Supplementary information

**MARCH8 inhibits HIV-1 infection by reducing virion incorporation of envelope glycoproteins**

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Supplementary Figures 1 to 15
Supplementary Figure 1  Inhibition of infectivity but not virion production of VSV-G-pseudotyped virus by MARCH8.

Left panels: HeLa or HOS cells were cotransfected with Env-defective HIV-1 luciferase reporter proviral DNA and either a control or HA-MARCH8 plasmid together with VSV-G plasmid. Virion production was analyzed by p24 antigen capture ELISA. Data are presented as a percentage of the virion production in the absence of MARCH8 (mean ± s.d., n = 3 technical replicates, representative of three experiments). Right panels: Infectivity of viruses prepared in the experiments shown in the left panels were analyzed by performing luciferase assays. Data are presented as a percentage of the infectivity of viruses produced without MARCH8 (mean ± s.d., n = 3 technical replicates, representative of three experiments).
**Supplementary Figure 2** MARCH8 expression in the target cells does not affect viral infectivity. (a) MARCH8 mRNA expression levels in M8166 cells stably expressing MARCH8. Data are shown as the fold increase of MARCH8/GAPDH mRNA expression relative to that in the original M8166 cells. (b) Cell proliferation of M8166 cells stably expressing MARCH8 (open circles) or the vector control (open squares). The assay was started with 100,000 cells. Data are mean ± s.d., n = 3 technical replicates. (c) Single-round infection of MARCH8-expressing M8166 cells with four different doses (0.125-1 ng p24Gag) of luc-reporter viruses pseudotyped with NL-Env. Data are mean ± s.d., n = 3 technical replicates, representative of three experiments. (d) Virus replication in M8166 cells stably expressing MARCH8 (closed circles) or the vector control (closed squares). Cells (1 x 10^5) were infected with 0.1 ng of p24 antigen of NL4-3 viruses. Supernatants were harvested at two-day intervals, and virus replication was monitored by p24 ELISA. Data shown are representative of three independent experiments.
Supplementary Figure 3  Inhibition of NL-Env- or VSV-G-pseudotyped virus infectivity by MARCH8. 293T cells were transfected with Env-defective NL4-3 luciferase reporter proviral construct, either NL-Env or VSV-G expression plasmid, together with or without HA-MARCH8. Produced viruses were normalized by p24 and subjected to a viral infectivity assay. Data are presented as a percentage of the infectivity of viruses produced without MARCH8 (mean ± s.d., n = 3 technical replicates, representative of three experiments).
Supplementary Figure 4  MARCH8 expression in producer cells decreases HIV-1 gp120 levels in viral supernatants. ELISA-based levels of Env gp120 in viral supernatants from 293T cells cotransfected with luc reporter proviral DNA and NL-Env plasmid, together with (a) increased amounts of MARCH8 expression plasmid (0–200 ng) or (b) either wild-type MARCH8 (WT) or its RING-CH mutants (CS and W114A) (120 ng). Representative data from three independent experiments are shown as percent gp120 Env/p24Gag in the supernatants relative to that from control cells.
Supplementary Figure 5  MARCH8 is incorporated into HIV-1 particles. (a) Virus particles were concentrated through a sucrose cushion by ultracentrifugation of the supernatants of HIV-1 produced from cells, and pelleted virions were treated with or without subtilisin. After the reaction was stopped with PMSF, virions were re-pelleted and first subjected to immunoblot analyses using antibodies against gp120 and p24 to verify that the samples were successfully digested with subtilisin (left panels). HIV-1 Env or VSV-G-pseudotyped viruses produced from cells expressing MARCH8 or the vector control were pelleted and treated with subtilisin as described above, and subjected to immunoblot analyses using antibodies against HA (MARCH8) and p24 (right panels). (b) Virion incorporation of MARCH8 RING-CH mutant proteins was also confirmed by subtilisin treatment. Note that the anti-HA antibody recognizes virion-incorporated MARCH8 that was HA-tagged at its N terminus located in the virion interior.
Supplementary Figure 6  Intracellular expression levels of HIV-1 Env and VSV-G with or without MARCH8 in producer cells. 293T cells were transfected with Env-defective NL4-3 proviral construct, either (a) NL4-3-Env or (b) VSV-G expression plasmid, with or without HA-MARCH8 expression plasmid. Cell extracts were subjected to immunoblot analyses using antibodies against gp120, VSV-G, p24, HA (MARCH8), and β-actin.
**Supplementary Figure 7** Cell-surface downregulation and intracellular retention of HIV-1 Env by MARCH8. 293T cells were cotransfected with HIV-1 Gag-Pol expression plasmid, Env expression plasmid, and Rev expression plasmid, with or without HA-MARCH8. Fixed cells were subjected to triple immunofluorescence staining (cell-surface or intracellular). Note that in cell-surface staining of HIV-1 Env (*right panels*), Gag and MARCH8 were stained after permeabilization.

*Bars*, 10 μm.
Supplementary Figure 8  Lack of rescue of VSV-G expression from MARCH8-induced degradation by a proteasome inhibitor. Immunofluorescence analyses were performed to determine intracellular levels of VSV-G with or without MARCH8 in the presence of MG-132 in transfected 293T cells. Bars, 10 μm.
Supplementary Figure 9  Interaction of both wild-type (WT) and RING-CH mutant (CS and W114A) MARCH8 proteins with either HIV-1 Env or VSV-G. Immunoprecipitated HA-MARCH8 (upper) and whole-cell lysates (lower) were analyzed by immunoblotting against either gp120 (left) or VSV-G (right). It should be noted that the transfection experiments in the right panels were performed in the presence of lysosomal protease inhibitors.
Supplementary Figure 10  Endogenous expression of MARCH8 in MDMs. (a) Levels of endogenous MARCH8 expression in MDMs from two donors were determined by real-time RT-PCR and normalized to those of RPL27 mRNA for comparison with those in cells transfected with increasing amounts of the MARCH8 expression plasmid. Note that we used 120 ng of MARCH8 expression plasmid for transfection throughout this study. (b) Cell extracts derived from different cell lines and MDMs from three donors were subjected to immunoblot analyses using an anti-MARCH8 polyclonal antibody. β-actin was used as a loading control.
**Supplementary Figure 11** Type I interferon-induced upregulation of expression of the restriction factors. To determine whether MARCH8 expression could be upregulated by type I interferon (IFN), MDMs and stimulated primary CD4+ T cells were incubated with or without 1,000 U ml⁻¹ IFN-α overnight, and total RNA was extracted from these cells and subjected to real-time RT-PCR. Data are presented as the fold increase of target gene/GAPDH expression induced by IFN-α relative to that in the absence of IFN-α.
Supplementary Figure 12 Knockdown or knockout of MARCH8 by shRNA or CRISPR/Cas9. 293T cells were transfected with HA-MARCH8 expression plasmid and either lentiviral shRNA plasmids or lentiviral CRISPR/Cas9 plasmids, and cell lysates were subjected to immunoblot analyses. Protein levels of MARCH8 were determined using an anti-HA monoclonal antibody. β-actin was used as a loading control.
Supplementary Figure 13  Virus replication in PHA/IL-2-stimulated primary CD4+ T cells in which MARCH8 was depleted (closed squares) or non-depleted (closed circles). Cells (5 x 10^5) were infected with 1 ng ("low-dose infection") or 25 ng ("high-dose infection") of p24 antigen of NL4-3 viruses. Supernatants were harvested at three-day intervals, and virus replication was monitored by p24 ELISA. Data shown are representative of three independent experiments. Note that the primary CD4+ T cells expressed very low levels of endogenous MARCH8 as shown in Fig. 4a.
**Supplementary Figure 14** Models depicting potential mechanisms by which virion incorporation of HIV-1 Env and VSV-G is inhibited. (a) MARCH8 downregulates VSV-G, leading to lysosomal degradation. (b) MARCH8 downregulates HIV-1 from the cell surface and probably sequesters it in endosomes and/or TGN without degradation.
Supplementary Figure 15 The lack of HIV-1 accessory proteins does not affect MARCH8-mediated inhibition of infection by HIV-1. (a) Infection of MAGIC5 cells with NL Env-pseudotyped Vpr(+) or Vpr(–) luc-reporter viruses produced from cells expressing HA-MARCH8 or the vector control. Data are presented as a percentage of the infectivity of viruses produced without MARCH8 (mean ± s.d., n = 3 technical replicates, representative of three experiments). Immunoblot images of the expression of Vpr, MARCH8 (HA) and β-actin in the producer cells are shown (lower panel). (b) Infection of MAGIC5 cells with HIV-1 Vpr-defective Env-intact Luc reporter viruses produced from MARCH8-depleted MDMs derived from three donors. Data are shown as the fold increase of viral infectivity relative to that of viruses produced from control MDMs (compare with Fig. 4c, left panel), mean ± s.d., n = 3 technical replicates, representative of four experiments. *P < 0.05 compared with the control vector using a paired two-tailed t-test. (c, d) Parallel infection experiments were performed as in the experiments shown in a, using either (c) NL Env-pseudotyped Vpu(+) or Vpu(–) luc-reporter viruses, or (d) NL Env-pseudotyped Nef(+) or Nef(–) luc-reporter viruses. Immunoblot images of the expression of either Vpu or Nef, MARCH8, and β-actin in the producer cells are shown (lower panel). Representative data are shown from three independent experiments (mean ± s.d. of 3 technical replicates).