid membranes of host and virus being brought together in close proximity. The membrane surrounding the virus then fuses with the host cell membrane and the viral genome, in association with viral proteins, is deposited into the cytoplasm of the cell. Fusion can occur at the plasma membrane or following receptor-mediated endocytosis. Upon entering the cytoplasm of the host cell, the virus first undergoes a fairly mysterious process termed "uncoating" in which the Capsid (CA) protein making up the outer shell of the viral core is shed.

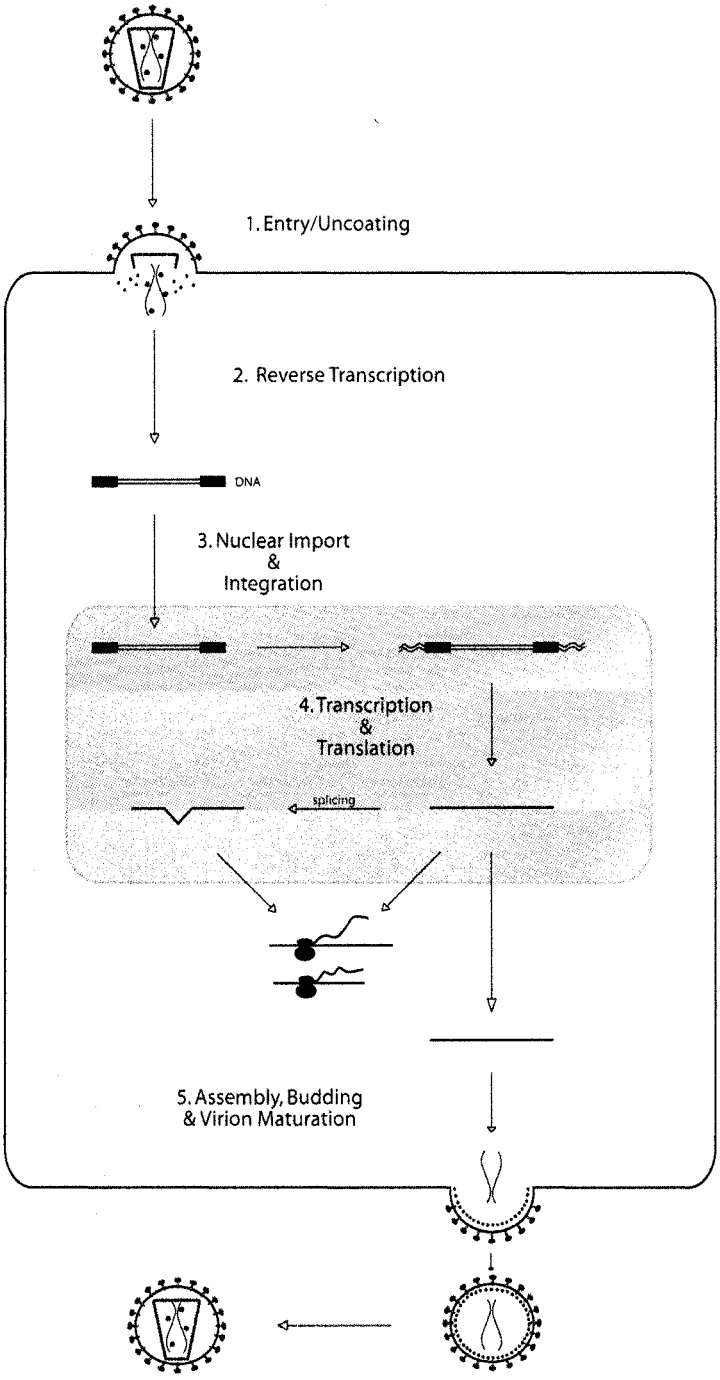


Figure 1 – The Retroviral Life Cycle
(Adapted from Ref 1).

2: Reverse Transcription

After uncoating, the virus then begins the process of converting its RNA genome into DNA through reverse transcription. The sequential steps of reverse transcription are shown in Figure 2 and outlined here:

1) DNA synthesis begins near the 5' end of the viral +sense RNA. A tRNA bound to the Primer Binding Site (PBS) serves as a primer for the virally encoded Reverse Transcriptase (RT) enzyme. The minus-strand DNA is extended through the U5 (5' Unique) and R (Repeat) regions of the genome. This short stretch of newly synthesized DNA is termed minus-strand strong-stop DNA (-sssDNA).
2) The RNase H activity of RT degrades the RNA that was used as template in the first round of minus-strand DNA synthesis. The activity of RNase H is specific for RNA/DNA duplexes. Therefore, RNA degradation occurs until the PBS is reached.
3) The newly synthesized minus-strand DNA is transferred to the 3' end of the viral genomic RNA, most likely facilitated by Nucleocapsid (NC). The annealing of the minus-strand DNA and genomic RNA is mediated by identical R sequences. This process is termed First Strand Transfer.
4) Using the annealed minus-strand DNA as a primer, RT continues the synthesis of minus-strand DNA. In tandem, RNase H degrades the RNA template. A stretch of RNA known as the Polypurine Tract (PPT) is excluded from degradation.

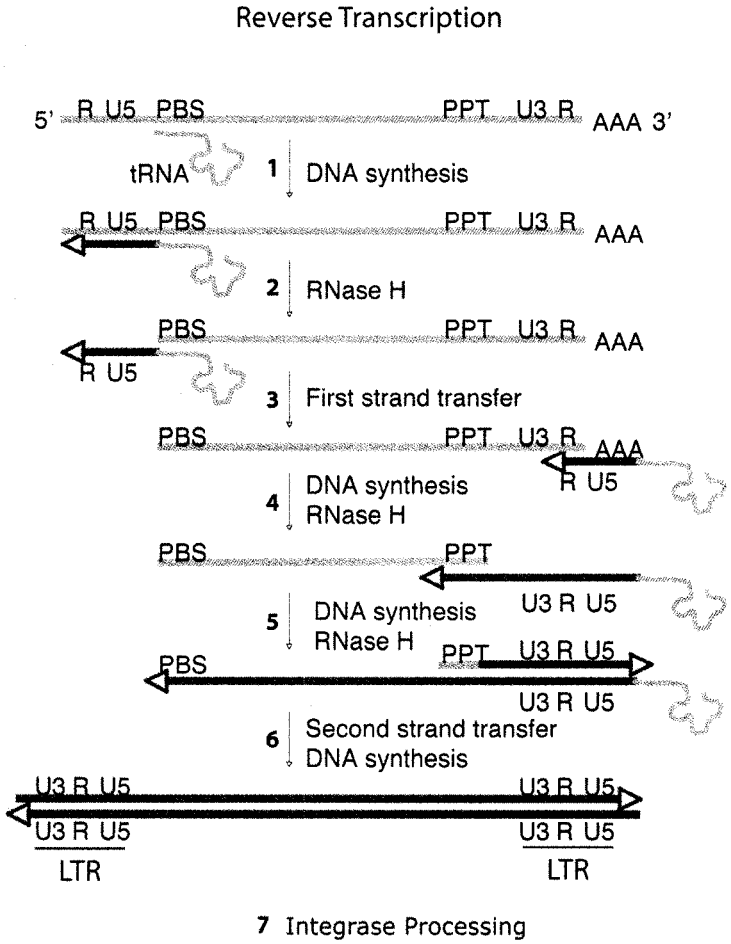


Figure 2 – Retroviral Reverse Transcription
The mechanism through which the viral ssRNA+ genome is converted into dsDNA. RNA is shown in green and DNA in blue (Adapted from Ref 1).

5) Minus-strand DNA synthesis through the PBS and plus-strand DNA synthesis initiates using the PPT as a primer. Plus-strand synthesis continues through a portion of the tRNA primer to create the plus-strand strong-stop DNA (+sssDNA). RNase H then degrades the tRNA primer, exposing sequence in the +sssDNA identical to the PBS in the minus-strand DNA.

6) The identical PBS sequences in the minus- and plus-strand DNA strands allows for circularization and annealing during the process of second strand transfer. Both plus- and minus-strands are extended using the opposing strand as a template to create the full-length, blunt-ended dsDNA genome that contains identical Long Terminal Repeats (LTRs) composed of U3, R and U5 sequences.
7) Finally, integrase processing results in cleavage of 2 (or sometimes 3) bases from both ends of the viral DNA. This gives rise to recessed 3'OH groups that will be available for attachment to host DNA in the subsequent steps of integration.

3: Nuclear Import and Integration

The preintegration complex (PIC), made up of newly synthesized viral DNA and both viral and cellular proteins, enters the nucleus. The integrase/viral DNA complex binds to the host DNA and integrase catalyzes the breaking of host DNA phosphodiester bonds and subsequent insertion of viral DNA into the host cell chromosome. The insertion sites targeted for retroviral integration are not random and different retroviruses show different propensities to integrate in certain genomic regions, such as actively-transcribed genes for HIV[7]. Finally, gaps at the insertion site are filled in and insertion of the retroviral provirus is complete.

4: Transcription and Translation

The LTRs of the integrated provirus contain *cis*-acting elements that drive viral gene expression by the cellular RNA polymerase II. Viral transcripts have a 5' cap

and a 3' polyA tail. Unspliced viral RNA is exported to the cytoplasm for packaging as genomic RNA. Unspliced RNAs can also serve as templates for multiple viral proteins that are synthesized from a single RNA through frameshifting, translational read-through and/or proteolysis. In addition, alternative splicing generates sub-genomic mRNAs that are translated to yield the remaining viral proteins.

5: Assembly, Budding and Virion Maturation

Viral Env, Gag and Gag-Pol proteins are transported to the plasma membrane where Assembly and Budding occur. Via interaction with Gag, two copies of unspliced viral genomic RNA are packaged into the budding virion. After release from the cell, the viral protease cleaves the Gag and Gag-Pol precursors, giving rise to the many structural and enzymatic protein components of the virion. Protease-mediated cleavage events lead to morphological changes in the virion shape that can be detected using electron microscopy (EM).

The Primate Lentiviruses

Human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) are related members of the lentiviral genus of retroviruses that infect humans and non-human primates, respectively. HIV infection in humans, as well as in experimental models such as infection of rhesus macaques, leads to progressive immunodeficiency and, ultimately, death. Many primate species in Africa are infected with SIV and, unlike HIV infection in humans, infection does not cause disease[8, 9]. The basis for this difference in outcome is largely unknown. The current HIV-1 pandemic has been shown to be the result of zoonotic infections of humans by

SIVcpz from wild populations of chimpanzees in Africa[10, 11]. The HIV-2 epidemic is the result of transmission of SIVsm from sooty mangabeys in West Africa[10, 12].

The HIV-1 genome is more complex than a simple retrovirus in that it encodes 15 proteins in 3 Open Reading Frames (ORFs): Tat, Rev, Matrix (MA), Capsid (CA), Nucleocapsid (NC), p6, Vpu, Nef, Protease, Vif, SU, TM, Vpr, Reverse Transcriptase and Integrase (Figure 3A)[6]. The arrangement of the major viral proteins in the mature virion is shown in Figure 3B. In addition to the major structural and enzymatic viral proteins that are conserved among retroviruses (described above), HIV-1 encodes various accessory proteins that assist in replication of the virus at various stages of the viral life cycle. These include Rev, allows for export of unspliced RNA from the nucleus, Nef, decreases cell-surface CD4 expression, and Vif, counteracts the antiviral effects of host APOBEC proteins.

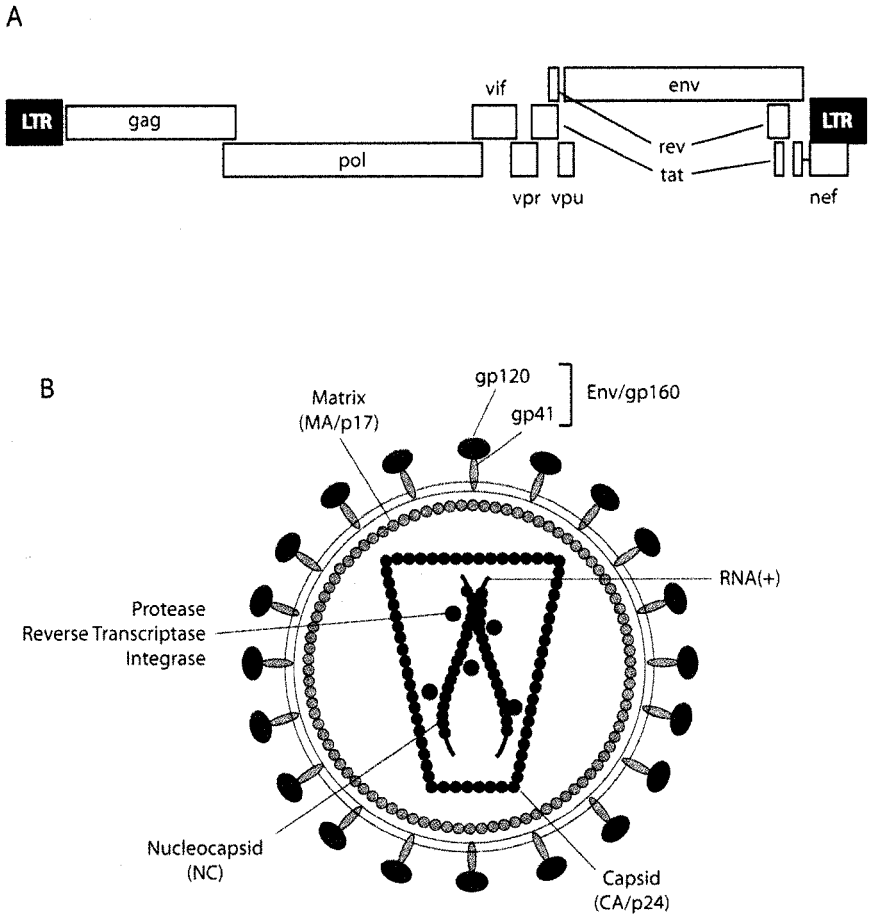


Figure 3 – The HIV-1 Genome and Viral Particle

LTR-Retrotransposons

LTR-Retrotransposons or "endogenous retroviruses" are LTR-containing retroelements that have successfully invaded the genome of their hosts. While retroviruses typically infect only the somatic cells of their host, they can on rare occasions infect germ line cells and subsequently are transmitted vertically from one generation to the next. Recent invasion of the koala genome by an exogenous retrovirus highlights the fact that retrovirus endogenization can occur on a relatively

short time-scale[13]. Approximately 8% of the human genome is made up of sequences from endogenous retroviruses (Fig 4) [14].

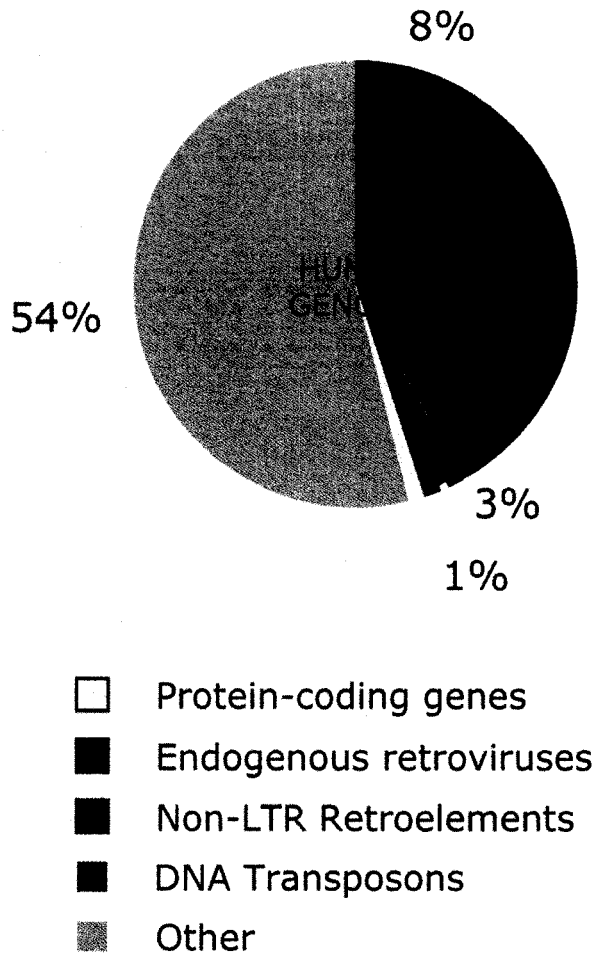


Figure 4 – Retroviral Sequence in the Human Genome
(Adapted from Ref. 15)

With some exceptions [15-17], endogenous retroviruses are generally simple retroviruses (i.e. they do not encode accessory proteins). Most families of endogenous retroviruses have a counterpart among the exogenous retroviruses and thus share many similarities with their exogenous retrovirus counterparts. They encode gag and pol gene products that facilitate all the structural and enzymatic

requirements of their replication, although endogenous retroviruses often do not encode intact *env* genes. They either do not encode an *env* open reading frame or their *env* gene has been inactivated through the accumulation of mutations. Loss of functional Env protein may not be detrimental to the replication of endogenous retroviruses if they are able to complete their life cycle entirely within a single cell, thereby re-infecting the genome from which the came. Less frequently, endogenous retroviruses do encode an intact *env* open reading frame[18] and these proviruses have the potential to replicate exogenously.

Non-LTR Retrotransposons

Non-LTR Retrotransposons are distinguished from both exogenous and endogenous retroviruses in that they do not contain an LTR and, subsequently, reverse transcription during replication of these elements is significantly different. Two major types of non-LTR Retrotransposons are defined based on whether they are able to replicate their genomes using only their own viral proteins (autonomously replicating) or if they require other viral proteins during their life cycle (non-autonomously replicating).

The largest family of autonomously replicating non-LTR retrotransposons found in mammals is the Long Interspersed Nucleotide Elements, LINE-1s. LINE elements make up approximately 27% of the human genome sequence (Fig 4)[14]. They are 4 – 6 kb in length and have two open reading frames, ORF1 and ORF2. ORF1 has nucleic acid binding activity and ORF2 encodes endonuclease and reverse transcriptase activities. Unlike their retrovirus relatives, LINE-1 elements do not undergo reverse transcription in the cytoplasm of cells. Instead, LINE-1s use a

"target-primed" reverse transcription (TPRT) mechanism in which the LINE-1-encoded endonuclease introduces a nick into the host DNA which is used to prime reverse transcription of the LINE-1 element, starting from the 3' polyA tail. LINE-1 endonuclease recognizes a short consensus sequence and, therefore, LINE-1 integrations are relatively promiscuous throughout the genome. While LINE-1 proteins show a strong cis-preference (they preferentially recognize and retrotranspose their own RNA), they can work in trans to retrotranspose other cellular RNAs. In particular, LINE-1s are responsible for the retrotransposition of the non-autonomous Short Interspersed Nucleotide Elements (SINEs)[19].

The most abundant SINE elements in the human genome are Alu elements. Alu elements have been very successful in colonizing the human genome with current estimates suggesting that Alu elements make up approximately 10% of the human genome (Fig 4)[14]. These primate-specific SINE elements are dimeric approximately 300 bp sequences that are derived from the cellular 7SL RNA. They contain two RNA polymerase III promoters and a polyA tail at their 3' end. Alu elements parastize the LINE-1 machinery to drive their own replication. They are retrotransposed through the same TPRT mechanism described above for LINE-1 elements. Interestingly, the amplification of both LINE-1 and Alu elements continues in the human population. Insertions that are polymorphic, not fixed in the human lineage, have been described for both Alu[20] and LINE-1 elements[21, 22].

INTRINSIC IMMUNITY

Host genomes have devoted significant resources to limit the successful replication of retroviral elements due to the selective pressure exerted on host

genomes by retroviral invaders. In addition to innate and adaptive immune responses, including T Cell, antibody and IFN-mediated antiviral systems, recent work in the retrovirology field has uncovered several novel types of antiviral defense genes termed "intrinsic immunity" genes [2, 23]. These 'intrinsic' immune effectors are distinguished from the innate and adaptive arms of the immune system in that they 1) are cell autonomous, 2) act to directly interfere with the viral life cycle within a single cell, 3) are ubiquitously expressed and 4) do not require induction to be active. These include the TRIM (TriPartite Motif) genes, such as Trim5α [24], and the APOBEC3 (Apolipoprotein B Editing Complex) gene family including APOBEC3H, the focus of the work presented here.

Discovery of APOBEC3G

APOBEC3G, the first member of the APOBEC3 family to be described as an antiviral gene, was discovered as a solution to the mystery of the function of the HIV protein, Vif, that had remained elusive for years [25, 26]. While wild-type HIV was able to productively infect both "permissive" and "non-permissive" cell types, HIV deleted for *vif* produced virions from "non-permissive" cells that were non-infectious. Therefore, it was hypothesized that a Vif-sensitive antiviral factor might be present in the "non-permissive" cells. This hypothesis was confirmed when heterokaryon experiments in which "non-permissive" and "permissive" cells were fused demonstrated that the factor was a dominant inhibitor as these cells retained the "non-permissive" phenotype [27, 28]. Finally, a subtractive hybridization screen in which a cDNA was found in "non-permissive" cells that was absent from permissive cells, uncovered APOBEC3G (initially named *CEM15* due to its discovery in the non-permissive CEM

cell line) [29]. APOBEC3G has subsequently been shown to inhibit the replication of a diverse array of retroviral elements including lentiviruses [30 31], gammaretroviruses [32], deltaretroviruses [33] and spumaretroviruses [34, 35], as well as murine endogenous retroviruses [36], yeast retrotransposons [37, 38] and non-LTR retrotransposons[39] (see Table 1 for a more complete summary).

To successfully infect APOBEC-expressing cells, retroviruses must be able to counteract this host barrier. In the case of primate lentiviruses, this is achieved by the Vif protein which causes the degradation of APOBEC proteins via the proteasome, thereby allowing efficient viral replication[29, 40, 41]. The mouse gamma retrovirus, MLV, evades the murine APOBEC3 enzyme by avoiding its incorporation into budding virions, most likely through a nucleocapsid-mediated exclusion[32]. Finally, foamy virus encodes the accessory protein Bet which has been shown to rescue retroviral infectivity in the presence of APOBEC3 proteins by binding to and, perhaps, sequestering the APOBEC3 proteins away from the sites of virus particle assembly[34,35].

The APOBEC Locus in Primates

APOBEC3G belongs to a large family (the APOBEC family) of enzymes that deaminate cytidines in varying polynucleotide contexts. Other APOBEC family members include Activation Induced Deaminase (AID), involved in immunoglobulin diversification[42], and APOBEC1, an enzyme that catalyzes the deamination of a host mRNA in the small intestine, resulting in a truncated Apolipoprotein B isoform[43]. All APOBECs have either one or two domains that contain the consensus cytidine deaminase motif, $HXEX_{23-28}PCX_{2-4}C$[44]. While the Histidine (H) and Cysteine (C)

residues are necessary for Zn^{++} ion coordination, the glutamate (E) is directly involved in catalysis of the deamination reaction that occurs through a proton shuttling mechanism[44].

The APOBEC3 family of genes underwent a large degree of duplication and expansion in primate genomes, giving rise to 7 genes arrayed in tandem on chromosome 22 in the human genome (Fig 5 and [44, 45]). The APOBEC3 locus in other primates is likely similar, although not necessarily identical to that found in humans and will be revealed as more primate genomes are sequenced. The APOBEC3 locus consists of 7 APOBEC3 genes, APOBEC3A – APOBEC3H, that contain either a single cytidine deaminase domain or two cytidine deaminase domains (represented by short and long arrows in Fig 5, respectively). APOBEC3A – APOBEC3G are all derived from duplication and expansion of a single ancestral cytidine deaminase domain (Fig 6; pink circles). In contrast, APOBEC3H, the most distal member of the locus is derived from a second type of ancestral cytidine deaminase domain (Fig 6; blue squares). The single APOBEC3 gene found in mice is a double-domained APOBEC3 gene, with one of each type of ancestral cytidine deaminase domain (Fig 6; far left).

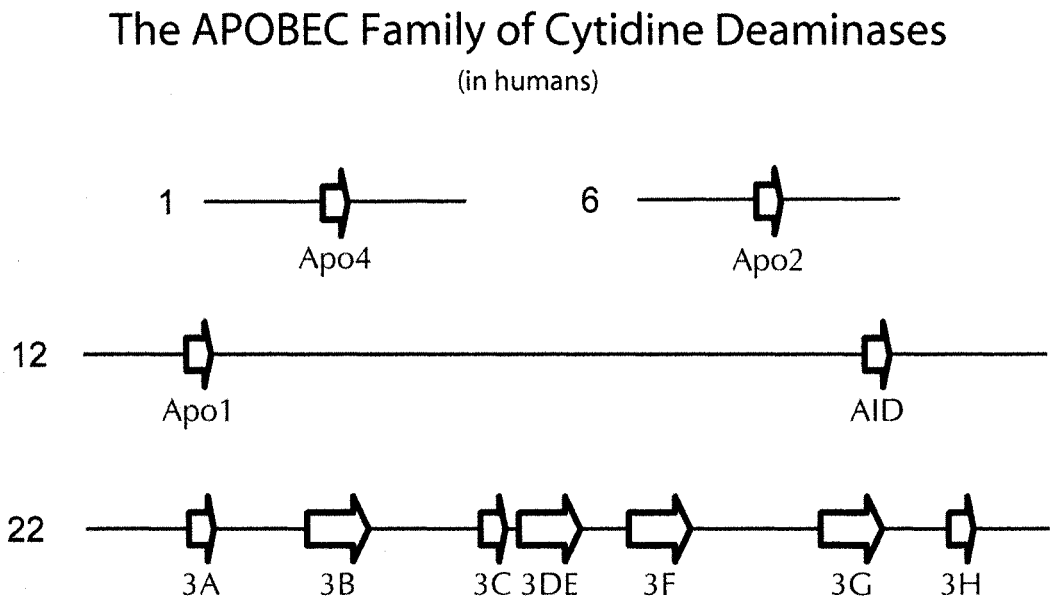


Figure 5: The APOBEC Family of Cytidine Deaminases
An ancestral cytidine deaminase domain has undergone significant amplification in mammalian and primate genomes (the human genes are shown here). The ancient duplication events have given rise to a total of 11 APOBEC genes in the human genome found on 4 different chromosomes (chr. 1, 6, 12 and 22). Double-domained APOBEC genes (denoted by larger arrows – 3B, 3DE, 3F and 3G) are found in the large expansion that occurred on human chromosome 22. All other APOBEC genes consist of a single cytidine deaminase domain (smaller arrows). This figure was adapted in part from Refs 1 and 44.

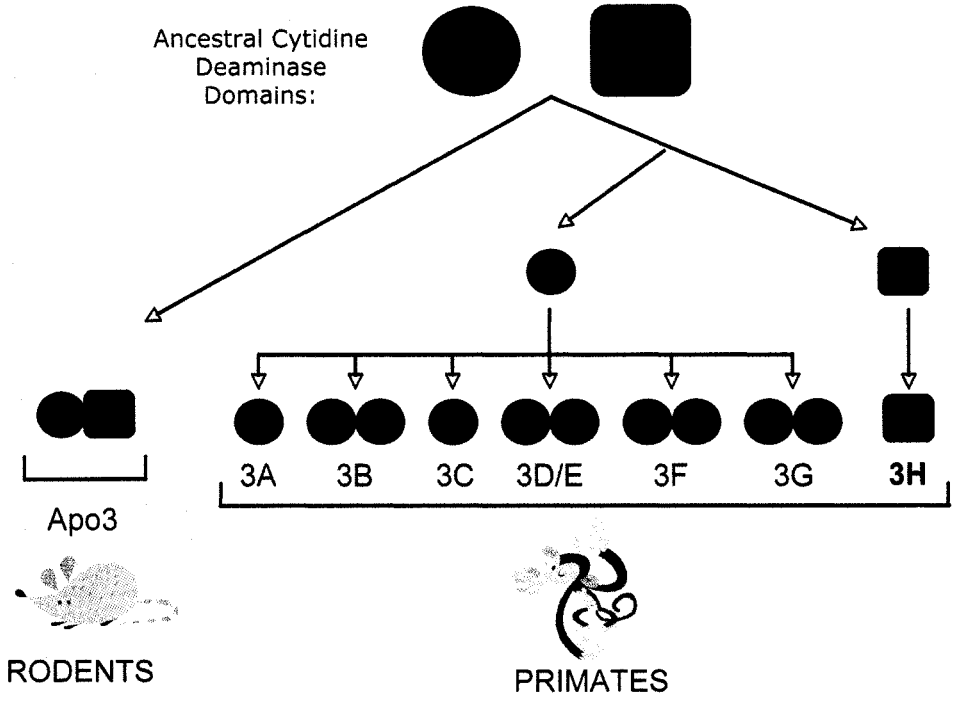


Figure 6 – Evolution of Primate APOBEC3 Genes
The two types of ancestral cytidine deaminase domains are represented by a pink circle and a blue square. Only a single APOBEC3 gene is found in mice, while a large expansion of one type of ancestral cytidine deaminase domain gave rise to the family of APOBEC3 genes found in primates (APOBEC3A – APOBEC3H) (Adapted from Ref 45).

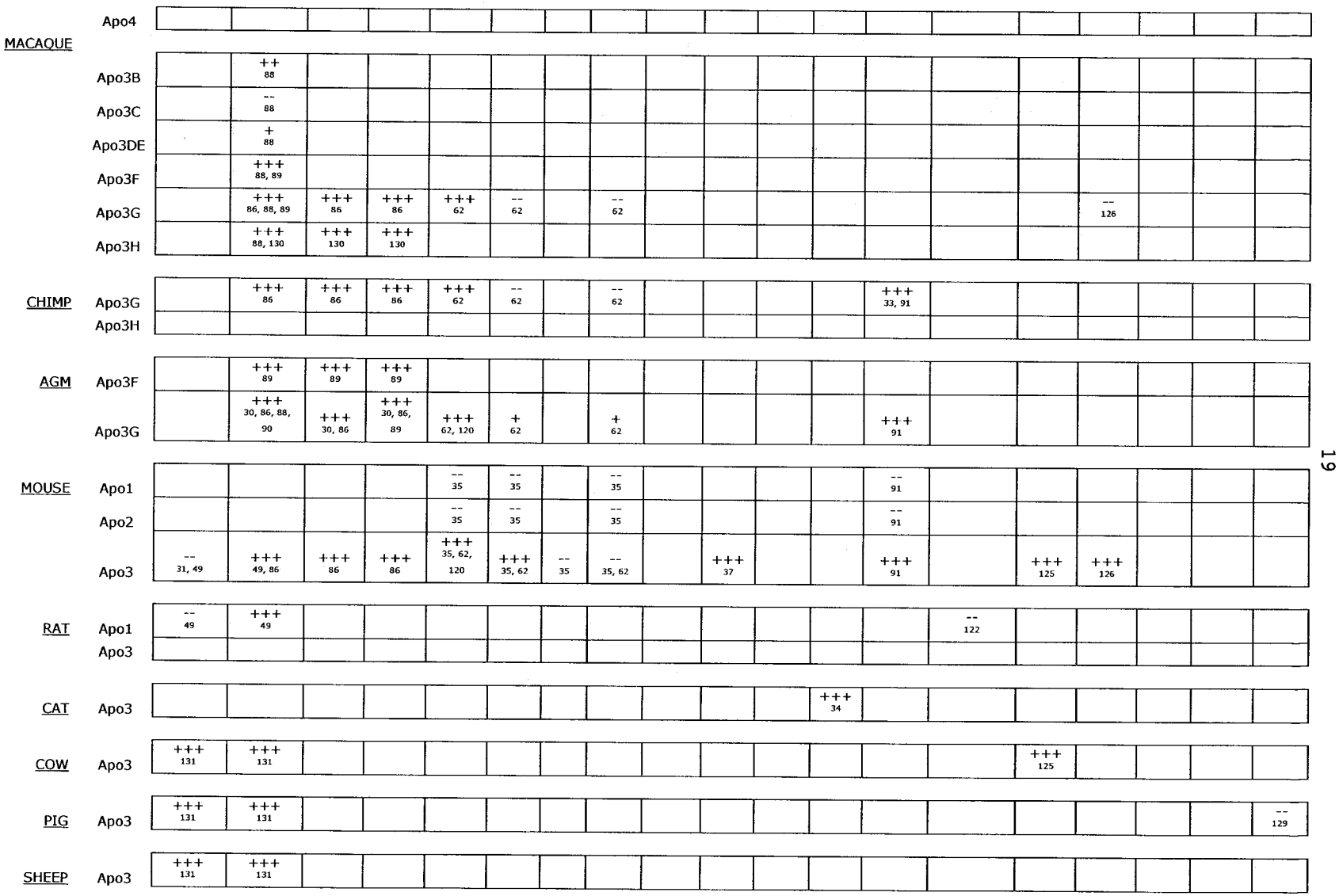
Although APOBEC3G is the most well characterized member of the family, all other APOBEC3 proteins, with the notable exception of APOBEC3H (the focus of the work presented here), have also been shown to have antiviral activities (see Table 1 for a summary). APOBEC3A lacks activity against the lentiviruses (HIV and SIV) as well as the mouse gamma retrovirus MLV, but it has been demonstrated to potenty inhibit endogenous retroelements (including IAP, MusD and LINE-1 elements) as well as adeno-associated virus (AAV).

Table 1 – Antiviral Activities of APOBEC3 Proteins

MLV = Murine Leukemia Virus
HIV = Human Immunodeficiency Virus
SIV = Simian Immunodeficiency Virus
(mac = SIV isolated from rhesus macaques)
(agm = SIV isolated from african green monkeys)
MusD = mouse type D retroelement
IAP = Intracisternal A Particle
L1 = Long Interspersed Nuclear Element (LINE-1)
Hu = human
Mu = mouse
Alu = Short Interspersed Nuclear Element (SINE)
Ty1 = Yeast LTR-retrotransposon
EIAV = Equine Infectious Anemia Virus
FFV = Feline Foamy Virus
PFV = Primate Foamy Virus
HBV = Hepatitis B Virus
HTLV-1 = Human T-Lymphotropic Virus
MPMV = Mason-Pfizer Monkey Virus
AAV = Adeno-Associated Virus
HERV-K = Human Endogenous Retrovirus K
PERV = Porcine Endogenous Retrovirus

+++ = Potent antiviral activity
+ = Weak antiviral activity
-- = No antiviral activity
+/- = Controversial

		MLV	HIV-1	SIV agm	SIV mac	MusD	IAP	Mu L1	HuL1	Alu	Ty1	EIAV	FFV	PFV	HBV	HTLV-1	MPMV	AAV	HERV-K	PERV
HUMAN	Apo1	-- 49	-- 49			-- 120								-- 91						
	Apo2		-- 30	-- 30	-- 30	-- 120								-- 91						
	AID	-- 49	-- 49	-- 30	-- 30	-- 120								-- 91						
	Apo3A	-- 31, 49	-- 49, 62, 121	-- 62		+++ 62	+++ 61, 62, 121		+++ 62, 121	+++ 121								+++ 62		
	Apo3B	+/- 30, 31, 49	+++ 30, 49, 98, 121	+++ 30	+++ 30	+/- 62, 120	+++ 61, 62, 121		+++ 62, 121	+++ 121				+ 91	+++ 122, 123			-- 62		
	Apo3C	-- 30, 31, 49	+/- 30, 49, 121	+++ 30	+++ 30	+/- 62, 120	+/- 61, 62		+ 62, 121	+ 121	+++ 37			+/- 33, 91	+++ 122			-- 62		
	Apo3DE	-- 76	+ 76	+ 76	+ 76															
	Apo3F	-- 31, 49	+++ 49, 121			+++ 62, 120	+/- 61, 62		+/- 62, 121		+++ 36, 37			+++ 91	+++ 122-124	+ 125	+++ 126	-- 62	+++ 127	
	Apo3G	+++ 29, 31, 49, 56	+++ 31, 49, 56, 86, 121	+++ 56, 86	+++ 86	+++ 35, 62, 120	+/- 35, 61, 62	-- 35	-- 35, 62, 121	+++ 38, 128	+++ 36, 37	+++ 56		+++ 91	+++ 94, 122-124	+++ 32, 125	+++ 126	-- 62	-- 127	+++ 129
	Apo3H		-- 76, 130	-- 130	-- 130															


MACAQUE
Apo4
Apo3B
Apo3C
Apo3DE
Apo3F
Apo3G
Apo3H
CHIMP
Apo3G
Apo3H
AGM
Apo3F
Apo3G
MOUSE
Apo1
Apo2
Apo3
RAT
Apo1
Apo3
CAT
Apo3
COW
Apo3
PIG
Apo3
SHEEP
Apo3

APOBEC3B, an APOBEC3 with broad antiviral activity, potently blocks the replication of the lentiviruses HIV and SIV as well as the endogenous retroelements MusD, IAP and LINE-1 elements. In addition, APOBEC3B has also been shown to block the replication of the hepadnavirus, HBV. APOBEC3C does not block the replication of HIV, but is able to potently inhibit SIV as well as HBV and the yeast LTR-retrotransposon Ty1. The activity of APOBEC3DE is rather limited as it has only been shown to have minor activity against the lentiviruses HIV and SIV. APOBEC3F potently blocks HIV replication as well MusD, Ty1 and HBV replication. In addition, APOBEC3F inhibits primate foamy virus (PFV) as well as the betaretrovirus Mason-Pfizer Monkey Virus (MPMV).

Viral Inhibition Through Deamination

The ability of APOBEC3G and other APOBEC family members to inhibit retroviral replication is at least partially due to their cytidine deaminase activity, causing hypermutation and degradation of invading retroviral genomes [2, 23]. The first described mechanism through which APOBEC3G blocks HIV-1 replication is through encapsidation into budding virions (mediated by binding to viral RNA and NC[70, 71]) and subsequent deamination of cytidines in the viral minus-strand DNA produced during reverse transcription (Fig 7). Deamination by APOBEC3G leads to either 1) hypermutation of proviral genomes or 2) degradation of viral cDNA transcripts before nuclear import and integration.

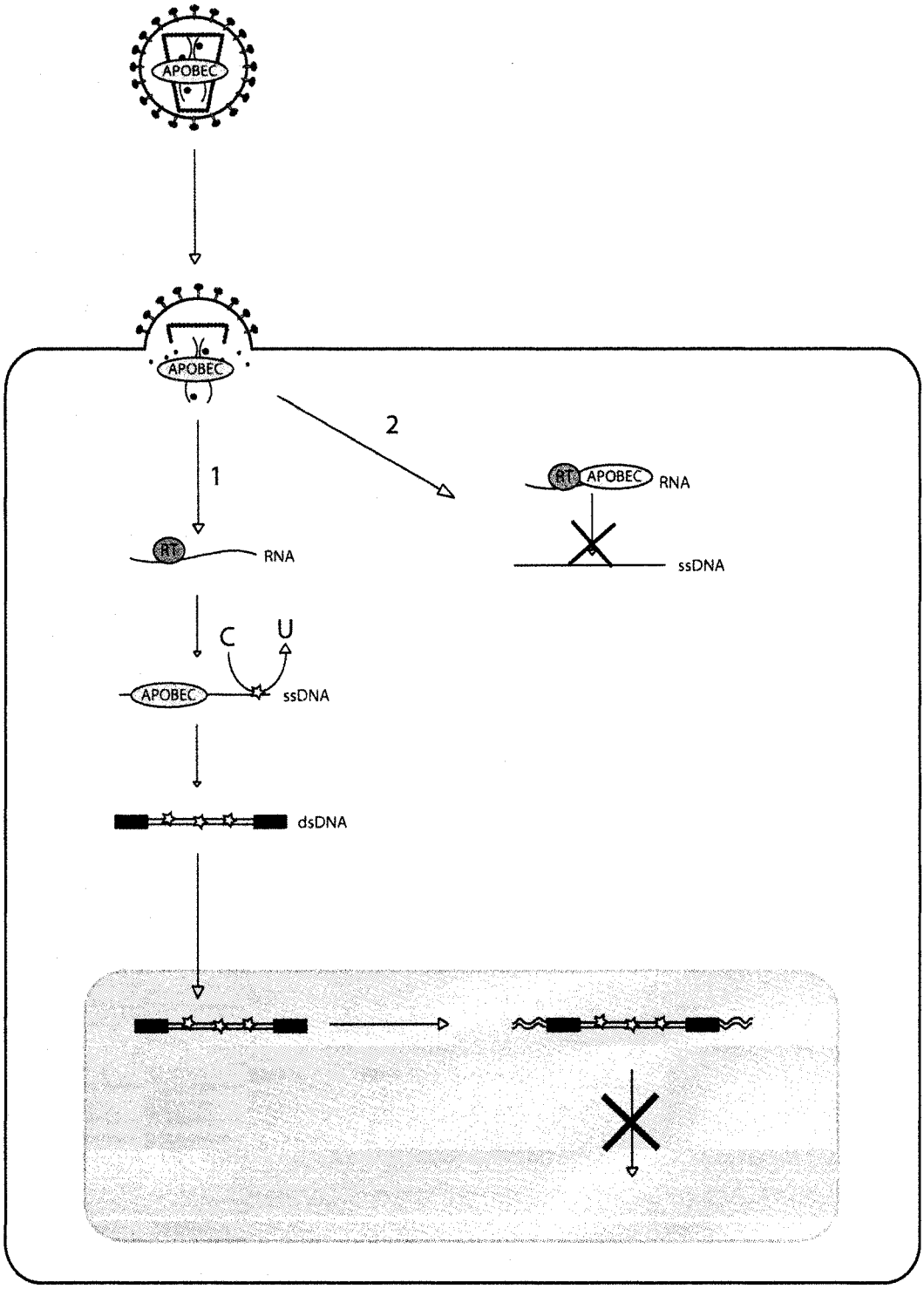


Figure 7 – Mechanism of APOBEC3-mediated Inhibition of HIV

APOBEC3G deamination of HIV genomes leads to G to A transition mutations on the plus-strand of the provirus, termed "G to A hypermutation"[72]. This implies that it is the cytidines in the minus strand DNA that are the targets of deamination. Therefore, APOBEC3G must discriminate between minus and plus strands of DNA. The minus strand specificity of APOBEC3G is likely due to a preference to deaminate single-stranded DNA. Accordingly, a gradient of deamination events occurs in direct correlation with the length of time a region of the genome remains single-stranded[73]. In addition, editing by different APOBEC3 enzymes occurs in specific polynucleotide contexts such that signatures of editing by particular APOBEC3 enzymes can be deduced. For example, APOBEC3G almost exclusively edits at cytidine bases preceded by another cytidine (a CC dinucleotide preference) while APOBEC3F and APOBEC3B prefer to edit cytidines preceded by thymidine residues (a TC dinucleotide preference) (see Table 2)[46, 74, 75].

The second possibility, that viral cDNAs containing APOBEC3-induced uracil lesions are degraded, has been hypothesized, but lacks any formal demonstration. Work from several groups has described a significant decrease in reverse transcription products and integration events in the presence of APOBEC3G[38, 76-78] and APOBEC3F[77]. The host Uracil DNA Glycosylase (UNG2) enzyme, a host cell protein that is incorporated into HIV-1 virions, has been proposed as a potential enzyme mediating the excision of uracils from newly-sythesized viral transcripts, followed by cleavage by host cell endonucleases and, therefore, degradation of viral transcripts[60, 79-81]. However, UNG2 has recently been demonstrated to be non-essential as the decreases levels of viral transcripts seen in the presence of APOBEC3G occurs independent of UNG2[76]. Instead, the defect in accumulation of

reverse transcription products may be due to APOBEC3G's ability to interfere directly with the reverse transcription process. This interference may be independent of APOBEC3G's enzymatic activity, although this is controversial, and will be described in more detail in the next section.

Deamination-Independent Mechanisms

In addition to inhibition of viral replication through APOBEC3-mediated editing, recent work suggests that the antiretroviral activity of APOBEC3 proteins also occurs through deamination-independent mechanisms. These include both target cell effects as well as antiviral activity without a requirement for catalytic activity.

There is a growing consensus in the field that editing may not be required for the inhibition of many retroviral elements by at least a subset of APOBEC3 proteins and/or for a subset of retroviral elements. Specifically, mutants of APOBEC3G in which the catalytic site has been inactivated still show residual antiviral activity and, in some cases, knocking out editing activity yields little reduction in antiviral activity[82]. The case for editing-independent inhibition by APOBEC3F is even more clear as a catalytically dead mutant of APOBEC3F retains near wild-type levels of inhibitory activity against HIV-1[78].

Nonetheless, some work suggests that deamination-independent mechanisms of inhibition may occur primarily at levels used *in vitro* that are well above physiologic APOBEC3G concentrations, whereas the deamination-dependent mechanisms may predominant at the levels of APOBEC3 protein found natively in most cells[83]. These studies are, however, often difficult to interpret as many "catalytic site mutants" likely affect much more than the protein's enzymatic activity and may instead also be

abolishing the ability of APOBEC3 proteins to bind nucleic acids. This is important as the proposed mechanisms of deamination-independent inhibition rely on nucleic acid binding. More careful and detailed analyses will have to be carried out to sort out the relative importance of either deamination-dependent or –independent mechanisms.

In contrast to the controversial deaminase-independent inhibition of HIV by APOBEC3G, it is generally accepted that APOBEC3A can act through deamination-independent mechanisms. APOBEC3A is clearly able to inhibit the mouse LTR-retrotransposon IAP even if its catalytic activity has been abolished[51] and no hypermutation can be detected in IAP, MusD or LINE-1 elements despite potent inhibition by APOBEC3A[49].

Along similar lines, APOBEC3G present in the target cells of HIV infection has recently been shown to be able to block efficient infection and appears to be responsible for the block to HIV infection in resting CD4+ T cells[84]. The target cell effect of APOBEC3G has subsequently been verified by other groups[85] and appears to function through a mechanism that is independent of editing as integrated proviruses show only barely detectable levels of G to A hypermutation. Instead, it is proposed that APOBEC3G may bind to reverse transcription complexes and affect the efficiency with which reverse transcription proceeds, leading to a decrease in the accumulation of reverse transcription products[84].

In accordance with the proposed mechanism of HIV inhibition in non-permissive resting CD4+ T cells, various editing-independent mechanisms of APOBEC3G inhibition of reverse transcription efficiency or integrity have recently been proposed. First, both APOBEC3G and APOBEC3F have been proposed to block the ability of the primer tRNA to initiate reverse transcription of HIV[86-88]. Second,

APOBEC3G has been shown to interfere with the proper processing and removal of the primer tRNA after minus-strand DNA synthesis[89]. Third, APOBEC3G can interfere with the strand transfer steps of reverse transcription, resulting in decreased levels of viral transcripts and integration events[90]. The relative importance of each of these reverse transcription defects is unclear and will be revealed as more detailed studies of APOBEC3 biology continue.

The defects in HIV reverse transcription seen for APOBEC3G and APOBEC3F antiviral activity have not been evaluated for other APOBEC3s and in only some instances will this be appropriate as only some APOBEC3 proteins have no impact on HIV replication. Instead, several deamination-independent mechanisms for inhibition of endogenous retroviral elements, in particular the SINE and LINE elements, have been proposed. The two major proposed mechanisms are outlined in Figure 8. Importantly, both LINE and SINE RNA exit the nucleus to the cytoplasm where they can associate with the LINE ORF1 and ORF2 proteins that are required for replication of these elements. It may be that APOBEC3 proteins inhibit LINE and SINE replication by binding to viral RNA transcripts and either block their ability to associate with necessary host and viral proteins or sequester the RNA away from sites necessary for efficient replication. The high molecular mass (HMM) complexes that APOBEC3G and other APOBEC3s are known to associate with may be such sites of sequestration[39]. Alternatively, APOBEC3s may bind to viral RNA/protein complexes in a manner similar to binding of Gag/viral RNA complexes of exogenous retroviruses and be present at the site of reverse transcription to impede the reverse transcription process through mechanisms analogous to what has been described for APOBEC3G's inhibition of the reverse transcription of HIV. The two mechanisms are

not mutually exclusive and detailed analyses of the true mechanisms will have to wait until further characterization.

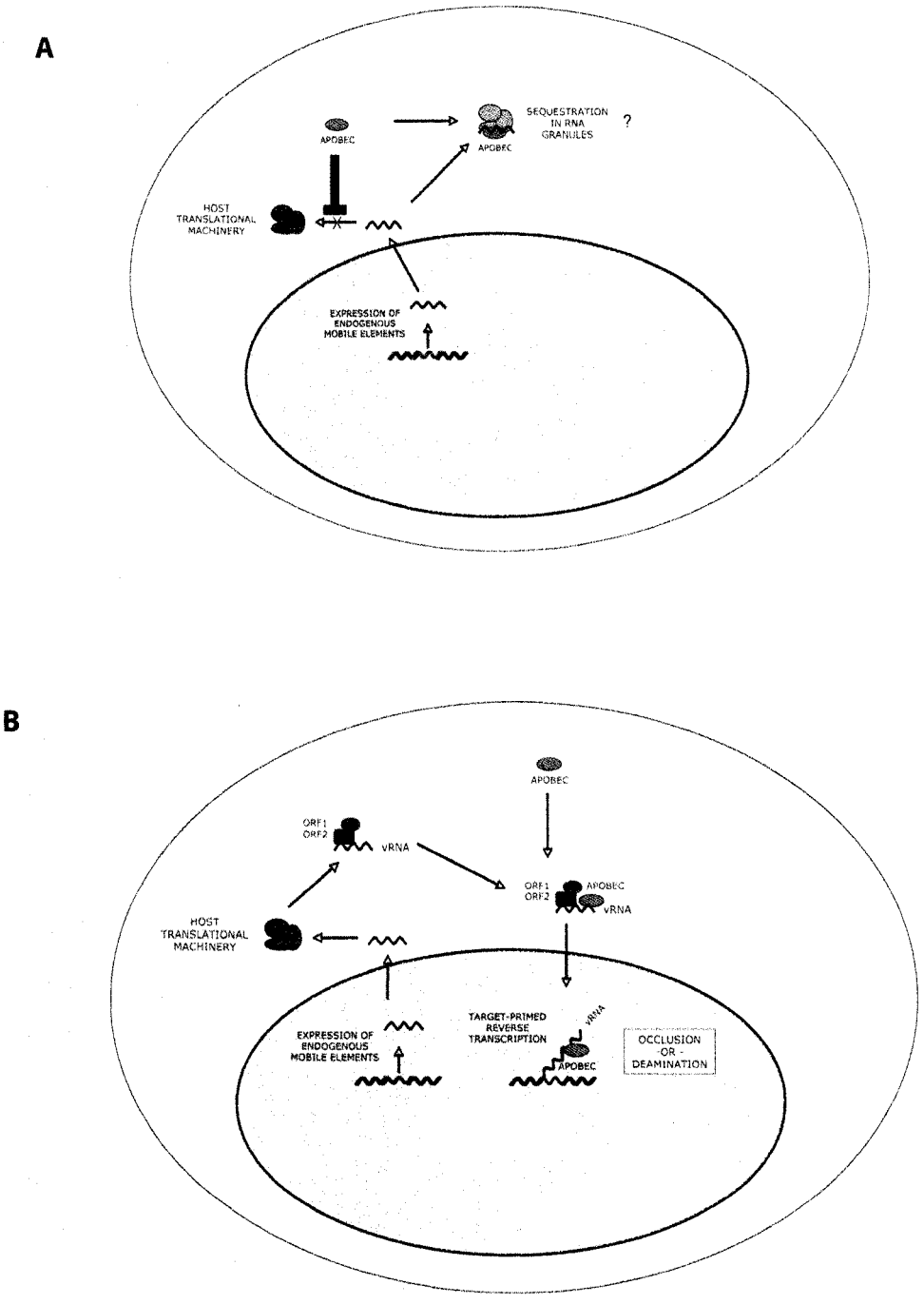


Figure 8 – Proposed Mechanisms of APOBEC3-mediated Inhibition of non-LTR Retroelements

Subcellular Localization of APOBEC3 Proteins

As more details of APOBEC3-mediated antiviral activity come to light it is clear that the mechanisms of inhibition may not necessarily be shared. This may not be totally unexpected as the subcellular localization of the various APOBEC3 enzymes is quite distinct from one another with some APOBEC3s being exclusively cytoplasmic (3G and 3F), some exclusively nuclear (3A and 3B) and some showing both nuclear and cytoplasmic staining (3C)[91, 92] (Table 2). Through pull-down experiments, APOBEC3G has been shown to associate with ribonucleoprotein (RNP) complexes in the cell cytoplasm, termed High Molecular Mass (HMM) complexes[92-94] (Table 2). Whether these complexes represent P-Bodies, stress granules or other, perhaps novel, types of RNA-containing protein complexes is unclear. Further, the importance of association with RNPs for antiviral activity is unclear.

Table 2 – Editing Context & Localization of APOBEC3 Proteins

	Editing Context (base @ -1)	Localization	RNP Association?
Apo3A		Nuc/Cyto [91, 95]	No [96]
Apo3B	T	Nuc [91, 95]	No
Apo3C	C/T [31]	Nuc/Cyto [91, 95]	No [96]
Apo3DE	T/A [55]	Cyto [91, 95]	Yes [96]
Apo3F	T	Cyto [91, 93, 95]	Yes [92, 93]
Apo3G	C	Cyto [91-93, 95]	Yes [92, 93]
Apo3H	?	?? [91, 95]	??

Rapid Evolution of Primate APOBEC3 Genes

Genetic conflict between host and pathogen drives rapid change in interacting host and pathogen proteins (rapid evolution) as they attempt to increase or decrease interactions with one another in the battle for dominance. Therefore, a signature of positive selection is often observed for host proteins that are directly involved in pathogen defense. Positive selection can be defined as any type of selection where new mutations are advantageous[97].

Previous work from our labs demonstrated that APOBEC3G shows a strong signature of positive selection throughout the primate lineage[98]. In this study, the entire APOBEC3G coding sequence was determined from a panel of primates including Hominoids, Old World monkeys and New World monkeys. The sequences were then compared computationally to ask if the APOBEC3G coding sequence shows any evidence of an excess of non-synonymous (changes the encoded amino acid) to synonymous (does not change the encoded amino acid) changes on any branches of the primate phylogeny. Such an excess of amino acid-altering mutations is the hallmark of rapidly evolving genes and is indicative of positive selection. Indeed, APOBEC3G shows a signature of positive selection on most branches of the phylogeny suggesting that this protein has been functioning as an antiviral gene throughout most, if not all, of primate evolution.

Importantly, the signal of positive selection is seen deep in the tree as well as on branches leading to existing primate species, suggesting that APOBEC3G has been in conflict with retroviral assailants for many millions of years. As primate lentiviruses are thought to have entered the primate lineage no more than 1 million years ago[10], this argues that the target of APOBEC3G's antiviral activity may be

something older and more pervasive. One possibility is that APOBEC3G and other APOBEC3 genes have been targeting the endogenous retroviruses and retroviral elements that have invaded our genome. These endogenous retroelements have been present throughout primate evolution and keeping their replication in check has been crucial to maintaining the genomic integrity of the primate hosts. The signature of rapid evolution we see in the APOBEC3G coding sequence may be the result of successive sweeps of APOBEC3G alleles with activity against the replication of a diverse array of endogenous retroelements.

The specific residues evolving under positive selection were found scattered throughout the protein sequence, implying that perhaps APOBEC3G has been interacting with retroviral elements at various locations in its primary sequence during its evolution in primates. Finally, since many members of the APOBEC3 family in primates show signals of positive selection in pairwise comparisons of sequences from only 2 primate species (a conservative barometer of positive selection), this was the first suggestion that other members of the family would also function as antiviral genes, a prediction that has been widely demonstrated in recent years (see Table 1).

A notable exception to these analyses is the most distal member of the locus, APOBEC3H. Due to limitations of the available human genome sequence at the time, APOBEC3H was not initially identified as a member of the APOBEC3 family and therefore its activity and evolutionary signatures were not evaluated in any previous studies. APOBEC3H turns out to be one of the most interesting APOBEC3 family members as its evolutionary history is distinct from that of the other APOBEC3 genes and, as I show in this work, studies of the function of APOBEC3H have uncovered several surprising findings not described for any other APOBEC3 genes.

Using Comparative Primate Genomics in APOBEC3 Biology

Given their role in frontline defense against invading pathogens, it is not surprising that we find a large degree of change has occurred in intrinsic immunity genes over relatively short periods of time. For the purposes of my work, I am especially interested in changes in the landscape of intrinsic immunity genes during the evolution of humans and other primates. The finding of positive selection of these genes is a barometer of exactly this type of diversification.

In the case of immunity genes, the rapid evolution of these genes is thought to be the result of changing pathogen pressures as primates have explored new environments or undergone lifestyle changes that have resulted in increased or decreased pathogenic loads. The most classic case of rapid evolution of components of the primate immune system is the MHC loci that have undergone large amounts of duplication, deletion and diversification throughout the primate lineage[99]. Specific regions of genes involved in pathogen defense may be rapidly evolving if the same domains or residues of the protein have been involved in pathogen interaction throughout evolution. For MHC molecules this means that the Antigen Recognition Sites (ARS) are the most rapidly evolving regions of the protein as this is the direct contact site between host and pathogen[99]. A similar concentration of diversifying selective pressure is found in the primate intrinsic immunity gene, TRIM5α. A single domain of TRIM5α, the B30.2 or SPRY domain, shows the strongest signal of positive selection and allowed for identification of the region of the protein responsible for binding to and recognizing viral capsids[100].

In addition to selective pressure from invading pathogens that drives the rapid evolution of host antiviral proteins, APOBEC3 genes are also undoubtedly subject to selective pressure to maintain their appropriate compartmentalization both in terms of localization within the cell and proper regulation of their deaminase activity. Mutations that lead to mis-localization or misregulation of APOBEC3 proteins represents a significant risk to host cell integrity as spontaneous deamination of genomic DNA or host cell mRNA transcripts could have disastrous consequences[101]. In fact, upregulation of APOBEC3 genes has been described in various cancer cell types[44]. Therefore, APOBEC3 coding sequences are subject to significant selective constraint (purifying selection) in addition to positive selection of specific domains and/or residues.

Taking a comparative genomics approach to understanding the biology of intrinsic immunity proteins can be particularly informative. Identifying sites or domains evolving under positive selection can reveal the sites of direct host and viral protein interaction. Further, identification of sites or domains that are evolving under purifying selection reveals components of the protein sequence that are fundamentally required for the function of the protein, such as maintenance of intact enzymatic activity for the APOBEC3 genes.

Tests commonly used to identify genes or specific amino acid residues that are or have recently been affected by natural selection include methods that compare sequence among related species (such as PAML, Phylogenetic Analysis by Maximum Likelihood[102, 103]) or methods that focus on human-specific evolution by using population genetics to ask what genes and/or sites have a signature of natural selection (see Nielsen 2005[97] for a review). For example, genes/sites that show

signatures of natural selection can be found by comparing levels of polymorphism and divergence in the human population since speciation from the chimpanzee, such as the MKA (McDonald-Kreitman) test[104] or the HKA (Hudson-Kreitman-Aguade) test[105]. Similarly, natural selection acting in certain world populations can be detected using F_{ST} analyses which compare allele frequencies between populations[106]. Finally, several tests examine the extent of linkage disequilibrium associated with particular haplotypes and allows for detection of very recent selection in the human population[107].

We know that all primate species have been subject to diverse and numerous invasions by different types of retroviral elements. Often, these invasions may be unique to distinct primate lineages. The selective pressures exerted on host genomes by these elements, therefore, have been highly variable and are the likely cause of the large diversification of primate intrinsic immunity genes. Comparisons of APOBEC3 orthologs from various primates species may reveal changes in specificity and, ultimately, some understanding of the varied selective pressures encountered by primates during evolution.

Finally, comparative analyses of intrinsic immunity proteins can lead to an understanding of the biology of these proteins that may be completely unexpected and that would have been impossible, or at least extremely difficult, without a comparative approach. The work I present here is exactly such a scenario in which my comparative analysis of APOBEC3H from various primates reveals a surprising story of the evolution of the function of this gene in humans that would not have been possible without an analysis of related sequences from other primates.

Chapter Two:

MATERIALS AND METHODS

APOBEC3H sequences for Evolutionary Analyses

Genomic DNA was obtained from Coriell (Camden, New Jersey, United States) with the exception of the sample from sooty mangabey which was a kind gift from Cristian Apetrei (Tulane National Primate Research Center). Exons were amplified from genomic DNA with PCR Supermix High Fidelity (Invitrogen) and PCR products were either sequenced directly or cloned into TA vector (Invitrogen) in which case multiple clones were sequenced. The human APOBEC3H sequence was obtained from the Ensembl database (ENSG00000100298) and from cDNA clones. Exon-intron boundaries are conserved across species that we sampled. Primers were designed using alignments between human genomic and rhesus monkey (*Macaca mulatta*) genomic sequence, obtained from a BLASTN search of the NCBI Trace Archive sequences. Individual exons were amplified from the various primate genomic DNAs using the following primers. Exon 2 (1^{st} coding exon): Forward primer, 5'-GAAACACGATGGCTCTGTTAACAG or 5'-TGAGCTGAGATCGGGAGAATGAG and Reverse primer, 5'-CAAGCACCCGCTTCCTGCC; Exon 3 and 4: Forward 5'-GAAGTGGGTGCTTGCCAGGC and Reverse 5'-GGGTTGAAAAACTACCTATTGGGTG; Exon 5: Forward 5'-AGCACCCAATAGGTAGTTTTTCAACCC and Reverse 5'-GGCAACTGACATGCCCCAGGG. For most primate genomes we only obtained sequence from exons 2 through 4, which cover all except the last 2 codons (including termination) of the human *APOBEC3H* gene. Sequences of other *APOBEC3* family

members were obtained from Genbank, and by searching EST and genomic databases using TBLASTN searches.

Alignments and Sequence Analysis

APOBEC3 protein sequences were aligned using CLUSTAL_X [108] followed by a manual adjustment of some gaps, and a neighbor-joining phylogeny was reconstructed (with bootstrapping analysis) by ignoring all gapped positions in the PAUP* suite of programs [109]. A Bayesian phylogenetic analysis was done using the MrBayes program version 3.1 [110]. Clade credibility was obtained from a consensus of 80,000 trees after a "burn-in" period of 20,000 trees (ie, the first 20,000 trees were discarded); these values were largely congruent with those obtained by the neighbor-joining bootstrap analysis. Maximum likelihood analysis was performed with the PAML software package [102]. Global dN/dS ratios for the tree were calculated by a free-ratio model, which allows this parameter to vary along different branches. To detect selection, the multiple alignments were fitted to the F61 model of codon frequencies (the F3x4 model gave similar results). We then compared the log-likelihood ratios of the data using different NSites models- Model 1 (two-state, neutral, dN/dS > 1 disallowed) to Model 2 (similar to Model 1 but dN/dS >1 allowed) and Model 7 (fit to a beta distribution, dN/dS > 1 disallowed) to Model 8 (similar to Model 7 but dN/dS >1 allowed). In both cases, permitting sites to evolve under positive selection gave a much better fit to the data ($p<0.0001$) with a significant fraction of the sites (>25%) predicted to evolve at average dN/dS ratios close to 5 (see Table S1 for details). A Bayes Empirical Bayes analysis also identified certain amino acid residues with high posterior probabilities (>0.95) of having evolved under positive selection.

Cloning of APOBEC Constructs

Human, rhesus macaque, and sooty mangabey homolog of APOBEC3H were cloned by RT-PCR from testis, spleen and unstimulated peripheral blood lymphocyte tissue, respectively. Each of the *APOBEC3H* genes was then transferred into the same mammalian expression vector, which drives gene expression under the control of the CMV IE94 promoter. An identical HA epitope tag was added at the N-terminus of each protein. The human *APOBEC3G* construct was made by PCR amplification from CEM15HA [29]. All constructs were confirmed by sequencing. The human *APOBEC3H* clone used in our studies matches the one in the NCBI Consensus CDS database for human *ARP10* (*APOBEC3H*) except for a single amino acid change at position 140 from K to E. We changed this amino acid back to the consensus and found no difference in function between the two proteins as determined by Western blot analysis and a single-round infectivity assay (data not shown).

Lentiviral Infectivity Assays

HIV-WT and HIVΔ*vif* were expressed from the pLai3ΔenvLuc2 [111] and pLai3ΔenvLuc2Δvif (constructed by NdeI-StuI deletion in pLai3ΔenvLuc2) proviruses. These proviruses contain a deletion in the *env* gene and have the firefly luciferase gene inserted into *nef*. The luciferase-expressing SIVagmTAN provirus was a gift from Ned Landau [61]. The vesicular stomatitis virus glycoprotein (VSV-G) was used for pseudotyping. Viruses were produced through lipid transfection (Fugene; Roche) of 293T cells plated in 1 mL in 12-well plates at 3.5×10^5 cells/mL one-day prior. Co-transfections consisted of 1 μg total APOBEC plasmid along with control pCS2

plasmid to normalize, 0.5 µg proviral plasmid, 0.25 µg L-VSV-G and 0.1 µg CMV-Tat in 100 µL Serum-Free media. Viral supernatants were harvested at 48 hours, clarified by centrifuging for 5 min at 1000 x g and the total amount of virus in supernatants was quantified by p24 gag (HIV-1) or p27 gag (SIVagm) ELISA (Coulter). Viral stocks were frozen at -80°C until use. 1 ng of virus in 50µL was used to infect 293T cells in a 96-well plate, plated at $1.5x10^5$ cells/mL in 150 µL 24 hours prior to infection. Polybrene at 5 µg/mL and spinoculation of plates at 1000 x g for 1 hour at 24°C was performed to increase levels of infection. After 48 hours, cells from triplicate infections were lysed in 80 µL Cell Culture Lysis Reagent (Promega). 10 µL of lysates were used for quantitation of luciferase activity with the Luciferase Assay Kit (Promega) and read on a luminometer.

Protein and mRNA Expression Assays

For mRNA expression studies, a cDNA panel (Primgen) consisting of 10 ng of first strand cDNA from various human tissues, was screened using primers specific for human *APOBEC3H* or human *APOBEC3G* (APOBEC3H: Forward 5'-ATGGCTCTGTTAACAGCCGAAACATTCCG-3' and Reverse 5'–CTCTCAAGCCGTCGCTTTATGGC-3'; APOBEC3G: Forward 5'–ATGCGCTCCACCTCATAACACAG-3' and Reverse 5'-TGGAGCCCCTGCACAAAGTG-3'). The same primers were used for semi-quantitative RT-PCR analyses of RNA isolated by the RNeasy Mini Kit (Qiagen) from primary cells or cell lines. Macrophages were isolated by plating PBMCs from the donor in RPMI containing 10% heat-inactivated human sera, 5% FBS and 1% Penicillin/Streptomycin. Cells were cultured for 10 days. To isolate CD4+ cells, the

Dynal CD4 Negative Isolation Kit (Dynal) was used as per the manufacturer's protocol. For stimulation, 5 µg/mL PHA was added overnight. For mammalian protein expression analysis, plasmids were transiently transfected into 293T cells by Fugene lipid transfection (Roche) and their expression levels were evaluated by Western blot analysis. Briefly, 3.5×10^5 cells were plated in 1mL in 12-well plates, transfected the next day with 0.5 µg APOBEC plasmid, lysed 24 hours later in NP40-doc buffer (20 mM Tris, pH 8.0, 120 mM NaCl, 1 mM EDTA, 1% NP-40, 0.2% NaDeoxycholate and Protease Inhibitors – either PMSF or Roche Complete Cocktail Tablets), incubated on ice for 5 minutes and centrifuged at 12000 x g for 10 minutes to clear cell debris and then frozen at -20°C. For analysis of different cell types expressing stably-integrated human APOBEC3H protein, the human APOBEC3H cDNA was cloned into an LPCX vector (using HindIII/ClaI sites), cell lines were infected with the retroviral vector and transduced cells were selected in 0.4 – 2.5 mg/mL Puromycin. Equivalent amounts of total protein as determined by Bradford assay (BioRad) were loaded onto an SDS-PAGE gel and transferred to Immobilon-P PVDF membrane (Millipore). Membranes were probed using a HA-specific antibody (HA.11; Babco) at a 1:1000 dilution or anti-Actin antibody (Sigma) @ 1:500, followed by Goat anti-mouse-HRP secondary antibody (Santa Cruz Biotechnologies) at 1:10,000 and detection with ECL Plus Reagent (Amersham Biosciences). For semi-quantitative RT-PCR analyses, cytoplasmic mRNAs were harvested with an RNeasy Kit (Qiagen) from cells transiently transfected with equivalent amounts of either human APOBEC3H or macaque APOBEC3H expression plasmid. Cytoplasmic RNA preps were treated with 2 µL DNase (Invitrogen) for 15 minutes to degrade contaminating plasmid DNA, followed by inactivation of DNase by adding EDTA to 2.5 mM and heating to 65°C for

10 minutes. Cytoplasmic transcripts were than reverse transcribed for 30 minutes at 50°C (Qiagen One-Step RT-PCR Kit) using an APOBEC3H-specific primer (HSM179 5'-GAGAGTTCGGCAGCGAAATACCG-3'). cDNAs were then serially diluted (1:2) and 5 µL was used for 30 cycles of PCR amplification (94°C – 30 seconds, 55°C – 30 seconds, 72°C – 1 minute) with a 5' HA-specific primer (HASense 5'-GGATACCCATACGATGTTCCAG-3') and the 3' APOBEC3H-specific primer (HSM179 – see above).

Proteasome Inhibitor Analyses

293T cells were plated in 1 mL in a 12-well plate at $3.5x10^5$ cells/mL one day prior to transfection. Individual wells were transfected with 0.5 µg APOBEC plasmid and, 24 hours after transfection, media was replaced with DMEM containing either 10.5 µM MG-132 (Calbiochem) or an equivalent volume of DMSO as a control. Lysates were harvested using NP40-doc buffer (see above) 2 and 20 hours after initiation of treatment. The DMSO-only control was harvested at the 20-hour timepoint. Lysates were normalized for total protein content via a Bradford assay, resuspended in SDS-Sample Buffer, boiled for 5 minutes and 10 µg total cellular protein was loaded per well on an SDS-PAGE gel for Western blot analysis as outlined above. Viral infectivity assays in the presence of proteasome inhibitors were done as above except that virus-producing cells were incubated in MG132 after transfection.

Virion Incorporation – Wild-type and vif-deficient HIV was produced from 12-well transfections of 293T cells in which 3µg empty vector, human APOBEC3G, human APOBEC3H or macaque APOBEC3H CS2HA plasmids were included. Supernatants

were collected and debris spun out for 5 minutes @ 1000g. Virions were pelleted by spinning at 24,000g for 1.5 hours through a 20% Sucrose cushion in Standard Buffer (0.001M EDTA, 0.01M Tris pH 7.4). Supernatants were removed, pellets were dried and resuspended in 1X Sample Loading Buffer and boiled for 5 minutes @ 95°C. Viral lysates were analyzed by Western Blot using both a p24-specific (mouse monoclonal anti-p24 CA; ShuLok Hu – Univ of WA) and an HA antibody (RAW HA.11) as described above.

Bacterial mutator assay - APOBEC cDNAs were cloned into the IPTG-inducible pTrc bacterial expression vector (pTrc-C; Invitrogen) and transformed into an ung-/- *E. coli* strain, BW310 (Genetic Stock Center, Yale University). Single colonies were used to inoculate independent cultures in LB with 150 µg/mL carbenicillin and 1mM IPTG and grown overnight at 37°C to saturation. The OD600 of 1:5 dilutions was measured to assess relative viability and cultures were then plated onto LB/Agar plates containing rifampicin (Calbiochem) to select for rifampicin-resistance. Results were analyzed using GraphPad Prism software. Single rifampicin-resistant colonies were picked from plates and used for colony PCR with primers specific to the bacterial RNA polymerase gene, *rpoB* [112]. 1µL of PCR reactions were used to directly sequence products using an internal primer (*rpoB*Seq 5'-ATCTGGATACCCTGATGCCACAG-3'). For bacterial expression analysis, 3 mL cultures in log phase (as determined by OD600) were induced with 1 mM IPTG and 100 µL of cultures was pelleted after 2.5 hours, resuspended in 100 µL 1X SDS-Sample Buffer (0.06M Tris-HCl, pH6.8, 10% Glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.0025% bromophenol blue) and boiled for 5 minutes. 10 µL of a 1:100 dilution of lysates was loaded on a SDS-PAGE

gel, transferred to Immobilon-P PVDF membrane and detected using a c-*myc* monoclonal antibody (9E10) at a 1:1000 dilution. Equal loading of total cell lysates was confirmed by Coomassie staining of the polyacrylamide gel after transfer.

Hypermutation Assay – VSV/G-pseudotyped HIVΔ*vif* was produced in 293T cells in the presence or absence of transiently transfected pCS2HA/APOBEC expression vectors. 10 ng p24 was used to infect 293T cells plated at $8x10^5$ cells/mL one day prior to infection. Genomic DNA was harvested with the DNeasy Tissue Kit (Qiagen) 48-hours post-infection, followed by treatment with 1 µL DpnI for 1 hour at 37°C to exclude contaminating plasmid DNA from being amplified in subsequent PCR amplifications. A 407 bp region of the HIV-1 *pol* sequence was amplified using the high-fidelity *Pfu* DNA polymerase and *pol*-specific primers (pol1721 5'-GAGCAGACCAGAGCCAAC-3' and pol2152 5'-AGTTTCAATAGGACTAATGGG-3'). The *pol* fragment was subsequently gel purified (Qiagen), cloned into the TOPO-TA vector (Invitrogen) and sequenced.

Accession Numbers – The sequences used in the evolutionary analyses have been submitted to GenBank and assigned accession no. DQ408605 – DQ408615.

Primate APOBEC3H cDNAs

APOBEC3H cDNAs were cloned by RT-PCR or nested RT-PCR (QIAGEN One-Step RT PCR Kit) from RNA derived from chimpanzees (*Pan troglodytes verus* – Coriell GM03448), gorilla (*gorilla gorilla* – Coriell AG05251), orangutan (*Pongo pygmaeus pygmaeus* - Coriell AG05252) and gibbon (*Nomascus leucogenys leucogenys* -

GM051496). Primers used were as follows: chimpanzee APOBEC3H was amplified using an initial round of RT-PCR (50 cycles) with primers JAK012 (5'-ggcaactgacatgccccaggg-3') and JAK015 (5'-gaaacacgatggctctgttaacagcc-3') followed by a second round of 30 cycles (*Pfu Turbo* – Stratagene) with primers JAK012 and ggA3H-Exon1_F (5'-atggctctgttaacagccgaaac-3'). Gorilla APOBEC3H was amplified with an initial round of RT-PCR using primers JAK012 and JAK015 followed by a second round of 30 cycles with JAK012 and ggA3H-pCS2HA_F (5'-cggggtaccggctctgttaacagccgaaacattcc-3'). Orangutan APOBEC3H was amplified in a single round of RT-PCR (50 cycles) using primers JAK012 and orangA3H5UTR/ATG (5'-gaaacacgatggctctcctaacagcc-3'). Gibbon APOBEC3H was amplified in a single round of 50 cycles using primers JAK012 and JAK015. RT-PCR products were gel purified, TOPO cloned (Invitrogen) and sequenced. Cloning of human and macaque APOBEC3H cDNAs were described previously [65].

TAIL Chimeras

The human APOBEC3H with the macaque "Tail" was constructed by PCR using primers that match both the macaque and human APOBEC3H sequences. The truncated macaque APOBEC3H protein was constructed using the human APOBEC3H 3' primer, resulting in truncation at the STOP found in the human sequence.

Sub-Cellular Localization

HeLa cells plated onto coverslips in a 6well dish @ 2e5 cells/mL were transfected the next day with 1.5µg pcDNA/HA-APOBEC vectors. 24 hours post-transfection slips

were fixed in 4% Paraformaldehyde, permeabilized in 0.3%Triton-X/PBS and blocked in 10%FBS/PBS for 30 minutes at room temp or overnight @ 4°C. HA-tagged proteins were detected using RAWHA.11 antibody @ a 1:100 dilution in 10%FBS/PBS for 1 hour @ RT, followed by secondary anti-mouse-TexasRed conjugate (Invitrogen) @ 1:1000 for 1 hour @ RT. Nuclei were stained with DAPI in mounting media (Vectashield; Vector Labs). Images were collected on an Olympus DeltaVision microscope.

Pulse-Chase Analysis

293Ts were plated at $2x10^5$ cells/mL in 6 cm poly-L-lysine plates (Becton Dickinson) one day prior to transfection. Plates were transfected with 5 µg pcDNA/APOBEC plasmids. One day post-transfection, Pulse was started by incubating cells in 1mL Starvation media - Met/Cys-free DMEM (Invitrogen)/5% Dialyzed FCS + L-Glutamine. After 30 minutes, starvation media was removed and 750µL Pulse media was added. Pulse media is Starvation media plus 440µCi EasyTag EXPRE35S35S Protein Label (PerkinElmer) per plate. Following a 30-minute pulse, 1mL warm Chase media (Met/Cys-free DMEM + 5%FCS + 2mM Met + 2mM Cys) was added to all plates. Cells were harvested at 0, 30min, 1hr, 2hr and 6hr timepoints by washing 1x in PBS and lysing cells directly in 500µL ice-cold NP40doc buffer (1% NP40, 0.2% Sodiumdeoxycholate, 0.12M NaCl, 20mM Tris pH 8.0) with Protease Inhibitors (Roche Complete Mini, EDTA-free tablets). Debris was removed by spinning 5 minutes at 16,000g @ 4°C. Supernatants were frozen @ -80°C until use. HA-tagged APOBEC3H proteins were Immunoprecipitated using Protein G-Sepharose beads (Zymed) conjugated to RAW HA.11 antibody. After pre-clearing lysates by incubating

lysates with unconjugated beads for 1 hour @ 4°C with rocking, antibody was pre-conjugated with beads for 1hr @ 4°C with rocking. Beads were washed twice in NP40doc and spun out for 2min @ 4000g @ 4°C. 0.01% BSA was added to antibody-conjugated beads which were incubated with pre-cleared lysates for 2 hours @ 4°C with rocking. Beads were spun out (2 min @ 4000g) and washed in a series of wash buffers with protease inhibitors (Roche Mini, EDTA-free tablets) as follows: RIPA (10mM Tris pH7.5, 150mM NaCl, 1% NP40, 1% sodiumdeoxycholate, 0.1% SDS), High Salt Buffer (2M NaCl, 10mM Tris pH7.4, 1% NP40, 0.5% sodiumdeoxycholate), Low Salt Buffer (0.5% NP40, 0.1% SDS in PBS) and RIPA. Bead slurries were resuspended in 20µL 2X Protein Loading Buffer (125mM Tris pH6.8, 4% SDS, 20% Glycerol and 10% β-mercaptoethanol), boiled for 10 minutes, beads spun out @ 16,000g for 1 min and supernatants used for 12% SDS-PAGE. Radioactivity was detected using a Typhoon Trio imager (Amersham Biosciences) and quantitated using ImageQuantTL software (Amersham Biosciences).

Ancestor Reconstruction

The human/chimpanzee ancestor APOBEC3H protein sequence was inferred using the gorilla sequence as an outgroup. The human/chimpanzee ancestor APOBEC3H cDNA was then constructed *in vitro* by site-directed mutagenesis (QuikChange Site Directed Mutagenesis Kit – Stratagene) using a chimpanzee/human_1 chimera (at a *BamHI* site) as a template. The human ancestor APOBEC3H protein was defined by determining all changes that are fixed in the human population and reconstructed *in vitro* using the human_A cDNA as a template by site-directed mutagenesis.

Human Polymorphism Analysis

The human APOBEC3H coding sequence was amplified/sequenced from genomic DNA samples with the following primer pairs: Ex1_For 5'-gaaacacgatggctctgttaacagcc-3'/Ex1_Rev 5'caagcacccgcttcctgcc-3', Ex2&3_For 5'-cacgggtgcgtgagtgcaggg-3'/Ex2&3_Rev 5'-gggttgaaaaactacctattgggtgc-3' and Ex4_For 5'-agcaccccaataggtagtttttcaaccc-3'/Ex4_Rev 5'-ggcaactgacatgccccaggg-3'. Missing data and additional sequencing was performed with the following primers: huA3H_Ex2_For (276) 5'- CTTTCTGTTGCACAGAAACACGATGG-3'/huA3H_Ex2_Rev (271) 5'- GCTTTCCCGAAGTAGTGACTGAGC -3', huA3H_Ex3_For (272) 5'- CAGGCCACGCACTAGAAAGTTCAC -3'/huA3H_Ex3_Rev (273) 5'- TCCCGGGTGGTGTCAGATCTTG -3' and huA3H_Ex4_For (274) 5'-AGATCTGACACCACCCGGGAG -3'/huA3H_Ex4_Rev (275) 5'-AGGACAGTGCCTCACCTTTATCC -3'. Human population samples sequenced from Coriell were: African Pygmy (GM10469 – 10473/GM10492 – 10494), South American (GM10965 – 10969), Central American (GM10975/6/8 & 9), Caucasian (GM893, 946, 1310, 1805, 1806, 1814, 1953, 8428, 9948 and 14492), African South of the Sahara (NA17341 – 17349) and African North of the Sahara (NA17378 – 17384). PHASE 2.1 software[113] was used to infer phasing of haplotypes over five iterations. Human haplotypes found at >10% frequency in the panel were reconstructed by site-directed mutagenesis.

Chapter Three:

ADAPTIVE EVOLUTION AND ANTIVIRAL ACTIVITY OF THE CONSERVED MAMMALIAN CYTIDINE DEAMINASE APOBEC3H

BACKGROUND

Discovery of APOBEC3G and its ability to block HIV replication[29] uncovered a novel mechanism through which primate cells could protect themselves from the detrimental effects of retroviral invasion of the genome. The majority of work has been focused on understanding the mechanism and retroviral targets of human APOBEC3G. While this work has been crucial to our understanding of the function of APOBEC3G in humans, there has been relatively little work done to understand how the role of APOBEC3G may be similar or different in other primates. To date, only a limited set of primate APOBEC3 homologs have been cloned and tested.

Several groups have cloned and tested chimpanzee, african green monkey and rhesus macaque APOBEC3G cDNAs and found that their antiviral activities largely mirror that of human APOBEC3G in terms of both the targets of their inhibition as well as their relative potency ([31, 48, 61, 66-68] and Table 1). Specifically, all primate APOBEC3G proteins tested so far potently inhibit the primate lentiviruses HIV, SIVagm and SIVmac. Further, the APOBEC3G homologs show differential sensitivity to the Vif proteins encoded by each of the lentiviruses as is required for these viruses to replicate in their primate hosts.

In addition to lentiviruses, all four of these primate APOBEC3G proteins are able to efficiently block the replication of both the mouse endogenous retrovirus, MusD,[49] as well as foamy virus[48]. Similar to what has been shown for human APOBEC3G, the other primate APOBEC3G homologs also demonstrate no inhibitory

activity against either the mouse endogenous retrovirus, IAP, or human LINE-1 elements[49].

In this chapter I present my work demonstrating that APOBEC3H, an APOBEC3G-related gene in primates, also plays a role in antiretroviral defense. The evolutionary history of APOBEC3H differs from that of the other APOBEC3 genes known to exist in primates as it is derived from an ancestral cytidine deaminase domain that is evolutionarily distinct (Figure 6 – Chapter 1 and [45]). Despite its unique evolutionary history, the presence of a conserved cytidine deaminase domain encoded by APOBEC3H and its close chromosomal proximity to the other known antiviral APOBEC3 family members suggested that APOBEC3H may also play a role in genome defense.

The work I present here is the first to describe the role of APOBEC3H in antiretroviral defense in primates. While a single APOBEC3H-like domain is found in placental mammals, the APOBEC3H coding sequence has been rapidly evolving in primates, an evolutionary signature that suggests a possible role in antiviral defense. I find that the rhesus macaque APOBEC3H homolog is able to block the replication of HIV-1, while the human APOBEC3H homolog shows no detectable antiviral activity. Sequencing of integrated proviruses demonstrates deamination of viral genomes by macaque but not human APOBEC3H despite the fact that the cytidine deaminase enzymatic activity of both homologs is conserved. The lack of activity of human APOBEC3H appears to be due to low steady-state expression levels that are not sufficient for incorporation into budding virions. Levels of human APOBEC3H protein can be increased by blocking proteasome-mediated degradation pathways, but this is not sufficient to confer antiviral activity. In sum, the results presented in this chapter

demonstrate that APOBEC3H is an antiretroviral effector in some, but not all primates.

RESULTS

The Evolution of APOBEC3H is Unique Among Primate APOBEC3 Genes

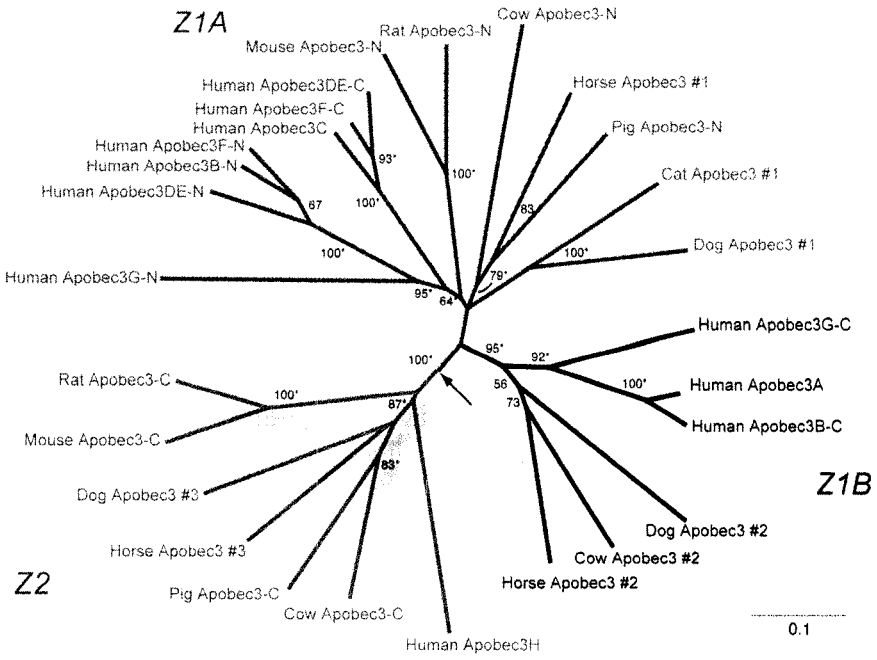
The APOBEC3 gene expanded during the evolution of primates from a single domain to 7 APOBEC3 genes in humans [44, 45, 98]. During this expansion, several scenarios could have given rise to the current architecture of the APOBEC3 locus in humans including repeated duplication of an ancestral domain, serial duplication of one domain after another or a more random duplication pattern. To ask how APOBEC3H arose and how it relates to the other APOBEC3 genes, APOBEC3 proteins from a number of species were aligned and a neighbor-joining tree was constructed using CLUSTAL_X [108]. Our results are consistent with those published by Conticello et al [45] and we have maintained the same nomenclature for consistency. Based on phylogenetic analyses, mammalian APOBEC3 cytidine deaminase domains can be classified as belonging to one of two types that appear to have been present in the common ancestor of the mammalian lineage (termed Z1A/Z1B in blue/purple and Z2 in red; Figures 9A and 9B [45]). While the APOBEC3A-G genes in primates are paralogous genes (related by duplication) of the Z1-type (blue in Fig. 9; also see Figure 6), the primate APOBEC3H gene is distinct as it is the only primate APOBEC3 of the Z2-type (indicated in red in Figures 9A and 9B). Our analysis of available sequence from completed and ongoing genome sequencing projects identifies the presence of an APOBEC3H-like ortholog throughout the mammalian lineage (Z2-type domains in red in Fig. 9A). While the Z1-like domains have

expanded in many mammalian lineages (most dramatically in humans), the APOBEC3H-like domain is always present in a single copy, either as part of a fusion gene (rodents, cows and pigs) or as a stand-alone, single-domained gene (dog, horse and human) (Fig. 9B). While any functional differences that may distinguish Z1 and Z2-type domains have yet to be described, it is clear that the evolutionary constraints acting on the two types of domains are distinct as the Z1 domain has expanded independently in a number of genomes while the Z2 domain has not.

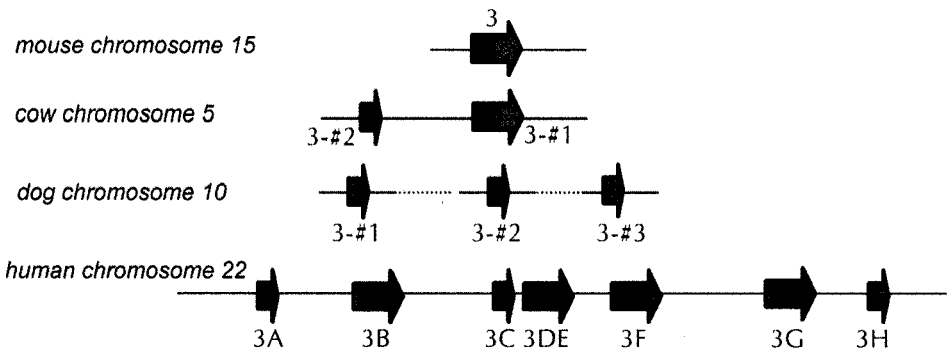
Figure 9: Evolution of APOBEC3H

APOBEC3 genes from human, primates and other mammals were used for an analysis of the evolutionary history and selective pressures affecting APOBEC3H throughout mammalian evolution. Panel A: A neighbor-joining tree was constructed in CLUSTAL_X based on a protein alignment of *APOBEC* sequences from humans and non-primate eutherian mammals. The N-terminal and C-terminal domains of double-domained APOBEC proteins have been split and are denoted as –N and –C, respectively. APOBEC3 proteins from mammals in which more than one APOBEC3 was identified are numbered. An APOBEC3H-like domain (red lineages, Z2) has been conserved in a number of mammalian species, including mouse, rat, dog, horse, pig, cow and primate. This phylogeny is rooted using the most closely related Activation Induced Deaminase (AID) cytidine deaminase as an outgroup lineage; the placement of this root is shown by an arrow. Bootstrap support for the groupings is indicated by numbers next to the relevant branches; those nodes that were supported by a Bayesian maximum-likelihood analysis are indicated with an asterisk. Panel B: Schematic of APOBEC3 genes identified in representative mammalian genomes, including the mouse, cow, dog and human genomes. Red denotes Z2 (APOBEC3H-like), while blue and purple denote Z1A and Z1B respectively (nomenclature system as suggested in [45]). While rodents encode only one APOBEC3 gene (a Z1-Z2 fusion), the cow and pig genomes all contain at least two APOBEC3 genes, one whose domain structure mirrors that of the rodent Z1-Z2 fusion gene, as well as an additional Z1-type gene. The horse and dog genomes contain at least 3 single-domained APOBEC3 genes, 2 Z1-type and a Z2-type; however, the dog genes have not been assigned to a genomic contig yet and hence the chromosomal assignment, and order of the genes, is still tentative (this uncertainty is represented as a dotted line).

A.


Z1A
Cow Apobec3-N
Rat Apobec3-N
Mouse Apobec3-N
Human Apobec3DE-C
Human Apobec3F-C
Human Apobec3C
Human Apobec3F-N
Human Apobec3B-N
Human Apobec3DE-N
Horse Apobec3 #1
Pig Apobec3-N
Cat Apobec3 #1
Dog Apobec3 #1
Human Apobec3G-N
Human Apobec3G-C
Human Apobec3A
Human Apobec3B-C
Z1B
Rat Apobec3-C
Mouse Apobec3-C
Dog Apobec3 #3
Horse Apobec3 #3
Pig Apobec3-C
Cow Apobec3-C
Human Apobec3H
Dog Apobec3 #2
Cow Apobec3 #2
Horse Apobec3 #2
Z2
0.1

B.


mouse chromosome 15
3
cow chromosome 5
3-#2
3-#1
dog chromosome 10
3-#1
3-#2
3-#3
human chromosome 22
3A
3B
3C
3DE
3F
3G
3H

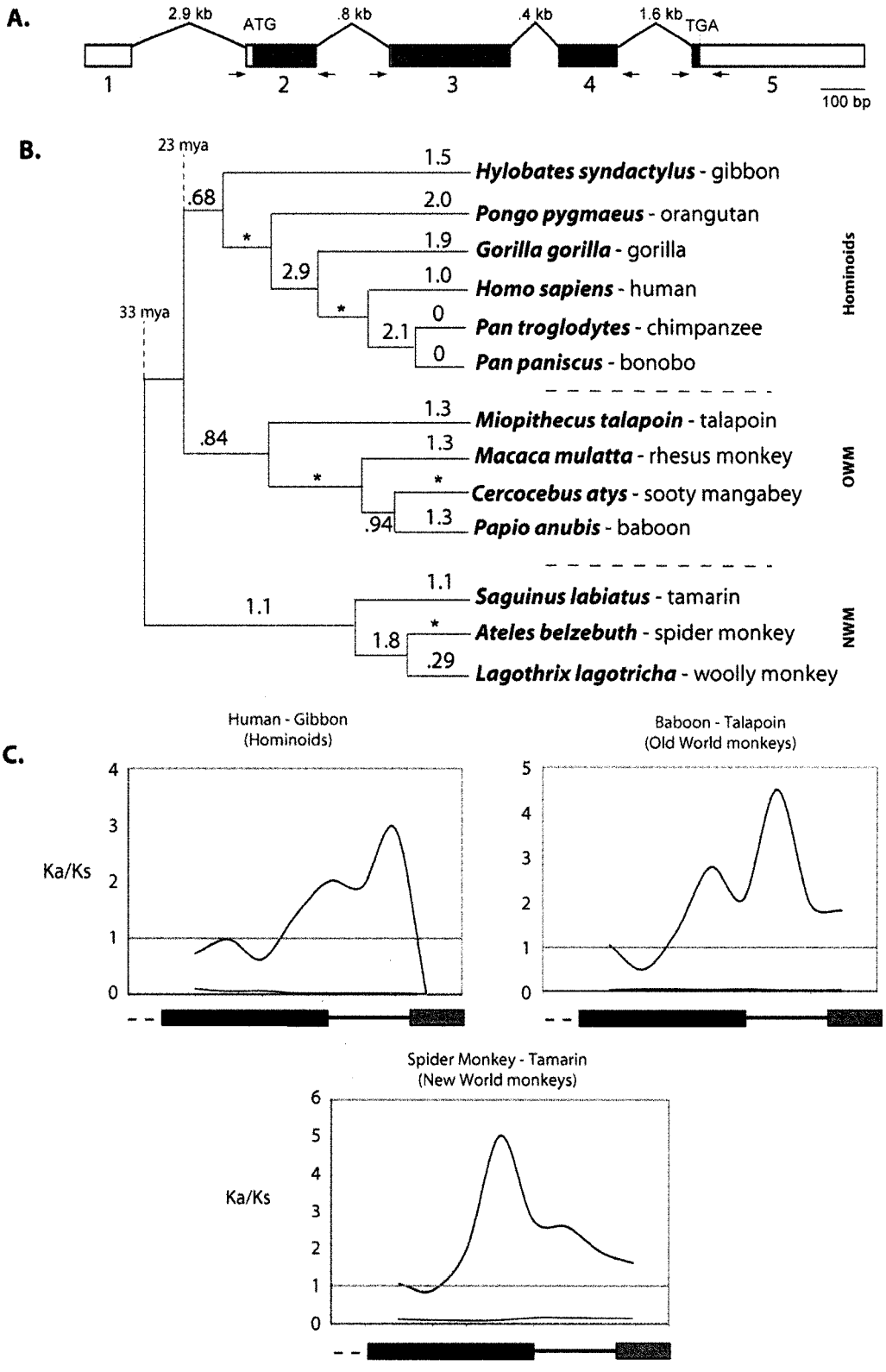
APOBEC3H is Rapidly Evolving in Primates

Previous work from our labs has shown that genes involved in genome defense, such as APOBEC3G and TRIM5α, are frequently subject to positive selection (also called adaptive evolution), indicated by an excess of replacement mutations which alter the encoded amino acid over synonymous mutations in which there is no amino acid change [98, 43]. In the case of proposed genome defense genes, the finding of positive selection has proven to be a reliable indicator of antiviral function. Functional studies have shown that the APOBEC genes that are adaptively evolving in primates [98, 100] are also those that have been shown to possess antiviral activity (APOBEC1, APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3DE, APOBEC3F and APOBEC3G - see Table 1 for a summary).

To ask if APOBEC3H may be involved in antiviral defense, we first undertook an evolutionary analysis to survey the evolutionary pressures acting on this gene during primate evolution. To investigate whether APOBEC3H has also evolved under positive selection (thereby implicating it as an antiviral gene), we sequenced the APOBEC3H coding exons from genomic DNA for a panel of New World monkey (NWM), Old World monkey (OWM) and Hominoid species (primers used for amplification of the APOBEC3H coding sequence are shown in Fig 10A). Using the PAML program (Phylogenetic Analysis by Maximum Likelihood) [102, 103], we calculated the number of observed changes per non-synonymous site (dN) and the number of observed changes per synonymous site (dS) over the entire length of the APOBEC3H gene and calculated the dN/dS ratios for each branch of the primate phylogeny (Fig. 10B). Despite the fact that whole gene values of dN/dS are often conservative measures of positive selection (due to averaging of values over the

entire gene length), many branches show a dN/dS value greater than 1, indicative of positive selection. We find that APOBEC3H has an average dN/dS ratio of 1.39 over primate evolution. Models in which codons are permitted to evolve under positive selection (NsSites models M2 and M8) fit the APOBEC3H data significantly better than those in which positive selection is not permitted (NsSites Models M1 and M7 respectively, $p<0.0001$). These results are similar to our finding for APOBEC3G (average primate dN/dS = 1.31) [98], implying that the evolutionary pressures driving the positive selection of APOBEC3H have been just as strong as those affecting APOBEC3G. We found no significant variation of dN/dS among different branches of the primate phylogeny, implying that like APOBEC3G, a relatively uniform selective constraint has acted on APOBEC3H for the past 33 million years.

Figure 10: APOBEC3H is Rapidly Evolving (whole-gene and domain analysis)
Panel A: Primer sets (small black arrows) flanking Exon 2, Exons 3/4 and part of Exon 5 (Exon numbers are shown below the protein schematic) were used to amplify APOBEC3H coding sequence from a panel of primates. Panel B: The relative rates of Replacement (Ka or dN) to Synonymous (Ks or dS) changes were calculated for APOBEC3H using PAML. We obtained sequences from a panel of six hominoids, four Old World monkeys and three New World monkeys. dN/dS ratios were calculated along each branch of the phylogeny with the free-ratio model in the PAML package that allows the dN/dS ratio to vary along each branch. dN/dS values are indicated on the APOBEC3H phylogeny, which is completely congruent with the accepted primate phylogeny (Purvis 1995). A dN/dS ratio greater than 1 is indicative of positive selection. In some instances, zero synonymous substitutions lead to an apparent dN/dS ratio of infinity (shown with an asterisk). mya, million years ago. Panel C: Ka/Ks ratios (y-axis; blue in Panel B) were calculated for three pairwise comparisons among Hominoids (human vs. gibbon), Old World monkeys (baboon vs. talapoin) and New World monkeys (spider monkey vs. tamarin) using K-Estimator (window size of 100bp, slide of 50bp). Values are plotted against nucleotide position (x-axis) with general domain structure of APOBEC3H shown below (core of cytidine deaminase domain is shown in black; a linker region connects core to the pseudocatalytic site[98]). Horizontal grey bar delineates a Ka/Ks = 1 which is indicative of neutral evolution. Values lower than one are under purifying selection; values greater than one indicate positive selection.


A.
2.9 kb
ATG
.8 kb
.4 kb
1.6 kb
TGA
1
2
3
4
5
100 bp
B.
23 mya
33 mya
1.5
Hylobates syndactylus - gibbon
.68
2.0
Pongo pygmaeus - orangutan
*
1.9
Gorilla gorilla - gorilla
2.9
1.0
Homo sapiens - human
*
0
2.1
Pan troglodytes - chimpanzee
0
Pan paniscus - bonobo
Hominoids
1.3
Miopithecus talapoin - talapoin
.84
1.3
Macaca mulatta - rhesus monkey
*
*
Cercocebus atys - sooty mangabey
.94
1.3
Papio anubis - baboon
OWM
1.1
Saguinus labiatus - tamarin
1.1
*
Ateles belzebuth - spider monkey
1.8
.29
Lagothrix lagotricha - woolly monkey
NWM
C.
Human - Gibbon
(Hominoids)
Baboon - Talapoin
(Old World monkeys)
Ka/Ks
Spider Monkey - Tamarin
(New World monkeys)
Ka/Ks

Specific Domains of APOBEC3H Are Evolving Under Positive Selection

The nature of genetic conflict often leads to specific domains/residues of proteins that are at the interface between antagonistic proteins and are, consequently, rapidly evolving. Other domains that have a conserved role required for the protein's function, such as interaction with other cellular factors or residues required for the protein's enzymatic activity, evolve under purifying selection as change is not tolerated at these sites. We did find a significant signal of positive selection in our whole gene analysis by PAML, but this does not give any information regarding which domains of the protein, specifically, are evolving under either positive or purifying selection. Therefore, in addition to whole gene analyses of selection pressures on APOBEC3H using PAML, pairwise comparisons of selected APOBEC3H homologs were done using K-Estimator[114]. By analyzing the Ka/Ks ratio by sliding window analyses, we are able to delineate which regions/domains of APOBEC3H show the strongest signals of positive selection (Fig. 10C). Three pairwise comparisons (Hominoid, Old World monkey and New World monkey) reveal two peaks of positive selection, falling just at the end of the conserved cytidine deaminase domain (Fig 10C in black) and another peak in the APOBEC3H "linker" region (Fig 10C connecting black to grey domains). While the strength of these signals varies over primate evolution (compare signals among primate lineages), this data suggests that these two domains/regions are at the interface between host and virus and, therefore, have been the sites of APOBEC3H's rapid evolution for the last ~40 million years.

Tissue and Cell-Specific mRNA Expression

We further asked if APOBEC3H had characteristics of an antiretroviral defense gene through an analysis of the tissue distribution of APOBEC3H mRNA expression. We would expect a gene important for antiretroviral defense to be expressed in tissues relevant to retroviral replication *in vivo*, similar to what has been found for the other APOBEC3 genes [31, 44]. A panel of cDNAs from various human tissues was screened for mRNA expression using primers that are specific for human APOBEC3G and APOBEC3H. Although overlapping in some tissues, the expression patterns of human APOBEC3G and APOBEC3H mRNA transcripts are not identical (Fig. 11A). For example, both human APOBEC3H and human APOBEC3G are heavily transcribed in the ovary and testis, potential sites of endogenous retrovirus mobilization, as well as in both stimulated and unstimulated peripheral blood lymphocytes (PBLs), sites of lentiviral (HIV and HTLV) replication *in vivo*. In contrast, human APOBEC3G mRNA is expressed at higher levels in the trachea and adipose tissue while human APOBEC3H mRNA is expressed at higher levels in the fetal liver and skin where APOBEC3G mRNA appears to be poorly transcribed. Thus, while the expression pattern of human APOBEC3H in retroviral target tissues supports a role for this gene in antiretroviral defense and is similar to the potent antiretroviral effector human APOBEC3G, there are significant tissue-specific differences suggesting that these two APOBEC3 genes are not functionally redundant.

In addition to the tissue-specific analysis, I surveyed several cell lines and primary cells by an end-point RT-PCR assay to ask which of these cell types express APOBEC3H mRNA (Fig 11B). APOBEC3G transcripts were amplified as a control as the expression of APOBEC3G mRNA in various cell lines and primary cells has

previously been described[29, 44]. This experiment was performed twice, with similar results (compare left and right panels of Fig 11B). I found expression of APOBEC3H mRNA in H9 and Jurkat cells (commonly used lab-adapted T cell lines) as well as freshly isolated macrophages and HeLa cells. No expression was detected in either SupTI (a T cell line that is known to be permissive to replication of vif-deficient HIV), 293T cells or primary CD4+ T cells. Therefore, similar to the tissue-specific analysis, I find that APOBEC3H mRNA is expressed in various primary and lab-adapted cell lines but it is not ubiquitously expressed as I was unable to detect expression in all cell types.

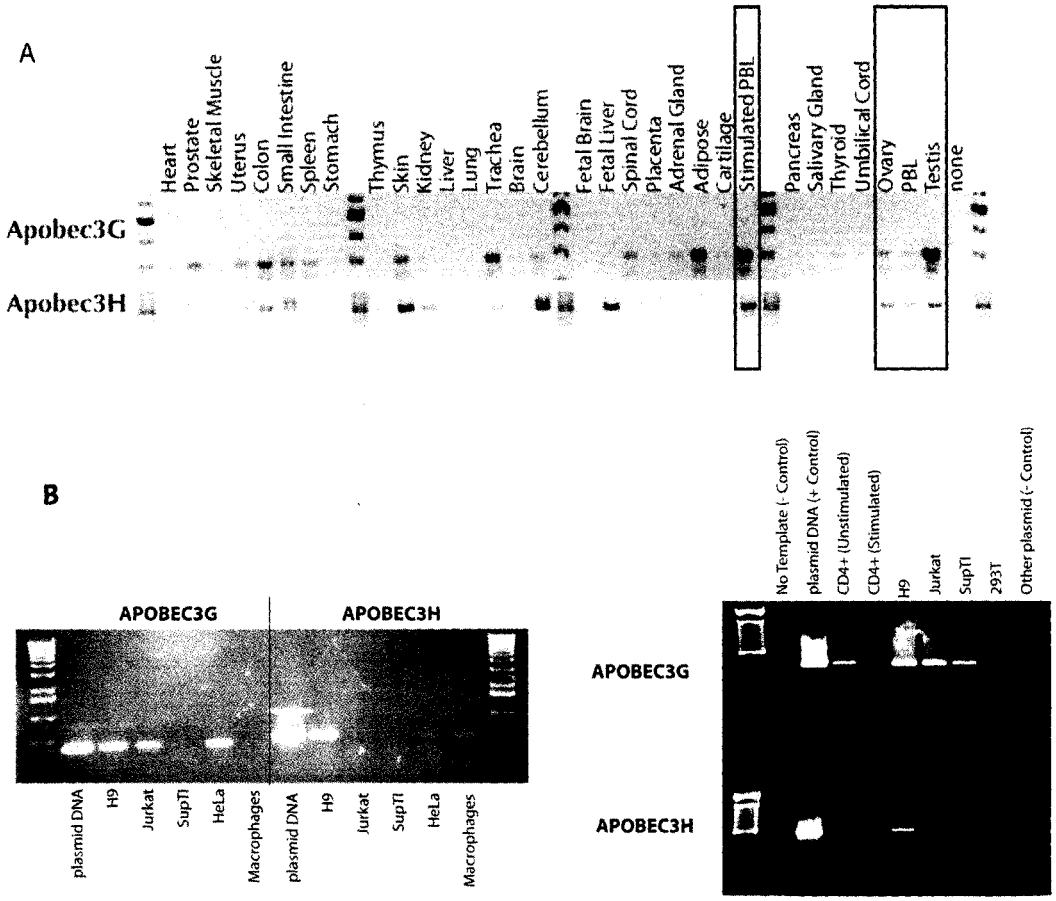


Figure 11: Tissue- and Cell Type-Specific Expression of APOBEC3H mRNA
Panel A: mRNA expression pattern of APOBEC3H in human tissues. Both APOBEC3G and APOBEC3H were PCR amplified with specific primers from cDNAs made from a panel of different human tissues. Molecular weight markers are shown every ninth lane, and the tissue of origin is marked above each lane. Boxes indicate expression of human APOBEC3G and human APOBEC3H in PBLs and germ line cells (sites relevant to retroviral replication). Panel B: Left - Freshly-isolated Macrophages and cell lines were screened by semi-quantitative RT-PCR for expression of human APOBEC3H and human APOBEC3G mRNA. The corresponding plasmid DNA was used a positive control. Right – RNA from Primary CD4+ T cells from a single donor (half the cells were treated with PHA overnight to stimulate) as well as several cell lines was used for semi-quantitative RT-PCR to amplify APOBEC3H mRNA. Negative controls consisted of either no template or amplification of the opposing (APOBEC3G or APOBEC3H) plasmid. APOBEC-specific plasmid DNA served as a positive control.

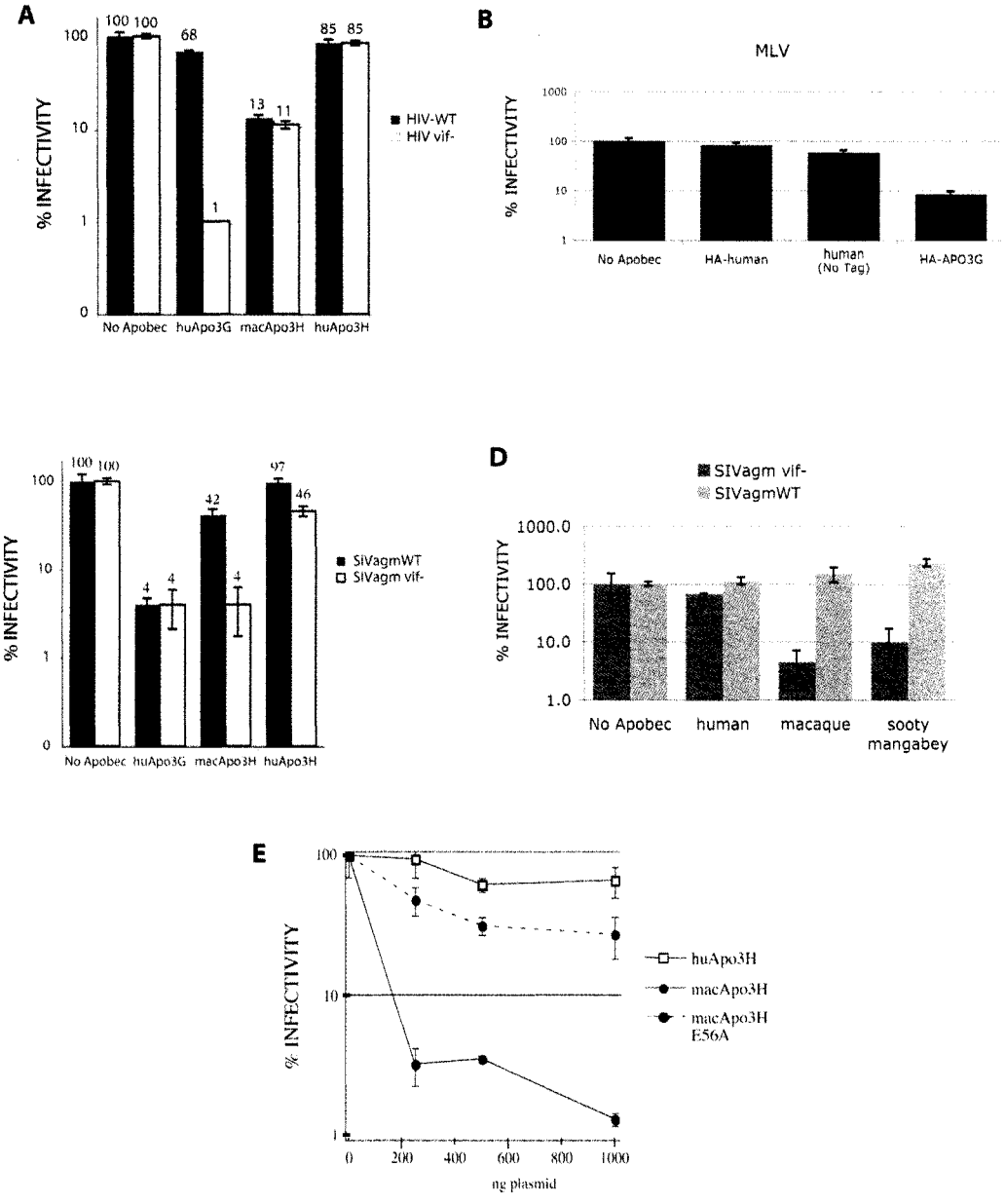
APOBEC3H is a Retroviral Restriction Factor

I next tested the antiretroviral activity of APOBEC3H in single-round viral infectivity assays. First, cDNAs for human (Hominoid), macaque (OWM) and sooty mangabey (OWM) APOBEC3H were cloned from testis, spleen and lymphoid cells, respectively. Plasmids expressing either APOBEC3H (human, macaque or sooty mangabey) or APOBEC3G (human) were then co-transfected with an HIV-1 (HIV-WT) genome that encodes the luciferase gene along with VSV-G expression plasmid for pseudotyping of virions. Viral supernatants were collected, normalized for p24 gag and used to infect 293T cells. As HIV/SIV Vif proteins can counteract the inhibitory effects of other APOBEC3 proteins [61], I also tested APOBEC3H for activity against HIV-1 lacking the *vif* gene (HIVΔ*vif*).

I found macaque APOBEC3H to be a potent inhibitor of both HIV-WT and HIVΔ*vif* (Fig. 12A). The resistance of macaque APOBEC3H to the effects of HIV-1 Vif is expected due to the known species-specificity of Vif/APOBEC interactions [61]. For instance, the macaque APOBEC3G protein is also unaffected by HIV-1 Vif, but is susceptible to SIV Vif [61]. In contrast to this antiretroviral activity of the macaque homolog, I found that human APOBEC3H had almost no effect on HIV replication even in the absence of the Vif protein (maintenance of 85% infectivity for both HIVΔ*vif* and HIV-WT) (Fig. 12A). The HA epitope tag at the N-terminus was not responsible for the lack of activity of human APOBEC3H as plasmids expressing human APOBEC3H without the HA tag were also tested for any activity against MLV and shown to be inactive (Fig 12B). Human APOBEC3G was used as a control in these experiments as it has the best characterized

Figure 12: APOBEC3H has Antiretroviral Activity

Single-round viral infectivity assays were used to assess the relative abilities of APOBEC3 proteins to inhibit retroviral replication, as described in the Materials and Methods. Panel A: Effect of human APOBEC3G (huApo3G), macaque APOBEC3H (macApo3H) and human APOBEC3H (huApo3H) on HIV-1 infectivity. Black boxes are HIV-1 WT and white boxes are HIV-1Δ*vif*. Results are shown as a percent infectivity of the control infection in which no APOBEC expression plasmid was included. Results from one representative experiment are shown (error bars reflect variation between triplicate infections within one experiment). Panel B: MLV was produced in the presence of human APOBEC3H either with or without an HA tag at the N-terminus. HA-tagged human APOBEC3G was used as a positive control as it is known to efficiently inhibit MLV. Panel C: The same as Panel A except that APOBEC proteins were assayed for activity against SIVagm, a related non-human primate lentivirus. Of note, the 2-fold decrease in SIVagmΔ*vif* infectivity in the presence of human APOBEC3H was not reproducible in independent experiments. Panel D: Sooty mangabey APOBEC3H was tested for the ability to inhibit both wild type (green) and Vif-deficient (blue) SIVagm. Panel E: Single-round viral infectivity assay of HIVΔ*vif* produced in the presence of increasing amounts of APOBEC3H expression plasmids. Human APOBEC3H (open squares) plasmids decreased HIV-1 infectivity less than 2-fold at all concentrations of transfected plasmid, while macaque APOBEC3H (solid circles; solid lines) decreased infectivity almost 50-fold. A catalytic site mutant of macaque APOBEC3H (macApo3H E56A; solid circles; dotted lines) was severely impaired in its ability to inhibit HIV-1 replication.


A
100 100
68
1
13 11
85 85
% INFECTIVITY
HIV-WT
HIV vif-
No Apobec
huApo3G
macApo3H
huApo3H
B
MLV
% INFECTIVITY
No Apobec
HA-human
human (No Tag)
HA-APO3G
C
100 100
4 4
42
4
97
46
% INFECTIVITY
SIVagmWT
SIVagm vif-
No Apobec
huApo3G
macApo3H
huApo3H
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1000.0
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0
200
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antiviral activity against a broad panel of retroviruses [32-34, 36, 38, 46, 60, 63]. As expected, human APOBEC3G was able to efficiently restrict HIVΔ*vif* but not HIV-WT (human APOBEC3G is known to be potently inhibited by HIV-1 Vif) [29] (Fig. 12A). The finding that human APOBEC3H is not able to inhibit HIV-1 despite the potent antiviral activity of the macaque homolog is surprising given that the APOBEC3G homologs from various primate species (human, chimp, macaque and african green monkey) do not demonstrate variable activity against HIV-1 [61].

Since the antiviral activity of APOBEC3 family members is sometimes specific to certain viral species [31], I also tested macaque and human APOBEC3H alongside human APOBEC3G for their ability to inhibit a related, non-human primate lentivirus, SIVagm. SIVagm endemically infects african green monkeys, but also is capable of replicating in the rhesus macaque suggesting that SIVagm Vif is sufficient to counteract APOBEC3 proteins encountered in this host. I found macaque APOBEC3H to be able to potently restrict SIVagmΔ*vif*, but not SIVagmWT (Fig. 12C). Thus, macaque APOBEC3H is specifically inhibited by SIVagm Vif, but not by HIV Vif (compare Fig. 12A with Fig. 12C) similar to what has been shown for macaque APOBEC3G [61]. In contrast to macaque APOBEC3H, human APOBEC3H was unable to significantly inhibit either SIVagmWT or SIVagmΔ*vif*, again suggesting that human APOBEC3H is incapable of inhibiting lentiviral replication. Consistent with previous findings, human APOBEC3G inhibited SIVagm and was not neutralized by SIVagm Vif (Fig. 12C). We also cloned the APOBEC3H cDNA from sooty mangabey and found that its restriction pattern was essentially identical to the macaque homolog (Fig 12D), as would be expected given the relatively short evolutionary distance between the two primates.

Next, I tested the ability of macaque and human APOBEC3H to inhibit HIVΔ*vif* in a dose-response experiment by increasing the amount of APOBEC3H plasmid in the transfection from 250 ng to 1000 ng. Even at the lowest levels of transfected plasmid, macaque APOBEC3H was able to reduce the infectivity of HIVΔ*vif* to less than 5% of the control (no APOBEC) infection (Fig. 12E). In contrast, human APOBEC3H had less than a 2-fold effect on HIVΔ*vif* even at the highest level of transfected plasmid. In addition, I introduced a mutation in the predicted active site of the macaque APOBEC3H cytidine deaminase domain by changing amino acid 56 from glutamate to alanine (macApo3H E56A in Fig. 12E). This mutant was severely impaired in its ability to inhibit HIVΔ*vif*, although even the residual antiviral activity of macApo3H E56A was greater than that of human APOBEC3H (Fig. 12E).

I conclude, therefore, that APOBEC3H in these Old World monkey species is able to restrict divergent primate lentiviruses with an expected species-specific sensitivity to the lentiviral *vif* gene, while the human APOBEC3H homolog is incapable of restricting any of the viruses tested here. These findings suggest an unexpected and fundamental difference in activity between APOBEC3H proteins from Old World monkeys and humans.

APOBEC3H Protein Expression in Mammalian Cells

To understand the difference in antiviral activity between macaque and human APOBEC3H, I examined steady-state expression levels of the proteins in transiently transfected cells. Western blot analysis of lysates from 293T cells (a human cell line) that were transfected with equivalent amounts of macaque APOBEC3H and human APOBEC3H expression plasmids revealed a large discrepancy between steady-state levels of the two proteins (Fig. 13A). At levels of transfected plasmid that correspond to efficient inhibition of primate lentiviruses by macaque APOBEC3H (the same levels used for the Infectivity Assays in Fig. 12A/B), I saw high levels of macaque APOBEC3H protein but was able to detect only low levels of human APOBEC3H protein. Through semi-quantitative RT-PCR analysis I found equivalent amounts of mRNA to be present in cells transiently transfected with either APOBEC3H homolog (Fig. 13B). Thus the inability of human APOBEC3H to restrict retroviral replication appears to reflect low protein levels in the virus-producing cell. Further, this low steady-state level of expression is not due to decreased transcriptional activity or mRNA instability. Work that will be described in Chapter 5 shows that the human APOBEC3H protein is expressed at low levels because the protein has a very short half-life relative to other primate APOBEC3H proteins.

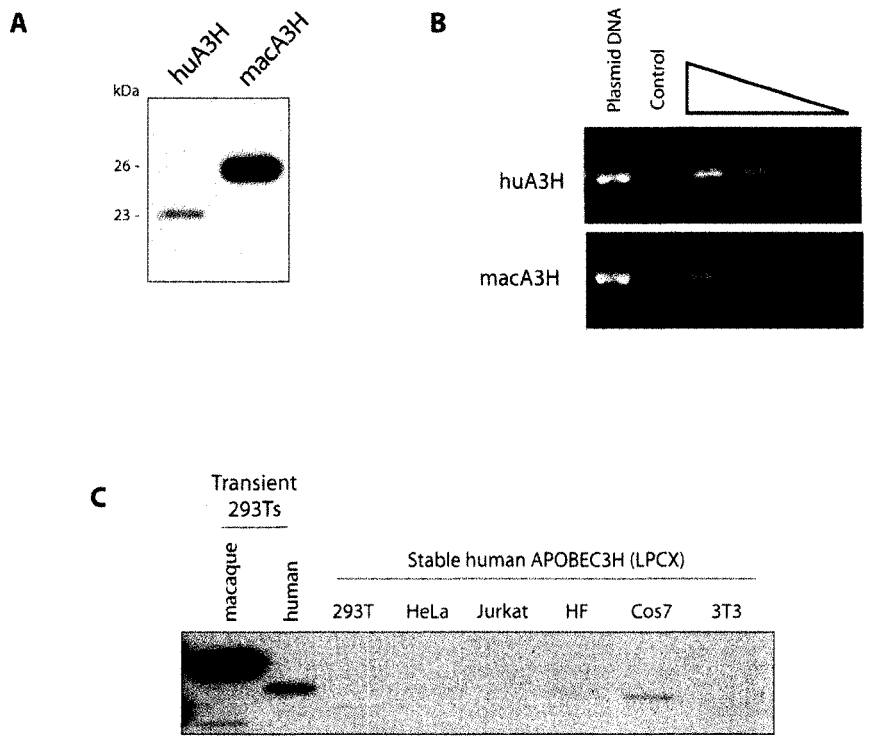


Figure 13: Steady-State Levels of APOBEC3H Proteins are Different
Cell lysates from 293T cells transiently transfected with APOBEC expression plasmids were analyzed by Western blot and semi-quantitative RT-PCR to determine relative levels of protein and mRNA, respectively. Panel A: APOBEC3H protein levels were detected in cell lysates 48 hours after transfection. The predicted size of the HA-tagged human APOBEC3H is ~23 kDa and the macaque APOBEC3H is ~26 kDa. Panel B: Cytoplasmic mRNAs were reverse transcribed using an APOBEC3H-specific primer and serial dilutions (1:2) were used as a template for PCR amplification. Amplification of APOBEC3H transcripts is equivalent for both the human and macaque APOBEC3H transfections. The negative control (no reverse transcriptase) was included to rule out the possibility of plasmid DNA contamination. Panel C: Human cell lines (293T, HeLa and Jurkat) as well as human primary cells (HF = Human Fibroblasts), an African green monkey cell line (Cos7) and a mouse cell line (NIH3T3) were transduced with a retroviral vector (LPCX) to create cells stably-expressing HA-human APOBEC3H protein. 293T cells transiently-transfected with either macaque or human APOBEC3H are shown for comparison. 10μg total protein was loaded per well, followed by SDS-PAGE and an anti-HA Western Blot.

Next, I looked for expression of stable human APOBEC3H protein in a variety of other human cells (primary human fibroblasts, HeLa cells, and Jurkat T cells) as well as non-human mammalian cells (mouse 3T3 cells) by retroviral transduction and found that there was low to undetectable human APOBEC3H protein expression in all

of them (Fig 13C). Therefore, the low steady-state expression of human APOBEC3H is not specific to only certain human cell lines and is seen in cells from other primates or mice.

Different Levels of Human and Macaque APOBEC3H Proteins is Independent of Tag Position or Expression Vector

The different levels of human and macaque APOBEC3H protein could be due to artifacts of the experimental system or due to relevant biological consequences of the evolution of the APOBEC3H coding sequence during primate evolution. To determine if this difference could be due to aspects of the experimental system, such as placement of the epitope tag relative to the protein sequence or the type of expression vector used, I evaluated the expression of both human and macaque APOBEC3H proteins with the HA tag at either the N-terminus or the C-terminus, as well as in two additional mammalian expression vectors.

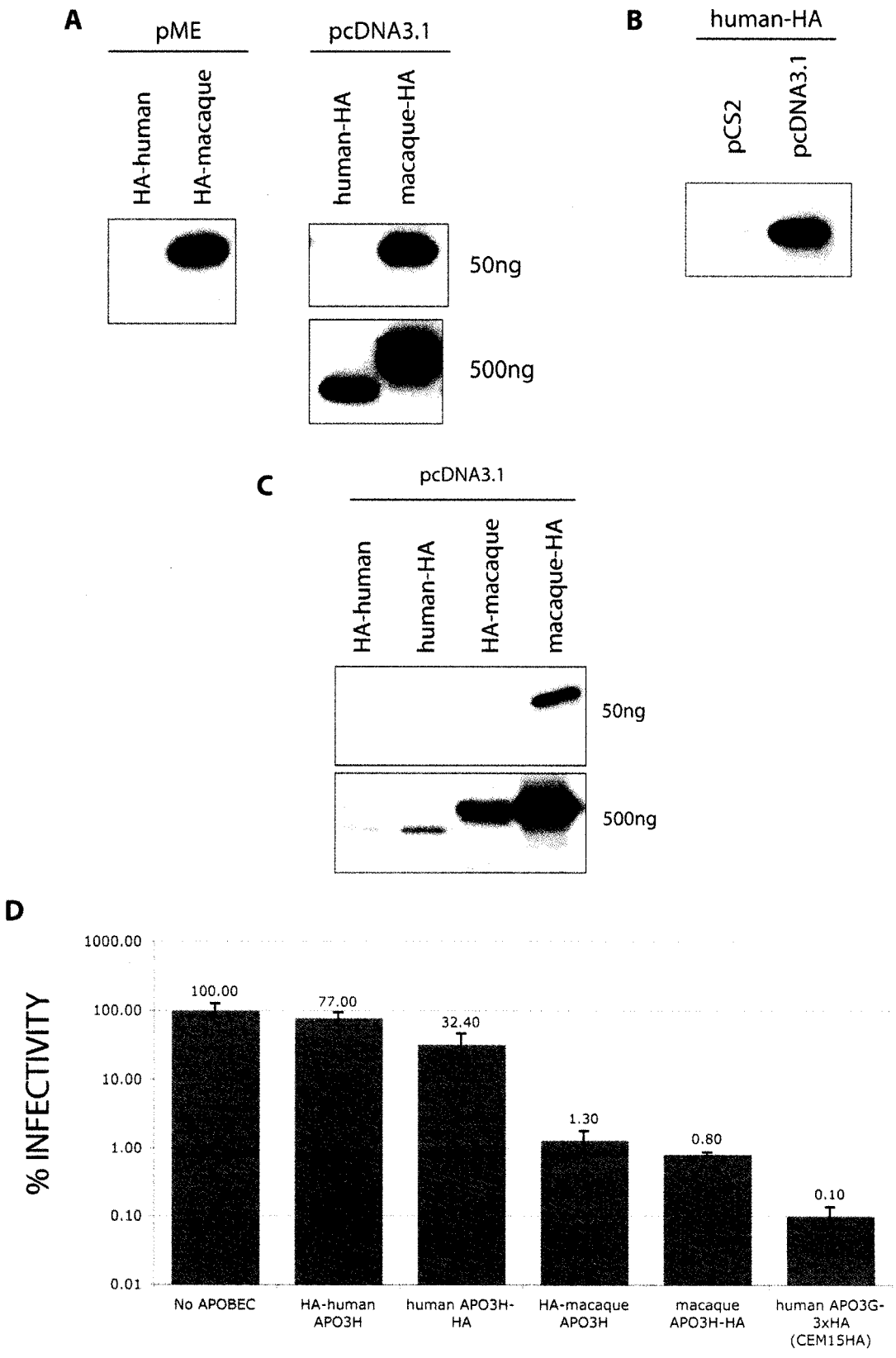
Expression of the human and macaque APOBEC3H proteins from the two additional mammalian expression vectors gives results identical to my previous results using the pCS2 vector as levels of human APOBEC3H protein are dramatically less than that of macaque APOBEC3H (Fig 14A). Expression of APOBEC3H protein from the pcDNA3.1 vector showed significantly higher levels of expression and this expression vector was used in all future experiments (Fig 14B). Moving the HA tag from the N-terminus to the C-terminus of the APOBEC3H protein results in an increase in steady-state expression levels of APOBEC3H proteins (Fig. 14C). However, the steady state levels of human APOBEC3H protein relative to macaque APOBEC3H protein are still dramatically different (Fig 14C; compare HA-human to HA-macaque or human-HA to macaque-HA). Therefore, these data

demonstrate that the difference in expression levels is inherent to the amino acid sequences of the human and macaque proteins rather than an artifact of tag placement or expression vector.

Finally, the antiviral potencies of human and macaque APOBEC3H proteins with either the N- or C-terminal tags was tested against HIVΔvif. Similar to my original findings, macaque APOBEC3H shows robust inhibtion of HIV replication (~100-fold) while the human APOBEC3H homolog shows little to no inhibition (Fig14D). Tag placement has only a very minor affect (< or = 2-fold) on the antiviral activities of either APOBEC3H homolog (Fig 14D; compare HA-human to human-HA and HA-macaque to macaque-HA). Therefore, macaque APOBEC3H is a potent antiviral independent of the polarity of the HA tag, while human APOBEC3H lacks robust antiviral activity in any expression system tested here.

Figure 14 – Macaque APOBEC3H protein Levels are higher regardless of Expression Vector or Tag Placement

Panel A: Human and macaque APOBEC3H cDNAs were cloned into 2 alternative mammalian expression vectors (pME – left or pcDNA3.1 – right). A transfection with 10-fold more transfected plasmid (500ng) is shown for the pcDNA3.1 experiement. Panel B: Relative expression levels of human APOBEC3H protein in either the pCS2 (vector used in all previous experiments) or the pcDNA3.1 expression vector (vector used in future experiments). Panel C: Human and macaque APOBEC3H proteins were cloned into pcDNA3.1 with the HA epitope tag at either the N-terminal or C-terminal end of the protein. The lower panel shows 10-fold more transfected plasmid (500ng). Panel D: HIV-1Δ*vif* produced in cells transfected with C- or N-terminally tagged human and macaque APOBEC3H proteins. Values are shown as % Infectivity relative to the control infection in which "No Apobec" was expressed in the virus-producing cells. The pcDNA3.1-based human APOBEC3G construct (CEM15HA) is shown as a positive control for comparison.


A
pME
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HA-human
HA-macaque
human-HA
macaque-HA
50ng
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B
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C
pcDNA3.1
HA-human
human-HA
HA-macaque
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10.00
1.00
0.10
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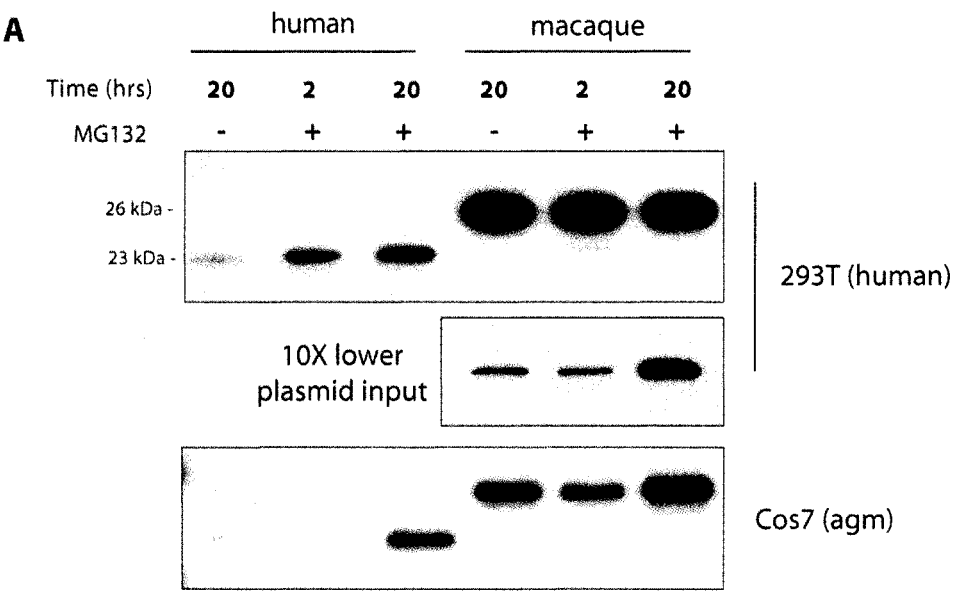
100.00
77.00
32.40
1.30
0.80
0.10
No APOBEC
HA-human APO3H
human APO3H-HA
HA-macaque APO3H
macaque APO3H-HA
human APO3G-3xHA (CEM15HA)

Proteasome-mediated Degradation of APOBEC3H proteins

To test the possibility that human APOBEC3H protein is actively degraded, I used a well-described proteasome inhibitor, MG-132, to determine if blocking proteasome-mediated protein degradation would lead to higher steady-state levels of human APOBEC3H in transiently transfected 293T cells. Indeed, I found that proteasome inhibitor treatment partially stabilized intracellular levels of human APOBEC3H at both the 2 and the 20-hour time points (a 5-fold increase over the DMSO-only control) (Fig. 15A; top panel), suggesting that human APOBEC3H is actively degraded by the proteasome. No increase was seen for macaque APOBEC3H when it was transfected at similar plasmid levels as that of the human APOBEC3H plasmid, but I did see an increase of macaque APOBEC3H protein in response to MG-132 when ten-fold lower amounts of plasmid were transfected (Fig. 15A, middle). However, the response of human APOBEC3H differed from that of macaque APOBEC3H since human APOBEC3H could be induced by a 2-hour incubation with proteasome inhibitor, while induction of macaque APOBEC3H required a 20-hour incubation (Fig 15A, compare top and middle panels). Thus, both proteins are likely subject to proteaseome-mediated degradation, but the human protein may be more sensitive to this degradation. Similar results were seen in Cos7 cells (Fig 15A; lower panel), an african green monkey cell line (OWM), ruling out the possibility that this degradation is specific to expression of the proteins in a human cell line.

Figure 15 – Human APOBEC3H levels are increased in the presence of proteasome inhibitors, but this is not sufficient to confer antiviral activity

Panel A: The proteasome inhibitor, MG-132, was added to transient transfections of human APOBEC3H and macaque APOBEC3H in 293T cells and lysates were used for SDS-PAGE/Western blot analysis. 2- and 20-hour time points were harvested and revealed ~5-fold stabilization of the human APOBEC3H protein even at the earliest time point. In the middle panel, 10-fold less macaque APOBEC3H was transfected. Similar results were obtained in the African green monkey (agm) cell line – Cos7 (bottom panel), although no significant increase in human APOBEC3H levels were seen at the 2-hour time point in these cells. Panel B: Both human (huA3H – black triangles) and macaque (macA3H – open squares) APOBEC3H proteins were tested for their ability to inhibit HIV-1Δ*vif* in the presence of the proteasome inhibitor, MG-132 (see Panel C: for levels of APOBEC proteins from the virus-producing cells). Inhibition of HIV-1 by macaque APOBEC3H increases as the amount of macA3H plasmid increases. Addition of proteasome inhibitors to cells producing virus from huA3H-expressing cells has no effect on infectivity, even at the highest amount tested (1000ng plasmid).


A
human
macaque
Time (hrs)
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2
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2
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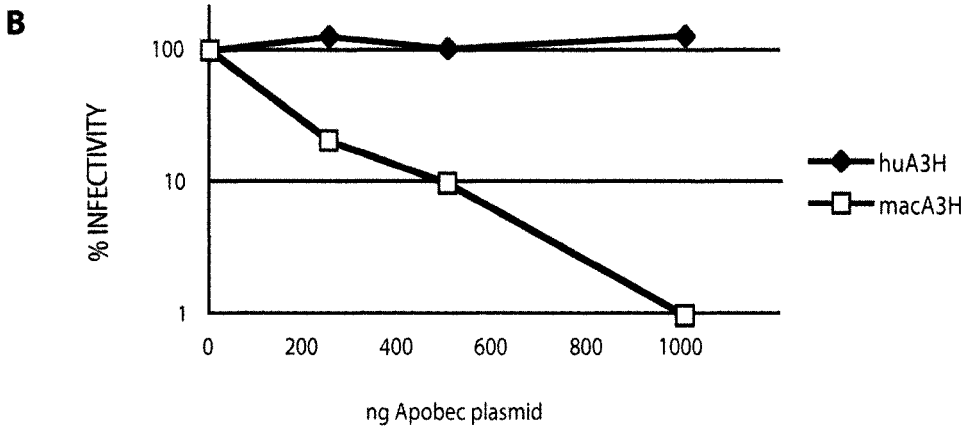
MG132
-
+
+
-
+
+
26 kDa -
23 kDa -
293T (human)
10X lower
plasmid input
Cos7 (agm)


B
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ng Apobec plasmid
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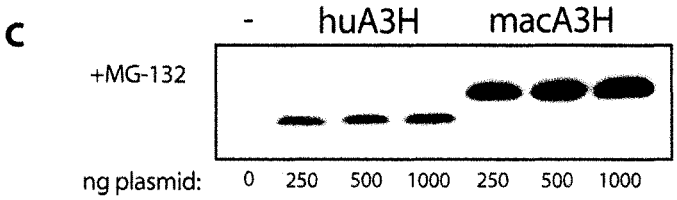

C
-
huA3H
macA3H
+MG-132
ng plasmid:
0
250
500
1000
250
500
1000

Despite the fact that cells treated with a proteasome inhibitor contain increased levels of human APOBEC3H, the levels are still lower than that of macaque APOBEC3H. To ask if the increased levels of human APOBEC3H seen in the presence of proteasome inhibitors might be sufficient to confer antiviral activity on the human protein I produced virus from these cells to use in a single-round infectivity assay. I find that human APOBEC3H produced at these levels has no antiviral effect on HIV-1Δ*vif* (Fig. 15C/D). These results suggest that the significantly different steady-state expression levels of the APOBEC3H proteins may explain the observed disparity in their antiviral activities.

Macaque but not Human APOBEC3H is Incorporated Into Virions

Given that the steady-state expression levels of human APOBEC3H are significantly lower than that of the rhesus macaque APOBEC3H homolog, I hypothesized that human APOBEC3H would not be incorporated into budding virions. This would support a model in which not enough human APOBEC3H protein is present in virus-producing cells to allow for efficient packaging. In contrast, I would expect to see efficient incorporation of macaque APOBEC3H as this protein is present at high levels and has demonstrated antiviral activity. To ask if macaque and human APOBEC3H proteins differ in their ability to be incorporated into budding virions, viral supernatants from virus producing cells expressing APOBEC3 proteins were collected and virion preparations were analyzed for incorporation of APOBEC3H by Western blot. Human APOBEC3G was used as a positive control as it is known to be efficiently packaged into vif-deficient HIV. Consistent with my hypothesis I was able to detect incorporation of macaque APOBEC3H into both wild-type and vif-

deficient HIV while human APOBEC3H protein was undetectable in either scenario (Fig 16). These data suggest that human and macaque APOBEC3H proteins are not incorporated to equivalent levels and are consistent with the hypothesis that the low levels of human APOBEC3H proteins in virus-producing cells are not sufficient for incorporation into budding virus.

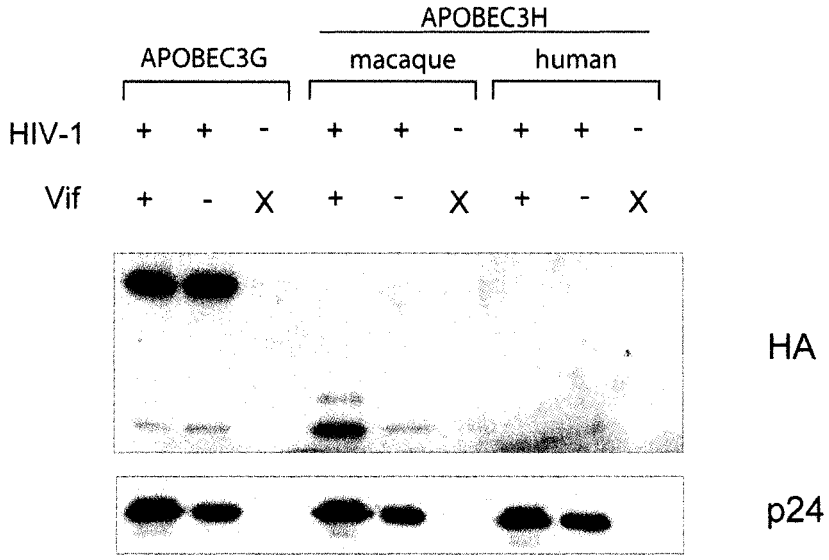


Figure 16 – Incorporation of Macaque but not Human APOBEC3H into HIV Virions
Wild type and Vif-deficient HIV was produced from cells expressing human APOBEC3G, human APOBEC3H or macaque APOBEC3H proteins. Samples in which no viral expression plasmid was transfected did not show detectable levels of HA-APOBEC, suggesting that detection of HA-APOBEC proteins in virus+ samples was specific to virus production. Viral preparations collected by high-speed centrifugation of supernatants through 20% sucrose were used for SDS/PAGE followed by Western Blot. The blot was probed for HA, stripped and re-probed to detect p24 levels.

APOBEC3H Encodes a Conserved Cytidine Deaminase Activity

The residues necessary for cytidine deamination, such as the consensus His-X-Glu-X_{23-28}-Pro-Cys-X_{2-4}-Cys cytidine deaminase motif [115], are conserved in both the human and macaque APOBEC3H homologs. To ask whether either APOBEC3H

homologs have functional enzymatic activity, I took advantage of a well-described bacterial mutator assay [116, 117]. In this assay, induced APOBEC proteins catalyze cytidine deamination of the bacterial genome. Mutations that occur at well-characterized positions of the RNA polymerase gene, *rpoB*, confer resistance to the antibiotic rifampicin, allowing us to screen for DNA mutator activity by assaying for increased frequency of rifampicin-resistance (Figure 17).

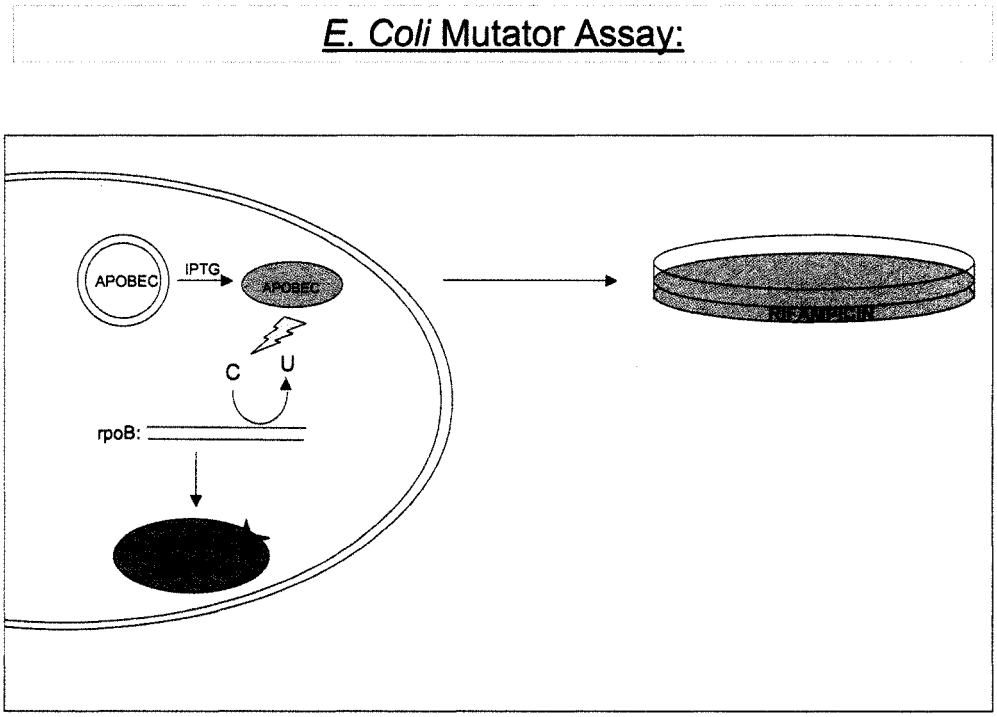


Figure 17 – E. Coli Mutator Assay

In contrast to my results in primate cells, human APOBEC3H can be stably expressed in the bacterial system (Fig. 18A). In this mutator assay, expression of either APOBEC3H homolog resulted in an increase in the median Rif^R frequency (human APOBEC3H: ~6.1-fold over background; macaque APOBEC3H: ~3.1-fold

over background in this experiment; $p<0.05$), suggesting that both APOBEC3H homologs have conserved DNA mutator activity. Similar to what other groups have shown [112], I saw an elevated median mutation frequency (~3-fold) when comparing human APOBEC3G to the vector-only control (Fig. 18B).

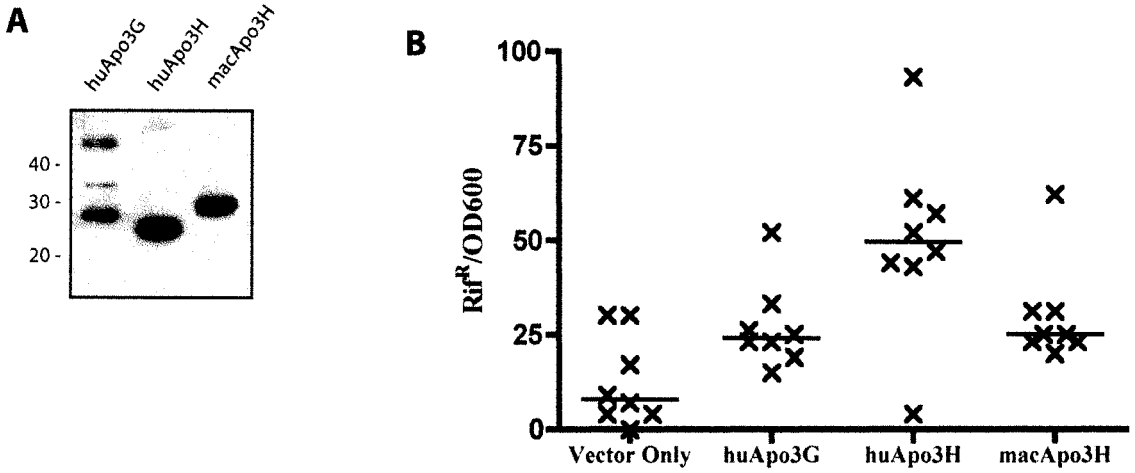


Figure 18: Cytidine Deaminase Activity is Conserved in APOBEC3H Homologs
Panel A: Macaque and human APOBEC3H homologs with a C-terminal Myc tag were expressed in a bacterial system to evaluate protein expression levels. Whole-cell lysates were used for Western blot analysis to evaluate expression levels of APOBEC proteins in bacteria. Sizes of myc-tagged APOBEC proteins are ~48 kDa for human APOBEC3G, ~23 kDa for human APOBEC3H and ~26 kDa for macaque APOBEC3H. All APOBECs were stably expressed in this system. Panel B: APOBEC proteins were evaluated for their ability to affect rates of acquired resistance to rifampicin in a bacterial cytidine deamination assay. Results are shown as the number of rifampicin-resistant colonies (Rif^R) normalized for cell number (OD_{600}). One representative experiment is shown in which 8 independent cultures were evaluated. Of note, both the human APOBEC3H and macaque APOBEC3H mutation frequencies were higher than the control (Vector only) in independent experiments ($p<0.05$). However, the potency of macaque and human APOBEC3H relative to one another varied in independent experiments and should not be compared.

To further this analysis, I sequenced a small region of the *E. coli* RNA polymerase gene isolated from Rif^R colonies. If cytidine deamination is the major mechanism through which mutations are conferring a resistant phenotype, sequencing should reveal more C to T and G to A transition mutations in the APOBEC-expressing samples as compared to the vector-only control. As expected, I

found that the mutations in the *rpoB* gene from human APOBEC3H, macaque APOBEC3H and human APOBEC3G were transition mutations 93%, 95% and 95% of the time, respectively, whereas transitions occurred only 84% of the time for the vector-only control. I conclude, therefore, that both APOBEC3H homologs have conserved cytidine deaminase activity that is capable of, although perhaps not limited to, DNA mutation.

G to A Hypermutation of Retroviral Genomes

APOBEC3-mediated antiviral activity has been shown to be due, at least in part, to cytidine deamination of nascently transcribed retroviral cDNAs following infection of target cells. Such cytidine to uracil deamination in minus-strand DNA is detected as replacement of guanine with adenine in integrated proviral genomes. I found that a mutation in the putative active site of macaque APOBEC3H disrupted most (but not all) of its antiviral activity (Fig. 12C). To further define the role of deamination in APOBEC3H-mediated antiviral activity we also looked for direct evidence of APOBEC3H-induced hypermutation of viral genomes. After infection of cells with HIVΔ*vif* produced in the presence or absence of APOBEC expression plasmids, a region of the viral genome was amplified, sequenced and analyzed for evidence of hypermutation. Significant G to A hypermutation was observed for both human APOBEC3G and macaque APOBEC3H (Fig. 19A), implying that both enzymes actively deaminate cytidines in minus-strand viral DNA. As might be expected from my viral infectivity, protein expression and virion incorporation data, human APOBEC3H infections showed no evidence of hypermutation. Further analysis of this sequence data allowed me to delineate the target site preference for

macaque APOBEC3H. Macaque APOBEC3H demonstrated a clear target site preference for thymine at the -1 position relative to the deaminated cytidine, an 85% TC dinucleotide preference (Figure 19B). This dinucleotide preference is similar to what has been reported for human APOBEC3B [52] and human APOBEC3F [46, 74], but is in contrast to the CC dinucleotide motif preferred by human APOBEC3G (Fig. 19B) [73]. These data suggest hypermutation of retroviral genomes as a likely mechanism of APOBEC3H-mediated antiviral activity.

A

	No Apobec	huApo3G	huApo3H	macApo3H
Clones Sequenced	2	4	6	11
Total bp Sequenced	814	1628	2442	4477
# of G to A Mutations	0	21	0	20
# Other Mutations	0	0	0	0

B

huA3G

n=21	-2	-1	C	+1
A	19	0	0	52
C	33	100	100	19
G	0	0	0	0
T	48	0	0	29
	T/C	C	C	A

macA3H

n=20	-2	-1	C	+1
A	30	10	0	15
C	15	5	100	35
G	15	0	0	25
T	40	85	0	25
	A/T	T	C	-

Figure 19: APOBEC3H Induces G-to-A Hypermutation of Retroviral Genomes
Retroviral hypermutation induced by APOBEC proteins was evaluated by sequencing HIVΔ*vif* proviral genomes following infection of a target cell line, as described in Materials and Methods. Panel A: Integrated proviruses were sequenced from infections with viral supernatants from transfections containing human APOBEC3G, macaque APOBEC3H, human APOBEC3H or no APOBEC expression plasmid. G to A changes were the only mutations observed in ~9000 bp of sequenced genomes, ruling out the likelihood of generalized reverse transcriptase errors or otherwise experimentally introduced mutations. Panel B: Hypermutated viral sequences were analyzed to determine the sequence context of deaminated cytidines. The number of deamination target sites evaluated for each APOBEC is noted (huA3G = 21, macA3H = 20). Shown are percentages of each nucleotide found at the -2, -1 and +1 positions relative to the deaminated cytidine (C). Macaque APOBEC3H demonstrates a clear TC dinucleotide preference.

DISCUSSION

In this chapter, I have described the initial characterization of a unique component of the primate APOBEC3 family of antiretroviral effectors, APOBEC3H. We find APOBEC3H orthologs to be conserved in mammalian genomes and undergoing sustained adaptive evolution in primates. As expected given the signal of

positive selection acting on APOBEC3H in primates, I find OWM APOBEC3H to be a potent inhibitor of lentiviral infectivity, through a mechanism that includes, at least in part, deamination of nascent viral minus-strand DNA. Consistent with this mechanism I found OWM APOBEC3H to be efficiently incorporated into HIV virions while the human APOBEC3H is incapable of retroviral inhibition and, accordingly, is not packaged into virions at detectable levels in my assay.

The fact that SIV Vif allows escape from OWM APOBEC3H-mediated restriction suggests that APOBEC3H plays a significant role in restriction of retroviral infection in these primates since Vif proteins must have evolved to recognize APOBEC3H in Old World monkeys. Moreover, our analysis of positive selection indicates that APOBEC3H may be as much an active participant in host defense as the prototypic antiviral gene APOBEC3G. Thus, in order to productively infect Old World monkeys like rhesus macaques, SIV Vif must be able to inactivate a panel of APOBEC3 proteins, including APOBEC3F, APOBEC3G and APOBEC3H. Thus, the ability of lentiviral Vifs to neutralize APOBEC3 proteins encountered in the host must involve complex interactions that allow Vif proteins to recognize diverse substrates [118], including divergent APOBEC3 proteins.

While orthologous APOBEC3 genes from different primates may encode similar antiviral functions (APOBEC3G, for example), it is clear from my study that other members of the APOBEC3 family, including APOBEC3H, may encode proteins that are not necessarily functionally redundant in different primates. This work, therefore, emphasizes the importance of testing orthologs from various primate species. While multiple human APOBEC3 genes have been tested for antiviral function, only APOBEC3F and APOBEC3G orthologs from other primates have been

tested [61, 67]. My APOBEC3H study suggests that a survey limited to only human genes may lead to an incomplete picture of antiviral potency, or lack thereof, encoded by the APOBEC3 cluster in primates.

APOBEC3H is a potent restriction factor likely to be important in preventing lentiviral infection *in vivo* in certain primate species, including the rhesus macaque and sooty mangabey but not humans. Despite its DNA mutator activity and in contrast to the antiretroviral activity of the macaque APOBEC3H homolog, I find that human APOBEC3H is poorly expressed at the protein level and has no detectable antiretroviral activity. The differential stability and subsequent change in antiviral activity of APOBEC3H homologs suggests that the ~35 million years of evolution that separates OWMs and Hominoids has resulted in significant functional divergence of this conserved antiviral cytidine deaminase. Although I found the instability of human APOBEC3H in a number of cell types, I still cannot rule out the possibility that the protein is differentially regulated in different cells (such as stabilization of the human homolog in an undetermined cell type or cellular environment).

The mechanism by which APOBEC proteins are degraded by lentiviral Vif proteins[40] could be a descendant of such a regulatory control system. This model supposes, however, that regulated degradation, a possible mechanism of protein regulation[119], serves as a major regulator of APOBEC3H stability.

My results demonstrate that an antiviral effector that is active in some primates has become inactive in humans. While this may seem incongruous with the finding of positive selection of APOBEC3H in primates (Fig. 10B), we cannot determine if positive selection has occurred specifically along the evolutionary branch leading to humans. The selective pressures acting on human APOBEC3H will be

discussed in more detail in Chapter 5 after I present data showing when the APOBEC3H protein became unstable during primate evolution. Why an unstable antiviral APOBEC3 gene would evolve specifically in humans, but not in OWMs, could be due to several reasons, but I discuss two strong possibilities. The first possibility is that the loss of function of human APOBEC3H is the result of relaxed selective pressures occurring specifically along the human lineage. Although it is hard to reconcile this possibility with the finding of robust antiviral activities of other human APOBEC3 genes, it is plausible that APOBEC3H may have become specialized to inhibit a particular retroviral invader and extinction of this retrovirus specifically in humans resulted in decreased selective pressure to maintain activity and allowed for the subsequent loss of APOBEC3H activity. Evidence for differential pressure from retroelements among primates is accumulating, particularly in the case of endogenous retroviruses[120, 121]. Given relaxed pressure to maintain antiviral activity, it may even have been advantageous for humans to evolve an unstable APOBEC effector as this would alleviate the cost associated with maintenance of a full repertoire of potentially hypermutagenic cytidine deaminases in the cell[101, 122, 123]. Similar relaxation of constraints may have led to the high frequency persistence of an impaired TRIM5α allele in the human population[124].

A second possibility is that the original function of APOBEC3H has been assumed by another APOBEC3 family member in humans. The rapid expansion of APOBEC3 genes in primate genomes supports this possibility. APOBEC3A, for example, appears to be a recent arrival in Hominoids, revealed in the short evolutionary distance separating it from the C-terminal domain of APOBEC3B (Fig. 9A). This evolutionary distance is less than that between human and macaque

APOBEC3H, implying its recent evolution. Recent work suggests that there may be no APOBEC3A ortholog in the rhesus macaque genome[66]. Since both APOBEC3A and APOBEC3H are single-domained cytidine deaminases, it is possible that APOBEC3A now performs a function in humans[51] that is usually undertaken by APOBEC3H in other primates.

The finding that humans now encode an unstable APOBEC3H protein is significant because orthologous genes are conserved in most mammals, implying an important although unknown function for this gene in mammalian cells. If the human homolog of APOBEC3H has indeed lost the ability to serve as an effective retroviral restriction factor, our current ability to combat retroviral infections might be significantly different from that of other primates, a finding that is both evolutionarily as well as medically important.

Chapter Four:

EVOLUTION OF APOBEC3H STABILITY AND ANTIVIRAL ACTIVITY IN PRIMATES

BACKGROUND

The work presented in Chapter Three was the first to describe the antiretroviral activity of APOBEC3H in primates. In this chapter I describe my work demonstrating the consequences of the rapid evolution of APOBEC3H function in the primate lineage. I find that the C-terminal domain contains a putative Nuclear Export Sequence and that this domain is required for strict cytoplasmic expression of APOBEC3H protein. Further, this domain is required for the potent antiretroviral activity of macaque APOBEC3H protein although it does not affect steady-state expression levels. I find that this domain was truncated from the human and chimp APOBEC3H coding sequences by the fixation of nonsense mutation during the evolution of the human/chimpanzee ancestor. Despite the loss of this domain that is required for macaque APOBEC3H antiviral activity, I find the chimpanzee APOBEC3H homolog to be capable of efficient inhibition of a range of retroviral elements, including HIV, SIV, MusD and LINE-1 elements while human APOBEC3H is not.

RESULTS

Truncation of APOBEC3H Occurred During the Evolution of the Human/Chimp Ancestor

Macaque and Human APOBEC3H proteins share only 83% identity, differing at 31 amino acid residues as well as a 27 amino acid domain present in the C-terminus (referred to as the "Tail") that is conserved in the macaque homolog but

missing in human APOBEC3H (Fig. 20A). Truncation of human APOBEC3H is the result of a nonsense mutation that was fixed at some point during human evolution. Comparison with the APOBEC3H coding sequence reveals that the same nonsense mutation is also present in the chimpanzee and bonobo genomes, but not other hominoid coding sequences (Fig 20B). Therefore, the loss of the TAIL domain in APOBEC3H protein was fixed in the lineage leading to humans and chimpanzees before speciation.

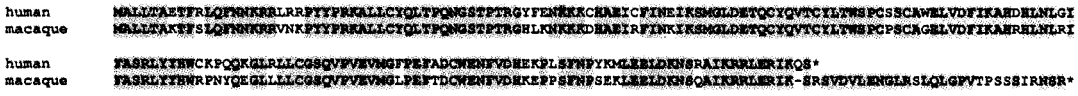
Macaque C-terminal "Tail" is Important for Its Antiviral Activity

To evaluate whether or not this domain controls the stability and, therefore, antiviral activity of macaque and human APOBEC3H, chimeric human/macaque proteins were constructed (Fig 20C). The native macaque protein was truncated at the residue in which the human APOBEC3H protein ends (Macaque –TAIL) while the macaque TAIL sequence was attached to the C-terminus of the native human APOBEC3H protein (Human +TAIL). Steady-state expression levels of both the native and truncated macaque APOBEC3H proteins are similar (Fig. 20D; compare Macaque to Macaque –TAIL), suggesting that the TAIL domain does not control the stability of APOBEC3H protein. Consistent with this data, adding the TAIL to the human APOBEC3H protein has no effect on the steady-state expression levels of the protein (Fig. 20D; compare Human to Human +TAIL).

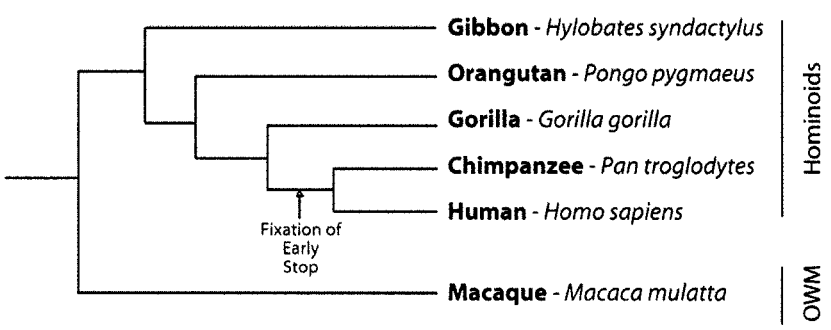
Figure 20: Macaque APOBEC3H "TAIL" Domain is Important for Antiviral Activity but not Stability

Panel A: Amino acid residues conserved between human and rhesus macaque APOBEC3H proteins are shaded in grey. In bold are the key catalytic site residues. The C-terminal domain present in macaque APOBEC3H but not human APOBEC3H is denoted as the "TAIL". Panel B: Primate APOBEC3H coding sequences were aligned and evaluated from presence of the nonsense mutation leading to the prematurely truncated protein. Panel C: Chimeric human/macaque APOBEC3H proteins were constructed in which the "TAIL" domain (shown in blue) was removed from the macaque protein (Macaque –TAIL) and added on to the human protein (Human +TAIL). Panel D: Native and chimeric APOBEC3 proteins were expressed from identical expression vectors with an N-terminal HA tag and evaluated for steady-state expression by Western Blot. Panel E: HIV-1Δ*vif* virions were produced from cells expressing transiently-transfected human and macaque wild type and chimeric proteins and used to infect 293T cells. Infectivities are shown as the % Infectivity of the No Apobec control (100%).

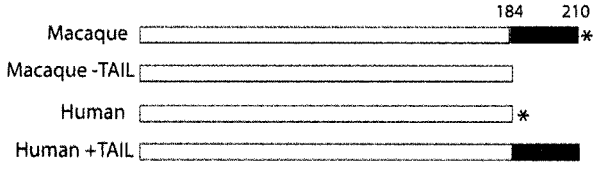
A


human
macaque
human
macaque

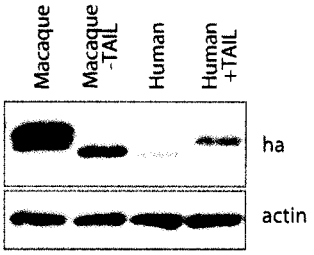
B


Gibbon - Hylobates syndactylus
Orangutan - Pongo pygmaeus
Gorilla - Gorilla gorilla
Chimpanzee - Pan troglodytes
Human - Homo sapiens
Fixation of Early Stop
Macaque - Macaca mulatta
Hominoids
OWM

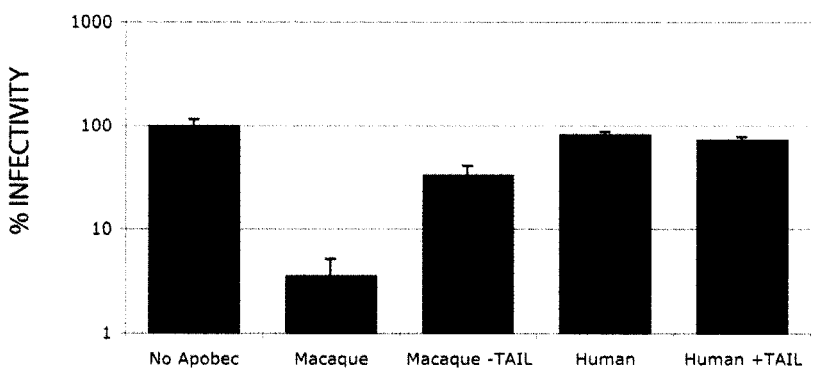
C


184
210
Macaque
Macaque -TAIL
Human
Human +TAIL

D


Macaque
Macaque -TAIL
Human
Human +TAIL
ha
actin

E


% INFECTIVITY
1000
100
10
1
No Apobec
Macaque
Macaque -TAIL
Human
Human +TAIL

Next, the chimeric proteins were tested for their ability to block the replication of HIV-1Δ*vif* in a single-round viral infectivity assay. We see no increase in the ability of human APOBEC3H to inhibit HIV with the addition of the TAIL domain (Fig. 20E), consistent with its low steady-state expression levels. However, truncation of macaque APOBEC3H does lead to a significant decrease in its antiviral activity (Fig 20E). Therefore, the APOBEC3H TAIL domain is important for the antiviral activity of macaque APOBEC3H but is not sufficient to restore the antiviral activity of human APOBEC3H. Together, these data suggest that the Tail domain plays an important role for macaque APOBEC3H antiviral activity but it is not the sole determinant of the loss of activity of the human protein.

The TAIL Domain Confers Strict Cytoplasmic Localization of APOBEC3H Proteins

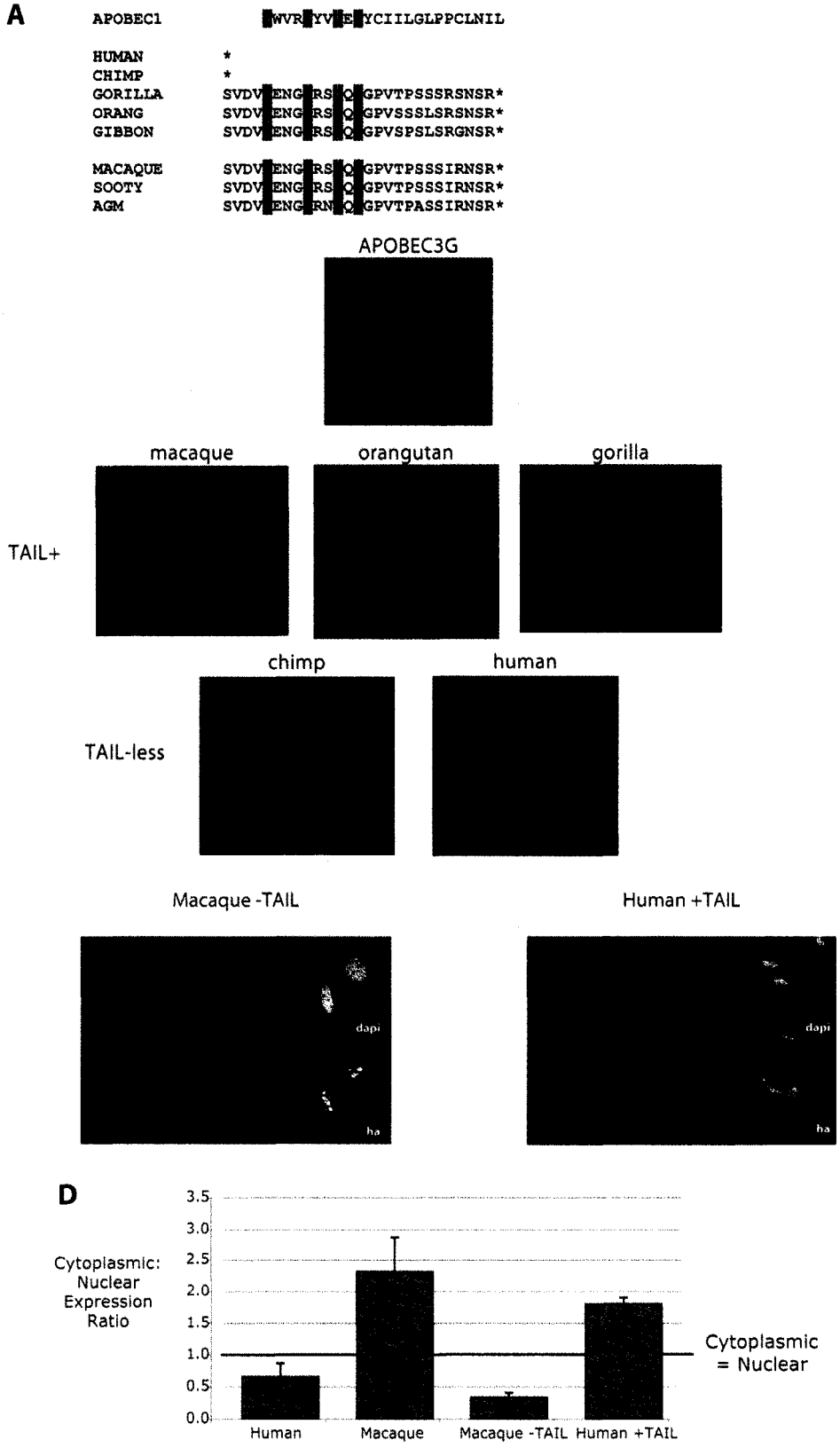
Truncation of the TAIL domain from the macaque APOBEC3H protein did not decrease its steady-state expression, but it did decrease the ability of the protein to block HIV replication. Therefore, I hypothesized that truncation of macaque APOBEC3H's TAIL domain may have detrimental effects on another aspect of APOBEC3H function such as the sub-cellular localization of the protein. Comparison of the TAIL sequence with related APOBEC1 sequence reveals complete conservation of a 4-Leucine Nuclear Export Sequence (NES) in all primate APOBEC3H TAIL sequences that is implicated in nucleocytoplasmic shuttling of APOBEC1 (Fig 21A; 4 Leucine residues are in blue)[125, 126]. Therefore, I hypothesized that APOBEC3H proteins with an intact TAIL domain will localize to the cytoplasm, while truncated (or TAIL-less) APOBEC3H proteins will not be exported to the cytoplasm and, therefore, will show nuclear or diffuse cellular staining.

Various primate APOBEC3H homologs were expressed in HeLa cells and evaluated by immunofluorescence to determine their subcellular localization pattern. Human APOBEC3G was included as a control as it is known to localize strictly to the cytoplasm in discrete foci (Fig 21B; top panel)[92-94]. The 3 APOBEC3H homologs tested here that have an intact TAIL domain (macaque, orangutan and gorilla) all localize to the cytoplasm with a staining pattern similar to that of APOBEC3G (Fig 21B; middle panel). In contrast, the human and chimpanzee APOBEC3H proteins that do not have a TAIL domain show significant nuclear staining (Fig 21B; bottom panel), suggesting that the TAIL domain is important for cytoplasmic localization of APOBEC3H proteins.

Consistent with this hypothesis, truncation of the TAIL domain from the macaque APOBEC3H protein causes it to mis-localize to the nucleus (Fig 21C; left). Further, I find that the TAIL domain of APOBEC3H is sufficient for cytoplasmic localization of the protein as addition of the macaque TAIL to the C-terminus of the human APOBEC3H protein results in its relocalization to the cytoplasm (Fig 21C; right). Finally, these results were quantitated to determine the expression levels in the cytoplasmic versus nuclear compartments. Proteins with equivalent cytoplasmic to nuclear expression would show a ratio of 1 in this assay.

Figure 21 – TAIL+ APOBEC3H Proteins Localize to the Cytoplasm

Panel A: The C-terminal regions of human APOBEC1 as well as primate APOBEC3H homologs were aligned using ClustalX. The consensus Nuclear Export Sequence (NES), Lx(2,3)[LIVFM]x(2,3)Lx[LI] – brackets indicate possible alternative residues, is completely conserved in all primate APOBEC3H homologs (highlighted in blue). Panel B: Constructs expressing HA-tagged human and non-human primate APOBEC3H proteins were transiently transfected into HeLa cells, fixed and HA-tagged proteins detected using an anti-HA antibody, followed with a TexasRed-conjugated secondary (in Red). Cells were co-stained with dapi to show the nucleus (blue). Panel C: The TAIL-less macaque mutant and the human APOBEC3H with the macaque TAIL sequence added on were introduced into HeLa cells and detected by immunoflourescence. Panel D: Relative ratios of cytoplasmic to nuclear expression of human and macaque proteins were quantitated. 10 cells were evaluated/sample and the average intensity of cytoplasmic to nuclear expression is shown with standard deviation.


A
APOBEC1 WVRYVEYCIILGLPPCLNIL
HUMAN *
CHIMP *
GORILLA SVDVENGTRSIQTGPVTPSSSRSNSR*
ORANG SVDVENGTRSIQTGPVSSSLSRSNSR*
GIBBON SVDVENGTRSIQTGPVSPSLSRGNSR*
MACAQUE SVDVENGTRSIQTGPVTPSSSIRNSR*
SOOTY SVDVENGTRSIQTGPVTPSSSIRNSR*
AGM SVDVENGTRNIQTGPVTPASSIRNSR*
B
APOBEC3G
macaque
orangutan
gorilla
TAIL+
chimp
human
TAIL-less
C
Macaque -TAIL
Human +TAIL
dapi
ha
dapi
ha
D
Cytoplasmic: Nuclear Expression Ratio
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Cytoplasmic = Nuclear
Human
Macaque
Macaque -TAIL
Human +TAIL

The quantitation further highlights the importance of the Tail domain for cytoplasmic localization of APOBEC3H as APOBEC3H proteins with the Tail domain show Cytoplasmic:Nuclear expression ratios greater than 1 while the Tail-less proteins have ratios less than 1 (Fig 21D). Together, these results suggest that conserved elements in the APOBEC3H C-terminus direct the protein to the cytoplasmic compartment of the cell.

Primate APOBEC3H Homologs Show Variable Stability and Evidence of Variable Post-Translational Modification

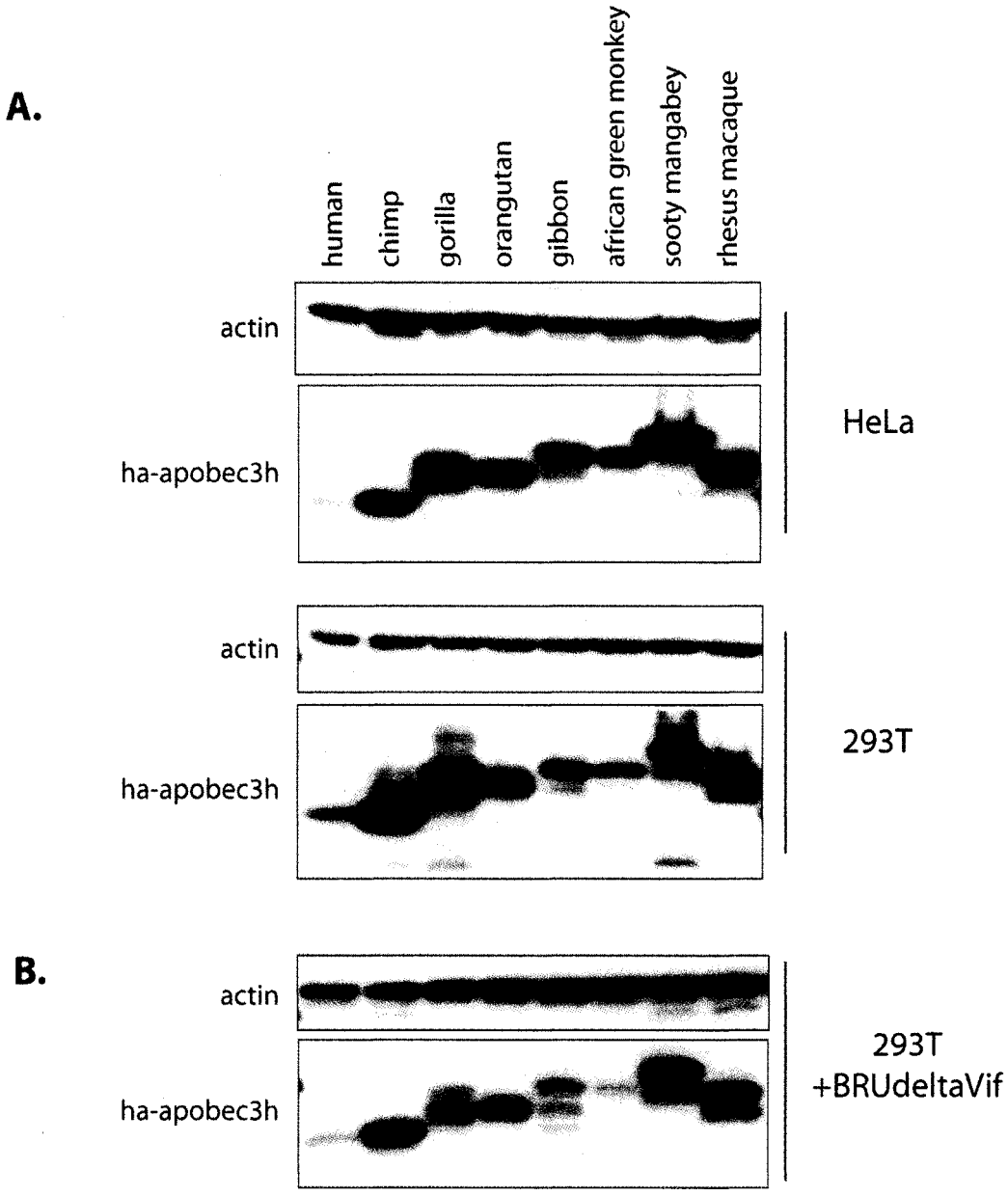
Given that the APOBEC3H Tail domain does not play a role in determining the steady-state expression levels of the APOBEC3H protein, I asked if the molecular determinants of expression could be mapped to other domains or residues of the APOBEC3H protein. Given the large degree of diversification that has occurred since humans and macaques last shared a common ancestor (20+MYA), mapping the residues responsible for the large difference in their steady-state expression is difficult. Therefore, I took an evolutionary approach to ask if APOBEC3H proteins from other Hominoid species are stable. Identification of a more-closely related (and therefore less divergent) APOBEC3H protein that shows high steady-state expression would allow me to map the residues that determine the instability of human APOBEC3H.

The APOBEC3H proteins from chimpanzee, gorilla, orangutan and gibbon (in addition to the Old World monkey homologs from the macaque, sooty mangabey and african green monkey) were expressed in transient transfections from identical expression vectors. The relative steady-state expression of the encoded proteins

were then evaluated by Western Blot. Relative expression levels were evaluated in both 293T and HeLa cells with similar results.

Most hominoid APOBEC3H proteins show variable levels of steady state expression, while the chimpanzee homolog shows a high level of expression similar to the rhesus macaque (Fig 22A). Similar to my previous findings of low steady-state expression of human APOBEC3H, african green monkey APOBEC3H is also expressed at relatively low levels (this is most pronounced in lysates derived from virus-producing cells; Fig 22B). Interestingly, several primate APOBEC3H proteins migrate at several molecular weights, suggesting some type of post-translational modification or, perhaps, truncation of the protein (Fig 22A and B: gorilla, gibbon, sooty mangabey or rhesus macaque). In addition, this pattern of multiple bands seems to be exaggerated in the presence of HIV (compare Fig 22A and Fig 22B). Whether or not this observation has any functional consequences has not been explored further. These data suggest that our closest primate relatives, the chimpanzee, have an APOBEC3H protein that is expressed at high levels. Therefore, orthologous antiviral proteins from the human and chimpanzee show dramatically different phenotypes.

Figure 22 – Primate APOBEC3H Homlogs Show Variable Stability and Modification
APOBEC3H cDNAs were cloned by RT-PCR for 5 Hominoid (human, chimpanzee, gorilla, orangutan and gibbon) and 3 Old World monkey (rhesus macaque, sooty mangabey and african green monkey) primate species. Each homolog was expressed from an identical expression vector with an HA tag at the N-terminus by transient transfection, lysates harvested at 48 hours post-transfection and relative levels of protein were evaluated by Western Blot. Panel A: Western blot of lysates produced in two different human cell lines (HeLa – top; 293T – bottom). Expression in either cell line is essentially equivalent. Panel B: Primate APOBEC3H homologs were expressed along with HIVΔ*vif* in 293T cells.


A.
human
chimp
gorilla
orangutan
gibbon
african green monkey
sooty mangabey
rhesus macaque
actin
ha-apobec3h
HeLa
actin
ha-apobec3h
293T
B.
actin
ha-apobec3h
293T
+BRUdeltaVif

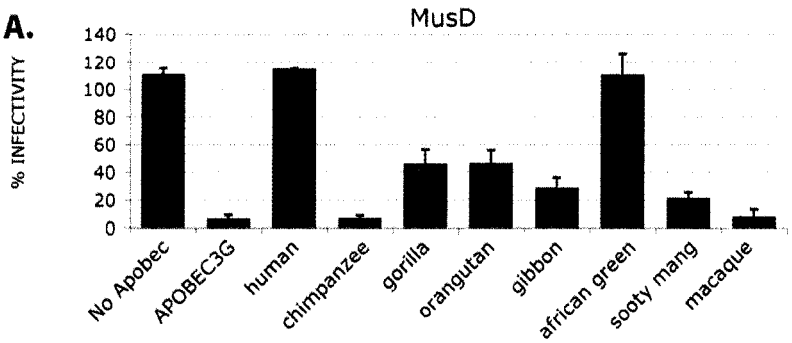
Primate APOBEC3H Proteins Have Variable Antiviral Activity

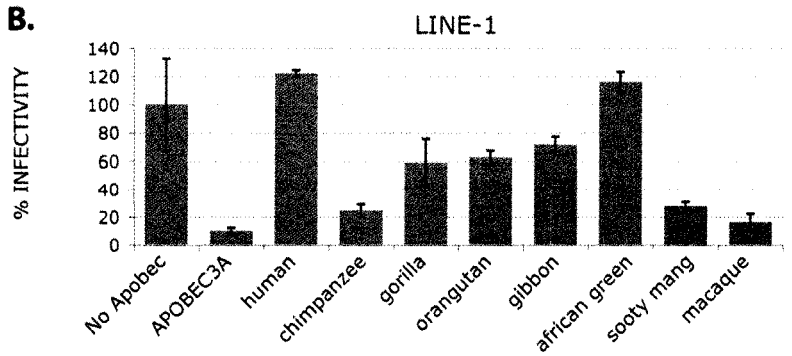
My finding of variable steady-state expression and apparent post-translational modifications of primate APOBEC3H homologs suggested that the function of this protein may have changed significantly during evolution. To ask if the primate APOBEC3H proteins have antiviral activity, they each were tested for their ability to inhibit a panel of retroviral elements representing diverse replication schemes. Replication assays in which no APOBEC expression plasmid was included serves as the negative ("No Apobec") control and APOBEC+ assays are expressed as a the percent infectivity relative to the No Apobec control (100% Infectivity).

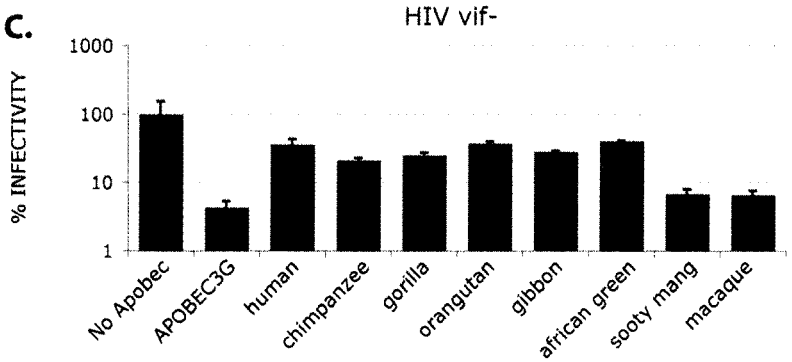
First, each protein was tested for the ability to inhibit the mouse endogenous retroelement, MusD. Human APOBEC3G, which has previously been shown to block MusD replication, was used as a positive control and shows strong (> 10-fold) inhibition of MusD replication (Fig 23A). Both human and african green monkey proteins are unable to affect MusD replication, consistent with their low levels of steady-state expression (Fig 23A). In contrast, inhibition by chimpanzee and macaque APOBEC3H proteins is robust (greater than 10-fold) similar to human APOBEC3G (Fig 23A). The other hominoid APOBEC3H proteins (gorilla, orangutan and gibbon) as well as sooty mangabey APOBEC3H proteins show an intermediate phenotype in which they are able to decrease the extent of MusD replication, but with a potency that is diminished relative to the more potent chimpanzee or macaque APOBEC3H proteins (Fig 23A).

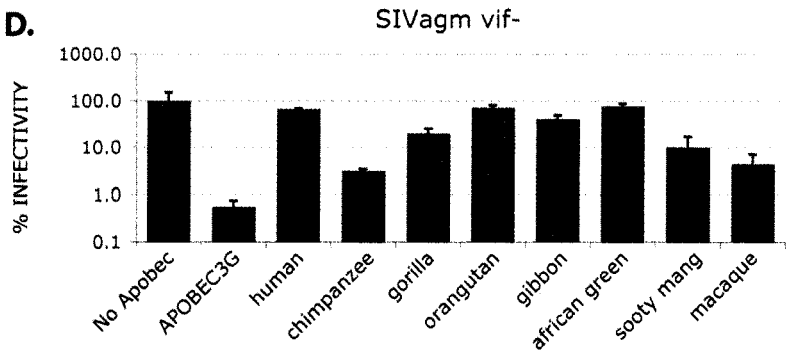
Figure 23 – Antiviral Activity of Primate APOBEC3H Proteins

Each primate APOBEC3H protein was tested for the ability to inhibit the replication of several retroviral elements. Panel A: Inhibition of MusD (mouse endogenous retrovirus) by primate APOBEC3H proteins. Human APOBEC3G (APOBEC3G) is shown as a positive control as it is known to have potent activity against MusD. Panel B: Inhibition of a human LINE-1 element by primate APOBEC3H homologs. Human APOBEC3A is shown as a positive control as it is known to have potent activity against LINE-1 elements (inhibition by primate APOBEC3H homologs relative to APOBEC3A should not be compared as APOBEC3A is expressed from a different expression vector). Panel C: Inhibition of HIVΔ*vif* by primate APOBEC3H homologs. Human APOBEC3G (Apo3G) is shown as a positive control as it is known to potently inhibit HIVΔ*vif*. Panel D: Inhibition of SIVagmΔ*vif* by primate APOBEC3H homologs. Human APOBEC3G (huApo3G) is shown as a positive control as it is known to potenly inhibit SIVagm. Panel E: Assays from Panels A – D were quantitated for the Fold Inhibition of each retroviral element by each primate APOBEC3H homolog. A value of 1 corresponds to zero inhibition relative to the No APOBEC control. APOBEC3H proteins with some inhibitor activity are marked with one asterisk (*) and those with potent inhibitory activity are marked with three asterisks (***).









E.

Fold Inhibition

	LINE-1	SIVagm	HIV-1	MusD	
human	1	1	3	1	
chimp					***
gorilla	2	5	4	2	*
orang	2	1	3	2	*
gibbon	1	3	4	4	*
agm	1	1	2	1	
sooty					***
macaque					***

Next, similar assays were carried out with more relevant primate pathogens, the human LINE-1 element as well as the primate lentiviruses HIV and SIVagm (SIV from african green monkeys). These results largely mirror the effects seen for inhibition of MusD as neither human nor african green monkey APOBEC3H proteins are able to block the replication of any of these retroviral elements (Fig 23A, B, C & D). In contrast, macaque and chimpanzee APOBEC3H proteins show potent inhibition of all the retroviral elements, with the exception of chimpanzee APOBEC3H's lack of potent inhibition of HIV (Fig 23C). There is some specificity to APOBEC3H-mediated inhibition as chimpanzee APOBEC3H is able to block the replication of most but not all the retroviral elements tested here (compare inhibition of the related primate lentiviruses HIV and SIVagm by chimpanzee APOBEC3H in Figure 23C and D). Similar to the results with MusD, the other hominoid APOBEC3H proteins (gorilla, orangutan and gorilla) show only intermediate inhibition of LINE-1 elements (Fig 23B) or either primate lentivirus (Fig 23 C & D). In contrast to the intermediate inhibition of MusD, sooty mangabey APOBEC3H shows potent inhibition of LINE-1 elements as well as both lentiviruses (Fig 23 B, C & D). The relative antiviral activities of all the primate APOBEC3H proteins are summarized in Fig 23E as the fold inhibition by each homolog.

In summary, I find chimpanzee, macaque and sooty mangabey APOBEC3H proteins to be generally potent antiviral effectors (Fig 23E in bright pink) while human and african green monkey proteins do not show significant inhibition of any of the retroviral elements tested here (Fig 23E in grey). The other hominoid APOBEC3H proteins (gorilla, orangutan and gibbon) show an intermediate phenotype (Fig 23E in light pink) as they show some activity against several retroviral elements, but this

activity is always relatively limited compared to more potent APOBEC3H proteins from chimpanzees, sooty mangabeys and macaques.

DISCUSSION

Here I describe the consequences of the rapid evolution of the APOBEC3H coding sequence in primates. I find that potent antiviral activity is conserved in the sooty mangabey and chimpanzee, similar to that I originally described for the rhesus macaque. In contrast, I have uncovered another example of a loss of steady-state expression and antiviral activity in the primate lineage in the african green monkey (agm). Similar to human APOBEC3H, the agm APOBEC3H homolog is poorly expressed at steady state and unable to inhibit any of the retroviral elements tested in my assays. The phenotype for the other Hominoid species (gorilla, orangutan and gibbon) is best described as intermediate as these proteins are readily detectable by Western blot and show a small ability to block retroviral replication in several assays although this activity is not nearly as robust as that seen for other APOBEC3H homologs (rhesus macaque, sooty mangabey and chimpanzee).

It is important to note that APOBEC3H sequences from all hominoids contain the 27-residue C-terminal domain that is present in Macaque APOBEC3H except humans, chimpanzees and bonobos (the bonobo sequence is identical to chimpanzee and, therefore, not included in our analysis). However, since the chimpanzee APOBEC3H protein is expressed at high steady-state levels, the determinants for higher steady-state expression of human APOBEC3H do not correlate with the loss of the C-terminal domain found in most primate APOBEC3H homologs, but is specific to differences between the human and chimpanzee versions

of this gene. Thus, the stability of APOBEC3H was lost sometime after human-chimpanzee divergence.

Loss of the Tail domain in the human/chimpanzee lineage may have allowed for the evolution of a new function of APOBEC3H in humans and chimpanzees. This is particularly intriguing as I found the other hominoid APOBEC3H proteins (gorilla, orangutan and gibbon) to have an intermediate phenotype. While their steady-state expression level as determined by Western Blot was relatively comparable to that of the macaque, sooty mangabey or chimpanzee proteins, they all showed only small inhibitory activity against any of the viruses tested here. Therefore, APOBEC3H appears to have lost its potent antiviral activity in each of these lineages. It seems that the APOBEC3H protein may be obsolete at least in Hominoid species, and therefore may have been co-opted for a different function during the evolution of the human/chimpanzee ancestor. For example, if most APOBEC3H proteins shuttle between the nucleus and cytoplasm analogous to APOBEC1 and AID (this model is supported by our finding that the Tail domain controls cytoplasmic localization), the loss of cytoplasmic localization that occurred with the loss of the Tail domain in the human/chimpanzee ancestor may have allowed for a new or altered function of APOBEC3H in these primates. We have not, however, detected any major differences in antiviral activity between chimpanzee and macaque APOBEC3H proteins, suggesting that the change in function may be subtle or may involve a phenotype other than antiviral activity.

One possibility that cannot be ruled out is that there may in fact be multiple alleles of APOBEC3H circulating in the various primate populations or even duplications of the APOBEC3H coding sequence in some primate lineages. I am

limited here in my analyses as I've cloned and tested only a single version of APOBEC3H from each primate. A more exhaustive analysis of coding sequence variation from several primate species may reveal that duplications or functional polymorphisms do exist at least in some primates.

In summary, the results presented in this chapter highlight the extent of functional diversification of APOBEC3H that has occurred during primate evolution. As we will see in the next chapter, the function of APOBEC3H has changed dramatically even in comparisons among closely related primates such as chimpanzees and humans.

Chapter Five:

ANTIVIRAL ACTIVITY OF APOBEC3H WAS LOST TWICE DURING RECENT HUMAN EVOLUTION

BACKGROUND

The function of APOBEC3H proteins has changed dramatically throughout the evolution of primates. These changes include truncation of a domain required for cytoplasmic localization of the protein, significant changes in the apparent molecular weights of the proteins (perhaps due to differential post-translational modifications), changes in steady-state expression and, ultimately, changes in antiviral activity. The work described in the previous chapter showed that chimpanzee APOBEC3H protein is well expressed at steady state and capable of efficient inhibition of a range of retroviral elements. This is in stark contrast to my finding of low steady state expression and a complete lack of antiviral activity of the human APOBEC3H homolog. Such examples of homologous proteins from humans and chimpanzees, our closest primate relatives, with distinct phenotypes are rare and warranted deeper investigation.

Here, I examine the evolution of the function of APOBEC3H specifically since humans and chimpanzees last shared a common ancestor. I find that a protein representing the hypothetical ancestral sequence of humans and chimpanzees is stable and capable of inhibiting a wide range of retroviral elements. Further, a sequence representing the hypothetical human ancestor APOBEC3H protein is also stable and has potent antiviral activity. Therefore, the loss of APOBEC3H function is specific to the human lineage. I map this loss of function to two independent mutations that have risen to high frequencies in the human population, but,

nonetheless, remain polymorphic. An analysis of APOBEC3H coding sequence reveals a significant subset of APOBEC3H alleles that lack either inactivating mutation and, therefore, are stable and able to block retroviral replication. This stable human APOBEC3H protein is capable of blocking the replication of HIV but is sensitive to the viral, APOBEC-blocking Vif protein. Further, this allele blocks the replication of endogenous retroelements, such as LINE-1 elements. Therefore, these data imply that active APOBEC3H alleles do exist in the human population and may contribute to altered susceptibilities of individuals to HIV, LINE-1 or Alu replication.

RESULTS

Human but not Chimpanzee APOBEC3H Proteins Are Unstable

Human and chimpanzee APOBEC3H proteins show significantly different levels of steady state expression. Given that relatively few changes distinguish the human and chimpanzee proteins, I asked if a second human APOBEC3H allele may contain the residues required for stable expression. Therefore, I cloned and tested the expression of a second human allele of APOBEC3H that differs in three amino acids (R18L, G105R and K121D) as well as a single amino acid deletion (N15) from the original human allele (amino acid numbering is maintained based on the original clone). The stability of the second human APOBEC3H protein (human_2) is similar to that of the original human APOBEC3H protein (human_1) and is significantly less than that of chimpanzee APOBEC3H (Fig 24A).

The difference in steady-state levels of human and chimpanzee APOBEC3H proteins could be due to differences in the turnover rates of the proteins or due to effects of the primary sequence on translation. To test protein stability directly, the

half-life of both human and chimpanzee APOBEC3H proteins were measured by a pulse-chase ^{35}S metabolic labeling experiment with a 6-hour time-course (Fig 24B). I found that both human APOBEC3H proteins turn over quickly ($t^{1/2}$ < 30 minutes) while the chimpanzee APOBEC3H protein turns over much more slowly ($t^{1/2}$ > 6 hours) (Fig 24C). Effects on the stability or translation of the mRNAs can be excluded as initial labeling during the pulse resulted in similar levels of labeled human and chimpanzee protein (Fig 24B, 0 hr lanes), suggesting that amounts of human and chimpanzee APOBEC3H proteins synthesized are equivalent. Therefore, the low levels of steady-state expression of both human APOBEC3H proteins are due to their rapid turnover.

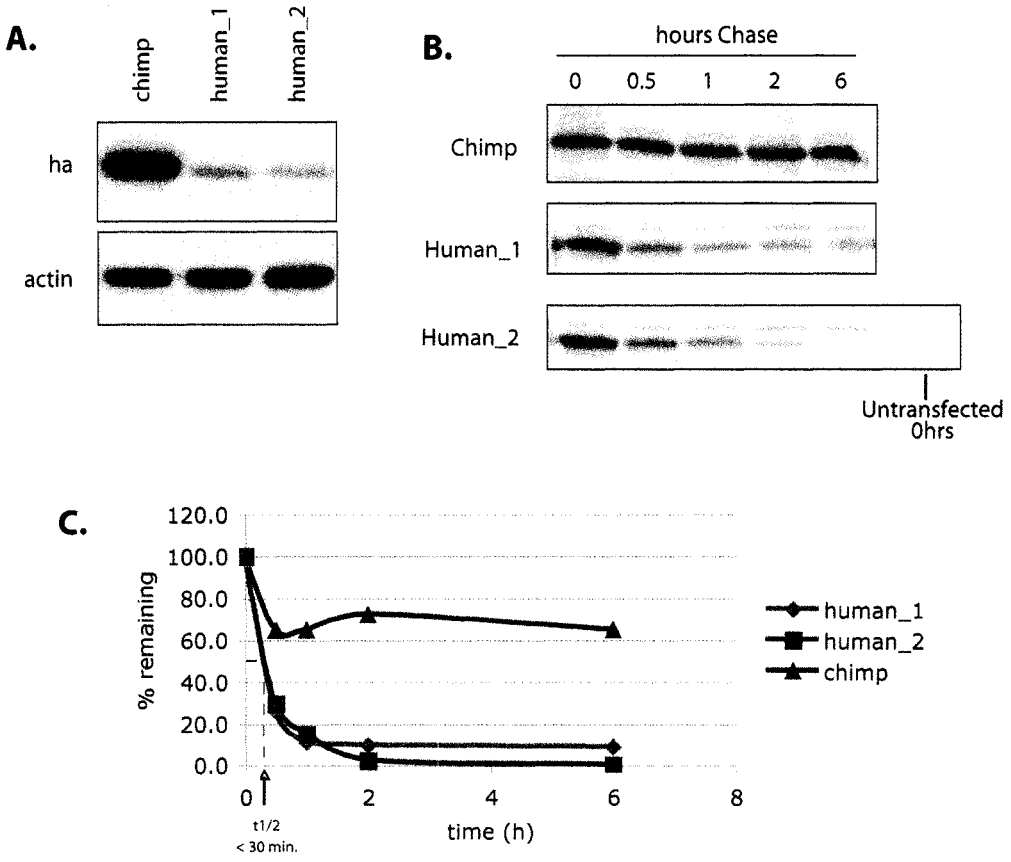


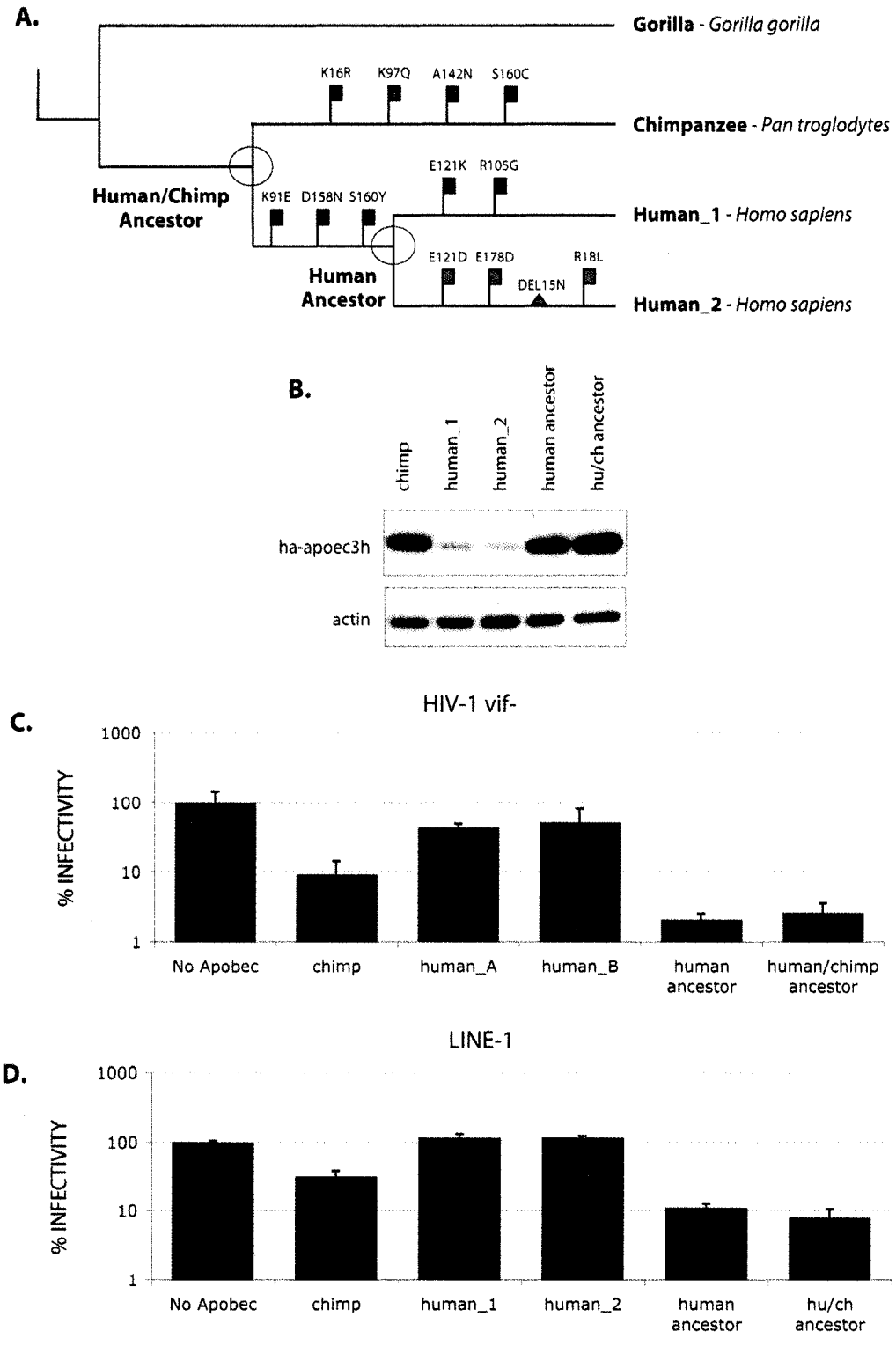
Figure 24 – Human APOBEC3H Proteins Have a Short Half-Life

Chimpanzee APOBEC3H as well as two human APOBEC3H alleles were expressed from identical expression vectors and used for Western Blot and Pulse-Chase Analyses. Panel A: Human and chimpanzee proteins were expressed in transient transfections in 293T cells and steady-state levels of protein were compared by Western Blot (anti-HA; anti-Actin probe of the same blot was used to demonstrate equivalent loading of total protein per well). Panel B: Human and chimpanzee APOBEC3H proteins were transiently transfected into 293T cells and used for a ^{35}S metabolic pulse-chase labeling analysis. Each sample was pulsed with ^{35}S-labeled Methionine and Cysteine amino acids for 30 minutes, followed by a chase with excess cold Methionine and Cysteine. Cells were lysed at several time points following the pulse: 0, 0.5, 1, 2 and 6 hours. Radiolabeled APOBEC3H proteins were immunoprecipatated using the HA tag and used for SDS/PAGE. Radiolabeled proteins were imaged on a phosphorimager. Panel C: Radiolabeled human and chimpanzee proteins were quantitated using ImageQuant software and the approximate half-life determined by assessing the time at which half the protein had been degraded.

Loss of APOBEC3H Function is Specific to the Human Lineage

Other primates, such as the rhesus macaque, have active antiviral APOBEC3H alleles. Therefore, I hypothesized that the antiviral activity of APOBEC3H was maintained in the chimpanzee lineage and lost specifically in the human lineage. To test this hypothesis, I asked if a reconstructed APOBEC3H protein corresponding to the common ancestor of chimpanzees and humans is stable and capable of retroviral inhibition. The protein sequence of the Most Recent Common Ancestor of human and chimpanzee APOBEC3H (Hu/Ch Ancestor) was predicted *in silico* using the Gorilla sequence as an outgroup. Using the human/chimpanzee ancestor sequence as a reference, 4 amino-acid altering or replacement changes can be mapped to have occurred in the chimpanzee lineage since human/chimpanzee speciation (Fig 25A in purple: K16R, K97Q, A142N and S160C). During the same evolutionary time frame, 3 replacement changes have been fixed in the human lineage (Fig 25A in blue: K91E, D158N and S160Y), while 4 polymorphic replacement changes (E121D/K, R105G, E178D and R18L) and a deletion (N15) have arisen in the human lineage (Fig 25A in green/pink). The Human/Chimp Ancestor cDNA was reconstructed *in vitro* by site-directed mutagenesis and tested for stability and antiviral activity.

Figure 25 – Evolution of Impaired APOBEC3H in the Human Lineage
Using the gorilla APOBEC3H sequence as an outgroup, the hypothetical ancestral APOBEC3H protein sequence was determined at two nodes: 1) the node representing the human/chimpanzee ancestor and 2) the node representing a hypothetical human ancestral sequence. Panel A: A cladogram depicting the amino acid changes that can be inferred to have occurred using the gorilla APOBEC3H sequence as an outgroup. In purple, four amino acid changes are likely to have occurred in chimpanzees following human/chimpanzee speciation (K16R, K97Q, A142N and S160C). Three changes (shown in blue) have occurred in the human lineage that have been completely fixed (shared by the entire human population) (K91E, D158N and S160Y). Finally, 6 amino acid changes are polymorphic in the human population (not fixed in humans) and are shown in pink (human_1; E121K and R105G) and green (human_2; E121D, E178D, deletion of N15 and R18L) respectively. The nodes used to reconstruct the human/chimp ancestor and human ancestor APOBEC3H proteins are marked. Panel B: Human, chimpanzee and reconstructed ancestral APOBEC3H proteins were expressed in 293T cells, lysates collected and used for Western blot analysis to determine relative expression levels. APOBEC3H proteins were detected with an HA antibody and the same blots were probed with an actin antibody to show equal loading of all wells. Panel C: Human, chimpanzee and ancestral APOBEC3H proteins were evaluated for their ability to inhibit HIVΔ*vif*. Panel D: Human, chimpanzee and ancestral proteins were tested for their ability to block LINE-1 replication.


A.
Gorilla - Gorilla gorilla
K16R
K97Q
A142N
S160C
Chimpanzee - Pan troglodytes
E121K
R105G
Human/Chimp Ancestor
K91E
D158N
S160Y
Human_1 - Homo sapiens
E121D
E178D
R18L
DEL15N
Human Ancestor
Human_2 - Homo sapiens
B.
chimp
human_1
human_2
human ancestor
hu/ch ancestor
ha-apoec3h
actin
C.
HIV-1 vif-
% INFECTIVITY
1000
100
10
1
No Apobec
chimp
human_A
human_B
human ancestor
human/chimp ancestor
D.
LINE-1
% INFECTIVITY
1000
100
10
1
No Apobec
chimp
human_1
human_2
human ancestor
hu/ch ancestor

Consistent with our hypothesis that APOBEC3H activity was maintained rather than gained in the chimpanzee lineage, human/chimpanzee ancestor APOBEC3H is stable (Fig 25B) and inhibits the replication of HIV-1 greater than 10-fold (Fig 25C). The hu/ch ancestor APOBEC3H protein is also able to block the replication of the human LINE-1 element (Fig 25D). Therefore, the evolution of APOBEC3H proteins with a high turnover rate and no antiviral activity occurred after human/chimpanzee speciation through the accumulation of inactivating mutation(s) that occurred specifically in the human lineage.

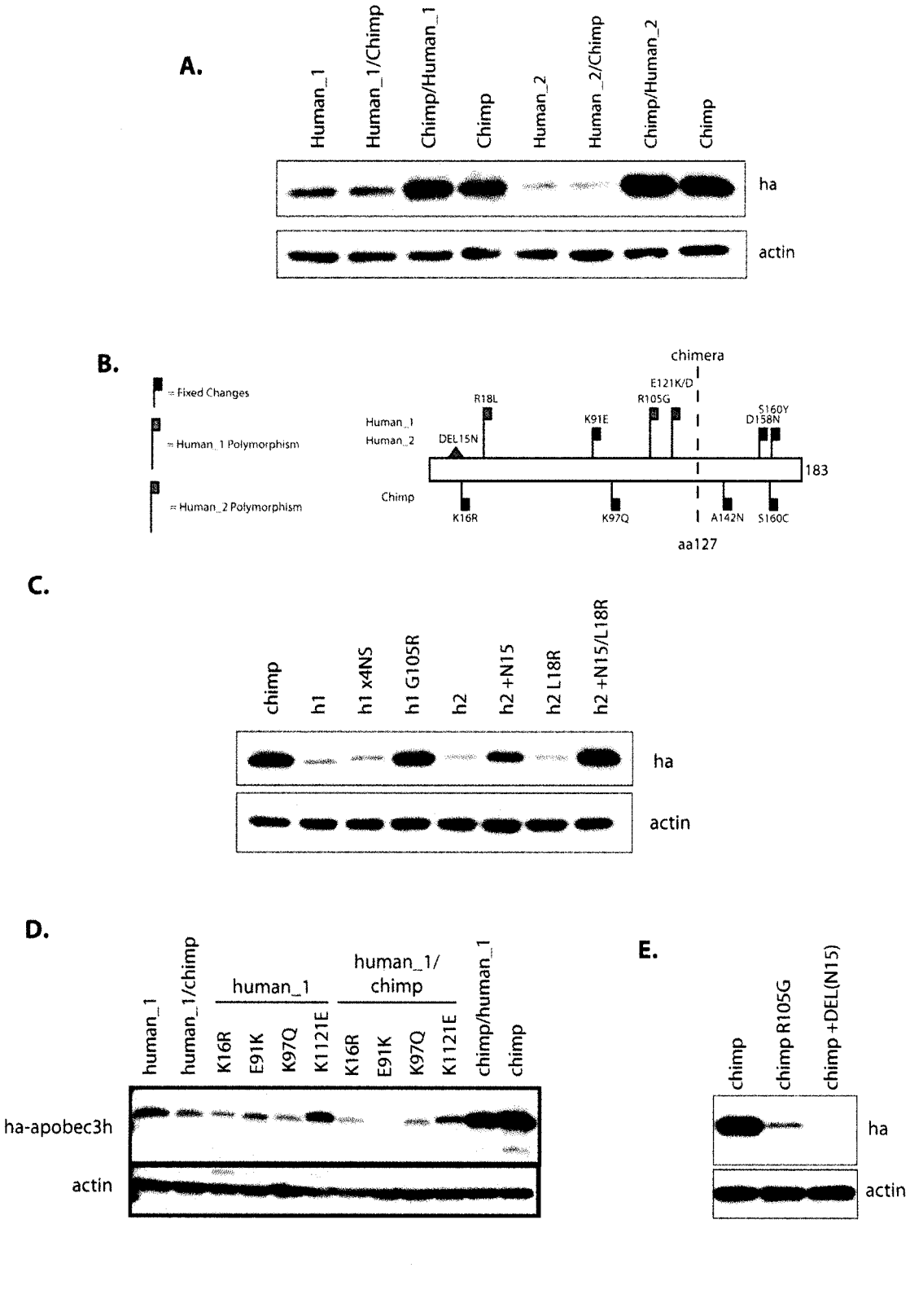
The Human Ancestor APOBEC3H is Stable and Active

Defining changes that are fixed versus polymorphic in humans allowed me to predict a hypothetical human ancestral sequence (Human Ancestor) that I then reconstructed *in vitro*. The Human Ancestor protein contains all 3 replacement changes that have been fixed in the human population (Fig 25A in blue: K91E, D158N and S160Y) but none of the polymorphic changes (delN15, R18L, R105G, E121K, E121D or E178D). The Human Ancestor protein is stable and, similar to the hu/ch ancestor protein, is able to potently inhibit HIV-1 and LINE-1 replication greater than 10-fold (Fig 25C & D). These data suggest that the loss of APOBEC3H stability and antiviral activity happened recently during human evolution and is not fixed in the human population.

The loss of stability of Human APOBEC3H occurred at least twice during human evolution

To ask which residues control the turnover rate of these APOBEC3H proteins, chimeric proteins were generated by combining human and chimpanzee APOBEC3H cDNAs, creating human_1/chimpanzee, chimpanzee/human_1, human_2/chimpanzee and chimpanzee/human_2 chimeras (see Fig 26B for position of the chimeras). Relative stability of chimeric human/chimpanzee proteins were then determined by Western Blot. The unstable phenotype of the human APOBEC3H proteins map to the N-terminal region of human APOBEC3H as both human/chimpanzee chimeras are unstable (Fig 26A). Human and chimpanzee proteins differ in this N-terminal region at 5 sites for human_1 (compare pink and blue human changes to blue chimp changes in Fig 26B) and 6 sites for human_2 (compare green and blue human changes to blue chimp changes in Fig 26B). To ask if mutating one or a combination of these residues may restore stable expression of human APOBEC3H the 5 chimpanzee-specific changes present in the N-terminal region of APOBEC3H were introduced into the human_1 cDNA. I found that the single G105R change is sufficient to restore the stability of human APOBEC3H while a cDNA containing all 4 other changes (x4NS = K16R, E91K, K97Q and K121E) shows very little change in stability relative to the wild-type human_1 protein (Fig 26C). Each amino acid was also tested individually and shown to have little to no effect on protein stability (Fig 26D).

Figure 26 – Instability of Human APOBEC3H Proteins Is Due to Independent Mutations
Panel A: Chimeric human (human_1 and human_2) and chimpanzee APOBEC3H proteins were constructed and evaluated for steady-state expression by Western blot. Panel B: Fixed (blue flags) and Polymorphic (Pink and Green flags) changes occurring in humans and chimpanzees are shown on a schematic of the APOBEC3H protein. The position of the chimeras is shown at amino acid 127. Panel C: Western blot analysis of wild type and mutant human APOBEC3H proteins. Four non-synonymous (NS) changes between the human_1 protein and the chimpanzee protein occurring the N-terminus of APOBEC3H were introduced into human_1 (h1 x4NS; K16R, E91K, K97Q and K121E) and shown to have no impact on the stability of the protein. In contrast, the single G015R change (h1 G105R) is sufficient to restore stable expression of human_1. Similarly, re-insertion of the deleted Asn (N) residue at position 15 in the human_2 protein give a significant rescue of human_2 stability that is further enhanced by the L18R change. Panel D: The four non-synonymous (NS) changes introduced into human_1 previously were also evaluated one-by-one for their ability to increase the stability of either the wild type human_1 or chimeric human_1/chimp protein. Panel E: The destabilizing R105G or N15 deletion were introduced into the chimpanzee APOBEC3H protein and evaluated for their effect on protein stability by Western blot.


A.
Human_1
Human_1/Chimp
Chimp/Human_1
Chimp
Human_2
Human_2/Chimp
Chimp/Human_2
Chimp
ha
actin
B.
= Fixed Changes
= Human_1 Polymorphism
= Human_2 Polymorphism
chimera
E121K/D
R18L
R105G
S160Y
D158N
Human_1
Human_2
DEL15N
K91E
183
Chimp
K16R
K97Q
A142N
S160C
aa127
C.
chimp
h1
h1 x4NS
h1 G105R
h2
h2 +N15
h2 L18R
h2 +N15/L18R
ha
actin
D.
human_1
human_1/chimp
human_1
human_1/ chimp
chimp/human_1
chimp
K16R
E91K
K97Q
K1121E
K16R
E91K
K97Q
K1121E
ha-apobec3h
actin
E.
chimp
chimp R105G
chimp +DEL(N15)
ha
actin

Remarkably, the G105R change that confers stability on the human_1 protein is present in the unstable second human allele (human_2). This suggests, therefore, that the human_2 APOBEC3H protein is unstable due to an independent change. To map the residue(s) controlling the stability of human_2 I introduced the chimpanzee-specific changes at the sites in human_2 that were not already made initially in human_1 (+N15, L18R and D121E). Re-introduction of the Asn residue deleted at position 15 shows a significant increase in stability (Fig 26C). However, levels of human_2 protein are not equivalent to that of chimpanzee, suggesting that either the L18R or D121E can also contribute to the protein's turnover. Therefore I tested a human_2 protein in which both the +N15 and L18R changes were made. The double mutation shows stability similar to that of the human_1 G105R mutant and the native chimpanzee protein (Fig 26C), suggesting that the arginine at position 18 is required but not sufficient for stability of human APOBEC3H. The N15 deletion, however, is sufficient to destabilize the human APOBEC3H protein regardless of the residue found at position 18 and, therefore, can be considered the dominant de-stabilizing mutation.

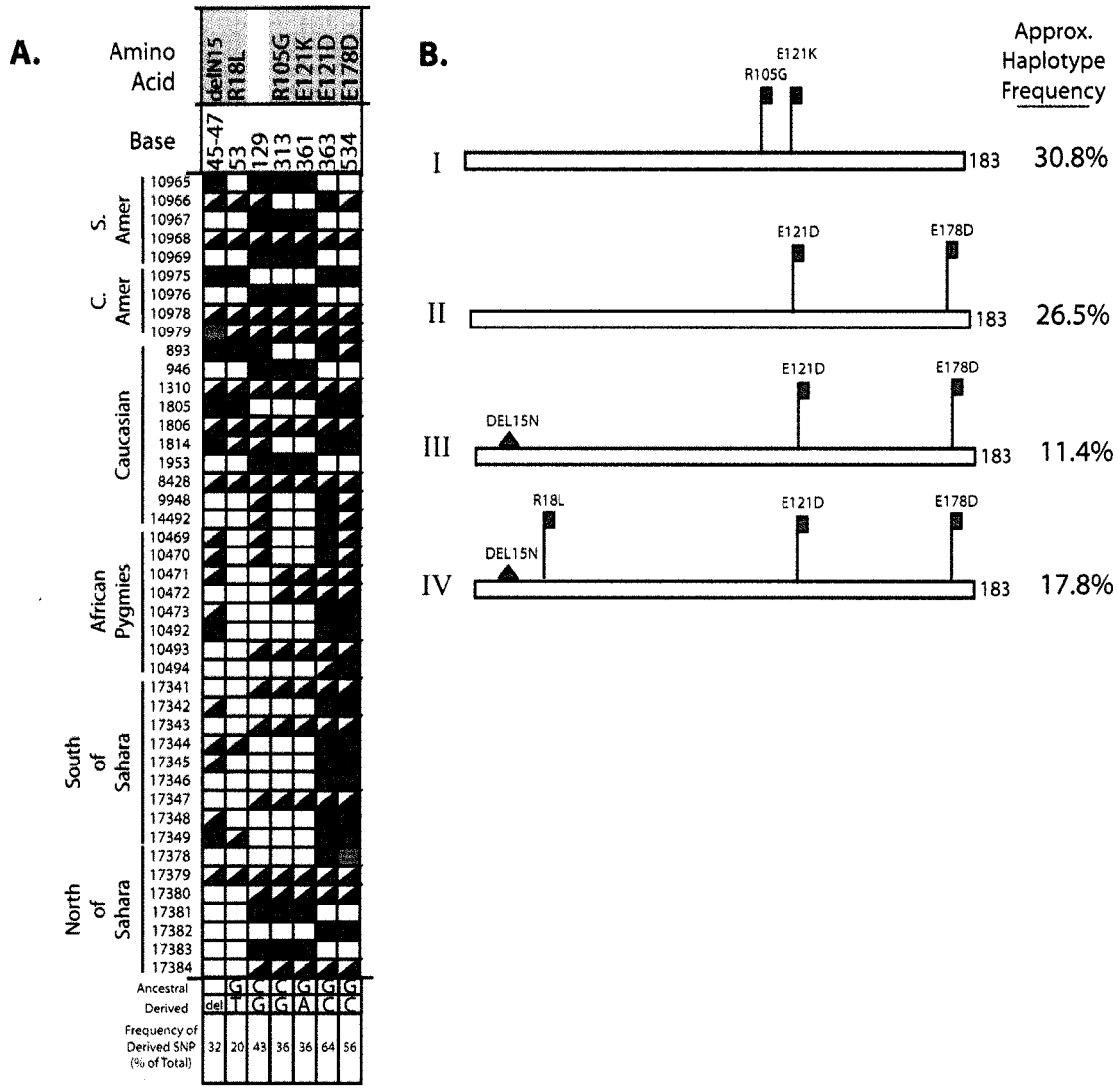
The importance of both N15 as well as the R105G mutation in determining the turnover of APOBEC3H was then confirmed by asking if these changes are sufficient to destabilize the chimpanzee APOBEC3H protein. Either deletion of N15 or introducing the R015G mutation are sufficient to decrease levels of chimpanzee APOBEC3H protein dramatically, suggesting that either change is sufficient to increase the turnover of APOBEC3H protein (Fig 26D). These data indicates that the human proteins are unstable due to different mutations that are independently sufficient to destabilize APOBEC3H.

Active APOBEC3H Alleles Are Present in the Human Population

Given that single changes at polymorphic positions in the human APOBEC3H proteins restore stable expression and antiretroviral activity, I hypothesized that stable and active APOBEC3H alleles might be found in the human population at a significant frequency. Specifically, alleles with an intact N15 as well as the ancestral 105R residue would be expected to be stable and active. Therefore, we analyzed the polymorphisms present in the APOBEC3H coding sequence among a panel of individuals from several world populations, including indigenous Latin American, African and Caucasian individuals (Fig 27A). We screened a set of 86 chromosomes that allowed me to calculate the frequencies of the 6 Single Nucleotide Polymorphisms (SNPs), 5 Replacement and 1 Synonymous, as well as the single codon deletion (N15) that are polymorphic within the APOBEC3H coding sequence. The ancestral coding sequence (Fig 27A; white) is used as a reference and all polymorphisms are listed as the derived sequence relative to the ancestral (Fig 27A; green – delN15, R18L, T43T, R105G, E121K, E121D and E178D). Of note, two nucleotide polymorphisms occur at the first and third positions of the same codon, resulting in a single polymorphic amino acid (121K or 121D).

Figure 27 – A Survey of Polymorphisms and Haplotypes of APOBEC3H in the Human Population

Panel A; The APOBEC3H ORF from Exons 2, 3 and 4 were sequenced for 43 individuals from several world populations (South American, Central American, Caucasian, African Pygmies, South of the Sahara and North of the Sahara; Coriell). Ancestral and derived states are shown at the bottom of the table. Green shading indicates the presence of the derived state, relative to the human/chimpanzee ancestor, at a position while white indicates presence of the ancestral nucleotide at that position. A filled in square indicates homozygosity; half-shading indicates heterozygosity. The base number at which the polymorphism occurs is shown along with the resulting amino acid change (delN15, R18L, R105G, E121K, E121D and E178D). The frequency of each polymorphism, as a percent of the total, is tabulated at the bottom of each column. Panel B: The polymorphism data from Panel A was used to infer haplotypes using PHASE2.1 software. The four most frequent haplotypes identified in the panel are shown as Haplotypes I, II, III and IV and are present in this panel at predicted frequencies of ~30.8%, 26.5%, 11.4% and 17.8%, respectively.


A.
Amino Acid
delN15
R18L
R105G
E121K
E121D
E178D
Base
45-47
53
129
313
361
363
534
S. Amer
C. Amer
Caucasian
African Pygmies
South of Sahara
North of Sahara
10965
10966
10967
10968
10969
10975
10976
10978
10979
893
946
1310
1805
1806
1814
1953
8428
9948
14492
10469
10470
10471
10472
10473
10492
10493
10494
17341
17342
17343
17344
17345
17346
17347
17348
17349
17378
17379
17380
17381
17382
17383
17384
Ancestral
Derived
del
T
G
A
C
G
G
C
C
Frequency of Derived SNP (% of Total)
32
20
43
36
36
64
56
B.
Approx. Haplotype Frequency
E121K
R105G
I
183
30.8%
E121D
E178D
II
183
26.5%
E121D
E178D
DEL15N
III
183
11.4%
R18L
E121D
E178D
DEL15N
IV
183
17.8%

The frequency of polymorphisms ranges from 20% to 64% (= percentage derived allele), with a subset of polymorphisms present at nearly equal frequency in the population as a whole (5 polymorphisms – T43T, R105G, E121K, E121D and E178D at frequencies between 35 and 65%) (Fig 27A – bottom of chart). I then used the SNP data to predict the major haplotypes (sets of polymorphisms that are inherited together) that exist in the human population using PHASE 2.1 software [113, 127]. Four haplotypes are estimated to occur at frequencies >10% with approximate frequencies of 30, 27, 11 and 18% in our sample of several world populations (Fig 27B; Haplotypes I, II, III and IV). Haplotype I corresponds to my cloned human_1 cDNA while human_2 is identical to Haplotype IV but without the E178D change. Importantly, Haplotype II (the second most frequent haplotype in my sample) has an intact N15 residue as well as the Arginine at position 105, suggesting that this allele should be stable and active.

A Human Allele is Stable and Inhibits HIV and LINE-1 Replication

The four most prevalent human haplotypes identified in our panel (Haplotypes I – IV) were constructed *in vitro* and tested for their stability and antiviral activity. Western blot analysis of the 4 human proteins demonstrates that only the N15-containing/R105 allele (haplotype II) is stably expressed to levels similar to that of the active chimpanzee protein (Fig 28A). Next I tested the stable human allele for its ability to inhibit the replication of HIV-1 with and without the APOBEC-blocking Vif protein. The stable Human APOBEC3H allele (HuA3H IV: Haplotype IV) is capable of potent inhibition of HIV replication (greater than 20-fold) and is at least partially sensitive to inhibition by the HIV Vif protein as there is a 10-fold increase in infectivity

in the presence of Vif (Fig 28B; compare white to black bars). In contrast, I see no significant inhibition of HIV by any of the other human haplotypes (Fig 28B).

Next, I tested the four human haplotypes for their ability to inhibit the replication of LINE-1 elements. Similar to the results with HIV, the stable human allele (Haplotype II) is capable of efficient inhibition of LINE-1 replication (Fig 28C). Therefore, a human APOBEC3H allele with antiviral activity against both HIV-1 and LINE-1 elements exists in the human population.

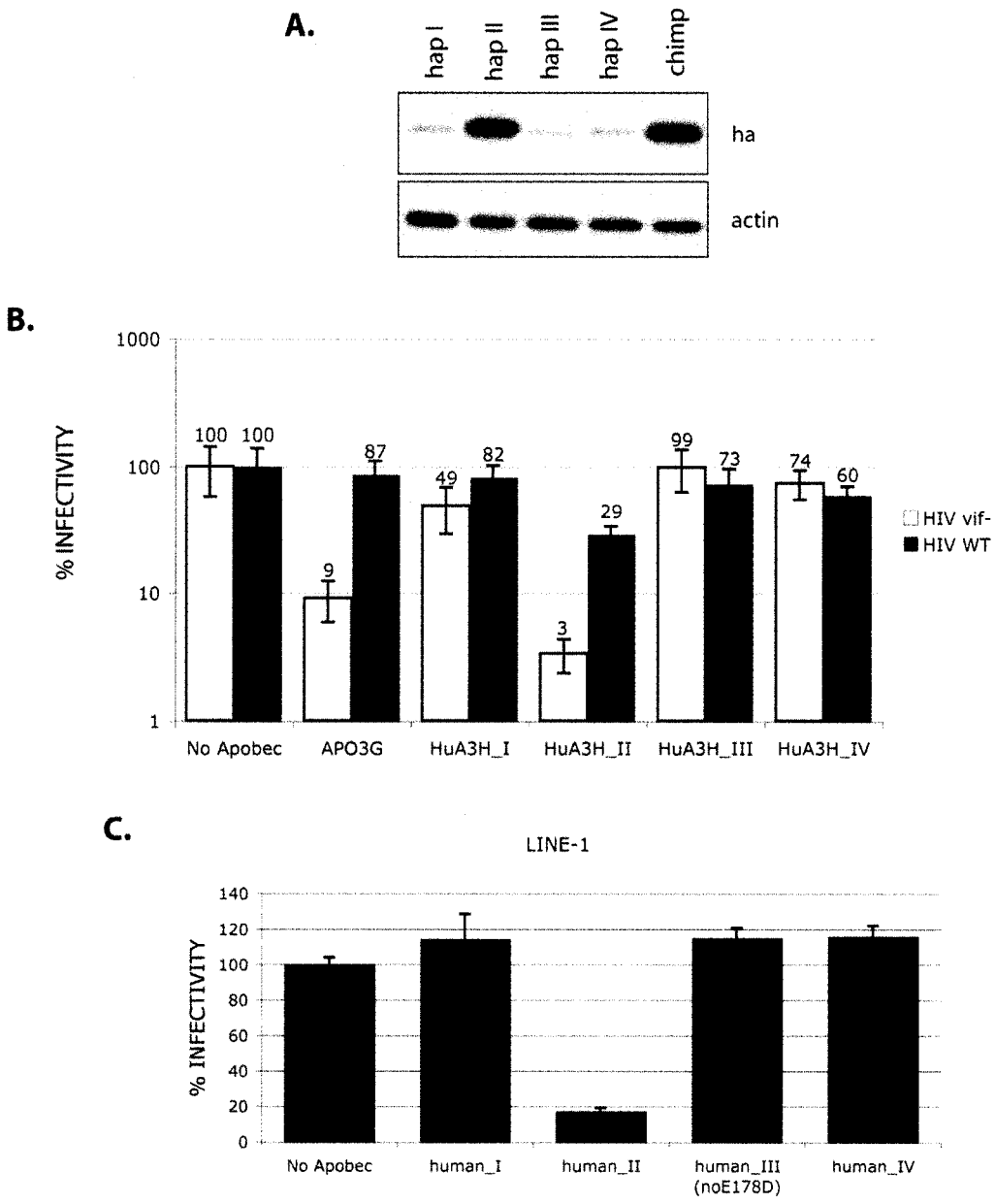


Figure 28 – A Human Allele Is Stable and Blocks Retroviral Replication

Panel A: Each of the most frequent human haplotypes identified in our panel was reconstructed, expressed in transient transfections and evaluated for stability by Western blot. Panel B: The four human Haplotypes (HuA3H_I, HuA3H_II, HuA3H_III and HuA3H_IV) were tested for their ability to block the replication of wild type HIV and HIVΔ*vif* in a single-round infectivity assay. Human APOBEC3G is shown as a control. Panel C: Each of the four human haplotypes was tested for the ability to block the replication of a human LINE-1 element. Note that haplotype III differs at a single residue (it does not contain the E178D SNP) compared to the true haplotype III allele shown elsewhere.

DISCUSSION

In this chapter I have shown that our closest primate relatives, the chimpanzee, encode a stable APOBEC3H protein capable of potent inhibition of several retroviral elements, including HIV-1 and LINE-1 elements. Human APOBEC3H lacks the stable expression and antiviral activity of chimpanzee APOBEC3H, an effect that appears to be due to the rapid turnover of the human proteins. I show that a reconstructed hypothetical human/chimpanzee APOBEC3H ancestor protein is also stable and an active antiretroviral effector, suggesting that the loss of activity of APOBEC3H occurred specifically in the human lineage. Further, the human ancestor APOBEC3H protein is also stable and active. Finally, I was able to map the loss of stability and antiviral activity of the two human APOBEC3H proteins to two distinct mutations (G105R and deletion of N15) that are polymorphic in the human population. Finally, a survey of APOBEC3H coding sequence from several world populations reveals a haplotype of human APOBEC3H that lacks either destabilizing mutation and, therefore, is stable and capable of blocking the replication of both HIV and LINE-1 elements.

Here, I present evidence of the complex history of APOBEC3H evolution in humans in which two independent mutations have arisen during recent human evolution that give rise to an unstable APOBEC3H protein with no detectable antiviral activity. Both mutations occur in distinct regions of APOBEC3H, but act to decrease the half-life of APOBEC3H protein. I have not explored further the mechanism for the loss of stability, but it is possible that these mutations affect the proper folding of nascent APOBEC3H protein. Alternatively, one or both mutations could affect dimerization of APOBEC3H proteins (other APOBEC3 proteins have been shown to

dimerize[128]) or association with other cellular factors that may be required for stable expression. Further, I cannot exclude the possibility that one or both of these nonfunctional alleles could be functional in certain contexts (ie, association with other cellular factors in certain cell types or at certain times). However, this model supposes that whatever factor may be contributing to the instability of human but not chimpanzee APOBEC3H proteins is conserved across the human and other mammalian cell lines that I've examined so far.

I find that chimpanzee APOBEC3H and human/chimpanzee Ancestor APOBEC3H proteins are stable and capable of blocking retroviral replication. This means that a relatively recent loss of function of APOBEC3H occurred after human/chimpanzee speciation. Furthermore, the fact that the reconstructed human Ancestor APOBEC3H allele is highly active suggests that our ancestors possessed an active APOBEC3H allele capable of blocking both HIV-1 and LINE-1 replication. Loss of this active allele through the accumulation of inactivating mutations has left the human population more susceptible to HIV-1 infection. In addition, my finding that the human Ancestor APOBEC3H protein is stable and active suggests that the loss of APOBEC3H activity in humans occurred recently during the evolution of modern humans as the changes conferring the unstable/inactive phenotype are not yet fixed in the human population.

The evolution of non-functional APOBEC3H alleles through two inactivating mutations has important implications for our current-day susceptibility to various retroviral assailants. Modern chimpanzees, host of the modern day pandemic HIV-1 progenitor, possess an active APOBEC3H allele, suggesting that selection pressure exerted on the virus by APOBEC3H may have been significantly reduced after

zoonosis to human hosts. Importantly, we did not find evidence of either inactivating mutation occurring in the chimpanzee lineage among 5 or more sequenced *P.t.verus* individuals (Fig 29). Further, it seems that our human ancestors possessed an APOBEC3H allele with activity against LINE-1 and Alu elements. Subsequent loss of this allele in the human population could lead to increased susceptibility to retrotransposition events.

My finding that both these mutations are polymorphic in the human population suggested that some fraction of the human population possesses APOBEC3H alleles with activity against relevant modern day retroviral assailants, such as HIV and LINE-1 elements. This is consistent with my finding of a stable and active APOBEC3H allele present in ~1/4 of the chromosomes sampled in this study.

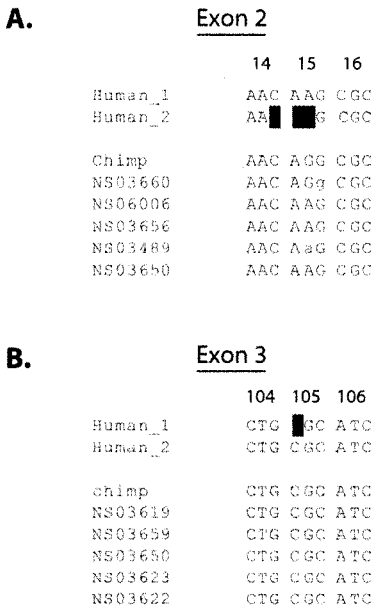


Figure 29 – The N15 Deletion of R105G SNP Were Not Detected in Several Chimpanzees
Panel A: Primers specific for Exon 2 of the APOBEC3H ORF were used to amplify and sequence the region surrounding the N15 deletion detected in the human population. This deletion was not detected in any of the 5 chimpanzees (*P. t. verus* subspecies). Panel B: Exon 3 was amplified and sequenced from 5 chimpanzees (also *P. t. verus*) and no R105G SNPs were detected.

This study represents the first finding of a single nucleotide polymorphism in an APOBEC3 coding sequence that shows an impact on the function of the protein. Previous work had revealed a polymorphic deletion of a portion of the APOBEC3 locus on chromosome 22 that is present at a significant frequency in the human population. This deletion results in functional loss of an APOBEC3B coding sequence[129]. However, identification of single nucleotide polymorphisms affecting the function of APOBEC3 proteins has been limited only to human APOBEC3G. Several groups identified a single polymorphism in the APOBEC3G coding sequence (H186R) that was, at least in one case, significantly associated with CD4 T cell decline and progression to AIDS[130, 131]. However, this mutation was not found to impact the activity of the protein[130]. Instead, several groups have hypothesized that it may be the expression levels of APOBEC3G that affect HIV/AIDS outcomes[132], although the likelihood of mRNA expression effects is controversial[133].

Another possibility is that any genetic association of APOBEC3G with HIV/AIDS outcomes instead reflects an association by Linkage Disequilibrium (LD) with another nearby gene. An intriguing possibility is that APOBEC3H, just distal to APOBEC3G in the APOBEC3 locus on human chromosome 22, may be responsible for the finding of differences in HIV/AIDS outcomes. Further investigation into association of the various APOBEC3H alleles with HIV/AIDS outcomes in cohort studies would be warranted.

My finding that the majority of the human population may encode non-functional APOBEC3H alleles suggests that perhaps non-functional APOBEC3H alleles have been actively selected for in recent human history. Adaptive selection for a non-functional allele in humans is supported by our finding that not one, but two mutations

have been selected for that give rise to unstable, inactive human APOBEC3H proteins. Further, the excess of replacement relative to synonymous mutations (both fixed and polymorphic) is suggestive of adaptive selection rather than neutral variation, although more formal analyses are necessary to demonstrate selection.

If, indeed, non-functional APOBEC3H alleles have been selected for (or at least tolerated) in humans, several scenarios can explain the presence of these high frequency, non-functional alleles. In addition to the possibility of genetic drift, I discuss two alternative possibilities here. First, the APOBEC3H gene could be in the process of pseudogenization in the human lineage. This would give further support to the "less-is-more" hypothesis in which loss of function mutations are actively selective for during a shift of environmental pressures in which loss-of-function mutations may actually be advantageous, particularly if the functional allele presents a significant cost to the host. In the case of APOBEC3H, we can make several arguments for the potential cost to the host. Unlike all other APOBEC3 genes in mammals, duplications of the APOBEC3H domain have never been tolerated as we cannot find evidence of a duplication of this domain in any mammalian genome analyzed so far[65], suggesting that dosage effects may constrain APOBEC3H. Further, APOBEC3H is a nucleic acid-mutating enzyme and presents a risk to the host if it were to act on the wrong targets, such as host mRNA or host genomic DNA[101]. Therefore, there may be significant evolutionary pressure to control levels of APOBEC3H protein in the cell and, in times of decreased pathogen pressure, to down-regulate the APOBEC3H protein. As there has not been complete deletion of the gene, reversion to an active allele is possible if there is another shift in environmental pressure, such as a novel retroviral pathogen that APOBEC3H is called upon to inhibit.

The second scenario in which we might expect to find persistence of a high-frequency detrimental allele is in a case of balancing selection. Balancing selection maintains two or more alleles at a single locus in a population with none of the alleles reaching extinction or fixation[134, 135]. In the case of APOBEC3H, balancing selection could be operating through a type of heterozygote advantage to maintain two alleles with varied pathogen recognition abilities, a possibility that is supported by our finding of ~50% frequency of a charge-change polymorphism at position 121 (K/D) that corresponds to the region of APOBEC3G that have been described to determine HIV packaging and vif-sensitivity[136].

Alternatively, balancing selection may be operating to maintain stable and unstable alleles at high frequencies. Maintenance of both alleles may operate to reduce the risk of decreased genomic integrity in those individuals heterozygous for both types alleles. In the case of APOBEC3H, maintaining a stable and active APOBEC3H protein capable of retroviral inhibition may be balanced with the costs associated with maintaining high doses of this protein in the cell.

Yet another possibility to explain a case of balancing selection of APOBEC3H alleles is that the unstable APOBEC3H protein may have a role whose function depends on the instability of the protein. Unstable APOBEC3H protein serves as a "sink" or "suicide decoy" for virally encoded anti-APOBEC proteins, such as the HIV Vif protein. In this scenario, individuals homozygous for stable and unstable APOBEC3H proteins would be at an advantage as the unstable APOBEC3H protein may clear the way for the stable APOBEC3H protein to mediate antiviral activities. An argument can be made for a function of unstable APOBEC3H protein as I find two, destabilizing mutations that do not disrupt the coding sequence. If absence of

APOBEC3H protein was selectively advantageous we may have expected a STOP codon to have been selected for as mutations giving rise to truncated APOBEC3H proteins have a high probability of occurring, but have not been tolerated or selected for.

In summary, my work demonstrates a double-loss of function of APOBEC3H that occurred during recent human evolution. Neither mutation, however, has reached fixation in the human population such that we would expect APOBEC3H to have a significant impact on variable current day susceptibilities of individuals to retroviral pathogens, such as HIV, LINE-1 and Alu elements.

Chapter Six:

SUMMARY & FUTURE DIRECTIONS

SUMMARY

The work presented here demonstrates the functional consequences of the rapid evolution of the primate antiviral intrinsic immunity gene, APOBEC3H. While some primate APOBEC3H homologs are stably expressed and capable of efficient retroviral inhibition, other primate homologs (such as the human and african green monkey) are unstable and are unable to block the replication of any of the retroviral elements tested here (Figure 30A & B). Further, the sub-cellular localization of primate APOBEC3H homologs has changed and reflects the loss of a putative nuclear export signal present in the N-terminal "TAIL" region of the protein (Figure 30C & D).

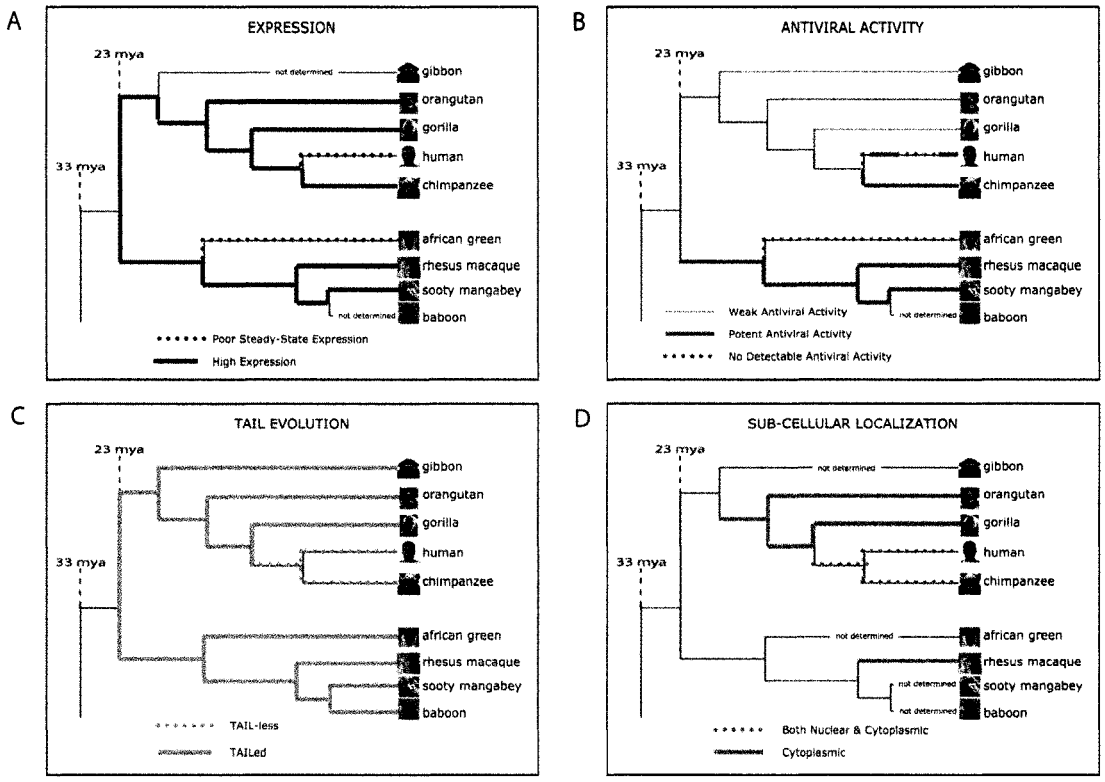


Figure 30 – Expression, Antiviral Activity & Subcellular Localization of Primate APOBEC3H Homologs

The relative expression levels (A), antiviral activity (B), TAIL evolution (C) and sub-cellular localization (D) of the primate APOBEC3H homologs examined in this study.

Further, we have characterized the evolution of APOBEC3H function, specifically in the human lineage. Two, independent polymorphic mutations have risen to high frequency in the human population and give rise to unstable and inactive APOBEC3H protein. Therefore, while our recent human ancestors possessed stable and active APOBEC3H alleles, the antiviral activity of APOBEC3H has been lost in the majority of the human population. Because neither inactivating mutation has reached fixation, individuals in the human population encode APOBEC3H alleles with different abilities to inhibit retroviral and retrotransposon replication (see Figure 31 for

a summary).

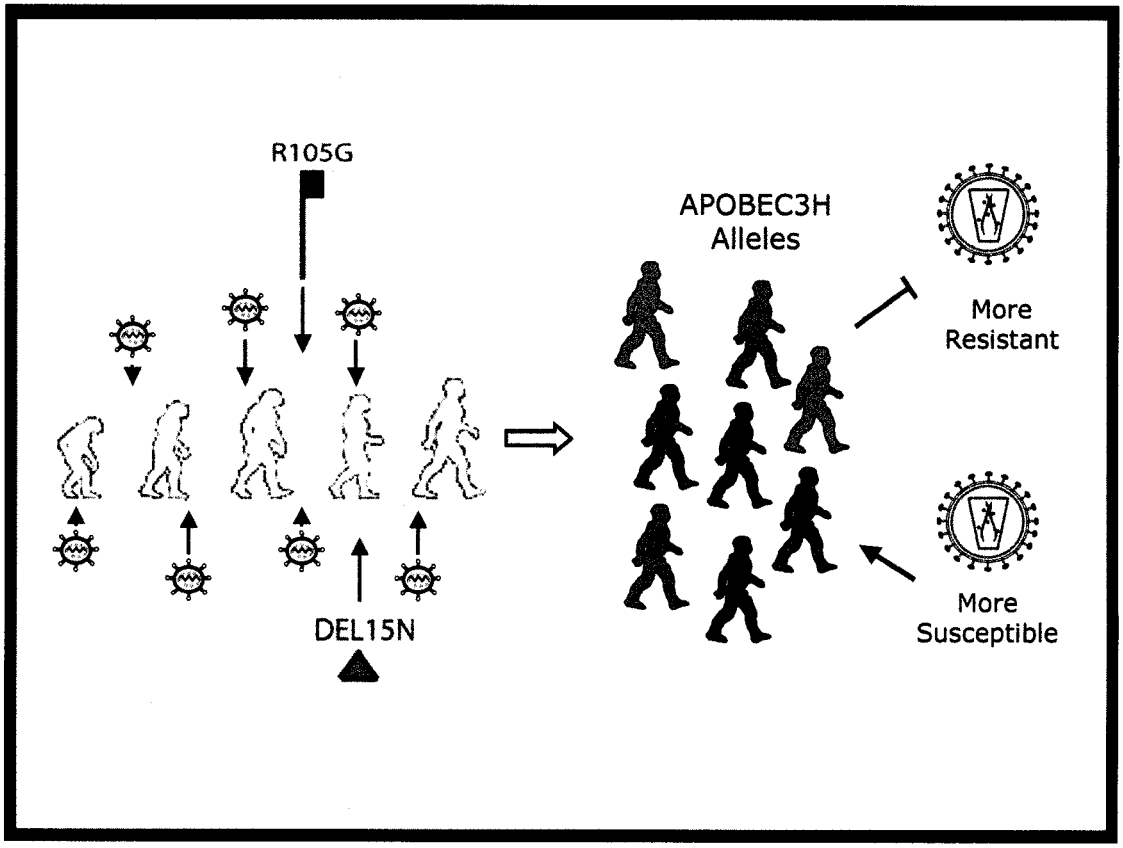


Figure 31 – Evolution of APOBEC3H Alleles in the Human Population
After speciation from chimpanzees, two inactivating mutations (R105G and DEL15N) arose specifically in the human lineage. Modern humans encode several types of APOBEC3H alleles, some of which may be protective against infection by retroviruses (such as HIV) or retrotransposons (such as LINE-1 elements) while unstable and inactive alleles may contribute to an increased susceptibility to these elements.

Therefore, APOBEC3H may contribute to the differential susceptibilities of humans to the replication of retroelements, such as HIV-1 and LINE-1 elements.

FUTURE DIRECTIONS

Our understanding of APOBEC3H and its role in containing/restricting retroviral replication is fairly limited and is, almost entirely, limited to my work presented here. Now that we know active APOBEC3H alleles to exist in the human

population, my hope is that our knowledge of APOBEC3H biology will rapidly increase as more researchers are drawn to the field. My work with APOBEC3H has been fascinating and full of surprises and, no doubt, more are in store in the future.

APOBEC3H Mechanism:

The mechanism(s) through which unstable human APOBEC3H are being degraded needs further investigation as this work could, theoretically, allow for stabilization of unstable APOBEC3H protein as a therapeutic intervention against HIV infection. One outstanding question is whether the mechanism of rapid turnover is the same for the two types of unstable human APOBEC3H protein. Further, a crystal structure of APOBEC3H or another related APOBEC3 protein would greatly inform this work as it would allow small molecules to be designed against exposed surfaces of APOBEC3H that may allow for stabilization/activation of the antiviral activity of the protein.

Further characterization of the localization and activities of primate APOBEC3H proteins may aid in defining the importance of subcellular localization of APOBEC3 proteins for antiviral activity. In particular, does APOBEC3H associate with High Molecular Mass ribonucleoprotein structures in the cell similar to what has been shown for APOBEC3G[92]? If so, is targeting to these cytoplasmic structures necessary for the function of the protein? To address these questions, mutations in potentially important localization determinants can be introduced to ask if there is an effect on protein localization and if so, does this affect the protein's antiviral activity?

Another avenue of APOBEC3H biology worth exploring relates to the *in vivo* impact of APOBEC3H. For example, an anti-APOBEC3H antibody could be used as

a tool to ask if the effects we see on protein stability *in vitro* translate to significant differences *in vivo* by screening cells from individuals homozygous for stable versus unstable alleles of APOBEC3H. Another outstanding question is whether APOBEC3H functions to block the replication of incoming virus as has been shown for APOBEC3G[84]? Studies could be designed to knockdown expression of APOBEC3H in resting CD4+ T cells from individuals homozygous for stable and active APOBEC3H alleles to ask if they contribute to the block to HIV infection of these cell types.

Still another avenue to explore comes from my finding that there is a significant amount of variation among primate APOBEC3H homologs, including changes in what appears to be post-translational modification of the protein. Work targeted at understanding these modifications may reveal mechanisms through which APOBEC3H, and possibly other APOBEC3 proteins, are regulated or how their cytidine deaminase activity is controlled inside the cell. A first step would be to construct chimeric APOBEC3H proteins to localize specific sites of a putative post-translational modification. If such sites can be identified, further studies to identify which modification may be occurring at this residue could be pursued. For example, antibodies specific to certain post-translational modifications could be used to probe immunoprecipitated APOBEC3H proteins. Alternatively, a mass spectrometry approach could be used to identify modified amino acids[137].

Finally, experiments to test the activity of APOBEC3H proteins against Alu elements would expand our knowledge of the host factors contributing to the limitation of the replication of this extremely successful primate parasitic element. The ability of APOBEC3H to block Alu replication is particularly interesting as Alu activity

in humans has been shown to be significantly higher than that in chimpanzees[138], a finding that correlates well with our finding of a significant loss of activity of an antiretroviral gene in a portion of the human population.

APOBEC3H Evolution & Genetics

My finding of APOBEC3H variants with functional consequences opens the door to studies aimed at determining if these variants play a role in determining susceptibilities of individuals to retroviral invasion. In terms of HIV biology, genetic association studies looking for association of active APOBEC3H alleles with HIV outcomes could be fruitful. This work could include statistical analyses of associations between APOBEC3H haplotypes and HIV viral load, kinetics of replication *in vitro*, CD4+ T cell dynamics and progression to AIDS. Similarly, associations between APOBEC3H and LINE-1 or Alu replication may exist, although these studies may be limited by the availability of data regarding the relatively rare amplification events of these retroviral elements. One possible approach would be to genotype those individuals in which we know novel retrotransposon insertions to have occurred and ask if they tend to possess inactive APOBEC3H alleles. Another approach would be to look at retrotransposon insertions at regions linked (close-by such that recombination rates are lower) to specific APOBEC3H haplotypes to ask if the density of insertions is lower in individuals with active APOBEC3H alleles.

In addition to genetic association studies, my work with APOBEC3H suggests that there may have been recent selection for particular alleles in the human population. Specifically, the frequencies of active versus inactive alleles appears to vary significantly among world populations and more detailed analyses may be

suggestive of selection rather than genetic drift as the cause for the spread of APOBEC3H variants around the globe. Specific comparative genomic and population genetic tests for signatures of selection on APOBEC3H polymorphisms and haplotypes can be used for such an analysis, such as HKA, F_{ST} and EHH tests[97].

Moving On From APOBEC3H

Further, this work highlights the dynamic evolution of the APOBEC3 gene family during the evolution of primates and other mammals. In addition to diversification of the APOBEC3 coding sequences, emerging studies suggest that even the expansion of the APOBEC3 locus itself may be variable among primates. Characterization and analysis of the structure of the APOBEC3 locus and the function of APOBEC3 proteins from other primates will lead to a greater understanding of the extent to which the function of these homologous proteins from relatively-closely related species has diverged. Specifically, we may begin to understand the scope of selective pressures exerted on host genomes by retroviral elements and how that may result in a landscape of APOBEC3 genes with distinct functions and specificities. In summary, we may find ourselves one step closer to understanding what makes each primate species unique.

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Curriculum Vitae

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EDUCATION

2003 – 2007	Ph.D., Molecular & Cellular Biology University of Washington/Fred Hutchinson Cancer Research Center, Seattle, WA
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SELECTED HONORS, AWARDS and FELLOWSHIPS

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2007	Keystone Symposia Scholarship, Molecular Determinants of HIV-1 Pathogenesis
2007	Young Investigator Award, Conference on Retroviruses and Opportunistic Infections
2006	International Fellowship, International AIDS Research & Training Program
2004 - 2005	Hearst Foundation Interdisciplinary Fellowship
2001	Dean's Honors, UC Davis
2000	Outstanding Senior Leadership Award, UC Davis
2000	Hoefer Scholar, Athletic & Academic Excellence
1997 – 1999	Academic All-American, Outstanding Achievement in Athletics & Academics

PUBLICATIONS

Molly OhAinle, Julie A. Kerns, Harmit S. Malik and Michael Emerman (2006) Adaptive Evolution and Antiviral Activity of the Conserved Mammalian Cytidine Deaminase *APOBEC3H*. Journal of Virology. 80: 3853-3862.

Meares, C.F., Chmura, A.J., **Orton, M.S.**, Corneille, T.M. and Whetstone, P.A. (2003) Molecular tools for targeted imaging and therapy of cancer. Journal of Molecular Recognition. 16: 255-259.

Chmura, A.J., **Orton, M.S.** and Meares, C.F. (2001) Antibodies with infinite affinity. PNAS. 98: 8480-8484.

PRESENTATIONS

2007	Oral Presentation	The 14th West Coast Retrovirus Meeting "Double-Loss of APOBEC3H Function During Human Evolution"
2007	Poster Presentation	Cold Spring Harbor Lab – Retroviruses "On-again/Off-again – The fantastic evolutionary dynamics of APOBEC3 genes in primates"
2007	Poster Presentation	Keystone Symposia, Molecular and Cellular Determinants of HIV-1 Pathogenesis, Whistler, B.C. "Rapid Evolution and Functional Divergence of APOBEC3H In Primates"
2007	Poster Presentation/ Discussion Panel	Conference on Retroviruses and Opportunistic Infections (CROI), Los Angeles, CA "Evolution of Antiretroviral Activity of APOBEC Genes in Primates"
2007	Invited Speaker	MCB Graduate Program Recruitment, Seattle, WA "Evolution of an Ineffective Antiretroviral Effector"
2005	Oral Presentation	The 12th West Coast Retrovirus Meeting "Monkeys Win and Humans Lose: Apobec3H from Old World Monkeys is an Active Antiviral Cytidine Deaminase while the Human Homologue is Unstable and Inactive"