

sequence in quasi-species existing in patients is relatively conserved in the face of a vigorous antibody response, especially in early HIV-1 infection. A recent study by Deeks et al. has important implications for understanding the NAb response against autologous virus (9): although NAb responses against contemporaneous autologous viruses are absent in early HIV infection, they can be detected at low levels in some patients with chronic infection. These data suggest the existence of an NAb response that overcomes the emergence of escape mutants. Further characterization of the response in humans who have potent and broadly neutralizing activities not affected by V1/V2 blocking effects may allow the identification of additional neutralization sites in HIV-1 Env, which might allow new targets to be identified for vaccine development.

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Wild type and *H43Y* variant of human *TRIM5 α* show similar anti-human immunodeficiency virus type 1 activity both in vivo and in vitro

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Abstract Polymorphisms in human genes have been shown to affect the rate of disease progression to acquired immune deficiency syndrome in human immunodeficiency virus type 1 (HIV-1)-infected individuals. Recently, tripartite motif 5 α (TRIM5 α) was identified as a factor that confers resistance to HIV-1 infection in Old World monkey cells. Subsequently, Sawyer et al. (Curr Biol 16:95–100, 2006) reported a single nucleotide polymorphism (H43Y) in the human *TRIM5 α* gene and TRIM5 α protein with 43Y was found to lose its

ability to restrict HIV-1. In the present study, we reevaluated effects of this allele on in vitro anti-HIV-1 activity as well as on HIV-1 disease progression in European and Asian cohorts of HIV-1-infected individuals. Our epidemiological and molecular biological findings clearly indicate H43Y has a very minor effect on anti-HIV-1 activity of TRIM5 α , suggesting that this allele is immaterial, at least in HIV-1-infected Europeans and Asians.

Keywords TRIM5 α · H43Y · RING domain · Polymorphism · HIV-1 disease progression · Anti-HIV-1 activity

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Human immunodeficiency virus type 1 (HIV-1) has a very narrow host range limited to humans and chimpanzees. In experiments, HIV-1 does not infect Old World monkeys, such as rhesus and cynomolgus monkeys. Recently, the screening of a rhesus monkey cDNA library identified tripartite motif 5 (TRIM5) as a factor that confers resistance to HIV-1 infection (Stremlau et al. 2004). Shortly afterwards, TRIM5 α of the African green monkey (AGM), another Old World monkey, was also shown to restrict HIV-1 infection (Hatzioannou et al. 2004; Keckesova et al. 2004; Nakayama et al. 2005), while human TRIM5 α reportedly restrict HIV-1 only weakly but potently restrict N-tropic murine leukemia virus (N-MLV; Hatzioannou et al. 2004; Keckesova et al. 2004; Perron et al. 2004; Yap et al. 2004). TRIM5 α is composed of two zinc-finger (RING and B-box), coiled-coil, and SPRY (B30.2) domains.

HIV-1 infection in humans is generally characterized by a long-term, chronic disease course gradually progressing to acquired immune deficiency syndrome (AIDS). Polymor-

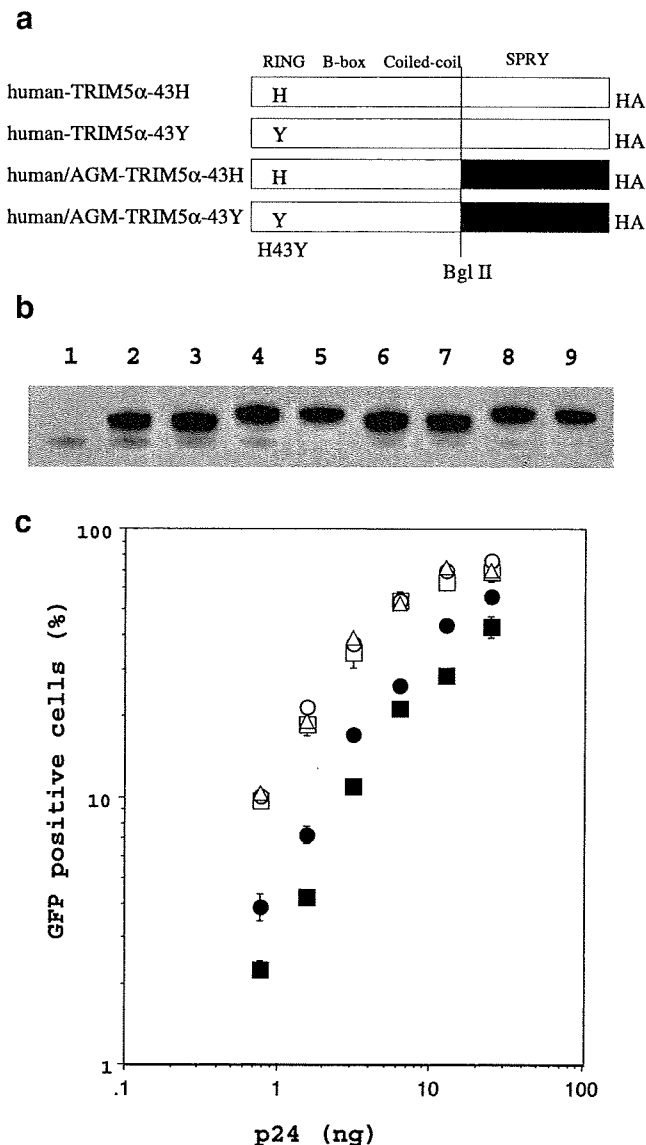


Fig. 1 **a** Schematic representation of TRIM5 α fused with HA-tag. Four domains of TRIM5 α are shown at the top. Black and white bars denote human and AGM sequences, respectively. A Bgl II site was used to swap SPRY domains between human and AGM TRIM5 α . *H* or *Y* denotes the amino acid residue at the 43rd position. **b** Expression levels of HA-tagged TRIM5 α proteins. C143 cells were transfected with an empty pCEP4 plasmid (lane 1) or pCEP4 carrying human-TRIM5 α -43Y (lanes 2 and 3), human/AGM-TRIM5 α -43Y (lanes 4 and 5), human-TRIM5 α -43H (lanes 6 and 7), human/AGM-TRIM5 α -43H (lanes 8 and 9) protein and hygromycin-resistant cells were selected for 14 days. One million cells of each transformant were lysed for immunoprecipitation with an anti-HA antibody. Two independent clones for each construct were evaluated for expression levels. **c** C143 cells expressing human-TRIM5 α -43H (open squares), human-TRIM5 α -43Y (open circles), human/AGM-TRIM5 α -43H (closed squares), human/AGM-TRIM5 α -43Y (closed circles), or cells transfected with an empty vector (open triangles) were exposed to the indicated p24 amounts of GFP-expressing HIV-1 vector. GFP-positive cells were counted with a flowcytometry (FACScan, Beckton Dickinson). Error bars indicated actual fractures of two independent cell cultures derived from independent clones. Representative data from three independent experiments are shown

phisms in human *CCR5* and other genes reportedly affect the rate of disease progression to AIDS (Kasper et al. 2005). Regarding the human *TRIM5 α* gene, Sawyer et al. (2006) reported a common histidine-to-tyrosine polymorphism at the 43rd amino acid residue (H43Y) of human *TRIM5 α* . This single nucleotide polymorphism (SNP) locates in the RING domain, and TRIM5 α protein with H43Y was found to lose its ability to restrict HIV-1. This SNP was also shown to greatly reduce the ability of TRIM5 α to restrict N-MLV. On the other hand, Speelman et al. (2006) sequenced the *TRIM5 α* gene from 110 HIV-1-infected and 96 exposed-seronegative European Americans and found 48 SNPs in their *TRIM5 α* genes. However, they did not observe any association between H43Y polymorphism in HIV-1-infected subjects and their set-point viral load after acute infection. Furthermore, they detected no difference in in vitro HIV-1 susceptibility of CD4⁺ cells between 43Y homozygote and the wild type. Sawyer et al. (2006) and Speelman et al. (2006) thus came to opposite conclusions; the former suggested that 43Y incapacitates even the modest human TRIM5 α resistance to HIV-1 infection, while the latter showed no difference between 43H and 43Y.

After that, two more groups published their observations. Goldschmidt et al. (2006) have published their analysis on Caucasians in Swiss cohort and reported lack of association of H43Y with rapid progression to AIDS in HIV-1-infected individuals. In this report, they showed that HeLa cells stably transduced with human TRIM5 α with 43Y do not differ from those with 43H in susceptibility to HIV-1 infection, whereas the 43Y variant failed to restrict N-MLV. Javanbakht et al. (2006) have published their analysis on large number of European Americans and African Americans and reported lack of any significant associations of TRIM5 α SNPs with different rate of disease progression in HIV-1-infected individuals, although they found a controversial protective effect of H43Y against HIV-1 transmission only in African Americans but not in European Americans. They showed the 43Y human TRIM5 α was less effective in restricting HIV-1 as well as N-MLV infections in vitro. Those reports agreed that 43Y variant failed to restrict N-MLV. However, the effect of H43Y substitution on HIV-1 restriction was not consistent among four reports. To make an addition to this debate, we conducted molecular biological and epidemiological studies in H43Y allele.

To reevaluate the effects of H43Y on in vitro anti-HIV-1 activity of TRIM5 α , we first established stable cell lines expressing recombinant TRIM5 α proteins. An expression plasmid carrying a hygromycin-resistant gene (pCEP4, Invitrogen) and hemagglutinin (HA)-tagged TRIM5 α genes with 43H or 43Y (Fig. 1a) were introduced into CD4-negative human osteosarcoma cell line C143 and hygromycin-resistant cells were selected. Equal levels of HA-tagged TRIM5 α expression were detected in those cell lines by

using immunoprecipitation followed by Western blot analysis (Nakayama et al. 2005; Fig. 1b). Serially diluted vesicular stomatitis virus (VSV)-pseudotyped HIV-1 vectors encoding green fluorescent protein (GFP) were then inoculated into the TRIM5 α expressing cells, and infected cells were counted by flow-cytometry 40 h after infection. As shown in Fig. 1c, there was no difference in anti-HIV-1 activity among empty vector, human TRIM5 α with 43H and that with 43Y, probably because human TRIM5 α did not show any anti-HIV-1 effect in C143 cells.

Several recombinant studies of human and monkey TRIM5 α revealed that the determinant of the species-specific restriction of HIV-1 lies in the SPRY domain of monkey TRIM5 α (Nakayama et al. 2005, 2006; Perez-Caballero et al. 2005; Sawyer et al. 2005; Stremlau et al. 2005; Yap et al. 2005). To enhance the weak anti-HIV-1 activity of human TRIM5 α , we introduced H43Y SNP in a chimeric version of TRIM5 α , which carried part of the SPRY domain of AGM-TRIM5 α and RING, B-box, and coiled-coil domains of human TRIM5 α (Fig. 1a). Equal levels of TRIM5 α expression were detected (Fig. 1b), and both chimeric TRIM5 α s with 43H and 43Y showed potent anti-HIV-1 activity. Although there was a small increase in HIV-1-infected cells in transfectants with 43Y TRIM5 α compared with those with 43H, we did not observe any complete loss of anti-HIV-1 activity for this variant.

To evaluate the effect of H43Y on multiple replication of HIV-1, we constructed recombinant Sendai viruses (SeVs) expressing human TRIM5 α with 43H or that with 43Y, or their chimeric versions. There were 10^5 cells of human T cell line MT4 infected with recombinant SeV expressing human TRIM5 α with 43H or that with 43Y at a multiplicity of infection at 10 plaque forming units per cell. Nine hours after infection, 20 ng of p24 of HIV-1 NL43 strain was challenged, and culture supernatants were periodically assayed for the levels of p24 by enzyme-linked immunosorbent assay (ZeptoMetrix). In this assay, we can observe multiple replications of HIV-1 and weak anti-HIV-1 activity of human TRIM5 α can be amplified. As expected, both human TRIM5 α s with 43H and 43Y showed weak but apparent anti-HIV-1 activity, although there was a small increase in HIV-1 titer in cells infected with SeV expressing human TRIM5 α with 43Y compared with those infected with SeV expressing human TRIM5 α with 43H (Fig. 2a). Equal levels of TRIM5 α expressions were detected in those SeV-infected cells (Fig. 2b). When we used SeVs expressing human/AGM chimeric TRIM5 α , both TRIM5 α : with 43H and 43Y completely suppressed HIV-1 replication (Fig. 2c). Again, equal levels of TRIM5 α expressions were detected in those SeV infected cells (Fig. 2d).

To exclude the possible effect of endogenous human TRIM5 α , we then used TK-tsl3 cells, a derivative of baby hamster kidney cell. As HIV-1 cannot complete the late step

of its replication in rodent cells, serially diluted VSV-pseudotyped HIV-1 vectors encoding GFP were inoculated into the cells infected with SeV expressing TRIM5 α s. GFP-positive cells were counted 40 h after infection. As shown in Fig. 2c, there was no difference in anti-HIV-1 activity among cynomolgus monkey TRIM5 α lacking SPRY domain [CM-SPRY(-)TRIM5 α], human TRIM5 α with 43H, and that with 43Y. Both chimeric TRIM5 α s with 43H and 43Y showed potent anti-HIV-1 activity, and there was no difference in anti-HIV-1 activity between chimeric TRIM5 α with 43H and that with 43Y. These results indicate that H43Y exerts only a minor effect on the anti-HIV-1 activity of TRIM5 α protein. They are in contrast with those reported by Sawyer et al. (2006), who found the 43Y SNP completely abolished the anti-HIV-1 activity of human TRIM5 α . Although the reason for this discrepancy is not clear at present, differences in the expression systems used may be involved.

To evaluate the effects of H43Y polymorphism on anti-HIV-1 activity of human TRIM5 α in Asian population, 49 HIV-1-infected Japanese subjects with different rates of disease progression were analyzed. Of the 49 patients, 21 were long-term non-progressors (LTNPs). They are all hemophiliacs and infected through contaminated blood products before 1985, and their CD4 counts were over 200 cells/ μ l without highly active anti-retroviral therapy until 2001. The LTNPs included ten cases with undetectable viral load without any kinds of anti-retroviral therapy by 2001. The remaining 28 cases were standard progressors (SPs) comprising 15 hemophiliacs, 9 homosexual, and 4 heterosexual cases. The homosexual and heterosexual cases were infected with HIV-1 after 1985. Among 28 SPs, 13 died of AIDS before 1999, and 15 developed AIDS before 1996. RING and B-box region was polymerase chain reaction (PCR)-amplified from genomic DNA by using primer pair forward (5'-TCAGGTCTATCATGACAAGG CAG-3') and reverse (5'-GGCAGGAGCAGTGGAAATG C-3'). Genotypes of the 43rd position were determined by direct sequencing of the resultant 542-bp PCR product with forward primer. Among 21 LTNPs, one subject was homozygous for 43Y allele, five were heterozygous, and 15 were homozygous for the wild type. Of the 28 SPs, one subject was homozygous for 43Y, five were heterozygous, and 22 were homozygous for the wild type. There was no statistically significant difference in 43Y allele frequencies between LTNPs and SPs (0.167, seven out of 42 chromosomes vs 0.125, seven out of 56 chromosomes $p=0.77$, Yates chi square test). As the number of HIV-1 infected patients studied here was relatively small, we performed statistical simulation with ten times more subject numbers. Nevertheless, difference did not reach statistical significance ($p=0.065$). To exclude possible confounding effect of *CCR2-64I*, which is known as a protective genetic factor

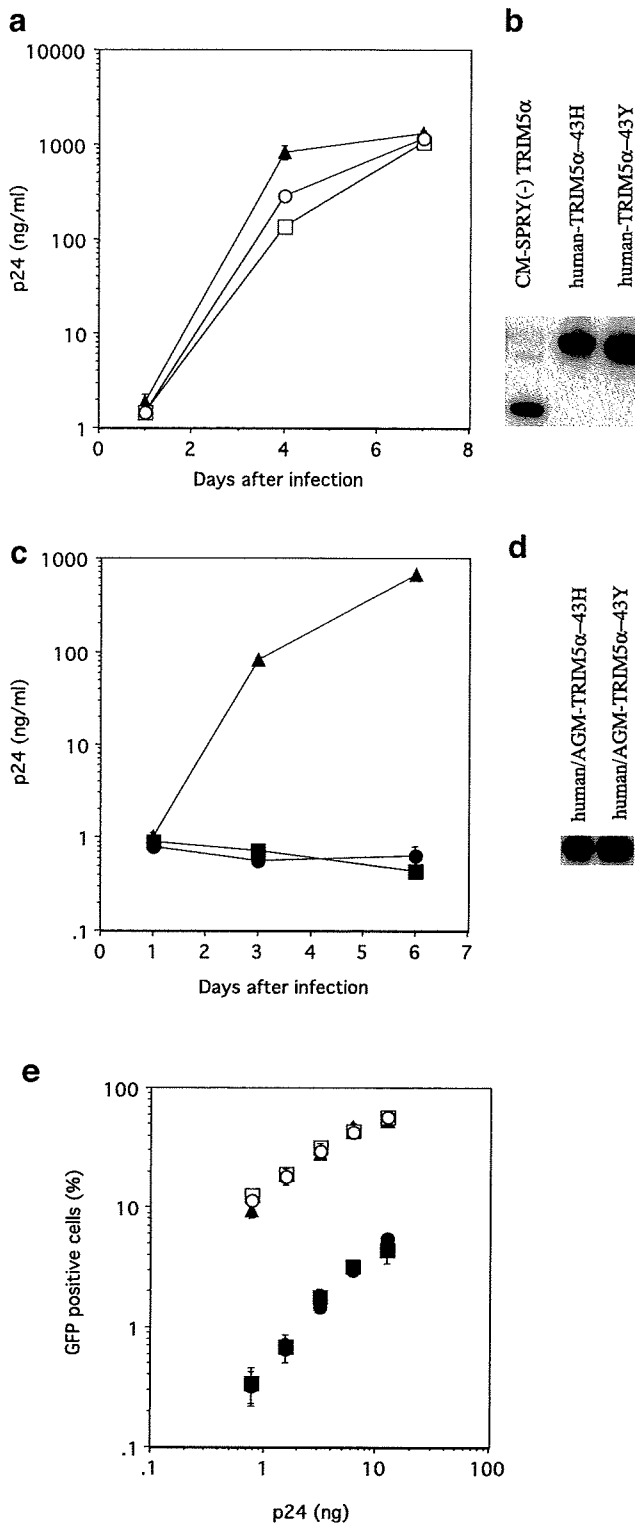


Fig. 2 a, c MT4 cells were infected with recombinant SeV expressing human-TRIM5α-43H (open squares), human-TRIM5α-43Y (open circles), human/AGM-TRIM5α-43H (closed squares) or a truncated form of CM-SPRY(-) TRIM5α as a negative control (closed triangles). Nine hours after infection, cells were inoculated with 20 ng of p24 of HIV-1 NL43, and culture supernatants were periodically assayed for levels of p24. Error bars showed actual fluctuations between measurements of p24 in duplicate samples. Representative data from two independent experiments are shown. b, d One million cells of each recombinant SeV-infected cells were lysed for immunoprecipitation with an anti-HA antibody. e TK-ts13 cells were infected with SeV expressing human-TRIM5α-43H (open squares), human-TRIM5α-43Y (open circles), human/AGM-TRIM5α-43H (closed squares), human/AGM-TRIM5α-43Y (closed circles), or CM-SPRY(-)TRIM5α (closed triangles) and then exposed to the indicated p24 amounts of GFP-expressing HIV-1 vector. GFP-positive cells were counted with a flow cytometry. Error bars indicated standard deviation of triplicate samples

numbers again failed to show statistically significant difference ($p=0.09$)

We also analyzed HIV-1-infected individuals in two well-characterized French cohorts: 45 subjects from the Agence Nationale de Recherches sur le Sida CO16 Long Term Non-Progressors Cohort (ALT) and 62 from the cohort of SPs (IMMUNOCO). The patients in ALT cohort were with no AIDS symptoms at the time of recruit in 1996 without any kinds of treatment, and their CD4 counts were more than 600/ μ l during last 5 years. The patients in IMMUNOCO cohort were at any stage, with or without treatment, and their CD4 counts were more than 150/ μ l at entry in 1991 to 1992 (Magierowska et al. 1999). Thirteen of the ALT subjects and 14 of the IMMUNOCO subjects were heterozygous for 43Y. Again, there was no statistically significant difference in the ratio of 43Y heterozygotes between ALT and IMMUNOCO subjects (0.29 vs 0.23, $p=0.460$, chi square test). The odds ratio was 0.72 with a 95% confidence interval of 0.30–1.73, indicating that H43Y did not exert any strong effect on HIV-1 disease progression in the French subjects. Our findings for Asians and Europeans indicated that the effects of H43Y SNP of the human *TRIM5α* gene on HIV-1 disease progression are minor, if any. Our results are consistent with the previous observation that 43Y does not have a protective effect against HIV-1 replication or disease progression in European Americans (Goldschmidt et al. 2006; Javanbakht et al. 2006; Speelman et al. 2006).

In conclusion, the results of our epidemiological and molecular biological studies clearly indicate that H43Y SNP in the human *TRIM5α* gene has a minor effect on the anti-HIV-1 activity of TRIM5α. Although we did not evaluate the effects of H43Y on the anti-MLV activity of human TRIM5α, this allele is immaterial, at least in cases with HIV-1 subtype B such as found in Europeans, European Americans, and Japanese hemophiliacs. It might be important to test the restriction capability of human TRIM5α in other subtypes of HIV-1 or HIV-2.

against AIDS progression, we excluded patients with this allele from the analysis. There was still no statistically significant difference of the 43Y allele frequency between 9 LTNPs and 15 SPs (0.111, two out of 18 chromosomes, vs 0.166, five out of 30 chromosomes, $p=0.69$, Fisher's exact test). Statistical simulation with ten times more subject

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Successful Efavirenz Dose Reduction in HIV Type 1–Infected Individuals with Cytochrome P450 2B6 *6 and *26

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Background. Efavirenz (EFV) is metabolized primarily by cytochrome P450 2B6 (CYP2B6), and high plasma concentrations of the drug are associated with a G→T polymorphism at position 516 (516G→T) of CYP2B6 and frequent central nervous system (CNS)–related side effects. Here, we tested the feasibility of genotype-based dose reduction of EFV.

Methods. CYP2B6 genotypes were determined in 456 human immunodeficiency virus type 1 (HIV-1)–infected patients who were receiving EFV treatment or were scheduled to receive EFV-containing treatment. EFV dose was reduced in CYP2B6 516G→T carriers who had high plasma EFV concentrations while receiving the standard dosage (600 mg). EFV-naïve homozygous CYP2B6 516G→T carriers were treated with low-dose EFV. In both groups, the dose was further reduced when plasma EFV concentration remained high.

Results. CYP2B6 516G→T was identified in the *6 allele (found in 17.9% of our subjects) and a novel allele, *26 (found in 1.3% of our patients). All EFV-treated CYP2B6 *6/*6 and *6/*26 carriers had extremely high plasma EFV concentrations (>6000 ng/mL) while receiving the standard dosage. EFV dose was reduced to 400 mg for 11 patients and to 200 mg for 7 patients with persistently suppressed HIV-1 loads. EFV-containing treatment was initiated at 400 mg in 4 CYP2B6 *6/*6 carriers and one *6/*26 carrier. Two of them still had a high plasma EFV concentration while receiving that dose, and the dose was further reduced to 200 mg, with successful HIV-1 suppression. CNS-related symptoms improved with dose reduction in 10 of the 14 patients, although some had not been aware of the symptoms at initial dosage.

Conclusions. Genotype-based EFV dose reduction is feasible in CYP2B6 *6/*6 and *6/*26 carriers, which can reduce EFV-associated CNS symptoms.

Efavirenz (EFV) is an important anti-HIV-1 agent in current combination treatment and is usually prescribed at a fixed dosage of 600 mg once daily [1, 2].

The plasma concentration of EFV varies widely in individuals, and the prevalence of CNS symptoms is higher in those with high concentrations [3]. EFV is metabolized mainly by cytochrome P450 2B6 (CYP2B6), and its concentration was reported to be associated with the CYP2B6 516G→T genetic polymorphism [4–8]. Previously, we reported that all Japanese patients with the 516TT genotype had extremely high EFV concentrations (>6000 ng/mL), without exception [4]. However, other studies reported some exceptional cases of subjects with the 516TT genotype with normal concentrations, although most of the

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516TT carriers had high concentrations [5–8]. The difference between our data and those of others may reflect polymorphisms other than 516G→T in *CYP2B6*. If this is the case, analysis of other polymorphisms and determination of the *CYP2B6* haplotype may be helpful in predicting EFV plasma levels. In the present study, we determined the *CYP2B6* haplotype of 456 HIV-1-infected patients and analyzed its relationship with EFV concentration in 111 of them. Furthermore, we reduced the EFV dose in 12 patients whose EFV concentrations had been high while receiving the standard dosage. We also used reduced doses of EFV in 5 EFV-naive patients in whom EFV concentration was predicted to become extremely high while receiving the standard dosage, on the basis of *CYP2B6* haplotype determination.

SUBJECTS, MATERIALS, AND METHODS

Patients. This analysis included 60 previously reported HIV-1-infected individuals at the International Medical Center of Japan (IMCJ) [4] and another group of 396 HIV-1-infected patients who were receiving treatment of the standard dosage (600 mg once daily) of EFV or were scheduled to begin receiving EFV-containing treatment at the following 11 hospitals in Japan: Hokkaido University (Sapporo), Sendai Medical Center (Sendai), Niigata University (Niigata), Higashi Saitama Hospital (Hasuda), IMCJ (Tokyo), Ishikawa Prefecture Central Hospital (Kanazawa), Nagoya Medical Center (Nagoya), Osaka National Hospital (Osaka), Hiroshima University (Hiroshima), Kyushu Medical Center (Fukuoka), and Kumamoto University (Kumamoto). The ethics committee of each hospital approved this study, and each participant gave written informed consent.

***CYP2B6* genotype.** DNA samples were extracted from peripheral blood specimens obtained from participants, and genotyping of *CYP2B6* 64C→T (*rs8192709*), 415A→G (*rs12721655*), 499C→G (*rs3826711*), 516G→T (*rs3745274*), 777C→A (*rs* number not available), 785A→G (*rs2279343*), 1375A→G (*rs* number not available), and 1459C→T (*rs3211371*) was performed by allele-specific fluorogenic 5' nuclease chain reaction assay with predesigned primers and TaqMan MGB probes (TaqMan SNP Genotyping Assay; Applied Biosystems) or previously published primers and MGB probes [4]. In subjects confirmed to carry 499C→G, all 9 exons of the *CYP2B6* gene were amplified with previously published primers [9], and their DNA sequences were directly determined. For haplotype analysis of the *CYP2B6* allele, PCR amplification of the genomic region (3130 bp) containing exons 4 and 5 was performed using sense primer 5'-AACTGTACTCACTCCCAGAGT-3' and antisense primer 5'-CTCCCTCTGTCTTTCATTCTGT-3'. The amplified PCR product was subjected to subcloning, and the DNA sequence of each clone was determined. For genotyping of *CYP2B6* 983T→C (*rs28399499*), new primers and probes were designed as follows: forward primer, 5'-GCCTGAAATGCCTCTTAAA-

ATGAGATTC-3'; reverse primer, 5'-GCCATGTGGGCCAATCAC-3'; VIC probe for 983T, 5'-CTGTTCAATCTCCC-3'; and FAM probe for 983C, 5'-CTGTTCAAGTCTCCC-3'. The obtained genotyping results of *CYP2B6* 983T→C for >10 patients were confirmed by direct sequencing of exons 7 and 8 with use of primers published elsewhere [9].

Plasma EFV concentration. Samples of peripheral blood were collected during a daytime office visit (9–16 h after the patient took EFV) from the patients who had received EFV treatment at 600-mg dose at bedtime for >4 weeks. EFV concentration was measured by the reverse-phase high-performance liquid chromatography (HPLC) method [10]. For cases of EFV-dose reduction, plasma concentration was measured >2 weeks after the change in EFV dose. Differences in EFV concentrations between groups were examined for statistical significance with Student's *t* test. A *P* value <.05 denoted the presence of a statistically significant difference.

RESULTS

Novel *CYP2B6* allele. The *CYP2B6* genotype was analyzed in 456 HIV-1-infected patients, including 442 Japanese, 8 other Asians, and 6 others. During the analysis, we noticed that some patients had the *CYP2B6* 499C→G polymorphism, substituting Ala for Pro at the 167th amino acid, which is already registered in the SNP Database, although the *CYP2B6* allele containing 499G had not been determined yet. TaqMan Genotyping Assay indicated that *CYP2B6* 449G was heterozygous with 499C in 12 individuals (2.6%), who were all Japanese (table 1). Direct sequencing of all the exons confirmed the results of TaqMan Genotyping Assay and showed that 8 subjects had 516GT, 785AG, and 1375AA genotypes; 3 had 516TT, 785GG, and 1375AA genotypes; and 1 had 516GT, 785AG, and 1375AG genotypes without any other mutation. Subcloning analysis of the PCR products confirmed that 499G always coexisted in the same allele with 516T and 785G (figure 1). Therefore, it was concluded that the novel haplotype containing 499C→G had 2 other single-nucleotide polymorphisms (SNPs): 516G→T and 785A→G. We formally registered this novel allele with the Human Cytochrome P450 Allele Nomenclature Committee, and it was designated "*CYP2B6* *26" (<http://www.cypalleles.ki.se/>). With use of this nomenclature, the *CYP2B6* haplotype of the twelve 499C→G carriers were identified as eight *1/*26 heterozygotes, three *6/*26 heterozygotes, and one *23/*26 heterozygote (table 1). The allelic frequency of *26 was 1.3% in our study participants.

***CYP2B6* haplotype determination.** In 456 HIV-1-infected individuals, we determined the genotypes of 9 SNP positions (64C→T, 415A→G, 499C→G, 516G→T, 777C→A, 785A→G, 983T→C, 1375A→G, and 1459C→T) in *CYP2B6* (table 1). No *CYP2B6* genetic polymorphism was detected in 211 patients, and their haplotype was determined to be *1/*1. The haplotypes

Table 1. CYP2B6 haplotype and allele frequencies in study participants.

CYP2B6 status	CYP2B6 genotype at nucleotide position								No. (%) of subjects	
	415	499	516	777	785	983	1375	1459	All ^a	Japanese
Haplotype:										
*1/*1	AA	CC	GG	CC	AA	TT	AA	CC	211 (46.3)	205 (46.4)
*1/*2	AA	CC	GG	CC	AA	TT	AA	CC	30 (6.6)	30 (6.8)
*1/*4	AA	CC	GG	CC	AG	TT	AA	CC	43 (9.4)	42 (9.5)
*1/*5	AA	CC	GG	CC	AA	TT	AA	CT	4 (0.9)	3 (0.7)
*1/*6	AA	CC	GT	CC	AG	TT	AA	CC	104 (22.8)	101 (22.9)
*1/*23	AA	CC	GG	CC	AA	TT	AG	CC	2 (0.4)	2 (0.5)
*1/*26	AA	CG	GT	CC	AG	TT	AA	CC	8 (1.8)	8 (1.8)
*2/*4	AA	CC	GG	CC	AG	TT	AA	CC	6 (1.3)	5 (1.1)
*2/*5	AA	CC	GG	CC	AA	TT	AA	CT	1 (0.2)	1 (0.2)
*2/*6	AA	CC	GT	CC	AG	TT	AA	CC	5 (1.1)	5 (1.1)
*4/*4	AA	CC	GG	CC	GG	TT	AA	CC	5 (1.1)	5 (1.1)
*4/*6	AA	CC	GT	CC	GG	TT	AA	CC	12 (2.6)	12 (2.7)
*5/*5	AA	CC	GG	CC	AA	TT	AA	TT	1 (0.2)	1 (0.2)
*5/*6	AA	CC	GT	CC	AG	TT	AA	CT	1 (0.2)	1 (0.2)
*6/*6	AA	CC	TT	CC	GG	TT	AA	CC	19 (4.2)	17 (3.8)
*6/*26	AA	CG	TT	CC	GG	TT	AA	CC	3 (0.7)	3 (0.7)
*23/*26	AA	CG	GT	CC	AG	TT	AG	CC	1 (0.2)	1 (0.2)
Total									456	442
Allele:										
*1	A	C	G	C	A	T	A	C	613 (67.2)	596 (67.4)
*2	A	C	G	C	A	T	A	C	42 (4.6)	41 (4.6)
*4	A	C	G	C	G	T	A	C	71 (7.8)	69 (7.8)
*5	A	C	G	C	A	T	A	T	8 (0.9)	7 (0.8)
*6	A	C	T	C	G	T	A	C	163 (17.9)	156 (17.6)
*23	A	C	G	C	A	T	G	C	3 (0.3)	3 (0.3)
*26	A	G	T	C	G	T	A	G	12 (1.3)	12 (1.4)
Total									912	884

^a Including 442 Japanese, 8 other Asians (5 Thai, 2 Koreans, and 1 Filipino), 4 Hispanics, and 2 non-Hispanic whites.

of single-SNP carriers with 64CT, 785AG, 1375AG, and 1459CT were determined to be *1/*2, *1/*4, *1/*23, and *1/*5, respectively. Those of homozygous polymorphism carriers with 785GG only, 1459TT only, and both 516TT and 785GG were determined to be *4/*4, *5/*5, and *6/*6, respectively. When the fact that *2 is the only allele harboring 64C→T is considered, patients with 64CT and 785AG; 64CT and 1459CT; and 64CT, 516GT, and 785AG were identified as *2/*4, *2/*5, and *2/*6 heterozygotes, respectively. Patients with both 516GT and 785GG genotypes but without other polymorphisms were determined to have *4/*6 heterozygotes. There were 104 patients (22.8%), including 101 Japanese, who held both 516GT and 785AG genotypes without other polymorphisms. There were 2 possible haplotypes, *1/*6 and *4/*9, in this genotypic pattern. When the fact that *9 had not been reported in Japanese subjects was considered [11], we found that all 101 Japanese were *1/*6 heterozygotes. Haplotype analysis by subcloning of PCR products described above was performed in the 3 others, and their haplotype was determined as *1/*6. One Japanese patient

had 516GT, 785AG, and 1459CT genotypes without other polymorphisms, and there were 2 possible haplotypes, *1/*7 and *5/*6, in this genotypic pattern. Because *7 had not been reported in Japanese subjects [11], the haplotype in this patient was determined to be *5/*6. Overall, the allelic frequency of *6 was 17.9% in our study participants. The 415A→G, 777C→A, and 983T→C polymorphisms, which are the determinants of *8, *3, and *18, respectively, were not observed in our subjects.

CYP2B6 and EFV concentration. We determined the CYP2B6 haplotype in 251 patients at IMCJ and in 205 patients at the other 10 hospitals. Of the 251 genotype-analyzed patients at IMCJ, 101 were being treated or were beginning treatment with a standard dose of EFV during this study period (figure 2). Plasma EFV concentrations were measured in all 101 patients, including sixty-seven 516GG holders, twenty-eight 516GT holders, and six 516TT holders. To clarify the effect of the 516TT genotype, EFV concentration was also measured in ten 516TT holders undergoing treatment with the standard dose of EFV at other hospitals. The mean concentration (\pm SD)

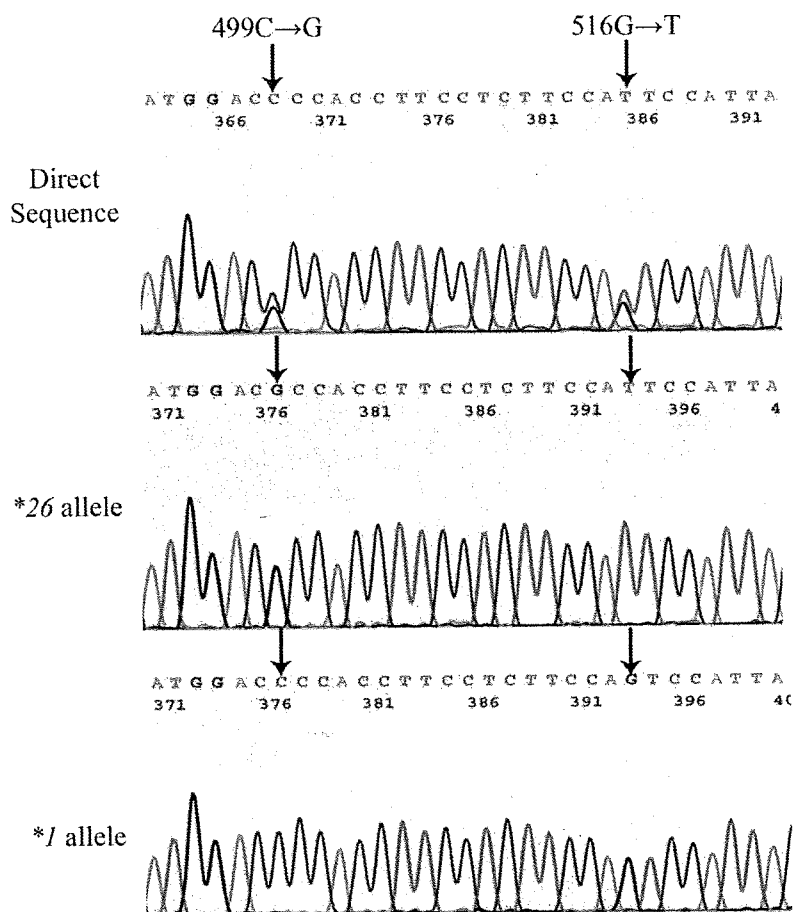


Figure 1. Direct (top panel) and subclonal (middle and bottom panels) sequences of *CYP2B6* in 499C→G carriers. The genotypes 499G, 516T, and 785G (not shown) exist in the same allele, newly designated as "*CYP2B6* *26." The same results were obtained in all 9 patients with the 499CG, 516GT, and 785AG genotypes, and the patients were identified as eight *1/*26 carriers and one *23/*26 carrier. Although shown are the sense-strand sequences only, both strands were sequenced. Arrows indicate the variant nucleotide positions 499 and 516.

of EFV in all patients was 3740 ± 2800 ng/mL. When divided by the genotype of position 516, striking discreteness was observed (figure 3). All (95% CI 91.1%–100%) of the 16 carriers of 516TT genotype, including fourteen *6/*6 carriers and two *6/*26 carriers, had extremely high EFV concentrations (>6000 ng/mL). Their mean concentrations (9500 ± 2580 ng/mL) were many orders of magnitude higher than those of the other genotype carriers ($P < 10^{-4}$). There was no significant difference in EFV concentration between *6/*6 carriers and *6/*26 carriers. On the other hand, there were only 2 patients who had such high EFV concentrations among the other genotype carriers. One was a *1/*6 carrier (7140 ng/mL), and the other was a *1/*26 carrier (9710 ng/mL). Direct sequencing of all *CYP2B6* exons showed no polymorphism other than 499C→G, 516G→T, and 785A→G in these individuals. The mean concentrations of EFV of the twenty-eight 516GT carriers, including twenty-five *6-heterozygotes (3320 ± 1240 ng/mL; $P < 10^{-4}$) and three *26-heterozygotes (5470 ± 3840 ng/mL; $P < 10^{-4}$), were signifi-

cantly higher than those of the sixty-seven 516GG genotype carriers (2450 ± 770 ng/mL). None (95% CI 0%–0.1%) of the 516GG carriers had a high EFV concentration (>6000 ng/mL). Considered together, it was concluded that high plasma EFV concentrations were associated with *CYP2B6* *6 and *26 and that *CYP2B6* *6/*6 and *6/*26 carriers had extremely high plasma EFV concentrations at standard dosage, without exception.

EFV dose reduction from 600 mg. To determine whether the EFV dose can be reduced in patients who have a high concentration while receiving the standard dose, a dose-reduction protocol was applied in 12 patients with high plasma concentrations (>6000 ng/mL [range, 6170–14,690 ng/mL]), including one *1/*26 heterozygote, nine *6/*6 homozygotes, and two *6/*26 heterozygotes. Before the dose reduction, plasma HIV-1 load was undetectable (<50 copies/mL) in all patients for >1 month with treatment of a standard antiretroviral regimen containing 600 mg of EFV. In these 12 patients,

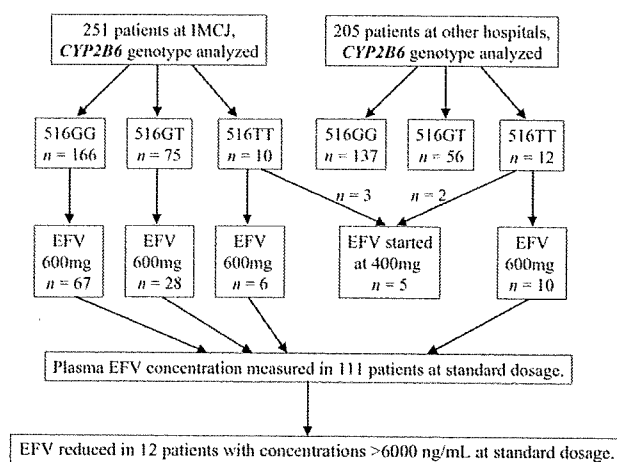


Figure 2. Flow diagram of study participants. The *CYP2B6* genotype was analyzed in 251 patients at the International Medical Center of Japan (IMCJ) and in 205 patients at other hospitals. Standard dosage of EFV was administered in 101 patients at IMCJ, including sixty-seven *CYP2B6* 516GG, twenty-eight 516GT, and six 516TT holders, whose EFV concentrations were measured. Ten 516TT holders at the other hospitals were administered standard dosages of EFV, and their EFV concentrations were also measured. A reduced-dose (400 mg) regimen of EFV was initiated in 5 other 516TT holders.

the EFV dose was reduced from 600 to 400 mg in 11 subjects and was further reduced to 200 mg in 7 of them who consented to further reduction. The plasma EFV concentrations decreased by approximately one-third (36%–46%), to 3720–6160 ng/mL, with dose reduction from 600 to 400 mg in 10 of 11 subjects, and further decreased by approximately one-half (51%–59%), to 1620–2960 ng/mL, with reduction from 400 to 200 mg in 6 of 7 subjects (figure 4). In one patient who had a markedly high EFV concentration (14,690 ng/mL) at the standard 600-mg dose, however, the concentration decreased unexpectedly by 69%, to 4500 ng/mL, with the reduction to 400 mg and further decreased by 82%, to 790 ng/mL, lower than the recommended range (>1000 ng/mL) [1], with the reduction from 400 to 200 mg. Therefore, the dose was increased in this patient back to 400 mg. In another patient who had reported severe dizziness during treatment with the standard dose (600 mg), the dose was reduced immediately to 200 mg at the patient's request. The plasma EFV concentration was also markedly high in this patient (14,360 ng/mL) during treatment with the standard dosage. However, it decreased by 83%, to 2410 ng/mL, with the dose reduction to 200 mg. Consequently, the final EFV dose was 400 mg in 5 subjects and 200 mg in 7 subjects. The determined dosage for each patient was continued for >6 months (the longest was 26 months for a patient who received the 200-mg dose), and the plasma HIV-1 load was continuously undetectable in all patients.

EFV initiation at 400-mg dose. Our analysis showed that *CYP2B6* *6/*6 and *6/*26 carriers had extremely high EFV concentrations, without exception (figure 3), and that dose reduction was possible in patients with high EFV concentration with retention of therapeutically effective anti-HIV-1 activity (figure 4). In the next phase of our study, we used an antiretroviral regimen containing a reduced dose (400 mg) of EFV in 5 EFV-naïve patients (four *6/*6 homozygotes and one *6/*26 heterozygote). Before the introduction of low-dose EFV-containing regimen, the plasma HIV-1 loads had been undetectable during receipt of the previous protease inhibitor-containing regimen in all 5 patients. Their EFV concentrations were 4080–9450 ng/mL, and all such concentrations (95% CI, 99.5%–100%) were therapeutically adequate (>1000 ng/mL) at the 400-mg dose (figure 5). One *6/*6 homozygote developed severe dizziness, necessitating discontinuation of EFV-treatment at day 16. His EFV concentration was 5430 ng/mL. In one *6/*26 heterozygote, severe thrombocytopenia emerged, probably because of overdosage of rifabutin prescribed for the treatment of coinfection with *Mycobacterium intracellulare*, and EFV treatment was stopped at day 15. The EFV concentration was 5770 ng/mL. Two of the remaining 3 patients still had extremely high EFV concentrations (6760 and 9450 ng/mL) at the 400-mg dose, and their dose was subsequently reduced to 200 mg. The plasma EFV concentrations decreased to 2690 and 3660 ng/mL (i.e., by 60% and 61%, respectively). Consequently, 2 subjects

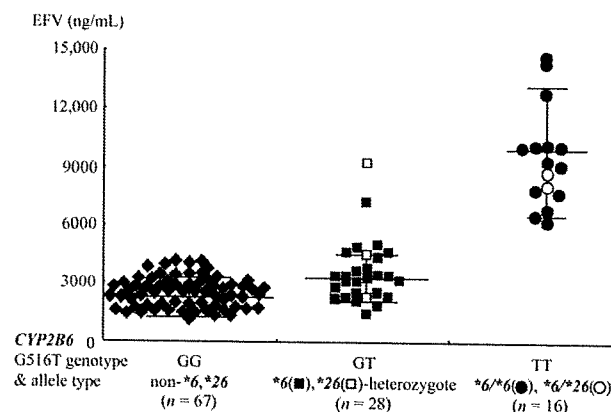


Figure 3. Plasma efavirenz (EFV) concentrations measured during EFV treatment with standard dose (600 mg). A total of 111 HIV-1-infected patients treated with EFV-containing regimens were divided into 3 groups on the basis of nucleotide genotype at *CYP2B6* position 516 (GG, GT, or TT), and their plasma EFV concentrations were compared. Blackened squares, *6 heterozygote with allele other than *26; unblackened squares, *CYP2B6* 499C→G carriers (*26 heterozygote with allele other than *6); blackened circles, *6 homozygote (*6/*6); unblackened circles, *CYP2B6* 499C→G carriers (*6/*26 heterozygotes); blackened diamonds, other genotype carriers. Horizontal lines represent the mean (±SD) plasma EFV concentrations for each group.

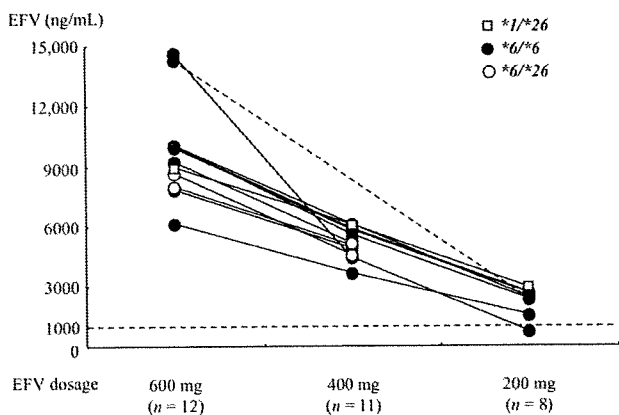


Figure 4. Dose reduction of efavirenz (EFV) in 12 patients whose concentrations were extremely high while receiving treatment with standard dose (600 mg). EFV dose was reduced from 600 to 400 mg in 11 patients and was further reduced, to 200 mg, in 7 patients. In one patient who had severe CNS symptoms while receiving treatment with standard dose, EFV dose was directly reduced to 200 mg (concentrations connected with a dotted line). The suggested minimum target concentration (1000 ng/mL) is indicated by the thin line.

discontinued the EFV-containing regimen, and 3 subjects continued low-dose EFV-containing regimen (400 mg for 1 patient and 200 mg for 2 patients). The low-dose regimen was continued for >6 months, and the plasma HIV-1 load was persistently undetectable in all 3 patients.

Improvement of CNS symptoms. As described above, the EFV dose was reduced from 600 to 400 and 200 mg as the final dose in 5 and 7 subjects, respectively (figure 4), and it was decreased from 400 mg as the initial dose to 200 mg for 2 other subjects (figure 5). To delineate the changes in CNS symptoms associated with the decrease in EFV concentration, a questionnaire survey of these 14 patients was conducted regarding 6 items: dizziness, strange dreams, depression, irritability, concentration problems, and sleep difficulty. More than 1 month after the dose had been reduced to the lowest dose, the patients were asked to judge the 6 CNS symptoms above at initial and final doses, with use of a 5-grade system (“none,” “slight,” “sometimes,” “often,” and “always”). Ten (71%) of the 14 patients had some of the aforementioned CNS symptoms during treatment with the initial dose (table 2). The most common symptom was dizziness (57%), followed by strange dreams (50%). Interestingly, all the symptoms improved after dose reduction in the 10 patients. Furthermore, dizziness and concentration problems disappeared during treatment with the final dose in one-half of the patients, although strange dreams and sleep difficulty were still reported by all the patients who had those difficulties at the initial dose. Finally, when the patients were asked whether they wanted to reincrease EFV to

the previous dose, all 10 patients with CNS symptoms at the initial dose answered “no” (9 answered “absolutely no”).

DISCUSSION

In this study, we identified a novel *CYP2B6* allele, *26, which includes 499C→G, 516G→T, and 785A→G in 12 Japanese patients, and we showed that, without exception, all *6/*6 and *6/*26 carriers, all holding 516TT, had extremely high plasma EFV concentrations while receiving the standard dose (600 mg) [4]. In other reports, however, there were some exceptional subjects with 516TT who had normal concentrations of EFV, and the discreteness of the EFV concentration with the position 516 genotype was not as clear as it was in our patients [5–8]. This difference may be because some of the 516TT carriers had other *CYP2B6* alleles, such as *7 (containing 516G→T, 785A→G, and 1459C→T), *9 (containing 516G→T only), and *13 (containing 415A→G, 516G→T, and 785A→G). Those alleles could not be found in our subjects, and their effects on EFV concentration were not well described. Because numerous additional *CYP2B6* variants with impact on expression and/or function were recently reported [12–18], correct determination of *CYP2B6* haplotype seems indispensable for prediction of EFV plasma levels.

We reduced the EFV dose in 12 patients whose plasma EFV concentrations were extremely high while receiving the standard dose, and we initiated EFV treatment at a 400-mg dose in 5 EFV-naïve *6/*6 and *6/*26 carriers. In most patients, the plasma EFV concentration decreased proportionally with the dose-reduction ratio. In 2 subjects, however, the concentrations decreased much more than expected, given the dose reduction

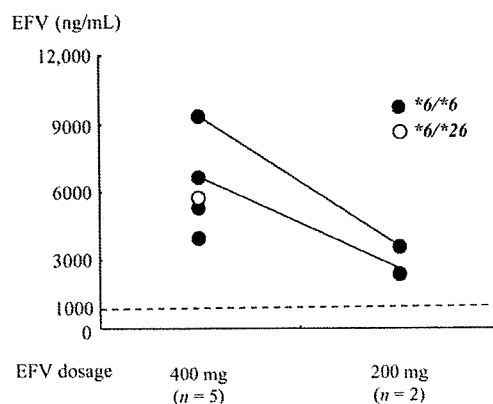


Figure 5. Introduction of low-dose efavirenz (EFV)-containing antiretroviral regimen to *CYP2B6* *6/*6 and *6/*26 carriers. Treatment was started in 4 EFV-naïve carriers *CYP2B6* *6/*6 and one *6/*26 carrier, with 400-mg EFV-containing regimens. EFV dose was further reduced, to 200 mg, in 2 patients whose EFV concentrations were >6000 ng/mL while receiving treatment with the 400-mg dose.

Table 2. Changes in CNS-related symptoms after reduction of efavirenz dosage.

Symptom	No. (%) of subjects who reported symptom status during efavirenz treatment		
	Present ^a (n=14)	Improved ^b	Disappeared ^b
Dizziness	8 (57)	8 (100)	4 (50)
Strange dreams	7 (50) ^c	7 (100) ^c	0 (0)
Depression	5 (36)	5 (100)	1 (20)
Irritability	5 (36)	5 (100)	1 (20)
Concentration problem	4 (29)	4 (100)	2 (50)
Sleep difficulty	3 (21)	3 (100)	0 (0)
Any of the above	10 (71) ^c	10 (100) ^c	4 (40)

^a Including the 4 grades "slight," "sometimes," "often," and "always" at the initial dosage. Includes 2 patients whose efavirenz treatment was originally 400 mg and was reduced to 200 mg.

^b Percentage of those who initially reported "present."

^c Including 1 patient whose efavirenz dose was originally 400 mg and was reduced to 200 mg.

ratio. Both of these patients had markedly high concentrations at standard dosage. Hasse et al. [19] reported a patient with excessively high plasma EFV concentration at standard dose, which decreased to one-thirtieth following dose reduction from 600 to 200 mg. Long-term exposure to such excessively high concentrations may induce CYP2B6 enzymatic expression in the liver, which could result in an unexpectedly large decrease in plasma EFV concentration by dose reduction if deinduction of the enzyme takes several weeks. At the 400-mg dose, the plasma concentrations of EFV were therapeutically adequate in all the treated $\Delta 6/\Delta 6$ and $\Delta 6/\Delta 26$ carriers in this study. Regarding the reduced dose, it is noteworthy that a phase II study during EFV development supported the use of a lower dose [20]. The same study indicated that the 600-mg dose of EFV is associated with a high rate of adverse events that could lead to discontinuation, which suggests that the lower dose of 400 mg may be almost as effective without the high discontinuation rate. In the present study, associated with the dose-reduction regimen, a significant number of patients experienced improvement of CNS symptoms, which was unexpected on the basis of previous reports [5, 21, 22]. Interestingly, some of these patients did not appreciate their clinical state and considered themselves to have no CNS-related symptoms during the standard-dose treatment. However, after the dose reduction, they reassessed the status and evaluated symptoms during the treatment with the standard dose as associated with CNS symptoms and indicated that the reduced dose of EFV relieved them of such symptoms. Because EFV-treated patients often stick to the regimen, previous reports of symptom questionnaires conducted during the standard treatment might have underestimated the EFV-associated CNS symptoms [5, 21, 22]. However, this finding might be confounded by placebo effect, because the patients were told

that their EFV levels were high while receiving the initial dose and decreased throughout the dose-reduction protocol. Because of this possible placebo effect, a double-blind, placebo-controlled study would best address this question.

EFV dose reduction and initiation of EFV treatment at reduced dose is possible with therapeutic anti-HIV-1 potency retained in CYP2B6 $\Delta 6/\Delta 6$ homozygotes and $\Delta 6/\Delta 26$ heterozygotes, which could relieve the patients of the EFV-associated CNS symptoms. It may also decrease the risk of development of EFV-resistant HIV-1 after mandatory treatment discontinuation, such as abdominal surgery [23], and reduce the treatment cost, an important issue in developing countries [24]. After dose reduction, however, careful monitoring is necessary until larger studies confirm the safety of reduced dose in such specific genotype carriers.

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Potential conflicts of interest. All authors: no conflicts.

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SOCS1 is an inducible host factor during HIV-1 infection and regulates the intracellular trafficking and stability of HIV-1 Gag

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Human immunodeficiency virus type 1 (HIV-1) utilizes the macromolecular machinery of the infected host cell to produce progeny virus. The discovery of cellular factors that participate in HIV-1 replication pathways has provided further insight into the molecular basis of virus–host cell interactions. Here, we report that the suppressor of cytokine signaling 1 (SOCS1) is an inducible host factor during HIV-1 infection and regulates the late stages of the HIV-1 replication pathway. SOCS1 can directly bind to the matrix and nucleocapsid regions of the HIV-1 p55 Gag polyprotein and enhance its stability and trafficking, resulting in the efficient production of HIV-1 particles via an IFN signaling-independent mechanism. The depletion of SOCS1 by siRNA reduces both the targeted trafficking and assembly of HIV-1 Gag, resulting in its accumulation as perinuclear solid aggregates that are eventually subjected to lysosomal degradation. These results together indicate that SOCS1 is a crucial host factor that regulates the intracellular dynamism of HIV-1 Gag and could therefore be a potential new therapeutic target for AIDS and its related disorders.

AIDS | pathogenesis | drug target | lysozyme

Human immunodeficiency virus type 1 (HIV-1) infection is a multistep and multifactorial process mediated by a complex series of virus–host cell interactions (1, 2). The molecular interactions between host cell factors and HIV-1 are vital to our understanding of not only the nature of the resulting viral replication, but also the subsequent cytopathogenesis that occurs in the infected cells (3). The characterization of the genes in the host cells that are up- or down-regulated upon HIV-1 infection could therefore provide a further elucidation of virus–host cell interactions and identify putative molecular targets for the HIV-1 replication pathway (4).

The HIV-1 p55 Gag protein consists of four domains that are cleaved by the viral protease concomitantly with virus release. This action generates the mature Gag protein comprising the matrix (MA/p17), capsid (CA/p24), nucleocapsid (NC/p7), and p6 domains, in addition to two small spacer peptides, SP1 and SP2 (5, 6). The N-terminal portion of MA, which is myristoylated, facilitates the targeting of Gag to the plasma membrane (PM), whereas CA and NC promote Gag multimerization. p6 plays a central role in the release of HIV-1 particles from PM by interacting with the vacuolar sorting protein Tsg101 and AIP1/ALIX (7–9). Several recent studies have implicated the presence of host factors in the control of the intracellular trafficking of Gag. AP-3 δ is a recently charac-

terized endosomal adaptor protein that binds directly to the MA region of Gag and enhances its targeting to the multivesicular body (MVB) during the early stages of particle assembly (10). The *trans*-Golgi network (TGN)-associated protein hPOSH plays another role in Gag transport by facilitating the egress of Gag cargo vesicles from the TGN, where it assembles with envelope protein (Env) before transport to PM (11). Although the involvement of these host proteins in the regulation of intracellular Gag trafficking has been proposed, the detailed molecular mechanisms underlying this process are still not yet well characterized.

In our current work, we demonstrate that the suppressor of cytokine signaling 1 (SOCS1) directly binds HIV-1 Gag and facilitates the intracellular trafficking and stability of this protein, resulting in the efficient production of HIV-1 particles. These results indicate that SOCS1 is a crucial host factor for efficient HIV-1 production and could be an intriguing molecular target for future treatment of AIDS and related diseases.

Results

SOCS1 Is Induced upon HIV-1 Infection and Facilitates HIV-1 Replication via Posttranscriptional Mechanisms. We and others have shown that HIV-1 infection can alter cellular gene expression patterns, resulting in the modification of viral replication and impaired homeostasis in the host cells (4, 12). Hence, to elucidate further the genes and cellular pathways that participate in HIV-1 replication processes, we performed serial analysis of gene expression (SAGE) using either a HIV-1 or mock-infected human T cell line, MOLT-4 (12). Further detailed analysis of relatively low-abundance SAGE tags identified *SOCS1* as a preferentially up-regulated gene after HIV-1 infection. This finding was validated by both semiquantitative RT-PCR and immunoblotting analysis with anti-SOCS1 anti-

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The authors declare no conflict of interest.

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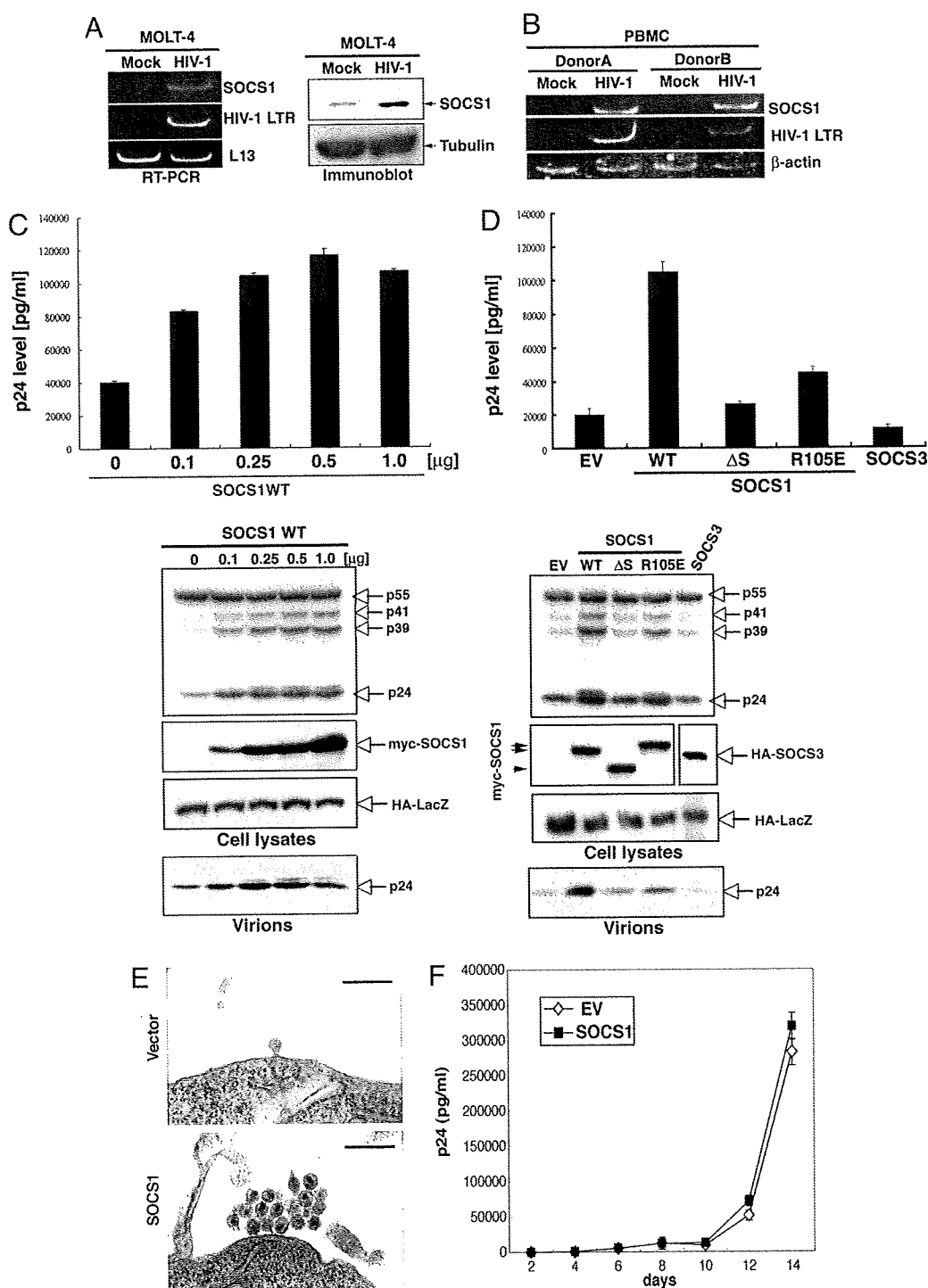


Fig. 1. SOCS1 is induced upon HIV-1 infection and enhances HIV-1 particle production. (A) MOLT-4 cells were mock-infected or infected with HIV-1_{NL4-3}, and then total RNA and protein extracts derived from these cells were subjected to semiquantitative RT-PCR (Left) and immunoblotting (Right), respectively. (B) PBMC from two healthy individuals were infected with HIV-1_{NL4-3} or were mock-infected, and SOCS1 expression was examined by semiquantitative RT-PCR. (C) 293T cells were transfected with pNL4-3 and cotransfected with various amounts of pcDNA-myc-SOCS1. Forty eight hours after transfection, p24 antigen release into the supernatant in each case was measured by antigen-capture ELISA (Upper), and the cell lysates and pelleted viruses were analyzed by immunoblotting (Lower). The data shown represent the mean \pm SD from three independent experiments. HA-LacZ is a transfection control. (D) 293T cells were transfected with pNL4-3 and cotransfected with control vector, SOCS1 (WT), SOCS1 Δ S (Δ SOCS box), SOCS1R105E, or SOCS3. Cell lysates and pelleted viruses were then collected after 48 h and subjected to ELISA (Upper) or immunoblotting (Lower), as described in C. (E) 293T cells cotransfected with either pNL4-3 plus control vector, or pNL4-3 plus myc-tagged SOCS1 were analyzed by TEM. Note that substantial numbers of mature virus particles can be observed in the myc-SOCS1-transfected cells. (Scale bars: 500 nm.) (F) Jurkat cells were infected with virions (adjusted by p24 levels) from either control vector (EV)- or SOCS1-transfected 293T cells. Supernatant p24 levels at the indicated time points were measured by ELISA.

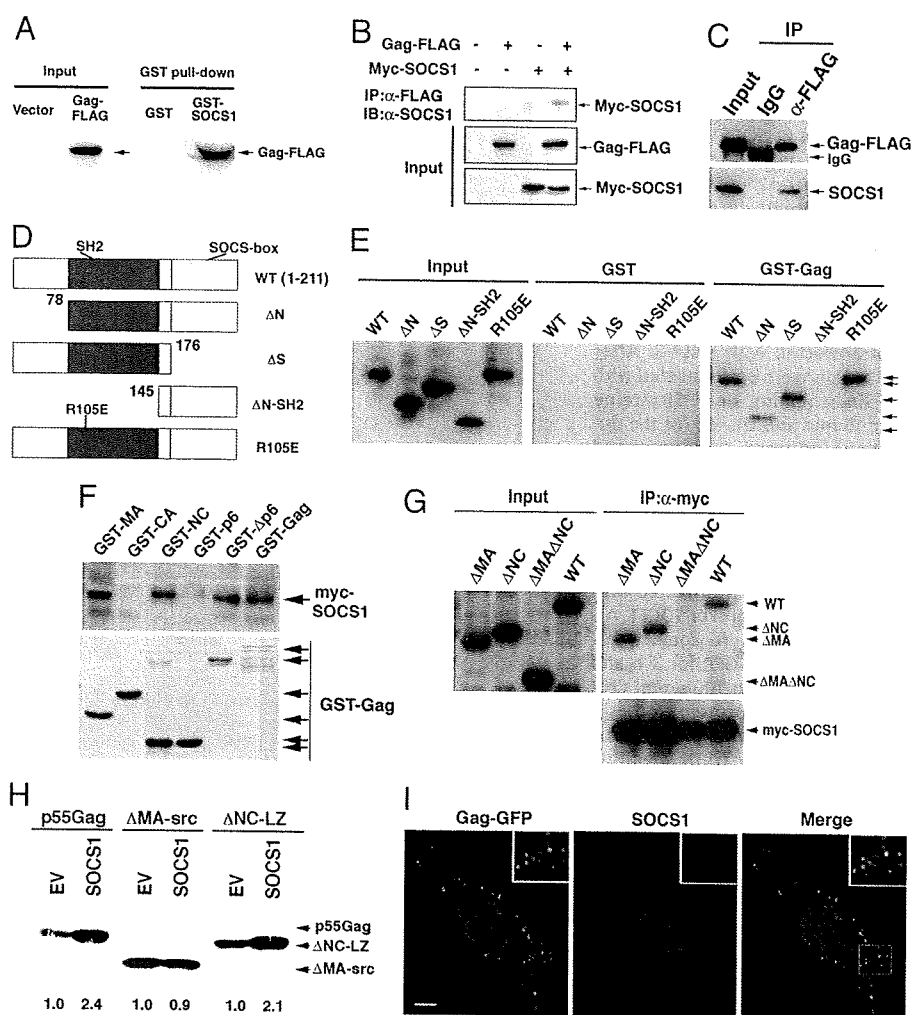
bodies (Fig. 1A). In addition, *SOCS1* was found to be up-regulated also in peripheral blood mononuclear cells (PBMC) from two different individuals (following HIV infection, Fig. 1B).

Our initial findings that SOCS1 is induced upon HIV-1 infection prompted us to examine whether this gene product affects viral replication. We first cotransfected 293T cells with a HIV-1 infectious molecular clone, pNL4-3 (13), and also pcDNA-myc-SOCS1, and then monitored the virus production levels in the resulting supernatant. We then performed ELISA using an anti-p24 antibody and found that wild-type SOCS1 significantly increases the production of HIV-1 in the cell supernatant in a dose-dependent

manner (Fig. 1C Upper). In contrast, neither the SH2 domain-defective mutant (R105E) nor the SOCS box deletion mutant (Δ S) of SOCS1 could promote virus production to the same levels as wild type, indicating that both domains are required for this enhancement (Fig. 1D Upper). Furthermore, another SOCS box protein, SOCS3, failed to augment HIV-1 replication in a parallel experiment (Fig. 1D Upper), indicating that the role of SOCS1 during HIV-1 replication is specific.

We next performed immunoblotting analysis using cell lysates and harvested virus particles in further parallel experiments (Fig. 1C and D Lower). Consistent with our ELISA analysis, the expres-

Fig. 2. SOCS1 interacts with HIV-1 Gag. (A) Extracts of 293T cells transfected with either empty vector or Gag-FLAG were subjected to pull-down analyses using glutathione-agarose beads with GST-SOCS1 in the presence of 10 ng/ml RNase followed by immunoblotting with anti-FLAG antibodies. (B) Extracts of 293T cells transiently expressing myc-SOCS1 and Gag-FLAG were subjected to immunoprecipitation (IP) with anti-FLAG monoclonal antibodies in the presence of 10 ng/ml RNase followed by immunoblotting (IB) analysis with either anti-FLAG or anti-myc polyclonal antibodies. (C) 293T cells were transiently transfected with Gag-FLAG, and cell lysates were then subjected to immunoprecipitation with anti-FLAG antibodies followed by immunoblotting with an antibody directed against endogenous SOCS1. (D and E) 293T cells expressing various myc-tagged SOCS1 mutants (schematically depicted in D) were analyzed by GST pull-down analysis with either GST or GST-Gag recombinant protein (E). (F) GST fusion proteins of the indicated regions of Gag were bound to glutathione beads and incubated with cell lysates from 293T cells expressing myc-SOCS1 in the presence of 10 ng/ml RNase followed by immunoblotting with anti-myc antibodies. (G) SOCS1 binds p55 Gag via either its MA or NC domains. 293T cells were transfected with myc-SOCS1 and cotransfected with Gag-FLAG, Gag Δ MA-FLAG, Gag Δ NC-FLAG, or Gag Δ MA Δ NC-FLAG. At 24 h after transfection, cell lysates treated with 10 μ g/ml RNase were subjected to coimmunoprecipitation with anti-myc monoclonal antibodies followed by immunoblotting with anti-FLAG or anti-myc polyclonal antibodies. (H) Functional interaction of SOCS1 with MA but not NC. 293T cells were transfected with wild-type Gag, Δ MA-src, or Δ NC-LZ (Z_{IL}-p6) and cotransfected with either control vector or SOCS1. Supernatant virus particles were then collected after 24 h and subjected to immunoblotting with anti-p24 antibody. Numerical values below the blots indicate fold induction of supernatant p55 signal intensities derived by densitometry. (I) Colocalization of SOCS1 with Gag. HeLa cells were transiently transfected with Gag-GFP. After 24 h, the cells were fixed, permeabilized, and immunostained with anti-SOCS1 polyclonal antibody followed by fluorescently labeled secondary antibodies before confocal microscopy. (Scale bar: 10 μ m.)



sion of wild-type SOCS1, but neither its SH2 nor SOCS box mutant counterparts, resulted in a marked and dose-dependent increase in the level of intracellular Gag protein, particularly in the case of CA (p24) and intermediate cleavage products corresponding to MA-CA (p41) and CA-NC (p39). This increase was found to be accompanied by an enhanced level of HIV-1 particle production in the supernatant (Fig. 1 C and D Lower). These results together indicated that SOCS1 facilitates HIV-1 particle production in infected cells and that this role of SOCS1 requires the function of both its SH2 and SOCS box domains. For further details about SOCS1 interaction with MA and NC and SOCS1-enhanced particle production, see supporting information (SI) Text.

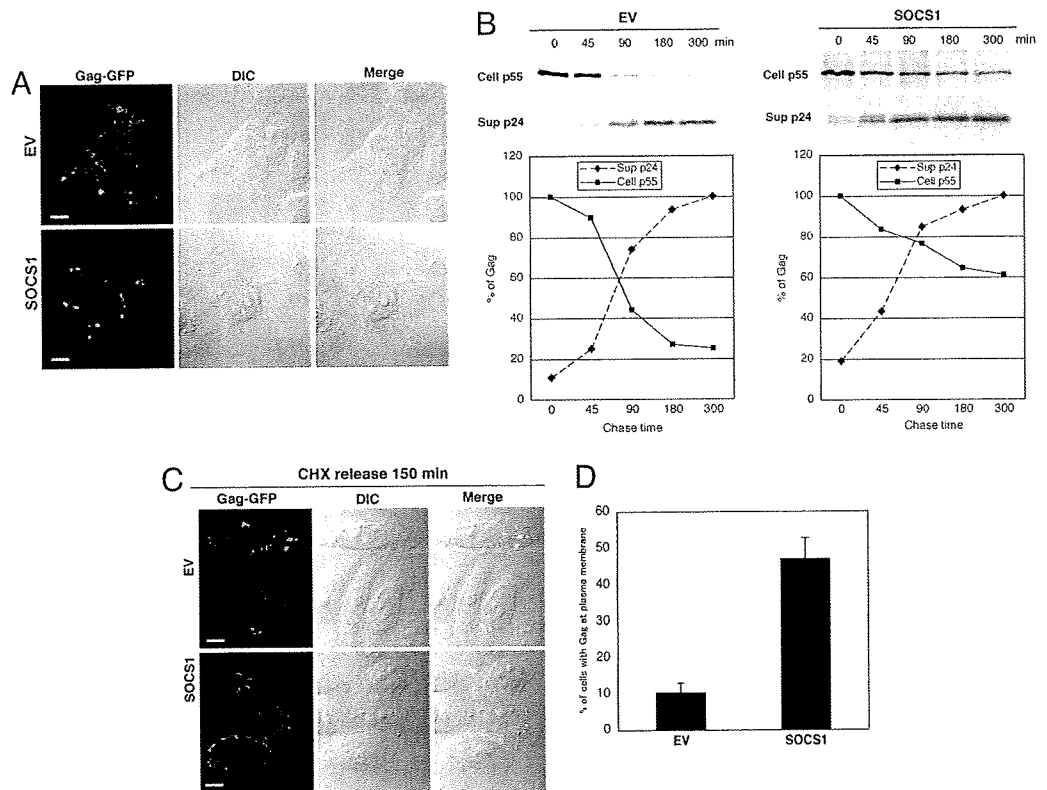
To examine the morphological aspects of HIV-1 particle production, transmission electron microscopy (TEM) was performed. 293T cells that had been cotransfected with pNL4-3, and either a control vector or a SOCS1 expression construct, were subjected to TEM analysis after fixation in glutaraldehyde. In SOCS1-transfected cells, a significantly increased number of mature virus particles was observed on the surfaces of PM compared with the control vector-transfected cells (Fig. 1E). There were also no obvious malformations of the virus particles in SOCS1-expressing cells, such as doublet formation or tethering to PM, which are characteristic of particle budding arrest (14) (Fig. 1E). Consistent with this observation, virions from SOCS1-transfected cells were found to be infectious as control viruses in Jurkat cells when the

same amounts of virus were infected (Fig. 1F). These results together indicate that SOCS1 enhances mature and infectious HIV-1 particle formation.

To elucidate the specific step in HIV-1 production that is enhanced by SOCS1, we next performed gene reporter assays using either luciferase expression constructs under the control of wild-type HIV-LTR (pLTR-luc), or a full-length provirus vector (pNL4-3-luc) (15). Interestingly, SOCS1 overexpression was found not to affect the transcription of these reporter constructs (data not shown), indicating that SOCS1 enhances HIV-1 replication via posttranscriptional mechanisms during virus production.

SOCS1 Interacts with the HIV-1 Gag Protein. The results of our initial experiments indicated that SOCS1 enhances HIV-1 production via a posttranscriptional mechanism. We therefore next tested whether SOCS1 could bind directly to HIV-1 Gag. GST pull-down analysis using C-terminal FLAG-tagged p55 Gag (codon-optimized) and GST-fused SOCS1 revealed that p55 Gag undergoes specific coprecipitation with GST-SOCS1 (Fig. 2A). Furthermore, both ectopically expressed myc-tagged SOCS1 and endogenous SOCS1 were found to undergo coimmunoprecipitation with Gag-FLAG in 293T cells (Fig. 2B and C). Additionally, GST pull-down analysis with various SOCS1 mutants, as depicted in Fig. 2D, further demonstrated that a mutant lacking the both N-terminal and SH2 domain (Δ N-SH2) could not bind

Fig. 3. SOCS1 enhances both the stability and trafficking of HIV-1 Gag. (A) HeLa cells cotransfected with pNL4-3 and either control vector (EV) or SOCS1 were immunostained with antibodies targeting anti-p24 (CA). Confocal microscopy with differential interference contrast (DIC) was then performed. (Scale bars: 10 μ m.) (B) 293T cells were transfected with either a control empty vector (EV) (Left) or myc-SOCS1 (Right) and cotransfected with pNL4-3. After 48 h, cells were pulse-labeled with [³⁵S]methionine or [³⁵S]cysteine for 15 min and chased for the durations indicated. Cell lysates and pelleted supernatant virions were immunoprecipitated with anti-p24 antibodies followed by autoradiography. (C and D) HeLa cells seeded on poly-L-lysine-coated cover slides were transfected with either vector control or SOCS1. After 24 h, cells were again transfected with Gag-GFP for 3 h and then treated with 100 μ g/ml CHX for 5 h to inhibit protein synthesis. This treatment was followed by incubation with fresh medium; then 150 min after the CHX release, cells were fixed and subjected to confocal microscopy (C). (Scale bars: 10 μ m.) Cells with Gag protein on the plasma membrane were scored out of 200 transfected cells (D).



p55 Gag, whereas an N-terminal or a SOCS box deletion did not affect the binding of SOCS1 to Gag in 293T cells (Fig. 2E). This finding indicates that the SH2 domain is important for the interaction of SOCS1 with HIV-1 Gag. Interestingly, the R105E mutant of SOCS1, which disrupts the function of the SH2 domain, still binds Gag (Fig. 2E), indicating that the Gag-SOCS1 association is independent of the tyrosine phosphorylation of Gag, as is the case for both HPV-E7 and Vav (16, 17).

To elucidate the SOCS1-binding region of the Gag protein, GST pull-downs with various GST-fused Gag domain constructs were performed. SOCS1 was detected in glutathione bead precipitates with GST-wild-type Gag, GST- Δ p6, GST-MA, and GST-NC, but not with other domain constructs (Fig. 2F), indicating that SOCS1 interacts with Gag via its MA and NC domains. Consistent with these results, the deletion of both the MA and NC domains of p55 Gag (Δ MA Δ NC) completely abolishes its interaction with SOCS1 in coimmunoprecipitation experiments (Fig. 2G). Furthermore, *in vitro* analysis with purified proteins also demonstrated that SOCS1 can indeed interact with both the MA and NC regions of HIV-1 Gag in the absence of nucleic acids or other proteins (SI Fig. 5).

We next wished to determine the functional interaction domain in HIV-1 Gag through which SOCS1 functions in terms of virus-like particle production. To this end, we used a MA-deleted Gag mutant with an N-terminal myristoyl tag derived from src (Δ MA-src) (18) and also an NC-deleted Gag mutant with a GCN4 leucine zipper in place of NC, which we herein denote as Δ NC-LZ but which has been described as Z_{IL}-p6 (19). Both of these mutants have been shown still to assemble and bud (18, 19). We found that SOCS1 overexpression can still augment the particle formation of both wild-type Gag and Δ NC-LZ but not Δ MA-src (Fig. 2H), indicating that the functional interaction between SOCS1 and HIV-1 Gag is in fact mediated through MA.

To confirm further the direct interaction between SOCS1 and Gag in cells, we examined the intracellular localization of these two proteins. Confocal microscopy revealed that endogenous SOCS1

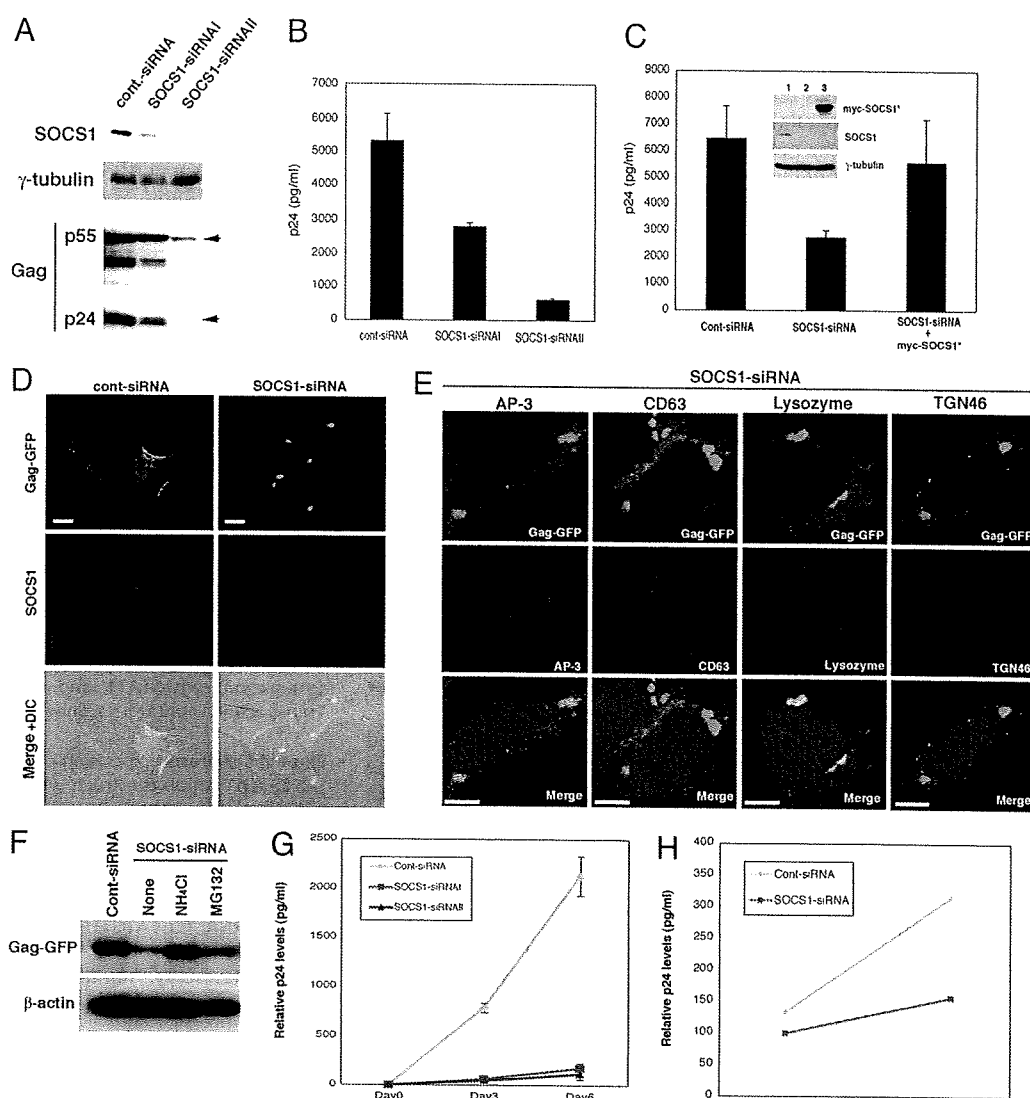
forms dotted filamentous structures in the cytoplasm and that Gag localizes in a very punctate pattern with SOCS1 from the perinuclear regions to the cell periphery (Fig. 2I). These data indicate that SOCS1 interacts with HIV-1 Gag in the cytoplasm during HIV-1 particle production.

SOCS1 Promotes both the Stability of Gag and Its Targeting to the Plasma Membrane. Because we had found from our initial data that SOCS1 increases HIV-1 particle production as a result of its direct interaction with intracellular Gag proteins, we next addressed whether SOCS1 positively regulates Gag stability and subsequent trafficking to PM. Our immunofluorescent analysis with the anti-p24 (CA) antibody initially revealed that SOCS1 overexpression increases the levels of Gag at PM when cotransfected with pNL4-3 at 48 h after transfection, although it was detected at PM in both control and SOCS1-expressing cells (Fig. 3A). Furthermore, the levels of cytoplasmic Gag were found to be much lower in the SOCS1-expressing cells compared with the control cells (Fig. 3A). These results indicate that SOCS1 enhances Gag trafficking to PM.

To examine next whether SOCS1 affects the stability and trafficking of newly synthesized Gag proteins, we performed pulse-chase analysis. This experiment revealed that SOCS1 significantly increases the stability of the intracellular p55 Gag polyprotein as well as the levels of p24 in the supernatant (Fig. 3B). Importantly, p24 was detectable at an earlier time point and reached maximum levels in a shorter period in the cell supernatant of SOCS1-transfected cells compared with control vector-transfected cells (Fig. 3B). This finding again suggests that SOCS1 facilitates the intracellular trafficking of newly synthesized Gag proteins to PM.

To confirm this hypothesis further, we performed cycloheximide (CHX) analysis with HeLa cells transfected using either vector control or SOCS1. After 24 h, cells were again transfected with Gag-GFP for 3 h and treated with CHX for 5 h to inhibit protein synthesis. Cells were then cultured in fresh medium without CHX for an additional 150 min and subjected to confocal microscopy. At

Fig. 4. The targeted inhibition of SOCS1 suppresses Gag trafficking and HIV-1 particle production and enhances Gag degradation in lysosomes. (A and B) 293T cells were transfected with either control siRNA or two different SOCS1-specific siRNAs (I or II) together with pNL4-3. At 48 h after transfection, cell lysates were subjected to immunoblotting analysis with the indicated antibodies (A). Cell supernatants were then subjected to ELISA analysis of p24 levels (B). (C) 293T cells were transfected with pNL4-3 and cotransfected with control-siRNA, SOCS1-siRNAI alone, or SOCS1-siRNAI plus siRNA-resistant myc-SOCS1 (myc-SOCS1*). After 48 h, cell supernatants were collected and subjected to p24 ELISA. (Inset) Immunoblots of the cell lysates. (D) HeLa cells were transfected with control or SOCS1-specific siRNA and cotransfected with GFP-Gag. At 48 h after transfection, the cells were subjected to confocal microscopy. (E) HeLa cells were transfected with Gag-GFP and SOCS1-siRNA constructs for 48 h. Cells were then fixed and subjected to immunofluorescent analysis with indicated antibodies followed by DAPI staining. (Scale bars: 10 μ m.) (F) HeLa cells were transfected with Gag-GFP and cotransfected with either control-siRNA or SOCS1-siRNA. After 36 h, the cells were treated with a mock solution, 10 mM NH_4Cl or 10 μM MG132 for another 16 h. Cells were then harvested and subjected to immunoblotting analysis with anti-GFP or anti- β -actin antibodies. (G) Jurkat cells were infected with a retroviral vector encoding control (Cont) or two different SOCS1-specific siRNAs (I or II). After selection with puromycin, the cells were then infected with HIV-1_{NL4-3} (multiplicity of infection, 0.1), and p24 antigen levels in cell supernatant were measured by ELISA at the indicated time points. (H) Human primary CD4 T cells were separated from healthy donors and infected with lentivirus vectors encoding either control- or SOCS1-siRNAI. The cells were then infected with HIV-1_{NL4-3} (multiplicity of infection, 0.1), and p24 antigen levels in cell supernatant were measured by ELISA at the indicated time points.



this time point, Gag-GFP was found to localize predominantly in a perinuclear region in the control cells (Fig. 3C), whereas almost half of the SOCS1-transfected cells exhibited Gag-GFP localization on PM (Fig. 3D). These results again indicate that SOCS1 efficiently enhances the trafficking of newly synthesized Gag protein to PM.

The Targeted Disruption of SOCS1 Inhibits Gag Trafficking and HIV-1 Particle Production. To delineate further the role of SOCS1 in the trafficking of Gag and in subsequent HIV-1 particle production, we depleted cellular SOCS1 by siRNA. The significant depletion of SOCS1 expression by two different SOCS1-specific siRNA constructs was confirmed by immunoblotting analysis (Fig. 4A and B). Significantly, in cells cotransfected with pNL4-3 and SOCS1-specific siRNAs, both HIV-1 particle release and the levels of intracellular Gag protein are significantly decreased compared with the control cells (Fig. 4A and B). Furthermore, the effects of SOCS1-siRNA on the inhibition of HIV-1 particle production was diminished by reexpression with a codon-optimized SOCS1 construct that is resistant to these siRNAs (Fig. 4C), indicating that the SOCS1 siRNA suppression of HIV-1 particle production depends on the availability of endogenous SOCS1.

Consistent with these observations, immunofluorescent analysis further revealed that the expression of SOCS1-siRNA dramatically inhibits Gag trafficking such that Gag proteins accumulate in the perinuclear regions as large solid aggregates, as has been reported (20) (Fig. 4D). This finding indicates that SOCS1 plays an essential role in the Gag trafficking from perinuclear clusters to PM. Interestingly, these discrete perinuclear clusters of Gag were found to colocalize with lysosome markers, lysozyme, and partly with AP-3, but neither with the late endosome MVB marker CD63 nor the *trans*-Golgi marker TGN46, indicating that Gag is targeted for degradation by lysosomes when the function of SOCS1 is inhibited (Fig. 4E). In support of this notion, the levels of intracellular Gag were found to be significantly increased by treatment with a lysosome inhibitor NH_4Cl but not by a proteasome inhibitor MG132 in SOCS1-siRNA cells (Fig. 4F), further indicating that the perinuclear clusters of Gag will undergo lysosomal degradation rather than proteasomal degradation when optimal Gag transport to PM is suppressed by the inhibition of SOCS1.

We next addressed whether targeted SOCS1 inhibition would affect HIV-1 particle production in human T cells. The effect of SOCS1 depletion was clearly evident in both HIV-1_{NL4-3}-infected