Figures and figure supplements

Angiomotin functions in HIV-1 assembly and budding

Gaelle Mercenne, et al.
Figure 1. AMOT p130 is a NEDD4L binding partner. (A) Schematic illustrations of the domain structures and motifs in NEDD4L, AMOT p130 and p80, AMOTL1, AMOTL2 and HIV-1 Gag. Here and throughout, NEDD4L refers to a naturally occurring protein isoform (isoform 2) that contains only the C-terminal 32 residues of the C2 domain (Genebank accession number AAM46201.1, denoted NEDD4L in other publications and NEDD4LΔC2 in our previous publication (Chung et al., 2008)). To maintain consistency with that publication, the NEDD4L numbering scheme used here corresponds to NEDD4L isoform 1, which is 121 residues longer at the N-terminus and contains the entire C2 domain (denoted NEDD4LWT in reference Chung et al., 2008). (B) Affinity co-purification and identification of AMOT p130 as a binding partner of OSF (One-STrEP-FLAG)-tagged NEDD4L. SDS-PAGE/Coomassie-stained gel showing STrEP-Tactin matrix affinity purified proteins from 293T cells transfected with an OSF-NEDD4L expression construct (lane 2) or an empty vector control (lane 1). Labels denote OSF-NEDD4L (bait) and AMOT p130 (prey). Trypsin-digested AMOT p130 peptides identified by mass spectrometric analyses are summarized in Figure 1—figure supplement 1. A representative image from three independent repetitions is shown. (C) NEDD4L WW domains are required for AMOT p130 binding. Western blots showing co-immunoprecipitations of endogenous 293T cell AMOT proteins (prey) with OSF-NEDD4L (bait). Panel 1: endogenous AMOT proteins (anti-AMOT blot) co-immunoprecipitated with OSF-NEDD4L baits from cells that lacked exogenous OSF-NEDD4L (lane 1, control), expressed wild type OSF-NEDD4L (lane 2), expressed OSF-NEDD4LΔWW (lane 3, construct has inactivating point mutations in all four WW domains), expressed OSF-NEDD4LΔHWT (lane 4, construct has an inactivating point mutation in the HECT E3 domain), or expressed OSF-NEDD4LΔC942AΔWW (lane 5, construct has inactivating point mutations in the WW and HECT E3 domains). Panel 2: same co-immunoprecipitation experiment blotted with anti-FLAG antibodies to detect OSF-NEDD4L proteins. Panel 3: input levels of endogenous AMOT (anti-AMOT blot, 10% of total). Panel 4: input levels of exogenous OSF-NEDD4L (anti-FLAG, 10% of total). Note that endogenous AMOT p80 was present in the input lysate, but did not co-immunoprecipitate with OSF-NEDD4L. Representative image from three independent repetitions.

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Figure 1—figure supplement 1. Identification of AMOT p130 as a NEDD4L binding partner. The protein band denoted 'AMOT p130' in Figure 1B, lane 2 was excised, digested with trypsin and the eluted peptides were identified by mass spectrometry as described in 'Materials and methods'. This analysis was performed three times and the identified peptides are mapped onto the AMOT p130 primary sequence, with peptides identified in experiments 1–3 color coded in black, blue and red, respectively. DOI: 10.7554/eLife.03778.004

Table 1: Peptide sequences identified in AMOT p130 from mass spectrometry analysis.

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Experimental Run</th>
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<tr>
<td>MRNSEEQPSG GTTVLQRLQ EQLRYGNPSE NRSLLAIHQE ATGNGPFPFS GSGNPQFQSD 60</td>
<td>-run1</td>
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Figure 1—figure supplement 2. The AMOT p130 PPXY motifs contribute to NEDD4L binding. Western blots show co-immunoprecipitations of OSF-NEDD4L (bait, captured on STreP-Tactin affinity matrices) with endogenous 293T cells AMOT and exogenously expressed HA-AMOT p130 proteins (prey). Co-immunoprecipitations with OSF-NEDD4L were from cells that lacked exogenous AMOT p130 (lane 1), expressed wild type HA-AMOT p130 (lane 2) or expressed a mutant HA-AMOT p130 protein carrying PP/AA mutations in the three N-terminal "PPXY" motifs (Wang et al., 2012; Yi et al., 2013). Panel 1: AMOT protein co-immunoprecipitations (anti-AMOT blot) with OSF-NEDD4L. Note that OSF-NEDD4L co-immunoprecipitated low levels of endogenous AMOT p130 (lanes 1 and 3, denoted p130) but not endogenous AMOT p80 (denoted p80 with a dashed line). Panel 2: The same co-immunoprecipitation experiment blotted with anti-HA antibodies to detect exogenously expressed HA-AMOT p130 proteins. Panel 3: The same co-immunoprecipitation experiment blotted with anti-FLAG antibodies to detect exogenously expressed OSF-NEDD4L. Panels 4–6 show the input quantities (10%) of endogenous AMOT and HA-AMOT p130 (panel 4, anti-AMOT), exogenous HA-AMOT p130 (panel 5, anti-HA), and exogenous OSF-NEDD4L (panel 6, anti-FLAG). A representative image from three independent repetitions is shown. DOI: 10.7554/eLife.03778.005
Figure 2. AMOT p130 binds directly and specifically to NEDD4L and HIV-1 GagΔp6.

(A) OSF-AMOT p130 binds NEDD4L. Recombinant OSF-GFP (specificity control, lanes 3–5) or OSF-AMOT p130 (lanes 6–8) were expressed, captured on STREP-Tactin affinity matrices, and incubated with either a buffer control (lanes 3 and 6) or buffers containing 0.5 μM wild type NEDD4L (lanes 4 and 7) or NEDD4LΔWW (lanes 5 and 8, inactivating point mutations in all four WW domains). Matrix-bound proteins were released by boiling in denaturing buffer and detected by SDS-PAGE with Coomassie blue staining (panel 1) or by western blotting (panel 2, anti-NEDD4L) to confirm the identities of the bound NEDD4L and help to distinguish background proteins from low-level binding in panel 1. Input levels of NEDD4L (lane 1, 1.5% of total) and NEDD4LΔWW (lane 2, 1.5% of total) are shown for reference. A representative image from three independent repetitions is shown.

(B) OSF-AMOT p130 binds HIV-1 GagΔp6. Recombinant OSF-GFP (control, lanes 3–6) or OSF-AMOT p130 (lanes 7–10) were expressed, captured on STREP-Tactin affinity matrices and incubated with a buffer control (lanes 3 and 7) or with buffers containing 1.0 μM HIV-1 GagΔp6 (lanes 3 and 8), 0.5 μM wild type NEDD4L (lanes 5 and 9) or both proteins (lanes 6 and 10). Matrix-bound proteins were released by boiling in denaturing buffer and detected by SDS-PAGE with Coomassie blue staining (panel 1) or by western blotting to confirm the identities of the bound Gag (panel 2, anti-MA and anti-CA) and NEDD4LΔWW (panel 3, anti-NEDD4L) proteins and help to distinguish background proteins from low-level binding in panel 1. Input GagΔp6 (lane 1, 2% of total) and NEDD4LΔWW (lane 2, 1.5% of total) are shown for reference. A representative image from three independent repetitions is shown.

DOI: 10.7554/eLife.03778.006
Figure 2—figure supplement 1. AMOT p130, AMOTL1 and AMOTL2 bind directly and specifically to NEDD4L and HIV-1 GagΔp6. (A) OSF-AMOT p130, OSF-AMOTL1, and OSF-AMOTL2 bind NEDD4L. Recombinant OSF-GFP (specificity control, lanes 2 and 6), OSF-AMOT p130 (lanes 3 and 7), OSF-AMOTL1 (lanes 4 and 8), and OSF-AMOTL2 (lanes 5 and 9) were expressed, captured on STREP-Tactin affinity matrices, and incubated with either a buffer control (lanes 2–5) or with a buffer containing 0.5 μM wild type NEDD4L (lanes 6–9). Matrix-bound proteins were released by boiling in denaturing buffer and detected by SDS-PAGE with Coomassie blue staining. Input NEDD4L (lane 1, 1.5% of total) is shown for reference. Asterisk denotes an OSF-AMOTL2 breakdown product that co-migrates with NEDD4L. A representative image from two independent repetitions is shown. (B) OSF-AMOT p130, OSF-AMOTL1, and OSF-AMOTL2 bind GagΔp6. Recombinant OSF-GFP (specificity control, lanes 2 and 6), OSF-AMOT p130 (lanes 3 and 7), OSF-AMOTL1 (lanes 4 and 8), and OSF-AMOTL2 (lanes 5 and 9) were expressed, captured on STREP-Tactin affinity matrices, and incubated with either a buffer control (lanes 2–5) or buffers containing 1 μM GagΔp6 (lanes 6–9). Matrix-bound proteins were released by boiling in denaturing buffer and detected by SDS-PAGE with Coomassie blue staining. Input GagΔp6 (lane 1, 2% of total) is shown for reference. A representative image from two independent repetitions is shown.

DOI: 10.7554/eLife.03778.007
AMOT p130 stimulates NEDD4L-dependent release of HIV-1ΔPTAP,ΔYP. Left panels are western blots showing 293T cellular levels of endogenous AMOT and exogenous HA-AMOT p130 proteins (panel 1, anti-AMOT), exogenous FLAG-NEDD4L (panel 2, anti-FLAG), endogenous GAPDH (panel 3, anti-GAPDH, loading control) and HIV-1 GagΔPTAP,ΔYP proteins (panel 4, anti-MA and anti-CA). Cells were co-transfected with expression vectors for HIV-1ΔPTAP,ΔYP (lanes 1–6), FLAG-NEDD4L proteins (lanes 3–6, with wild type (WT) FLAG-NEDD4L in lanes 3 and 4 and FLAG-NEDD4LΔWW in lanes 5 and 6), and wild type HA-AMOT p130 (lanes 2, 4 and 6) or an empty vector control (lanes 1, 3, and 5). Right panels show corresponding levels of extracellular, virion-associated CAGag and MAGag proteins (panel 1, anti-MA and anti-CA) and viral titers (panel 2, IU denotes ‘infectious units’). Here and in subsequent figures: (1) error bars denote standard deviations between independent repetitions of the experiment, n = 5 in this case, and (2) numbers within the blots show integrated intensities of the CA band intensities (relative to the value in the control experiment, set to 1.0). Here and throughout significance is denoted by: NS, not significant (p > 0.05); *0.05 > p > 0.01; **p < 0.01.

DOI: 10.7554/eLife.03778.008
Figure 3—figure supplement 1. Dose-dependent AMOT p130 stimulation of NEDD4L-dependent release of HIV-1ΔPTAP, ΔYP. Left panels are western blots showing 293T cellular levels of endogenous AMOT and exogenous HA-AMOT p130 proteins (panel 1, anti-AMOT), exogenous FLAG-NEDD4L (panel 2, anti-FLAG), GAPDH (panel 3, anti-GAPDH, loading control) and HIV-1 GagΔPTAP, ΔYP proteins (panel 4, anti-MA and anti-CA). Cells were co-transfected with expression vectors for HIV-1ΔPTAP, ΔYP (lanes 1–8), FLAG-NEDD4L (lanes 2–8), and increasing quantities of an AMOT p130 expression construct (lanes 3–8, 0.25–1.5 μg DNA). Cells were also co-transfected with empty vectors as necessary to keep total DNA levels constant (3 μg DNA total). Right panels show corresponding levels of extracellular virion-associated Gag proteins (upper panel, anti-MA and anti-CA) and viral titers (lower panel), n = 3.
DOI: 10.7554/eLife.03778.009
Figure 3—figure supplement 2. The AMOT p130 isoform specifically stimulates NEDD4L-dependent release of HIV-1ΔPTAP,ΔYP. Left panels are western blots showing 293T cellular levels of endogenous AMOT, exogenous p80 and exogenous HA-AMOT p130 proteins (panel 1, anti-AMOT), exogenous FLAG-NEDD4L (panel 2, anti-FLAG), GAPDH (panel 3, anti-GAPDH, loading control) and HIV-1 GagΔPTAP,ΔYP proteins (panel 4, anti-MA and anti-CA). Cells were co-transfected with expression vectors for HIV-1ΔPTAP,ΔYP (lanes 1–6), AMOT p80 (lanes 2 and 5), AMOT p130 (lanes 3 and 6) and FLAG-NEDD4L (lanes 4–6). Cells were also co-transfected with empty vectors as necessary to keep total DNA levels constant (2.5 μg DNA total). Right panels show corresponding levels of extracellular virion-associated Gag proteins (upper panel, anti-MA and anti-CA) and viral titers (lower panel), n = 4. DOI: 10.7554/eLife.03778.010
**Figure 4.** AMOT p130 is required for NEDD4L-stimulated release of HIV-1ΔPTAP,ΔYP. Left panels are western blots showing 293T cellular levels of endogenous AMOT and exogenous HA-AMOT p130 proteins (panel 1, anti-AMOT), exogenous FLAG-NEDD4L (panel 2, anti-FLAG), endogenous GAPDH (panel 3, anti-GAPDH, loading control) and HIV-1 GagΔPTAP,ΔYP proteins (panel 4, anti-MA and anti-CA). Cells were co-transfected with a control siRNA (lanes 1 and 3) or with an siRNA that depleted both AMOT p130 and p80 (lanes 2 and 4–6), with expression vectors for HIV-1 GagΔPTAP,ΔYP (lanes 1–6), and with FLAG-NEDD4L (lanes 3–6) or an empty vector control (lanes 1 and 2), and with siRNA-resistant expression constructs for HA-AMOT p130 proteins (with wild type HA-AMOT p130 in lane 5 and an HA-AMOT p130ΔPPXY protein carrying inactivating point mutations in the three PPXY motifs in lane 6). Right panels show corresponding levels of extracellular virion-associated Gag proteins (upper panel, western blot, anti-MA and anti-CA) and viral titers (lower panel), n = 6.

DOI: 10.7554/eLife.03778.011
Figure 4—figure supplement 1. Dose-dependent AMOT p130 rescue of HIV-1 release from cells depleted of endogenous AMOT. Left panels are western blots showing 293T cellular levels of endogenous AMOT and exogenous HA-AMOT p130 proteins (panel 1, anti-AMOT), exogenous FLAG-NEDD4L (panel 2, anti-FLAG), GAPDH (panel 3, anti-GAPDH, loading control) and HIV-1 Gag proteins (panel 4, anti-MA and anti-CA). Cells were co-transfected with a control siRNA (lanes 1 and 3) or with an siRNA that depleted both AMOT p130 and p80 (lanes 2 and 4–10), with expression vectors for HIV-1ΔPTAP,ΔYP (lanes 1–10), for FLAG-NEDD4L (lanes 3–10), and with increasing quantities of an siRNA-resistant AMOT p130 construct (lanes 5–10, 0.25–1.5 μg DNA). Cells were also co-transfected with empty vectors as necessary to keep total DNA levels constant (3 μg DNA total). Right panels show corresponding levels of extracellular virion-associated Gag proteins (upper panel, western blot, anti-MA and anti-CA) and viral titers (lower panel), n = 3. DOI: 10.7554/eLife.03778.012
Figure 4—figure supplement 2. The AMOT p130 isoform specifically stimulates NEDD4L-dependent release of HIV-1ΔPTAP,ΔYP. Left panels are western blots showing 293T cellular levels of endogenous AMOT and exogenous HA-AMOT p130 or AMOT p80 proteins (panel 1, anti-AMOT), exogenous FLAG-NEDD4L (panel 2, anti-FLAG), endogenous GAPDH (panel 3, anti-GAPDH, loading control) and HIV-1 GagΔPTAP,ΔYP proteins (panel 4, anti-MA and anti-CA). Cells were transfected with a control siRNA (lanes 1 and 3) or with an siRNA that depleted both AMOT p130 and p80 (lanes 2 and 4–7). Cells were co-transfected with expression vectors for HIV-1ΔPTAP,ΔYP (lanes 1–7), FLAG-NEDD4L (lanes 3–7), and siRNA-resistant expression constructs for AMOT p80 (lane 5), HA-AMOT p130 (lane 6) or both (lane 7). Right panels show corresponding levels of extracellular virion-associated Gag proteins (upper panel, western blot, anti-MA and anti-CA) and viral titers (lower panel), n = 6.

DOI: 10.7554/eLife.03778.013
Figure 5. AMOT p130 is required for efficient release of wild type HIV-1. Left panels are western blots showing 293T cellular levels of endogenous ALIX (panel 1, anti-ALIX), TSG101 (panel 2, anti-TSG101), AMOT (panel 3, anti-AMOT), GAPDH (panel 4, anti-GAPDH, loading control) and levels of HIV-1 Gag proteins (panel 5, anti-MA and anti-CA). Cells were co-transfected with a control siRNA (lane 1) or with siRNA that depleted ALIX (lane 2), TSG101 (lane 3) or AMOT p130 and p80 (lanes 4–6), with expression vectors for wild type HIV-1 (lanes 1–6) and with siRNA-resistant expression constructs for HA-AMOT p130 proteins (with wild type HA-AMOT p130 in lane 5 and an HA-AMOT p130ΔPPXY protein with inactivating point mutations in all three PPXY motifs in lane 6). Right panels show corresponding levels of extracellular virion-associated Gag proteins (upper panel, western blot, anti-MA and anti-CA) and viral titers (lower panel), n = 4.

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**Figure 6.** AMOT p130 stimulates release of HIV-1 from HeLa and Jurkat T cells. (A) Left panels are western blots showing HeLa cellular levels of endogenous AMOT p130 and exogenous HA-AMOT p130 (panel 1, anti-AMOT), endogenous GAPDH (panel 2, anti-GAPDH, loading control) and HIV-1 Gag proteins (panel 3, anti-MA and anti-CA). HeLa cells were co-transfected with expression vectors for HIV-1 (lanes 1–5), and with increasing concentrations of an expression construct for HA-AMOT p130 (0–4 μg DNA in lanes 1–5). Right panels show corresponding levels of extracellular virion-associated Gag proteins (upper panel, western blot, anti-MA and anti-CA) and viral titers (lower panel), n = 4. (B) Levels of extracellular virion-associated Gag proteins (upper panel, western blot, anti-MA and anti-CA) and viral titers (lower panel). Jurkat T cells were co-transfected with expression vectors for HIV-1 (lanes 1–5), together with a control vector (lane 1) or expression vectors for wild type HA-AMOT p130 (lanes 2 and 3, 5 and 10 μg DNA respectively) or an HA-AMOT p130ΔPPXY protein with inactivating point mutations in the three PPXY motifs (lanes 4 and 5, 5 and 10 μg DNA respectively), n = 3. 
DOI: 10.7554/eLife.03778.015

**Figure 6—figure supplement 1.** HeLa cells express little or no AMOT. Western blots showing endogenous AMOT p130 and p80 expression levels in 293T cells (lanes 1–4) and in HeLa cells (lanes 5–8). Increasing numbers of cell equivalents were added as indicated (ranging from 1.5 x 10^5 cells in lanes 1 and 5 to 1.2 x 10^6 cells in lanes 4 and 8). Upper and lower panels show lighter and darker exposures of the same blot. Image is representative of two independent repetitions of this experiment. 
DOI: 10.7554/eLife.03778.016
Figure 7. AMOTL1 and AMOTL2 can substitute for AMOT p130 in HIV-1 release. Left panels are western blots showing 293T cellular levels of endogenous AMOT and exogenous HA-AMOT p130 proteins (panel 1, anti-AMOT), exogenous HA-AMOT p130, HA-AMOTL1 or HA-AMOTL2 proteins (panel 2, anti-HA), endogenous GAPDH (panel 3, anti-GAPDH, loading control) and HIV-1 Gag proteins (panel 4, anti-MA and anti-CA). Cells were co-transfected with a control siRNA (lane 1) or with an siRNA that depleted AMOT p130 and AMOT p80 (lanes 2–5), and with expression vectors for HIV-1 (lanes 1–5), with siRNA-resistant expression constructs for AMOT p130 (lane 3), AMOTL1 (lane 4) or AMOTL2 (lane 5). Right panels show corresponding levels of extracellular virion-associated Gag proteins (upper panel, western blot, anti-MA and anti-CA) and viral titers (lower panel), n = 4.
DOI: 10.7554/eLife.03778.017
Figure 7—figure supplement 1. AMOTL2 can stimulate NEDD4L-dependent release of HIV-1ΔPTAP,ΔYP and rescue HIV-1ΔPTAP,ΔYP release from cells depleted of endogenous AMOT. (A) AMOTL2 stimulates NEDD4L-dependent release of HIV-1ΔPTAP,ΔYP. Left panels are western blots showing 293T cellular levels of exogenous HA-AMOT p130, HA-AMOTL1 or HA-AMOTL2 proteins (panel 1, anti-HA), exogenous FLAG-NEDD4L (panel 2, anti-FLAG), GAPDH (panel 3, anti-GAPDH, loading control) and HIV-1 Gag proteins (panel 4, anti-MA and anti-CA). Cells were co-transfected with expression vectors for HIV-1ΔPTAP/ΔYP (lanes 1–8), FLAG-NEDD4L (lanes 5–8), and either HA-AMOT p130 (lanes 2 and 6), HA-AMOTL1 (lanes 3 and 7) or HA-AMOTL2 (lanes 4–8). Right panels show HIV-1 infectivity in the absence (lanes 1–4) or presence (lanes 5–8) of HA-AMOT p130, HA-AMOTL1 or HA-AMOTL2. 

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Figure 7—figure supplement 1. Continued

and 8). Right panels show corresponding levels of extracellular virion-associated Gag proteins (upper panel) and viral titers (lower panel), n = 3. (B) AMOTL2 can rescue HIV-1\textsubscript{ΔPTAP,ΔYP} release from 293T cells depleted of endogenous AMOT. Left panels are western blots showing cellular levels of endogenous AMOT and exogenous HA-AMOT proteins (panel 1, anti-AMOT), exogenous HA-AMOT p130, HA-AMOTL1 or HA-AMOTL2 (panel 2, anti-HA), exogenous FLAG-NEDD4L (panel 3, anti-FLAG), GAPDH (panel 4, anti-GAPDH, loading control) and HIV-1 Gag proteins (panel 5, anti-MA and anti-CA). Cells were co-transfected with a control siRNA (lanes 1 and 3) or with an siRNA that depleted both AMOT p130 and p80 (lanes 2 and 4–7), with expression vectors for HIV-1 (lanes 1–7), FLAG-NEDD4L (lanes 3–7), and either an siRNA-resistant HA-AMOT p130 expression construct (lane 5), or expression constructs for HA-AMOTL1 (lane 6), or HA-AMOTL2 (lane 7). Right panels show corresponding levels of extracellular virion-associated Gag proteins (upper panel, western blot, anti-MA and anti-CA) and viral titers (lower panel), n = 4.

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Figure 8. AMOT p130 is required for HIV-1 assembly/budding. Scanning electron microscopic (SEM) images of HIV virions budding from 293T cells depleted with a control siRNA (A–C), depleted of TSG101 (D–F) or depleted of AMOT (G–I). Successive images across each row show expansions of the adjacent boxed regions, and scale bars are 500 nm in all cases. Arrows in panel (C) highlight budding wild type HIV-1 virions.

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Figure 9. AMOT p130 is required for an early stage of HIV-1 assembly/budding. (A) Transmission electron microscopic (TEM) images of HIV virions budding from 293T cells depleted of TSG101. Panel 1: wide view. Panel 2: expanded view. Scale bars here and in part B are 100 nm. (B) TEM images of HIV virions budding from 293T cells depleted of AMOT p130 and p80. Panel 1: wide view. Panel 2: expanded view. (C) Quantification of the release and maturation status of HIV-1 virions associated with cells that were treated with a control siRNA (black bars), depleted of TSG101 (red), depleted of AMOT (teal), or depleted of AMOT and re-expressing AMOT p130 from an siRNA-resistant construct (light blue). This experiment was repeated twice with similar results, and error bars were derived by quantifying three separate sets of >100 virions each from one of the experiments. Quantification of the completeness of virions budding from cells treated with a control siRNA (black triangles), depleted of TSG101 (red squares), or depleted of AMOT (teal diamonds). The extent of the Gag shell arc (in degrees) was measured from TEM images of 500 budding virions of each type, the measurements were binned into the intervals shown below the x-axis, and the virion numbers in each bin are shown in the plot.

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