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Effects of human *TRIM5α* polymorphisms on antiretroviral function and susceptibility to human immunodeficiency virus infection

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Abstract

TRIM5α acts on several retroviruses, including human immunodeficiency virus (HIV-1), to restrict cross-species transmission. Using natural history cohorts and tissue culture systems, we examined the effect of polymorphism in human TRIM5α on HIV-1 infection. In African Americans, the frequencies of two non-coding SNP variant alleles in exon 1 and intron 1 of *TRIM5* were elevated in HIV-1-infected persons compared with uninfected subjects. By contrast, the frequency of the variant allele encoding TRIM5α 136Q was relatively elevated in uninfected individuals, suggesting a possible protective effect. TRIM5α 136Q protein exhibited slightly better anti-HIV-1 activity in tissue culture than the TRIM5α R136 protein. The 43Y variant of TRIM5α was less efficient than the H43 variant at restricting HIV-1 and murine leukemia virus infections in cultured cells. The ancestral *TRIM5* haplotype specifying no observed variant alleles appeared to be protective against infection, and the corresponding wild-type protein partially restricted HIV-1 replication *in vitro*. A single logistic regression model with a permutation test indicated the global corrected *P* value of <0.05 for both SNPs and haplotypes. Thus, polymorphism in human *TRIM5* may influence susceptibility to HIV-1 infection, a possibility that merits additional evaluation in independent cohorts.
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Introduction

A major barrier to cross-species transmission of retroviruses is mediated by the TRIM5α protein (Bieniasz, 2003; Stoye, 2002; Stremlau et al., 2004). Variants of TRIM5α in different primate species block the early, post-entry phase of infection of cells by particular retroviruses (Hatziioannou et al., 2004b; Keckesova et al., 2004; Perron et al., 2004; Yap et al., 2004b). For example, even when expressed at comparable levels, human TRIM5α (TRIM5α_{hu}) is less potent at suppressing infection of human immunodeficiency virus (HIV-1) than the rhesus monkey ortholog, TRIM5α_{rh} (Hatziioannou et al., 2004b; Keckesova et al., 2004; Stremlau et al., 2004; Yap et al., 2004a). On the other hand, TRIM5α_{hu} more potently restricts infection by the N-

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tropic murine leukemia virus (N-MLV) than TRIM5α_{rh} (Hatziioannou et al., 2004b; Perez-Caballero et al., 2005; Perron et al., 2004; Song et al., 2005c; Stremlau et al., 2004; Yap et al., 2004a).

TRIM5 is a member of a family of proteins that contain a *tri*partite *m*otif, hence the designation TRIM (Reymond et al., 2001). TRIM proteins have also been called RBCC proteins because the tripartite motif includes a *R*ING domain, *B*-box 2 domain and *c*oiled *c*oil domain. TRIM proteins exhibit the propensity to assemble into cytoplasmic or nuclear bodies (Reymond et al., 2001). Many cytoplasmic TRIM proteins contain a C-terminal B30.2 or SPRY domain. Differential splicing of the *TRIM5* primary transcript gives rise to the expression of several isoforms of the protein product. The TRIM5α isoform is the largest product (~493 amino acid residues in humans) and contains the B30.2(SPRY) domain. The B30.2(SPRY) domain of rhesus monkey TRIM5α is essential for anti-HIV-1 activity (Stremlau et al., 2005). Moreover, the difference in the anti-HIV-1 potency of rhesus and human TRIM5α proteins is determined by B30.2(SPRY) sequences (Perez-Caballero et al., 2005; Stremlau et al., 2005; Yap et al., 2005). An intact RING domain also contributes, either directly or indirectly, to the antiretroviral activity of TRIM5α_{rh} and TRIM5α_{hu} (Javanbakht et al., 2005; Perez-Caballero et al., 2005; Stremlau et al., 2005). The B-box 2 domain appears to be essential for efficient retrovirus restriction (Javanbakht et al., 2005; Perez-Caballero et al., 2005).

Interspecies differences in primate TRIM5α proteins dictate the potency of restriction against particular retroviruses (Hatziioannou et al., 2004b; Keckesova et al., 2004; Song et al., 2005c; Stremlau et al., 2004; Yap et al., 2004a). The effect of intraspecies variation in TRIM5α on antiretroviral potency is unknown. Moreover, it is uncertain whether the modest inhibitory effect of TRIM5α_{hu} on HIV-1 infection in tissue-cultured cells translates into any limiting effect on HIV-1 infection of humans. Functional polymorphisms in human *TRIM5* that correlate with differential susceptibility to HIV-1 infection or disease progression would support an interaction of TRIM5α_{hu} with HIV-1 *in vivo*. We therefore studied the influence of human *TRIM5* polymorphisms on HIV-1 infection and progression to AIDS in five U.S.-based natural history cohorts, an approach that has identified several other AIDS-modifying host variants (An et al., 2004; Dean et al., 1996; O'Brien et al., 2000). We also investigated the antiretroviral activity in tissue-cultured cells of common and several rare *TRIM5*α_{hu} variants.

Results

Identification of human TRIM5 single nucleotide polymorphisms (SNPs)

Common and rare *TRIM5* SNPs were identified in the human *TRIM5* gene using the SNP discovery panel (n=188), representing the extremes of the distribution for HIV-1 progression and infection, and from 359 normal blood donors and CEPH family founders, the Human Diversity Panel (n=368), at-risk HIV-1-uninfected individuals (n=344), and HIV-1-infected persons from the 5 U.S.-based natural history cohorts (n=698) and from South African HIV-1-infected and -uninfected Xhosa (n=272). The SNP discovery panel of 188 DNA samples was re-sequenced across all exons and intron–exon boundaries, and the other groups were re-sequenced for exon 2 encoding the RING domain and exon 8 encoding the B30.2(SPRY) domain. In addition, DNAs from persons progressing to AIDS in 5 or less years were re-sequenced for coding exons 2, 3, 8 and intron 6 and at-risk HIV-1-uninfected individuals were re-sequenced for exons 2, 3 and 8.

Twelve *TRIM5* SNPs having allele frequencies of >5% in either European Americans (EA) or African Americans (AA) were identified either by re-sequencing and/or from the NCBI dbSNP database; these common SNPs are listed in Table 1 and their locations indicated in Fig. 1. Of the 6 open reading frame (ORF) SNPs, 5 were non-synonymous and one was synonymous. For SNP4, SNP9 and SNP11, the minor alleles in AA were the major alleles in EA. These three SNPs also had the highest F_{st} (a measure of population differentiation) ranging from 0.23 to 0.35 (Supplementary Table 1). The remaining SNPs had F_{st} values ranging from 0.002 to 0.16 compared to an average F_{st} of 0.12 across the autosomes (Altshuler et al., 2005).

We also re-sequenced a large number of subjects to identify variant alleles in other populations (human diversity panel and the South African Zhosa) as well as participants enrolled in the AIDS cohorts with unusual patterns of progression (unusually rapid or slow progression). Nearly all of these additional variant alleles were infrequent and are listed in Table 2. In the Zhosa, only one variant allele had a frequency in the 4–5% range. Two non-synonymous SNPs were observed in only the Xhosa population. P479L was polymorphic in both HIV-1-infected (minor allele frequency (MAF) ≈5%) and -uninfected Xhosa (MAF ≈4%) but was observed only once in a European American. Because these SNPs are rare, their possible effects on infection or progression could not be assessed: SNP distribution between HIV-1 seronegatives and seropositives are listed in Table 2.

Selection on TRIM5

Perturbations in the allele frequency spectrum can reflect relatively recent selective episodes. The SNP discovery panel (unambiguous DNA sequences for all exons were available for 155 subjects) suggests a scarcity of low frequency polymorphism (range 1–5%) *TRIM5α* SNP alleles (6.5% for H43Y, 6.1% for Y112F, 26.8% for R136Q, 20% for L159L, 12.9% for G249D and 1.9% for H419Y) throughout the 1.5-kb protein-coding segment. However, the large screen (Table 2) contained a number of rare variant alleles at frequencies less than 1%. The allele spectrum does not deviate significantly from neutrality—Tajima's $D = -1.306$ ($P > 0.1$).

Past or ongoing selection might also be revealed in the ratio of synonymous to non-synonymous changes. In humans, only 5 of 24 coding region variants are synonymous (Tables 1 and 3) and only 2 of 17 substitutions found between human and

Table 1
Characterization of *TRIM5α* SNPs and their allele frequencies in HIV-1 risk groups

No. [a]	SNP [b]	nt change [c]	aa change [d]	Location	Domain	Minor allele frequency in AA					Allele frequency in EA				
						SC [e]	SN [f]	HREU [g]	*P* value [h]	*Q* value [i]	SC	SN	HREU	*P* value	*Q* value
1	rs3802980	T/C		5′ Upstream		0.438	0.398	0.424	0.427	0.861	0.483	0.475	0.475	0.734	0.861
2	rs16934386	T/C		Exon 1		0.112	0.080	0.045	0.006	0.130	0.002	0.000	0.004	0.762	0.861
3	rs7127617	T/C		Intron 1		0.479	0.441	0.391	0.038	0.280	0.466	0.474	0.467	0.845	0.875
4	rs3824949	C/G		5′ UTR		0.165	0.187	0.203	0.211	0.861	0.549	0.550	0.504	0.254	0.861
5	rs3740996	CAC>TAC	H43Y	Exon 2	RING	0.060	0.087	0.095	0.063	0.350	0.114	0.114	0.101	0.608	0.861
6	rs11601507	GTC>TTC	V112F	Exon 2	B-box 2	0.014	0.009	0.013	0.690	0.861	0.078	0.063	0.089	0.950	0.875
7	rs10838525	CGG>CAG	R136Q	Exon 2	Coil	0.114	0.131	0.188	0.024	0.260	0.354	0.372	0.352	0.744	0.861
8	rs3740995	TTG>TTA	L159L	Exon 3	Coil	0.285	0.308	0.228	0.424	0.861	0.143	0.134	0.141	0.779	0.861
9	rs904375	A/G		Intron 4		0.448	0.457	0.423	0.757	0.861	0.823	0.841	0.810	0.934	0.875
10	rs11038628	GGT>GAT	G249D	Exon 5	Linker 2	0.274	0.259	0.237	0.329	0.861	0.063	0.060	0.074	0.651	0.861
11	rs11820502	G/C		Intron 5		0.216	0.210	0.215	0.922	0.875	0.703	0.730	0.707	0.543	0.861
12	rs28381981	CAT>TAT	H419Y	Exon 8	B30.2	0.010	0.013	0.013	0.719	0.861	0.060	0.055	0.057	0.733	0.861

[a] Number corresponds to SNPs in Fig. 1, tables and text.
[b] Reference number from NCBI dbSNP.
[c] Nucleotide (nt) change is ordered by major/minor allele in AA and based on the forward sequences (AC109341 and NM_033034).
[d] Predicted amino acid replacement for non-synonymous SNPs. The amino acid encoded by the major allele in AA precedes the residue number, which is followed by the amino acid encoded by minor allele. Note that for SNPs 4, 9 and 11, the minor allele in AA is the major allele in EA.
[e] HIV-1+ seroconverter.
[f] HIV-1 seronegative belonging to an HIV-1 risk group.
[g] High-risk exposed HIV-1 uninfected.
[h] *P* values are from Mantel–Haenszel test for trend for the three groups (SC, SN and HREU), in AA or EA, respectively. The *P* values have not been corrected for multiple comparisons.
[i] The false discovery *Q* value is the proportion of statistical tests deemed significant that are actually false positives.

chimpanzee sequences are synonymous. The amount of non-synonymous substitution (0.0146 substitutions per site by Li's 1993 method for human–chimpanzee comparisons) exceeds that of synonymous (0.00383 substitutions per site). However, the excess of non-synonymous substitution is not sufficient to implicate diversifying selection ($P>0.1$ in Fisher's exact test using the modified Nei–Gojobori method of synonymous site counting).

Effect of TRIM5 SNPs on susceptibility to HIV-1 infection

The effects of the 12 *TRIM5* SNPs on susceptibility to HIV-1 infection were tested by comparing allele frequencies among three HIV-1 risk groups with increasing HIV-1 risk levels: high-risk exposed HIV-1-uninfected individuals (HREU), HIV-1 seronegatives (SN) and HIV-1-infected seroconverters (SC) using the Mantel–Haenszel trend test. SNP2, SNP3 and SNP7 had differences ($P=0.006$, 0.038 and 0.024, respectively) in allele frequencies among the three groups and SNP5 (43Y) showed a tendency to be more frequent in SN ($P=0.063$) (Table 1) in African Americans. No differences in allele frequencies were observed in European Americans ($0.25<P\leq 0.95$).

To assess the influence of multiple tests shown in Table 1, we estimated the false discovery rate (FDR) *Q* value, which is defined as the proportion of statistical tests deemed significant that are actually false positives. The *Q* values are shown in Table 1 for all the SNPs. For the three SNPs showing uncorrected *P* values less than 0.05, the FDR ranged from 13% to 28%, and for H43Y the FDR was 35%. For all other SNPs in AA and EA, the FDR was greater than 86%.

The genotypic distribution of the four SNPs showing the lowest *P* values for all at-risk HIV-1 SN or HREU compared to HIV-1+ seroconverters in AA, in each case comparing

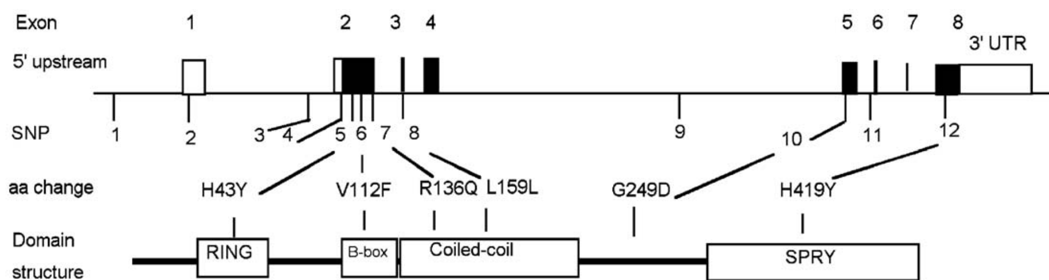

Fig. 1. SNP locations in the human *TRIM5* gene. Black boxes correspond to the *TRIM5* gene open reading frame, and white boxes represent the untranslated regions (not drawn to scale) with exon numbers indicated above. Locations of SNPs with nucleotide changes and corresponding amino acid (aa) changes in protein sequence are indicated by vertical lines. Domain structure of the TRIM5α protein is shown below. The genomic structure of *TRIM5* was predicted based on the comparison of a genomic sequence (accession number AC109341) and the mRNA reference sequence (NM_033034).

Table 2
TRIM5α variants identified by re-sequencing selected exons[a]

Codon[b]	Exon	South African Xhosa[c]		Large screen[d]		
		HIV+	Blood donors	HIV+	HIV−	Donors
		$n=193$ (frequency)	$n=79$ (frequency)	$n=611$	$n=616$	$n=820$
C58Y	2	ND[e]	ND	0	1	0
V77A	2	ND	ND	1	0	0
D93V	2	ND	ND	1	0	0
G110E	2	ND	ND	2	6	1
R119W	2	ND	ND	1	0	0
Q143R	3	ND	ND	1	0	ND
V140L	3	ND	ND	0	1	ND
R229R	4	ND	ND	1	0	ND
128625G/C[f]	Intron 5	ND	ND	1[g]	0	ND
K396K	6	ND	ND	0	0	2
V423F	8	0	0	1	0	0
V438G	8	0	0	0	0	1
Y444C	8	0	0	1	0	0
A446S[h]	8	2 (0.005)[i]	1 (0.006)	0	0	0
I461L	8	0	0	0	2	0
S470P	8	0	0	1	0	0
P475P[h]	8	3 (0.008)[i]	1 (0.006)	0	0	0
P479L	8	20 (0.052)[i]	7 (0.044)	1	0	0

[a] The lists of all variant alleles observed by re-sequencing that are not listed in Table 1. With the exception of P479L in the Zhosa, all the variants have frequencies <1%.

[b] Predicted amino acid replacement for non-synonymous SNPs. The wild-type (wt) TRIM5α_{hu} protein has the sequence reported in Stremlau et al. (2004). The amino acid residue found in wt TRIM5α_{hu} precedes the residue number, which is followed by the amino acid residue found in the variant TRIM5α_{hu} protein.

[c] 193 HIV-1-seropositive patients from clinics in the Western Cape Province of South Africa and 79 HIV-1-seronegative controls who are blood donors from the Western Province Blood Transfusion Service (Petersen et al., 2005).

[d] See Materials and methods for the number of individuals re-sequenced for each exon. The HIV-1-seropositive and -seronegative groups are from the 5 U. S.-based natural history cohorts. The donors are from the CEPH founders, Human diversity panel, and normal blood donors.

[e] Not determined.

[f] Located at nt. 128625 on sequence AC109341.

[g] This individual survived AIDS free for >16 years.

[h] Observed only in the Xhosa population.

[i] The Fisher exact test was used for determining allele frequency differences between Xhosa HIV-1-seropositive and -seronegative groups, with $P>0.80$.

homozygotes for the major allele to heterozygotes or homozygotes for the minor allele (Table 3). The frequencies for the minor alleles of SNP2 and SNP3 were elevated in the SC group relative to either the SN group or the HREU group, implicating these SNPs as possible risk factors for HIV-1 infection (Table 3). Two non-synonymous SNPs were more frequent in uninfected persons. The 43Y allele (SNP5) was elevated in uninfected persons (OR = 0.61, $P=0.043$) and the 136Q allele (SNP7) was elevated in the HREU group (OR = 0.52, $P<0.02$, dominant model) (Table 3). SNP5 (H43Y) and 7 (R136Q) are both in exon 2 encoding the RING and coiled coil domains, respectively (Table 1; Fig. 1). The same analyses were also performed in EA; however, alleles and genotype frequencies were evenly distributed in each of three infection status groups, with no associations observed with HIV-1 infection (Table 1).

Effect of TRIM5 haplotypes on susceptibility to HIV-1 infection

Pairwise LD of the 12 common *TRIM5* SNPs in AA and EA is presented in Supplementary Figs. 1A and B and inferred haplotypes are listed in Supplementary Table 1. A low degree of D', a measure of linkage disequilibrium (range = 0–1.0, mean comparing to SNP1 = 0.50 in AA; range = 0.03–1.0, mean = 0.69 in EA), and r^2, a measure of correlation, (range = 0.001–0.43 and 0.001–0.75 for AA and EA, respectively) were observed for many of the SNP pairs.

An analysis was performed for haplotypes inferred from the 12 SNPs. In AA, a protective effect on infection was observed only for haplotype 2 when comparing SC to SN or HREU (OR = 0.50, 0.59, $P=\sim 0.02$) (Supplementary Table 2). In AA, there are 7 haplotypes that carry all of the major frequency amino acids, but varying at the non-coding SNPs. Haplotype 2, the most frequent, also carries major frequency nucleotides at the non-coding sites, suggesting that this haplotype may be ancestral. In EA, haplotype 2 was too infrequent (freq. ≈0.6%) to be tested. No other significant associations between haplotypes and either progression or infection were observed for either EA or AA.

To determine if the susceptibility-enhancing effects of the SNP2 T/C and SNP3 T/C and the protective effects of SNP5 C/T (H43Y) and SNP7 G/A (R136Q) variant alleles were independent, we considered the haplotype structure for the SNP pairs. The variant alleles for SNP pair 2 and 3 and for SNP pair 5 (43Y) and 7 (136Q) are in strong negative LD ($D'=0.9$, 1.0, respectively) and therefore rarely occur together on the same haplotype (for SNP2–SNP3, the haplotypes are T-T, C-T and T-C); for SNP5–SNP7, the haplotypes are C-G, T-G and C-A) (Table 4). This indicates that the susceptibility-enhancing effects of the SNP2 and SNP3 variant alleles and the protective effects of 43Y and 136Q are independent. We formally tested the independent predictive value of the variant haplotypes using multivariate logistic regression, testing association with increasing resistance to HIV-1 infection (HREU < SN < SC) under the proportional odds model. For each 2-SNP haplotype, the model was adjusted for the effects of the other variant haplotype (i.e., the effects of haplotype C-T was adjusted for the effects of haplotype T-C). For SNP2 and SNP3, the variant alleles were independent risk predictors of infection (SNP2 C: OR = 1.85; $P=0.007$; SNP3 C: OR = 1.56, $P=0.01$). Similarly, both SNP5 and SNP7 variant alleles were associated with decreased risk of infection (SNP5 T: OR = 0.61, $P=0.03$; SNP7 A: OR = 0.65, $P<0.02$).

As correction for multiple comparisons is not straightforward when there are multiple significant results before correction, in particular when the results are for genetic factors in LD (thus correlated), we tested for overall significance of the associations by a permutation test, simulating the null hypothesis of *TRIM5* variants having no effect on infection. Here we tested the simplest *a priori* alternate hypothesis, that any effects would be additive for the genetic factors and progressive in susceptibility to infection. We ran a proportional odds logistic regression with *TRIM5* SNPs as explanatory variables and ordinal susceptibility to infection as the outcome variable. SNPs with minor allele frequency <0.05 were omitted.

Table 3
The effects of selected *TRIM5α* SNPs[a] on HIV-1 infection in AA

SNP	Group	Number			Total	Genotype frequency			SC cases vs. SN or HREU controls	
						11	12	22	OR (95% CI)[b]	$P_{uncorrected}$[c]
		TT	TC	CC					TC+CC vs. TT	
SNP2 rs16934386	SC	222	57	4	283	0.785	0.201	0.014	1	
	All SN	266	40	2	308	0.864	0.130	0.007	1.74 (1.12–2.70)	0.011
	HREU	70	7	0	77	0.909	0.091	0.000	2.75 (1.20–6.25)	0.013
		TT	TC	CC					TC+CC vs. TT	
SNP3 rs7127617	SC	65	131	55	251	0.259	0.522	0.219	1	
	All SN	98	121	63	282	0.348	0.429	0.223	1.52 (1.05–2.22)	0.027
	HREU	29	30	15	74	0.392	0.405	0.203	1.85 (1.06–3.22)	0.027
		CC	TC	TT					TC+TT vs. CC	
SNP5 H43Y	SC	250	30	2	282	0.887	0.106	0.007	1	
	All SN	250	50	2	302	0.828	0.166	0.000	0.61 (0.38–0.99)	0.043
	HREU	63	14	0	77	0.818	0.182	0.000	0.57 (0.29–1.15)	0.110
		GG	G/A	AA					AG+AA vs. GG	
SNP7 R136Q	SC	224	59	4	295	0.759	0.200	0.014	1	
	All SN	310	101	9	420	0.738	0.240	0.021	0.79 (0.56–1.16)	0.198
	HREU	52	26	2	80	0.650	0.325	0.025	0.52 (0.30–0.89)	0.017

[a] All SNPs showing an uncorrected *P* value of <0.050.
[b] OR is the odds ratio of being an HIV-1-infected seroconverter compared to being either HIV-1-seronegative (All SN) or high-risk exposed HIV-1 uninfected (HREU).
[c] *P* values are not corrected for multiple comparisons.

Comparing the AIC for the actual data with that for random permutations of the data, 65 out of 5000 permutations showed an AIC as low as that (1257.98) observed for the actual data, indicating an overall significance of *TRIM5* associations with infection in AA of 0.013. A corresponding test of haplotype associations, using all haplotypes with frequency >0.05 in place of the SNPs as additive explanatory variables in the logistic regression, indicated a significance of 0.0224.

Correcting for the two races tested (but not for the two analyses, SNP and haplotype, as these are non-independent analyses with equivalent results), overall significances of 0.03 for an effect of *TRIM5* SNPs and of 0.04 for *TRIM5* haplotypes on HIV-1 infection are indicated.

Table 4
Effects of two-SNP haplotypes of *TRIM5α* on HIV-1 infection in African Americans

Haplotype	Haplotype frequency	HREU<SN<SC[a]	
		OR (95%CI)	$P_{uncorrected}$
SNP2–SNP3			
T-T	0.47	1	–
C-T	0.09	1.85 (1.18–2.90)	0.007
T-C	0.44	1.56 (1.11–2.20)	0.01
SNP5–SNP7 H43Y–R136Q			
C-G (H-R)	0.79	1	–
T-G (Y-R)	0.07	0.61 (0.40–0.94)	0.03
C-A (H-Q)	0.14	0.65 (0.46–0.93)	0.02

P values are not corrected for multiple comparisons.
[a] HREU, SN and SC were coded as ordinal levels of increasing susceptibility to HIV-1 infection in a proportional odds logistic regression. The independent effects of the variant SNP2–SNP3 and SNP5–SNP7 haplotypes on susceptibility to infection were assessed by considering them as regression covariates in this model. The odds ratio (OR) is the effect of the haplotype on the odds of being in a being in a more susceptible risk category.

Effect of TRIM5 SNPs on AIDS progression

No associations were found for any of 12 common *TRIM5* SNPs on progression to CD4<200, AIDS-defining conditions or AIDS-related death, using Cox model analysis in AA (n=295) or EA (n=644) seroconvertors (data not shown).

Effect of TRIM5α polymorphisms on antiretroviral activity

The contribution of each of the common and several of the rare polymorphisms in the TRIM5α_{hu} protein to antiretroviral activity was studied. The "wild-type" TRIM5α_{hu} protein for these studies was that encoded by the *TRIM5* allele reported in Stremlau et al. (2004) and the amino acid change associated with each variant was introduced into this wild-type (wt) protein (Fig. 2A). The amino acid residues in the wild-type TRIM5α protein are those encoded by the more frequent *TRIM5* alleles. The TRIM5α_{hu} proteins possess C-terminal epitope tags derived from influenza hemagglutinin (HA). LPCX retroviral vectors expressing the TRIM5α_{hu} variants were used to transduce Cf2Th canine thymocytes. Recombinant viruses were produced in 293T cells by cotransfecting the pLPCX plasmids with the pVPack-GP and pVPack-VSV-G packaging plasmids. The pVPack-VSV-G plasmid encodes the vesicular stomatitis virus (VSV) G envelope glycoprotein, which allows efficient entry into a wide range of vertebrate cells (Yee et al.,

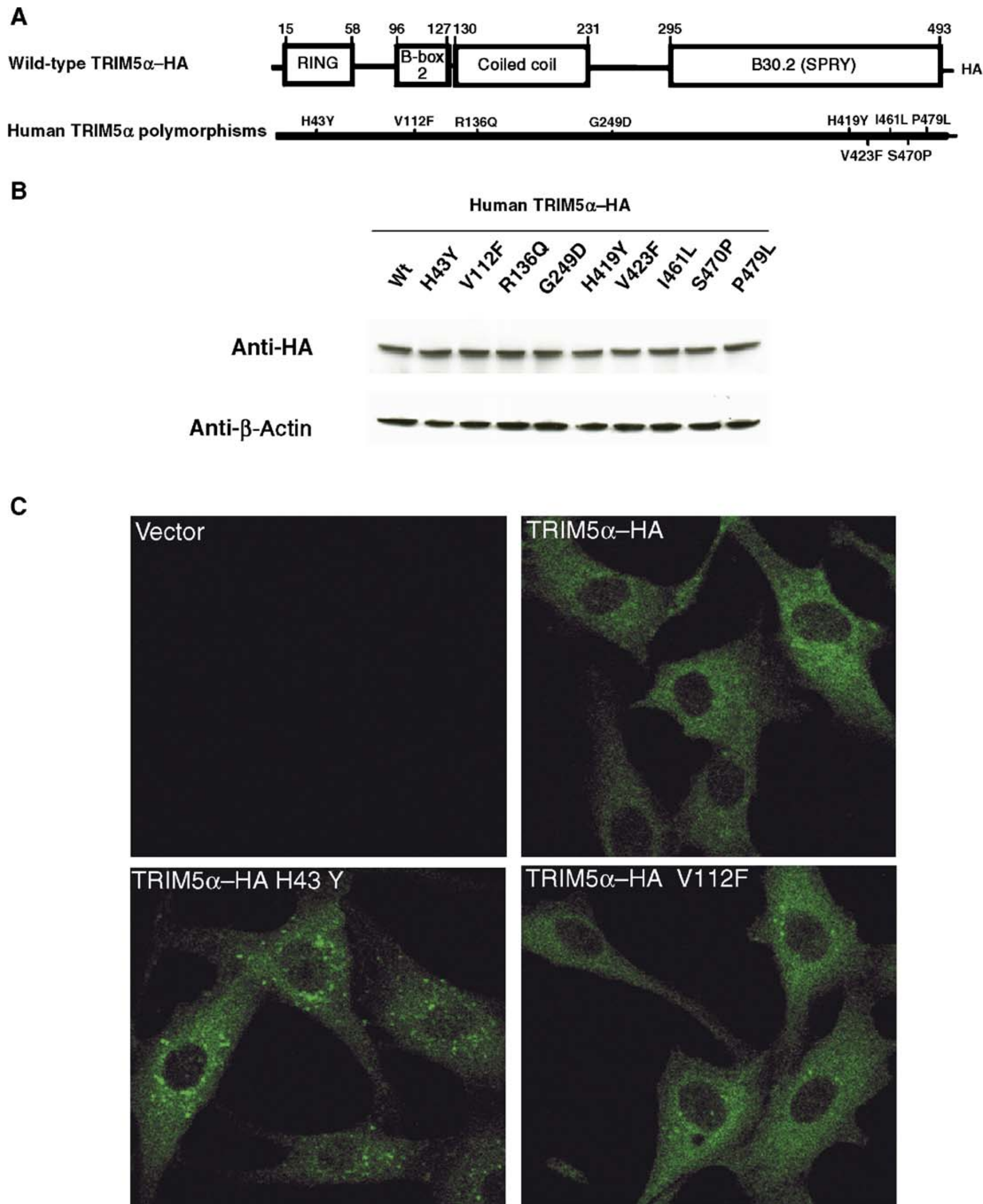

Fig. 2. Sequence and expression of wild-type and mutant TRIM5α_{hu} proteins. (A) A representation of the TRIM5α_{hu} protein with the carboxy-terminal hemagglutinin (HA) tag is shown, with the domains labeled and domain boundaries numbered according to the amino acid residue. Human TRIM5α polymorphic variants tested for antiviral function are depicted beneath the wild-type TRIM5α_{hu} protein. (B) Cf2Th cells stably expressing the wild-type and variant TRIM5α_{hu} proteins were lysed. Cell lysates were Western blotted and probed with antibodies directed against either the HA epitope tag (top panel) or β-actin (bottom panel). (C) Cf2Th cells expressing the wild-type or variant TRIM5a proteins were grown overnight on 8-well chamber slides, fixed, permeabilized and incubated with rat anti-HA 3F10 antibody (Roche), followed by anti-rat IgG conjugated with FITC (Santa Cruz). Subsequently, samples were mounted for fluorescence microscopy.

1994). The TRIM5α_{hu} variants were expressed stably in the transduced Cf2Th canine thymocytes. Because dogs do not have a *TRIM5* gene (Song et al., 2005a, 2005c), the use of Cf2Th cells provides a neutral background for the assessment of TRIM5α_{hu} function. Western blotting of the cell lysates with an anti-HA antibody indicated that all of the TRIM5α_{hu} variants were expressed at comparable levels (Fig. 2B). Staining of the Cf2Th cells with the anti-HA antibody revealed diffuse cytoplasmic staining as well as scattered cytoplasmic bodies for all of the variants (Fig. 2C and data not shown). All of the TRIM5α_{hu} variants exhibited staining patterns similar to that of the wt TRIM5α_{hu} protein, with one exception. The 43Y TRIM5α_{hu} variant formed more prominent cytoplasmic bodies than the other TRIM5α_{hu} variants (Fig. 2C).

To examine the susceptibility of the Cf2Th cells expressing the TRIM5α_{hu} variants to N-MLV infection, we incubated the cells with the recombinant N-MLV-GFP virus. This recombinant, single-round virus expresses green

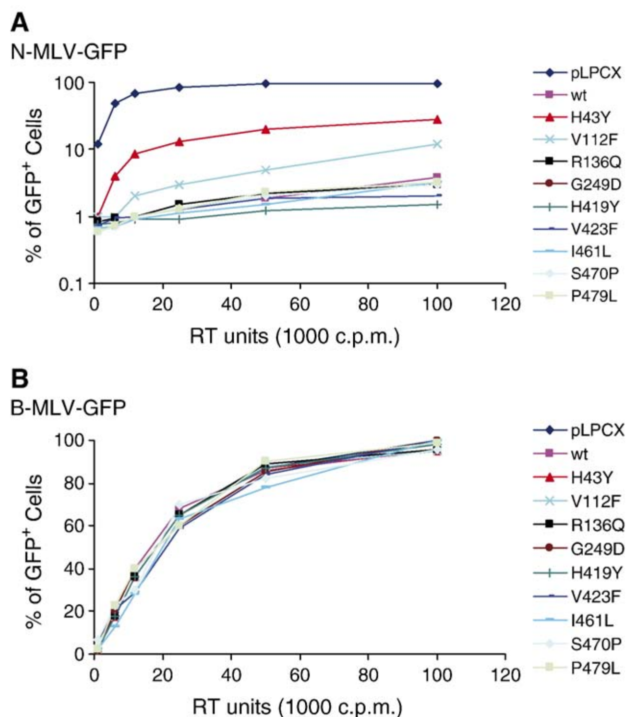

Fig. 3. Effect of expression of the TRIM5α_{hu} variants on N-MLV and B-MLV infection. Cf2Th cells expressing the wild-type (wt) and mutant TRIM5α proteins, or control Cf2Th cells transduced with the empty pLPCX vector, were incubated with various amounts of N-MLV-GFP (A) or B-MLV-GFP (B). Cells were washed and returned to culture for 48 h, and then subjected to FACS analysis with a FACScan (Becton Dickinson). The results of a typical experiment, which was repeated twice with comparable outcomes, are shown.

fluorescent protein (GFP), allowing successfully infected cells to be quantified (Fig. 3). Compared with Cf2Th cells transduced with the empty pLPCX vector, Cf2Th cells expressing the wt TRIM5α_{hu} protein were strongly resistant to N-MLV-GFP infection (Fig. 3A). Most of the TRIM5α_{hu} variants restricted N-MLV-GFP infection as efficiently as wt TRIM5α_{hu}. However, Cf2Th cells expressing the 43Y TRIM5α_{hu} variant were more susceptible to N-MLV-GFP infection than cells expressing wt TRIM5α_{hu}. Cf2Th cells expressing the 112F TRIM5α_{hu} variant were slightly more infectible by N-MLV-GFP than cells expressing wt TRIM5α_{hu}. Thus, although both the 43Y and 112F variants of TRIM5α_{hu} retained the ability to restrict N-MLV-GFP infection, these mutants were less efficient than the wt TRIM5α_{hu} protein and the other human variants tested.

To examine the ability of the TRIM5α_{hu} variants to inhibit HIV-1 infection, we incubated the Cf2Th cells expressing the wt and mutant TRIM5α_{hu} proteins and the control Cf2Th cells transduced with the empty pLPCX vector with HIV-1-GFP. Compared with the Cf2Th cells transduced with the pLPCX vector, Cf2Th cells expressing the wt and mutant TRIM5α_{hu} proteins exhibited a modest reduction in susceptibility to HIV-1-GFP infection. The level of HIV-1 restriction was similar for the wt and mutant TRIM5α_{hu} proteins, although the 43Y and 112F proteins were slightly less effective at blocking HIV-1 infection than the other variants (Fig. 4A). Apparently, the 43Y and 112F variants of TRIM5α_{hu} exhibit mild decreases in the ability to restrict N-MLV and HIV-1 infection, compared with other TRIM5α_{hu} variants. Conversely, the 136Q TRIM5α_{hu} variant consistently exhibited slightly more potent HIV-1-restricting ability compared with the wild-type TRIM5α_{hu} protein (Fig. 4A). To corroborate these results, we established three independent Cf2Th cell lines expressing each of the wt TRIM5α_{hu}, TRIM5α_{hu} 43Y and TRIM5α_{hu} 136Q proteins. The cells, along with control cells transduced with the empty vector, were incubated with HIV-1-GFP. As shown in Fig. 4B, the anti-HIV-1 activity of the 43Y mutant was consistently weaker than that of the wt TRIM5α_{hu} protein and the anti-HIV-1 activity of the 136Q protein was consistently more potent than that of wt TRIM5α_{hu}. All of the TRIM5α_{hu}-mediated decreases in susceptibility to N-MLV or HIV-1 infection were specific, as the susceptibility of the Cf2Th cells expressing the TRIM5α_{hu} variants to B-MLV infection was similar (Fig. 3B).

TRIM5α_{rh} exhibits more potent anti-HIV-1 activity than TRIM5α_{hu} (Stremlau et al., 2004). Residue 138 in TRIM5α_{rh}, which is equivalent to residue 136 in TRIM5α_{hu}, is glutamine. To determine whether the glutamine at this position contributes to TRIM5α_{rh} antiviral potency, we altered it to arginine. This change, however, had little effect on the ability of TRIM5α_{rh} to restrict HIV-1 infection (Fig. 4B).

We conclude that naturally occurring variation in residues 43 and 136 of TRIM5α_{hu} results in subtle but reproducible effects on anti-HIV-1 potency.

Discussion

Variation among the TRIM5α proteins of primate species governs the ability to restrict infection by particular retroviruses (Hatziioannou et al., 2004a; Hofmann et al., 1999; Keckesova et al., 2004; Song et al., 2005c; Stremlau et al., 2004; Yap et al., 2004a). Evidence of positive selection operating on *TRIM5* supports the possibility that this gene has evolved to contribute to antiviral immunity (Sawyer et al., 2005; Song et al., 2005b; Ortiz et al., 2006). In this genetic epidemiological study of five HIV-1 natural history cohorts, we identified 12 common *TRIM5* SNPs, five of which result in amino acid changes. Several other, presumably rare, SNPs were also identified. A high proportion of the identified SNPs in the *TRIM5*-coding region involved non-synonymous changes; however, this tendency was short of significant.

In African Americans, four *TRIM5* alleles exhibited different frequencies in HIV-1-infected and -uninfected individuals, raising the possibility that they influence the risk of acquiring HIV-1 infection. SNP2 in the non-coding exon 1 and SNP3 in intron 1 were each associated with increased risk of infection. Although the functional role of these SNPs is unknown, it is possible that they are either in LD with causal SNPs elsewhere in the gene or are themselves causal and may affect gene regulation or alternative splicing. Because the variant alleles for SNP2 and SNP3 are in negative LD and rarely occur together on the same haplotype, their effects on HIV-1 susceptibility are independent.

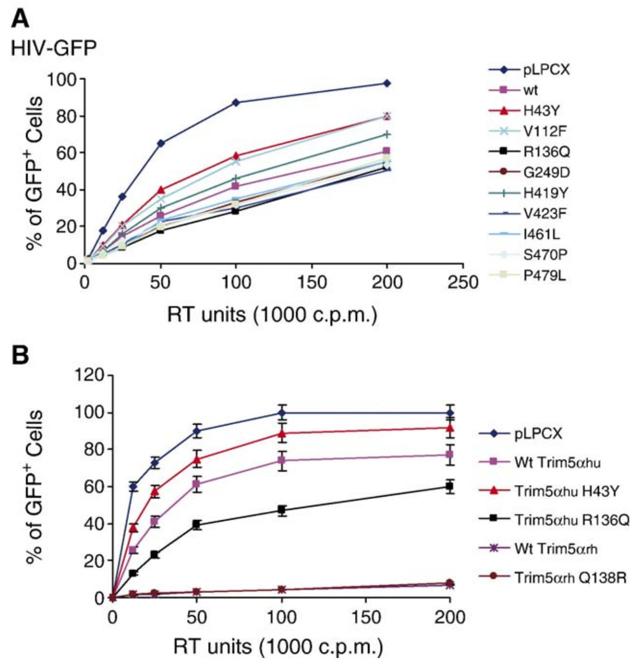

Fig. 4. Effect of expression of the TRIM5α_{hu} variants on HIV-1 infection. (A) Cf2Th cells expressing the wild-type (wt) and mutant TRIM5α proteins, or control Cf2Th cells transduced with the empty pLPCX vector, were incubated with various amounts of HIV-1-GFP. Cells were washed and returned to culture for 48 h, and then subjected to FACS analysis with a FACScan (Becton Dickinson). (B) For wt TRIM5α_{hu} and each mutant TRIM5α_{hu} protein (H43Y or R136Q), three independent Cf2Th cell lines were created. Three Cf2Th lines were also made by transduction with the empty pLPCX control vector. Cf2Th cells expressing wt TRIM5α_{rh} or TRIM5α_{rh} Q136R were also created. Cells were incubated with the indicated amounts of HIV-1-GFP. Cells were washed and returned to culture for 48 h and then subjected to FACS analysis with a FACScan (Becton Dickinson). The means and standard deviations derived from the experiments using the three independently established cell lines are indicated.

The *TRIM5* allele encoding a TRIM5 protein with glutamine 136 was more frequent in both HREU and SN groups than in the SC group. The elevated frequency of this allele in both HREU and SN groups suggests that an HIV-1-protective effect may be afforded by this allele—and that this protection applies to individuals who are subject to a range of exposure frequencies. The *TRIM5* allele encoding a TRIM5 protein with tyrosine 43 tended to be more frequent in uninfected individuals. Any effects of 43Y and 136R are independent because the two alleles do not occur together on the same haplotype.

The effects of these four SNPs were observed only for the African American group, almost all of whom reported injecting drugs, but not for European Americans in our cohorts, although the latter group has greater sample size. SNP2 was nearly absent from EA; however, SNP3, SNP5 and SNP7 had either similar or greater allele frequencies in EA. This suggests that any modification of infection susceptibility afforded by particular *TRIM5* alleles may be restricted to particular population group (s) or types of exposure. The diverse frequency distribution of almost all SNPs and different haplotype structure in populations could be responsible for the discrepancy. Such population-specific effects have previously been observed for other AIDS-modifying genes (An et al., 2002, 2004; Gonzalez et al., 1999; Winkler et al., 2004).

It is difficult to assess the significance of haplotypes and correlated SNPs given the number of statistical comparisons. We measured the overall significance of *TRIM5* SNPs and haplotypes by testing a multivariant logistic regression model by a permutation test. The permutation test determines how frequently one would see an overall association as strong as that seen for the actual data under the null hypothesis of no association of SNPs or haplotypes with HIV-1 infection. The global corrected P value <0.05 for both SNPs and haplotypes indicates that polymorphism in *TRIM5* may affect HIV-1 infection susceptibility although no individual SNP or haplotype reaches significance after correction for false discovery (Storey and Tibshirani, 2003; Benjamini and Hochberg, 1995).

Ultimately, associations between *TRIM5* genotypes and HIV-1 infection in humans require confirmation in multiple studies. Recently, a study of the effects of *TRIM5* polymorphisms on HIV-1 infection was conducted in a cohort consisting primarily of European American men having sex with men (Speelmon et al., 2006). None of the individual *TRIM5* SNPs identified in that study demonstrated an association with HIV-1 susceptibility, consistent with our results in European Americans. Speelmon and colleagues found that a *TRIM5* haplotype containing 136Q exhibited increased frequency among HIV-1-infected subjects compared with exposed seronegative individuals. This contrasts with the apparently protective effect of 136Q in African Americans in our study. Explanations for these different results include differences in genetic background and type of exposure, linkage with other genetic sequences that are responsible for the phenotype, and spurious associations. Additional studies should provide a more complete picture of the role of *TRIM5* variation in susceptibility to HIV-1 infection. In agreement with our results, Speelmon and colleagues (2006) found no association between *TRIM5* genotype and markers of clinical progression. Apparently, once HIV-1 infection is established, the contribution of variation in TRIM5α_{hu} to outcome is negligible.

The 136Q variant of TRIM5α_{hu} was associated with protection from HIV-1 infection in African Americans in our study. Although we observed no significant difference in the ability of TRIM5α_{hu} proteins differing in residue 136 to restrict N-MLV infection of tissue-cultured cells, the 136Q TRIM5α_{hu} variant was consistently superior to wt TRIM5α_{hu} in blocking HIV-1 infection. Although this HIV-1-restricting phenotype is weak, it is consistent with the epidemiologic observations. The mechanistic basis of this phenotype is uncertain; the coiled coil domain in which arginine/glutamine 136 resides has been implicated in TRIM5α oligomerization (Javanbakht et al., 2005; Mische et al., 2005; Perez-Caballero et al., 2005). Of note, glutamine (or, in one case, glutamic acid) is commonly found at residue 136 in the TRIM5α proteins of most non-human primates, with the exception of chimpanzees (Song et al., 2005b). Thus, most primates may

preserve a somewhat more active form of TRIM5α with respect to antiretroviral activity.

The 43Y variant was less effective at restricting HIV-1 and N-MLV infections of tissue-cultured cells than the other $TRIM5\alpha_{hu}$ variants tested. This result corroborates those recently reported by (Sawyer et al., 2006). The superior antiretroviral activity of the $TRIM5\alpha_{hu}$ protein with a histidine 43 residue, compared with that of the tyrosine 43 residue, in this in vitro system is not consistent with the trend towards an HIV-1-protective effect of 43Y observed in our the epidemiological study. Of note, histidine is found at residue 43 of most other non-human primate TRIM5α proteins (Song et al., 2005b). Residue 43 is located in the RING domain of $TRIM5\alpha_{hu}$; the RING domain contributes to, but is not absolutely required for, the anti-HIV-1 activity of rhesus monkey TRIM5α (Javanbakht et al., 2005). Although no structural information is available for TRIM5α, the structure of the RING domain of TRIM19 (PML) has been solved (Borden et al., 1995). Assuming that the TRIM5 and TRIM19 RING domains fold similarly, residue 43 of TRIM5α is predicted to be well-exposed on the surface of the protein. Substitution of a tyrosine for histidine in this surface-exposed location could add hydrophobic character, increasing the tendency for aggregation. $TRIM5\alpha_{hu}$ 43Y formed more prominent cytoplasmic bodies in the transfected Cf2Th cells than the other $TRIM5\alpha_{hu}$ proteins. Other studies have suggested that TRIM5α cytoplasmic bodies represent pre-aggresomal structures that are not essential for antiretroviral activity (Diaz-Griffero et al., 2006; Song et al., 2005a). Thus, relative to other $TRIM5\alpha_{hu}$ variants, 43Y may exhibit increased propensity to coalesce into cytoplasmic bodies and may be less available in the forms required for antiretroviral activity. The consequences of the observed modest decreases in the ability of $TRIM5\alpha_{hu}$ 43Y to restrict HIV-1 or N-MLV infection in our experimented system will need to be examined in more natural contexts. Factors such as the level of $TRIM5\alpha_{hu}$ expression and the co-expression of other *TRIM5α* alleles might influence the phenotype of $TRIM5\alpha_{hu}$ H43Y, for example. Such variables may account for the inconsistencies between the functional and epidemiological results for 43Y.

The $TRIM5\alpha_{hu}$ 112F variant exhibited a slight decrease in antiretroviral activity in tissue-cultured cells, relative to the wt $TRIM5\alpha_{hu}$ protein. This fairly common variant involves the B-box 2 domain, which has been shown to be essential for the ability of rhesus monkey TRIM5α to block HIV-1 infection (Javanbakht et al., 2005). The precise contribution of residue 112 to antiretroviral activity of TRIM5α remains to be determined. We did not observe any evidence of an *in vivo* effect of changes in this TRIM5α residue on susceptibility to HIV-1 infection or progression to AIDS, consistent with the results of another study (Speelmon et al., 2006).

Future studies may clarify the circumstances in which *TRIM5* polymorphisms exert effects on susceptibility to HIV-1 infection, and the mechanisms of these effects. Awareness of variation in human TRIM5α proteins or in the regulation of this protein may be important in attempts to manipulate this antiretroviral factor to our advantage.

Materials and methods

Study participants

Study subjects were enrolled in five natural history HIV/AIDS cohorts: AIDS Link to the Intravenous Experience (ALIVE; Vlahov et al., 1998), Multicenter AIDS Cohort Study (MACS; Buchbinder et al., 1994; Kaslow et al., 1987), the San Francisco City Clinic Study (SFCC; Buchbinder et al., 1994), Hemophilia Growth and Development Study (HGDS; Goedert et al., 1989; Hilgartner et al., 1993) and the Multicenter Hemophilia Cohort Study (MHCS; Goedert et al., 1989). The subjects used in this report consisted of HIV-1 seroconverters (SC), seroprevalents (SP) and seronegatives (SN) for a total of 3604 participants (2171 EA and 1433 AA). The number of subjects studies in each disease category was as follows: SC = 644 EA, 295 AA; SN = 571 EA, 420 AA; and HREU = 145 EA, 80 AA. Seroprevalents were included for allele frequency determination and haplotype inference but were not used in the association analysis.

The date of seroconversion after study enrollment was estimated as the midpoint between the last seronegative and first seropositive HIV-1 antibody test; only individuals with less than 2 years elapsed time between the two tests were included. The censoring date was December 31, 1995, for the MACS, MHCS, HGDS or SFCC or July 31, 1997, for the ALIVE cohort to avoid potential confounding by highly active anti-retroviral therapy (HAART). Because uptake of HAART was delayed in the ALIVE cohort (Celentano et al., 2001), the later censoring date was used.

High-risk exposed uninfected (HREU) subjects were those 80 African Americans and 145 European Americans with high-risk exposure through sharing of injection equipment (Vlahov and Polk, 1988), who had anal receptive sex with multiple partners (Detels et al., 1994) or transfusions with Factor VIII clotting factor prior to 1984, when heat treatment was initiated (Salkowitz et al., 2001). Seronegative subjects ($n = 420$ and 571 for AA and EA, respectively) are those enrolled in the cohorts who remained HIV seronegative despite ongoing or prior risk activity. In addition, DNAs from 193 HIV-1-seropositive individuals and 79 seronegative blood donors from the Western Cape of South African of Xhosa descent were included for polymorphism discovery (Petersen et al., 2005). We included DNA from 92 normal Chinese Han to determine *TRIM5* allele frequencies for this major Asian population.

The study protocols were approved by the Institutional Review Boards of participating institutions and informed consent was obtained from all study participants.

Identification of DNA polymorphisms

Genetic variation in the *TRIM5* gene was identified by several methods. SNPs were identified from the Center for Biotechnology Information (NCBI) dbSNP Database (Sherry et al., 2001), by re-sequencing, and by a heteroduplex assays designed to detect nucleotide mismatches. To discover unreported *TRIM5* polymorphisms, we used a screening panel

consisting of 94 DNA samples from European Americans (EA) and 94 from African Americans (AA) comprised of rapid progressors (clinical AIDS within 3 years of seroconversion), slow progressors (CD4<200 at 12 or more years after seroconversion) and HREUs (confirmed multiple exposures). A non-isotopic RNA cleavage assay (NIRCA) in conjunction with re-sequencing was performed to detect polymorphisms (Goldrick et al., 1996): PCR primers were designed to cover the putative promoter, 5′ untranslated region (UTR), all exons and 3′ UTR, based on the genomic sequence ac109341 (Supplementary Table 3a). The 5′ upstream region (or putative promoter region) sequenced covers 1.2 kb upstream of the first base of the *TRIM5* mRNA sequence (GenBank accession number NM_033034). The exons were amplified with primers located in the introns.

To discover rare genetic variants associated with HIV-1 disease or polymorphisms in diverse populations, we re-sequenced DNA ($n=2293$) using primers listed in Supplementary Table 3b for coding exons 2 and 8 (unless otherwise indicated) from the following groups: U.S.-based normal donors ($n=359$); unrelated founders from the CEPH families ($n=93$); the Human Diversity panel ($n=368$); and participants from the 5 natural history AIDS cohorts including seroconverters ($n=420$); at-risk seronegatives for all exons except exons 1 and 5, and intron 7 ($n=344$); and seroprevalent progressing to AIDS in within 5 years of study entry for all exons except exons 1, 5 and 7 ($n=188$) or who remaining AIDS-free for 10 or more years ($n=90$). Exon 8 containing the B20.3 (SPRY) domain was also re-sequenced in South Africans of Xhosa descent ($n=272$) (Petersen et al., 2005).

Genotyping of genetic variants

Genotypes were determined by TaqMan or PCR-restriction fragment length polymorphism (RFLP) assays for participants enrolled in the 5 natural history cohorts. TaqMan allelic discrimination assays to determine genotypes were designed by using the Primer Express software or by the assay-by-demand or the assay-by-design service offered by Applied Biosystems (Foster City, CA). Genotyping was performed using TaqMan assays according to the manufacturer's protocols (Applied Biosystems, Foster City, CA). Primer and probe combinations are listed in Supplementary Table 4. TaqMan genotypes were automatically called and plotted using the SDS 2.1 software (Applied Biosystems, Foster City, CA), based on a two-parameter plot using fluorescence intensities of FAM and VIC dye-labeled MGB probes. Eight water controls were included on each plate to monitor potential PCR contamination and 10% of the DNA samples were duplicated. Three SNPs were genotyped by PCR- RFLP assays. PCR was performed in 20 μl reactions consisting of 20 ng of genomic DNA using TaqGold polymerase protocols (Applied Biosystems, Foster City, CA) and PCR buffer (Roche, Indianapolis, IN). PCR was carried out with 35 cycles of denaturing at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 45 s. PCR primer sequences and restriction enzymes used for each SNP are listed in Supplementary Table 4.

Statistical analysis

The genetic effects of SNPs on HIV-1 infection susceptibility were assessed by comparing allelic and genotypic frequencies between (1) HIV-1 high-risk exposed uninfected (HREU) individuals and HIV-1+ seroconverters (SC) and (2) HIV-1 seronegatives (SN) belonging to HIV-1 risk groups and seroconverters (SC), by using the chi-square test or Fisher's exact test. The significance level was $P<0.05$ for two-tailed tests. Genetic risk magnitudes were measured by calculating odds ratios (ORs) with 95% confidence intervals (CIs). The Mantel–Haenszel test and proportional odds logistic regression were used to test for the trend among three groups: SC, SN and HREU. Individuals carrying the CCR5 Δ32/Δ32 genotype were excluded from the analysis.

A permutation test was used to compare the null hypothesis of no effect of *TRIM5* SNPs or haplotypes with the most natural *a priori* alternate hypothesis, that there are effects of TRIM5 variants that are additive for the genetic factors and progressive in susceptibility to infection. A proportional odds logistic regression was done (the polr function in *R*, using automatic forward selection by the Aikike information criterion (AIC) (stepAIC)) with *TRIM5* SNPs or haplotypes as explanatory variables, and ordinal susceptibility to infection (level 1: HREU; level 2: other uninfected; level 3: seroconverters) as the outcome variable. SNPs with minor allele frequency <0.05 were omitted. The results with actual data were compared with those from 5000 random permutations of the infection data, with the genotype data held fixed. Significance was measured as the proportion analyses of permuted for which the AIC was ≤ to the AIC for the actual data.

Kaplan–Meier survival statistics and the Cox proportional hazards model were used to assess the effects of SNPs and haplotypes on the rate of progression to AIDS. Three endpoints were evaluated: time to less than 200 $CD4^+$ cells/mm^3 (CD4<200); AIDS-1987 (Centers for Disease Control, 1987); and the expanded AIDS-1993 CDC case definitions (Centers for Disease Control, 1992) and AIDS-related death. AIDS-93 definition is defined as CD4<200 or any AIDS-defining condition in an HIV-1-infected person. The significance of genotypic associations and relative hazards (RH) for dominant and recessive genetic models were determined. Participants were stratified by sex and by age at seroconversion: 0–20, >20–40 and over 40 years (O'Brien et al., 2000). All P values are 2-tailed.

To assess the impact of the multiple comparison tests performed in our analysis, we estimated the Q value measuring false discovery rate (FDR), which is defined as the proportion of statistical tests called significant that are actually false positive (Storey and Tibshirani, 2003). An FDR of 5% means that 5% of the significant associations are false positive. We calculated Q value considering all the SNP association analyses of each SNP in AA and EA (Table 1) (Storey et al., 2003).

Linkage disequilibrium (LD) between all pairs of biallelic loci was quantified using the absolute value of Lewontin's D' (Lewontin, 1964; Long et al., 1995). Absolute values of D' range from 0 for independence to 1 for complete

linkage disequilibrium between the pairs. Haplotypes were inferred by the expectation maximization algorithm (Long et al., 1995). F_{st} estimates between EA and AA for the SNP alleles were calculated as described by Weir and Cockerham (1984).

Cells and antibodies

Cf2Th canine thymocytes were obtained from the American Type Culture Collection. The generation of a cDNA containing the amino-acid-coding sequence of the wild-type (wt) human *TRIM5*α gene was described previously (Stremlau et al., 2004). Cells stably expressing TRIM5α_{hu} variants were created using a retroviral transduction system. Retroviral vectors encoding the TRIM5α_{hu} variants were created using the pLPCX vector (Stratagene). The pLPCX-TRIM5α vectors contain only the amino-acid-coding sequence of the TRIM5α_{hu} cDNA. The TRIM5α_{hu} protein possesses a C-terminal epitope tag derived from influenza virus hemagglutinin (HA). Recombinant viruses were produced in 293T cells by cotransfecting these pLPCX plasmids with the pVPack-GP and pVPack-VSV-G packaging plasmids (Stratagene). The pVPack-VSV-G plasmid encodes the vesicular stomatitis virus (VSV) G envelope glycoprotein, which allows efficient entry into a wide range of vertebrate cells (Beyer et al., 2002; Hofmann et al., 1999; Yee et al., 1994). The resulting virus particles were used to transduce $\sim 1 \times 10^5$ Cf2Th cells in the presence of polybrene (5 μg/ml). Cells were selected in medium supplemented with 1 μg/ml of puromycin (Sigma).

The rat anti-HA antibody 3F10 was obtained from Roche. The anti-rat IgG conjugated with FITC was obtained from Santa Cruz.

Western blotting

Steady-state levels of TRIM5α proteins in the cell were determined by Western blotting with an anti-HA antibody, as described previously (Song et al., 2005c).

Immunofluorescence confocal microscopy

Cf2Th cells expressing the wild-type or variant TRIM5α_{hu} proteins were cultured overnight on Lab-Tek II Chamber Slides (Nalge Nunc International). Following fixation for 15 min in Cytofix/Cytoperm (BD Biosciences) and permeabilization for 15 min in Perm/Wash (BD Biosciences), the cells were incubated for 1 h with rat anti-HA 3F10 antibody (Roche). The cells were then incubated with an anti-rat IgG conjugated with FITC (Santa Cruz) and examined by confocal fluorescence microscopy.

Infection with viruses expressing GFP

Recombinant viruses (HIV-1-GFP, N-MLV-GFP and B-MLV-GFP) expressing the humanized Renilla reniformis green fluorescent protein (GFP) were prepared as previously described (Perron et al., 2004; Stremlau et al., 2004). The viral stocks were quantified by measuring reverse transcriptase activity, as described previously (Rho et al., 1981). Cf2Th cells expressing the wild-type (wt) and mutant TRIM5α proteins, or control Cf2Th cells transduced with the empty pLPCX vector, were incubated with various amounts of the recombinant viruses, as described (Perron et al., 2004; Stremlau et al., 2004). For infections, 3×10^4 Cf2Th cells seeded in 24-well plates were incubated with virus for 24 h. Cells were washed and returned to culture for 48 h and then subjected to FACS analysis with a FACScan (Becton Dickinson).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2006.06.031.

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