Dynamic subunit turnover in ESCRT-III assemblies is regulated by Vps4 to mediate membrane remodelling during cytokinesis

Beata E. Mierzwa1,7, Nicolas Chiaruttini2,7, Lorena Redondo-Morata1,7, Joachim Moser von Fileseck2, Julia König4,8, Jorge Larios2, Ina Poser2, Thomas Müller-Reichert4, Simon Scheuring3,8, Aurélien Roux2,6,9 and Daniel W. Gerlich1,9

The endosomal sorting complex required for transport (ESCRT)-III mediates membrane fission in fundamental cellular processes, including cytokinesis. ESCRT-III is thought to form persistent filaments that over time increase their curvature to constrict membranes. Unexpectedly, we found that ESCRT-III at the midbody of human cells rapidly turns over subunits with cytoplasmic pools while gradually forming larger assemblies. ESCRT-III turnover depended on the ATPase VPS4, which accumulated at the midbody simultaneously with ESCRT-III subunits, and was required for assembly of functional ESCRT-III structures. In vitro, the Vps2/Vps24 subunits of ESCRT-III formed side-by-side filaments with Snf7 and inhibited further polymerization, but the growth inhibition was alleviated by the addition of Vps4 and ATP. High-speed atomic force microscopy further revealed highly dynamic arrays of growing and shrinking ESCRT-III spirals in the presence of Vps4. Continuous ESCRT-III remodelling by subunit turnover might facilitate shape adaptions to variable membrane geometries, with broad implications for diverse cellular processes.

The endosomal sorting complex required for transport-III (ESCRT-III) promotes membrane fission from the inner side of membrane necks in various cellular processes1, including the biogenesis of multivesicular bodies2, cytokinetic abscission3–27, nuclear envelope sealing28–31, plasma membrane repair32, human immunodeficiency virus (HIV) budding13,14, and exosome or microvesicle shedding15–17. ESCRT-III forms polymers that are thought to constrict membrane necks until they split18–25, but the mechanism underlying constriction is unknown.

ESCRT-III is evolutionarily conserved from humans to archaea, and is composed of four structurally related core subunits with distinct functions18–27. Budding yeast Vps20 (human homologue is CHMP6) functions as a nucleation factor, Snf7 (human homologue CHMP3 has three isoforms, A–C) serves as a main polymer subunit, Vps24 (CHMP3 in humans) and Vps2 (CHMP2A and B isoforms in humans) inhibit Snf7 polymerization38–42 and recruit the ATPase Vps4, which is thought to predominantly disassemble ESCRT-III polymers30,31,33–38. How different ESCRT-III components coordinately assemble and remodel polymer structures has remained unclear.

Purified ESCRT-III subunits polymerize into filaments that form spirals on flat membranes or helices on membrane tubes36,39–45. ESCRT-III also forms filament spirals and helices in intact cells44,46,47, and it is required for the assembly of large filament helices that constrict the intercellular bridge during cytokinetic abscission4.

Prevailing models propose that ESCRT-III mediates membrane fission by sequential assembly of distinct subunits18–23,25, whereby late-binding Vps2/Vps24 (CHMP2/CHMP3) subunits might form a rigid dome-shaped scaffold to guide attached membranes towards the fission site36 or induce changes in the curvature of pre-assembled Snf7 filament spirals to promote membrane neck constriction41. These models rely on the sequential addition of distinct subunits and the persistence of ESCRT-III polymers, yet this has not been directly observed under physiological conditions. We hence set out to systematically quantify the assembly kinetics, dynamics and structure of ESCRT polymers in live human cells and in an in vitro reconstitution system.

1Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA), Vienna Biocenter (VBC), AT-1030 Vienna, Austria. 2Department of Biochemistry, University of Geneva, CH-1211 Geneva, Switzerland. 3U1006 INSERM, Aix-Marseille Université, 13009 Marseille, France. 4Experimental Center, Medical Faculty Carl Gustav Carus, Technische Universität Dresden, D-01307 Dresden, Germany. 5Max Planck Institute of Molecular Cell Biology and Genetics, D-01307 Dresden, Germany. 6Swiss National Centre for Competence in Research Programme Chemical Biology, CH-1211 Geneva, Switzerland. 7These authors contributed equally to this work. 8Present addresses: Electron Microscopy Unit, Francis Crick Institute, London NW1 1AT, UK (J.K.); Departments of Anesthesiology and Physiology & Biophysics, Weill Cornell Medicine, New York, New York 10065, USA (S.S.). 9Correspondence should be addressed to A.R. or D.W.G. (e-mail: aurelien.roux@unige.ch or daniel.gerlich@imba.oeaw.ac.at)

Received 22 January 2017; accepted 19 May 2017; published online 12 June 2017; DOI: 10.1038/ncb3559
RESULTS
ESCRRT-III assemblies continuously turn over their subunits with the cytoplasm

To investigate the dynamics of ESCRRT-III polymers at the abscission site, we generated stable HeLa cell lines expressing fluorescently tagged subunits. We found that CHMP4B tagged with GFP via a long flexible linker (localization and affinity purification tag, LAP) and expressed close to endogenous levels did not perturb abscission (Fig. 1a). To probe the functionality of LAP-tagged CHMP4B, we depleted endogenous CHMP4B in wild-type HeLa cells or in HeLa cells stably expressing short interfering RNA (siRNA)-resistant mouse CHMP4B-LAP (Supplementary Fig. 1a). Cytokinetically abscission was substantially perturbed following depletion of endogenous CHMP4B in wild-type HeLa cells, but was not affected in mouse-CHMP4B-LAP-expressing HeLa cells (Fig. 1a), validating the functionality of CHMP4B-LAP.

We next investigated the dynamics of midbody-localized ESCRRT-III by fluorescence recovery after photobleaching (FRAP) experiments. Unexpectedly, we found that CHMP4B-LAP rapidly re-accumulated at the midbody following photobleaching (Fig. 1b,c and Supplementary Video 1). A single exponential function constrained to initial fluorescence values did not fit the FRAP kinetics (Fig. 1c), indicating the presence of two populations of CHMP4B-LAP with distinct residence times at the midbody. We determined the residence times for the two midbody-localized fractions by a double-exponential fit (Fig. 1b,h,i). A highly mobile fraction of 64 ± 6% (mean ± s.e.m. as in the rest of the paper, if not otherwise noted) of CHMP4B-LAP had a residence time of 19.5 ± 2.7 s, whereas a stably bound fraction of 36 ± 2% had a residence time of 716.0 ± 91.3 s. Modelling the stably bound fraction as completely immobile also yielded a good approximation (Supplementary Fig. 1b,c). Importantly, both methods of model fitting yielded consistent values for the fraction and residence time of the highly mobile pool (Supplementary Fig. 1d). Thus, the majority of CHMP4B-LAP molecules at the midbody continuously turn over with a cytoplasmic pool—at a rate up to two orders of magnitude faster than the macroscopic accumulation of ESCRRT-III at the midbody.

ESCRRT-III localizes initially within two cortical regions adjacent to the midbody, which later constrict to split the plasma membrane. Photobleaching of CHMP4B-LAP prior to or during constriction stages revealed similar recovery kinetics (Fig. 1b,d,e and Supplementary Video 1), indicating that midbody-localized ESCRRT-III dynamically turns over subunits with cytoplasmic pools during its macroscopic accumulation and constriction.

We next investigated the localization and dynamics of other ESCRRT-III subunits. We tagged human CHMP2B, CHMP3 and CHMP4B with the same design used for tagging mouse CHMP4B. All three LAP-tagged ESCRRT-III subunits localized to the midbody (Fig. 1f,g and Supplementary Video 2) and did not perturb abscission (Supplementary Fig. 2a–d). CHMP2B, CHMP3 and CHMP4B subunits accumulated at the midbody with indistinguishable kinetics (Fig. 1f,g and Supplementary Video 2). Hence, ESCRRT-III assemblies at the midbody with a fairly constant proportion of different core subunits during the progression of abscission.

We next probed the dynamics of human CHMP2B, CHMP3 and CHMP4B at the midbody. FRAP experiments showed that all three subunits had highly mobile fractions with residence times similar to mouse CHMP4B (Fig. 1h,i). We noticed variable kinetics at late stages of FRAP recovery, which resulted in inaccurate fitting of single exponential functions (Supplementary Fig. 2e–g). Given the technical difficulty to accurately measure long residence times, the relevance of the observed variations remains unclear. Importantly, however, all FRAP experiments consistently show that highly mobile fractions of CHMP2B, CHMP3 and CHMP4B dynamically turn over with similar residence times. Overall, our experiments show that ESCRRT-III forms highly dynamic assemblies at the midbody.

Dynamic subunit turnover in ESCRRT-III assemblies depends on VPS4

We wondered whether VPS4 could be responsible for ESCRRT-III turnover, as it is the only known nucleotide hydrolase in the ESCRRT-III pathway. VPS4 was previously detected at the midbody only during late stages of abscission, which would be inconsistent with its contribution to the high ESCRRT-III turnover observed during early stages. However, previous measurements of VPS4 accumulation were based on overexpression from a viral promoter, which could limit its detection at the midbody owing to high cytoplasmic background. We thus re-examined VPS4 accumulation in cells stably expressing LAP-tagged murine VPS4B from its endogenous promoter (Supplementary Fig. 3a). In these cells, VPS4B-LAP indeed accumulated at the midbody simultaneously with CHMP4B-LAP (Fig. 2a,b and Supplementary Video 3). Thus, VPS4 is present at early stages and could contribute to ESCRRT-III dynamics throughout the entire abscission process.

To investigate the role of VPS4 in ESCRRT-III dynamics, we depleted both isoforms VPS4A and VPS4B in CHMP4B-LAP-expressing cells using RNA-mediated interference (RNAi). Depletion of endogenous VPS4A/B to undetectable levels at 48 h after siRNA transfection (Supplementary Fig. 3b) substantially reduced the amount of CHMP4B-LAP at the midbody (Fig. 2c, and Supplementary Fig. 3c). FRAP experiments revealed that under this condition, CHMP4B-LAP turnover at the midbody was almost completely suppressed (Fig. 2c,f). We considered that this phenotype may arise from the strong reduction of the cytoplasmic CHMP4B concentration, owing to the accumulation of ESCRRT-III at endosomes following complete VPS4 depletion (Fig. 2d). However, partial depletion of VPS4A/B at 20 h after siRNA transfection also reduced the FRAP (Fig. 2e,f) without altering the cytoplasmic CHMP4B-LAP concentration (Fig. 2c,d). The cytoplasmic levels of CHMP2B-LAP or CHMP3-LAP were also not affected following partial VPS4A/B depletion (Supplementary Fig. 3d,e). Furthermore, microinjection of recombinant human CHMP4B protein into telophase cells resulted in rapid accumulation at the midbody in control cells, but a much slower rate in VPS4A/B-depleted cells (Supplementary Fig. 4). Together, these experiments show that VPS4 is required for dynamic turnover of ESCRRT-III at the midbody.

VPS4 is required for constriction of the intercellular bridge

We next studied how VPS4 contributes to abscission. RNAi depletion of VPS4A/B delayed abscission and frequently caused cleavage furrow regression (Fig. 3a,b). These abscission failures did not occur in cells stably expressing murine VPS4B-LAP, which is resistant to siRNA targeting human VPS4A/B (Fig. 3b and Supplementary Fig. 3a), validating that this phenotype is caused by on-target depletion. To gain further insight into the underlying defect, we investigated the...
Figure 1 ESCRT-III assemblies at the midbody dynamically turn over subunits in early and late abscission stages. (a) Validation of mmCHMP4B-LAP functionality by RNAi phenotype rescue. Cumulative histograms indicate duration from complete cleavage furrow ingression until abscission for wild-type HeLa cells and for HeLa cells expressing mmCHMP4B-LAP at 55–80 h after siRNA transfection (3 independent experiments with combined sample numbers of n=48 cells for wild-type + siControl, n=38 cells for wild-type + siCHMP4B, n=60 cells for mmCHMP4B-LAP + siControl, and n=46 cells for mmCHMP4B-LAP + siCHMP4B). siCHMP4B (hs) targets only endogenous human CHMP4B but not mmCHMP4B-LAP. (b) FRAP of mmCHMP4B-LAP at a HeLa cell midbody at early and late abscission stages, stained with SiR-tubulin. The dashed circles indicate the photobleaching region; time 0 indicates the first image after photobleaching. High-resolution example of experiment in c–e. (c,d) Fluorescence recovery curves for early abscission (n=18 cells from 4 independent experiments) (c) or late abscission stages (n=17 cells from 4 independent experiments) (d). Single exponential function f(t) = 1 – e^{-kt}, or double exponential function f(t) = A1·(1 – e^{-k1·t}) + (1 – A1)·(1 – e^{-k2·t}) were fitted to the data. The points and shaded areas indicate mean ± s.e.m. of fluorescence; the dashed lines indicate fits of exponential functions. (e) Quantification of highly mobile fractions by fitting double exponential functions to data from c,d. Dots represent individual cells. (f) Three-dimensional live-cell confocal microscopy of the intercellular bridge during telophase, in HeLa cells expressing hsCHMP2B-LAP or hsCHMP3-LAP, respectively. The arrowheads indicate abscission. High-resolution example of experiment in f. (g) Quantification of hsCHMP2B-LAP (n=17 cells from 4 independent experiments), hsCHMP3-LAP (n=13 cells from 3 independent experiments), and hsCHMP4B-LAP (n=17 cells from 3 independent experiments) midbody accumulation. Points and shaded areas indicate mean ± s.e.m., normalized to intercellular bridge fluorescence after cleavage furrow ingression, and temporally aligned to abscission (time point 0). (h) Highly mobile fractions of LAP-tagged ESCRT-III subunits derived from double-exponential fits to FRAP curves. Each dot represents a single FRAP experiment acquired in 3 independent experiments; bars indicate medians. (i) Residence times of highly mobile fractions for cells shown in h. Scale bars, 1 μm in b,f.
Figure 2  VPS4 is required for ESCRT-III accumulation and turnover. (a) Confocal microscopy of the intercellular bridge in HeLa cells expressing mmCHMP4B-LAP or mmVPS4B-LAP, respectively. The arrowheads indicate abscission. High-resolution example of experiment in b. (b) Midbody accumulation of mmCHMP4B-LAP (n = 15 cells from 3 independent experiments) or mmVPS4B-LAP (n = 16 cells from 3 independent experiments) relative to abscission (time point 0). Points and shaded areas indicate mean ± s.e.m. (c) Live-cell images of telophase cells expressing mmCHMP4B-LAP after transfection of a non-targeting control siRNA, or siRNAs targeting hsVPS4A/B, 20 h or 48 h after siRNA transfection. The insets show enlarged midbody regions. The same contrast settings were used for all panels. Quantification of midbody accumulation shown in Supplementary Fig. 3c. (d) Quantification of cytoplasmic mmCHMP4B-LAP levels from data in e,f. Dots represent individual cells from 3 independent experiments; bars indicate medians. (e) FRAP curves and double-exponential fits for mmCHMP4B-LAP at pre-constriction stages transfected with control siRNAs (n = 18 cells from 3 independent experiments) or siRNAs targeting VPS4A/B (n = 18 cells for siVPS4A/B 20 h, and n = 17 cells for siVPS4A/B 48 h from 3 independent experiments). Points and shaded areas indicate mean ± s.e.m. (f) Highly mobile fractions of mmCHMP4B-LAP determined by double-exponential fits to FRAP curves shown in e (3 independent experiments with combined sample numbers of n = 9 cells for siControl 48 h, n = 18 cells for siVPS4A/B 20 h, and n = 13 cells for siVPS4A/B 48 h). Statistical test using the two-sided Kolmogorov–Smirnov test yielded P = 6.562 × 10⁻³ for siControl 48 h relative to siVPS4A/B 20 h, and P = 4.021 × 10⁻⁶ for siControl 48 h relative to siVPS4A/B 48 h. Dots represent individual cells; bars indicate medians. **P < 0.01, ****P < 0.0001. Scale bars, 1 μm in a; 5 μm or 1 μm (inset) in c.

Ultrastructure of intercellular bridges in cryo-immobilized telophase cells. Most of the control cells contained constriction zones adjacent to the midbody with regularly spaced 17-nm-diameter filaments and compressed bundles of microtubules (Fig. 3c; 4 out of 7 cells), as previously observed4. After partial VPS4A/B depletion at 26 h after siRNA transfection, only a small fraction of cells had 17-nm-diameter filaments (Fig. 3d; 4 out of 27 cells), and narrow constriction zones were never observed. Together, these data indicate that VPS4 is required for the formation and constriction of a functional ESCRT-III apparatus at intercellular bridges.
Vps2 and Vps24 inhibit Snf7 polymerization in vitro

The high ESCRT-III dynamics in cells prompted us to dissect the specific contribution of each subunit in vitro. As purified human ESCRT-III proteins are difficult to spontaneously polymerize on flat membranes under physiological concentrations49, we considered the use of the evolutionarily conserved budding yeast proteins. We first tested whether budding yeast Snf7 (homologue of human CHMP4) can in principle recapitulate the cellular dynamics observed for human proteins and therefore expressed a LAP-tagged version in HeLa cells. Budding yeast Snf7-LAP specifically localized to the midbody during abscission and rapidly recovered after photobleaching similar to human and mouse CHMP4B-LAP (Supplementary Fig. 5ad), validating the use of yeast proteins for in vitro analysis of ESCRT-III dynamics.

Previous work showed that Snf7 spontaneously polymerizes on membrane-covered areas of coverslips to form large patches composed of densely packed filament spirals43. We studied patch growth kinetics only in central areas of membrane-covered regions, as patches stop growth at the edge of membrane-covered regions (Supplementary Fig. 5e–g). We first investigated how Vps24 (homologue of human CHMP3) and Vps2 (CHMP2) affected the kinetics of Snf7 polymerization. We therefore incubated supported lipid bilayers with fluorescently labelled Snf7 until patches formed and then simultaneously added Vps2 and Vps24 (Fig. 4a,b and Supplementary Video 4; 22 min). Following rapid binding, Vps2 and Vps24 suppressed patch growth and strongly reduced further accumulation of Snf7 in patches (Fig. 4c,d and Supplementary Fig. 6ac). Sequential injection of Vps2 and Vps24 into the fluid chamber further showed that these subunits depend on each other in their Snf7 growth-inhibitory function (Fig. 4e and Supplementary Fig. 6d,e and Supplementary Videos 5 and 6). Thus, prolonged phases of ESCRT-III assembly, as observed during cytokinetic abscission4,5, are not recapitulated by mixed solutions of Snf7, Vps2 and Vps24.

The inhibition of Snf7 patch growth by Vps2/Vps24 could be caused by lower rates of Snf7 subunit accumulation or by an increase of the Snf7 dissociation rate. To investigate this, we incubated supported lipid bilayers with fluorescently labelled Snf7 until patches formed and then

Figure 3 VPS4 is required for constriction of the intercellular bridge. (a,b) Transfection of siRNAs targeting hsVPS4A/B causes abscission failure in wild-type HeLa cells, but not in HeLa cells stably expressing mmVPS4B-LAP. (a) Progression from cleavage furrow ingression (time point 0) until abscission in wild-type HeLa cells at 30–50 h after transfection of the indicated siRNAs (n = 84 cells for wild-type + siControl, and n = 80 cells for wild-type + siVPS4A/B for 3 fields of view from 2 independent experiments). (b) Rescue of abscission failure in HeLa cells stably expressing mmVPS4B-LAP (data from a, n = 54 cells for mmVPS4B-LAP + siControl, and n = 45 cells for mmVPS4B-LAP + siVPS4A/B for 3 fields of view from 2 independent experiments). Bars and error bars indicate mean ± s.e.m. (c) Representative electron micrograph of an intercellular bridge of a control cell (n = 10 cells, out of which 3 cells had filaments without constriction, and 4 showed filaments with constriction). The arrowheads indicate 17-nm-diameter filaments. (d) Intercellular bridge of a cell 26 h after transfection of VPS4A/B siRNA (n = 26 cells, out of which 4 cells showed filaments without constriction). The arrowheads indicate 17-nm-diameter filaments. Scale bars, 200 nm in c,d.
Vps2 and Vps24 cooperatively bind Snf7 patches and inhibit ESCRT-III polymerization. (a) Time-lapse microscopy of ESCRT-III polymerization on supported lipid membranes in a microfluidic flow chamber. Recombinant Snf7-AlexaFluor-488 was injected at $t=0$ min; Vps2-Atto-565 and Vps24 were added at $t=22$ min. (b) Kymograph of a single ESCRT-III patch from a. (c,d) Quantification of mean fluorescence (c) and patch diameters (d) from 24 patches as in a,b (quantified from 4 fields of view within a representative experiment, and consistent results in 3 independent experiments using differently labelled proteins, for example, Supplementary Fig. 6a–c). Curves and shaded areas represent mean ± s.e.m. (e) Kymograph of an experiment where Snf7-AlexaFluor-647N was added at $t=0$ min, followed by sequential addition of Vps24-AlexaFluor-488 and Vps2-Atto-565 (representative image from 24 patches within the shown experiment, and 1 additional independent experiment). (f) Kymograph of an ESCRT-III patch, where Snf7-AlexaFluor-488 was added at $t=0$ min, and then washed out during 28–32 min (shaded area), followed by addition of Vps2-Atto-565 and Vps24 at $t=47$ min. The transient increase of Vps2 signal during washout resulted from background ambient light. (g) Fluorescence quantification of 37 patches as in f (analysed from 4 fields of view within the shown experiment, and 3 additional independent experiments). Curves and shaded areas represent mean ± s.e.m. Scale bars, 5 μm in a; 5 μm (vertical) and 5 min (horizontal) in b,e,f.

We subsequently added fluorescently labelled Vps2 and Vps24, which enriched at the edge of the patch, where newly growing Snf7 filament spirals localize (Fig. 4f,g and Supplementary Video 7). Snf7 remained stably bound to patches throughout the entire imaging period, indicating that Snf7 polymers have extremely low intrinsic subunit dissociation rates irrespective of their association.
Figure 5 Vps2 and Vps24 polymerize side-by-side with Snf7 to form filament bundles. (a) Transmission electron microscopy of Snf7 spirals polymerized on liposomes. Coloured overlays indicate the number of parallel filament strands. (b) Distribution of filament bundle lengths quantified in 11 spirals from 3 independent experiments as in a. (c) Snf7 was polymerized on liposomes, followed by Vps2 and Vps24 addition. Coloured overlays indicate the number of parallel filament strands. (d) Quantification of 17 spirals from 2 independent experiments as in c. (e) Examples of filament morphologies with different strand numbers, corresponding to coloured overlays used in a–d. (f) Averaged line profiles across ESCRT-III filament bundles from a–d (n = 3 filaments for 1 strand, n = 8 filament bundles for 2 strands, n = 3 filament bundles for 3 strands, n = 8 filament bundles for 4 strands; and n = 3 filament bundles for 6 strands). Curves and shaded areas indicate mean ± s.e.m. (g–i) HS-AFM imaging of ESCRT-III polymers on supported lipid membranes. Snf7 was polymerized on lipid membranes, followed by addition of Vps2 and Vps24 at t = 0. (g,h) Spiral morphology before and after addition of Vps2 and Vps24 (g). Green and magenta lines indicate spiral cross-sections used to measure height variability (h). (i) Height variability was measured as the coefficient of variation along radial line profiles within spirals before and after addition of Vps2 and Vps24, respectively, as shown in g,h (n = 28 spirals for Snf7, and n = 26 spirals for Snf7 + Vps2 + Vps24 from 2 independent experiments). Statistical test using the two-sided Kolmogorov–Smirnov test yielded P = 2.875 \times 10^{-14} for Snf7 relative to Snf7 + Vps2 + Vps24. Dots represent individual line profile measurements; bars indicate medians. ****P < 0.0001. Scale bars, 50 nm in a,c,e; 200 nm in g.

Vps2 and Vps24 form filament bundles with Snf7

We next investigated whether growth inhibition by Vps2 and Vps24 could arise from an ultrastructural change in ESCRT-III polymers. Negative stain electron microscopy showed that Snf7 alone polymerized on liposomes to form one-start spirals containing a single 4.5-nm-wide filament, which occasionally paired between neighbouring turns (Fig. 5a,b), as previously observed. When Vps2 and Vps24 were added after Snf7 polymerization, filaments appeared double-stranded and neighbouring spiral turns occasionally bundled to form quadruple strands with an approximate width of 15 nm (Fig. 5c,d) — close to the width of ESCRT-III-dependent filaments observed at the abscission site in vertebrate cells (Fig. 5c–f; compare Fig. 3c). Given the one-start single-stranded geometry of Snf7 spirals prior to addition of Vps2/Vps24, the paired filaments probably represent lateral copolymers of Vps2/Vps24 along Snf7.

To further characterize the morphological changes of Snf7 filaments following addition of Vps2/24, we visualized ESCRT-III assemblies by high-speed atomic force microscopy (HS-AFM) (Fig. 5g). Snf7 alone formed spirals with pronounced filaments, but subsequent addition of Vps2/Vps24 induced a compact disc-like morphology (Fig. 5g–i). This is consistent with a filament thickening and bundling limiting access of the AFM tip in between neighbouring spiral turns.

with Vps2 and Vps24. The inhibition of Snf7 patch growth imposed by Vps2 and Vps24 is thus independent of Vps4 and caused by a reduced rate of Snf7 subunit incorporation.
Together, these data suggest that Vps2/Vps24 might reduce the rate of Snf7 polymerization through the formation of bundled filaments.

**Vps4 induces subunit turnover and net growth of ESCRT-III assemblies in vitro**

In cells, Vps2 and Vps24 are both present in the cytoplasm, raising the question of how ESCRT-III polymerization is sustained over prolonged periods. Our *in vivo* observations imply the possibility that Vps4 might leverage Vps2/Vps24-mediated growth inhibition by ESCRT-III turnover. To explore whether Vps4 promotes ESCRT-III turnover *in vitro*, we separately measured ESCRT-III subunit association and dissociation kinetics. We first determined the rate by which Vps4 disassembles Snf7 patches in the absence of Vps2 and Vps24. We polymerized Snf7 on supported lipid membranes, washed out soluble Snf7, and then added Vps4 and ATP. This did not cause detectable disassembly of Snf7 patches even at very high Vps4 concentrations (Fig. 6a,b), indicating that Vps4-mediated Snf7 depolymerization strictly depends on Vps2/Vps24, as shown before. We thus quantified the rate of Vps4-mediated ESCRT-III patch disassembly in the presence of Vps2 and Vps24. We first polymerized Snf7 patches on supported lipid membranes, then removed the soluble pool of Snf7, and subsequently added a mix of fluorescently labelled Vps2 and unlabelled Vps24, Vps4 and ATP. Vps2 first bound to Snf7 patches and subsequently partially disassembled together with Snf7 (Fig. 6c,d and...
Figure 7 Vps4 induces subunit turnover and net growth of ESCRT-III assemblies. (a) Time-lapse microscopy of ESCRT-III polymerization on supported lipid membranes. Snf7-AlexaFluor-488 was injected at t = 0 min. Vps2-Atto-565 and Vps24 were added at t = 36 min while maintaining Snf7 in the solution. At t = 45 min, Snf7-AlexaFluor-488 was removed and a mix containing Snf7-Atto-647N, Vps2-Atto-565, Vps24 and Vps4 was added, followed by addition of ATP at t = 54 min. (b) Kymograph of a single patch from a. (c) Mean fluorescence quantification of 35 patches as in b (4 fields of view within the shown experiment, and consistent results in 2 additional independent experiments using differently labelled proteins). Curves and shaded areas represent mean ± s.e.m. (d,e) Time-lapse microscopy of in vitro polymerization as in a, but for a mixed solution containing Snf7-AlexaFluor-488, Vps2-Atto-565, Vps24 and ATP in the presence of Vps4 (d), or without Vps4 (e). Representative images of 2 independent experiments per condition are shown. Scale bars, 10 μm in a,d,e; 5 μm (vertical) and 5 min (horizontal) in b.

Supplementary Fig. 7a,b and Supplementary Video 8). Thus, Vps4-mediated Snf7 depolymerization is fast enough to account for dynamic turnover of its homologue CHMP4B at the midbody in human cells.

To characterize the Snf7-disassembly process at the molecular level, we visualized morphological changes of individual ESCRT-III spirals by HS-AFM. We polymerized Snf7 patches on supported lipid bilayers, then added Vps2 and Vps24, and subsequently washed out the soluble components. We then added Vps4 and ATP and acquired HS-AFM videos, which showed that ESCRT-III spirals reduced their diameter (Fig. 6e,f and Supplementary Video 9). When omitting ATP from the reaction, Vps4 did not disassemble ESCRT-III spirals (Fig. 6g and Supplementary Video 10), confirming that ESCRT-III disassembly is an energy-consuming process. Given that the ESCRT-III spirals did not depolymerize below a certain diameter, these data suggest that Vps4 mediates Snf7 filament disassembly predominantly from the outer regions of spirals, whereby inner spiral segments are refractory to disassembly.

We next tested whether Vps4 can mediate Snf7 turnover in vitro. For this purpose, we used Snf7 subunits labelled with two distinct fluorophores. We first incubated supported lipid bilayers with AlexaFluor-488-labelled Snf7 until patches formed and then added Vps24 and ATP to inhibit further patch growth (Fig. 7a–c, see 0–45 min, and Supplementary Video 11). We then exchanged the soluble pool of Snf7-AlexaFluor-488 with Snf7-Atto-647N while maintaining Vps2 and Vps24 in the reaction. At this point, we also added Vps4. Snf7 polymer patches maintained constant size and did not incorporate Atto-647N-Snf7, presumably because ATP was not yet present (Fig. 7a–c, see 45–54 min). Indeed, subsequent ATP addition caused dissociation of AlexaFluor-488-Snf7 from patches, but also concomitant binding of Atto-647N-Snf7 (Fig. 7a–c, see 54–88 min). Thus, an ATP-dependent activity of Vps4 promotes not only disassembly of ESCRT-III but also the formation of new ESCRT-III polymers on membranes.

Interestingly, Vps4 and ATP also restored the macroscopic growth of Snf7 patches despite the continued presence of Vps2 and Vps24 (Fig. 7a,b, see 58–88 min, and Supplementary Video 11). We thus tested whether a solution containing Vps4, ATP and all three ESCRT-III subunits supports nucleation and growth of ESCRT-III polymers as observed in vivo. Strikingly, this combination resulted in efficient nucleation and growth of ESCRT-III patches, in contrast to a mix that lacked Vps4 (Fig. 7d,e). Furthermore, the net ESCRT-III assembly rate increased with higher concentrations of Vps4 (Supplementary
ATP (Fig. 8c and Fig. 6g). Thus, Vps4 induces dynamic steady state growth and shrinkage of ESCRT-III filaments in the presence of growth-inhibiting Vps2 and Vps24 subunits.

Vps4 induces dynamic growth and shrinkage of ESCRT-III filament spirals

To study Vps4-mediated polymer remodelling at the molecular level, we visualized ESCRT-III spirals by AFM. We assembled Snf7 spirals on supported lipid bilayers and then added Vps2, Vps24, and ATP. This induced a drastic reorganization of ESCRT-III polymers: pre-existing spirals reduced their size, while many new spirals nucleated and grew in between the original spirals (Fig. 8a,b and Supplementary Video 12). The innermost parts of ESCRT-III spirals appeared refractory to disassembly and thus might represent the stably bound fraction of ESCRT-III observed by fluorescence microscopy. In contrast, pre-existing spirals remained unchanged in the absence of ATP (Fig. 8c and Fig. 6g). Thus, Vps4 induces a dynamic steady state with both growing and shrinking ESCRT-III spirals when Snf7, Vps2 and Vps24 are present in solution.

**DISCUSSION**

Our study shows that membrane-bound ESCRT-III polymers rapidly turn over subunits with cytoplasmic pools while they assemble into larger structures. The dynamic subunit turnover is driven by Vps4 and is necessary to sustain efficient net growth of ESCRT-III assemblies in the presence of inhibitory Vps2 and Vps24 subunits.

Most previous models for ESCRT-III-mediated membrane fission imply sequential recruitment of different ESCRT-III subunits and Vps4 to establish distinct phases of polymer growth, reorganization/maturation and disassembly. However, our study shows that different ESCRT-III subunits and Vps4 accumulate at the midbody with indistinguishable kinetics. This does not rule out
sequential subunit binding at the single-filament level, but indicates that the distinct stages of ESCRT-III accumulation, constriction and disassembly during abscission cannot be explained by a sequence of subunit binding.

Our findings call for re-interpretation of the terminal phenotype resulting from Vps4 depletion—the accumulation of ESCRT-III polymers in endosomes (termed class E compartment in yeast) that has led to the model of Vps4 serving predominantly as a disassembly factor.26,33,47 We show that Vps4 is important for efficient ESCRT-III assembly, yet we also note that residual slow ESCRT-III polymerization in the absence of Vps4 is sufficient to ultimately capture all cytoplasmic subunits in class E compartments—owing to the extremely low intrinsic subunit dissociation rates.

At the molecular level, Vps4 might promote the net growth of ESCRT-III assemblies by constant turnover of Vps2 and Vps24 in side-by-side copolymers along Snf7 filaments—to create growth-competent-free Snf7 filament tips (Fig. 8g). Furthermore, Vps4 might induce subunit turnover at the core of ESCRT-III filament bundles, in analogy to interaction of the Vps4 homologue Spastin with the lattice of microtubules.50 In contrast to previous models,5,31 however, Vps4 does not cut an ESCRT-III helix during a single definite time point to induce tension release, but rather continuously remodels filaments. Within bundled filaments, such turnover does not necessarily lead to a complete breakage of larger structures.

The innermost parts of ESCRT-III spirals appear refractory to disassembly even at high concentrations of Vps4, suggesting that ESCRT-III interaction with Vps4 might depend on mechanical stress owing to filament curvature or on filament polarity. Potential stochastic fluctuations in subunit turnover rates could then lead to dynamic growth and shrinkage of ESCRT-III spirals.

Vps4-induced subunit turnover in ESCRT-III assemblies might directly contribute to membrane constriction. Indeed, macroscopic shape changes of many other cellular polymer structures critically depend on dynamic subunit turnover within the constituent filaments, as for example mitotic spindles or actomyosin rings.53 In ESCRT-III assemblies, Vps4-induced subunit turnover might facilitate sliding of adjacent helix turns, thereby promoting constriction of mechanically pre-stressed, low-curved filaments into more relaxed high-curvature states.34 The underlying bending forces could be generated by binding of Vps2 and Vps24 to Snf7 (ref. 41), or by shortening-induced increase of filament rigidity. By revealing dynamic subunit turnover in ESCRT-III assemblies, our study provides a framework for understanding how this highly conserved membrane fission machinery adapts to diverse membrane geometries.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

Note: Supplementary Information is available in the online version of the paper.

**ACKNOWLEDGEMENTS**

D.W.G. has received financial support from the European Community’s Seventh Framework Programme FP7/2007-2013 under grant agreements no. 241548 (MitoSys) and no. 258068 (Systems Microscopy), from an ERC Starting Grant (agreement no. 261198), from the Wiener Wissenschaft- , Forschungs- und Technologiefonds (WWTF; project no. LS14-009), and from the Austrian Science Fund (FWF; project no. SFB F34-06). B.E.M. has received a PhD fellowship from the Boehringer Ingelheim Fonds. A.R. acknowledges funding from: Human Frontier Science Program (HFSP), Young Investigator Grant no. RGY0076-2008: the European Research Council (ERC), starting (consolidator) grant no. 311536-MEMPRO: the Swiss National Fund for Research, grants no. 131003A_130520 and no. 131003A_149975. N.C. acknowledges the European Commission for the Marie-Curie post-doctoral fellowship CYTOCUT no. 200352-2011. J.M.v.F. acknowledges funding by an EMBO long-term fellowship (ALTF 1065-2015). T.M.-R. has received funding from the Deutsche Forschungsgemeinschaft (DFG) grant MU1423/4-1. S.S. acknowledges funding by an ANR grant ANR-Nano (ANR-12 BSIO-009-01) and a European Research Council (ERC) Starting Grant (no. 310080, MEM-STRUCT-FEM). The authors thank D. Tris, M. Alonso Y Adell, C. Campsteijn and J. Gruenberg for comments on the manuscript, the IMBA/IMP/GMI BioOptics core facility for technical support, the EM Facility of the Vienna Biocenter Core Facilities (VBCF), who performed parts of the sample preparation and electron microscopy, E. Humbert for protein purification, C. Sommer and R. Höller for statistical advice, C. Blaukopf for technical support, W. Reiter for providing S. cerevisiae genomic DNA, and Life Science Editors for editing assistance.

**AUTHOR CONTRIBUTIONS**

B.E.M. designed, conducted and analysed all cell biological experiments, and analysed part of the HS-AFM data. N.C. designed, conducted and analysed in vitro reconstitution experiments based on fluorescence microscopy. L.R.-M. designed, conducted and analysed HS-AFM experiments. J.M.v.F. and N.C. designed, conducted and analysed electron microscopy of in vitro-assembly ESCRT-III polymers. J.K. and T.M.-R. designed, conducted and analysed electron microscopy experiments of intercellular bridges. I.L. established the CHMP4B purification and produced labelled CHMP4B. I.P. generated HeLa cells stably expressing mmtVPS4B-LAP.

B.E.M., N.C., D.W.G., A.R. and S.S. conceived the project, analysed data and wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Published online at http://dx.doi.org/10.1038/ncb3559

Reprints and permissions information is available online at www.nature.com/reprints

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Hurley, I. H. ESCRTs are everywhere. *EMBO J.* 34, 2398–2407 (2015).

2. Hanson, P. I. & Cashikar, A. Multivesicular body morphogenesis. *Annu. Rev. Cell Dev. Biol.* 28, 337–362 (2012).

3. Carlton, J. G. & Martin-Serrano, J. Parallels between cytokinesis and retroviral budding: a role for the ESCRT machinery. *Science* 316, 1908–1912 (2007).

4. Guizetti, J. et al. Cortical constriction during abscission involves helices of ESCRT-III-dependent filaments. *Science* 331, 1616–1620 (2011).

5. Elia, N., Sougrat, R., Spurlin, T. A., Hurley, J. H. & Lippincott-Schwartz, J. Dynamics of endosomal sorting complex required for transport (ESCRT) machinery during cytokinesis and its role in abscission. *Proc. Natl Acad. Sci. USA* 108, 4846–4851 (2011).

6. Lafaurie-Janvore, J. et al. ESCRT-III assembly and cytokinetic abscission are induced by tension release in the intercellular bridge. *Science* 339, 1625–1629 (2013).

7. Mierzwa, B. & Gerlich, D. W. Cytoytickinetic abscission: molecular mechanisms and temporal control. *Dev. Cell* 31, 525–538 (2014).

8. Vietri, M. et al. Spastin and ESCRT-III coordinate mitotic spindle disassembly and nuclear envelope sealing. *Nature* 522, 231–235 (2015).

9. Olmos, Y., Hodgson, L., Manteli, J., Verkade, P. & Carton, J. G. ESCRT-III controls nuclear envelope reformation. *Nature* 522, 236–239 (2015).

10. Raab, M. et al. ESCRT III repairs nuclear envelope ruptures during cell migration to limit DNA damage and cell death. *Science* 352, 359–362 (2016).

11. Denis, C. M. et al. Nuclear envelope rupture and repair during cancer cell migration. *Science* 352, 353–358 (2016).

12. Jimenez, A. J. et al. ESCRT machinery is required for plasma membrane repair. *Science* 343, 1247134 (2016).

13. von Schwedler, U. K. et al. The protein network of HIV budding. *Cell* 114, 701–713 (2003).

14. Bleck, M. et al. Temporal and spatial organization of ESCRT protein recruitment during HIV-I budding. *Proc. Natl Acad. Sci. USA* 111, 12211–12216 (2014).

15. Baetti, M. F. et al. Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. *Nat. Cell Biol.* 16, 677–685 (2012).

16. Choudhuri, K. et al. Polarized release of T-cell-receptor-enriched microvesicles at the immunological synapse. *Nature* 507, 118–123 (2014).

17. Mazets, T. et al. The ESCRT machinery regulates the secretion and long-range activity of Hedgehog. *Nature* 516, 99–103 (2014).

18. Henne, W. M., Steinsmark, H. & Emr, S. D. Molecular mechanisms of the membrane sculpting ESCRT pathway. *Cold Spring Harb. Perspect. Biol.* 5, a016766 (2013).
METHODS Cloning and generation of stable cell lines. A Gateway recombination cassette was inserted into pcDNA3/FRT/TO (Invitrogen) to generate a Tet-inducible pTO_H2B-mRED in medium containing 0.5 μg ml⁻¹ puromycin (Merck), 400 μg ml⁻¹ zeocin (Invitrogen), but no G418. Cells were then FACs-sorted for the absence of red fluorescence, and single clones were isolated. Inducible ESCRT-III constructs were then engineered into the FRT site by co-transfection with pOG44 (Invitrogen) at a ratio of 1:10, and selected in medium containing 300 μg ml⁻¹ hygromycin B (Roche) and 0.5 μg ml⁻¹ puromycin (Merck).

To generate a cell line expressing mpVPS4B-LAP, the bacterial artificial chromosome (BAC) RP24-139J22 was used to GEP-tag mouse VPS4B using BAC-TransGeneOmics®. Homologous recombination yielded BAC DNA containing VPS4B-LAP, which was isolated and transfected into HeLa cells using Effectene (Qiagen), and selected in medium containing 400 μg ml⁻¹ G418 (Invitrogen). All HeLa BAC-GFP cell lines were cultured in medium supplemented with 300 μg ml⁻¹ G418 (Invitrogen). No cell line used in this study is listed in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI Biosample. The HeLa wild-type cell line (Kyoto strain) was obtained from S. Narumiya (Kyoto University, Japan) and validated by a Multiplex human Cell line Authentication test (MCA, 21.04.16). All cell lines have been regularly tested for mycoplasma, and contamination has never been detected.

Cell culture, live-cell microscopy and image analysis. HeLa cells were cultured at 37 °C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 10% (v/v) fetal calf serum (FCS, Invitrogen) and 1% (v/v) penicillin–streptomycin (Sigma-Aldrich). For live-cell imaging, cells were plated in LabTek II chambered coverslips (ThermoFisher Scientific) or glass-bottom dishes (μ-Dish 35mm×100mm Glass Bottom, Ibidi) in cell culture medium without ribonulbin and phenol red to minimize background fluorescence. Microtubules were stained with 100 nM SIr-tubulin and 10 nM verapamil (Sigma-Aldrich; to inhibit influx of SIr-tubulin from cells), at least 1 h before imaging. Protein expression in cell lines bearing H2B-mRED-LAP, hCHAmp3-LAP or hsCHMP4B-LAP was induced with 1 μg ml⁻¹ doxycycline (Sigma-Aldrich), titrated to match levels of mmCHMP4B-LAP from the BAC construct. For expression of yeast Snf7-LAP, HeLa Kyoto FRT were transfected with pTO_SNf7_LAP using X-tremeGENE 9 (Roche) 24 h prior to imaging, and induced with 0.05 ng ml⁻¹ doxycycline (Sigma-Aldrich).

Cells were imaged on a Zeiss LSM780 confocal microscope controlled by ZEN 2011 and an AutoFocussScreen kindly provided by J. Ellenberg (EMBL). Imaging was performed in a humidified atmosphere of 5% CO₂ and 37 °C in an incubation chamber (EMBL).

For analysis of accumulation kinetics, cells were imaged with a 63 × 1.4 NA oil DIC Plan-Apochromat objective (Zeiss), ESCRT-III levels at the midbody were quantified in single optical sections using Fiji®. For normalization, the fluorescence signal prior to ESCRT-III accumulation was set to zero. Due to the variable duration of abscission, we chose the earliest time point for which we had data in all cells, which was at 48 min before abscission. Peak signals were normalized to the average midbody fluorescence from 12 min before to 6 min after abscission. Accumulation curves in VPS4A/B depletion experiments were normalized to 18–30 min after complete cleavage furrow ingestion.

For quantification of abscission timing, cells were imaged with a 40 × 1.4 NA oil DIC Plan-Apochromat objective (Zeiss). Abscission timing was measured from the duration from complete cleavage furrow ingestion until the first disassembly of the midbody microtubules. For VPS4B rescue experiments, only cells expressing mmVPS4B-LAP were analysed.

FRAP experiments. Midbodies were imaged using a 63 × 1.4 NA oil DIC Plan-Apochromat objective (Zeiss). Photobleaching was performed in circular regions with 2.5 μm diameter at 42-fold higher laser intensity compared with that used for imaging. Fluorescence was measured in midbody regions following by background subtraction. For normalization, acquisition photobleaching and ESCRT-III net accumulation during the recording interval was measured separately for early and late-stage midbodies. FRAP curves were then normalized to the mean of the pre-bleach fluorescence and to the first frame after photobleaching. For all different constructs, cells with expression levels similar to that of mmCHMP4B-LAP cells were used.

To determine FRAP recovery kinetics, we fitted single exponential functions 
\[ f(t) = 1 - e^{-kt}, \] 
single exponential functions with a variable immobile fraction 
\[ f(t) = A - (1 - e^{-kt}), \] 
and double exponential functions 
\[ f(t) = A_1 (1 - e^{-k_1 t}) + A_2 (1 - e^{-k_2 t}), \] 
using the Levenberg–Marquardt nonlinear least-squares algorithm (minpack.1m package for R, version 3.0.2). Convergence to minimal residuals was reached in ~70% of the cases. Residence times were calculated from the dissociation rates 1/k₁ and 1/k₂. Because the slow dissociation rate k₂ was frequently fitted as 0 due to a completely stable fraction, we derived the slow residence times from the Taylor expansion to calculate the reciprocal mean from 
\[ E[Y] = E[1/X] = \mu_e = (1/\mu_0 + (\sigma^2/\mu_0^2)), \] 
and standard deviation from 
\[ \sigma = (\sigma^2/\mu_0^2), \] 
where μ is the mean and σ is the standard deviation. Since the long residence time could not be measured accurately due to the relatively short duration of measurements, we constrained k₂ to the mean ± s.d. of mmCHMP4B-LAP in all experiments. For comparison of highly mobile fractions, we additionally constrained k₂ to the mean of mmCHMP4B-LAP, which corresponds to a residence time of 19.5 s.

siRNA treatment. Cells were transfected using Lipofectamine RNAiMax (Invitrogen) with non-targeting siRNA silConControl (Ambion, sense strand: UCUCC-GAACUGUACCAAGU), or siRNAs targeting CHMP4B (Microsynth, sense strand: AGAAAGAGAGGAGGAGGAGGAT(5′)-3′), VPS4A (Microsynth, sense strand: CGCA-GACAGCAGACAGCAAGAAGT(5′)-3′), or VPS4B (Microsynth, sense strand: CCAA-GAGGACUCUGAAGAGAT(5′)-3′), at a final concentration of 80 nM. Samples were analysed at the indicated times after transfection.

Generation and microinjection of CHMP4B. To generate a CHMP4B bacterial expression plasmid containing an N-terminal 6×His tag followed by a maltose binding protein (MBP) and a tobacco etch virus (TEV) protease cleavage site, human CHMP4B was cloned into pMBP-HIS2-Sn7 (Addgene no. 21492), by replacing Sn7 with PCR-amplified CHMP4B using BamHI and NotI.

For production of recombinant protein, the plasmid was transformed into Escherichia coli Rosetta cells and expressed for 3 h at 30 °C with induction with 0.5 mM IPTG. Bacteria were lysed by sonication at 4 °C in lysis buffer containing 20 mM Hesper pH 8, 100 mM NaCl, 1% Triton X-100, and cOmpule Protease Inhibitor Cocktail (Roche). CHMP4B was purified using affinity chromatography (MBFTrap HP 5 ml column, GE Healthcare), washed with 20 mM Hesper pH 8, 250 mM NaCl, 0.1% Triton X-100, and eluted in 20 mM Hesper pH 8, 10 mM maltose, followed by removal of maltose using Zeba Spin Desalting Columns (7K MWCO 5 ml, ThermoFisher Scientific). After cleavage with TEV protease, the resulting His-MBP fragment was removed by incubation with Ni-NTA Agarose beads (ThermoFisher Scientific) for 1 h, followed by incubation with Dextrin Sepharose beads (Pharmacia) for 1 h to remove remaining protein that was not cleaved by TEV protease.

Purified CHMP4B was labelled with Atto-565-NHS ester (Sigma-Aldrich), by incubation with two molar excesses of reactive dye in the presence of 100 mM NaHCO₃, pH 9. Excess free dye was removed by dialysis against 20 mM Hepes pH 8 at 4 °C using a MWCO membrane of 12–14kDa, and the sample was further purified using a Zeba Spin desalting column (ThermoFisher Scientific). The resulting labelling density was ~80%. Protein aggregates were removed by ultracentrifugation at 100,000g for 10 min at 4 °C, and the protein was stored at ~80 °C after flash freezing.

For microinjection into cells, CHMP4B was ultracentrifuged for 30 min at 80,000g at 4 °C and loaded into Femtotips (Eppendorf). Microinjection was performed using an InjectMan 4 micromanipulator (Eppendorf) and the FemtoJet 4i (Eppendorf) microinjection system, with an injection pressure of 100 kPa, an injection time of 0.2–0.5 s, and a maintenance pressure of 20 kPa. The microinjection system was mounted on the Zeiss LSM780 system described above, and cells were imaged with a 40 × 1.4 NA oil DIC Plan-Apochromat objective (Zeiss) immediately before and after injection. Fluorescence intensity of injected CHMP4B-Atto-565 was measured in midbody regions containing a CHMP4B-LAP signal. The mean intensity of the surrounding region was subtracted for background correction, and the first time point after injection was normalized to 0.

Western blot analysis. Cells were seeded and transfected as described above in LabTek II chambered coverslips (ThermoScientific). Protein extracts were separated on 4–12% NuPAGE Bis-Tris gradient gels (Life Technologies) and transferred to nitrocellulose (Protran, GE Healthcare) or PVDF membranes (Amersham HybridBond, GE Healthcare). Western blotting was performed by standard methods using antibodies against CHMP4B (1:1,000, Abcam, ab105767), actin (1:30,000, Merck Millipore, MA1B501), VPS4B (1:500, Abcam, ab137027), GFP (1:5,000, Abcam, ab290) and GAPDH (1:2,500, Abcam, ab9485).

DOI: 10.1038/ncb3559
Electron microscopy of high-pressure frozen cells. HeLa cells were seeded in T75 flasks and transfected with siRNAs using Lipofectamine RNAiMAX (Invitrogen). At 23 h post-transfection, a primary shake-off was performed to remove dead cells and debris. Mitotic cells were harvested ~2 h later with a second shake-off, and seeded on 3 mm diameter coverslips. Coffee filter paper (Sigma) was placed on top. Cell suspensions were filtered through a 50 mm aluminium specimen carrier (type B, flat side up) and covered with a second carrier (type A), which had a 50 mm cavity filled with 10% (w/v) BSA (AppliChem) in cell culture medium, followed by high-pressure freezing in an HPF Compact 01 (M. Wohlwend GmbH). Freeze substitution was performed using a Leica EM AF5-2, in a medium of anhydrous acetone (Merck) containing 1% osmium tetroxide (Electron Microscopy Sciences) and 0.1% uranyl acetate (Merck) according to the following protocol: 21 h at ~90 °C, warm up at a rate of 5 °C per hour to ~30 °C, 6 h at ~30 °C, warm up at a rate of 5 °C per hour to 0 °C, 2 h at 0 °C. Specimens were washed 3 times in anhydrous acetone (Merck) at 4 °C and embedded in an Epon/Araldite resin (Electron Microscopy Sciences). Serial thin sections of a nominal thickness of 100 nm were collected on Formvar-coated (Electron Microscopy Science) copper slot grids (PLANO). Sections were post-stained with 2% aqueous uranyl acetate (Polysciences) and Reynold’s lead citrate (Electron Microscopy Science), and imaged on an FEI Morgagni 268D transmission electron microscope (FEI) at 80 kV with a Morada CCD camera (Olympus-SIS), or on an EM 906 transmission electron microscope (Zeiss) at 80 kV with a slow-scan CCD camera (Albert-Tröndle Systems).

ESCRT-III protein purification. ESCRT-III yeast proteins were expressed from plasmids encoding Snf7 (Addgene No. 21492), Vps2 (Addgene No. 21494), Vps24 (gift from the J. Hurley laboratory, Molecular Biophysics and Integrated Biomaging Division, Lawrence Berkeley National Laboratory), Vps4 (Addgene No. 21495) and purified as previously described71. Snf7 with a cysteine residue at the N terminus of the protein was purified as in ref. 61. Snf7 was labelled either with maleimide-Atto-647N (Atto-Tec AD 647N-4), or with TFP-AlexaFluor-488 (ThermoFisher Scientific, A30005). Vps2 was labelled with Atto-565 (Atto-Tec AD 565-3), and Vps24 with Atto-488 (Atto-Tec AD 488-3). The proteins were used at the following concentrations: [Snf7], 300 nM; [Vps2], 100 nM; [Vps24], 100 nM; [Vps4], 2 μM, except in Fig. 6a,b and Supplementary Figs 5e,g and 7, where concentrations are indicated in the figures. When added to the reaction, ATP concentration was 2 mM. Fluorescent proteins were mixed with unlabelled proteins at the following ratios: Snf7-Atto-647N, Vps2-Atto-565, Vps24-Atto-488: 1/3 labelled with 2/3 unlabelled; Snf7-AlexaFluor-488: 2/3 labelled with 1/3 unlabelled.

Preparation of giant unilamellar vesicles (GUVs). GUVs were prepared by electrospraying using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycerol-3-phosphoamine (DOPG) mixtures (Avanti Polar Lipids). Twenty microlitres of 1 mg ml⁻¹ lipid mix (DOPC/DOPG, 6:4, mol/mol) were deposited on two indium tin oxide-coated glass slides (70–100 Ω resistivity, Sigma-Aldrich) and placed in a vacuum drying oven for 1 h for complete solvent evaporation. An open tor rubber joint of ~1 mm thickness was used as a non-leaky spacer between the two ITO slides, and the chamber was formed by compressing the two slides with spring metal tweezers. The chamber was filled with 400 μl of 500 mM sucrose solution and exposed to 1 V a.c. current (10 Hz sinusoidal) for 20 min, followed by addition of 1 μl of the GUVs suspension. GUVs were incubated for 10 min, and gently rinsed with imaging buffer (10 mM Tris-HCl, 150 mM KCl, pH 7.4), before immersion into the HS-AFM fluid chamber (volume: ~120 μl). After SLB assessment and image acquisition area positioning, Snf7 was added to the fluid chamber to a final concentration of 1 μM. After ~20 min, Snf7 filaments started to form on the SLB and assembled into spiral structures in the following minutes. Vps2 and Vps24 were then added to the AFM fluid chamber to a final concentration of 100 nM. Next, Vps4 was injected to a final concentration of 1–2 μM. Finally, ATP and MgCl₂ were added to the fluid chamber to a final concentration of 200 μM and 1 mM, respectively.

HS-AFM and image analysis. Imaging was performed at room temperature on an HS-AFM 1.0 (RIBM) using ~8-μm-long cantilevers (NanoWorld) with resonance frequency of ~0.7 MHz in liquid and a spring constant of ~0.15 Nm⁻¹. Typically, membrane-bound patches were selected and imaged using the 0.75 μl tip of 1 ml. Imaging was performed only in membrane-covered areas of the coverslip, which were distinguishable from the non-membrane-covered glass surface on the basis of an unspecified background fluorescence of the labelled ESCRT-III subunits adsorbing onto glass. For all experiments, a 2-μm-thick volume stack surrounding the coverslip surface was acquired with 0.5 s per pixel acquisition rate and a 1 s minute time lapse.

The raw data were processed in three steps. First, the three-dimensional stacks were projected in two dimensions by a Fiji plugin, which determines the right focal surface plane along a z-stack. Second, the field illumination inhomogeneity was corrected by dividing all of the images by an image of a homogeneously labelled surface. Third, the x–y stage drift throughout the image acquisition was corrected using the plugin Turboreg. For each experiment, patches that did not fuse completely with neighbouring patches (manually selected for quantification). For each patch, a slice was manually selected to extract a kymograph. The integrated fluorescence intensity of these kymographs was measured and maximum values were normalized to 1 for all experiments, except for Fig. 7c, where curves were adjusted to match the end point of the experiment. To calculate kinetics of patch disassembly, Snf7 mean patch intensity curves were fitted to exponential decay function $f(t) = (1 - a) \cdot e^{-t/t_1} + a$.

Electron microscopy of ESCRT-III filaments. For electron microscopy, multilamellar vesicles made of DOPC/DOPS, 6:4 (mol/mol), were prepared at 5 mg ml⁻¹. For Snf7-only experiments, vesicles at 0.5 mg ml⁻¹ were incubated for 1 h with Snf7 at 750 nM. For Snf7 + Vps2 + Vps24 experiments, Snf7 was first incubated as before, and then Vps2 and Vps24 were added, each at 500 nM final concentration for 1 h. All samples were then centrifuged for 4 min at 4000g, washed, resuspended in fresh Formvar-coated EM grids, and then negatively stained for 30 s with 2% uranyl acetate. Images were acquired using a Tecnai G2 Sphera (FEI) electron microscope.

Sample preparation for HS-AFM. One microlitre of adsorption buffer (10 mM Hepes, 220 mM NaCl, 25 mM MgCl₂, pH 7.4) was added to freshly cleaved mica, followed by addition of 1 μl of the GUVs suspension. GUVs were incubated for 10 min, and gently rinsed with imaging buffer (10 mM Tris-HCl, 150 mM KCl, pH 7.4), before immersion into the HS-AFM fluid chamber (volume: ~120 μl). After SLB assessment and image acquisition area positioning, Snf7 was added to the fluid chamber to a final concentration of 1 μM. After ~20 min, Snf7 filaments started to form on the SLB and assembled into spiral structures in the following minutes. Vps2 and Vps24 were then added to the AFM fluid chamber to a final concentration of 100 nM. Next, Vps4 was injected to a final concentration of 1–2 μM. Finally, ATP and MgCl₂ were added to the fluid chamber to a final concentration of 200 μM and 1 mM, respectively.

HS-AFM and image analysis. Imaging was performed at room temperature on an HS-AFM 1.0 (RIBM) using ~8-μm-long cantilevers (NanoWorld) with resonance frequency of ~0.7 MHz in liquid and a spring constant of ~0.15 Nm⁻¹. Typically, membrane-bound patches were selected and imaged using the 0.75 μl tip of 1 ml. For each 0.04 s, 300 pixels and 200 x 200 pixels and frame acquisition rates ranged between 1 s⁻¹ and 2 s⁻¹.

For HS-AFM video analysis, we corrected stage drift using the StackReg plugin in Fiji, and performed a moving average of 2 to 4 consecutive time frames. ESCRT-III spiral diameters and line profiles were manually measured using Fiji. Coefficients of variation were determined for 45 nm-long sections of the line profiles from the raw data, used to mean. TrackMate was used for manual and semi-automated tracking of spiral centres with a ~5 s interval. Tracks shorter than 55 s were omitted from the analysis.

Statistics and reproducibility. To account for non-normal distributions and unequal variances, statistical analysis was performed using the two-sided Kolmogorov–Smirnov test using R (version 3.0.2) or GraphPad Prism 7. Numbers of repeats and experiments are indicated in the respective figure legends, and always refer to independent biological experiments, with all samples showing the same behaviour. All experiments were performed three or more times independently under identical or similar conditions, except experiments shown in Figs 3a,b, 4e, 5d–g, 6a–d and 7e,d and Supplementary Figs 1a,3a,b,d and 6d,e, which were performed twice, and experiments in Fig. 3c,d, and Supplementary Fig. 7a,b, which were performed once.

Data availability. All data supporting the findings of this study are available from the corresponding author on request.

54. Zemp, I. et al. Distinct cytoplasmic maturation steps of 40S ribosomal subunit precursors require hRi202. J. Cell Biol. 185, 1167–1180 (2009).
55. Posen, I. et al. BAC TransgeneOmics: a high-throughput method for exploration of protein function in mammals. Nat. Methods 5, 409–415 (2008).
56. Hein, M. Y. et al. A human interactome in three quantitative dimensions organized by stoichiometries and abundances. Cell 163, 712–723 (2015).
57. Schmitz, M. H. & Gerlich, D. W. Automated live microscopy to study mitotic gene function in fluorescent reporter cell lines. *Methods Mol. Biol.* **545**, 113–134 (2009).
58. Lukinavicius, G. et al. Fluorogenic probes for live-cell imaging of the cytoskeleton. *Nat. Methods* **11**, 731–733 (2014).
59. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676–682 (2012).
60. Morita, E. et al. Human ESCRT-III and VPS4 proteins are required for centrosome and spindle maintenance. *Proc. Natl Acad. Sci. USA* **107**, 12889–12894 (2010).
61. Wollert, T. & Hurley, J. H. Molecular mechanism of multivesicular body biogenesis by ESCRT complexes. *Nature* **464**, 864–869 (2010).
62. Aguet, F., Van De Ville, D. & Unser, M. Model-based 2.5-d deconvolution for extended depth of field in brightfield microscopy. *IEEE Trans. Image Process* **17**, 1144–1153 (2008).
Supplementary Figure 1 Validation of CHMP4B depletion and FRAP fitting of a single exponential model with a variable mobile fraction. (a) Western blot of whole cell lysates from HeLa cells stably expressing mmCHMP4B-LAP from an endogenous promoter, probed by anti-CHMP4B antibody, 48 h after transfection of an siRNA specifically targeting hsCHMP4B, or non-targeting negative control siRNA. Anti-α-actin antibodies were used as a loading control. Unprocessed scans are shown in Supplementary Fig. 8a, b. Western Blot was repeated in 2 independent experiments. (b-d) Comparison of a single exponential fit that allows an immobile fraction $f(t) = A^* (1 - e^{-k*t})$, and a double exponential fit $f(t) = A1^* (1 - e^{-k1*t}) + (1 - A1^*) (1 - e^{-k2*t})$ to data in Fig. 1c ($n = 18$ cells from 4 independent experiments). (b) Overlay of the fitted curves. Points and shaded area indicate mean ± SEM of fluorescence; dashed lines indicate fits of single or double exponential functions. (c) Residuals of the fits from b. (d) Residence times and mobile fractions derived from single exponential fits and highly mobile pools from double exponential fits. Dots represent individual cells.
**Supplementary Figure 2** Different LAP-tagged ESCRT-III subunits do not perturb abscission and have similar dynamics. (a–d) Abscission timing was measured by time-lapse microscopy and related to expression levels based on GFP fluorescence. Expression was induced in HeLa cell lines carrying stable integrations of (a) hsCHMP2B-LAP (n = 50 cells from 4 independent experiments), (b) hsCHMP3-LAP (n = 57 cells from 3 independent experiments), (c) hsCHMP4B-LAP (n = 21 cells from 3 independent experiments), or (d) mmCHMP4B-LAP (n = 25 cells from 3 independent experiments). Each dot represents a single cell. (e–g) Quantification of FRAP in telophase cells at pre-constriction stages for (e) hsCHMP2B-LAP (n = 19 cells from 3 independent experiments), (f) hsCHMP3-LAP (n = 16 cells from 3 independent experiments), or (g) hsCHMP4B-LAP (n = 24 cells from 3 independent experiments). Points and shaded areas indicate mean ± SEM; dashed lines indicate optimal fits in the upper panels of single exponential functions $f(t) = A^* (1 - e^{-k_1 t})$ and double exponential functions $f(t) = A_1^* (1 - e^{-k_1 t}) + (1 - A_1^*) (1 - e^{-k_2 t})$ to the mean recovery curves, with the residuals plotted in the lower panels.
Supplementary Figure 3 LAP-tagged VPS4B is functional and VPS4A/B depletion perturbs ESCRT-III assembly at the midbody. (a-b) Western blot of whole cell lysates from cells stably expressing mmVPS4B-LAP from an endogenous promoter, probed by (a) anti-GFP or (b) anti-VPS4B antibodies, 48 h after transfection of siRNAs specifically targeting hsVPS4A and hsVPS4B, or non-targeting negative control siRNA. Anti-GAPDH antibodies were used as a loading control. The asterisk marks a non-specific band. Unprocessed scans are shown in Supplementary Fig. 8, c-f. Western Blots were repeated in 2 independent experiments. (c) Quantification of mmCHMP4B-LAP accumulation at the midbody 48 h after transfection of siRNAs targeting hsVPS4A/B (n = 19 cells from 3 independent experiments), or non-targeting negative control siRNAs (n = 15 cells from 3 independent experiments), normalized and temporally aligned to the time point of complete cleavage furrow ingression (time point 0). Curves and shaded areas represent mean ± SEM. (d-e) Cytoplasmic levels of (d) hsCHMP2B-LAP or (e) hsCHMP3-LAP 20 h after transfection of siRNAs targeting hsVPS4A/B (n = 39 cells from 2 independent experiments, or n = 79 cells from 4 independent experiments, respectively), or non-targeting negative control siRNAs (n = 76 cells from 2 independent experiments, or n = 59 cells from 4 independent experiments, respectively). Dots represent individual cells; bars indicate medians.
Supplementary Figure 4 VPS4 is required for rapid incorporation of microinjected recombinant CHMP4B into midbody-localized ESCRT-III. (a) Recombinant human CHMP4B-Atto-565 was microinjected into telophase HeLa cells stably expressing mouse CHMP4B-LAP from an endogenous promoter, stained with SiR-tubulin. Time 0 indicates first image after injection. (b) As in a but 48 h after transfection of siRNAs specifically targeting hsVPS4A/B. (c) Quantification of hsCHMP4B-Atto-565 midbody incorporation in untreated cells (n = 8 cells from 3 independent experiments), or 48 h after transfection of siRNAs targeting hsVPS4A/B (n = 10 cells from 3 independent experiments). Curves and shaded areas indicate mean ± SEM. Fluorescence intensity of injected CHMP4B-Atto-565 was measured in midbody regions based on the CHMP4B-LAP signal. To correct for cytoplasmic background within the midbody region, the mean intensity of the surrounding area was subtracted from the region of interest. Scale bars, 5 µm, or 1 µm (inset) in a, b.
**Supplementary Figure 5** Validation of *in vitro* ESCRT-III reconstitution assay. (a) Localization of budding yeast Snf7-LAP in live HeLa cells during abscission. (b) FRAP of Snf7-LAP at a midbody at late abscission stages. (c) Fluorescence recovery and double exponential fit for early and late abscission as in b (n = 10 cells from 3 independent experiments). Points and shaded area indicate mean ± SEM. (d) Highly mobile fraction and residence time for data shown in c. Dots represent individual cells; bars indicate medians. (e-g) ESCRT-III patches form exclusively on lipid membranes. Lipid membranes were labelled with DOPE-Atto647N (left) in a flow chamber. Snf7-Alexa488 was then added, followed by addition of Vps2-Atto565 and Vps24. (e) Regions covered by burst GUVs are visible as dark regions in the Vps2 channel because they lack unspecific background fluorescence. Images show a representative experiment, with consistent observations in 2 additional independent experiments with differently labeled proteins. (f-g) Kymographs of single ESCRT-III patches as indicated by lines in e. Patch 1 (f) localizes to central area on the membrane and shows unconstrained growth, whereas patch 2 (g) shows that growth stops when ESCRT-III patches reach the edge of the membrane. Vps2-Atto565 and Vps24 were injected together in two steps, first at 1 nM where they did not prevent Snf7 growth, second at 10 nM where they bind and prevent growth. Scale bars, 5 µm or 1 µm (inset) in a; 1 µm in b; 10 µm in e; 5 µm (vertical) and 5 min (horizontal) in f, g.
Supplementary Figure 6 Vps2 and Vps24 cooperatively bind Snf7 patches and inhibit Snf7 polymerization. (a) Long-term time-lapse microscopy of fluorescently labeled budding yeast ESCRT-III subunits during polymerization on supported lipid membranes in microfluidic chambers. A solution of Snf7-AlexaFluor-488 was injected into the flow chamber at t = 0 min; Vps2-Atto-565 and unlabeled Vps24 were added at t = 22 min. (b) Quantification of patch fluorescence as in a (n = 7 patches from 4 fields of view within the shown experiment, and additional independent experiment is shown in Fig. 4a-d). Curves and shaded areas indicate mean ± SEM. (c) The addition of Vps2 and Vps24 inhibits patch growth independently of their size. Kymograph of multiple patches of a representative experiment where Snf7-AlexaFluor-647N was added at t = 0 min, followed by addition of Vps2-Atto-565 and unlabeled Vps24 at t = 29 min. Similar experiments are shown in a, and Fig. 4a-d. (d) Snf7-AlexaFluor-647N was added at t = 0 min, followed by addition of Vps2-Atto-565 at t = 26 min, and Vps24-AlexaFluor-488 at t = 33 min. (e) Kymograph of an ESCRT-III patch from d (representative example from 24 patches in 4 different fields of view within the shown experiment, and 1 additional independent experiment with differently labelled proteins). Scale bars, 5 µm (vertical) or 5 min (horizontal) in a, c, e; 10 µm in d.
Supplementary Figure 7 ESCRT-III patch growth and disassembly kinetics under various conditions. (a) Representative patches of three experiments with different Vps4 concentrations. For each experiment, Snf7-AlexaFluor-488 patches were polymerized, then Snf7 was washed out while Vps2-Atto-565 and Vps24 were added for ~10 min. ATP and Vps4 (at 1, 2, or 4 µM, respectively) were then added to the solutions. Scale bars, 5 µm (vertical) or 5 min (horizontal). (b) Quantification of mean fluorescence of several patches shown in e (n = 16 patches for 1 µM Vps4, n = 14 patches for 2 or 4 µM Vps4, from 4 fields of view per condition from 1 independent experiment). Curves and shaded areas indicate mean ± SEM; dotted lines indicate optimal fits to . Fitted parameters for 1 µM Vps4: τ = 4.7 ± 1.0 min, a = 37 ± 3%; 2 µM Vps4: τ = 2.6 ± 1.5 min, a = 38 ± 2%; and for 4 µM Vps4: τ = 1.4 ± 0.5 min, a = 30 ± 3%. (c) Quantification of patch radial growth speed in presence of Snf7/Vps2/Vps24/Vps4+ATP as a function of Vps4 concentration. Patches were pre-nucleated with 500 nM Snf7, followed by addition of Vps2/Vps24, ATP and different concentrations of Vps4 (0.5, 1, 2, or 4 µM). Dots represent individual patches from 4 independent experiments per condition; bars and error bars indicate mean ± SD.
Supplementary Figure 8 Unprocessed images of Western Blots. Full scans of Western Blots shown in (a-b) Supplementary Fig. 1a, (c-d) Supplementary Fig. 3a, and (e-f) Supplementary Fig. 3b. Red rectangles indicate cropped regions shown in figures.
Supplementary Video 1 Dynamic subunit turnover in CHMP4B at the midbody before and during constriction. FRAP of mmCHMP4B-LAP (green) at the midbody, stained with SiR-tubulin (magenta). Left panel shows early abscission (before constriction), and right panel shows late abscission (during constriction). Circles indicate photobleaching region; time point 0 is the first frame recorded immediately after photobleaching. Scale bars, 1 μm.

Supplementary Video 2 Accumulation of CHMP2B and CHMP3 at the midbody. 3-D live cell confocal microscopy of the intercellular bridge during telophase in HeLa cells expressing hsCHMP2B-LAP (left) or hsCHMP3-LAP (right), stained with SiR-tubulin. Time point 0 indicates complete disassembly of the midbody-associated microtubule bundle during abscission. Scale bars, 1 μm.

Supplementary Video 3 Accumulation of CHMP4B and VPS4B at the midbody. Telophase intercellular bridges of HeLa cells expressing mmCHMP4B-LAP (left) or mmVPS4B-LAP (right), stained with SiR-tubulin. Time point 0 indicates complete disassembly of the midbody-associated microtubule bundle during abscission. Scale bars, 1 μm.

Supplementary Video 4 Vps2 and Vps24 inhibit Snf7 polymer growth. Time-lapse microscopy of fluorescently labeled budding yeast ESCRT-III subunits during polymerization on supported lipid membranes in a microfluidic flow chamber. A solution of Snf7-AlexaFluor-488 was injected into the flow chamber at t = 0 min. Vps2-Atto-565 (magenta) together with unlabeled Vps24 were added after 22 min while maintaining initial concentration of Snf7 in the solution, as depicted in the timeline above the movie. Insets show kymographs of a selected patch indicated by a yellow line. Dashed yellow line indicates time point of solution exchange in the flow chamber. Scale bar, 10 μm.

Supplementary Video 5 Vps2 requires Vps24 to bind Snf7 polymers. Time-lapse microscopy of fluorescently labeled budding yeast ESCRT-III subunits during polymerization on supported lipid membranes in a microfluidic flow chamber. A solution of Snf7-AlexaFluor-488 (green) was injected into the flow chamber at t = 0 min. Vps2-Atto-565 was added after 25 min, and Vps24-AlexaFluor-488 was added after 32 min while maintaining the initial Snf7 concentration in the solution, as depicted in the timeline above the pictures. Insets show kymographs of a selected patch indicated by a yellow line. Dashed yellow lines indicate time points of solution exchange in the flow chamber. Scale bar, 10 μm.

Supplementary Video 6 Vps24 requires Vps2 to bind Snf7 polymers. Time-lapse microscopy of fluorescently labeled budding yeast ESCRT-III subunits during polymerization on supported lipid membranes in a microfluidic flow chamber. A solution of Snf7-AlexaFluor-647N was injected into the flow chamber at t = 0 min. Vps24-AlexaFluor-488 was added after 19 min, and Vps2-Atto-565 was added after 30 min while maintaining the initial Snf7 concentration in the solution, as depicted in the timeline above the pictures. Insets show kymographs of a selected patch indicated by a yellow line. Dashed yellow lines indicate time points of solution exchange in the flow chamber. Scale bar, 10 μm.

Supplementary Video 7 Snf7 polymers have very low intrinsic subunit dissociation rates. Time-lapse microscopy of Snf7 patch polymerization on supported lipid membranes in a microfluidic flow chamber. Snf7-AlexaFluor-488 was added, and then removed from the buffer solution at t = 34 min. Vps2-Atto-565 and unlabeled Vps24 were simultaneously added to the solution at t = 50 min, as depicted in the timeline above the pictures. Insets show kymographs of a selected patch indicated by a yellow line. Dashed yellow lines indicate time points of solution exchange in the flow chamber. Scale bar, 10 μm.

Supplementary Video 8 Vps4 rapidly disassembles ESCRT-III patches in the absence of soluble Snf7. Time-lapse microscopy of Snf7 polymer patches on supported lipid membranes in a microfluidic flow chamber. A solution of Snf7-AlexaFluor-488 was injected into the flow chamber and incubated until patches polymerized on the supported lipid membrane. At t = 34 min Snf7 was removed from the solution and Vps2, Vps24, Vps4, and ATP were injected, as depicted in the timeline above. Insets show kymographs of a selected patch indicated by a yellow line. The dashed yellow line indicates the time point of solution exchange in the flow chamber. Scale bar, 10 μm.

Supplementary Video 9 Vps4 induces shrinkage of ESCRT-III spirals in the presence of ATP. HS-AFM movie showing disassembly of ESCRT-III spirals by Vps4. ESCRT-III patches were assembled by polymerization of Snf7, and subsequent addition of Vps2 and Vps24. After washout of all soluble components, Vps4 was injected. Then, ATP and Mg2+ were added and imaging was started 22 s later (t = 0). Scale bar, 100 nm.

Supplementary Video 10 ESCRT-III spirals form an immobile and stable array in presence of Vps4 and absence of ATP. Snf7 was polymerized into supported lipid membranes, followed by addition of Vps2 and Vps24. Then, Vps4 but no ATP was added, and imaging was started 30 s later (t = 0). Scale bar, 200 nm.

Supplementary Video 11 Vps4 counteracts growth-inhibition imposed by Vps2/Vps24 and promotes rapid turnover of Snf7 subunits. Visualization of subunit exchange in ESCRT-III polymers by time-lapse microscopy using two distinctly labeled Snf7 pools. Snf7-AlexaFluor-488 was injected into the flow chamber until polymer patches formed on supported lipid membranes. Vps2-Atto-565 and Vps24 were added at t = 36 min while maintaining Snf7-AlexaFluor-488 in the solution. At t = 45 min, soluble Snf7-AlexaFluor-488 was removed from the solution, and a mix containing Snf7-Atto-647N, Vps2-Atto-565, Vps24 and Vps4 was injected. At t = 54 min, the same mix plus ATP was added to the solution. Insets show kymographs of a selected patch indicated by a yellow line. Dashed yellow lines indicate time points of exchanging the solution in the flow chamber. Scale bar, 10 μm.

Supplementary Video 12 Vps4 induces dynamic reorganization of ESCRT-III assemblies in the presence of soluble ESCRT-III subunits. HS-AFM movie showing dynamic reorganization of ESCRT-III spirals by Vps4. ESCRT-III patches were assembled by polymerization of Snf7, and subsequent addition of Vps2 and Vps24, Vps4, and ATP/Mg2+. Imaging was started 5.5 min later (t = 0). Scale bar, 200 nm.