## MATERIALS AND METHODS

### Plasmids.

**Retroviral reporter vectors.** pNL4-3 contains a full infectious provirus, HIV-$1_{NL4-3}$ (3). pNL4-3 G89V encodes a mutant CA that disrupts the CypA binding site (123). It was created by replacing the BssHII-SpeI fragment in pNL4-3 with a fragment that contained the G89V mutation. pNL4-$3_{GFP}$ is pNL4-3 with an *env*-inactivating mutation and enhanced green fluorescent protein (eGFP) replacing *nef* (57). pNL4-$3_{GFP}$ G89V carries a mutation that disrupts the CypA binding site.

CSGW is an HIV-1 vector expressing eGFP the control of the spleen focus-forming virus (SFFV) long terminal repeat (LTR) (34). CSGWΔN was modified from the parent CSGW vector by blunting a NotI restriction site.

The pCIG3-N and pCIG3-B are Murine leukemia virus (MLV)-derived constructs expressing N- and B-tropic CA, respectively (13). pCNCG is an MLV-based expression vector containing eGFP under the control of the cytomegalovirus immediate early (CMVie) promoter. pMIG is an MSCV-based expression vector containing an IRES-GFP cassette (124).

**Retroviral expression vectors.** p8.9NΔSB expresses HIV-1 *gag* and *pol* from the (CMVie) promoter and bears inactivating mutations in the RNA packaging region, *env*, and nonessential accessory genes (12). It was generated by cutting a 1.8-kbp BglII-SpeI fragment from the parent vector p8.9N (a gift from Greg Towers), and recircularizing after blunt ending the extremities using the Klenow enzyme. HIV-$1_{GFP}$G89V, HIV-$1_{GFP}$ P90A and HIV-1 $_{GFP}$A92E and are p8.9NΔSB with G89V, P90A or A92E mutations in CA (12). To generate these mutant versions of p8.9NΔSB, an 821-bp fragment was PCR-

amplified from the relevant mutated pNL4-3 clones (ref!), using the oligos 1 and 2. The PCR products were subcloned into the p8.9NΔSB vector using the NotI and SpeI sites. HIV-$1_{GFP}$ CA9 and HIV-$1_{GFP}$ MVP5180 are p8.9NΔSB in which the CypA binding region has been replaced with the CypA binding regions of HIV-1 Group O isolates CA9 or MVP5180, respectively. These versions of p8.9ΔSB were generated by PCR using the oligos 3 and 4 for CA9, and oligos 5 and 6 for MVP5180. The introduced mutations were verified by sequencing.

To restore CypA synthesis in HeLa CypA knockdown (KD) cells, a CypA cDNA bearing silent mutations that render it nontargetable by the CypA147 shRNA (103) was subcloned from pcDNA-ntCypA into CSGWΔN using BamHI and NotI sites to generate CSW-ntCypA. An otherwise isogenic vector CSW-ntCypA/R55A carrying the R55A mutation in the hydrophobic pocket of CypA that abolishes interaction with HIV-1 CA (20) was also engineered.

pCL-Eco contains *psi*-minus MLV expressed from the CMVie promoter (81). pMDG encodes the vesicular stomatitis virus glycoprotein (VSV-G) (13). PCR3.1-HXb2, pcDNA3.1-VCP, pcDNA3.1-IIIb express envelope glycoproteins from designated HIV-1 strains. pcDNA3.1-Mac251 expresses SIVmac251 envelope glycoproteins. All env-encoding constructs were a gift from Dr. Jim Hoxie, University of Pennsylvania.

**RNAi vectors.** To express siRNA from a plasmid we engineered pSUPER as described by others (25). pSUPER encodes short hairpin RNAs that are processed in the cell to generate siRNAs. The oligos 7 and 8 were used to generate pSUPER plasmid for CypA protein knockdown (pSUPER-CypA), with the following CypA target sequence: 5'-GGGTTCCTGCTTTCACAGA-3'. pMH-CypA147 is a retroviral Murine stem cell

leukemia (MSCV)-based shRNA vector that targets cyclophilin A expression. To generate it, the H1 promoter and the hairpin cassette were cut from pSUPER-CypA using EcoRI and SalI and subcloned into pMSCV that contains an inactivating deletion in the 3'LTR (MSCVΔU3) (5). pSUPER.retro.puro (pSRP; OligoEngine, Seattle) is an MSCV-derived vector expressing short hairpin RNAs (shRNAs) from the H1 pol III promoter as in pSUPER and a puromycin resistance gene driven by the PGK promoter (25). pSRP-CypA targets cyclophilin A expression and was generated similarly to pMH-CypA147. To target CypB expression, pSRP-CypB was generated by subcloning the pSUPER shRNA cassette into pSRP using Sal I and EcoR I, after subcloning the oligos 9 and 10 into pSUPER (CypB target sequence: 5'-GATGTAGGCCGGGTGATCT-3'). pSRP-$TRIM5_{hu}$ expresses an shRNA targeting human TRIM5 mRNA. It was generated similarly, using the oligos 11 and 12 (TRIM5hu target sequence: 5'-GCTCAGGGAGGTCAAGTTG-3') (114). The control plasmid, pSRP-Luc, encodes an shRNA targeting luciferase (103). Oligos 13 and 14 were used to generate this construct.

pshRNA.lenti.puro (pshRLP) is an HIV-1 based vector derived from pCSGWΔN. It contains an H1-shRNA cassette as in pSUPER (25) and a puromycin resistance gene driven by the PGK promoter. pshRLP-$TRIM5_{hu}$ expresses short hairpin RNAs (shRNAs) targeting expression of $TRIM5_{hu}$, and pshRLP-Luc is a control construct targeting firefly luciferase. To engineer pshRLP-$TRIM5_{hu}$ and pshRLP-Luc, oligos 15 and 16 were used for PCR, with pSRP-$TRIM5_{hu}$ or pSRP-Luc as templates. The PCR products were subcloned into pCSGWΔN using EcoRI and NotI sites.

**Expression plasmids.** pcDNA-CD4 expresses CD4 under the control of the CMVie promoter.

**Cells and drugs.** Adherent cells (293T, HeLa, TE671, *Mus dunni* tail fibroblasts) were maintained in DMEM (Cellgro), and suspension cells (Jurkat, CEM-SS, SupT1) were maintained in RPMI (Cellgro), each supplemented with 10% fetal bovine serum and antibiotics. PBL were isolated from whole blood using Ficoll-Paque™ PLUS (Amersham), stimulated with 2μg/ml Phytohemmagglutinin (PHA, Sigma), and maintained in RPMI supplemented with 15% fetal bovine serum (Cellgro), antibiotics and 20 IU/ml recombinant human IL2 (Invitrogen).

CsA (Bedford Laboratories) was prepared in dimethyl sulfoxide at 10 mg/ml and diluted in tissue culture medium to the indicated concentrations prior to each experiment. MeIle$^4$-CsA and Sanglifehrin (gifts from Novartis, Basel, Switzerland) were prepared in dimethyl sulfoxide at 10 mg/ml and 10 mM, respectively, and diluted further in tissue culture medium to 2.5 μM for each experiment. $As_2O_3$ (Sigma) was prepared as described previously (13) and diluted further in phosphate-buffered saline to the indicated concentrations prior to use. Dextran sulfate (5 mg/ml; Sigma) was prepared in $H_2O$ and was used at 100 μg/ml, a concentration that completely precludes Env-mediated entry, as described previously (13).

**Viruses.** Vectors and viruses were produced by transfecting $10^6$ 293T or $0.5x10^6$ HeLa cells per well in 6-well plates using Lipofectamine 2000 (Invitrogen). Full-length infectious HIV-1 viruses were produced by transfecting 293T cells with 4 $\mu$g of pNL4-3 or pNL4-3 G89V. For production of NL4-$3_{GFP}$ viruses, 293T cells were co-transfected with 0.3$\mu$g pMDG and 3.7 $\mu$g of either pNL4-$3_{GFP}$ or pNL4-$3_{GFP}$ G89V. HIV-$1_{GFP}$ vectors were produced by co-transfecting 293T cells with 2 $\mu$g CSGW, 0.3 $\mu$g pMDG and 1.7 $\mu$g of either wild-type or mutant p8.9NΔSB. To produce vectors delivering non-targetable

CypA cDNA, 293T cells were co-transfected with 1.7 $\mu$g of wild type p8.9NΔSB, 0.3 $\mu$g pMDG and 2 $\mu$g of either CSW-ntCypA, or CSW-ntCypA R55A.

To produce MSCV-based shRNA transducing vectors, 293T cells were co-transfected with 1.7 $\mu$g of pCL-Eco, 0.3 $\mu$g of pMDG, and 2 $\mu$g of either pMH-CypA, pSRP-CypA, pSRP-TRIM5$_{hu}$ or pSRP-Luc. To produce HIV-1-based shRNA vectors, 293T cells were co-transfected with 1.7 $\mu$g of p8.9NΔSB, 0.3 $\mu$g of pMDG and 2 $\mu$g of either pshRLP-TRIM5$_{hu}$ or pshRLP-Luc.

For production of N- or B-tropic MLV vectors, cells were co-transfected with 0.3 $\mu$g of pMDG, 2 $\mu$g of either pCNCG or pMIG, and 1.7 $\mu$g of either pCIG3-N or pCIG3-B.

The medium was changed 6 hours post transfection. If drugs were present at the time of virus production, they were added after the medium has been changed. All virus- and vector-containing supernatants were harvested 48 hrs post transfection, spun at 300 x *g* for 5 min to remove cell debris, filtered through an 0.45-µm pore-size filter (Pall Acrodisc) and stored at –80°C. For infectious NL4-3 viruses, or if drugs were added during virus production, clarified supernatant was layered onto a 25% sucrose cushion and accelerated at 100,000 x g for 2 hrs in an SW41 rotor (Beckman). The pelleted virions were resuspended in RPMI with 10% fetal bovine serum and stored at –80°C. Virions were normalized prior to infection by measuring reverse transcriptase (RT) activity as previously described .

Virus like particles (VLPs) were produced by transfecting 4.5 x $10^6$ 293T cells in a 10cm diameter plate using $Ca_2PO_4$. To produce HIV-1 VLPs, cells were co-transfected with 2$\mu$g of pMDG and 18$\mu$g of either wild-type p8.9NΔSB or p8.9NΔSB G89V. MLV

VLPs were produced by co-transfecting the cells with 2$\mu$g of pMDG and 18 $\mu$g of either pCIG3-N or pCIG3-B. All VLP-containing supernatants were harvested 48 hrs post transfection, spun at 300 x *g* for 5 min to remove cell debris, and filtered through an 0.45-$\mu$m pore-size filter (Pall Acrodisc). The clarified supernatant was then layered onto a 25% sucrose cushion and accelerated at 100,000 x g for 2hrs in an SW41 rotor. The pelleted VLPs were resuspended in RPMI with 10% fetal bovine serum and used immediately. Subsequently, RT activity was measured in all VLP samples to confirm that amounts of VLPs used for each experiment were equal among each other.

**Infections.** Adherent cells were plated in 48 well plates (4 x $10^4$ cells/well), cells in suspension were seeded in 48 well plates at $10^5$ cells/well. Virion stocks were added to the cells in a final volume of 250 $\mu$l/well. GFP expression in infected cells was analyzed 48 hrs post-infection by flow cytometry. For infections with full-length virus, dextrane sulfate was added 16 hrs post-infection (100 $\mu$g/ml) to preclude spread of the virus. 48 hrs post-infection, cells were fixed in 4% formaldehyde/PBS and assayed for intracellular p24 using KC57, an RD1-conjugated a-p24 antibody (Coulter Immunology), or using a monoclonal anti-p24 antibody (183-H12-5C, NIH AIDS Research & Reference Reagent Program).

To generate HeLa or TE671 cells with stable expression from either pMH - CypA147 or the pSRP vectors, cells were seeded into 24-well plates at 3 x $10^4$ cells/well, covered with 500 µl of clarified 293T supernatant, and spinoculated for 70 min at 1,200 x *g*. Single cell clones expressing pMH-CypA147 were screened by western blot for the best knockdown of CypA expression. 48 hrs transduction, pools of cells expressing pSRP were subjected to puromycin selection with quantum increases in drug concentration (1,

5, 10, 15, 20, 30, 40, 50 ng/μl) over a period of 10 days.

To generate Jurkat and CEM-SS cells with stable expression from pshRLP-vectors, cells were seeded into 24-well plates at $10^5$ cells/well and infected with 50 μl of clarified 293T supernatant in a total volume of 500 μl. All cells were subjected to puromycin selection 48 hrs post-transduction as described above.

**Flow Cytometry.** FACSScan and Cellquest Pro software (Becton Dickinson) were used to record GFP or FITC fluorescence in the FL1 and dsRed fluorescence in the FL2 channels. $10^4$ live cells were analyzed per sample.

**Western blotting.** Cells were normalized by number or protein concentration, and lysates were subjected to SDS-PAGE and immunoblotting with antibodies against CypA (rabbit polyclonal; Biomol) and β-actin (mouse monoclonal; Sigma). Coomassie gels were used to confirm that loading of samples had been properly normalized.

Virions produced as described above were pelleted through a sucrose cushion, resuspended in 2x SDS-loading buffer, and subjected to western blotting using antibodies against CypA and p24 (see above).