ZNRD1 (Zinc Ribbon Domain–Containing 1) Is a Host Cellular Factor That Influences HIV-1 Replication and Disease Progression

Ester Ballana,[1] Jordi Senserrich,[1] Eduardo Pauls,[1] Rosa Faner,[2] Josep Maria Mercader,[3] Frédéric Uyttebroeck,[4] Eduard Palou,[2] Maria Pau Mena,[1] Eulalia Grau,[1] Bonaventura Clotet,[1] Lidia Ruiz,[1] Amalio Telenti,[4] Angela Ciuffi,[4] and José A. Esté[1]

[1]Retrovirology Laboratory IrsiCaixa, Hospital Universitari Germans Trias i Pujol, Universitat Autònoma de Barcelona, and [2]Laboratori d'Immunobiologia per a la Recerca i Aplicacions Diagnòstiques, Banc de Sang i Teixits, Badalona, and [3]Barcelona Supercomputing Center, Barcelona, Spain; and [4]Institute of Microbiology, University Hospital Center and University of Lausanne, Lausanne, Switzerland

Background. Human immunodeficiency virus (HIV) takes advantage of multiple host proteins to support its own replication. The gene *ZNRD1* (zinc ribbon domain–containing 1) has been identified as encoding a potential host factor that influenced disease progression in HIV-positive individuals in a genomewide association study and also significantly affected HIV replication in a large-scale in vitro short interfering RNA (siRNA) screen. Genes and polymorphisms identified by large-scale analysis need to be followed up by means of functional assays and resequencing efforts to more precisely map causal genes.

Methods. Genotyping and *ZNRD1* gene resequencing for 208 HIV-positive subjects (119 who experienced long-term nonprogression [LTNP] and 89 who experienced normal disease progression) was done by either TaqMan genotyping assays or direct sequencing. Genetic association analysis was performed with the SNPassoc package and Haploview software. siRNA and short hairpin RNA (shRNA) specifically targeting ZNRD1 were used to transiently or stably down-regulate ZNRD1 expression in both lymphoid and nonlymphoid cells. Cells were infected with X4 and R5 HIV strains, and efficiency of infection was assessed by reporter gene assay or p24 assay.

Results. Genetic association analysis found a strong statistically significant correlation with the LTNP phenotype (single-nucleotide polymorphism rs1048412; $P = .0004$), independently of *HLA-A10* influence. siRNA-based functional analysis showed that ZNRD1 down-regulation by siRNA or shRNA impaired HIV-1 replication at the transcription level in both lymphoid and nonlymphoid cells.

Conclusion. Genetic association analysis unequivocally identified *ZNRD1* as an independent marker of LTNP to AIDS. Moreover, in vitro experiments pointed to viral transcription as the inhibited step. Thus, our data strongly suggest that ZNRD1 is a host cellular factor that influences HIV-1 replication and disease progression in HIV-positive individuals.

Humans show remarkable variation in their vulnerability to infection with human immunodeficiency virus (HIV), especially with respect to clinical outcome after infection [1]. The considerable heterogeneity in the epidemic is at least partially determined by variations in genes that influence virus replication and immunity [1]. The HIV type 1 (HIV-1) genome codes for only 15 proteins, requiring many cellular factors to complete the virus replication cycle [2]. Thus, a large number of host factors are likely to contribute to the variability of HIV-related phenotypes, including genetic variants that may be more or less advantageous for viral replication.

Recent advances in genomics and RNA interference have led to the conduct of genomewide surveys to identify cellular genes that affect human disease [3–5] and HIV replication [6–10]. The first genomewide association analysis for determinants of HIV-1 host control in humans identified a number of genomic regions associated with HIV load set point and/or disease pro-

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Reprints or correspondence: Dr José A. Esté, Fundació irsiCaixa, Laboratori de Retrovirologia, Hospital Universitari Germans Trias i Pujol, Ctra. Del Canyet s/n, Badalona, Barcelona 08916, Spain (jaeste@irsicaixa.es).

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Table 1. Phenotype Data on Patients Included in the Study

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gression [7]. Among them, a locus on human chromosome 6, close to the *ZNRD1* (zinc ribbon domain–containing 1) and *RNF39* (ring finger protein 39) genes but also to *HLA* loci, strongly correlated with HIV disease progression [7]. A genomewide functional screen also identified ZNRD1 among >250 host factors required for HIV-1 replication [6]. Knowledge of cellular factors and their interaction within the HIV-1 life cycle are essential for a better understanding of virus replication and disease pathogenesis, as well as for finding novel potential therapeutic targets [11]. At least one identified cellular gene, *CCR5* (CC chemokine receptor 5), has been successfully exploited as a target for anti-HIV intervention [12, 13].

Despite their enormous power and interest, large-scale genomewide screens should be taken only as starting points. Ideally, the identified genes should be analyzed in a physiologically relevant cell system, and genetic associations should be validated in independent populations.

ZNRD1 encodes a protein consisting of 2 zinc ribbon domains [14]. The C-terminal domain is well conserved in many organisms as a transcription-associated motif [15]. Homology searches identified *ZNRD1* as encoding an RNA polymerase I subunit [15]. By typing previously described and new polymorphisms in the *ZNRD1* genomic region together with *HLA-A10*, we unequivocally identify *ZNRD1* as an independent marker of long-term nonprogression (LTNP). In vitro experiments showed that knockdown of ZNRD1 expression inhibited HIV replication at transcription in both lymphoid and nonlymphoid cells.

METHODS

Patients and samples. In total, 208 HIV-infected patients (119 who experienced LTNP and 89 who experienced normal disease progression) were included in the study (Table 1). Progressors from the HIV unit of the Hospital Universitari Germans Trias i Pujol were selected on the basis of having a CD4 cell count of <200 cells/μL with 10 years or fewer of reported infection. Samples from LTNP patients were provided by the HIV BioBank, using eligibility criteria modified from that reported elsewhere [16]. Eligibility criteria were confirmed HIV infection for >10 years, CD4 cell counts of >500 cells/μL throughout the course of infection, and viral loads of <5000 copies/mL without antiretroviral therapy.

Blood samples were processed in accordance with current procedures. All patients who participated in the study provided informed consent, and protocols were approved by the Scientific Committee of Fundació IrsiCaixa.

Genotyping of DNA samples. The entire coding sequence of the *ZNRD1* gene was amplified by polymerase chain reaction (PCR) and sequenced using an ABI Prism 3100 genetic analyzer (Applied Biosystems). Primers were as follows: for fragment 1, 5′-CGAGACACGGTTCGCAATTA-3′ (forward) and 5′-CAACCCAACCGATCTTGAGT-3′ (reverse); for fragment 2, 5′-GGCGGTTGTACATTTGGTCT-3’ (forward) and 5′-AATAAGGGATGGGACCAAGG-3′ (reverse); and for fragment 3, 5′-GTTTAGGGGAGCCAGTCCTCC-3′ (forward) and 5′-GCCTCATTCCTGACTCTACTTTT-3′ (reverse).

The rs3869068 single-nucleotide polymorphism (SNP) was typed using the TaqMan SNP genotyping assay (C 26544924_10; Applied Biosystems). *HLA-A10* typing of DNA samples was performed using the HLA-B low-resolution bulk SSP kit (Olerup) [17].

Cells. HeLa-P4R5-MAGI, TZM-bl, SupT1, and MOLT-CCR5 cells (AIDS Research and Reference Reagent Program, US National Institutes of Health) were cultured in Dulbecco modified Eagle medium (DMEM; Gibco) or Roswell Park Memorial Institute (RPMI) 1640 L-glutamine medium (Gibco). Culture media were supplemented with 10% heat-inactivated fetal calf serum and antibiotics.

Drugs. Zidovudine was purchased from Sigma-Aldrich. HIV integrase inhibitor L-731988 was obtained from Merck [18]. Fusion inhibitor T-20 was synthesized by the Service of Peptide Synthesis, University of Barcelona (Spain).

Short interfering RNAs. Short interfering RNAs (siRNAs) targeting *ZNRD1* transcript (ZNRD1_1, UCGCUGUGGCUUCAACAUCA; ZNRD1_2, CUCGAUGUGGUCAUGAAGGAA) and *RNF39* transcript (RNF39_1, ACGCCCATTGCAGGAGTATTA; RNF39_2, CCGCCGCAGCCTGAGGTCTAA) were purchased from Qiagen. Nontargeting siRNA control (siNT) was a commercially available pool from Dharmacon; siRev/Env (siRNA targeting a viral sequence) has been described elsewhere [19].

Generation of cell lines stably expressing short hairpin RNAs. Commercial self-inactivating lentiviral expression vectors (pLKO.1-puro; Sigma) were used to express a short hairpin RNA (shRNA) targeting *ZNRD1* (shZNRD1, UGUGGCUUCAACAUCAACGUU). Briefly, shRNA-expressing lentiviruses were generated by cotransfecting in 293-T cells the pLKO-1 vector, a helper plasmid (psPAX2), and a vesicular stomatitis virus G protein–expressing plasmid. Target cells were infected and shRNA-expressing cells selected as described elsewhere [19].

Western blot. Cells were harvested, washed, and lysed in chilled hypotonic buffer (cell lysis buffer; Invitrogen). After running the samples, membranes were blocked, incubated with primary antibodies (ZNRD1, 1:1000, Abnova; Actin, 1:2000,

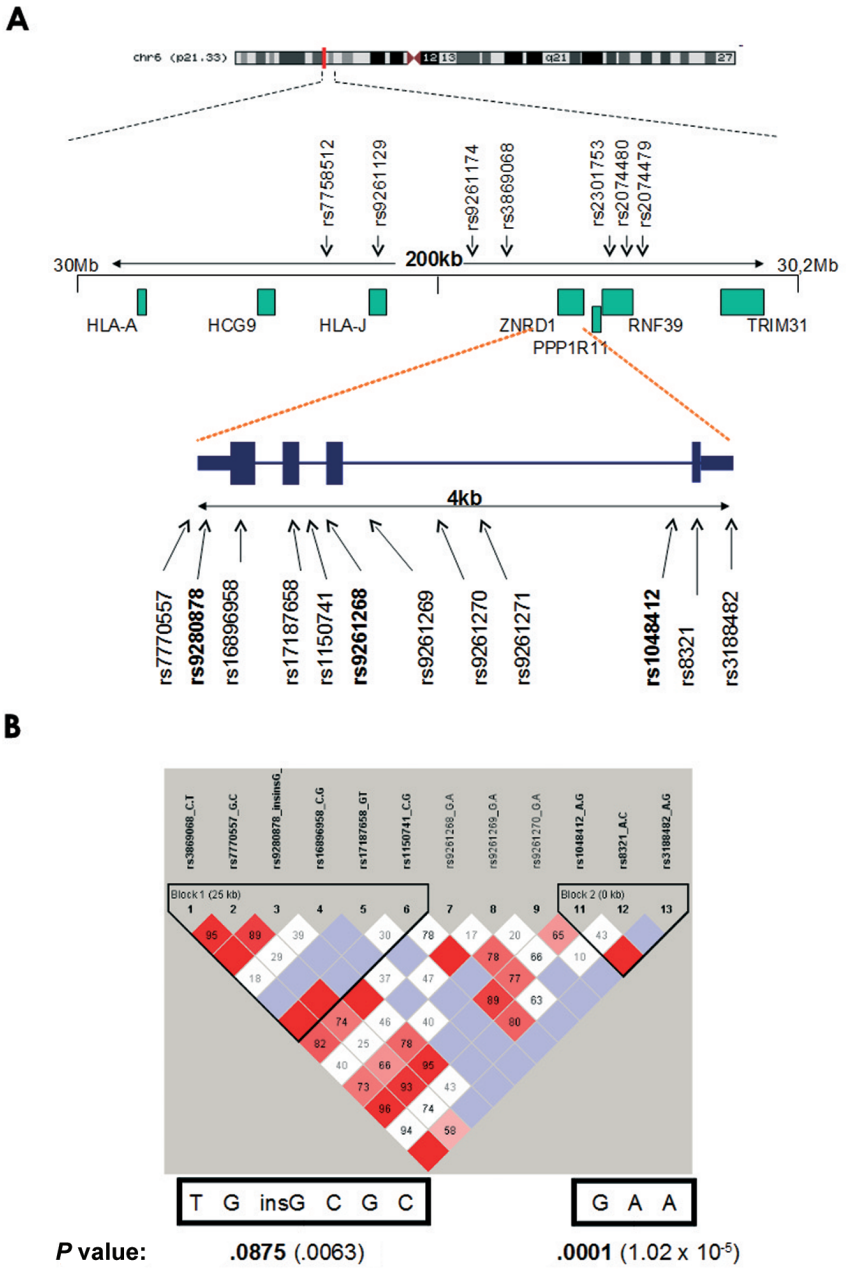

Figure 1. Polymorphisms analyzed and the linkage disequilibrium (LD) pattern of the *ZNRD1* gene. *A*, Genomic location of genes (*green boxes*) and single-nucleotide polymorphisms (SNPs) (*arrows*) present on chromosome 6p21. In the upper panel, the relative location of SNPs previously associated with differential human immunodeficiency virus type 1 clinical outcomes is represented [7, 30, 31]. Below, the *ZNRD1* gene region screened with relative positions of analyzed SNPs is shown. SNPs with positive associations after Bonferroni correction are shown in boldface type. *B*, Linkage disequilibrium (LD) blocks in the *ZNRD1* gene, estimated by means of Haploview software following the 4-gamete-rule algorithm. Haplotypes associated with long-term nonprogression are indicated in the boxes, along with corresponding corrected *P* values (nominal *P* values are shown in parentheses). Correction for multiple testing was performed by permutation analysis. Colors and values within the LD plot correspond to D′/LOD and D′ LD values, respectively (D′ is the value of D [Hedridgeos multiallelic D] between the 2 loci; LOD is the log of the likelihood odds ratio, a measure of confidence in the value of D′).

Santa Cruz Biotechnology) overnight at 4°C, and then incubated with a horseradish peroxidase–conjugated antibody [20]. Membranes were revealed with SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology). For RNF39, different commercial antibodies were tested, but none of them could specifically detect RNF39 protein (data not shown).

Real-time quantitative PCR. Total RNA was obtained using the RNeasy Mini kit (Qiagen). Relative levels of *ZNRD1*

The figure is available in its entirety in the online edition of *Clinical Infectious Diseases*.

Figure 2. Linkage disequilibrium (LD) pattern of the chromosome 6 region associated with human immunodeficiency virus (HIV) disease progression.

and *RNF39* messenger RNAs (mRNAs) were measured by real-time quantitative PCR (qPCR) and normalized to *GUSB* mRNA expression. For human genes, primers and DNA probes were commercially purchased (assay-on-demand Hs00205908_m1 for *ZNRD1*, Hs00961882_m1 for *RNF39*, and Hs99999908_m1 for *GUSB*; Applied Biosystems).

Viral DNA was extracted using a QiaAmp DNA extraction kit (QIAmp DNA Blood Mini kit; Qiagen). Quantitative amplification of the long terminal repeat [LTR] for viral entry detection was performed as described elsewhere (primers, 5′-CAAGCAGCCATGCAAATGTT-3′ [forward] and 5′-TGCACTGGATGCAATCTATCC-3′ [reverse]; probe, 5′-AAAGAGACCATCAATGAGGAAGCTGCAGA-3′) [21]. Viral integration was detected by Alu-LTR preamplification followed by LTR qPCR [22].

For analyzing HIV-1 transcription, complementary DNA was used for qPCR of total viral RNA, unspliced RNA, and multiply spliced RNA, as previously elsewhere [23].

HIV infection and replication. HIV-1 stocks of NL4-3 and clinical isolate 92UG024 were grown in lymphoid MT-4 cells, as described elsewhere [24, 25]. The R5 HIV-1 strain BaL was grown in peripheral blood mononuclear cells stimulated with phytohemagglutinin and interleukin 12.

HeLa-P4R5-MAGI or TZM-bl cells were seeded in 96-well plates and infected; 48 h after infection, cells were lysed and kept frozen until the β-galactosidase assay was performed. SupT1 cells [25] were infected for 4 h, washed, and resuspended in RPMI 1640 medium. HIV production was analyzed 3, 5, or 7 days after infection by HIV p24 enzyme-linked immunosorbent assay (Innotest HIV p24 antigen; Innogenetics) in culture supernatants.

β-Galactosidase detection assay. β-Galactosidase activity in 30 μL of cell extract was quantified by a colorimetric assay, as described elsewhere [20, 26]. Absorbance was measured at 405–620 nm.

siRNA and plasmid transfection. TZM-bl cells (1.5×10^5) were seeded in 24-well plates. A day later, siRNA or plasmids were mixed with Lipofectamine 2000 reagent (Invitrogen) in serum-free medium (Invitrogen) and then added to previously washed cells. After 4 h, fresh DMEM was added [20, 26]. Plasmid DNA was transfected in SupT1 cells by means of Amaxa Nucleofection technology (Lonza), in accordance with the manufacturer's recommendations [20, 26]. Then, cells were recovered and seeded in prewarmed 24-well plates. Cell viability and expression were monitored 2 days after transfection.

RNA from Jurkat cells was extracted using the RNeasy kit (Qiagen), in accordance with the manufacturer's instructions. After reverse transcription with hexamers (Expand RT; Roche), *ZNRD1* was amplified using the following primers, which introduced, respectively, *Eco*RI and *Cla*I (shown in boldface type): 5′-**GAATTC**gccaccATGTCTGTCATGGACCTCGCCAATAC-3′ and 5′-**ATCGAT**ggctcaAGAGTCTTCCTTCTCCTGGAACTTG-3′. PCR products were digested with *Eco*RI and *Cla*I, introduced into the pLPCX expression vector (Clontech), and checked by sequencing.

Statistical analysis. The paired Student *t* test was used for comparison between groups. Associations for each SNP and odds ratios were computed using logistic regression, as implemented in SNPassoc R library software [27]. The reference class was homozygosity for the major allele among controls. Analyses were done under 4 different genetic models: codominant, dominant, recessive, and additive [28]. The best model was chosen using the Akaike information criteria. Bonferroni correction for 13 nonmonomorphic SNPs was used to correct for multiple comparisons. An additional factor of correction of 2.5 was applied to account for the use of 4 different genetic models. Using this criterion, the corrected level of statistical significance was set to .0023.

Haplotype blocks were estimated using the 4-gamete-rule algorithm, as implemented in Haploview software [29]. Haplotype comparisons between groups were performed using the χ^2 test. Correction for multiple testing was performed by permutation analysis (100,000 random permutations).

RESULTS

Association between polymorphisms in the* ZNRD1 *gene and the LTNP phenotype. The genetic location of *ZNRD1* is shown in Figures 1 and 2. To dissect and map the putative causal gene influencing HIV disease progression on the human chromosome 6 locus, the entire coding region of *ZNRD1* was resequenced in HIV-positive progressor patients and LTNP patients. In addition, previously associated polymorphisms—the rs3869068 SNP, near rs9261174 [7], and HLA-A10 [32]—were typed.

Fifteen SNPs were found throughout the gene. Twelve SNPs were informative enough to undergo genetic association anal-

Table 2. Position, Hardy-Weinberg (HW) Equilibrium, and Genotype Frequency of the Analyzed Single-Nucleotide Polymorphisms

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Table 3. Associations between *ZNRD1* Single-Nucleotide Polymorphisms (SNPs) and Long-Term Nonprogression (LTNP)

SNP	Patients, no. (%)		Crude		P^a after adjustment for HLA-A10
	LTNP	Progression	P^a	OR (95% CI)	
rs9280878 (dominant model)			.00074[b]		.03145[c]
NI/NI	85 (73.9)	82 (92.1)		1.00 (reference)	
NI/insG, insG/insG	30 (26.1)	7 (7.9)		0.25 (0.1–0.6)	
rs9261268 (additive model)			.00049[b]		.00944[c]
GG	72 (74.3)	80 (92.0)		1.00 (reference)	
GA	22 (21.0)	7 (8.0)		0.27 (0.11–0.63)	
AA	5 (4.8)	0 (0)			
rs1048412 (recessive model)			4.37×10^{-5}[b]		.00046[b]
AA, AG	90 (87.4)	89 (100)		1.00 (reference)	
GG	13 (12.6)	0 (100)		0.0375 (0.002–0.64)	

NOTE. Shown are frequencies, *P* values, and odds ratios (ORs) for the 3 SNPs with statistically significantly associations with the LTNP phenotype, before and after adjustment for the presence of HLA-A10. All data are adjusted for sex. CI, confidence interval; insG, G insertion; NI, no insertion.

[a] *P* value for SNP in best-fitting genetic model.

[b] Statistically significant after Bonferroni correction ($P < .0023$).

[c] Statistically significant by $P < .05$ only.

ysis, together with rs3869068 (Figure 1*A*). All SNPs were in Hardy-Weinberg equilibrium and had a successful genotyping frequency of >90% (Table 2).

Three of the SNPs—rs9280878 ($P = .00074$, dominant model), rs9261268 ($P = .00049$, additive model), and rs1048412 ($P = 4.37 \times 10^{-5}$, recessive model)—were found to be statistically significantly associated with the LTNP phenotype after Bonferroni correction (defined as $P < .0023$) (Table 3). The rs3869068 SNP showed a positive nominal *P* value (recessive $P = .0029$). To complete our analysis, HLA-A10 genotypes were added to the model. Despite the high linkage disequilibrium of the genomic region, the rs1048412 SNP still remained statistically significantly associated with disease progression ($P = .00046$) (Table 1), whereas HLA-A10 was not associated with progression after Bonferroni correction ($P = .00938$).

The linkage disequilibrium structure of the *ZNRD1* genomic region was determined, with 2 haplotype blocks determined (Figure 1*B*). The larger block contained 6 SNPs and included 3 *ZNRD1* exons. The second block contained 3 SNPs and included the last exon of the *ZNRD1* gene (Figure 1*B*). Positive associations, with analysis performed using all possible marker haplotypes contained in the blocks, were found and involved a haplotype in each of the blocks (Figure 1*B*) [29].

Inhibition of HIV-1 replication due to down-regulation of ZNRD1 expression but not of RNF39 expression. The effect of *ZNRD1* and *RNF39* on HIV-1 infection was evaluated using RNA interference. HeLa-P4R5-MAGI cells were transiently transfected with siRNAs targeting ZNRD1 (siZNRD1_1 and siZNRD1_2) and RNF39 (siRNF39_1 and siRNF39_2), and their effect was assessed by real-time qPCR; siZNRD1_1 and RNF39_2 were the sequences that achieved the best silencing (Figure 3*A*). Western blot analysis of ZNRD1 protein levels showed a correlation with mRNA levels (Figure 3*B*).

siRNA-transfected HeLa-P4R5-MAGI cells were infected with the X4-tropic NL4-3 strain. Transfection with siZNRD1_1 inhibited HIV-1 replication (Figure 3*C*), compared with that in mock-transfected or siNT-transfected cells, whereas neither of the siRNAs targeting RNF39 was able to inhibit replication. siZNRD1_2, which produced only a mild reduction in ZNRD1 expression levels, did not statistically significantly inhibit HIV-1 replication (Figure 3*C*).

The role played by ZNRD1 in HIV-1 infection was confirmed by infecting siRNA-treated HeLa-P4R5-MAGI cells with the R5-tropic laboratory-adapted strain BaL and the clinical isolate UG024 (Figure 3*D*). ZNRD1 silencing impaired viral replication of BaL and UG024 to an extent similar to that of NL4-3 (mean inhibition ± standard deviation [SD], 63% ± 15% for NL4-3, 70% ± 15% for BaL, and 72% ± 6% for UG024) (Figure 3*D*).

TZM-bl cells stably expressing a shRNA targeting ZNRD1 ($TZM_{shZNRD1}$) or a nontargeting control shRNA (TZM_{shCTRL}) were generated. Specific down-regulation of *ZNRD1* gene expression was confirmed at the mRNA (Figure 3*E*) and protein (Figure 4) levels. Acute infection of $TZM_{shZNRD1}$ cells inhibited HIV-1 NL4-3 replication (mean decrease ± SD, 60% ± 0.1%), compared with that in wild-type TZM-bl cells or TZM_{shCTRL} cells (mean ± SD, 101% ± 3%) (Figure 3*F*). No changes were observed in cell viability or in CD4 receptor and HIV coreceptor cell-surface expression (data not shown).

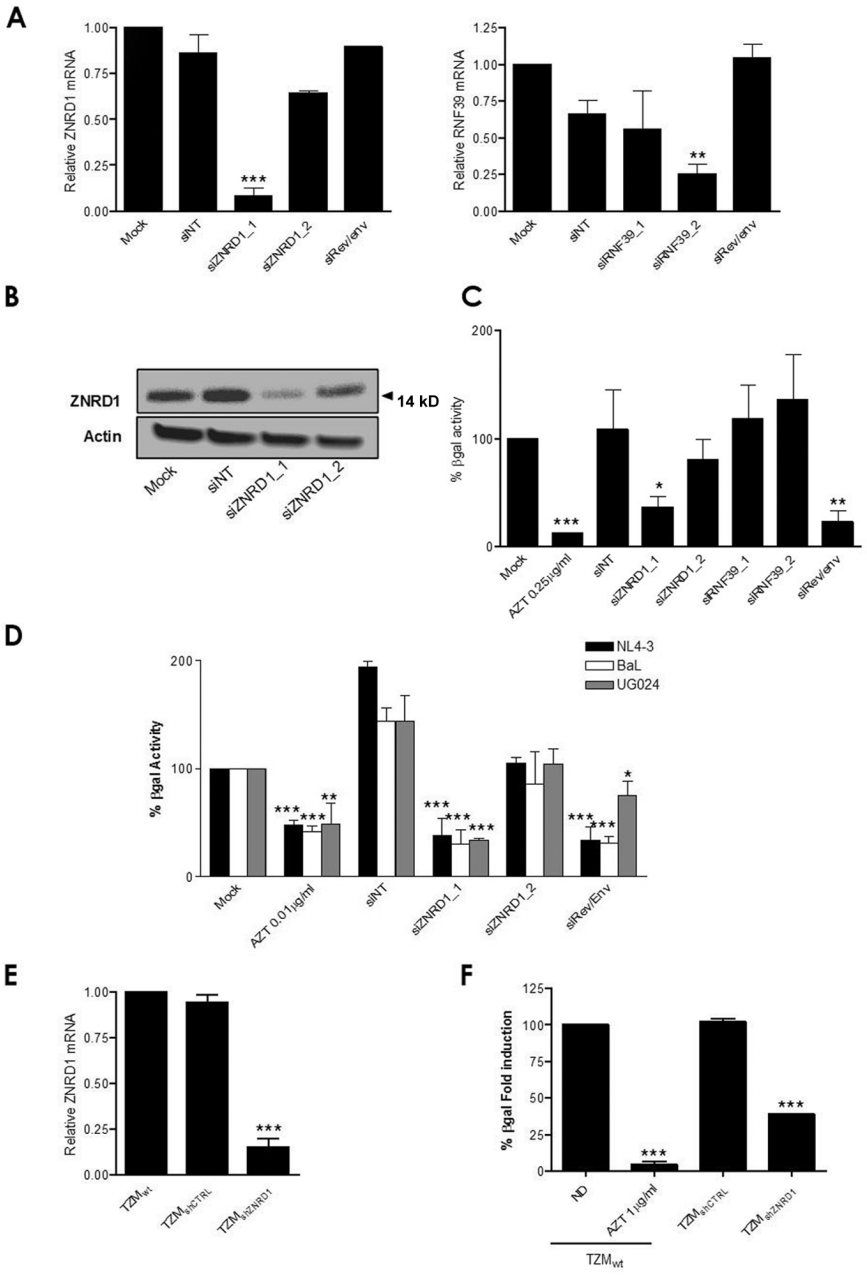

Figure 3. Inhibition of human immunodeficiency virus type 1 (HIV-1) replication due to *ZNRD1* RNA interference–mediated silencing in HeLa-derived cell lines. *A*, RNA interference–mediated inhibition of ZNRD1 and RNF39 transcript expression. Relative messenger RNA (mRNA) quantification of ZNRD1 and RNF39 in HeLa-P4R5-MAGI cells transfected with different short interfering RNAs (siRNAs) targeting these cellular transcripts is shown. A nontargeting siRNA (siNT) and a siRNA targeting a viral sequence (siRev/Env) were used as controls. Values were normalized to those of mock-transfected cells. Means ± standard deviations (SDs) for 3 independent experiments are represented. *B*, Western blot of ZNRD1 in HeLa-P4R5-MAGI cells transfected with siRNA targeting ZNRD1 transcript. *C*, Inhibition of HIV-1 replication in siRNA targeting ZNRD1, but not RNF39, mRNA. The percentage of HIV-1 NL4-3 replication compared with mock control is shown. Means ± SDs for at least 3 independent experiments are represented. *D*, Inhibition of replication of R5 and X4 HIV-1 strains by siZNRD1-treated cells. The percentage of viral replication for 3 different viruses compared with the mock control is shown. Means ± SDs for 3 independent experiments are represented. *E*, ZNRD1 mRNA stable down-regulation by short hairpin RNA (shRNA) in TZM-bl cells. Relative ZNRD1 mRNA quantification in wild-type TZM-bl cells (TZM_{wt}), cells harboring a nontargeting control shRNA (TZM_{shCTRL}), and stably silenced ZNRD1 cells ($TZM_{shZNRD1}$) is shown. Values were normalized to those of wild-type cells. Means ± SDs for 3 independent experiments are represented. *F*, Inhibition of HIV-1 replication in $TZM_{shZNRD1}$ cells. The percentage of HIV-1 NL4-3 replication compared with that in wild-type cells is shown. Means ± SDs for at least 3 independent experiments are represented. βgal, β-galactosidase; ND, no drug. $^{*}P < .05$; $^{**}P < .005$; $^{***}P < .0005$.

The figure is available in its entirety in the online edition of *Clinical Infectious Diseases*.

Figure 4. Inhibition of ZNRD1 protein expression in TZM-bl cells.

Effect of ZNRD1 on HIV-1 replication in lymphoid cells. A SupT1 lymphoid cell line stably down-regulating ZNRD1 expression ($SupT1_{shZNRD1}$) or harboring a shRNA targeting the luciferase gene ($SupT1_{shLUC}$) were generated (Figure 5*A*). Changes in the expression of CD4, CXCR4, or CD8 were not detected (data not shown), nor were any detrimental effects on cell viability or proliferation observed in a growth kinetics curve (Figure 5*B*).

Acute infection of the engineered shRNA-expressing SupT1 cells with NL4-3 showed that ZNRD1 down-regulation inhibited HIV-1 replication (mean inhibition ± SD, 64% ± 19%), compared with that in wild-type or control cells (mean inhibition ± SD, 5% ± 20%) (Figure 5*C* and 5*D*).

Restoration of HIV replication due to complementation of wild-type ZNRD1 expression in shZNRD1 cells. SupT1 and TZM-bl cell lines stably down-regulating ZNRD1 expression were transfected with a wild-type ZNRD1 expression plasmid (pZNRD1) or, alternatively, with a green fluorescent protein expression plasmid as a control. Recovery or overexpression of wild-type ZNRD1 was measured by real-time qPCR, which showed an increase in ZNRD1 expression in pZNRD1-transfected cells, compared with that in mock-transfected cells (Figure 6). Overexpression of the wild-type protein completely restored *ZNRD1* mRNA expression levels in SupT1 cells, in which shZNRD1 inhibition was more modest, but only partially restored them in TZM-bl cells, in which inhibition was more potent (Figure 6). Acute infection with NL4-3 restored HIV-1 replication levels in both $TZM_{shZNRD1}$ and $SupT1_{shZNRD1}$ cells transfected with the ZNRD1 expression plasmid (mean ± SD, 51% ± 4% for $TZM_{shZNRD1}$ and 150% ± 40% for $SupT1_{shZNRD1}$), compared with those in mock-transfected cells (mean ± SD, 13% ± 10% for $TZM_{shZNRD1}$ and 42% ± 3% for $SupT1_{shZNRD1}$) or cells transfected with the control plasmid (mean ± SD, 8% ± 1% for $TZM_{shZNRD1}$ and 29% ± 5% for $SupT1_{shZNRD1}$) (Figure 7).

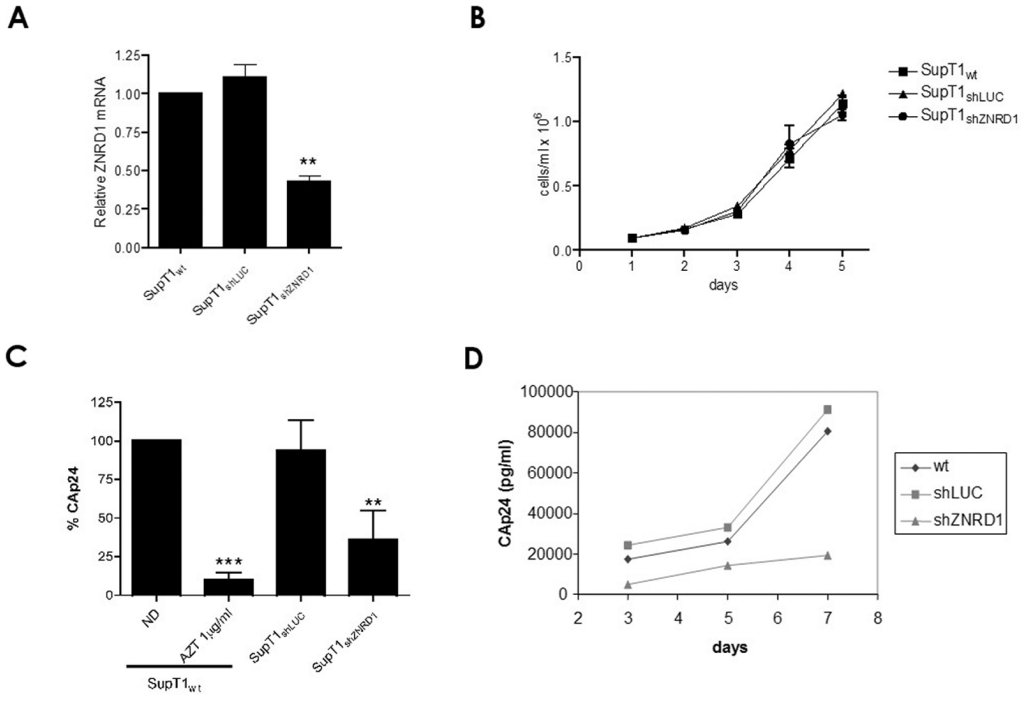

Figure 5. Impairment of human immunodeficiency virus type 1 (HIV-1) replication in lymphoid cells due to *ZNRD1* inhibition by short hairpin RNA (shRNA). *A*, ZNRD1 messenger RNA (mRNA) stable down-regulation by shRNA in SupT1 cells. Relative ZNRD1 mRNA quantification in wild-type SupT1 cells ($SupT1_{wt}$), cells harboring a control shRNA targeting luciferase ($SupT1_{shLUC}$), and stably silenced ZNRD1 cells ($SupT1_{shZNRD1}$) is shown. Values were normalized to those of wild-type cells. Means ± standard deviations (SDs) for 3 independent experiments are represented. $^{**}P < .005$. *B*, Growth kinetics of SupT1 cells containing shRNAs. Cell growth during 5 days was measured as the number of cells per milliliter. Means ± SDs for 2 different measurements are represented. *C*, Inhibition of HIV-1 replication in $SupT1_{shZNRD1}$ cells. Percentage of HIV-1 NL4-3 replication compared with wild-type cells is shown. Means ± SDs for at least 3 independent experiments are represented. ND; no drug. $^{**}P < .005$; $^{***}P < .0005$. *D*, Time course of p24 production in SupT1 cells. p24 production in cell supernatants was measured 3, 5, and 7 days after infection. Inhibition of HIV-1 NL4-3 replication was maintained during the course of infection. A representative experiment is shown. CAp24, HIV-1 capsid 24 antigen.

The figure is available in its entirety in the online edition of *Clinical Infectious Diseases*.

Figure 6. Restoration of ZNRD1 expression in TZM and SupT1 cell lines.

Effect of ZNRD1 on viral transcription from the LTR promoter. MOLT-CCR5 cells persistently infected with HIV-1 strain NL4-3 were cocultured together with HeLa-P4R5-MAGI cells transfected with siZNRD1_1 or siNT (nontargeting siRNA control). Fusion was monitored by intracellular β-galactosidase staining of overnight cocultures. ZNRD1 depletion did not alter HIV-1 envelope–mediated fusion (Figure 8).

Proviral DNA was measured 8 h after infection in $TZM_{shZNRD1}$ and $SupT1_{shZNRD1}$ cells together with their respective control and parental cell lines. No differences were observed between shZNRD1 and control cells (Figure 9*A*), indicating that ZNRD1 down-regulation did not affect viral entry or reverse transcription [21]. Viral integration at 24 h was quantified by Alu-LTR PCR followed by proviral DNA PCR [22]. Again, ZNRD1 inhibition did not alter HIV-1 proviral DNA integration (Figure 9*A*). Similar results were obtained with HeLa-P4R5-MAGI cells transfected with siZNRD1 (data not shown).

Viral transcription was measured 3 days after infection by real-time qPCR of different viral mRNA species (*gag*, *nef*, and multiply spliced *tat/rev/nef*). In all cases, *ZNRD1* silencing led to a statistically significant decrease in the relative amount of viral mRNA, compared with that in control or wild-type cells (Figure 9*B*). Taken together, these results pointed to viral transcription as the step inhibited by *ZNRD1* silencing.

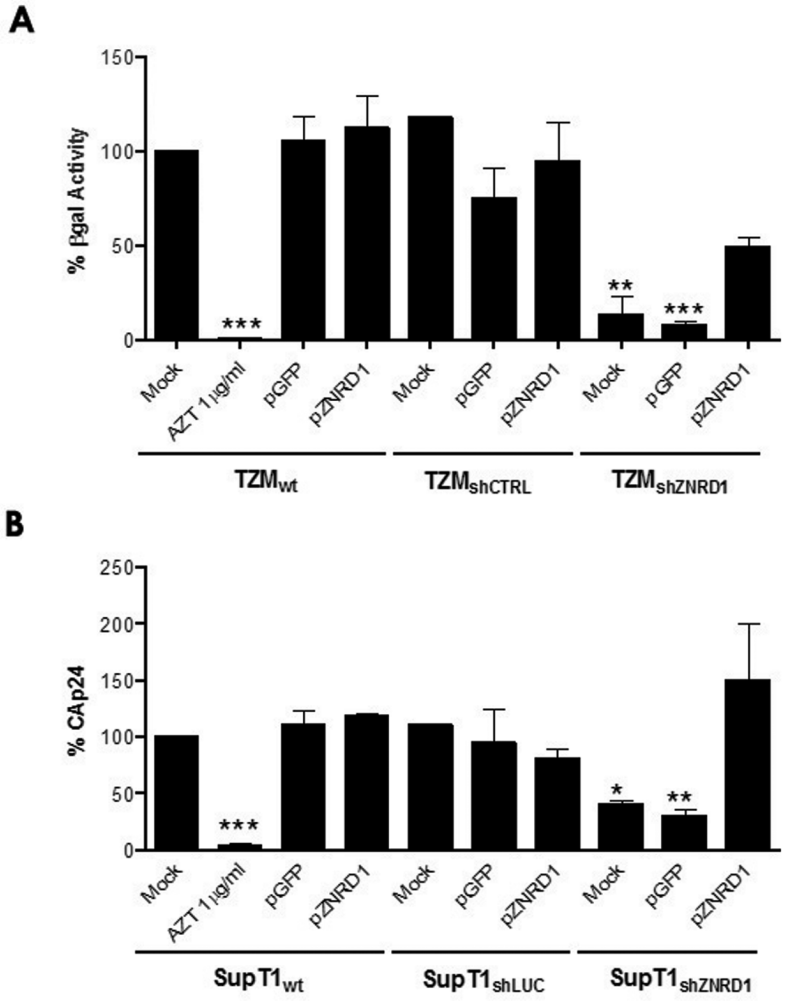

Figure 7. Recovery of human immunodeficiency virus type 1 (HIV-1) replication due to restoration of ZNRD1 expression in TZM-$bl_{shZNRD1}$ and $SupT1_{shZNRD1}$ cell lines. The percentage of HIV-1 NL-43 replication in the different TZM-bl cells (*A*) or SupT1 cells (*B*) transfected with a green fluorescent protein–expressing control plasmid (pGFP) or a plasmid expressing wild-type ZNRD1 (pZNRD1) is shown. HIV-1 replication was recovered in shZNRD1 cells when ZNRD1 expression was restored. Means $\pm$ standard deviations for at least 3 independent experiments are represented. βgal, β-galactosidase; CAp24, HIV-1 capsid 24 antigen. $^*P < .05$; $^{**}P < .005$; $^{***}P < .0005$.

The figure is available in its entirety in the online edition of *Clinical Infectious Diseases*.

Figure 8. No inhibition of human immunodeficiency virus (HIV) fusion due to ZNRD1 silencing.

DISCUSSION

The results of different genomewide screens have been published, which have aimed to describe genes that confer different susceptibilities to AIDS or cellular genes that are functionally required for viral replication [6–8, 10, 30, 33]. Strikingly, little overlap can be identified between them [4], therefore making essential the replication of genetic data in independent cohorts and further functional validation and characterization of selected genes [34].

Here, taking as a starting point the results of genomewide analyses [6, 7], we validated, clarified, and functionally characterized the implication of ZNRD1 in HIV-1 replication. Using RNA interference, we clearly demonstrated that ZNRD1 depletion, but not RNF39 depletion, impaired HIV replication. Importantly, ZNRD1 down-regulation hampered HIV repli-

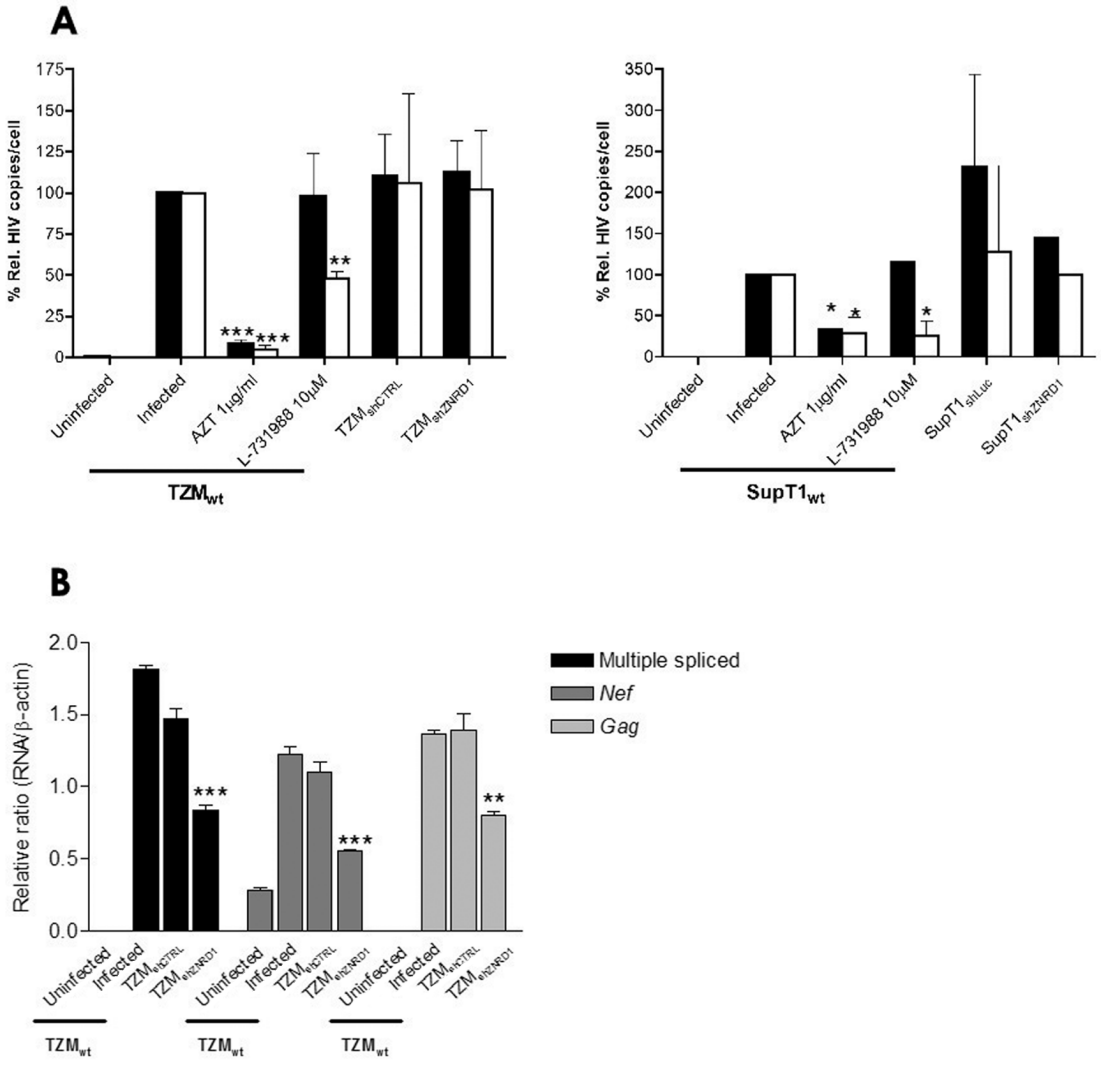

Figure 9. Inhibition of human immunodeficiency virus type 1 (HIV-1) transcription due to down-regulation of ZNRD1 expression. *A*, HIV-1 proviral DNA, measured by quantitative polymerase chain reaction (qPCR), in TZM-bl and SupT1 cells at 8 (*black bars*) and 24 (*white bars*) h after infection, in this case preceded by a first round of Alu–long terminal repeat PCR. The number of HIV-1 proviral copies per cell was obtained using a standard curve and quantification of the cellular gene *RNAseP*. Values are expressed as percentages compared with those in wild-type cells. Means ± standard deviations (SDs) for 3 independent experiments are represented. AZT, zidovudine. *B*, Relative ratios of different HIV-1 transcripts (*nef*, *gag*, and multiply spliced messenger RNAs) measured by real-time qPCR and normalized to β-actin in TZM-bl cells 3 days after infection. Means ± SDs for 3 replicates of a representative experiment are represented. $*P < .05$; $**P < .005$; $***P < .0005$.

cation in lymphoid cell lines without altering cell viability or proliferation, expanding its relevance to cells that are natural HIV targets. Moreover, we narrowed the molecular step at which ZNRD1 affects viral replication to the transcriptional level. Importantly, we found that genetic polymorphisms located in the *ZNRD1* gene were statistically significantly associated with the LTNP phenotype independently of HLA-A effects, thus providing further indication that the *ZNRD1* gene is a novel cofactor that affects the course of HIV infection both in vitro and in vivo.

Every step of the HIV life cycle depends on the cellular machinery [35]. For ZNRD1, we have narrowed down its mode of action to viral transcription from the LTR promoter, discarding any effect on early events of virus replication. In agreement with our data, Brass et al [6] have suggested that ZNRD1 could act before Gag translation. Additional work will be needed to clearly understand the mode of action of ZNRD1 in HIV-1 infection. An important element to elucidate would be the apparent partial effect of transient and stable RNA interference of ZNRD1. In addition, the potency of RNA-dependent knockdown appeared to correlate with the potency of inhibition of virus replication, but total knockout could not be achieved. These results, together with the observation that complementation of ZNRD1 expression in wild-type cells did not increase virus replication, suggest that a low level of expression of ZNRD1 may be sufficient to allow replication and that complete gene knockout should be required to abolish virus production.

Human susceptibility to viral infection can be seen as the final result of a dynamic interplay between the genetic makeup of the individual host and the pathogen, with environmental influences adding another layer of complexity. In the middle of such an intricate picture, however, several genetic polymorphisms have been described that clearly influence the outcome of HIV infection, specially in the *HLA* region, where *ZNRD1* is located [7, 31, 36, 37]. Genetic complexity and the high linkage disequilibrium of the region challenges the effect of *ZNRD1* on HIV disease progression [32, 37]. Unlike the findings of these studies, our data point to a role for the *ZNRD1* gene in HIV disease progression independent of the HLA-A10 allele, a factor proposed to be responsible for much of the effect of *ZNRD1* [32]. Polymorphisms in the *ZNRD1* gene remained statistically significantly associated with the LTNP phenotype when HLA-A10 was introduced as a covariable in the analysis. In addition, the effect of HLA-A10 was not statistically significantly associated with progression, suggesting a more discrete effect (if any) and an independent role for *ZNRD1*, in accordance with the findings of other association studies [7, 30, 31, 37]. Although the earlier studies were based on the complex spatial structure and linkage disequilibrium pattern of a relatively large region, we took a more focused approach on individual polymorphisms around the *ZNRD1* gene, because of its demonstrated effect on HIV-1 replication in cell culture.

Observations of determinants for host control of infection based on SNPs need to be replicated in relevant populations, followed by deeper genomic and functional analyses of the associated region to verify putative causal variants. Identification of polymorphisms in relevant genes might be the key to unraveling the basis of the pathogenesis of HIV-1 disease. We did not identify any point mutations or polymorphisms with clear functional implications, either at the mRNA or protein level. However, the rs9280878 SNP has been predicted to create a novel splice site that leads to a different mRNA isoform without altering the gene coding sequence. Moreover, the SNP with the lowest *P* value (rs1048412) is located in the 3′ untranslated region of the *ZNRD1* gene, a region putatively involved in posttranscriptional mRNA regulation by, for example, microRNAs [38]. Thus, the possibility that the identified SNPs might have an effect on mRNA properties could not be discarded [39]. Polymorphisms or haplotypes that affect such features as mRNA biogenesis, transport, or stability may have an effect on ZNRD1 and lead to altered expression levels, with effects on HIV replication resembling the results obtained in vitro. Future work will shed light on the putative regulatory mechanisms (if any) that underlie the identified SNPs.

A key therapeutic strategy for treating HIV-positive patients has been to simultaneously target multiple virus-encoded proteins to overcome the emergence of resistance. The identification and characterization of cellular cofactors represent a promising alternative. Indeed, the use of cellular genes as targets for antiviral drugs is already becoming a reality, as shown for the CCR5 coreceptor and its antagonist, maraviroc [40, 41]. Targeting multiple host cell gene products would minimize the acquisition of drug resistance and provide long-lasting blocking of virus replication.

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References

1. O'Brien SJ, Nelson GW. Human genes that limit AIDS. Nat Genet **2004**; 36(6):565–574.
2. Frankel AD, Young JA. HIV-1: fifteen proteins and an RNA. Annu Rev Biochem **1998**; 67:1–25.

3. Glazier AM, Nadeau JH, Aitman TJ. Finding genes that underlie complex traits. Science **2002**; 298(5602):2345–2349.
4. Goff SP. Knockdown screens to knockout HIV-1. Cell **2008**; 135(3): 417–420.
5. Goldstein DB. Genomics and biology come together to fight HIV. PLoS Biol **2008**; 6(3):e76.
6. Brass AL, Dykxhoorn DM, Benita Y, et al. Identification of host proteins required for HIV infection through a functional genomic screen. Science **2008**; 319(5865):921–926.
7. Fellay J, Shianna KV, Ge D, et al. A whole-genome association study of major determinants for host control of HIV-1. Science **2007**; 317(5840):944–947.
8. Konig R, Zhou Y, Elleder D, et al. Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication. Cell **2008**; 135(1):49–60.
9. Zhou H, Xu M, Huang Q, et al. Genome-scale RNAi screen for host factors required for HIV replication. Cell Host Microbe **2008**; 4(5): 495–504.
10. Siddiqui RA, Sauermann U, Altmuller J, et al. X chromosomal variation is associated with slow progression to AIDS in HIV-1-infected women. Am J Hum Genet **2009**; 85(2):228–239.
11. Greene WC, Debyser Z, Ikeda Y, et al. Novel targets for HIV therapy. Antiviral Res **2008**; 80(3):251–265.
12. Lederman MM, Este J. Targeting a host element as a strategy to block HIV replication: is it nice to fool with Mother Nature? Curr Opin HIV AIDS **2009**; 4(2):79–81.
13. Este JA, Telenti A. HIV entry inhibitors. Lancet **2007**; 370(9581):81–88.
14. Hong L, Piao Y, Han Y, et al. Zinc ribbon domain-containing 1 (ZNRD1) mediates multidrug resistance of leukemia cells through regulation of P-glycoprotein and Bcl-2. Mol Cancer Ther **2005**; 4(12): 1936–1942.
15. Coriton O, Lepourcelet M, Hampe A, Galibert F, Mosser J. Transcriptional analysis of the 69-kb sequence centromeric to HLA-J: a dense and complex structure of five genes. Mamm Genome **2000**; 11(12): 1127–1131.
16. Garcia-Merino I, de Las Cuevas N, Jimenez JL, et al. The Spanish HIV BioBank: a model of cooperative HIV research. Retrovirology **2009**; 6: 27.
17. Scheltinga SA, Johnston-Dow LA, White CB, et al. A generic sequencing based typing approach for the identification of HLA-A diversity. Hum Immunol **1997**; 57(2):120–128.
18. Hazuda DJ, Felock P, Witmer M, et al. Inhibitors of strand transfer that prevent integration and inhibit HIV-1 replication in cells. Science **2000**; 287(5453):646–650.
19. Senserrich J, Pauls E, Armand-Ugon M, et al. HIV-1 resistance to the anti-HIV activity of a shRNA targeting a dual-coding region. Virology **2008**; 372(2):421–429.
20. Ballana E, Pauls E, Senserrich J, et al. Cell adhesion through alphaV-containing integrins is required for efficient HIV-1 infection in macrophages. Blood **2009**; 113(6):1278–1286.
21. Armand-Ugon M, Gutierrez A, Clotet B, Este JA. HIV-1 resistance to the gp41-dependent fusion inhibitor C-34. Antiviral Res **2003**; 59: 137–142.
22. O'Doherty U, Swiggard WJ, Jeyakumar D, McGain D, Malim MH. A sensitive, quantitative assay for human immunodeficiency virus type 1 integration. J Virol **2002**; 76(21):10942–10950.
23. Ciuffi A, Bleiber G, Munoz M, et al. Entry and transcription as key determinants of differences in CD4 T-cell permissiveness to human immunodeficiency virus type 1 infection. J Virol **2004**; 78(19):10747–10754.
24. Moncunill G, Armand-Ugon M, Clotet-Codina I, et al. Anti-HIV activity and resistance profile of the CXC chemokine receptor 4 antagonist POL3026. Mol Pharmacol **2008**; 73(4):1264–1273.
25. Moncunill G, Armand-Ugon M, Pauls E, Clotet B, Este JA. HIV-1 escape to CCR5 coreceptor antagonism through selection of CXCR4-using variants in vitro. AIDS **2008**; 22(1):23–31.
26. Pauls E, Senserrich J, Clotet B, Este JA. Inhibition of HIV-1 replication by RNA interference of p53 expression. J Leukoc Biol **2006**; 80(3): 659–667.
27. Gonzalez JR, Armengol L, Sole X, et al. SNPassoc: an R package to perform whole genome association studies. Bioinformatics **2007**; 23(5):644–645.
28. Balding DJ. A tutorial on statistical methods for population association studies. Nat Rev Genet **2006**; 7(10):781–791.
29. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics **2005**; 21(2):263–265.
30. van Manen D, Kootstra NA, Boeser-Nunnink B, Handulle MA, van't Wout AB, Schuitemaker H. Association of HLA-C and HCP5 gene regions with the clinical course of HIV-1 infection. AIDS **2009**; 23(1): 19–28.
31. Limou S, Le Clerc S, Coulonges C, et al. Genomewide association study of an AIDS-nonprogression cohort emphasizes the role played by HLA genes (ANRS Genomewide Association Study 02). J Infect Dis **2009**; 199(3):419–426.
32. Catano G, Kulkarni H, He W, et al. HIV-1 disease-influencing effects associated with ZNRD1, HCP5 and HLA-C alleles are attributable mainly to either HLA-A10 or HLA-B*57 alleles. PLoS One **2008**; 3(11): e3636.
33. Dalmasso C, Carpentier W, Meyer L, et al. Distinct genetic loci control plasma HIV-RNA and cellular HIV-DNA levels in HIV-1 infection: the ANRS Genome Wide Association 01 study. PLoS One **2008**; 3(12): e3907.
34. Bushman FD, Malani N, Fernandes J, et al. Host cell factors in HIV replication: meta-analysis of genome-wide studies. PLoS Pathog **2009**; 5(5):e1000437.
35. Goff SP. Host factors exploited by retroviruses. Nat Rev Microbiol **2007**; 5(4):253–263.
36. Telenti A, Goldstein DB. Genomics meets HIV-1. Nat Rev Microbiol **2006**; 4(11):865–873.
37. Trachtenberg E, Bhattacharya T, Ladner M, Phair J, Erlich H, Wolinsky S. The HLA-B/-C haplotype block contains major determinants for host control of HIV. Genes Immun **2009**; 10(8):673–677.
38. Carthew RW, Sontheimer EJ. Origins and mechanisms of miRNAs and siRNAs. Cell **2009**; 136(4):642–655.
39. Stranger BE, Nica AC, Forrest MS, et al. Population genomics of human gene expression. Nat Genet **2007**; 39(10):1217–1224.
40. Dorr P, Westby M, Dobbs S, et al. Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. Antimicrob Agents Chemother **2005**; 49(11):4721–4732.
41. Gulick RM, Lalezari J, Goodrich J, et al. Maraviroc for previously treated patients with R5 HIV-1 infection. N Engl J Med **2008**; 359(14): 1429–1441.