Single molecule fate of HIV-1 envelope reveals late-stage viral lattice incorporation

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Human immunodeficiency virus type 1 (HIV-1) assembly occurs on the inner leaflet of the host cell plasma membrane, incorporating the essential viral envelope glycoprotein (Env) within a budding lattice of HIV-1 Gag structural proteins. The mechanism by which Env incorporates into viral particles remains poorly understood. To determine the mechanism of recruitment of Env to assembly sites, we interrogate the subviral angular distribution of Env on cell-associated virus using multicolor, three-dimensional (3D) superresolution microscopy. We demonstrate that, in a manner dependent on cell type and on the long cytoplasmic tail ofEnv, the distribution of Env is biased toward the necks of cell-associated particles. We postulate that this neck-biased distribution is regulated by vesicular retention and steric complementarity of Env during independent Gag lattice formation.
Virus assembly involves a choreographed coalescence of viral and host biomolecules to create new infectious particles, which propagate infection. In the case of HIV-1 assembly, the structural polypeptide Gag anchors to the inner leaflet of the plasma membrane through the matrix (MA) domain and oligomerizes to create a lattice which deforms the membrane. The HIV-1 Env glycoprotein complex traffics through the secretory pathway to the plasma membrane, where it is displayed as a heterotrimer composed of three molecules each of the surface glycoprotein gp120 and the transmembrane glycoprotein gp41. Determinants driving Gag and Env to efficiently co-assemble remain unclear, but numerous studies have implicated the long cytoplasmic tail of gp41 (Env-CT) in virus particle incorporation and oligomerization to create a lattice which deforms the membrane. Further compounding the complexity of HIV-1 assembly is the relative sparsity of Env on individual released particles (7–14 trimers). This suggests that Env incorporation into nascent Gag lattices is tightly regulated, but the mechanisms of regulation are also poorly understood. Specific Env retention at the virus assembly site is believed to be due to steric trapping of the long Env-CT between hexamers of Gag-MA trimers. In support of this model, a small deletion in the second predicted helix of Env-CT (LLP-3), d8, imposes Env incorporation defects that can be rescued by complementary mutations in the Gag-MA domain. Furthermore, deletion of the Env-CT (CTΔ144) results in a reduction in virus incorporation of Env, but this reduction is cell-type-dependent, suggesting that host cell factors regulate HIV-1 assembly.

Herein, we demonstrate that steric trapping fixes the angular distribution of Env clusters at virus budding sites, thereby driving the incorporation of Env into assembling HIV-1 particles. We show that Env encounters the Gag lattice late in lattice assembly and that this is cell-type-dependent as well as dependent on the Env-CT.

Results

Measurement of Env angular distribution at assembly sites. We hypothesized that, by interrogating the angular distribution of Env on the surfaces of cell-associated virus particles, we could determine when Env encounters the Gag lattice (Fig. 1a). To determine if this timing is dictated by host cell factors, we employed two cell lines: CEM-A, a T-cell line permissive for HIV-1 replication, and COS7, a fibroblast-like cell line. In both cell types, single-round infection with VSV-G pseudotyped virus (NL4–3 reference genome) allowed for expression of native levels of Gag and Env. Infection was performed with particles deficient for protease (ΔPol) in order to prevent premature processing of

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the cell-associated Gag lattice and to keep the angular distribution of Env about the budding particle surface fixed. Release-deficient virus containing a late-domain mutation (ΔPTAP)18-20 was used to arrest particles at a late stage of budding, strictly limiting analysis to cell-associated particles. Collectively, this system enables production of HIV-1 assembly sites for which the budding axis can be identified and used to measure the angular arrangement of Env.

Three-dimensional superresolution imaging of infected cells was achieved using interferometric photo-activated localization microscopy (iPALM)21,22, which allowed us to localize both Gag and Env molecules on a subviral scale (with a localization precision of 10–20 nm; Supplementary Fig. 1), along with the host cell plasma membrane, which was mapped by co-expression of a myristoylated photoswitchable fluorescent protein23 (S15-PSCFP2; Fig. 1b). Single assembly sites were segmented for analysis if a cluster of Gag was proximal to Env clusters, the local plasma membrane was sufficiently sampled, and the segmented area was resolved from other assembly sites (see “Methods” section, Supplementary Fig. 2). Gag cluster centroids were estimated as described previously22 (Supplementary Fig. 3). Env clusters that were localized less than a particle radius from the Gag centroid and residing extracellularly relative to the local plasma membrane were classified as assembly site proximal. Principle component analysis of the local plasma membrane topography at each assembly site (~200 × 200–400 × 400 nm) was used to define the viral budding axis, to which the angular positions of Env clusters could be referenced, (Fig. 1c and Supplementary Figs. 4 and 5) and to confirm that bud neck lengths were consistent with cell-associated particles (Supplementary Fig. 6). Due to the sparsity of Env on individual particles9, we derived statistical power from single-particle averaging of hundreds of aligned individual HIV-1 assembly sites. Single assembly site averaging was used to determine the probability density of Env with respect to the elevation angle (φ), defined relative to the virus bud equator. We tested this approach using simulated data mimicking the three models presented in section, Supplementary Fig. 2). Gag cluster centroids were additionally employed an orthogonal approach to explicitly measure the angular position (φ) of individual Env clusters at single virus assembly sites (Supplementary Figs. 8 and 9).

In order to assess the uncertainty in measurement of the angular probability distribution for Env, we used Monte Carlo simulation to generate synthetic virus assembly sites using empirically derived seed values (see Methods section). By analysis of this simulated data set, we estimate our angular uncertainty to be ±0.12° (s.d. ± s.e.m., n = 2500; Supplementary Fig. 10). To complement the error estimates from simulation data, we devised an additional analysis method utilizing phase correlation between the membrane channels of aligned random half data sets to estimate that our plane fitting method resulted in no more than 3° of uncertainty in rotational alignment (Supplementary Fig. 11), which is comparable to our simulated error in Env angular measurements. Phase correlation between the Env channels of random half data sets suggested, on average, 9° of uncertainty in angular sampling of Env (σφ) on the surfaces of virus particles (Supplementary Fig. 12). These results collectively suggest that a conservative upper limit for the angular resolution of our system is 9°.

Probability densities of averaged wild-type (WT) Env showed that the T-cell line, CEM-A, produces virus assembly sites with an angular distribution biased toward the necks of budding particles (Fig. 2a, b and Supplementary Fig. 13). Herein, we defined the elevation angle (φ) at the equator of the particle as 0° (orthogonal to the budding axis), the neck of the particle (southern hemisphere) from 0° to −90°, and the crown of the particle (northern hemisphere) from 0° to 90° (Fig. 2a cartoon). Using our orthogonal approach of directly measuring individual Env clusters at single assembly sites, we observed comparable angular bias of Env toward virus necks, with a mean value of φ = −17.1 ± 0.20° (s.e.m., n = 338) and a skewness of 0.523 relative to the equator of the particle (Fig. 2c and Supplementary Fig. 14). In contrast, particles produced in the COS7 fibroblast-like cell line showed an unbiased angular distribution of Env using the single-particle averaging approach (Supplementary Fig. 13), in agreement with a mean φ = 2.3 ± 1.3° (n = 813) and a skewness of −0.0970 obtained by individual cluster measurements (Fig. 2c and Supplementary Table 1). Attempts to confirm these observations by immunogold transmission electron microscopy failed to produce robust statistical sampling due to low-density Env labeling and frequent off-axis sectioning of budding profiles (Supplementary Fig. 15).

Next, we tested whether the Env-CT was responsible for the neck bias of Env in the CEM-A cell line. Indeed, removal of the Env-CT (CTΔ144) produced an unbiased distribution of Env signal on the surfaces of budding particles (mean φ = 4.1 ± 2.1°, n = 367; skewness −0.221). These results show that the Env-CT is responsible for the neck bias measured for WT-Env (Fig. 2 and Supplementary Figs. 13 and 14). As expected, the CTΔ144 mutation had no effect on the already unbiased distribution of Env in particles produced by COS7 (mean φ = −3.07 ± 2.3°, n = 260; skewness −0.0279).

We next sought to determine if inhibition of Gag lattice trapping could induce a neck bias for particles produced in the COS7 cell line. Strikingly, deletion of residues in the Env-CT (d8) that lead to steric clashing with the Gag-MA lattice and reduced Env incorporation14 resulted in a neck-biased distribution of Env in COS7 cells (mean φ = −9.4 ± 1.6°, n = 582; skewness 0.236), where no bias was observed for WT-Env. These results show that steric clashing imposed by the d8 mutation regulates Env to the periphery of the budding Gag lattice in COS7 cells (Fig. 2 and Supplementary Figs. 13 and 14). As anticipated, the d8 mutation did not produce a significant change in the Env neck-distributed phenotype observed for WT-Env in CEM-A cells (mean φ = −23.6 ± 2.9°, n = 134; skewness 0.530).

Single-molecule tracking of Env diffusivity on cell surfaces. Given that removal of the Env-CT (CTΔ144-Env) led to an unbiased angular distribution, we hypothesized that CTΔ144-Env may not be trapped by the Gag lattice, leading to sampling of the entire bud surface by diffusing Env trimers. This lack of trapping would also account for the substantial incorporation defect we observed with this genotype in both cell types (Supplementary Fig. 16). To test this hypothesis, we performed single-molecule tracking of WT-, CTΔ144-, and d8-Env trimers on the surfaces of CEM-A and COS7 cells using an anti-Env Fab fragment (b12), conjugated to Atto565 dye, in order to interrogate the nanoscale dynamics of these molecules during virus assembly (Fig. 3). We imaged Env on sparsely labeled cells, measured single-molecule positions, and used the uTrack software24 to link single-molecule trajectories and compute the diffusion coefficients of each track (Supplementary Fig. 17). Individual tracks were then classified as either mobile or confined/immobile, based on the estimated slope of the moment scaling spectrums In CEM-A cells, we found that the mean diffusion coefficients of WT-, CTΔ144-, and d8-Env tracks classified as mobile did not significantly differ from each other, with mean diffusion coefficients of 0.097 ± 0.049 (s.d., n = 1514 across four biological replicates), 0.149 ± 0.054 (n = 9694 across four biological replicates), and 0.107 ± 0.067 μm² s⁻¹ (n = 2139 across four biological replicates), respectively (Fig. 3b).
agreeing well with published results of cell-associated WT- and CTΔ144-Env diffusion using an orthogonal technique.11. However, the proportion of tracks classified as confined/immobile was significantly higher for WT-Env (weighted mean of 80 ± 6% confined/immobile tracks across four biological replicates, s.d., n = 7862 tracks) when compared with both CTΔ144- (27 ± 5% confined/immobile, n = 13,338 tracks) and d8-Env (56 ± 15% confined/immobile, n = 5434 tracks, Fig. 3c), in agreement with previous microscale FRAP data for WT- and CTΔ144-Env12. Our single-molecule measurements of Env dynamics suggest that the reduced incorporation of CTΔ144- and d8-Env into virus assembly sites derives from a reduced likelihood of steric trapping within Gag lattices, rather than from differential diffusion behavior. In COS7 cells, mean diffusion coefficients of Env did not significantly differ from those of Env in CEM-A cells, and the same trend of mobile versus immobilized populations was observed between genotypes (Fig. 3d–f), suggesting that the observed differences in diffusion behavior are not cell-type-dependent and do not, on their own, account for the cell-type-dependent differences in Env angular distributions measured by superresolution.

**Intracellular retention correlates to Env distribution bias.** We hypothesized that the cell-type-dependent neck-biased phenotype observed with WT-Env in CEM-A cells may result from Env retention within intracellular compartments while Gag autonomously assembles on the plasma membrane. To test this hypothesis, we performed live cell pulse-chase labeling of surface-exposed Env with an anti-Env Fab (b12) conjugate to determine the relative levels of internalization of Env and associated mutants. In addition, we also performed fixed cell surface staining of Env to compare relative surface-exposed populations for each genotype. Spinning disk confocal microscopy revealed a large...
**Fig. 3** Single-molecule tracking of HIV-1 Env trimers reveals that the Env-CT does not significantly alter diffusivity on the nanoscale, but determines the fraction of Env that is confined/immobile.  

**a, d** Single-particle tracking of WT-Env (left), CTΔ144-Env (middle), and d8-Env (right) labeled with Fab b12-Atto565 in a CEM-A cells and d COS7 cells. Scale bars are 2 μm (top) and 500 nm (red inset region, bottom). Tracks were classified as immobile and confined (dark green) versus mobile (light green).  

**b, e** Mean diffusion coefficients were computed based on individual molecular tracks in CEM-A (WT-Env, n = 1514; CTΔ144-Env, n = 9694; and d8-Env, n = 2139, each from four biological replicates) and COS7 (WT-Env, n = 274; CTΔ144-Env, n = 2238; and d8-Env, n = 1019 each from ≥4 biological replicates).  

In CEM-A cells, the mean diffusion coefficients of tracks classified as mobile for the CTΔ144-Env ($D_{\text{mobile}} = 0.149 \pm 0.054 \, \text{μm}^2 \, \text{s}^{-1}$) and d8-Env ($D_{\text{mobile}} = 0.107 \pm 0.067 \, \text{μm}^2 \, \text{s}^{-1}$) are not significantly different when compared to WT-Env ($D_{\text{mobile}} = 0.097 \pm 0.049 \, \text{μm}^2 \, \text{s}^{-1}$). For COS7 cells, mean diffusion coefficients of tracks classified as mobile indicate that the CTΔ144 ($D_{\text{mobile}} = 0.108 \pm 0.053 \, \text{μm}^2 \, \text{s}^{-1}$) and d8 mutations ($D_{\text{mobile}} = 0.144 \pm 0.087 \, \text{μm}^2 \, \text{s}^{-1}$) do not significantly alter the diffusion rates of Env when compared to WT-Env ($D_{\text{mobile}} = 0.085 \pm 0.056 \, \text{μm}^2 \, \text{s}^{-1}$).  

**c, f** The Env-CT dictates the confined/immobile fraction of Env on the plasma membrane. In CEM-A cells, the confined/immobile fractions of the CTΔ144-Env and (27 ± 5%) and d8-Env (56 ± 15%) were both found to be significantly smaller relative to WT-Env (80 ± 6%). In COS7 cells, the confined/immobile fractions of the CTΔ144-Env (57 ± 5%) and d8-Env (72 ± 13%) were significantly lower relative to WT-Env (92 ± 4%), suggesting that steric trapping of Env is dependent on the Env-CT. Error bars indicate s.d. ***$P < 0.0001$ and n.s. indicates not significant using one-way ANOVA with Tukey’s post-test. CEM-A (WT-Env, n = 7862; CTΔ144-Env, n = 13,338; and d8-Env, n = 5434) and COS7 (WT-Env, n = 3790; CTΔ144-Env, n = 5283; and d8-Env, n = 4384)
steady-state population of endocytosed Env, which localized with recycling endosomes co-labeled with fluorescent transferrin (Supplementary Fig. 18). We observed a reduction in the intracellular pool and higher levels of CTΔ144-Env on the plasma membrane in CEM-A relative to WT-Env (Fig. 4a, b), consistent with a role for the Env-CT in Env endocytosis25. In COS7 cells, however, the intracellular pool did not differ significantly between WT-Env and CTΔ144-Env, and surface levels of WT-Env were slightly higher than CTΔ144-Env, suggesting that endocytosis or intracellular retention of Env is less prevalent in this cell line (Fig. 5). In contrast, the d8 mutation led to an increase in the intracellular pool of Env relative to WT and lower levels on the plasma membrane in both CEM-A and COS7 cells (Figs. 4a, b and 5).

Given that the steady-state levels of Env mutants show drastic differences, we next quantified the rates of Env internalization and recycling to the plasma membrane by performing fluorescence recovery after photobleaching (FRAP) on the intracellular pool of Env in CEM-A cells. We found that internal pools of CTΔ144-Env recovered at rates more than tenfold slower than WT-Env and more than fivefold slower than d8-Env (Fig. 4c, d), confirming that impairment of endocytosis and lattice incorporation alter the flux of Env intracellular trafficking. The total percent recovery of CTΔ144-Env was considerably lower than either WT- or d8-Env; however, the intracellular pool of d8-Env recovered to a similar extent as WT-Env, despite having a smaller pool of un-bleached plasma membrane Env that could be endocytosed (Fig. 4b–d). This result suggests that larger intracellular pools of d8-Env may result not only from endocytosis of a larger population of freely diffusing Env trimers, but also from an accumulation of endocytosed trimers that are unable to properly return to the plasma membrane (Figs. 4a and 5a).

**Fig. 4** Intracellular retention of Env in CEM-A cells correlates with angular distributions of Env at assembly sites. **a** Pulse-chase-labeled Env (anti-Env Fab b12-Atto565; 15 min) demonstrates greater intracellular accumulation of d8-Env (43 ± 1%, n = 123 cells) and WT-Env (24 ± 1%, n = 142 cells) relative to CTΔ144-Env (14 ± 1%, n = 99 cells) in CEM-A cells. Scale bars are 10 μm (representative images above). **b** Labeling of fixed cells with anti-Env 2G12- and b12-AF647 probes demonstrates reduced levels of surface-exposed WT- and d8-Env relative to CTΔ144-Env. Scale bars are 10 μm (representative images above). **a**, **b** Bar and error bars represent mean and s.e.m. **c** Representative FRAP time-lapse highlighting the rates of recovery of intracellular Env pools. Time points are relative to photobleaching (bleach region, white dashed circle). Scale bars are 20 μm. **d** FRAP experiments demonstrate reduced internal compartment recovery rates for CTΔ144-Env (0.016 ± 0.01 s−1) relative to WT-Env (0.21 ± 0.01 s−1, relative to CTΔ144-Env P < 0.0001) and d8-Env (0.092 ± 0.01 s−1, relative to CTΔ144-Env P = 0.0036). Error bars indicate s.d. (n = 4 cells per genotype). *** indicates significance from WT with P < 0.0001. All statistical tests used a two-tailed unpaired t-test.
Collectively, our experimental results demonstrate a complex spatiotemporal pathway for HIV-1 Env incorporation into assembling Gag lattices. Our single-molecule approach, specifically imaging the subviral angular distribution of native levels of Env on the surface of nascent budding HIV-1 particles, enabled accurate quantification (at ~9° resolution) of a neck-biased phenotype present on particles produced in the T-cell line CEM-A, but not on those produced in the fibroblast-like line COS7. In this study, we chose to use a late-domain mutant Gag to trap and preserve the angular orientation of Env during virus budding, as particle release would result in scrambling of the angular distribution of Env. A potential consequence of using release-deficient Gag is that late-domain mutants will create assemblies.

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which continue to polymerize and overfill the particle. Additional Gag lattice polymerization will act to fix Env trimers that partition into the neck of the virus bud. If stochastic trapping of Env trimers was enhanced with late-domain mutant Gag lattices, we would expect to observe a larger number of Env clusters by superresolution imaging than previous studies observing released virus (only 1–4 clusters per particle)\(^{26}\). Finally, we were able to measure statistically significant differences in the angular distributions of Env mutants relative to WT-Env as well as observe cell-type differences, suggesting that mutant late-domain assembly sites do not dominantly determine Env distributions.

The measured neck bias of WT-Env in CEM-A T cells suggests that Gag lattices on average begin to form prior to Env encounter, statistically relegating trapped Env to the periphery of assembling lattices and limiting the amount of Env that can be incorporated prior to particle abscission. Strikingly, with assembling particles produced in COS7 cells, the timing of Env acquisition appears to be more synchronous with Gag lattice formation, resulting in an unbiased angular distribution about the viral particle surface, and this discrepancy suggests a role for host cell factors in regulating the timing of encounters. We show that the temporal regulation of Env incorporation is dependent on the presence of the long Env-CT, without which CTA144-Env trimers appear to freely diffuse through Gag lattices, enabling continued access to high-angular spaces in the head of the budding particle.

Our results further demonstrate that the angular distribution of Env can be engineered by the introduction of mutations in the Env-CT, without which CT-mediated Env incorporation acts to regulate virus association. These results suggest that complementarity between the Gag lattice and the Env-CT is a critical factor in Env incorporation and supports previous studies implicating residues in the Gag matrix domain (Gag-MA) for mediating Env acquisition\(^{14,15}\).

Interrogating the nanoscale diffusivity of single Env trimers on the cell surface was critical to confirm that removal of the Env-CT leads to increased nanoscale mobility of Env trimers in the presence of active HIV-1 assembly sites. We demonstrate using single-particle tracking of Env that, indeed, CTA144-Env trimers are far less confined/immobilized relative to WT-Env, on the tens of nanometers resolution scale, and measured over a large population. Furthermore, Env trimers possessing the \(d8\) mutation, unable to achieve high-angular distributions in the Gag lattice of budding particles on COS7 cells, showed a marked increase in population mobility relative to WT-Env, supporting the hypothesis that complementarity between the Env-CT and Gag lattice is critical for trapping and particle incorporation.

An alternative interpretation of the high-angular distributions achieved by CTA144-Env mutants could also be explained by the presence of a larger quantity of Env on the plasma membrane (Fig. 4b). Within this interpretive framework, the higher plasma membrane CTA144-Env trimer density would enable statistical sampling of the Gag lattice during the lifetime of assembly and achieve higher angular distributions on budding particles. We feel
that this scenario is not plausible, however, because it does not explain the potent particle incorporation defect observed with CTΔ144-Env, despite higher cell surface densities. In turn, the increased mobile fraction of CTΔ144-Env cannot be explained by saturation of the finite number of Gag lattices on the cell surface because previous studies have shown that CTΔ144-Env incorporation is nonsaturable over a wide range of surface densities, unlike WT-Env\(^{16}\). We suggest instead that increased mobility through the Gag lattice reduces the likelihood of Env being present as a particle is absorbed from the cell. Collectively, our data support previous interpretations of the role of the Env-CT in regulating Gag lattice incorporation and, through direct visualization and quantification, enables a nanoscale perspective of the encounter between these two viral molecules.

Our investigation into the nanoscale spatiotemporal dynamics of Env assembly suggested that additional mechanisms beyond lattice trapping and plasma membrane diffusion act to regulate the temporal incoherence of Env encounter with the Gag lattice. Specifically, single-molecule tracking could not explain the differences in angular distributions of WT-Env produced in CEM-A versus COS7 cells. We hypothesized that endocytosis and intracellular retention of Env trimers could be utilized by HIV-1 to regulate the timing of Env and Gag encounter in CEM-A cells. We demonstrate that the steady-state levels of intracellular WT-Env were much higher than CTΔ144-Env in CEM-A cells, while intracellular levels of WT- and CTΔ144-Env were very similar in COS7 cells, and surface levels of WT-Env were even slightly higher than CTΔ144-Env. These results suggest that CEM-A cells possess host cell trafficking machinery responsible for interaction with the Env-CT, which leads to intracellular retention in a transferrin-positive compartment (Fig. 4a and Supplementary Fig. 18a), whereas COS7 cells are altered in this trafficking machinery and thus, never significantly sequester a large fraction of intracellular Env. This significantly reduced intracellular retention in COS7 cells, by failing to sequester Env during early Gag lattice assembly, explains the unbiased angular distribution of WT-Env observed on particles produced by COS7 cells, as well as the higher density of Env incorporation per particle (Fig. 6).

Unlike CTΔ144-Env, the d8-Env mutation does not appear to impede specific internalization of Env, and its cell surface density is depleted compared to WT-Env in both cell types. The relatively small percentage of confined/immobilized d8-Env, combined with the high levels of intracellularly retained d8-Env compared with WT-Env, suggests that this Env mutant does not become trapped by Gag lattices and is then largely endocytosed and trafficked to an intracellular compartment. It is possible, however, that d8-Env trimers are still unable to recycle to the plasma membrane upon internalization, which would exacerbate particle incorporation defects and increase the steady-state levels of intracellular d8-Env relative to WT-Env. The loss of cell-type-dependent differences in d8-Env incorporation (Fig. 6b) seems to support a cell-type-independent occlusion of d8-Env from the Gag lattice, irrespective of the apparently cell-type-dependent intracellular retention of WT-Env. When the intracellular pool of d8-Env is photobleached and allowed to recover, a larger fraction of d8-Env recovers relative to WT-Env (63.6 ± 1.1% versus 44.6 ± 3.4%, respectively) despite having a smaller plasma membrane pool (Fig. 4), suggesting that the higher mobile fraction of d8-Env trimers, relative to WT-Env, on the plasma membrane may contribute to this recovery. This result could also be explained by a defect in recycling of d8-Env trimers. It is important to note that these data do not differentiate between flux due to endocytosis and flux due to exocytosis, and thus cannot conclusively prove a defect in d8-Env recycling to the plasma membrane, but these results are consistent with previous studies of related Env-CT mutants\(^{14,27}\). This mechanism of exaggerated intracellular retention would explain our observation of an induced neck bias for d8-Env distribution in COS7 cells and is analogous to the intracellular retention of WT-Env and neck bias we observe in CEM-A cells. Further study is warranted to define the exact host cell machinery responsible for intracellular retention of Env and
...to conclusively determine whether it is specifically impaired by the d8 mutation. The discovery that the host cell vesicular recycling protein, FIP1C, is responsible for regulating Env particle incorporation 27, 28 suggests that this factor may regulate intracellular retention and temporal modulation of the encounter between Env and Gag. As there are numerous steps in vesicular trafficking and endosomal cargo recycling, it is possible that other cell-type-specific machinery exists to regulate Env intracellular retention.

Collectively, this study supports a model wherein rapid Env-C-CT-dependent internalization and sequestration of Env in endocytic compartments, followed by limited recycling of Env back to the plasma membrane, restricts extracellular display and delays the encounter of Env with budding Gag lattices until late in the assembly process. This mechanism sterically consigns the long Env-C-CT to the peripheries of Gag lattices and the necks of budding particles, limiting the density of Env incorporation to individual virions (Fig. 7). Importantly, this trafficking itinerary for Env trimers appears to be cell-type-specific, as angular Env distributions were unchanged when expressed in fibroblast-like cells. We propose that the combination of host cell trafficking factors and Gag lattice complementarity are crucial for maintaining steady-state distributions between internally retained and virus-associated Env. These findings have significant implications for understanding the mechanisms of HIV-1 immune evasion and point to a tightly regulated assembly pathway limiting cell surface exposure of HIV-1 Env. While limiting the incorporation of Env trimers into virus particles reduces viral infectivity 28, this mechanism potentially contributes to the lack of antibody diversity generated by an infected host and reduced intensity of antibody-dependent cell-mediated cytotoxicity during HIV-1 infection 29.

Methods

Antibodies. Anti-Env antibodies b12 30–33 and 2G12 34, 35 were acquired from the NIH AIDS Reagent Program (Germantown, MD) or purchased from Polymun (Klosterneuburg, Austria), respectively (the b12 reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: anti-HIV-1 gp120 monoclonal (lgG1 b12) from Dr. Dennis Burton and Dr. Carlos Barbas). Anti-FLAG clone M2 antibody was purchased from Sigma-Aldrich (#F3165). Anti-capsid (Gag-CA) KC57 antibody was purchased from Beckman Coulter (Brea, CA). All anti-Env (b12 and 2G12) antibodies were coupled directly to AlexaFluor 647 N- succinimidyl ester (#A37566; Life Technologies). The anti-FLAG M2 and anti-CA KC57 antibodies were coupled directly to AlexaFluor750 N-succinimidyl ester (#A20111; Life Technologies). Typically, a labeling ratio 1.8:2.8 AlexaFluor dyes per IgG Fab Fab molecule was achieved. Anti-Env 2G12 antibody was used as primary and goat anti-human IgG with 6 nm Au (Electron Microscopy Sciences, Hatfield, PA) was used for immunogold labeling.

Recombinant antibody fragment production. The anti-Env b12 Fab fragment recombinant expression vector was a kind gift from Dr. Dennis Burton 36. The pCOMB3H-b12 expression vector was deleted for the pilI gene by digestion with Spel and Nhel (New England Biolabs, Ipswich, MA) and ligation of the vector backbone. Expression of b12 was carried out in Escherichia coli XL1 Blue competent cells (Strategene; San Diego, CA) as previously described 36. To purify b12, bacterial cell pellets were resuspended in PBS pH 7.4 containing a final concentration of 0.2 mM PMSF (#P-470-10; Gold Biotechnology, Inc.; St. Louis, MO) and sonicated to produce cellular lysate. Clarified cell lysates were purified by protein G affinity chromatography (#P-430; Gold Biotechnology, Inc.; St. Louis, MO). Eluates were pooled and dialyzed overnight in PBS, pH 7.4 at 4 °C. The b12 Fab, typically 99% pure, was conjugated with Atto565 N-succinimidyl ester (#72464; Sigma-Aldrich; St. Louis, MO). Typically, a labeling ratio of 1 Atto565 dye per b12 Fab molecule was achieved. This monovalent reagent was capable of labeling HIV-1 Env expressing cells without the concern of receptor crosslinking, unlike bivalency crosslinking in the case of full IgG molecules.

Cell lines. The COS7 parental cell line was obtained from ATCC (CRL-1651; Manassas, VA). The HEK293T cell line was also obtained from ATCC (CCL-1326). The CEM-A cell line was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: CEM-A from Dr. Mark Wainberg and Dr. James McMahon, CEM-CL1017. Cells were maintained at 37 °C with 5% CO2.

Growth medium. Complete growth medium was prepared with 10% fetal bovine serum (#35-011-CV), 2 mM l-glutamine (#25-005-CI), and 1% penicillin-streptomycin (#300-002-CI) in RPMI (#170-1525) or in RPMI (#170-1505) for the CEM-A cell line (Corning; Herndon, VA). The CEM-A cell line growth medium was additionally supplemented with 1% hypoxanthine, thymidine (HT) solution (25-047-CI; Corning).

Production of replication and release defective HIV-1. The modified NL-4–3 recombinant strain of HIV-1 env was cloned into an SV40 ori-containing backbone (pP1 vector; Clontech/Takara Bio USA, Mountain View, CA) and used as a transfer plasmid. Briefly, the following modifications were made to the NL-4–3 reference genome for the iPALM experimental imaging approach: (i) addition of a coding C-terminal FLAG tag to the gag open reading frame (GDSPSSSSSSGKDYDDDKK*; replacing Env Gag in the manuscript); 32 (ii) mutation of the p6 PTAP motif (acp6PTAPacp6-LIRL); 22 (iii) deletion of p6 by removal of the Bcl-Nal restriction site in this open reading frame, and (iv) removal of the S′ portion of the nef open reading frame and replacement with a probe containing the photoswitchable cyan fluorophore protein 2 (PSCFP2) open reading frame fused to a myristoylation coding sequence 32. Mutational modifications to the Env cytoplasmic tail were created on the aforementioned genetic background: WT– wild-type env, CtI del144 – ACT removal of the last 144 amino acids of the Env cytoplasmic tail by premature stop codon, 33 or – d8 - deletion of 5 amino acids (802-806, numbering references NL4-3 gp160) from the second predicted alpha helical domain of the Env-C-CT, LPP-34. Single-round infectious viruses were produced by transfecting HEK293T cells with the pPAX2 packaging vector (a gift from Didier Trono, Addgene plasmid #12260) and pseudotyped with pVSV-G (gift from Dr. Xuedong Liu, University of Colorado, Boulder). HEK293T producer cells were allowed to express for ~48 h before virus was collected, passed through a 0.45 μm filter, and frozen at ~80 °C.

Production of infectious viruses and biochemical analysis. Infectious viruses were produced by transfecting HEK293T cells with pNL4-3 WT, CtI del144-, or d8-Env and pseudotyped with pVSV-G. HEK293T producer cells were allowed to express for ~48 h before virus was collected, passed through a 0.45 μm filter, and frozen at ~80 °C. To quantitatively assess Env incorporation and particle production biochemically (Fig. 6 and Supplementary Fig. 16), single-round infection was performed on COS7 and CEM-A cells using ~1.5 – 4 RT-CPM/µL of virus. Approximately 40 h post infection, virus was collected, passed through a 0.45 μm filter, layered on a 20% (w/v) sucrose cushion, and concentrated at 20,000 × g for 2 h at 4 °C. Env gp41 and gp160 were detected with anti-Env 10E8 37 at 0.5 mg per ml, anti-Env 16H, 0.2 mg per ml, and p24(AA) and Pr55Gag were detected with HIV-IgG at 10 mg per ml (the following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH; anti-HIV-1 gp41 monoclonal (10E8), from Dr. Mark Conners and anti-HIV-1 Env 16H3 mono- clonal antibody from Drs Barton F. Haynes and Hua-Xin Liao 38). Secondary detection of anti-HIV antibodies was performed with anti-mouse HRP (16H3) and sheep anti-human HRP (HIV-IgG and 10E8). Full western blots are shown in Supplementary Fig. 19.

Interferometric photoactivation localization microscopy. Instrumentation associated with the iPALM method is described by Shtengel et al. 21, but with the z-axis measurement range extended to 750 nm with phase unwrapping using astigmatism 39. Images were collected in frame transfer mode using three iXon DU 897W EM-CCD cameras (Andor Technology, Belfast, Northern Ireland). An additional 300 mW 750 nm fiber-coupled diode laser (Edmund Optics; Barrington, NJ) with custom collimation optics was added to the microscope setup for imaging of AlexaFluor750 (Life Technologies, Carlsbad, CA). The 750 nm laser line was filtered with a 740/35 band-pass filter (Chroma Technology; Bellows Falls, VT), and the blue excitation laser at 488 nm was filtered through a 795/50 emission filter (Chroma Technology; Bellows Falls, VT). Filters used for the 488 nm and 647 nm channels were as described previously 22.

Transmission electron microscopy. Ultra-thin sections (60 nm) were cut on a Reichert Ultracut S from a small trapezoid positioned over the tissue and were picked up on copper grids (EMS). Sections were imaged on a FEI Tecnai G2 transmission electron microscope (Hillsboro, OR) with an AMT digital camera (Woburn, MA).

Spinning disk confocal microscopy. Imaging was performed with a customized inverted Nikon Ti-E microscope (Nikon Instruments Inc., Melville, NY). The three fiber-coupled 488 nm, 561 nm, and 640 nm lasers (OLIS CW solid-state lasers, Coherent; Santa Clara, CA) were used in combination with a CSU-X A1 spinning disk unit (Yokogawa Electronics; Tokyo, Japan) to excite and collect confocal sections of AlexaFluor488, Atto565, or AlexaFluor 647 fluorescence, respectively. Laser powers were measured at the sample stage. For FRAP experiments, the 561 nm laser was operated at 40 ± 5 mW cm–2. For fixed cell experiments (see surface stain and internalization assays), the 561 nm laser was operated at ~20 ± 5 μW cm–2. The 488 nm laser was...
measured at ~12 ± 2 µl·cm⁻²·s⁻¹, and the 640 nm laser was measured at ~315 ± 5 µl·cm⁻²·s⁻¹. A quad-band dichroic mirror (Chroma Technology) was used to reflect the excitation light, which was then transmitted through two 20× water immersion objective lenses and filtered using a 405 nm band-pass (ET525/50 m), 570 long-pass (ET570lp) emission filter for AlexaFluor488 and Atto565 imaging, respectively (Chroma Technology). For imaging of AlexFluor647 in separate experiments, a 700/45 band-pass filter was used (T660lp; Chroma Technology). Fluorescence emission was detected using an EM-CCD electronic multiplying charged-coupled device (EM-CCD, C9100-23B; Hamamatsu, Hamamatsu City, Japan). Rapid point photobleaching was performed on specimens using a rear-port coupled iLas laser illuminator (BioVision Technologies, Exton, PA) coupled with a 100 mW 405 nm OBIS laser (Coherent; Santa Clara, CA). A 405 nm reflecting dichroic and long-pass transmitting broadband dichroic mirror was used for laser coupling into the rear-aperture of the objective (ZT405rdc; Chroma Technology).

**Cell preparation for interferometric-PALM measurements.** Bare 25 × 45 mm Au nanorods (#A125-2700, Nanopartz, Loveland, CO) were used as fiducial markers, and were deposited onto 25 mm #1 coverslips (Warner Instruments, Hamden, CT). A 20–50 nm layer of SiO₂ was then sputtered over the gold-bearing coverslips (Denton Vacuum, Moorestown, NJ). Coverslips were cleaned for single-molecule imaging as described. After air drying, coverslips were placed in 3.5 cm culture dishes and coated with fibronectin in PBS (100 μg/ml; Millipore, Billerica, MA). COS7 or CEM-A cells were seeded onto Au-, SiO₂-treated coverslips with ~2 × 10⁴ cells in complete growth medium. Cells were typically imaged 1–2 h after plating. Pseudovirions VSV-G pseudotyping with a full-length HIV-1-LTR. An 8×10⁴ cells in complete growth medium. Cells were then pelleted and resuspended in complete media containing 0.2% BSA and then labeled with goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA) coupled with a 100 mW 405 nm OBIS laser (Coherent; Santa Clara, CA). A 405 nm reflecting dichroic and long-pass transmitting broadband dichroic mirror was used for laser coupling into the rear-aperture of the objective (ZT405rdc; Chroma Technology).

**Immunogold labeling and transmission electron microscopy.** CEM-A cells were infected with attenuated single-round virus for the appropriate NL4–3 genotype and allowed to express viral proteins for ~40 h, as described above. Cells were fixed with 4% paraformaldehyde for 15 min, then pelleted and resuspended in 1% glutaraldehyde (#16220) for 30 min (Electron Microscopy Science). The fixed cell suspension was then pelleted and resuspended four times in PBS containing 0.2% BSA. After the final wash, cells were pelleted and post fixed in 2% glutaraldehyde/PBS. The pellets were resuspended in 5% agarose that was allowed to harden and then cut into small pieces for processing. Cells were then fixed three times in 0.1 M sodium cacodylate buffer (pH 7.4), then post fixed in 2% osmium tetroxide for 1.5 h. After three washes in distilled water, the cells were dehydrated through a graded acetone series (3× 25%, 50%, 75%, 90%, 95%, 100%) for 10 min each, and infiltrated with LX112 resin. The cells were embedded and cured for 48 h at 60°C in an oven. Ultra-sectioned samples were cut as described above (60 nm) and imaged on copper mesh grids.

**Immunogold labeling and transmission electron microscopy.** CEM-A cells were infected with attenuated single-round virus for the appropriate NL4–3 genotype and allowed to express viral proteins for ~40 h, as described above. Cells were stained with antibodies to Gag and were fixed in 3% glutaraldehyde and 2% formaldehyde. After three washes in water, the cells were dehydrated through a graded acetone series (3× 25%, 50%, 75%, 90%, 95%, 100%) for 10 min each, and infiltrated with LX112 resin. The cells were embedded and cured for 48 h at 60°C in an oven. Ultra-sectioned samples were cut as described above (60 nm) and imaged on copper mesh grids.

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Supplementary Fig 15e).

Agreement with estimated neck lengths observed by thin-section transmission
assembly sites whose centroids resided at a distance larger than 300 nm from the
were performed as previously described. Typical regions of interest were
carried (typically <1.25 x
planes, representing early stages of Gag-induced membrane curvature, were dis-
lattices of Gag, prior to induction of membrane curvature, were discarded in early
fringe were discarded from the segmented volumes. All cell type and genotype
density were reassigned to the real cluster of localizations. All segmented
volume, a custom 3D angular averaging algorithm was used. Briefer, the
channels (750, Gag, Env; and 488, membrane) along the entire Z-depth.
Each HIV Gag cluster was then inspected for collisions with other HIV-1 Gag
clusters, circularity, presence of Env, and definability of the local membrane
topology. The Gag localizations describing the Gag volume were discarded.
Virus assembly sites which lacked sufficient local membrane sampling (<50 localizations
per segmented area) were filtered from the total pool in each cell and virus gen-
type. This discarded pool typically consisted of less than 5% of the segmented
data.

Rotational alignment by local membrane plane fitting. To enable angular
probability density measurements of Env distributions on averaged single HIV-1
assembly sites, we performed weighted principle component analysis (PCA) using
the Matlab function: pca with the default singular value decomposition algorithm
option and centration of the data. The localization uncertainty in all three
dimensions, \( \sigma_{\text{localization}(x,y,z)} \), for single virus assembly sites was averaged and nor-
malized to one. The normalized error was then used as weights for the PCA
function. The normal vector for the plane was extracted from the principle compo-
ent coefficients and the residual error was determined from computing the
real-space distances of each localization centroid to the fit plane. These values were
typically below 5 nm for each dimension. The angular deviations of the computed
normal vectors were used to perform a rigid affine transform of the aligned Gag
shell (AF750 channel), the corresponding Env signal (AF647 channel) and
the corresponding membrane plane (PSCFP2 channel). All channels were aligned to
the real-space optical axis (Z) using the weighted PCA estimated normal vectors
representing the virus bud polarity.

Viral bud neck normalization. We observed a normally distributed range of neck
lengths for each viral bud, estimated from the centroid of the Gag shell and the
centroid of the membrane plane during alignment with the optical axis (150 ± 92 nm;
see Supplementary Fig. 6). Those Gag centroids residing too close to the membrane
planes, representing early stages of Gag-induced membrane curvature, were dis-
carried (typically <1.25 x
length). Typically, however, the flat
lattices of Gag, prior to induction of membrane curvature, were discarded in early
stages of processing due to poor fitting to the Gag shell model. Those Gag
assembly sites whose centroids resided at a distance larger than 300 nm from the
membrane centroid, likely representing sheared or shed particles, were observed
infrequently and represented less than 6% of all assembly site measurements.
Importantly, the distribution of neck lengths we observed were in good
agreement with estimated neck lengths observed by thin-section transmission
electron microscopy (127 ± 38 nm from the proximal membrane plane;
Supplementary Fig 15e).

For purposes of visualizing the Gag and Env probability densities (see Fig. 2a
and Supplementary Fig. 13), we performed distance normalization based on the
mean neck length measured in each data set. Importantly, normalization of the
neck lengths was only performed for visualization purposes. However, the absolute
axial distance of the membrane plane from the Gag centroid has no effect on
determination of the fitted plane normal vectors, and thus, will not affect the
aligned angular probability of Env about the Gag lattice.

Single cluster averaging. For translationally and rotationally aligned single virus
assembly sites, the centroid coordinates and localization uncertainties for each
channel (Gag, Env, and membrane) were converted into 3D probability
densities using 1 nm voxels. This volumization algorithm was parallelized
using CPU multithreading and the Matlab parallel-processing toolbox.
The pre-aligned probability volumes were then integrated to calculate the total
probability density over each genotype or cell-type per data set and per volume site.
The final probability densities were then trimmed into cubes of 601 x 601 x 601 nm
with the center voxel (at 301 x 301 x 301 nm) representing the centroid of the Gag
shell.

Probability density analysis. Integrated probability densities for the aligned
HIV-1 Env channel were used to determine the angular probability distributions
for each Env genotype and for each cell type. Using the center voxel for each
data set, a custom 3D angular averaging algorithm was used. Briefer, the
algorithm integrates along a conical azimuthal angular interval: the angle \( \phi \), on the interval \(-90°:1°:90°\), where the elevation angle from \(-90°\) to \(0°\)
represents the southern hemisphere (neck) of the volume and \(0°:90°\) represents the
northern hemisphere (crown) of the volume. The probability density for each
elevation angle was integrated along the length of a vector originating at the center
voxel and having a fixed length of 20 nm. The directional probability density is then
integrated about the polar angle, \( \theta \), to compute the probability density of the cone described for each azimuthal angle, \( \phi \).

In order to determine the angular resolution for the angle \( \theta \) using this method
(and thus the appropriate bin size for the angular histograms), we generated
randomized half data sets for each Env genotype and the producing cell type,
computed the angular \( \theta \) probability of Env about the Gag shell for half data
set, and then phase correlated the one-dimensional angular probability vectors
of the two half data sets (see Supplementary Fig. 12). Briefer, the coordinates for
individual virus assembly sites were randomly divided in half. The half data sets
were then aligned, rotated, and computed two-dimensional density volumes for each genotype or cell type. The half data set volumes for each data set
were then passed to the 3D averaging routine described in the previous paragraph.
These two resulting integrated angular \( \phi \) probability vectors were then cross-
correlated using the Matlab function xcorr. The lag at which correlation was
maximum between the half data set vectors was used as a measure of the sampling
density for the full data set. The lags were never larger than 20° with an average of
9° between any random half data set for all Env genotypes and producing cell types
tested. This result indicates sufficient sampling of Env at HIV-1 assembly sites to
conclude fine spatial differences in probability density under specific biological
perturbations (genotypic or cell-type changes).

Visualization of probability densities. The 3D matrices of aligned and
averaged virus assembly sites were insouresed using the indicated thresholding
algorithm (see individual figures) using Matlab. Insouresed probability densities were insouped on the sliced open face of the shell using an identical threshold value. To enable
direct comparison for three-channel probability densities, each subfigure was
rendered using an identical insoussure and isoscap threshold for the respective
channels.

Simulation of angularly biased Env clusters. We developed a particle simulation
algorithm, which utilized seed parameters closely matching the geometric para-
meters of HIV-1 assembly sites, localization densities, estimated re-appearance
(blinking) parameters, and localization uncertainty values for AF647-labeled Env,
AF750-labeled Gag, and the S15-PSCFP2 membrane probe.

Monte Carlo simulation of errors in angular measurement. To assess the overall
accuracy measurement of the angular probability distribution of Env from aligned
multicolor 3D superresolution imaging data sets, we simulated the Gag shell, local
membrane plane, and Env localization centroids residing precisely at the equator
(\( \theta = 0° \)). The Env centroid and localization uncertainty were modeled as a Dirac \( \delta \)
function \( \rho_{\text{localization}} \) to fit the effects of both Gag shell fitting and uncertainties in
the principle component analysis of the approximated membrane plane. The
localization precisions in the x, y, and z dimension for all simulated channels were
selected pseudorandomly from a normal distribution centered at the estimated
mean localization precisions measured for each fluorescent probe averaged over all
data sets (Supplementary Fig. 1). We additionally simulated the small registration
total kinetic cycle time per frame for each experiment ranged from 17 to 19 ms and
the camera was operated in frame transfer mode.

Superresolution image analysis. InPALM superresolution images were generated
as previously described. Raw localizations in each data set were filtered by
positional uncertainty (\( \sigma_{\text{localization}(x,y,z)} \)) to less than 40 nm in the X, Y-dimensions and 30 nm in the Z-dimension (Supplementary Fig. 1) and all three channels were aligned by
transform of broadly fluorescent gold fiducial markers (Supplementary Fig. 2).
Segmentation of individual clusters of localization in the Gag and Env channel
were performed as previously described. Typical regions of interest were
–1.5–2.5x the 2D area of each HIV-1 Gag cluster depending on the HIV-1 Gag
density in the cell. A custom written Matlab program (Mathworks, Natick, MA) then extracted the segmented localizations coordinates and uncertainties for all
three channels (750, Gag, Env; and 488, membrane) along the entire Z-depth.
Each HIV Gag cluster was then inspected for collisions with other HIV-1 Gag
clusters, circularity, presence of Env, and definability of the local membrane
topology. The Gag localizations describing the Gag volume were discarded.
Virus assembly sites which lacked sufficient local membrane sampling (<50 localizations
per segmented area) were filtered from the total pool in each cell and virus gen-
type. This discarded pool typically consisted of less than 5% of the segmented
data.

Filtering assembly sites. Those segmented virus assembly sites which performed
poorly in the Gag centroid and shell fitting algorithm due to a lack of single
molecule localization per cell were discarded. Those assembly sites which failed
all filtering criteria were then converted to probability

Visualization of probability densities. The 3D matrices of aligned and
averaged virus assembly sites were insouresed using the indicated thresholding
algorithm (see individual figures) using Matlab. Insouresed probability densities were insouped on the sliced open face of the shell using an identical threshold value. To enable
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localization precisions in the x, y, and z dimension for all simulated channels were
selected pseudorandomly from a normal distribution centered at the estimated
mean localization precisions measured for each fluorescent probe averaged over all
data sets (Supplementary Fig. 1). We additionally simulated the small registration
total kinetic cycle time per frame for each experiment ranged from 17 to 19 ms and
the camera was operated in frame transfer mode.
The mean $\Delta$centroid,$\sigma_{\text{distribution}}$(x,y,z) averaged over all data sets ($\sigma_{\text{distribution}}$):

\[
\Delta_{\text{centroid}}(x,y,z) = \left( \sigma_{\text{blue,green}}^2 + \sigma_{\text{green}}^2 \right)^{1/2}
\]

(1)

To simulate the uncertainty in the fit radius, localizations for the red Gag channel were first expanded pseudorandomly on a sphere with a radius estimated from our measured mean cluster radius, in good agreement with the actual shell radius of HIV-1 Gag. All Gag localizations were then pseudorandomly displaced from the sphere surface using a lognormal distribution estimated from the mean standard error measurement for the Gag shell fit. The number of Gag localizations per Gag cluster was pseudorandomly selected from the mean and standard deviation of the empirically derived number of Gag localizations per particle (mean = 147.65 ± 6.90 localizations per cluster; $n = 2183$ clusters). To simulate the uncertainty in the principle component analysis of the membrane plane probability densities, we permuted each of the individual membrane planes as Gaussian clouds with $\sigma_{x,y}$ estimated from the empirically measured mean of the standard deviations of localization positions for all individual single HIV-1 assembly sites in this study (mean $\sigma_{x,y}$ = 76.91 nm and $\sigma_z$ = 29.72 nm; see Supplementary Fig. 5). The randomized localization centroids were bounded in the X- and Y-dimensions at an area of $4.0 \times 10^{-6}$ mm$^2$ ($200 \times 200$ nm) to simulate the boxcar segmentation of individual assembly sites adjacent to one another. We used the empirically measured mean localizations per plane (527 ± 11 localizations; see; $n = 1980$ planes) for pseudorandomly seeding localizations into each plane. Env localizations and membrane plane point clouds were affine transformed about the Gag cluster center pseudorandomly to simulate alterations in the virus bud polarity axis relative to the microscopic reference axis. Membrane planes and the single Env localization were displaced pseudorandomly from the Gag centroid, along the simulated budding axis, to mimic the lognormal distribution of bud neck lengths observed with empirically derived data (Supplementary Fig. 6; 127 ± 38 nm from the centroid of the membrane plane probability density of the Gag cluster). Simulated single HIV-1 assembly sites with a fixed Env angular orientation (Dirac function), which were pseudorandomly modified to incorporate the stochastic parameters described above, were then processed (identical to empirically derived data) using our particle processing software.

Additional synthetic data sets were constrained for the placement of single Env clusters at defined angular intervals ($\phi$) to mimic the occurrence of Env angular distribution bias about the budding Gag particle. Data sets biasing Env to the crown of the particle ($\phi = 0$ – $90^\circ$), the neck of the particle ($\phi = 90$ – $0^\circ$), or a random distribution about the particle ($\phi = -90$ – $90^\circ$) were generated. The probability density was used to single virus averaging faithfully recapitulates the angular bias constraints from each data set as assessed by integrated angular densities (see Supplementary Fig. 7).

Individual envelope cluster measurements. As an orthogonal measure of the angular distributions of Env clusters measured by iPALM for different Env genotypes or viruses-producing cell types, a 3D cluster segmentation algorithm was designed to investigate the variability of Env cluster density distributions in single HIV-1 assembly sites (Env clusters).

Briefly, from each data set, the Env of localizations from a single segmented HIV-1 assembly site was converted into a 3D probability density. This routine, as described above, was parallelized to enable facile throughput for probability density calculations of hundreds of Env clusters using CPU multi-threading and the Matlab parallel-processing toolbox. A template matching 3D convolution algorithm was implemented in the Fourier domain to find Env clusters. Manual inspection of over 400 segmented Env clusters, which contained a high density of Env trimer signals were normally distributed and clustered with a tight (less than $\sigma_{90^\circ}$) distribution of individual Env clusters referenced from aligned membrane plane centroids and Gag cluster centroids.

Total iPALM error assessment. The propagation of independent error arising from three-channel iPALM measurements and data processing are treated in Eq. 2 below:

\[
\sigma_{\text{total}} = \sigma_{\text{blue,green}} + \sigma_{\text{green}} + \sigma_{\text{red}} + \sigma_{\text{translational}} + \sigma_{\text{rotation}}
\]

(2)

The sample drift (X, Y, and Z) typically ranged from 5 to 50 nm for a given experiment. The residual X, Y, and Z-uncertainty after drift correction ranged from $\sigma_{\text{translational}} = 5$ to $\sigma_{\text{translational}} = 20$ nm. Two independent random sets of localizations were evaluated in this analysis. The residual uncertainty of 3–8 nm ($\sigma_{\text{rotation}}$) in X-, Y-, and Z-dimensions depending on the density and quality of Au nanoparticles present in the field of view. Using the described iPALM method, localization uncertainties for all single-molecule detection events followed a lognormal distribution. The mean of the localization uncertainty for the AlexaFluor 750 probe ($\sigma_{\text{translational}}$) was typically around 10–15 nm in the X- and Y-dimensions and 5–12 nm in the Z-dimension. The mean of the localization uncertainty distribution for the AlexaFluor 647 probe ($\sigma_{\text{translational}}$) was typically around 5–10 nm in the X- and Y-dimensions and 3–8 nm in the Z-dimension. Mean localization uncertainties for the PSCFP2 probe ($\sigma_{\text{translational}}$) ranged from 10 to 20 nm in X- and Y-dimensions and 10–15 nm in the Z-dimension. The residual translational uncertainty of alignment between the centroids of single HIV Gag clusters ($\sigma_{\text{translational}}$) was no greater than 5–15 nm. The residual tilt uncertainty of alignment between the local plasma membrane planes ($\sigma_{\text{translational}}$) was typically no greater than 3° as assessed by phase correlation lags of random half-sheets of membrane plane probability densities (see Supplementary Fig. 11) or 4.39 ± 0.12° from Monte Carlo simulations (see Supplementary Fig. 10). Combined with the standard error estimated from weighted principal component analysis, we computed the Euclidean space error in rotationally aligned data sets to be about 10 nm in plane tilt uncertainty.

It is important to note that the real-space sampling density (Nyquist) for Env clusters is a convolution of the true Env density per particle (biologically sparse; 7–14 trimers per particle) and the number of particles averaged. Cross-correlation analysis of Env angular distributions (see Probability density analysis section above) suggested that sufficient sampling was present to distinguish surface density distributions in, at minimum, 20° intervals (largest cross-correlation lag in one data set). This is sufficient to conclude that Euclidean space shifts in Env angular probability densities between hemispheres are derived from biological changes in Env trimmer distributions and therefore we do not consider the sampling resolution in this error assessment. Finally, the native Gag density per particle (3000–5000 molecules) yields sufficient sampling upon cluster averaging (typically up to 1–2 localizations per nm$^2$; see ref.27) to render the sampling criteria negligible when compared with other errors in the system.

Collectively, under these data acquisition conditions, the localization uncertainties for the X- and Y-dimensions dominate the propagated errors. Specifically, those for the PSCFP2 fluorescent probe provides the largest uncertainty. The PSCFP2 probe was only used for calculation of the normal membrane plane vector, however, which is a parameter we found to be less sensitive to X- and Y-positional uncertainties because these errors were also affinely transformed using the estimated normal vector for plane alignment. This Euclidean space error transformation served to lower the overall localization error by averaging $\sigma_{\text{translational},X,Y}$ with the much smaller interferometric $\sigma_{\text{translational},Z}$. The total error measurement, $\sigma_{\text{total}}$ = 33.5 nm, is an estimate of the resolution of the system for the three-channel iPALM measurements.

Single-molecule tracking analysis. Sparse labeling of HIV-1 Env on the surface of cellular specimens presented as well-resolved diffusion-limited spots, which displayed the expected single-step photobleaching characteristics of quantized fluorophore bleaching (average degree of labeling was 1 Atto565 dye per Fab b12). Detection of single-molecule centroids and residual uncertainty was computed identically to the protocol described in the iPALM method. Localization position estimates over the frame interval were then linked using modified scripts from the tracking algorithms found in the Matlab package uTrack$^{27}$. The mean localization uncertainty for these experimental conditions was 14 nm, but the uncertainty of each localization was utilized on a per-track basis for linking localizations. Only tracks with greater than 20 positions were used to compute the short-range diffusion coefficient for each track ($D = <4 \times 10^{-10} \text{ m}^2/\text{s}$). Tracks were classified as mobile or confined/immobile based on the uTrack classification system. Briefly, uTrack classifications were assigned using moment orders of 0 through 6, and a MSS analysis algorithm as described previously$^{24,25}$. Tracks were conservatively grouped as immobile if uTrack classification assigned either 0 (immobile) or 1 (trapped) to the track. Tracks that were consistently marked "pure Brownian" motion were grouped as mobile for the purposes of our analysis.
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**Author contributions**

C.B. and S.B.V. created superresolution constructs. C.B., J.A., and S.B.V. performed iPALM. C.B. and S.B.V. wrote the 3D analysis software package for particle averaging and error analysis. N.P. and S.B.V. created single-particle tracking probes and C.B., and S.B.V. performed single-particle tracking experiments. S.N. created expression constructs for both iPALM and single-particle tracking. M.V.F. performed

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**Code availability** Custom MATLAB code is available from the authors on request, in total or in modules.

**Data availability** All data supporting the findings of this study are available within the article and its Supplementary Information files, or are available from the authors on request.

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biochemical analysis of virus production. C.B., N.P., M.V.F., S.B.V., and E.F. wrote the manuscript. All authors discussed results and commented on the manuscript.

**Additional information**

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