1   **MARCH8 inhibits viral infection by two different mechanisms**

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1 **ABSTRACT**

2 Membrane-associated RING-CH 8 (MARCH8) inhibits infection with both HIV-1 and vesicular

3 stomatitis virus G-glycoprotein (VSV-G)-pseudotyped viruses by reducing virion incorporation of

4 envelope glycoproteins. The molecular mechanisms by which MARCH8 targets envelope

5 glycoproteins remain unknown. Here, we show two different mechanisms by which MARCH8

6 inhibits viral infection. Viruses pseudotyped with the VSV-G mutant, in which cytoplasmic lysine

7 residues were mutated, were insensitive to the inhibitory effect of MARCH8, whereas those with

8 a similar lysine mutant of HIV-1 Env remained sensitive to it. Indeed, the wild-type VSV-G, but

9 not its lysine mutant, was ubiquitinated by MARCH8. Furthermore, the MARCH8 mutant, which

10 had a disrupted cytoplasmic tyrosine motif that is critical for intracellular protein sorting, did not

11 inhibit HIV-1 Env-mediated infection, while it still impaired infection by VSV-G-pseudotyped

12 viruses. Overall, we conclude that MARCH8 reduces viral infectivity by downregulating envelope

13 glycoproteins through two different mechanisms mediated by a ubiquitination-dependent or

14 tyrosine motif-dependent pathway.

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INTRODUCTION

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2   Membrane-associated RING-CH (MARCH) 8 is one of 11 members of the MARCH family of

3   RING-finger E3 ubiquitin ligases, which consist of an N-terminal cytoplasmic tail (CT) domain

4   containing a C4HC3 RING finger (RING-CH finger) motif, two transmembrane (TM) domains,

5   between which a short ectodomain is located, and a C-terminal CT domain[1,2]. MARCH8

6   downregulates a variety of cellular transmembrane proteins, such as MHC-II[3], CD86[4], CD81[5],

7   CD44[6], TRAIL receptor 1[7], CD98[6], IL-1 receptor accessory protein[8], and transferrin receptor[9]. We

8   have recently reported that MARCH8 reduces HIV-1 infectivity by downregulating HIV-1

9   envelope glycoproteins (Env) from the cell surface, resulting in a reduced incorporation of Env

10   into virions[10]. Intriguingly, vesicular stomatitis virus G-glycoprotein (VSV-G) was even more

11   sensitive to the inhibitory effect of MARCH8. In the case of HIV-1 Env, it is retained

12   intracellularly without degradation after cell-surface downregulation. In contrast, VSV-G is not

13   only downregulated from the cell surface but also undergoes lysosomal degradation by

14   MARCH8[10]. In this regard, we hypothesized that VSV-G, whose cytoplasmic tail is lysine-rich (5

15   out of 29 amino acids), could be readily ubiquitinated by the E3 ubiquitin ligase MARCH8 and

16   therefore undergo lysosomal degradation, whereas HIV-1 Env carries only two lysines (out of 151

17   amino acids) in its cytoplasmic tail and may rarely undergo degradation after being trapped by

18   MARCH8. In this study, we created lysine mutants of both HIV-1 Env and VSV-G, together with

19   newly generated MARCH8 mutants to explore the hypothesis described above. The results with

20   these mutants show that MARCH8 targets HIV-1 Env and VSV-G by two different inhibitory

21   mechanisms.

22

RESULTS AND DISCUSSION

We have recently reported that MARCH8 inhibits lentiviral infection by reducing virion incorporation of both HIV-1 Env and VSV-G in a RING-CH domain-dependent manner. Because the RING-CH domain is known to be essential for the E3 ubiquitin ligase activity of MARCH8, we asked whether these envelope glycoproteins are susceptible to MARCH8-mediated ubiquitination. To investigate this, we first created the VSV-G mutant CT5K/R in which five arginine residues were introduced in place of cytoplasmic lysine residues that could be ubiquitination targets (Fig. 1A, upper). We also generated the HIV-1 Env gp41 mutant CT2K/R harboring two arginines in place of the cytoplasmic lysines (Fig. 1A, lower). Then, we prepared HIV-1 luciferase reporter viruses pseudotyped with the mutant envelope glycoproteins (VSV-G CT5K/R and HIV-1 Env CT2K/R) from 293T cells transiently expressing MARCH8, and compared their viral infectivity with that of control viruses pseudotyped with wild-type (WT) envelope glycoproteins. The infectivity of viruses harboring either VSV-G CT5K/R or HIV-1 gp41 Env CT2K/R was almost comparable to WT-enveloped viruses (Figs. 1B and 1C). As expected, the virus pseudotyped with VSV-G CT5K/R was completely resistant to MARCH8 (Fig. 1B). In contrast, the HIV-1 Env CT2K/R-pseudotyped virus was still susceptible to the inhibitory effect of MARCH8 (Fig. 1C). Consistent with these results, immunofluorescence staining showed that the five lysine mutations in the CT domain of VSV-G conferred resistance to MARCH8-mediated intracellular degradation (Fig. 1D), whereas the two lysine mutations in the CT domain of HIV-1 Env had no effect on its cell-surface downregulation by MARCH8 (Fig. 1E). It should be noted that MARCH8-resistant VSV-G CT5K/R colocalized with MARCH8 (Fig. 1D). We thus

1 speculated that MARCH8 ubiquitinates lysine residues of VSV-G but not of HIV-1 Env at their

2 CT domains.

3 To investigate this possibility, we performed immunoprecipitation (IP)/Western-based

4 ubiquitination assays. In cells coexpressing WT HA-MARCH8, VSV-G was efficiently

5 ubiquitinated, whereas in cells coexpressing the RING-CH mutant of HA-MARCH8, the

6 ubiquitination of VSV-G was lost. More importantly, the VSV-G lysine mutant CT5K/R did not

7 undergo MARCH8-mediated ubiquitination, as expected (Fig. 1F), suggesting that the lysine

8 residues at the CT domain of VSV-G are specifically ubiquitinated by MARCH8. These findings

9 are consistent with the immunofluorescence results (Figs. 1D and 1E). We therefore conclude that

10 lysine residues at the CT domain of VSV-G are ubiquitinated by MARCH8, which determines the

11 difference in the MARCH8-mediated intracellular fate between these viral glycoproteins.

12 Because unlike VSV-G, HIV-1 Env was retained intracellularly without degradation, as we

13 previously reported[10], we hypothesized that these viral envelope glycoproteins might undergo

14 endocytosis with different mechanisms of action. It has been reported that another MARCH family

15 member, MARCH11, has a conserved tyrosine-based motif, YXXϕ, which is known to be

16 recognized by the adaptor protein (AP) μ-subunits in the C-terminal CT domain[11]. We therefore

17 looked for the same motif(s) in MARCH8 and found the ^{222}YxxL^{225} and ^{232}YxxV^{235} sequences in

18 the CT domain at the C-terminus (Fig. 2A). Based on this finding, we generated tyrosine motif

19 mutants of MARCH8, in which either ^{222}Y or ^{232}Y was mutated to alanine (designated ^{222}AxxL^{225}

20 or ^{232}AxxV^{235}). The protein expression in cells transfected with each MARCH8 plasmid was

21 confirmed by immunoblotting using an anti-hemagglutinin (HA) antibody (Fig. 2B). Then, we

22 examined whether these YXXϕ motifs are important for the antiviral activity of MARCH8. The

1   infectivity of VSV-G–pseudotyped viruses was still inhibited by the expression of both ^{222}AxxL^{225}

2   and ^{232}AxxV^{235} MARCH8 mutants (Fig. 2C). In contrast, the infectivity of HIV-1 Env–

3   pseudotyped viruses was not impaired by ^{222}AxxL^{225} MARCH8 expression but was reduced by

4   that of ^{232}AxxV^{235} and WT MARCH8 (Fig. 2D), suggesting that the first tyrosine motif

5   (^{222}YxxL^{225}) is involved in the antiviral activity of MARCH8 against HIV-1 Env but not VSV-G.

6   We previously reported that the MARCH8-mediated reduction in viral infectivity was due to

7   reduced entry efficiency, resulting from a decreased virion incorporation of envelope

8   glycoproteins through their downregulation from the cell surface[10]. Therefore, by performing a β-

9   lactamase (BlaM)-fused viral protein R (Vpr)-based entry assay, we first focused on whether the

10   loss of function of ^{222}AxxL^{225} MARCH8 against HIV-1 Env but not VSV-G would indeed be due

11   to the loss of its inhibitory activity on viral entry. Whereas the entry of VSV-G–pseudotyped HIV-

12   1 prepared from cells expressing either WT or ^{222}AxxL^{225} MARCH8 was reduced compared with

13   that of the control virus (Fig. 2E), the inhibition of the entry of whole HIV-1 virions was abrogated

14   in viruses produced from cells expressing ^{222}AxxL^{225}, as expected (Fig. 2D). We further analyzed

15   whether this motif of MARCH8 is indeed involved in the reduced virion incorporation of HIV-1

16   Env, which results from its cell-surface downregulation. To address this, we conducted flow

17   cytometric analysis and quantified the levels of cell-surface and intracellular expression of Env

18   glycoproteins. In accordance with the results obtained in infectivity assays (Fig. 2C), ^{222}AxxL^{225}

19   MARCH8 still reduced intracellular VSV-G expression as well as WT MARCH8 did (Fig. 2G).

20   On the other hand, the mutant MARCH8 had a completely abrogated ability to downregulate cell-

21   surface HIV-1 Env, whereas WT expression led to the downregulation of HIV-1 Env from the cell

22   surface and its intracellular retention (Fig. 2H), as we previously observed[12]. The results were

6

1   consistent with those of the inhibitory activity of MARCH8 against the virion incorporation of

2   HIV-1 Env (Fig. 2I). We therefore conclude that ^{222}YxxL^{225} is critical for the MARCH8-mediated

3   downregulation of HIV-1 Env but not VSV-G, which results in its reduced virion incorporation

4   leading to impaired viral entry.

5   In summary, we first show the two different mechanisms by which MARCH8 inhibits viral

6   infections, one being a ubiquitin-dependent downregulation that mediates lysosomal degradation

7   of VSV-G whose cytoplasmic lysine residues are recognized by the RING-CH domain of

8   MARCH8 (Fig. 3, left), and the other, a YxxΦ motif-dependent downregulation that could explain

9   the intracellular retention of HIV-1 Env without degradation after cell-surface downregulation (Fig.

10   3, right). In terms of the latter mechanism, although this could be attributed to the AP-dependent

11   trafficking that requires the YxxΦ motif, which binds to AP μ-subunits, our preliminary results

12   showed no interaction between these subunits and MARCH8. We have recently reported that

13   MARCH1 and MARCH2 are also antiviral MARCH family members that inhibit HIV-1 infection,

14   although their antiviral activity is less robust, which is probably due to the lower protein expression

15   and/or stability than that of MARCH8[12]. Because MARCH1 and MARCH2 also harbor the Yxxϕ

16   motif in their C-terminal CT domains, it would be intriguing to verify the importance of the motif

17   in these proteins. Overall, our present findings are consistent with our previous studies showing

18   that MARCH8-induced downregulation of VSV-G leads to lysosomal degradation, while that of

19   HIV-1 Env results in intracellular retention without degradation. Thus, we conclude that MARCH8

20   targets HIV-1 Env and VSV-G by two different inhibitory mechanisms (either ubiquitin-dependent

21   or YxxΦ motif-dependent downregulation). Further investigations will clarify the more detailed

22   host defense mechanisms of this protein.

1

METHODS

3 **DNA constructs.** The Env-deficient HIV-1 proviral indicator construct pNL-Luc2-E(-), HIV-1

4 Gag-Pol expression plasmid pC-GagPol-RRE, HIV-1 Env-expression vector pC-NLenv, HIV-1

5 Rev expression plasmid pCa-Rev, VSV-G expression plasmid pC-VSVg and its C-terminally T7-

6 epitope-tagged version pVSVg-T7E, GFP expression plasmid pCa-EGFP, Vpr/β-lactamase

7 (BlaM) expression plasmid pMM310, and MARCH8 expression plasmid either pC-MARCH8 or

8 pC-HA-MARCH8 and its RING-CH mutant pC-HA-MARCH8-CS have previously been

9 described elsewhere[10]. The HIV-1 Env gp41 mutant CT2K/R, in which cytoplasmic lysine residues

10 at positions 792 and 806 were mutated to arginine, and the VSV-G mutant CT5K/R, in which

11 cytoplasmic lysine residues at positions 491, 493, 496, 497, and 511 were mutated to arginine

12 residues, were created by inserting overlapping PCR fragments into *Mfe*I/*Xho*I-digested pC-

13 NLenv and by inserting PCR fragments into *Kpn*I/*Not*I-digested pC-VSVg (or pVSVg-T7E),

14 respectively. The MARCH8 mutant ^{222}AxxL^{225} or ^{232}AxxV^{235}, in which a tyrosine residue at

15 position 222 or 232 was mutated to an alanine residue, was generated by inserting overlapping

16 PCR fragments into the *Kpn*I/*Xho*I-digested pCAGGS or *Xho*I-*Not*I-digested pCAGGS-NHA to

17 create an untagged or N-terminally HA-tagged expression plasmid. All constructs were verified

18 by a DNA sequencing service (FASMAC).

19

20 **Cell maintenance and generation of stable cell lines.** 293T, MT4, HeLa, MAGIC5 (HeLa

21 derivative[13]), and HOS cells were maintained under standard conditions. Cells were originally

22 obtained from ATCC (except MAGIC5 cells) and routinely tested negative for mycoplasma

1   contamination (PCR Mycoplasma Detection kit, Takara).

2

3   **Virion production and infectivity assays.** To prepare VSV-G-pseudotyped or HIV-1-Env-

4   pseudotyped luciferase reporter viruses, 2.5×10^5 293T cells were cotransfected with 120 ng of

5   the MARCH expression plasmid (WT or RING-CH mutants), 20 ng of pC-VSVg, pC-VSVg-

6   CT5K/R, pC-NLenv, or pC-NLenv-CT2K/R, 500 ng of pNL-Luc2-E(-), and an empty vector up

7   to 1 μg of total DNA, using FuGENE 6 (Promega). Sixteen hours later, the cells were washed with

8   phosphate-buffered saline, and 1 ml of fresh complete medium was added. After 24 h, supernatants

9   were treated as described above and then harvested. The p24 antigen levels in viral supernatants

10   were measured by an HIV-1 p24 antigen capture ELISA (XpressBio). Transfection efficiencies

11   were normalized to the luciferase activity. To determine viral infectivity, 1×10^4 MAGIC5 cells

12   were incubated with 1 ng of p24 antigen from the HIV-1 supernatants. After 48 h, cells were lysed

13   in 100 μl of One-Glo Luciferase Assay Reagent (Promega). The firefly luciferase activity was

14   determined with a Centro LB960 (Berthold) luminometer.

15

16   **Immunoblotting assays.** Protein expression of constructs was confirmed by Western blot analyses

17   as described elsewhere[10,12]. Briefly, cells transfected as described above were lysed in 500 μl of

18   lysis buffer containing 1.25% n-octyl-β-D-glucoside (Dojindo), and Complete protease inhibitor

19   cocktail (Roche Applied Science). Cell extracts were then subjected to gel electrophoresis and

20   transferred to a nitrocellulose membrane, followed by probing with an anti-HA mouse monoclonal

21   antibody (Sigma-Aldrich, H9658) or an anti-β-actin mouse monoclonal antibody (Sigma-Aldrich,

22   A5316). Proteins were then visualized by chemiluminescence using an ECL Western blotting

9

1 detection system (GE Healthcare) and monitored by using a LAS-3000 imaging system (FujiFilm).

2

3 **Viral entry assays.** A β-lactamase (BlaM)-fused viral protein R (Vpr)-based entry assay was

4 performed as described elsewhere [10,12]. Briefly, HIV-1 particles containing a fusion protein of Vpr

5 and BlaM-Vpr were produced by cotransfection of 293T cells with pNL4-3, pMM310[14] encoding

6 BlaM-Vpr, and either pC-MARCH8, pC-MARCH8-^{222}AxxL^{225}, pC-MARCH8-^{232}AxxV^{235}, or the

7 control vector. Similarly, VSV-G-pseudotyped HIV-1 particles containing BlaM-Vpr were

8 prepared by cotransfection with pNL-Luc-E(-), pC-VSVg, pMM310, and either pC-MARCH8,

9 pC-MARCH8-^{222}AxxL^{225}, pC-MARCH8-^{232}AxxV^{235}, or the control vector. The produced viruses

10 were normalized to the p24 antigen level (100 ng) and used for infection of the CD4$^+$ T cell line

11 MT4 (5 x 10^5 cells) at 37 °C for 4 h to allow viral entry. After extensive washing with Hank's

12 balanced salt solution (HBSS; Invitrogen), cells were incubated with 1 μM CCF2-AM dye

13 (Invitrogen), a fluorescent substrate of BlaM, in HBSS containing 1 mg ml^{-1} Pluronic F-127

14 surfactant (Invitrogen) and 0.001% acetic acid for 1 h at room temperature and then washed with

15 HBSS. Cells were further incubated for 14 h at room temperature in HBSS supplemented with

16 10% FBS, washed three times with PBS and fixed in a 1.2% paraformaldehyde solution.

17 Fluorescence was monitored at 520 and 447 nm by flow cytometry using BD FACS Canto II (BD

18 Bioscience), and the data were collected and analyzed with BD FACS Diva Software (BD

19 Bioscience).

20

21 **Env incorporation assays.** HIV-1 gp120 ELISA-based Env incorporation assays were performed

22 by using an HIV-1 gp120 antigen capture ELISA kit (Advanced BioScience Laboratories) as

1   described elsewhere[12].

2

3   **Ubiquitination assays.** 293T cells (5×10^5) were cotransfected with 0.8 µg of pC-VSVg-T7E, 0.2

4   µg of the empty vector, and 0.8 µg of pC-HA-MARCH8. After 48 h, cells were lysed in TBS-T

5   buffer (50 mM Tris-HCl buffer (pH 7.5), 0.15 M NaCl, 1% Triton X-100, and 0.5% deoxycholic

6   acid) containing a protease inhibitor cocktail and 10 mM N-ethylmaleimide, as an inhibitor of

7   deubiquitination enzymes. The mixture was centrifuged at $21,500 \times g$ for 15 min, and the

8   supernatant was used as total cell lysate for immunoblotting or immunoprecipitation. Fifty

9   microliters of Protein A-coupled Sepharose 4B (GE Healthcare, 17-0780-01) was preincubated for

10   2 h at 4 °C with 4 µg of the appropriate antibody (anti-T7 epitope rabbit polyclonal antibody, MBL,

11   PM022; anti-ubiquitin mouse monoclonal antibody Clone FK2, Cayman, 14220). Total cell lysate

12   was incubated with antibody-coupled Sepharose for 20 h at 4 °C. The Sepharose was washed three

13   times with TBS-T buffer and one time with PBS before the immunoprecipitated proteins were

14   eluted with SDS sample buffer. To evaluate the ubiquitination states of the immunoprecipitated

15   proteins, proteins immunoprecipitated with an anti-T7 epitope rabbit antibody were subjected to

16   Western blotting with an anti-ubiquitin mouse antibody, whereas proteins immunoprecipitated

17   with the anti-ubiquitin mouse antibody were subjected to Western blotting with the anti-T7 epitope

18   rabbit antibody. Immunoreactive bands were detected using an ECL detection kit (EzWestLumi

19   plus, ATTO) with a ChemiDoc imaging system (Bio-Rad).

20

21   **Immunofluorescence microscopy.** HOS cells were plated on 13-mm coverslips, cotransfected

22   with the indicated plasmids, 0.5 µg of either pC-NLenv or pC-VSVg-T7E, 0.1 µg of pC-Gag-Pol,

11

1   0.05 μg of pCa-Rev, and 0.3 μg of the pC-HA-MARCH8 expression plasmids (WT or CS-mutant)

2   using FuGENE6 and cultured for 24 h. For the total staining of both VSV-G and MARCH8, cells

3   were fixed with 4% paraformaldehyde for 30 min on ice and permeabilized with 0.05% saponin.

4   The fixed cells were incubated with both primary antibodies anti-T7 epitope mouse monoclonal

5   antibody (Novagen, 69522-4) and anti-HA goat polyclonal antibody (GenScript, A00168-40). The

6   secondary antibodies, Alexa 488 donkey anti-mouse IgG (Molecular Probes, A-21202) and Alexa

7   647 donkey anti-goat IgG (Molecular Probes, A-21447) were used for the double staining assay.

8   For the cell surface staining of gp120 protein, cells were incubated with an anti-gp120 goat

9   polyclonal antibody (Abcam, Ab21179) at 4 °C for 5 min and washed with PBS at 4 °C before

10   fixation. Fixation was performed with 4% paraformaldehyde for 30 min on ice, and fixed cells

11   were permeabilized with 0.05% saponin (Sigma-Aldrich) to detect the intracellular expression and

12   localization of MARCH8 proteins. The coverslips were incubated with the anti-HA mouse

13   monoclonal antibody (Sigma Aldrich) for 1 h, washed with PBS and incubated for 30 min with

14   the secondary antibodies Alexa 488 donkey anti-goat IgG (Molecular Probes, A-11055) and Alexa

15   647 donkey anti-mouse IgG (Molecular Probes, A-31571) for the double staining assay. Confocal

16   images were obtained with a FluoView FV10i automated confocal laser-scanning microscope

17   (Olympus; Tokyo).

18

19   **Statistical analyses.** Column graphs that combine bars and individual data points were created

20   with GraphPad Prism version 8.04. *P*-values generated from two-tailed paired *t*-tests for data

21   represented in Figures 2C, 2D, and 2I.

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1 **ACKNOWLEDGMENTS**

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5 **AUTHOR CONTRIBUTIONS**

6 Y.Z., T.T., S.O., H.F. and K.T. performed the experiments and analyzed the data. Y.Z., H.F. and

7 K.T. discussed the data. S.K. provided reagents. K.T. conceived the study, supervised the work

8 and wrote the paper. All authors read and approved the final manuscript.

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10 **COMPETING FINANCIAL INTERESTS**

11 The authors declare no competing financial interests.

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1   **FIGURE LEGENDS**

2   **Figure 1** (**A**) Schematic structure of the lysine mutants of VSV-G (CT5K/R; upper) and HIV-1

3   Env (CT2K/R; lower). SP, signal peptide; EC, extracellular domain; TM, transmembrane domain;

4   CT, cytoplasmic tail; SU, surface subunit. (**B**) Infectivity of viruses prepared from 293T cells

5   cotransfected with Env-defective HIV-1 luciferase (luc) reporter proviral DNA and either the

6   VSV-G wild-type (WT) or CT5K/R mutant plasmid together with either a control (Ctrl) (black) or

7   HA-MARCH8 (gray) plasmid. Data are shown as a percentage of the viral infectivity in the

8   absence of MARCH8 when WT VSV-G was used (mean + s.d. from three independent

9   experiments). (**C**) Infectivity of viruses prepared as shown in *B*, except for using either the WT

10   HIV-1 Env or its CT2K/R mutant plasmid (mean + s.d. from three independent experiments). (**D**)

11   The VSV-G lysine mutant is resistant to MARCH8-mediated intracellular degradation. Shown are

12   immunofluorescence-based analyses of the intracellular levels of either the WT or lysine mutant

13   VSV-G with or without MARCH8 in transfected HOS cells. Scale bars, 10 μm. (**E**) The lysine

14   mutant of HIV-1 Env is still sensitive to MARCH8-induced downregulation from the cell surface.

15   Immunofluorescence images show cell-surface levels of either the WT or lysine mutant HIV-1

16   Env with or without MARCH8 in transfected HOS cells. Scale bars, 10 μm. (**F**) Lysine residues at

17   the CT domain of VSV-G are ubiquitinated by MARCH8. The ubiquitination of the WT or lysine

18   mutant VSV-G tagged with T7-epitope (T7e) in cells expressing control or MARCH8 (WT or

19   RING-CH mutant (CS)) was examined by immunoprecipitation of either ubiquitinated proteins

20   with an anti-ubiquitin antibody (left panel) or of T7e-tagged VSV-G with an anti-T7e antibody

21   (right panel), followed by immunoblotting with an antibody to either T7e (left panel) or ubiquitin

22   (right panel), respectively.

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2 **Figure 2** (**A**) Schematic structure of YxxΦ motif mutants of MARCH8 (^{222}YxxL^{225} and

3 ^{232}YxxV^{235}). (**B**) Western blot analysis was performed by using extracts from 293T cells

4 transfected with HA-tagged MARCH8 expression plasmids. Antibodies specific for HA were used

5 to detect MARCH8 proteins. (**C**, **D**) Infectivity of viruses prepared from 293T cells cotransfected

6 with Env-defective HIV-1 luciferase (luc) reporter proviral DNA and either a control (Ctrl), HA-

7 WT, HA-^{222}AxxL^{225} or HA-^{232}AxxV^{235} MARCH8 plasmid, together with either (**C**) the VSV-G

8 expression plasmid or (**D**) the HIV-1 Env expression plasmid. Data are shown as a percentage of

9 the viral infectivity in the absence of MARCH8 (mean + s.d. from three independent experiments).

10 ns; ***P < 0.0005 compared with the Ctrl using two-tailed unpaired t-tests. (**E, F**) BlaM-Vpr-

11 based viral entry assay using VSV-G-pseudotyped viruses (**E**) or NL4-3 whole viruses (**F**)

12 produced from cells expressing either control, WT MARCH8, or the ^{222}AxxL^{225} mutant.

13 Representative FACS dot plots are shown from four independent experiments. (**G**) VSV-G is

14 downregulated by both WT and ^{222}AxxL^{225} mutant MARCH8, (**H**) whereas the cell-surface

15 expression of HIV-1 Env is not affected by the mutant MARCH8. (**I**) ^{222}AxxL^{225} MARCH8

16 expression in producer cells is unable to decrease HIV-1 gp120 levels in viral supernatants.

17 ELISA-based levels of Env gp120 in viral supernatants from 293T cells cotransfected with luc

18 reporter proviral DNA and NL-Env plasmid, together with either MARCH8 WT or its ^{222}AxxL^{225}

19 mutant. Representative data from three independent experiments are shown as percent gp120

20 Env/p24 Gag in the supernatants relative to that from control cells. (mean + s.d. from three

21 independent experiments). ns; ***P < 0.0005 compared with the Ctrl using two-tailed unpaired t-

22 tests.

17

1

2   **Figure 3** Schematic diagram of two different molecular mechanisms by which MARCH8

3   inhibits viral infection. *Left*, MARCH8 (red) downregulates VSV-G (violet) in a ubiquitin-

4   dependent manner. The RING-CH domain (pink) of MARCH8 recognizes VSV-G's

5   cytoplasmic lysine residues, which results in ubiquitin conjugation (shown as orange beads),

6   leading to lysosomal degradation; *Right*, MARCH8 downregulates HIV-1 Env (green) in a

7   YxxΦ motif-dependent manner. The tyrosine motif located in the C-terminal CT of

8   MARCH8 likely interacts with the adaptor protein μ-subunits (navy) (if this is the case with

9   μ2 or μ1, clathrin (brown) is involved in this step), resulting in the intracellular retention of

10 HIV-1 Env without degradation. It should be noted that the downregulation of these viral

11 glycoproteins might not necessarily occur at the plasma membrane. The nucleus and other
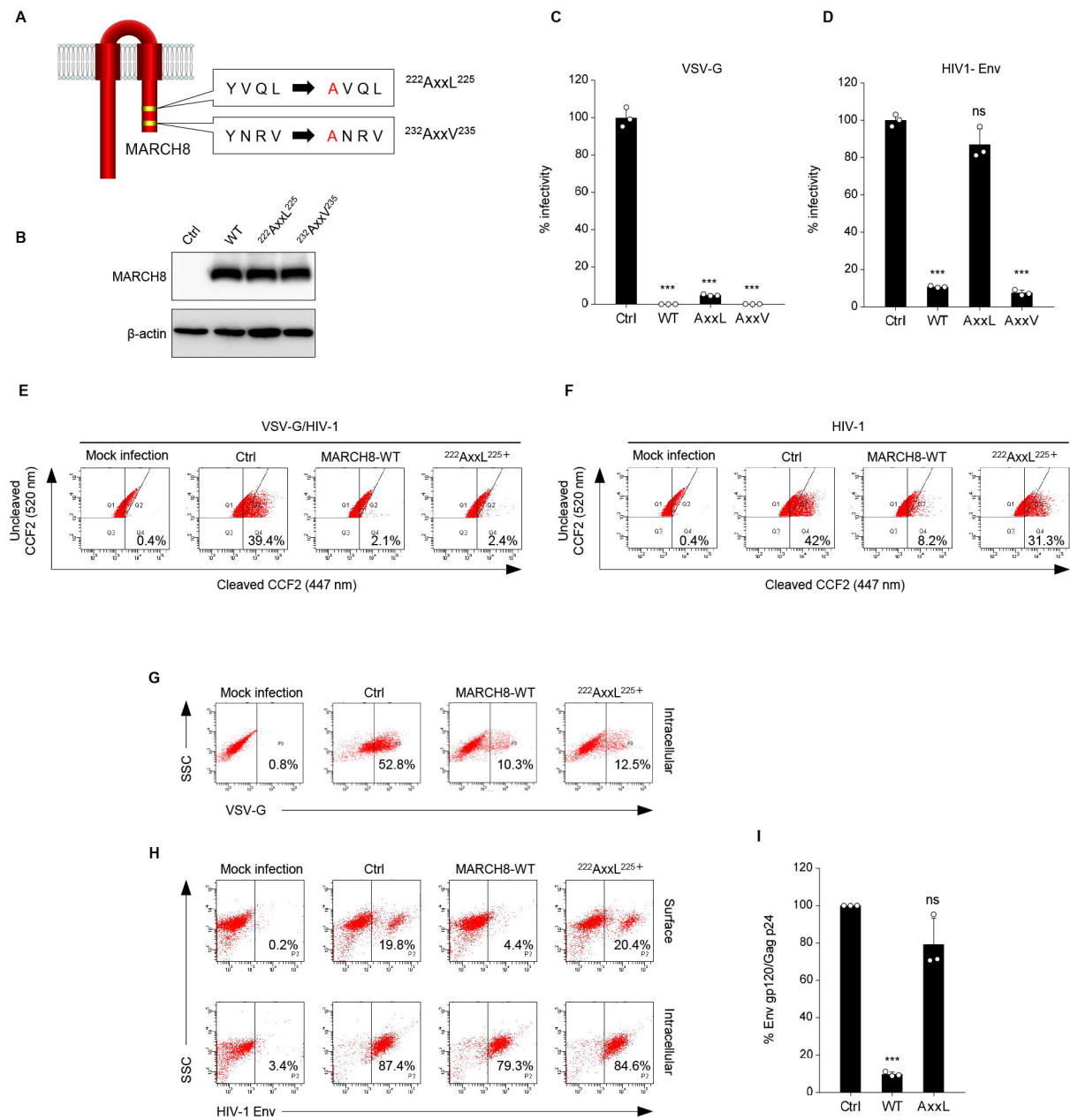
12 organelles are not shown.

Figure 1

Figure 2

Figure 3