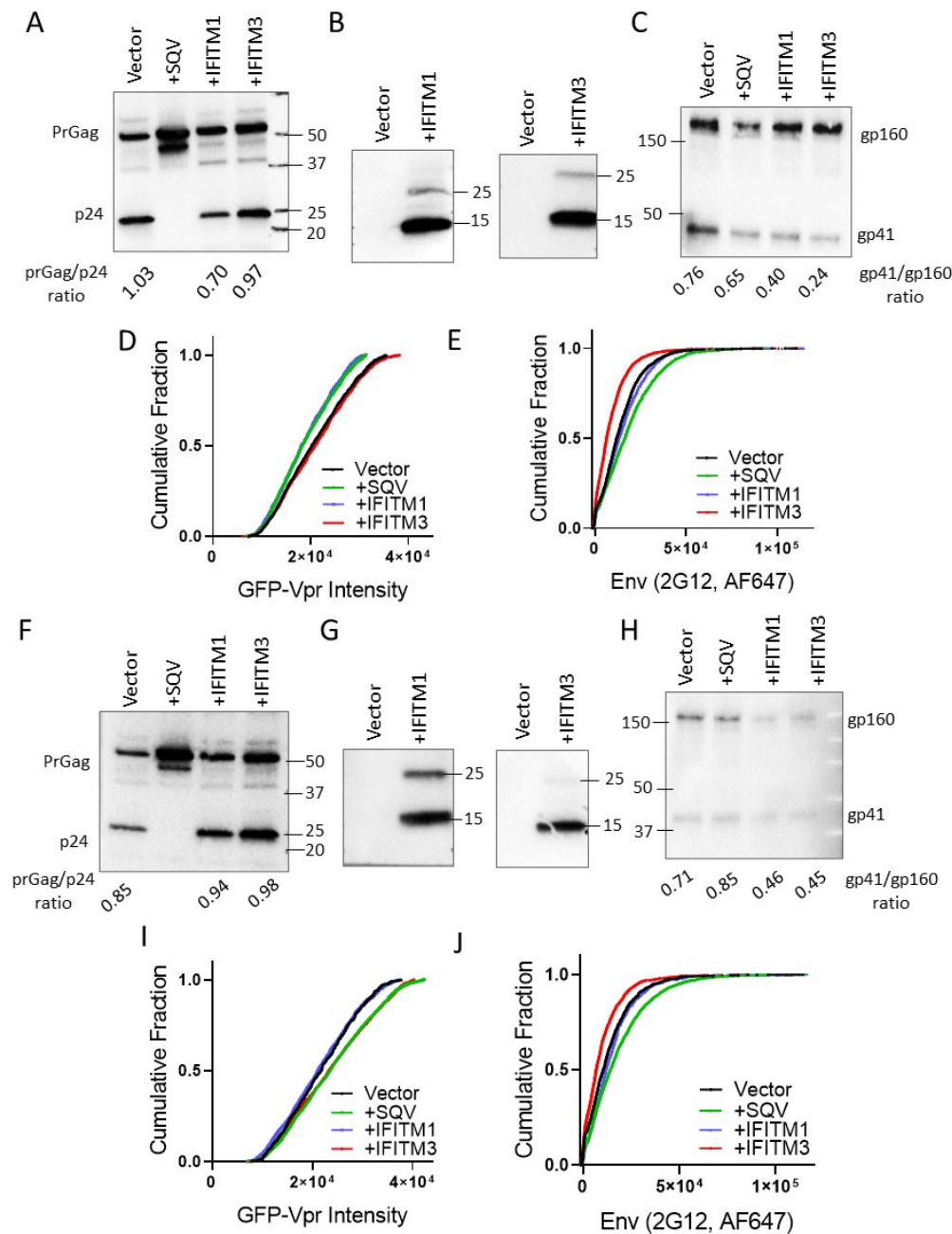**IFITM1 and IFITM3 Proteins Inhibit Infectivity of Progeny HIV-1 without Disrupting Env Clusters**

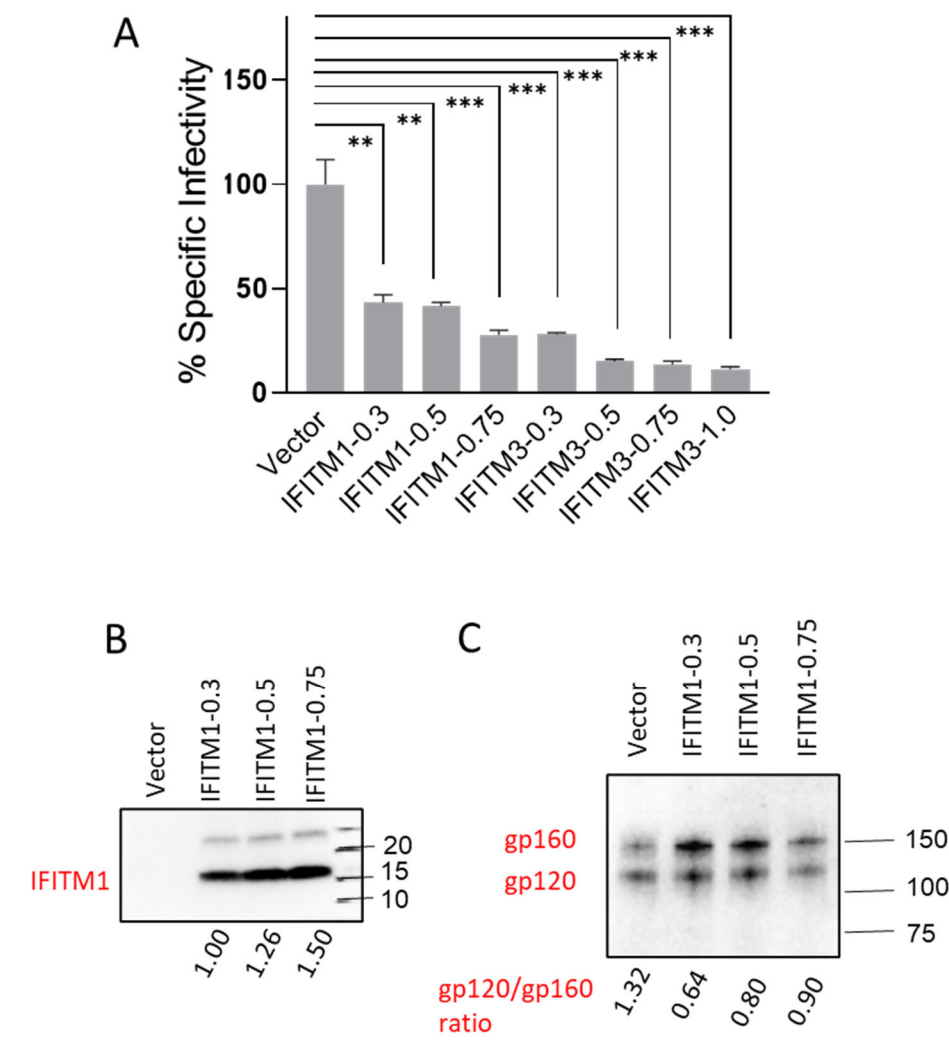Smita Verma[1], Yen-Cheng Chen[1,§], Mariana Marin[1,2], Scott E. Gillespie[1] and Gregory B. Melikyan[1,2,*]

[1] Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia, USA
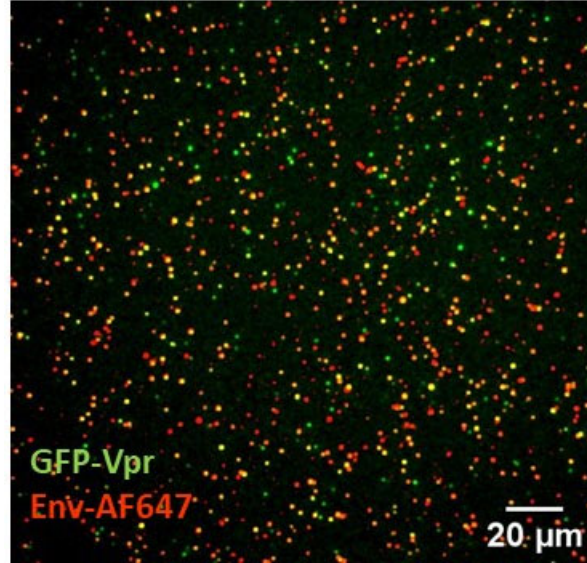
[2] Children's Hospital of Atlanta, Atlanta, Georgia, USA

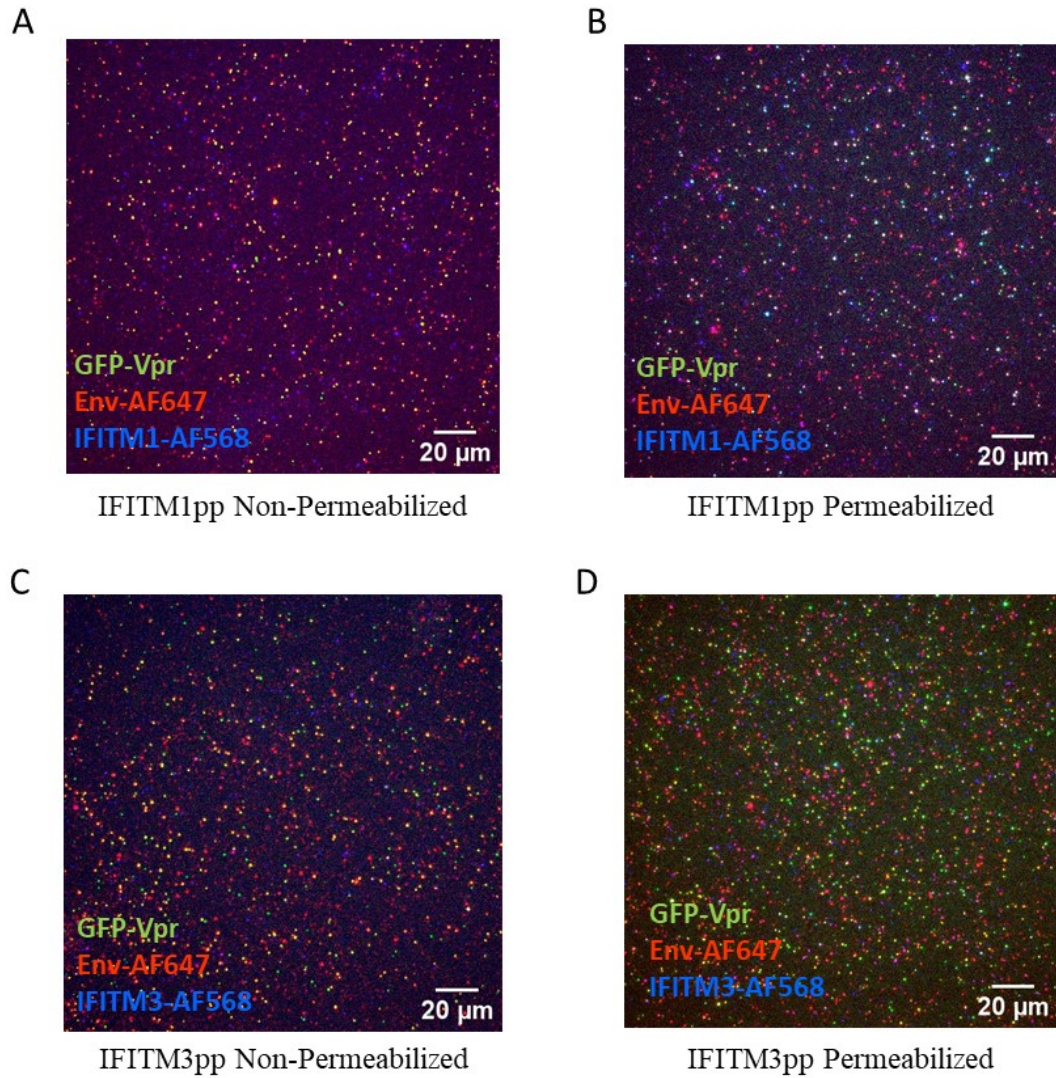**Supplemental Figure Legends**

Figure S1. Immunoblotting and immunofluorescence analysis of two independent HXB2 HIV-1 pseudovirus panels. Two pseudovirus panels, each consisting of four preparations: Control (Vector), +SQV (saquinavir treated, immature), IFITM1, and IFITM3 viruses were produced by transfection of HEK293T/17 cells, as described in Methods. (A, F) Characterization of pseudovirus maturation by Western blotting for p24. (B, G) Assessment of IFITM incorporation into pseudoviruses by Western blotting. (C, H) Analysis of HIV-1 Env incorporation and cleavage. Immunofluorescence analysis of GFP-Vpr incorporation (D, I) and HIV-1 Env incorporations (E, J) into single virions using anti-gp120 2G12 primary antibody and anti-human AF647-conjugated secondary antibody.
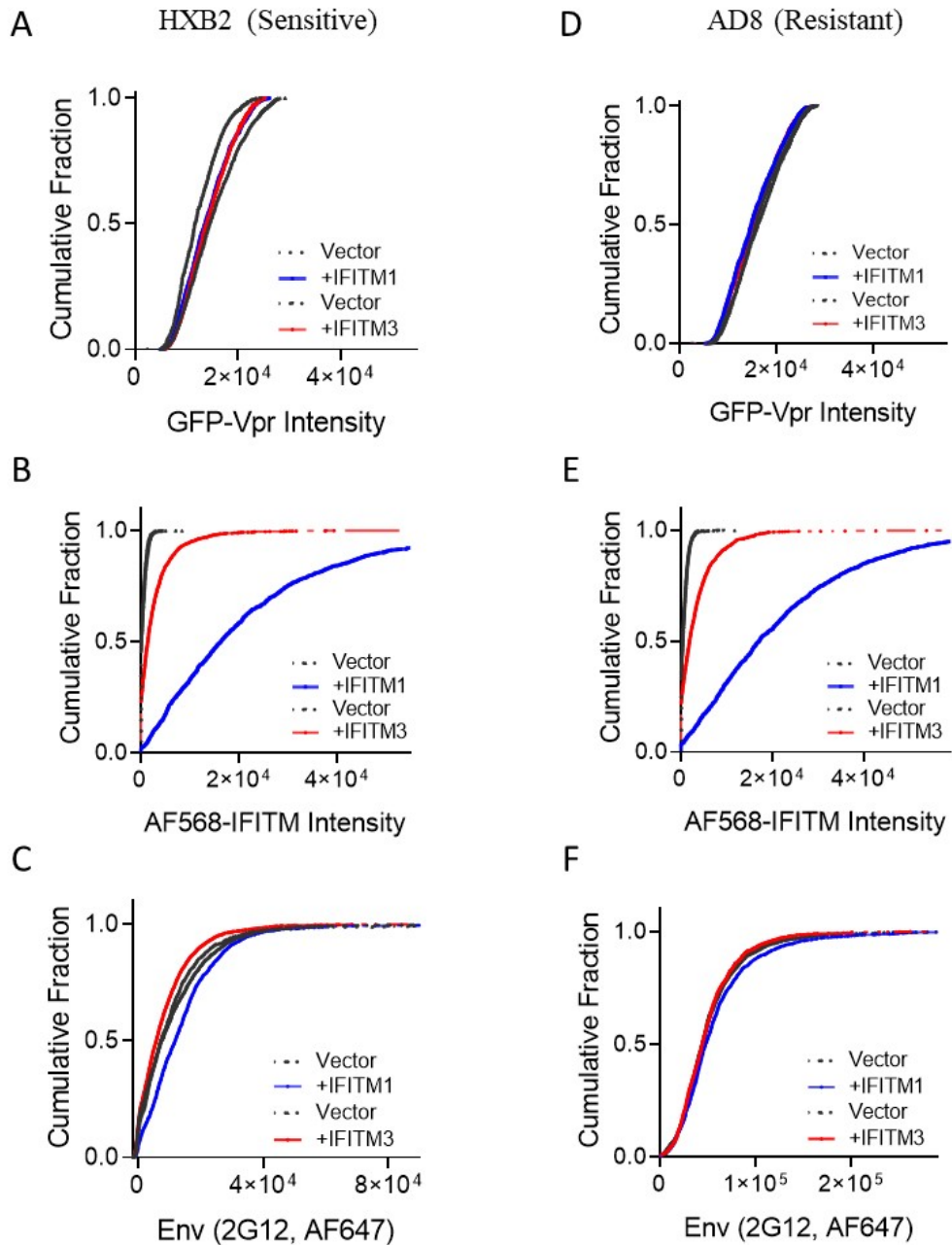
Figure S2. Titration of HIV-1 HXB2 pseudoviruses with increasing amounts of IFITM plasmids. (A) A panel of three pseudoviruses – Vector (Control), IFITM1, and IFITM3 containing particles – was produced in parallel by transfection of HEK293T/17 cells. (A) Dependence of HIV-1 pseudovirus infectivity on the amount of IFITM plasmids used for transfection. TZM-bl cells were infected for 48 h with the indicated pseudovirions using the same amount of viral p24. The resulting luciferase signal is normalized to control (Vector) particles. The statistical analysis was performed using Student's t-test. Significance: n.s., $p>0.05$; **, $0.01>p>0.001$; ***, $p<0.001$. (B) Western blotting analysis of IFITM1 incorporation into virions upon increasing the plasmid concentration from 0.3-0.75 µg. (C) Immunoblot analysis of Env incorporation and processing for a representative pseudovirus panel produced using increasing amounts of IFITM plasmids.
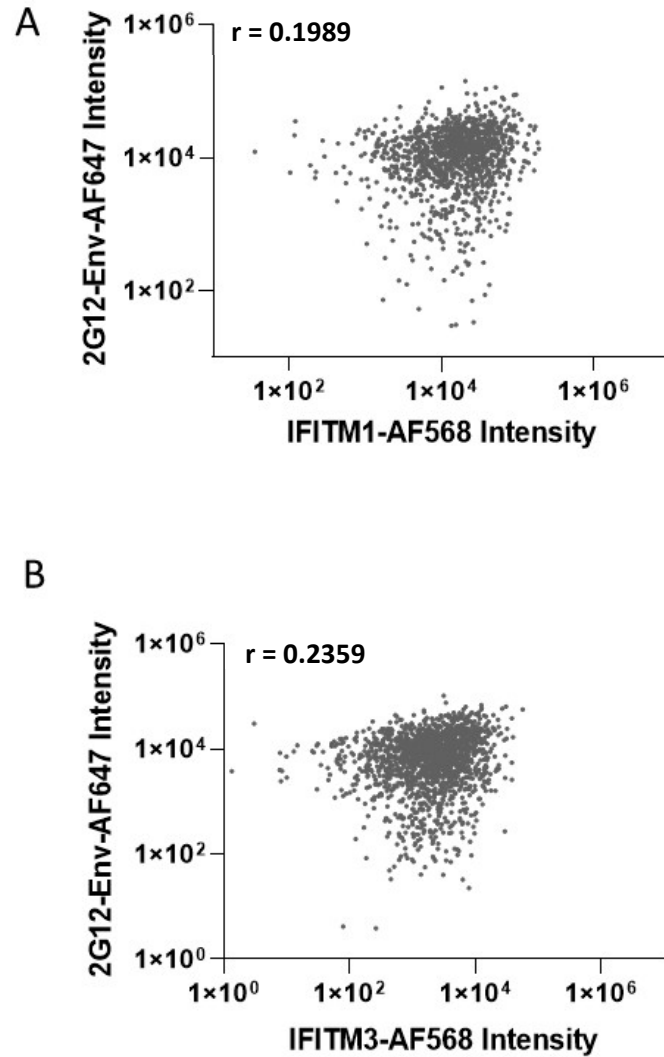
Figure S3. Representative immunofluorescence image of HXB2 pseudoviruses labeled with GFP-Vpr and immunostained for Env. Images of coverslip-immobilized single pseudoviruses show the extent of colocalization of GFP-Vpr (green) with HXB2 Env glycoproteins (red) after immunostaining for Env glycoproteins using anti-gp120 2G12 antibody and AF647-conjugated secondary antibody.

Figure S4. Single virus-based immunofluorescence analysis of IFITM and HXB2 Env incorporation. The virions were fixed and permeabilized using 0.2% Triton-X100. Single virus colocalized image of GFP-Vpr (green) labeled pseudovirions stained for Env glycoproteins (red) and IFITM (blue) proteins.
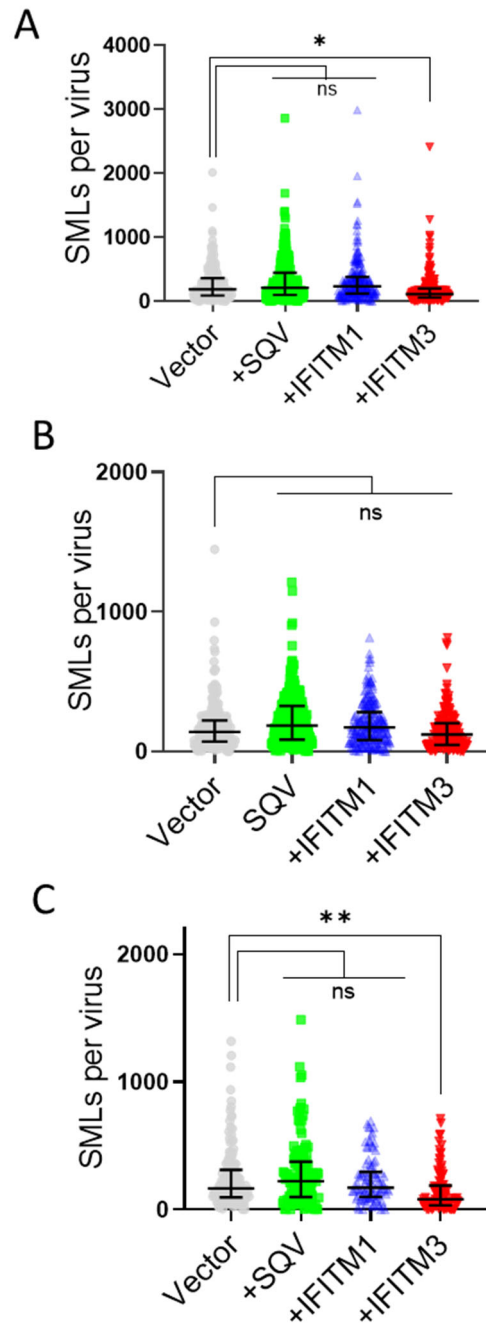
Figure S5. Assessment of IFITM and Env incorporation into single pseudoviruses. Immunofluorescence analysis of HIV-1 GFP-Vpr (A, D), IFITM (B, E), and Env (C, F) fluorescence intensity distributions using single pseudoviruses bearing sensitive (HXB2) and resistant (AD8) Env after fixation and permeabilization with 0.2% Triton-X100.

Figure S6. **Incorporation of sensitive Env into pseudoviruses does not correlate with IFITM incorporation**. (A) Lack of correlation between Env and IFITM1 signals per single virions. (B) Lack of correlation between Env and IFITM3 signals per single virion. Correlation coefficients were calculated using a Pearson correlation coefficient in GraphPad Prism.
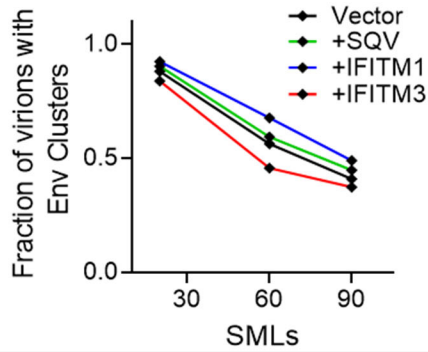
Figure S7. Single-molecule localization analysis of Env incorporation into pseudovirions after optimal binning of SML data. (A-C) Distributions of single-molecule localizations (SMLs) per virion measured by 2D dSTORM for three independent pseudovirus panels. Statistical analysis was performed by a two-sample Kolmogorov–Smirnov (KS) test with optimal binning of data using a custom MATLAB script. Significance: n.s., p>0.05; *, 0.05>p>0.01; **, 0.01>p>0.001.

A

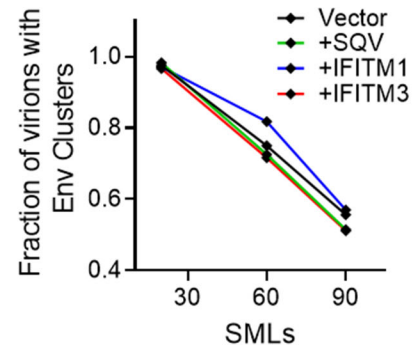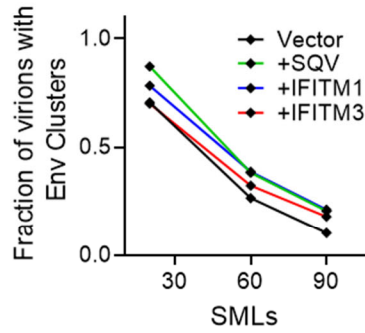| FE test	≥20SMLs	≥60SMLs	≥90SMLs
Control vs SQV	n.s.	n.s.	n.s.
Control vs IFITM1	n.s.	**	n.s.
Control vs IFITM3	**	***	**
IFITM1 vs IFITM3	***	***	***

B

FE test	≥20SMLs	≥60SMLs	≥90SMLs
Control vs SQV	n.s.	n.s.	n.s.
Control vs IFITM1	n.s.	n.s.	n.s.
Control vs IFITM3	n.s.	n.s.	n.s.
IFITM1 vs IFITM3	n.s.	n.s.	n.s.

C

FE test	≥20SMLs	≥60SMLs	≥90SMLs
Control vs SQV	***	***	***
Control vs IFITM1	n.s.	***	***
Control vs IFITM3	n.s.	n.s.	*
IFITM1 vs IFITM3	n.s.	n.s.	n.s.

Figure S8. The effect of IFITMs on Env clustering on HIV-1 pseudoviruses imaged by dSTORM using 2-category analysis. Env clusters were defined by the DBSCAN algorithm using a fixed search radius of 15 nm and varied minimum number of SMLs from ≥20 to ≥90. (A, B, and C) Fractions of pseudoviruses with and without Env clusters as a function of DBSCAN single molecule localization threshold for three independent panels of pseudoviruses. Statistical significance was determined by the Fisher's Exact test. Significance: n.s., p>0.05; *, 0.05>p>0.01; **, 0.01>p>0.001; ***, p<0.001.

Figure S9. Env-Env pairwise distance distribution analysis on single pseudoviruses. Comparison of the probability density function (PDF) of Env-Env pairwise distances between AF647 SMLs in control (gray), SQV (green), IFITM1 (blue), and IFITM3 (red) pseudoviruses obtained by dSTORM. Statistical analysis was performed by a two-sample Kolmogorov–Smirnov (KS) test with optimal binning of data using a custom MATLAB script. Significance: n.s., p>0.05; *, 0.05>p>0.01; ***, p<0.001.