SUPPLEMENTAL INFORMATION INVENTORY

Hultquist *et al*. 2016. *Cell Reports*. “A Cas9 Ribonucleoprotein Platform for Functional Genetic Studies of HIV-Host Interactions in Primary Human T Cells”

Supplemental Figures (5)

Supplemental Figure 1 | Knock-out of HIV co-receptor *CXCR4* confers resistance to infection in a tropism-dependent manner in 4 independent donors, related to **Figure 2**.

Supplemental Figure 2 | Knock-out of HIV co-receptor *CCR5* confers resistance to infection in a tropism-dependent manner in 6 independent donors, related to **Figure 2**.

Supplemental Figure 3 | LEDGF knock-out confers resistance to HIV infection in 4 independent donors, related to **Figure 3**.

Supplemental Figure 4 | Double knock-out of *CXCR4* and *LEDGF* in primary T cells in 3 donors, related to **Figure 4**.

Supplemental Figure 5 | Knock-out validation of selected genes targeted in the HIV integrase interactor screen in primary $CD4^+$ T cells, related to **Figure 5**.

Supplemental Tables (2 – uploaded separately)

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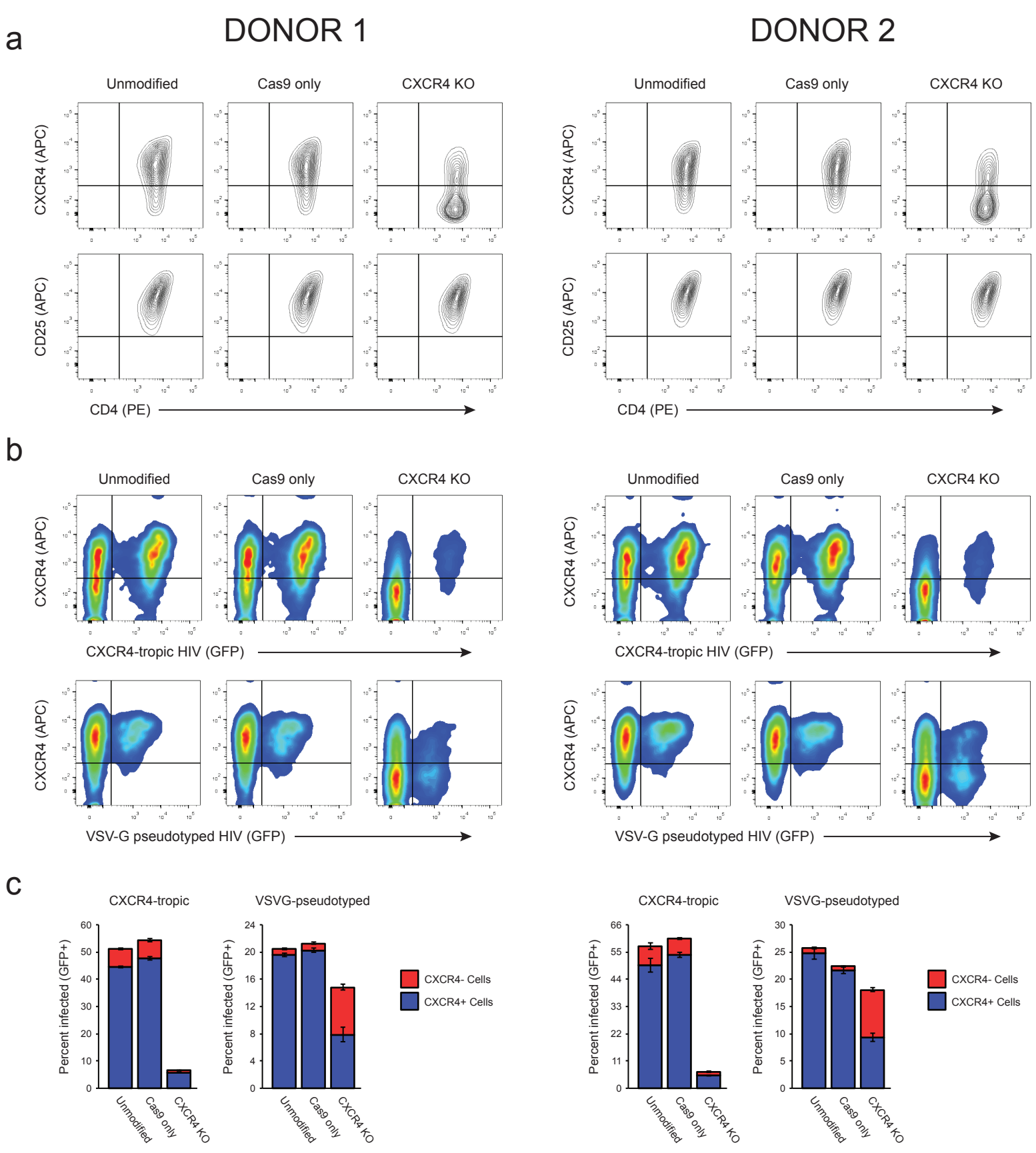
Supplemental Table 2 | CRISPR RNAs, raw data, and normalized data for the HIV integrase interactor screen, related to **Figure 5**.

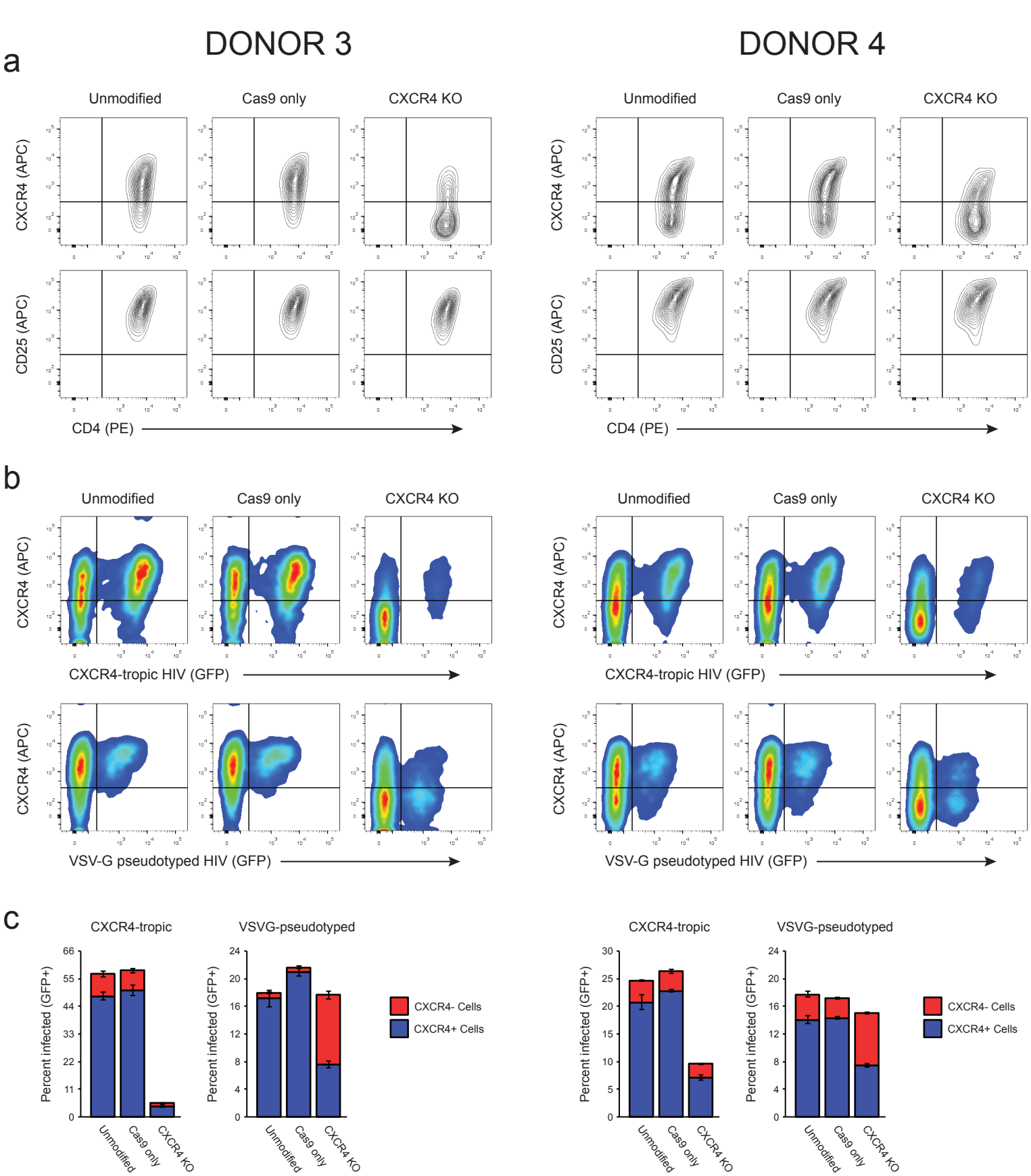
Supplemental Figure/Table Legends

Supplemental Experimental Procedures

Supplemental References



Supplemental Figure 1



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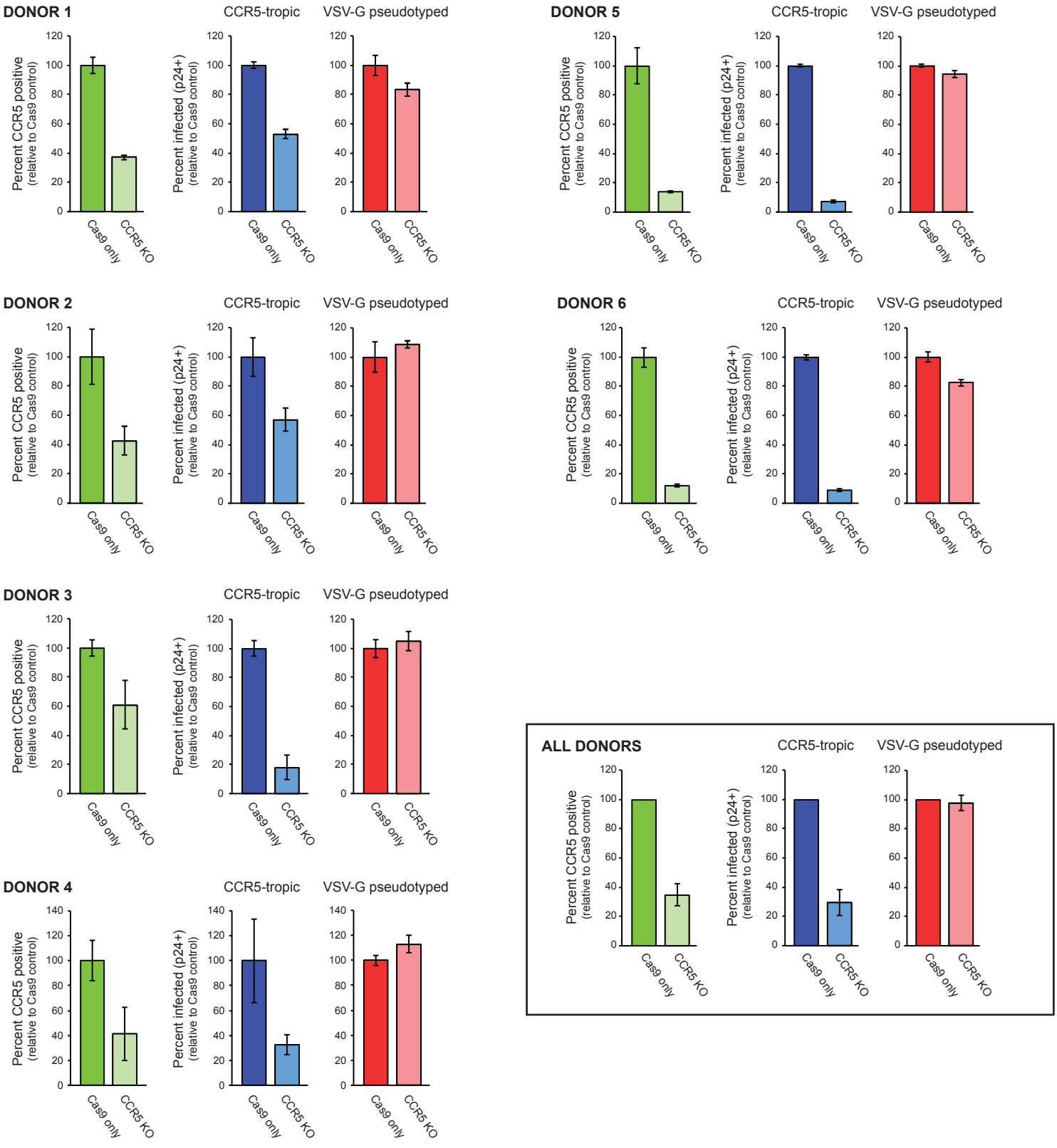
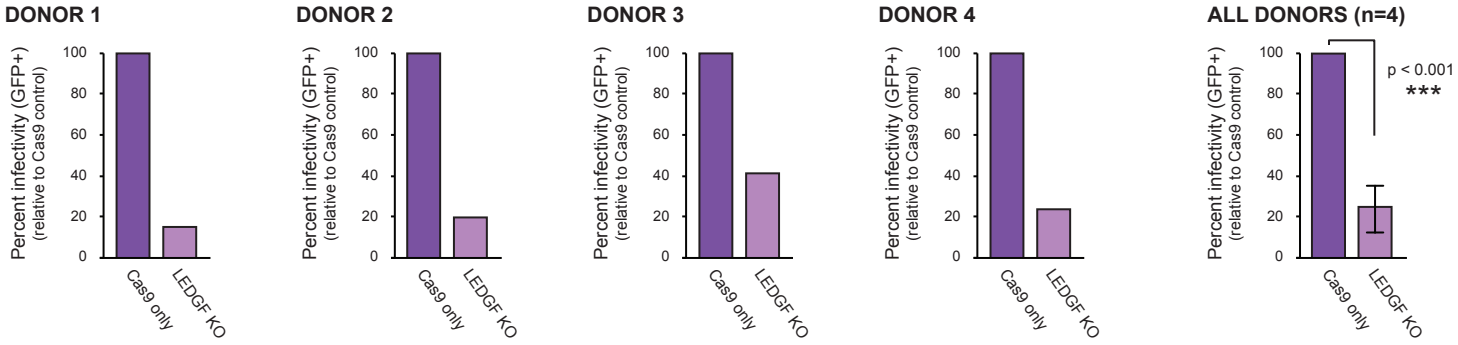


Supplemental Figure 2

a



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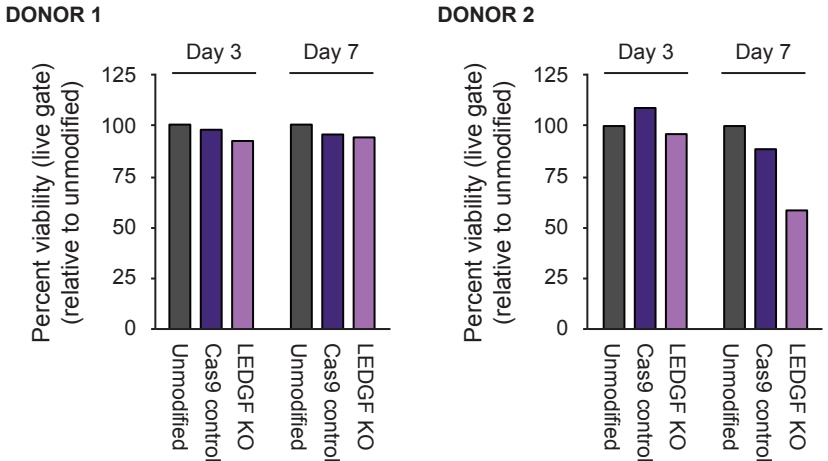

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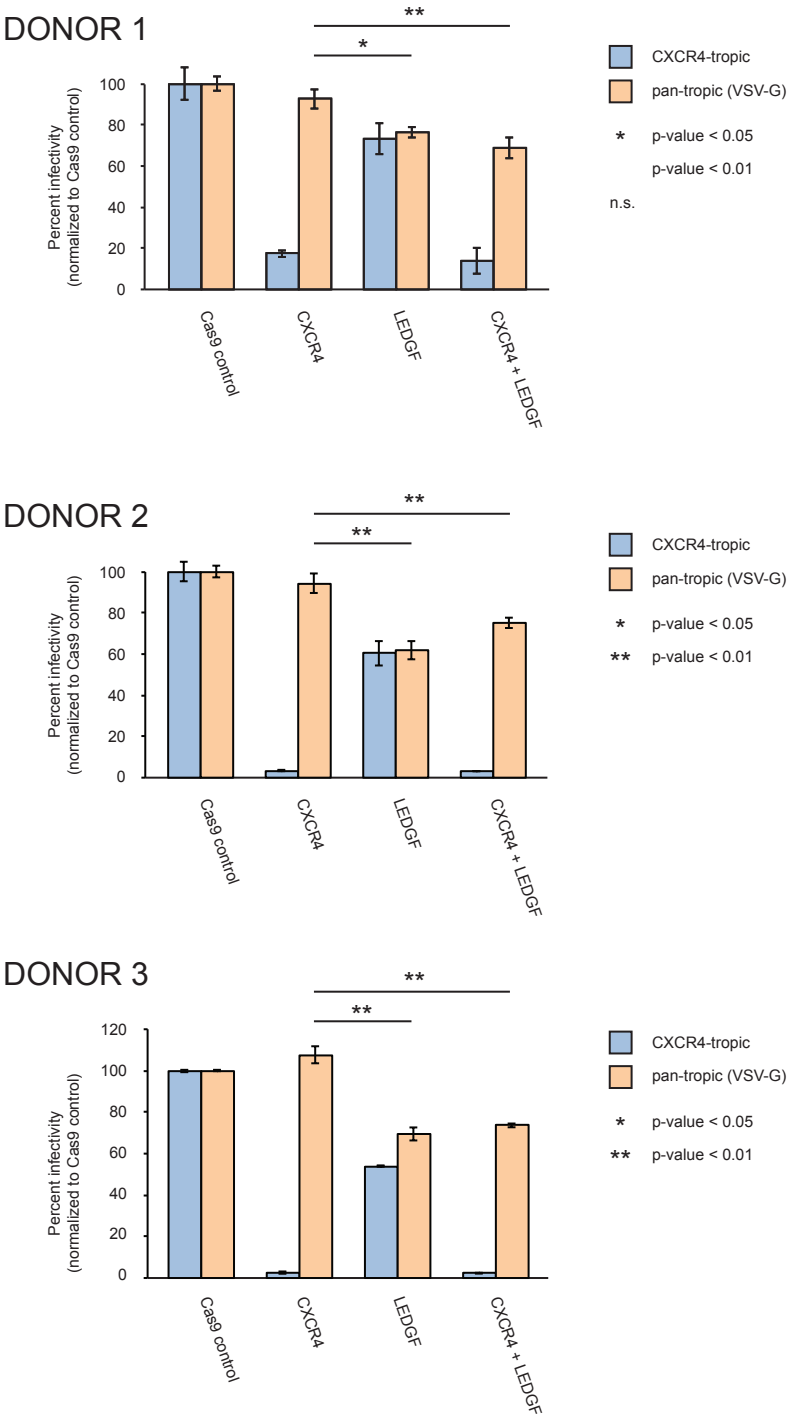

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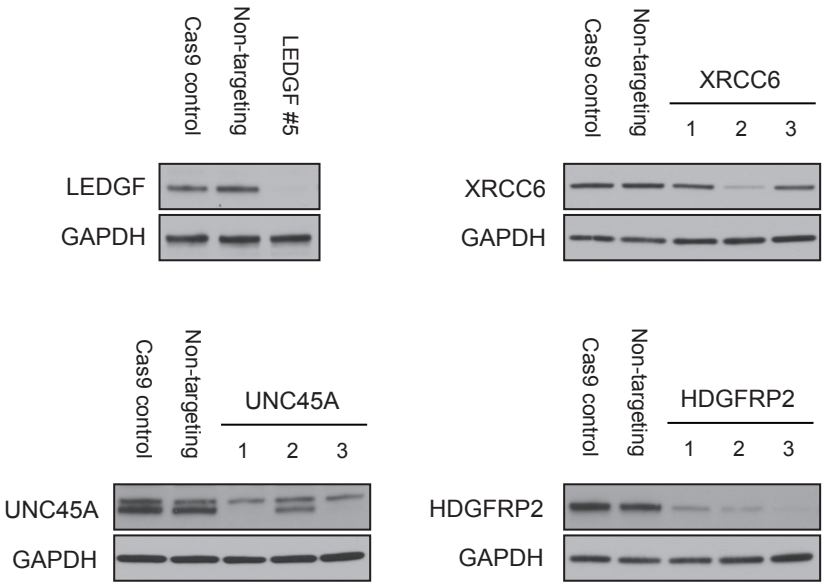

Supplemental Figure 5

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1 | Knock-out of HIV co-receptor *CXCR4* confers resistance to infection in a tropism-dependent manner in 4 independent donors, related to **Figure 2**. **(a)** Primary T cells were isolated from four different donors and electroporated with either nothing, Cas9 protein alone, or Cas9 RNPs targeting *CXCR4*. Four days after electroporation, these cells were stained with anti-CD4-PE and anti-CXCR4-APC, or anti-CD4-PE and anti-CD25-APC. **(b)** These cells were then infected with a CXCR4-tropic (LAI strain) GFP reporter virus or an identical VSV-G pseudotyped, Env-deficient virus in technical triplicate. After 36 hours, cells were co-stained with anti-CXCR4-APC and analyzed by flow cytometry. **(c)** Bar graphs depicting the average percent infected cells across technical triplicates +/- standard deviation, including both $CXCR4^-$ cells (red) and $CXCR4^+$ cells (blue). Donor 3 is presented in **Figure 2**.

Supplemental Figure 2 | Knock-out of HIV co-receptor *CCR5* confers resistance to infection in a tropism-dependent manner in 6 independent donors, related to **Figure 2**. Primary T cells were isolated from six different donors and electroporated with either nothing, Cas9 protein alone, or Cas9 RNPs targeting *CCR5*. Two days after electroporation, cells were stained with anti-CCR5-APC and analyzed by flow cytometry. Cells were then infected with a CCR5-tropic (JR-CSF strain) virus or a VSV-G pseudotyped reporter virus in technical triplicate. After 48 hours, cells were stained with p24-FITC before analysis. Bar graphs depict the average percent $CCR5^+$ cells (green), CCR5-tropic infected cells (blue), and VSV-G pseudotyped infected cells (red) across technical triplicates +/- standard deviation relative to the Cas9 control. All donor data was collected in the inset bar graph and is reported as the mean of averages +/- standard error of the mean. Donors 5 and the summation graph are presented in **Figure 2**.

Supplemental Figure 3 | LEDGF knock-out confers resistance to HIV infection in 4 independent donors, related to **Figure 3**. **(a)** Primary T cells were isolated from four different donors and electroporated with Cas9 protein alone or the most effective Cas9 RNP targeting *LEDGF* (crRNA #5 above). Cells were spinoculated with HIV-$1^{R3/7}$ and percent infection determined by flow cytometry after 3 days. Percent infection was normalized to the Cas9 control. The final bar graph depicts the average percent infection observed in the Cas9 control and LEDGF KO cells across all four donors at day 3 +/- standard deviation. Statistical significance was determined using a two-tailed Student's t-test. **(b)** Primary T cells were isolated from two different donors and electroporated with buffer alone, Cas9 alone, or the most effective Cas9 RNP targeting *LEDGF* (crRNA #5). Cells were infected as indicated in **Figure 3B** and percent infection determined by flow cytometry. Percent of cells in the live gate was

used as a rough estimate for cell viability in the population and is plotted here at days 3 and 7 normalized to the unmodified control. These data correspond to the infectivity data presented in **Figure 3C**.

Supplemental Figure 4 | Double knock-out of *CXCR4* and *LEDGF* in primary T cells in 3 donors, related to **Figure 4**. Primary T cells were isolated from three different donors and electroporated with the indicated Cas9 RNPs. CXCR4 and LEDGF crRNPs were mixed at a 1:1 ratio for the double knock-out condition. After four days, cells were infected with a CXCR4-tropic (LAI strain) GFP reporter virus or an identical VSV-G pseudotyped, Env-deficient virus in technical triplicate. After 36 hours, percent infected cells (GFP^+) were determined by flow cytometry. The bar graphs depict the average percent infected cells across technical triplicates +/- standard deviation. p-values were calculated by pairwise Student's t-test.

Supplemental Figure 5 | Knock-out validation of selected genes targeted in the HIV integrase interactor screen in primary $CD4^+$ T cells, related to **Figure 5**. Of the 15 genes to score significantly in the screen, antibodies were ordered for 4 proteins to validate knock-out at the protein level by immunoblot. Immunoblots are shown probing for LEDGF (as a positive control), UNC45A, XRCC6, and HDGFRP2 in a Cas9 control sample, a non-targeting control treated sample, and knock-out experimental samples for the respective RNPs targeting the respective genes. Blots were probed again for GAPDH as a loading control.

Supplemental Table 1 | Editing efficiency and variability by individual RNPs, pertaining to all **Results**. This table summarizes all crRNAs used in these studies, including the target gene, crRNA number, crRNA sequence, editing efficiency in two independent donors by TIDE analysis (when available), effect on protein expression (when available), and sequence of the TIDE primers used for verification.

Supplemental Table 2 | CRISPR RNAs, raw data, and normalized data for the HIV integrase interactor screen, related to **Figure 5**. This excel workbook contains the design, raw data, and processed data for the screen of HIV integrase interactors presented in **Figure 5** of the main text. The 'Gene List' tab includes the curated gene list of HIV integrase interactors pulled from the HIV-1, human interaction database and the PMID references supporting them. The 'Plate Set Up' tab depicts the crRNA array for each donor along with the targeting sequence. The next two tabs contain the raw data of HIV infected (GFP+) cells determined by flow cytometry after live gating at days 2, 4, and 6. The final two tabs contain the raw data arrayed by plate followed by data transformation to normalize values across

plates. A correction for observed edge effects was also applied as indicated and all standard deviations were transformed accordingly.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Human T Cell Isolation and Culture

Whole blood was collected from human donors in sodium heparinized vacutainer tubes (Becton Dickinson) with approval by the UCSF Committee on Human Research and processed within 12 hours. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation. Briefly, fresh blood was mixed in a 1:1 ratio with Ca^{2+} and Mg^{2+} free Hank's Balanced Salt Solution (HBSS). Buffy coats were diluted in a 1:10 ratio with HBSS. 30mL of the respective HBSS/blood solution were transferred to 50mL Falcon tubes and underlaid with 12mL Ficoll-Paque PLUS (Amersham/GE healthcare). After density gradient centrifugation (1,000*g, 20 minutes, no brakes) the PBMC layer was carefully removed and the cells were washed twice with Ca^{2+} and Mg^{2+} free HBSS. $CD4^+$ T cells were enriched with the Easysep Human $CD4^+$ T-cell enrichment kit (Stemcell Technologies).

Isolated $CD4^+$ T cells were suspended in complete Roswell Park Memorial Institute (RPMI), consisting of RPMI-1640 [UCSF Cell Culture Facility (CCF)] supplemented with 5mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, UCSF CCF), 2mM Glutamine (UCSF CCF), 50µg/mL penicillin/streptomycin (P/S, UCSF CCF), 5mM nonessential amino acids (UCSF CCF), 5mM sodium pyruvate (UCSF CCF), and 10% fetal bovine serum (FBS, Atlanta Biologicals). These cells were immediately stimulated on anti-CD3 coated plates [coated overnight with 10µg/mL αCD3 (UCHT1, Tonbo Biosciences)] in the presence of 5µg/mL soluble anti-CD28 (CD28.2, Tonbo Biosciences). Cells were stimulated for 48 hours prior to electroporation.

Cas9 RNP mediated Editing of Primary Human T cells

Electroporation was performed using the Amaxa P3 Primary Cell 96-well Nucleofector kit and 4D-Nucleofecter (Lonza). Recombinant *S. pyogenes* Cas9 protein used in this study expresses a C-terminal HA tag and two nuclear localization signal (NLS) peptides that facilitate transport across the nuclear membrane. The protein was expressed and purified as described (Anders and Jinek, 2014) and obtained from the QB3 Macrolab, University of California, Berkeley. Purified Cas9 protein was stored in 20 mM HEPES at pH 7.5 plus 150mM potassium chloride, 10% glycerol, and 1mM tris(2-carboxyethyl)phosphine (TCEP) at −80°C. crRNA for each gene were designed using the online tool developed by the Zhang lab at the Massachusetts Institute of Technology (http://crispr.mit.edu/)(Hsu et

al., 2013). Each crRNA and the tracrRNA were chemically synthesized (Dharmacon) and suspended in 10mM Tris-HCl pH 7.4 to generate 80μM RNA stocks.

Cas9 RNPs were prepared fresh for each experiment. crRNA and tracrRNA were first mixed 1:1 and incubated 30 minutes at 37°C to generate 40μM crRNA:tracrRNA duplexes. An equal volume of 40μM *S. pyogenes* Cas9-NLS was slowly added to the crRNA:tracrRNA and incubated for 15 minutes at 37°C to generate 20μM Cas9 RNPs. For each reaction, roughly 3*10^5 stimulated T cells were pelleted and re-suspended in 20μL P3 buffer. 3μl 20μM Cas9 RNP mix was added directly to these cells and the entire volume transferred to the 96-well reaction cuvette. For double editing reactions, 3μL of each Cas9 RNP was added to 20μL cells. Cells were electroporated using program EH-115 on the Amaxa 4D-Nucleofector (Lonza). 80μL pre-warmed, complete RPMI was added to each well and the cells were allowed to recover for 30 minutes at 37°C. Cells were then re-stimulated on plates coated overnight with 10μg/ml anti-CD3 (UCHT1, Tonbo Biosciences) and 10μg/ml anti-CD28 (CD28.2, Tonbo Biosciences) for 24 hours prior to infection.

HIV Virus Stocks

CXCR4-tropic virus stocks were generated from an HIV-1^{LAI} molecular clone wherein the viral *nef* gene had been replaced by a Green Fluorescent Protein (GFP) marker (kindly provided by Reuben S. Harris, University of Minnesota, Twin Cities). CCR5-tropic virus stocks were generated from an HIV-$1^{JR\text{-}CSF}$ full molecular clone (kindly provided by Tim Wang, Massachusetts Institute of Technology). Virus stocks for LEDGF spreading infection were generated from an HIV-$1^{R7/3}$ molecular clone with an IRES-GFP cassette after the *nef* reading frame (kindly provided by Cecilia Cheng-Mayer, Aaron Diamond AIDS Research Center). Replication-competent reporter virus stocks for the intregrase interactor screen were generated from an HIV-$1^{NL4\text{-}3}$ molecular clone wherein GFP had been cloned behind an Internal Ribosomal Entry Site (IRES) cassette following the viral *nef* gene.

Briefly, 10μg of molecular clone was transfected (polyJet, SignaGen) into 5*10^6 Human Embryonic Kidney 293 plus T cell antigen (HEK293T, CRL-3216, ATCC) cells according the manufacturer's protocol. 25mL supernatant was collected at 48 and 72 hours and combined. Virus-containing supernatant was filtered through 0.45μm polyvinylidene fluoride (PVDF) filters (Millipore) and precipitated in 8.5% polyethylene glycol (PEG, average M_n 6000, Sigma), 0.3M sodium chloride for 4 hours at 4°C. Supernatants were centrifuged at 3500rpm for 20 minutes and virus resuspended in 1mL phosphate buffered saline (PBS) for a 50x effective concentration. Aliquots were stored at -80C for later use. For VSV-G pseudotyped virus, an Env-deficient HIV-1^{LAI} *nef*:GFP molecular clone (identical to the CXCR4-tropic virus, but without native envelope expression, also provided by Reuben S. Harris) was transfected alongside the VSV-G envelope expressing plasmid pMD2.G (#12259, Addgene) at a 1:3

ratio of VSV-G:molecular clone.

Virus stocks were titered directly on activated primary human $CD4^+$ T cells (3-7 days post-stimulation with αCD2/CD3/CD28 beads, Miltenyi Biotec) over a 100-fold dilution by direct addition to the culture media in technical triplicate. Percent infected cells was determined after 48 hours by flow cytometry directly for GFP reporter viruses or after intracellular p24-FITC (KC57, Beckman Coulter) staining for non-reporter viruses (see Immunostaining below).

CXCR4-tropic HIV Infection

24 hours post-electroporation, cells were transferred to uncoated 48-well plates and maintained at roughly 2.5*10^6 cells/mL in RPMI supplemented with 5mM HEPES (UCSF CCF), 50µg/mL P/S (Corning), 5mM sodium pyruvate (Corning), and 10% FBS (UCSF CCF). Cells were restimulated with either αCD2/CD3/CD28 beads (Miltenyi Biotec, according to the manufacturer's protocol) or 5µg/mL phytohemagglutinin (PHA, ThermoFisher) in the constant presence of 20U/mL IL-2 (Miltenyi Biotec). 4-7 days post-electroporation, cells were counted and suspended at 1*10^6 cells/mL. At this time, 1*10^5 cells were removed and lysed in 2.5x Laemmli Sample Buffer (25mM Tris pH 6.8, 8% glycerol, 0.8% SDS, 2% 2-mercaptoethanol, 0.02% bromophenol blue) for immunoblotting. 1*10^5 cells were also removed and lysed in QuickExtract DNA Extraction Solution (Epicentre) for sequencing and TIDE analysis. Per infection, 1*10^5 cells were then plated in technical triplicate in a 96-well plate. According to viral titer, an appropriate amount of concentrated virus was added directly to the culture medium to achieve a roughly 10-25% infection rate along with sufficient supplemented RPMI + 20U/mL IL-2 to bring the total volume to 200µL per well. 36 hours post-infection, cells were pelleted at 400*g for 3 minutes in a swinging bucket rotor and the supernatant was removed. Cells were fixed directly in 1% formaldehyde PBS (Sigma) prior to analysis. Flow cytometry was performed on a Becton Dickinson FACSCanto II through the UCSF Laboratory for Cell Analysis. Analysis of flow data was performed in FlowJo v10.1.

CCR5-tropic HIV Infection

Immediately post-electroporation, cells were transferred to anti-CD3 coated 48-well plates [coated overnight with 2µg/mL anti-CD3 (HIT3a, BioLegend)] and maintained at roughly 2.5*10^6 cells/mL in supplemented RPMI in the presence of 20U/mL IL-2 (Miltenyi Biotec) and 2µg/mL soluble αCD28 (CD28.2, BioLegend). 48 hours later, cells were transferred to an uncoated plate. The next day, cells were counted and suspended at 1*10^6 cells/mL. 5*10^4 cells were removed and lysed in 2.5x Laemmli Sample Buffer for immunoblot while 5*10^4 cells were removed and lysed in QuickExtract DNA Extraction Solution (Epicentre) for sequencing and TIDE analysis. Per infection, 5*10^4 cells were

then plated in technical triplicate in a 96-well plate. 20µL concentrated virus was then added directly to the culture medium along with sufficient supplemented RPMI + 20U/mL IL-2 to bring the total volume to 200µL per well and sufficient polybrene to achieve a final concentration of 5µg/mL. These plates were spun in a swinging bucket rotor for 45 minutes at 800xg and subsequently transferred to an incubator for 4 hours to allow for viral adsorption. The supernatant was removed and cells suspended in 200µL supplemented RPMI + 20U/mL IL-2. 48 hours post-infection, cells were pelleted at 400*g for 3 minutes in a swinging bucket rotor and the supernatant was removed. These cells were first stained with CCR5-APC (REA354, Miltenyi Biotec) prior to fixation in 1% formaldehyde PBS (Sigma). After fixation, cells were co-stained for intracellular p24-FITC (KC57, Beckman Coulter) and again suspended in 1% formaldehyde PBS. Flow cytometry was performed on a Becton Dickinson FACSAria II through the Gladstone Flow Cytometry Core. Analysis of flow data was performed in FlowJo v10.1.

LEDGF Spreading Infection

24 hours post-electroporation, cells were transferred to uncoated 48-well plates and maintained at roughly 2.5*10^6 cells/mL in supplemented RPMI in the presence of 20U/mL IL-2 (Miltenyi Biotec). After roughly 3 days of expansion, cells were shipped to Mt. Sinai School of Medicine, where they were further expanded in RPMI supplemented with 10% FBS, 50µg/mL P/S, 0.1 M HEPES, 2mM L-glutamine, and 20U/mL IL-2 (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH; human rIL-2 was obtained from M. Gately, Hoffmann-La Roche, Inc.). Cells were re-stimulated with Human T-Activator CD3/CD28 Dynabeads (Life-Technologies) as per the manufacturer's instructions for 48 hours prior to infection. Per infection, 1*10^6 cells were then plated in a 48-well plate. 10ng p24 equivalent of HIV-$1^{R7/3}$ *nef*-IRES-GFP was then added to each well along with sufficient polybrene to achieve a final concentration of 2µg/mL. These plates were spun in a swinging bucket rotor for 2 hours at 1200rpm and subsequently transferred to an incubator overnight to allow for viral adsorption. Fresh supplemented media containing 20U/mL IL-2 was replaced the following morning and every two days. At 3 and 7 days post-infection, 1*10^5 cells were removed and fixed directly in 1% formaldehyde PBS (Sigma) prior to analysis by flow cytometry.

Cell Viability

Cell viability was determined qualitatively by culture survival and expansion over time after nucleofection relative to other populations treated in parallel from the same donor. Percent viability was determined by gating on the population as visualized by forward and side scatter during flow cytometry at 3-7 days post-nucleofection. In the case of the LEDGF knock-out cell populations, cell viability was also determined by exclusion of LIVE/ DEAD® Fixable Aqua Dead Cell Staining (ThermoFisher) according to the manufacturers protocol.

Statistical Analysis

Statistical analysis was performed using Graph-Pad Prism 5 or Excel 2011 software. P-values for the two-tailed Student's t-test are shown with values less than 0.05 considered significant. When calculating across donors, the mean of averages was reported with standard error of the mean. See figures for stated statistical values.

Immunostaining

Extracellular staining was performed on infected or uninfected, live primary T cell populations with anti-CD4-PE (M-T466, Miltenyi Biotec), anti-CCR5-APC (REA354, Miltenyi Biotec), anti-CXCR4-APC (12G5, Miltenyi Biotec), anti-CD25-APC (3G10, Miltenyi Biotec), or combinations of the above according to the manufacturer's instructions. Briefly, cells were pelleted at 400*g for 3 minutes and the media removed. Cells were suspended in a 1:10 dilution of the appropriate antibody(ies) in MACS buffer (PBS plus 0.5% bovine serum albumin (BSA) and 2 mM EDTA) at a concentration of roughly 1*10^3 cells/μL and incubated for 10 minutes at 4°C. Cells were pelleted again, washed with MACS buffer, and suspended in 1% formaldehyde PBS for fixation prior to flow cytometry or intracellular staining.

Intracellular staining was performed on infected, fixed primary T cell populations with p24-FITC (KC57, Beckman Coulter). After allowing at least 30 minutes for fixation, cells were pelleted at 400*g for 3 minutes and the supernatant was removed. Cells were suspended in PBS plus 1% BSA, 0.1% saponin at a concentration of roughly 1*10^3 cells/μL. These sat at room temperature for 20 minutes to block. An equal volume of 1:50 antibody in PBS plus 1% BSA, 0.1% saponin was added for a final effective antibody dilution of 1:100. Cells incubated in antibody in the dark at room temperature for an additional 30 minutes. Cells were then pelleted again, washed with PBS plus 1% BSA, and suspended in 1% formaldehyde PBS for fixation prior to flow cytometry.

Immunoblotting

Cell lysates were prepared by suspension of cell pellets directly in 2.5x Laemmli Sample Buffer followed by homogenization at 98°C for 30 minutes. Samples were run on 4-20% Tris-HCl SDS-PAGE gels (BioRad Criterion) at 90V for 40 minutes followed by separation at 150V for 70 minutes. Proteins were transferred to PVDF membranes by methanol-based electrotransfer (BioRad Criterion Blotter) at 90V for 2 hours. Membranes were blocked in 4% Milk in PBS, 0.1% Tween-20 for 1 hour prior to primary antibody incubation overnight at 4°C. LEDGF (C57G11, Cell Signaling), TNPO3 (3152C2a, Abcam), XRCC6 (D10A7, Cell Signaling), UNC45A (15479, Proteintech), and HDGFRP2 (15134, Proteintech) levels were probed relative to GAPDH (71.1, Sigma) as a protein loading control. Anti-

mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (BioRad) were detected using Pierce™ ECL Western Blotting Substrate (ThermoFisher). Blots were incubated in a 1xPBS, 0.2M glycine, 1.0% SDS, 1.0% Tween-20, pH 2.2 stripping buffer before reprobing.

PCR Amplification of Target Regions and TIDE Analysis

Following the protocols above, a total of 5*10^4 to 1*10^5 cells were re-suspended in 100µL of QuickExtract DNA Quick Extraction solution (Epicentre) to lyse the cells and extract genomic DNA. The cell lysate was incubated at 65°C for 20 min, 95°C for 20 min, and then stored at −20°C until PCR could be performed across the CRISPR/Cas9 target sites. Unique genomic primers to amplify across the proposed cut sites were designed using the Primer3 online web tool (http://bioinfo.ut.ee/primer3/), chemically synthesized (IDT), and suspended at 100µM.

Each PCR reaction contained 2µl 10x High-fidelity PCR buffer (Life Technologies), 3µl 2mM dNTPs (Bioline), 0.8µl 50mM $MgCl_2$ (Life Technologies), 0.6µl 10µM forward primer, 0.6µl 10µM reverse primer, 0.2µl 5U/µl Platinum HIFI Taq (Life Technologies), 1µl extracted DNA, and 11.8µl H2O. The primer sets used for each crRNA can be found in **Supplemental Table 1**. The thermocycler setting consisted of one step at 95°C for 5 minutes, followed by 14 cycles at 94°C for 20 seconds, 65°C for 20 seconds, and 72°C for 1 minute (wherein the annealing temperature was decreased by 0.5°C per cycle), followed by 35 cycles at 94°C for 20 seconds, 58°C for 20 seconds, and 72°C for 1 minute with one final step at 72°C for 10 minutes. PCR cleanup and capillary sequencing was performed by Quintarabio (San Francisco, CA). Sequencing traces were analyzed with the Tracking of Indels by DEcomposition (TIDE) webtool (http://tide.nki.nl/) (Brinkman et al., 2014).

Arrayed Screening of CRISPR RNPs

Targets were identified using the NCBI 'HIV-1, human protein interaction database' webtool (https://www.ncbi.nlm.nih.gov/genome/viruses/retroviruses/hiv-1/interactions/) (Ako-Adjei et al., 2015). All proteins with a documented interaction with Integrase in at least 2 publications were included. This was supplemented with the full set of physical interactors identified by systematic affinity-purification mass spectrometry (Jager et al., 2012), for a total of 45 gene targets (see **Supplemental Table 2**, 'Gene List' tab). crRNAs were designed as described above and ordered in a 96-well arrayed format (Dharmacon) alongside non-targeting and positive controls, including crRNAs against *CXCR4*, *LEDGF*, and *CDK9*. The full list of crRNA sequences is available in **Supplemental Table 1**.

Editing reactions were carried out as above in two donors with a 12-well, multichannel pipet and PCR machine for incubations. Electroporations were carried out on the Amaxa 96-well Shuttle for the 4D Nucleofector. After electroporation, 80µL pre-warmed, complete RPMI was added to each well and the cells were allowed to recover for 30 minutes at 37C. They were then transferred directly to a 96-well

plate with 100μL complete RPMI +20U/mL IL-2 +2.5μL anti-CD2/CD3/CD28 beads (Miltenyi Biotec). 48 hours later, 100μL complete RPMI +20U/mL was added to each well. At 4 days post-electroporation, cells were counted, and 75,000 cells replica plated to each of five 96-well plates in a total of 150μL. Three plates were infected with 2.5μL concentrated HIV-1^{NL4-3} *nef*:IRES:GFP as described above plus sufficient RPMI + 20U/mL IL-2 to bring the total volume to 200μL per well in order to achieve an initial infection rate of approximately 1-2%. Cells from the other two plates were lysed in either 2.5x Laemmli Sample Buffer for immunoblot or QuickExtract DNA Extraction Solution (Epicentre), heated, and stored at -20°C for later validation by sequencing and TIDE analysis, respectively.

At 2, 4, and 6 days post-infection, cells were suspended by pipet and 95μL culture removed to a fresh 96-well plate. 100μL fresh media +20U/mL IL-2 was added back to the infected plates and these were returned to the incubator. The removed samples were pelleted at 400*g for 3 minutes in a swinging bucket rotor and the supernatant was removed. Cells were fixed directly in 150μL 1% formaldehyde PBS (Sigma) prior to analysis. Flow cytometry was performed on a Becton Dickinson FACSCanto II through the UCSF Laboratory for Cell Analysis. Analysis of flow data was performed in FlowJo v10.1. See **Supplemental Table 2** for all raw and processed flow data.

SUPPLEMENTAL REFERENCES

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