Endocytic Activity of HIV-1 Vpu:
Phosphoserine-dependent Interactions with Clathrin Adaptors

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Supplemental Materials

SUPPORTING INFORMATION

Figure S1

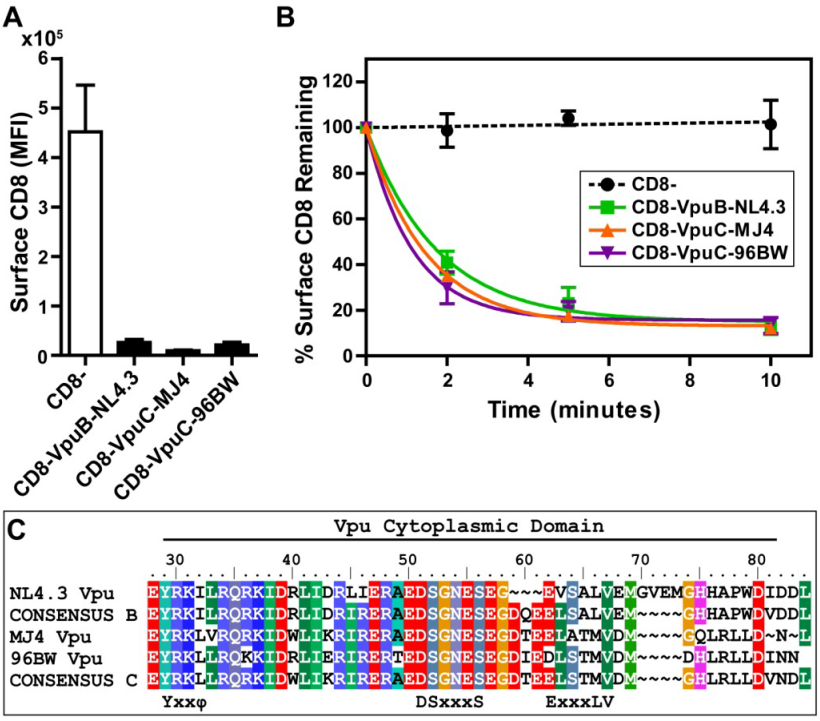

Figure S2

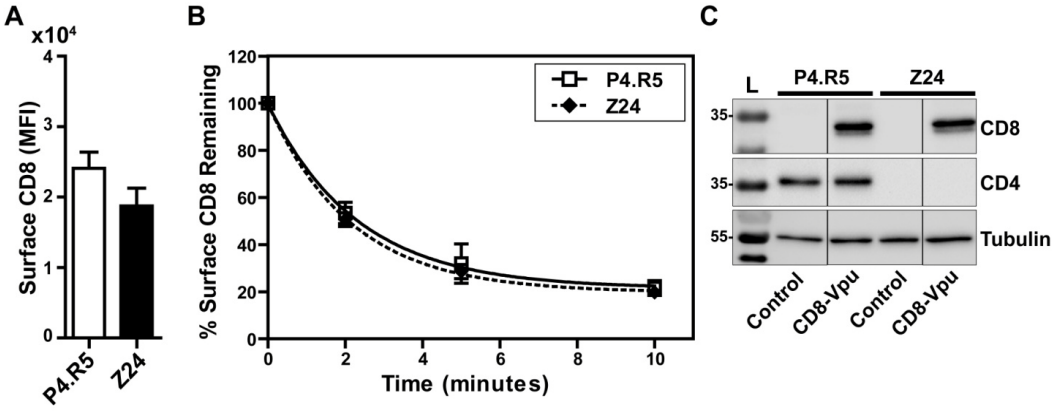
A
x10⁴
Surface CD8 (MFI)
P4.R5
Z24
B
% Surface CD8 Remaining
Time (minutes)
P4.R5
Z24
C
L
P4.R5
Z24
CD8
CD4
Tubulin
Control
CD8-Vpu
Control
CD8-Vpu

Figure S3

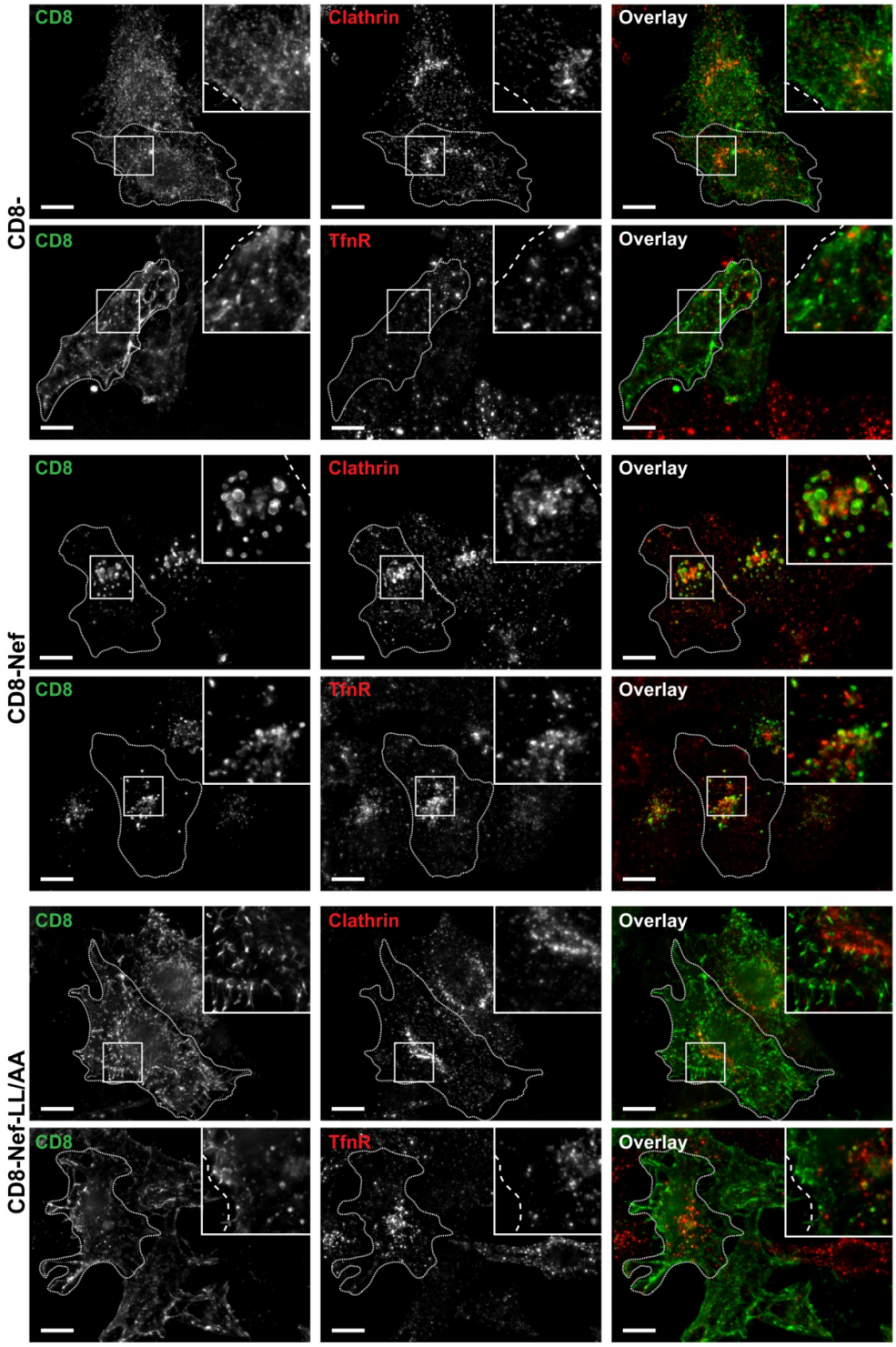
CD8-
CD8
Clathrin
Overlay
CD8
TfnR
Overlay
CD8-Nef
CD8
Clathrin
Overlay
CD8
TfnR
Overlay
CD8-Nef-LL/AA
CD8
Clathrin
Overlay
CD8
TfnR
Overlay

Figure S4

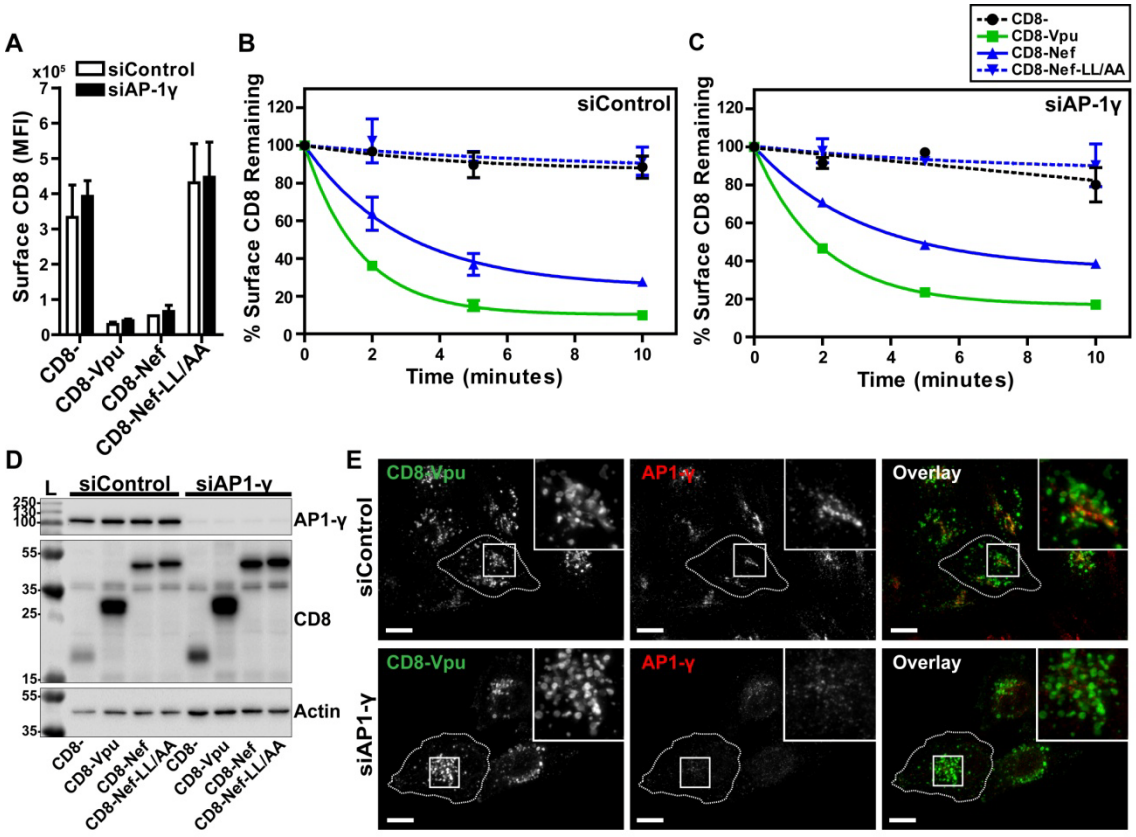
A
x10⁵
siControl
siAP-1γ
Surface CD8 (MFI)
CD8-
CD8-Vpu
CD8-Nef
CD8-Nef-LL/AA
B
siControl
% Surface CD8 Remaining
Time (minutes)
C
CD8-
CD8-Vpu
CD8-Nef
CD8-Nef-LL/AA
siAP-1γ
% Surface CD8 Remaining
Time (minutes)
D
L
siControl
siAP1-γ
AP1-γ
CD8
Actin
CD8-
CD8-Vpu
CD8-Nef
CD8-Nef-LL/AA
E
CD8-Vpu
AP1-γ
Overlay
siControl
siAP1-γ

Figure S5

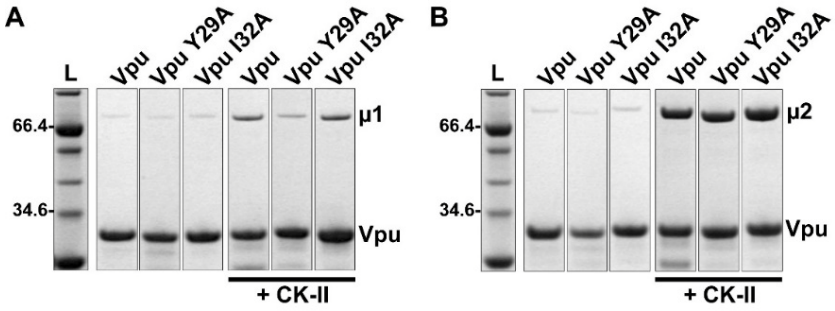
A
L
Vpu
Vpu Y29A
Vpu I32A
Vpu
Vpu Y29A
Vpu I32A
66.4-
34.6-
μ1
Vpu
+ CK-II
B
L
Vpu
Vpu Y29A
Vpu I32A
Vpu
Vpu Y29A
Vpu I32A
66.4-
34.6-
μ2
Vpu
+ CK-II

Figure S1. Similar endocytic activity is observed in both clade B and clade C Vpu. (**A**) Steady-state surface expression of CD8 constructs in HeLa P4.R5 cells. Cells were co-transfected to express CD8-, CD8-Vpu clade B (NL4.3), CD8-Vpu clade C (MJ4) or CD8-Vpu (96BW), in combination with a GFP expression construct as a transfection marker. Cell surface CD8 was stained and analyzed by two-color flow cytometry. The graph indicates the mean fluorescence intensity of surface CD8 staining in the GFP-positive cell population; error bars represent the standard deviation of two independent experiments. (**B**) The endocytic rates of the CD8-Vpu chimeras were measured in HeLa P4.R5 cells. Endocytosis assays were performed as detailed in Figure 1. Data are presented as the percentage of surface CD8 remaining over time. Error bars indicate the standard deviation of two independent experiments. (**C**) Sequence alignment showing location of residues conserved across clade B and C Vpu.

Figure S2. Endocytosis of CD8-Vpu-CD is independent of CD4. (**A**) Steady-state surface expression of CD8-Vpu-CD was measured in HeLa P4.R5 cells and HeLa Z24 cells, which do not express CD4. Cells were co-transfected to express CD8-Vpu and GFP as a transfection marker. Cell surface CD8 was stained and analyzed by two-color flow cytometry. The graph indicates the mean fluorescence intensity of surface CD8 staining in the GFP-positive cell population; error bars represent the standard deviation of three independent experiments. (**B**) The endocytic rate of CD8-Vpu was measured in HeLa P4.R5 and Z24 cells. Endocytosis assays were performed as detailed in Figure 1. Data are presented as the percentage of surface CD8 remaining over time. Error bars indicate the standard deviation of three independent experiments. (**C**) Expression of CD8-Vpu and CD4 was measured in the two cell lines by western blot, control cells were transfected to express GFP only.

Figure S3. Colocalization of CD8-fusion controls with markers of clathrin-mediated endosomal trafficking pathways. HeLa P4.R5 cells were either co-transfected with plasmids encoding CD8-, CD8-Nef, CD8-Nef-LL/AA and DSRed-Clathrin, or transfected to express the CD8-fusion proteins alone. The cells were fixed, permeabilized, blocked and stained for CD8 (green) and the endosomal marker transferrin receptor (TfnR, red). The cells were examined by widefield, deconvolution microscopy; images are z-stack projections of the total cell volumes. Scale bars = 10 μm.

Figure S4. Endocytosis of CD8-Vpu-CD is insensitive to siRNA-mediated depletion of AP-1. (**A**) The rates of internalization of the truncated CD8-, CD8-VpuCD, CD8-Nef, or CD8-Nef-LL/AA were measured in AP-1-depleted HeLa P4.R5 cells by flow cytometry. The cells were transfected with siRNA oligonucleotides directed against AP-1γ subunit (AP-1γ) or a non-targeting control (siControl). After 48 hours, the cells were transfected to express the indicated CD8-chimeras and a GFP expression construct. 24 hours later, the internalization rates of the CD8- chimeras were measured by antibody labeling and flow cytometric analysis. Steady-state surface levels of CD8-chimeras are shown (0-timepoint control). Data were normalized to the 0-timepoint control and presented as percentage surface CD8 remaining over time, in either siControl (**B**) or siAP-1γ (**C**) treated cells. Error bars indicate the standard deviation of three independent experiments. (**C**) Protein from cell lysates was separated by SDS-PAGE and AP-1γ depletion confirmed by western blotting. (**D**) The intracellular localization of CD8-VpuCD was determined by widefield microscopy of HeLa P4.R5 cells transfected with negative control or clathrin siRNA. Cells were transfected with CD8-VpuCD, and 24 hours later fixed and stained for immunofluorescence. AP-1γ depletion in these cells was determined by primary antibody labeling of AP-1γ and fluorescent secondary detection (red); CD8 staining is shown in green. Scale = 10μm.

Figure S5. The membrane-proximal Yxxφ motif is dispensable for μ binding. Substitutions Y29A and I32A were introduced into GST-tagged VpuCD. Phosphorylation of serines 52 and 56 was controlled through production of recombinant GST-VpuCD in the absence or presence of Casein Kinase II (CK-II). GST-pulldown was performed between μ1 (**A**), μ2 (**B**) and phosphorylated or non-phosphorylated GST-Vpu mutants. Bound proteins were analyzed by SDS-PAGE and Coomassie staining.