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Distribution of ESCRT machinery at HIV assembly sites reveals virus scaffolding of ESCRT subunits

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Abstract

The human immunodeficiency virus (HIV) hijacks the endosomal sorting complexes required for transport (ESCRT) to mediate virus release from infected cells. The nanoscale organization of ESCRT machinery necessary for mediating viral abscission is unclear. Here, we apply three-dimensional superresolution microscopy and correlative electron microscopy to delineate the organization of ESCRT components at HIV assembly sites. We observe ESCRT subunits localize within the head of budding virions and released particles, with head-localized levels of CHMP2A decreasing relative to Tsg101 and CHMP4B upon virus abscission. Thus, the driving force for HIV release may derive from initial scaffolding of ESCRT subunits within the viral bud interior followed by plasma membrane association and selective remodeling of ESCRT subunits.

One of the key host factors human immunodeficiency virus (HIV) relies on to complete its infection cycle is the endosomal sorting complexes required for transport (ESCRT). By recruiting this machinery, HIV is able to mediate the final step of virus particle fission from the membrane. Otherwise, virus egress is severely inhibited (1–5). Despite great progress in demonstrating the ESCRT machinery's role in mediating HIV abscission, the nanoscale organization, and thus function, of ESCRT subcomplexes at native HIV assembly sites remains poorly defined.

ESCRT machinery participates in important cellular membrane remodeling events, such as cytokinesis and multivesicular body biogenesis (MVB), organizing into three general sets of

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Supplementary Materials

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Materials and Methods

Figs. S1–S10

References (22–33)

subcomplexes. One set includes ESCRT-0, -I, and -II, which interact directly with cargo or scaffold proteins to direct assembly of downstream ESCRT complexes. Another set includes ESCRT-III subunits, which are thought to polymerize on membranes into a helical architecture to constrict and abscise membrane buds formed by early ESCRT/cargo complexes (6, 7). The final set consists of the AAA+ ATPases Vps4A/B, which are thought to be necessary for HIV abscission through ESCRT-III filament remodeling and or recycling (7–9). Together, the three sets of ESCRT subcomplexes cooperate to drive cellular membrane remodeling. Structural information obtained by in vitro assembly (7, 10) and cellular overexpression studies (11) suggest a model of bud formation and abscission where ESCRT-III filaments encircle and constrict the aperture of a membrane protrusion, acting on the base of the neck, in trans from the protrusion head. On the other hand, when directed by the midbody scaffold during cytokinesis (12, 13), ESCRT-III filaments appear to polymerize from the scaffold and constrict the membrane, acting in cis with respect to the scaffolding structure. Whether ESCRT-III filaments polymerize at HIV bud sites in cis or trans to the scaffolding structure is not known because of the small dimensions of a budding HIV virion (120–140 nm diameter). Consequently, it remains unclear how ESCRT machinery acts to mediate viral membrane abscission and propagate HIV infection.

Here, we used interferometric photoactivated-localization microscopy (iPALM) (14) to decipher the three-dimensional (3D) nanoscale organization of ESCRT components at HIV assembly sites, and thereby gain insight into the mechanism for viral membrane abscission (see supplementary online text). ESCRT subcomplexes were selected for our analysis based on their direct interaction with the structural HIV protein Gag (ESCRT-I subunit tumor susceptibility gene 101 (Tsg101)) (15) and their role in HIV membrane abscission (ESCRT-III and Vps4; CHMP2A/-4B and Vps4A) (16). The ESCRT proteins were modified with either green fluorescent protein (GFP) or photo-switchable cyan fluorescent protein (PSCFP2) and stably expressed in COS7 cells. Stable cell lines expressing fluorescent ESCRT probes were validated for proper function (see supplementary online text).

Superresolution image analysis of HIV Gag demonstrated the ability of the iPALM method to sensitively locate and resolve subviral details of membrane enveloped particles emanating from the plasma membrane of expressing cells (Figure 1; see supplementary online text). With the capacity to differentiate subviral protein organization within HIV bud structures, we next performed two-channel iPALM imaging of both HIV Gag and ESCRT subcomplexes. Imaging of HIV Gag and ESCRT-I (PSCFP2-Tsg101) revealed a small cytosolic pool of Tsg101 protein punctuated by clustering at Gag assembly sites (Figure 2A and S6A). PSCFP2-Tsg101 was localized within the interior of the Gag lattice of assembling particles (Figure 2D), consistent with the direct interaction between the p6 domain of HIV Gag and the UEV domain of Tsg101 (3). PSCFP2-CHMP2A and -CHMP4B (ESCRT-III) cell lines expressing HIV Gag showed high cytosolic pools of PSCFP2 followed by membrane-localized co-clustering with Gag clusters (Figure 2A–D and Figure S6B). When co-clustering was observed, the 3D distribution of ESCRT-III and Gag at assembly sites was similar and distinctly separable from the cytosolic pool of PSCFP2-CHMP2A or PSCFP2-CHMP4B (Figure 2A–B, and 2D). To correlate the apparent observation of ESCRT-III signal residing beyond the plane of the plasma membrane, we visualized the plasma membrane using a lipidated fluorescent probe. PSCFP2-CHMP2A signal was observed in

discrete distributions extending away from the cytosol and beyond the membrane plane in Gag expressing cells (Figure 2C). Co-clusters of HIV Gag and PSCFP2-CHMP2A were confirmed by scanning electron microscopy to be membrane budding structures resembling virus-like particles (Figure 2E). We further confirmed our observations of ESCRT-III localization to the HIV Gag interior using immuno-gold transmission electron microscopy (Figure S7).

To facilitate a robust statistical analysis of ESCRT organization at HIV assembly sites we performed two-color single-particle averaging. The resulting probability distributions of PSCFP2-ESCRT probes and HIV Gag revealed significant signal from each ESCRT subunit within the interior of the viral lattice (Figure 3A–C, Figure S8, and supporting online text). Radially averaged probability density plots of HIV Gag and ESCRT probes revealed 92% of PSCFP2-Tsg101, 61% of PSCFP2-CHMP4B, 80% of PSCFP2-CHMP2A, and 78% of PSCFP2-Vps4A integrated probability residing within the estimated radius of the HIV Gag shell (Figure 3E). This analysis highlighted a pool of PSCFP2-CHMP4B residing beyond the HIV Gag lattice (39% remaining probability), potentially indicating a signature of ESCRT-III polymer extending towards the neck of the budding structure. As control, we observed an order of magnitude less PSCFP2-CHMP4B signal residing within the probability shell of mutant Gag particles, unable to recruit ESCRT-I (Gag^{PTAP}) (1, 2), compared to wild-type Gag (Figure 3D and 3E). This suggests the Gag PTAP motif is required for localizing ESCRT-III subunits to the particle interior. These results support our single-particle observations and highlight a unique scaffolding mechanism of ESCRT-III and Vps4A subunits within the interior of the Gag lattice.

The presence of ESCRT-III subunits within the Gag lattice suggests that a portion of this pool may become trapped within the viral particle while mediating bud neck constriction and abscission. To test this possibility, we purified virus-like particles (VLPs) from GFP-ESCRT-expressing cells and performed quantitative fluorescence microscopy (Figure 4A). Using HIV Gag-GFP as a molecular standard, we estimated that on average hundreds of copies of GFP-ESCRT probes reside within released particles. Qualitative coincidence detection between Gag-mCherry and GFP-ESCRT proteins showed that GFP-ESCRT probes were detected in the majority of released particles (50–90%, Figure S9). Correlative two-color iPALM and SEM imaging confirmed the presence of ESCRT probes within released VLPs (Figure 4B). Western blot analysis showed GFP-Tsg101 and GFP-CHMP4B were readily detected in purified VLP fractions (Figure 4C). Levels of GFP-CHMP2A, but not GFP-CHMP4B or GFP-Tsg101 were reduced in released virus particles relative to membrane associated particles (see Fig. 3A–C). This suggested CHMP2A subunits may undergo selective remodeling upon virus abscission. This may be mediated by the ATPase activity of Vps4, given the established interaction between Vps4 and CHMP2A (17).

Current models have been unable to distinguish whether ESCRTs localize to the base of the neck of a virion (in trans) or to the head of the virion (in cis) in order to constrict and release the viral particle from the plasma membrane. Our results show that ESCRT-III and Vps4A probes concentrate on the interior of the HIV Gag lattice and occupy a similar volume to that of ESCRT-I. This architecture is consistent with previous cytokinesis observations that ESCRT-III subunits initiate proximal to the midbody scaffold protein CEP55 and ESCRT-I

(12). Indeed, HIV Gag and CEP55 share features, such as dual Tsg101 and ALIX recruitment domains (18–20), as well as an apparent independence of ESCRT-II for mediating membrane abscission (19–21). Collectively, these observations suggest that structural proteins, like HIV Gag and CEP55, act to nucleate structures composed of ESCRT-I, ESCRT-III, and Vps4 subunits (see supporting online text). These subunits then serve as distinct templates (acting in cis relative to the structural scaffold) for ESCRT-III polymerization and Vps4-mediated disassembly/remodeling of CHMP2A, ultimately leading to membrane abscission (Figure 4C). Further studies will be required to dissect the interplay between scaffolding and polymerizing/depolymerizing pools of ESCRT-III and the role this dynamism plays in mediating viral membrane constriction and abscission.

Supplementary Material

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Acknowledgments

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Figure 1. Interferometric superresolution microscopy resolves the three-dimensional organization of cell-associated HIV particles
(A) Diffraction-limited (left) and iPALM-based superresolution (right) images of COS7 cells expressing HIV Gag-FLAG labelled with AlexaFluor 647 (AF647)-conjugated antibody. Gag clusters from different surfaces of the membrane are clearly resolved (pseudocolored for height, white arrows). Scale bars 10 μ m (full image) and 100 nm (right panel). **(B)** Correlative iPALM (left panel) and scanning electron microscopy (middle panel) demonstrate HIV Gag-FLAG clusters to be membrane enveloped particles (overlay, right panel). Scale bars 250 nm. **(C)** Probability density of aligned clusters from HIV Gag-internal-FLAG (top, n=1181) and HIV Gag-FLAG (bottom, n=1398) highlight the shell-like

lattice (isosurface threshold = $0.5 \text{ localizations} \times \text{nm}^{-3}$). **(D)** Probability slices (10 nm) display a ring-like distribution for Gag-internal-FLAG clusters (top, middle) compared with the more compact Gag-FLAG clusters (bottom, middle). Distance between crosshatch, 100 nm (**D**, right panels). Radial averages highlight the density differences due to probe position within the HIV Gag molecule (inset model).

Figure 2. Two-color 3D superresolution imaging localizes ESCRT-I and ESCRT-III subunits within the HIV Gag lattice of budding particles

(A,B) HIV Gag fused internally to mEOS2 protein (Gag-imEOS2) detects assembly sites containing ESCRT-I subunits Tsg101 (top panel, A) and ESCRT-III subunits CHMP4B (bottom panel, A) and CHMP2A (B) fused to PSCFP2 protein. (B, top panel) iPALM measurements resolve the cytoplasmic pool of PSCFP2-CHMP2A (top arrow), peripheral membrane-associated Gag-imEOS2 (middle arrow), and densely coclustered Gag and CHMP2A structures extending from the membrane (bottom arrow). Scale bar 100 nm (A) and 500 nm (B). (C) PSCFP2-CHMP2A coexpressed with unlabeled HIV Gag and membrane marker tdEOS-farnesyl (red) show displacement of a CHMP2A cluster (green) extending beyond the plane of the plasma membrane. Scale Bar 500 nm. (D) Single-clusters representative of AF647 labeled Gag-FLAG and PSCFP2-ESCRT probes. Axial (top) and lateral (bottom) views show localization of PSCFP2-ESCRT probes within the HIV Gag lattice. Scale Bars 50 nm. (E) Correlative two-color iPALM and scanning electron microscopy of two virus-like particles emanating from a cell expressing Gag-FLAG (red) and PSCFP2-CHMP2A (green). The left membrane bud shows confinement of PSCFP2-CHMP2A to the head of the particle. The particle on the right is at an earlier stage of budding, apparent from the extended lateral fluorescence distribution (white arrows). Scale bar 250 nm.

Figure 3. Single-cluster averaging demonstrates ESCRT subunits within the HIV Gag lattice

(A–D) Cell Associated HIV Gag-FLAG clusters from PSCFP2-ESCRT expressing cells were subjected to 3D single-cluster averaging ((A) PSCFP2-Tsg101; n = 313, (B) PSCFP2-CHMP2A; n = 460 (C) PSCFP2-CHMP4B; n = 509 (D) PSCFP2-CHMP4B/HIV Gag PTAP; n = 278). Two-color isosurfaced probability densities show a high-probability core of PSCFP2-Tsg101 (A) and ESCRT-III subunits PSCFP2-CHMP2A/CHMP4B (B,C)

(green) with respect to the HIV Gag lattice (red) (isosurface threshold: HIV Gag-FLAG = 2.5×10^{-4} nm⁻³ and PSCFP2-ESCRTs = 2.5×10^{-5} nm⁻³). Right insets (A–D) depict 40 nm sections along the z-axis (top HIV Gag (red), bottom PSCFP2-ESCRT (green)). (D) A marked reduction in the probability density of PSCFP2-CHMP4B signal was observed upon expression of release defective HIV Gag-FLAG Δ PTAP. (E) Radial average plots of sections from (A–D) define the highest probability densities of ESCRT (green) subunits

residing within the HIV Gag probability shell (red). Approximately 90% of PSCFP2-Tsg101 (top left), 80% of PSCFP2-CHMP2A (top right), and 61% of PSCFP2-CHMP4B (lower right) integrated probability reside within the half-maximum Gag probability shell, while 10% of the integrated probability for PSCFP2-CHMP4B remains when ESCRT-I recruitment is inhibited (Gag-FLAG PTAP; lower right panel). Line thickness represents S.E.M and is < 1 nm.

Figure 4. Released Virus-Like Particles (VLP) contain ESCRT-I and ESCRT-III subunits, but reduced levels of CHMP2A relative to CHMP4B

(A) Quantitative fluorescence microscopy analysis of GFP-ESCRT and Gag-GFP containing single particles. The fluorescence intensity of Gag-GFP is predicted to arise from ~600–1000 molecules (assuming 3000–5000 Gag molecules per particle) suggesting that signal from GFP-ESCRT particles corresponds to hundreds of molecules per particle (data represent 25,000 particles from 2 independent samples for each probe). (B) Correlative two-color iPALM and scanning electron micrographs of a single released Gag (red; top) and CHMP4B (green; top) positive virus-like particle. Image registration residual uncertainty of 11 nm. Scale bar 50 nm. (C) Western blot analysis of purified VLP fraction from GFP-ESCRT stable cell lines. GFP-Tsg101 and GFP-CHMP4B are detected in released particles, however, GFP-CHMP2A levels are dramatically reduced from VLPs upon particle release (left panel; (*) indicates proteolysis product). Reduction in the levels of GFP-CHMP2A in released particles correlates between the biochemical and fluorescence data. (D) Proposed virus-scaffolding model for ESCRT-mediated HIV particle release from host cells. A pre-scission pool of ESCRT-I subunit Tsg101 (purple) interacts with HIV Gag (red) leading to assembly of ESCRT-III subunits CHMP4B and CHMP2A (green). Hypothetical ESCRT-III polymerization beyond the Gag lattice and Vps4 (orange) remodeling of CHMP2A subunits leads to membrane aperture constriction and particle abscission (middle and right panel).