

Structural basis for ESCRT-III protein autoinhibition

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Endosomal sorting complexes required for transport-III (ESCRT-III) subunits cycle between two states: soluble monomers and higher-order assemblies that bind and remodel membranes during endosomal vesicle formation, midbody abscission and enveloped virus budding. Here we show that the N-terminal core domains of increased sodium tolerance-1 (IST1) and charged multivesicular body protein-3 (CHMP3) form equivalent four-helix bundles, revealing that IST1 is a previously unrecognized ESCRT-III family member. IST1 and its ESCRT-III binding partner, CHMP1B, both form higher-order helical structures *in vitro*, and IST1-CHMP1 interactions are required for abscission. The IST1 and CHMP3 structures also reveal that equivalent downstream $\alpha 5$ helices can fold back against the core domains. Mutations within the CHMP3 core- $\alpha 5$ interface stimulate the protein's *in vitro* assembly and HIV-inhibition activities, indicating that dissociation of the autoinhibitory $\alpha 5$ helix from the core activates ESCRT-III proteins for assembly at membranes.

The endosomal sorting complexes required for transport (ESCRT) pathway functions in a series of important membrane-remodeling processes, including multivesicular body vesicle formation at endosomal membranes, enveloped virus budding from the plasma membrane and midbody abscission during cytokinesis (reviewed in refs. 1–4). In performing these functions, ESCRT components cycle between two different states: an inactive state, in which they are dispersed throughout the cytoplasm, and an active state, in which they are recruited to specific membranes by upstream adaptor proteins that induce tight membrane binding and component assembly. ESCRT recruitment and assembly ultimately lead to membrane fission, producing endosomal vesicles, budded viruses or daughter cells. The ESCRT pathway is therefore apparently a mobile machine that can be recruited to different membranes to help mediate membrane remodeling and fission.

Most of the approximately 30 proteins of the human ESCRT pathway function as components of one of five different complexes (termed the ESCRT-0–III and VPS4 complexes). These complexes are recruited sequentially to sites of action, with each successive complex recruiting and activating downstream complexes for membrane association and assembly^{1–4}. Recent studies have revealed that subunits of the late-acting ESCRT-III complex can form rings, helical filaments and tubes *in vitro* and *in vivo*. Thus, ESCRT-III complexes may form ‘collars’ within the cytoplasm-filled necks of membrane vesicles and tubules that can constrict the neck and promote membrane fission^{5–10}. Variants of this general model suggest that one specific subset of ESCRT-III proteins, those of the CHMP4/Snf7p (Snf7p is a yeast CHMP4 homolog) family, may create an encircling ring or spiral, and the other ESCRT-III proteins serve primarily to nucleate and cap the CHMP4 filament^{8–10}. ESCRT-III proteins also recruit VPS4 ATPases

and induce them to assemble into active complexes. Enzyme recruitment and activation are further promoted by the accessory factors IST1^{11–14} and LIP5 (Vtalp in yeast)^{1–4}, both of which can bind ESCRT-III and VPS4 proteins. Once assembled, VPS4 uses the energy of ATP hydrolysis to release the assembled ESCRT-III machinery back into the cytoplasm, thereby allowing multiple rounds of membrane deformation and fission¹⁰.

The 11 known human ESCRT-III-like proteins can be divided into seven different families, six of which (CHMP1–6) correspond to the six ESCRT-III-like proteins of *Saccharomyces cerevisiae*. Most ESCRT-III proteins can form both homo- and hetero-oligomers and can pair preferentially at membrane sites of action (for example, CHMP4–CHMP6 and CHMP2–CHMP3)^{6,15}. ESCRT-III proteins vary considerably in primary sequence, but all share common N-terminal core domains of ~150 residues. The core domain of one ESCRT-III protein, CHMP3_{8–183}, has been crystallized and shown to form a four-helix bundle¹⁶. The longer first two helices form an extended hairpin, and the shorter two helices pack at the open end of the hairpin. Sequences located beyond the helical core have autoinhibitory activities, and there is now considerable biochemical and genetic evidence that these downstream sequences prevent premature membrane binding and assembly^{6,16–21}. Downstream sequence elements also bind various late-acting ESCRT factors, including ALIX²² and a series of microtubule-interacting and transport (MIT) domain-containing proteins and enzymes such as ubiquitin hydrolases (for example, AMSH and UBPY)^{23,24}, proteases (calpain 7)^{25,26}, ATPases (Spastin and VPS4 proteins)^{21,27–30} and ATPase activators (LIP5 (Vtalp))^{31–33}. Different ESCRT-III subunits have distinct binding partner specificities, helping to explain the need for so many different ESCRT-III proteins.

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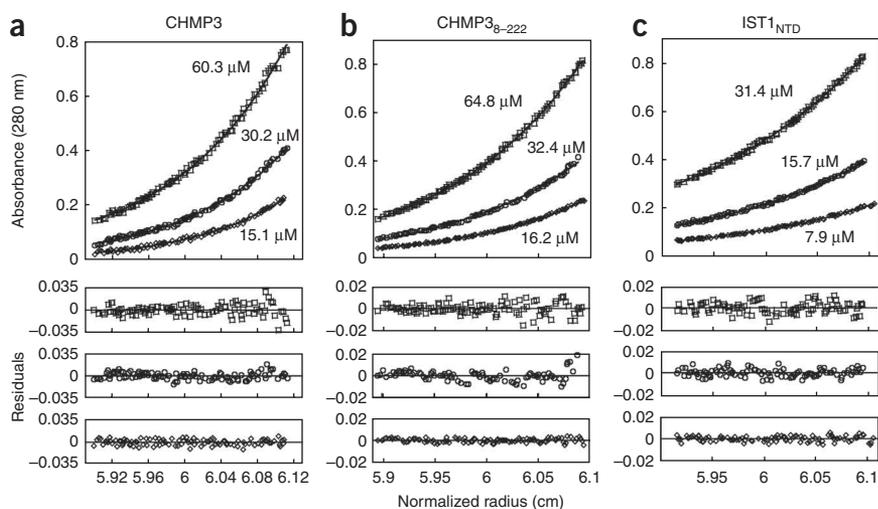


Figure 1 CHMP3, CHMP3₈₋₂₂₂ and IST1_{NTD} are monomers in solution. (a–c) Equilibrium sedimentation distributions of recombinant CHMP3 (a), CHMP3₈₋₂₂₂ (b) and IST1_{NTD} (c) (above), and residual differences (below), with data points shown in open symbols and the single species models shown as solid lines. Rotor speeds were 20,000 r.p.m. and the initial subunit protein concentrations are shown. Data sets were also collected at 24,000 r.p.m. (not shown) and all of the data were globally fit to single species models in which the molecular weights were allowed to float during the refinement. Estimated molecular weights were: CHMP3, 25,840 Da (molecular weight of the monomer (MW_{monomer}) = 25,267 Da, $M_{\text{obs}}/M_{\text{calc}}$ = 1.02); CHMP3₈₋₂₂₂, 24,390 Da (MW_{monomer} = 24,663 Da, $M_{\text{obs}}/M_{\text{calc}}$ = 0.99); IST1_{NTD}, 20,520 Da (MW_{monomer} = 21,791 Da, $M_{\text{obs}}/M_{\text{calc}}$ = 0.94).

IST1 is a recently identified, late-acting ESCRT pathway factor that binds LIP5, VPS4 and CHMP1 (Did2p in yeast)^{11–14}. The precise function(s) of IST1 are not well understood and, indeed, IST1 can exert both positive^{11–14} and negative¹² effects on ESCRT pathway functions. Yeast Ist1p has synthetic interactions with the Vta1p–Vps60p (LIP5–CHMP5) complex in endosomal protein-sorting reactions, implying that Ist1p functions in the formation of multivesicular bodies (MVB), albeit in a non-essential role^{12,13}. Human IST1 localizes to midbodies, and its depletion inhibits abscission but not HIV budding^{11,14}. IST1 proteins therefore seem to have important roles in cytokinesis, auxiliary roles in endosomal protein sorting and no essential role in HIV budding. Biochemical and secondary-structure analyses indicate that IST1 is composed of a helical N-terminal domain (residues 1–189, here termed IST1_{NTD}, but also called the ‘ELYC’ domain after a conserved tetrapeptide sequence¹²), C-terminal MIT-interacting motifs (MIM) (residues 321–366) that can bind cooperatively to the MIT domains of VPS4 (refs. 11,14) and a central linker region that is predicted to lack persistent secondary structure (residues ~190–320)^{11,14}.

Extensive structural and biochemical analyses have revealed many of the relevant molecular interactions that allow different ESCRT components to associate with one another, with phosphatidylinositol-3-phosphate (PI(3)P)-containing membranes and with ubiquitylated protein cargoes^{1–4}. In contrast, the mechanisms that regulate ESCRT pathway assembly and disassembly are much less well understood. Here we have determined crystal structures of the human IST1 and CHMP3 proteins. Furthermore, we have performed complementary biochemical analyses that characterize the functional importance of the IST1–CHMP1 binding interaction in cytokinesis, and we suggest how ESCRT-III proteins can switch between their inactive, soluble states and their active, membrane-bound assemblies.

RESULTS

Characterization of recombinant CHMP3 and IST1 proteins

We developed methods for expressing and purifying recombinant human IST1 and CHMP3 proteins for structural and biochemical studies. Full-length CHMP3 and an N-terminally truncated construct (CHMP3₈₋₂₂₂) were both monomeric in solution, as analyzed by equilibrium sedimentation (Fig. 1a,b), in good agreement with a previous report¹⁶. Full-length IST1 was also monomeric under high-ionic-strength conditions (>300 mM NaCl)¹¹, but aggregated at lower ionic strength. The purified N-terminal domain of IST1

(IST1_{NTD}) behaved similarly to the full-length protein in that it was also monomeric under high-ionic-strength conditions (Fig. 1c) and insoluble in lower-ionic-strength solutions. This construct was particularly amenable for structural and biochemical studies, presumably because it lacked the proline-rich linker region present in the full-length protein.

Crystal structures of IST1_{NTD}

We crystallized IST1_{NTD} under high-ionic-strength conditions and determined the structure in space groups $P2_1$ (1.8-Å resolution) and $P4_32_12$ (2.6-Å resolution) (Table 1 and Supplementary Fig. 1). The structures were similar, and the higher-resolution $P2_1$ structure is described here. IST1_{NTD} is composed of eight helices that pack to create a flat, elongated structure, with overall dimensions of $21 \times 30 \times 60$ Å (Fig. 2a). The two longest helices, $\alpha 1$ and $\alpha 2$, form a helical hairpin that packs against the smaller $\alpha 3$ and $\alpha 4$ helices to create an asymmetric four-helix bundle (termed the ‘core’). Beyond the core, the segment between helices $\alpha 4$ and $\alpha 5$ forms a flap that tracks along the length of the molecule, packing against the $\alpha 2$ – $\alpha 3$ edge. The flap contains two short helices, designated αA and αB to maintain a consistent numbering scheme for conserved ESCRT-III helices. Helix $\alpha 5$ packs perpendicularly across helices $\alpha 1$ and $\alpha 2$ near the closed end of the hairpin and is termed the ‘autoinhibitory helix’. A final turn takes the polypeptide across the hairpin loop and into $\alpha 6$, which projects away from the core. This helix was seen in only the $P2_1$ crystal form, where it is stabilized by lattice contacts. As discussed below, the IST1_{NTD} structure seems to represent a closed, autoinhibited ESCRT-III conformation in which the flap and autoinhibitory helix fold against the core to prevent higher-order interactions.

IST1_{NTD} bears an unexpected resemblance to the published CHMP3₈₋₁₈₃ structure¹⁶ (Fig. 2a–c), and the four helices of the IST1_{NTD} core superimpose with the first four helices of CHMP3 with an r.m.s. deviation of 2.4 Å (128 C α positions). Muziol *et al.* noted that the $\alpha 1$ edge of CHMP3 is distinctly cationic and proposed that this surface binds membranes¹⁶. The equivalent IST1 surface is also basic, although the $\alpha 3$ – $\alpha 4$ face is even more so (Fig. 2d–f).

In addition to their similar core structures, the autoinhibitory $\alpha 5$ helices of CHMP3₈₋₁₈₃ and IST1_{NTD} sit in equivalent positions (Fig. 2a–c, $\alpha 5$, orange). Thus, all of the ordered helices in the CHMP3₈₋₁₈₃ structure have analogs in IST1_{NTD}. However, the assigned connectivity of the autoinhibitory helices differ between the two structures because this helix is connected in *cis* to the IST1_{NTD}

Table 1 IST1_{NTD} and CHMP3 crystallographic statistics

	IST1 _{NTD} : Native 1	IST1 _{NTD} : Peak 1	IST1 _{NTD} : Inflection 1	IST1 _{NTD} : Remote 1	IST1 _{NTD} : Native 2	CHMP3 ₈₋₂₂₂	CHMP3 ₁₋₁₅₀
Data collection							
Space group	<i>P</i> ₄ ₃ ₂ ₁ ₂	<i>P</i> ₂ ₁	<i>P</i> ₂ ₁	<i>P</i> ₆ ₁			
Cell dimensions							
<i>a</i> , <i>b</i> , <i>c</i> (Å)	57.2, 57.2, 157.2	57.6, 57.6, 157.1	57.6, 57.6, 157.2	57.7, 57.7, 157.3	29.3, 92.4, 33.25	36.5, 131.5, 48.5	111.5, 111.5, 30.6
α , β , γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 97.0, 90.0	90.0, 108.1, 90.0	90.0, 90.0, 120.0
Resolution (Å) ^a	40–2.6 (2.7–2.6)	40–3.4 (3.5–3.4)	40–3.4 (3.5–3.4)	40–3.4 (3.5–3.4)	50–1.80 (1.86–1.80)	50–4.0 (4.14–4.0)	25–3.7 (3.83–3.7)
<i>R</i> _{sym}	6.8 (34)	7.5 (16)	5.9 (15)	6.5 (14)	5.8 (15)	0.16 (15)	0.063 (55)
<i>I</i> / σ <i>I</i>	13.5 (1.7)	23.9 (5.0)	24.3 (4.4)	24.4 (3.8)	19.2 (4.6)	9.3 (2.9)	18.4 (2.2)
Completeness (%)	97.7 (88.3)	88.5 (56.5)	87.7 (55.4)	83.4 (42.8)	97.2 (79.1)	78.7 (43.9)	99.2 (96.8)
Redundancy	3.8 (2.8)	6.7 (5.8)	3.9 (3.6)	3.9 (4.0)	3.1 (1.8)	4.0 (2.8)	3.2 (3.1)
Refinement							
Resolution (Å)	40–2.6				50–1.80	38–4.0	24–3.7
No. reflections	7,968				15,213	2,936	2,449
<i>R</i> _{work} / <i>R</i> _{free} (%)	25.9 / 29.8				19.5 / 24.7	37	41
No. atoms							
Protein	1,438				1,558	2,274	973
Water	35				163	0	0
<i>B</i> -factors (Å ²)							
Protein	87.2				26.0	n/a ^b	n/a ^b
Water	69.0				39.4	n/a ^b	n/a ^b
r.m.s. deviations							
Bond lengths (Å)	0.0007				0.011	n/a ^b	n/a ^b
Bond angles (°)	1.2				1.3	n/a ^b	n/a ^b

^aValues in parentheses are for the highest-resolution shell. ^b*B*-factors and individual atomic parameters were not refined and are unchanged from the molecular replacement model.

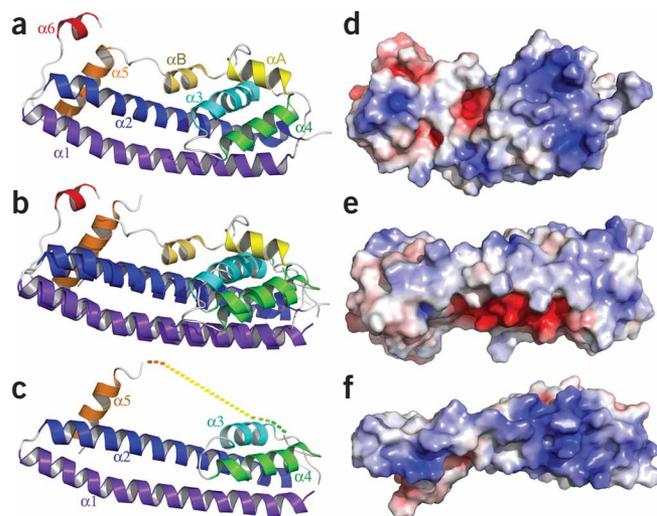
core via the flap segment, whereas it was assigned to an adjacent molecule in the CHMP3₈₋₁₈₃ lattice¹⁶. Muziol *et al.* noted, however, that the CHMP3₈₋₁₈₃ connectivity could not be defined unambiguously owing to a lack of visible electron density for the flap region¹⁶. We therefore speculate that the CHMP3₈₋₁₈₃ connectivity may actually match that of IST1_{NTD} and that the CHMP3₈₋₁₈₃ structure may also represent a closed conformation.

Crystal structures of CHMP3

The CHMP3₈₋₁₈₃ construct lacked the final 39 CHMP3 residues, which may have favored an open conformation¹⁶. We therefore attempted to remove this ambiguity by crystallizing both the full-length monomeric CHMP3₁₋₂₂₂ protein and a slightly truncated CHMP3₈₋₂₂₂ protein. Both proteins crystallized isomorphously in space group *P*₂₁, but CHMP3₈₋₂₂₂ diffracted to slightly higher resolution (4.0 Å), and this structure is therefore reported (Fig. 2c and Table 1). We determined the CHMP3₈₋₂₂₂ structure by molecular

replacement, using the core of CHMP3₈₋₁₈₃ as a search model, and then subjected the structure to rigid body refinement. Discernible features beyond the search model were limited, but the structure solution is supported by the molecular replacement statistics (see Online Methods) and by unbiased electron density maps (Supplementary Fig. 2). In addition to the four core helices, density for the autoinhibitory helix was evident in unbiased electron density maps, particularly for one of the two independent molecules in the asymmetric unit (Supplementary Fig. 2c,d). Although the connectivity between α 4 and α 5 was not defined by experimental electron density,

Figure 2 Structures of IST1_{NTD} and CHMP3. (a) Ribbon diagram and helix-labeling scheme for IST1_{NTD}. (b) Overlay of the ordered regions of IST1_{NTD} and CHMP3₈₋₁₈₃. (c) Ribbon diagram of CHMP3₈₋₂₂₂. (d) Space-filling model of IST1_{NTD}, color coded to show the surface charge distribution (blue, basic; red, acidic; ± 7 kV)³⁵ (created using PyMOL (<http://pymol.sourceforge.net>)). The molecule is shown in the same orientation as in a. (e) Same as d with the view toward α 1. Figure generated from d by rotation about the horizontal so that the bottom edge of d faces the viewer. (f) Space-filling model of CHMP3₈₋₂₂₂ shown in an equivalent orientation to the view of IST1_{NTD} shown in e, emphasizing the basicity of the α 1 surface of CHMP3 (ref. 16).



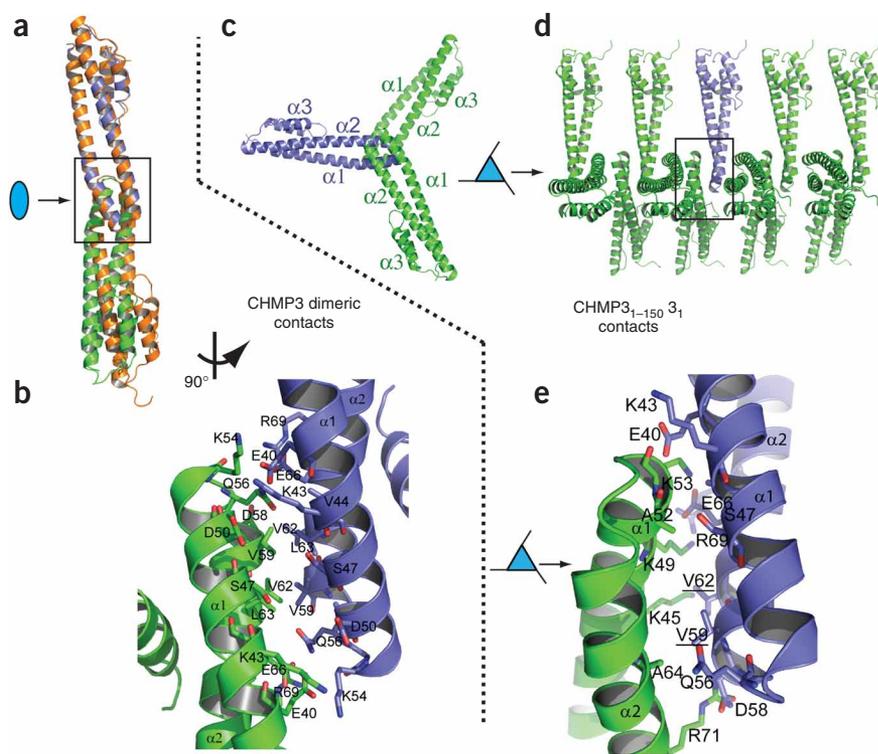


Figure 3 CHMP3 crystal packing interactions. **(a)** Overlay of the cores of the tip-to-tip dimers in the crystal lattices of CHMP3₈₋₁₈₃ (orange)¹⁶ and CHMP3₈₋₂₂₂ (blue and green). The upper subunits were aligned, and the lower subunits diverge owing to small differences in their tip-to-tip interfaces. **(b)** Detailed expansion of the boxed region of CHMP3₈₋₂₂₂ shown in **a**. Most side chain conformations are shown as defined in the CHMP3₈₋₁₈₃ search model, with some minor adjustments to avoid clashes. Precise description of these side chain conformations is not possible at current resolutions. **(c)** Subunit packing down the 3₁ screw axis of the CHMP3₁₋₁₅₀ crystal lattice, with a single CHMP3₁₋₁₅₀ molecule highlighted in blue. **(d)** Same assembly as in **c** but viewed perpendicularly to the 3₁ screw axis. **(e)** Detailed expansion of the boxed area shown in **d**. As in **b**, most side chain conformations are shown as defined in the CHMP3₈₋₁₈₃ search model, with some minor adjustments to avoid clashes.

Crystal packing interactions

We analyzed lattice contacts in the different IST1 and CHMP3 crystal structures to determine whether they were strong candidates for authentic interactions used in ESCRT-III protein assembly (see **Supplementary Figure 3** for a complete catalog of the different lattice interactions). Overall, there are no compelling reasons to believe that any of the various crystal contacts represent authentic assembly interfaces. Moreover, both of our CHMP3 structures lacked the ‘side-to-side’ dimer interface seen in the CHMP3₈₋₁₈₃ structure¹⁶ and postulated as a possible ESCRT-III assembly interface^{6,7}.

Two of the new CHMP3 interfaces merit comment because they are mediated by a conserved, exposed hydrophobic surface on the closed ‘tip’ of the $\alpha 1$ - $\alpha 2$ hairpin. In the CHMP₈₋₂₂₂ structure, two adjacent molecules associate through a small (359 Å²) two-fold symmetric ‘tip-to-tip’ dimer interface (**Fig. 3a,b**). A similar tip-to-tip interface was also present in the CHMP3₈₋₁₈₃ lattice¹⁶, and this is the only interface that has been seen in more than one crystal form.

$\alpha 5$ seems to sit in the same position in both the CHMP3₈₋₁₈₃ and the CHMP3₈₋₂₂₂ structures, despite different crystal packing interactions in the two lattices. We have connected $\alpha 4$ and $\alpha 5$ in *cis* rather than in *trans* to create a structure with the same topology as IST1_{NTD}, and this assignment is consistent with biochemical analyses described below.

Finally, we reasoned that a construct corresponding to the CHMP3 core alone could not adopt an autoinhibited conformation and might therefore reveal authentic protein-protein interactions used for ESCRT-III assembly. We crystallized the CHMP3 core construct (CHMP3₁₋₁₅₀) in space group P₆₁ and solved the structure by molecular replacement (3.7-Å resolution; **Table 1** and **Supplementary Fig. 2b**). As expected, the CHMP3₁₋₁₅₀ core was similar to the cores of the other CHMP3 and IST1_{NTD} structures, although, as described below, the lattice interactions were unique.

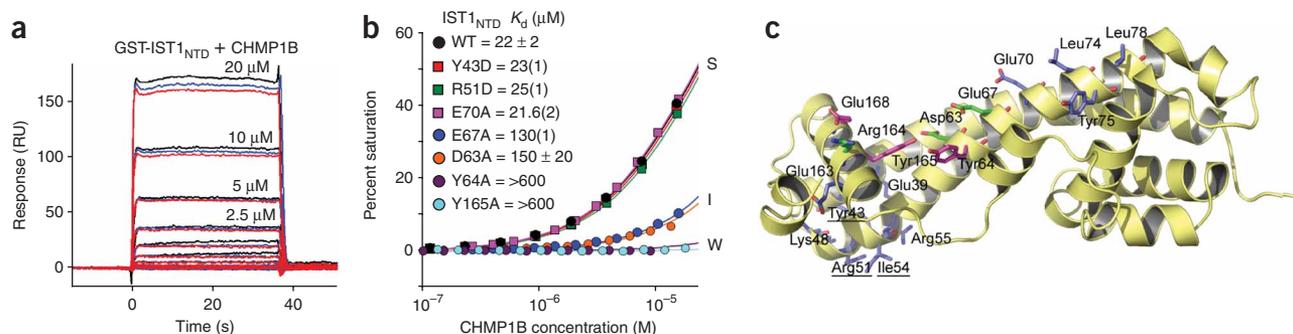


Figure 4 Mutational analyses of IST1-CHMP1B interactions. **(a)** Sensorgrams showing different concentrations of purified CHMP1B binding to immobilized GST-IST1_{NTD}. Triplicate measurements in response units (RU) are shown for each CHMP1B concentration. **(b)** Representative biosensor binding isotherms showing CHMP1B binding to wild-type (WT) and mutant GST-IST1_{NTD} proteins with strong (S), intermediate (I) and weak (W) binding affinities. IST1_{NTD} mutations and estimated dissociation constants are given in the inset. Errors represent either s.d. from multiple independent measurements ($n \geq 3$) or s.d. derived from single isotherms measured in triplicate (values in parentheses report the s.d. in the final digit of the measurement). **(c)** Ribbon diagram showing the location of all IST1_{NTD} mutations tested for CHMP1B binding. Mutated residues are shown explicitly, and the binding affinities of the mutant proteins are color coded as follows: blue, strong (S) binding (binding affinities within 1.5-fold of wild-type IST1_{NTD}); green, intermediate (I) binding (binding affinity reduced 1.5-fold to 8-fold); magenta, weak (W) binding (binding affinity reduced ≥ 8 -fold).

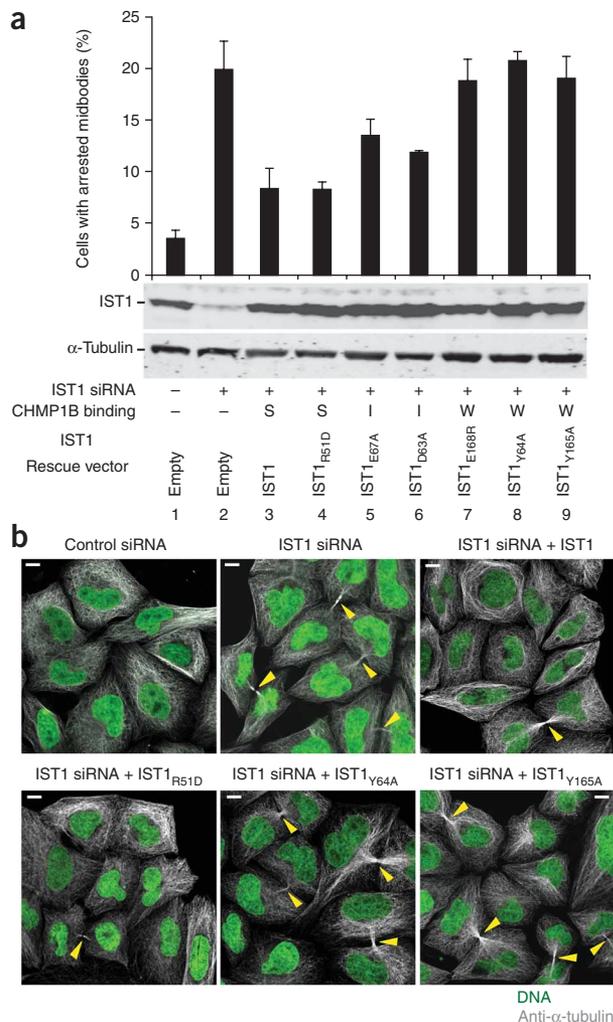


Figure 5 Requirement for IST1-CHMP1 interactions during abscission. (a) Above, quantified abscission defects as reflected in the percentages of HeLa M cells with visible midbodies following siRNA treatment to deplete endogenous IST1 (lanes 2–9) and rescue with an empty vector control (lane 2, negative control) or with vectors expressing wild-type IST1 (lane 3, positive control) or the designated IST1 mutants (lanes 4–9). Untreated cells are shown in lane 1. Error bars show s.d. from three independent repetitions of the experiment. Middle, western blot (anti-IST1) showing levels of soluble endogenous IST1 (lanes 1 and 2) or exogenously expressed IST1 proteins (lanes 3–9). Below, a western blot (anti- α -tubulin) showing expression levels of endogenous α -tubulin (loading control). CHMP1B binding phenotypes of the different IST1 proteins are shown below: strong (S), intermediate (I) or weak (W). (b) Immunofluorescence images showing the midbody phenotypes of cells from a designated subset of the experiments from a. Microtubules (anti- α -tubulin, gray) and nuclei (SYTOX Green) were stained for reference, and yellow arrowheads highlight midbodies. Scale bars, 10 μ m.

CHMP1B bound wild-type IST1_{NTD} with an estimated dissociation constant of $22 \pm 2 \mu\text{M}$ (Fig. 4a,b). CHMP1B binding was unaltered by most mutations tested, including mutations within the closed tip of the α 1- α 2 hairpin (for example, E39A, Y43D, K48D, R51D, I54D and R55D) and in the IST1_{NTD} surface that corresponded to the side-to-side dimer interface seen in the crystal structure of CHMP3_{8–183} and included the signature 73-ELYCEL-78 motif (for example, E70A, L74R, Y75A and L78R). However, one mutation along helix 2 (E67A) reduced CHMP1B binding in the immunoprecipitation and pull-down experiments and lowered the CHMP1B binding affinity six-fold in the biosensor binding assay, suggesting that this residue contributed to the CHMP1B binding site. We therefore tested mutations in adjacent surface-exposed residues to identify the CHMP1B binding site more precisely. Three IST1 mutants bound CHMP1B weakly (reductions in affinity of more than eight-fold; IST1_{Y64D}, IST1_{Y165A} and IST1_{E168R}), three bound CHMP1B with intermediate affinities (two- to eight-fold reductions; IST1_{D63A}, IST1_{E67A} and IST1_{R164D}) and one still bound strongly to CHMP1B (IST1_{E163R}; data not shown).

Given the proximity of the apparent CHMP1B binding site to the IST1 autoinhibitory helix α 5, we also tested the effects of a series of IST1 mutations predicted to destabilize the α 5-core interface (V56D, I60D, L166D and I169D). All of these IST1 mutations blocked CHMP1B binding entirely (data not shown), indicating that CHMP1B binds the closed conformation of IST1_{NTD} seen in the crystal structures. As summarized in Figure 4c, mutations that inhibited CHMP1B binding clustered within or near a groove created by packing of autoinhibitory helix α 5 against core helix α 2. The groove features a pair of exposed tyrosine residues (Tyr64 and Tyr165) that probably contribute to CHMP1B binding.

The IST1-CHMP1B interactions function in cytokinesis

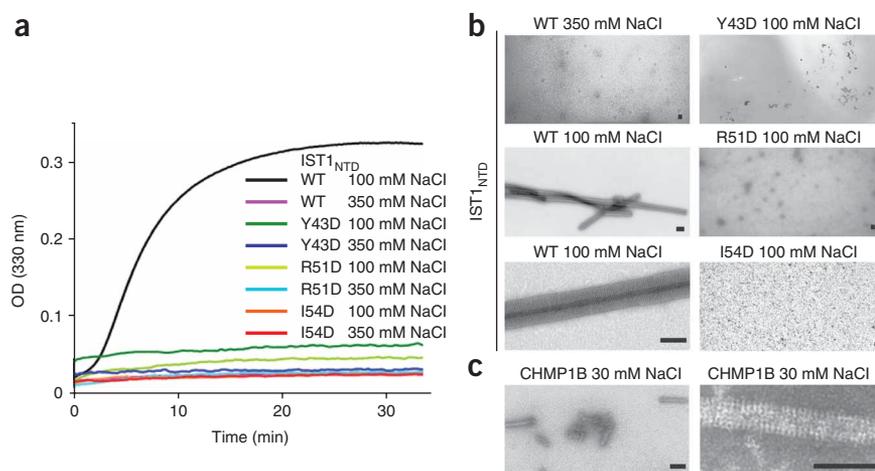
To determine whether CHMP1B binding was necessary for IST1 cytokinesis function(s), we tested whether IST1 mutations that impaired CHMP1B binding also inhibited abscission. Depletion of endogenous IST1 increased the percentage of HeLa cells with visible midbodies from $4 \pm 1\%$ to $20 \pm 3\%$ (Fig. 5a, compare lanes 1 and 2, and Fig. 5b, compare image above left with image above middle), and this midbody arrest defect was largely (but not entirely) rescued by re-expression of a wild-type, small interfering RNA (siRNA)-resistant IST1 construct ($8 \pm 2\%$; Fig. 5a, lane 3, and Fig. 5b, above right)¹¹. Midbody arrest was also corrected by an IST1 protein with a mutation in the tip of the α 1- α 2 hairpin that retained strong (S) CHMP1B binding (IST1_{R51D}, $8 \pm 1\%$; Fig. 5a, lane 4,

A different type of tip-to-tip interaction is present in the CHMP3_{1–150} lattice. In this case, the interaction lacks two-fold rotational symmetry and instead follows a crystallographic 3₁ screw axis that creates an infinite CHMP3_{1–150} filament with interfaces on both sides of the tip (Fig. 3c–e). The yeast homolog of CHMP3 (Vps24p) can assemble into three-stranded filaments *in vitro*⁷, although the relationship between the Vps24p filaments and the crystallographic CHMP3 filaments remains to be determined. In summary, the crystal packing interactions in our IST1 and CHMP3 structures do not obviously correspond to authentic biological interfaces, but do reinforce the idea that the exposed hydrophobic surface located at the tip of the helix 1-helix 2 loop is a preferred site for protein-protein interactions^{6,7,16}.

IST1_{NTD} binds CHMP1 proteins

Many ESCRT-III proteins bind one another, and previous studies have shown that the N-terminal domain of IST1 binds preferentially to the C-terminal region of the CHMP1 subset of ESCRT-III proteins^{11–14}. To map the IST1 binding surface more precisely, we created an ensemble of IST1 proteins with point mutations at conserved, surface-exposed residues and tested them for CHMP1A and CHMP1B interactions using: (i) immunoprecipitation reactions in 293T cells (not shown), (ii) glutathione S-transferase (GST) pull-down experiments with recombinant proteins (not shown) and (iii) biosensor binding experiments in which pure recombinant CHMP1B bound immobilized GST-IST1_{NTD} (Fig. 4).

Figure 6 IST1_{NTD} and CHMP1B tube assembly. (a) Assembly of wild-type (WT) IST1_{NTD} or three different IST1_{NTD} mutants with the designated amino acid substitutions at the tip of the $\alpha 1$ - $\alpha 2$ hairpin (mutated residues are underlined in **Figure 4c**). IST1_{NTD} proteins were diluted from concentrated protein stocks in high-salt buffers to final concentrations of 62 μ M. Salt concentrations in the assembly buffers are provided in the inset key and protein assembly was followed by light scattering at 330 nm. (b) TEM images of a subset of the wild-type and mutant IST1_{NTD} assembly reactions from a. Note that IST1_{NTD} assembled only under low-salt conditions (compare upper and middle left images) and that IST1_{NTD} proteins with different point mutations at the tip of the $\alpha 1$ - $\alpha 2$ hairpin did not assemble in either high-salt (not shown) or low-salt conditions (right). An expanded view of a single IST1_{NTD} tube is shown in the lower left image. Scale bars, 500 nm. (c) TEM images of CHMP1B tubes assembled under low-salt conditions. A field of tubes is shown at left and an expanded view of a single CHMP1B tube is shown at right. Scale bars, 500 nm.



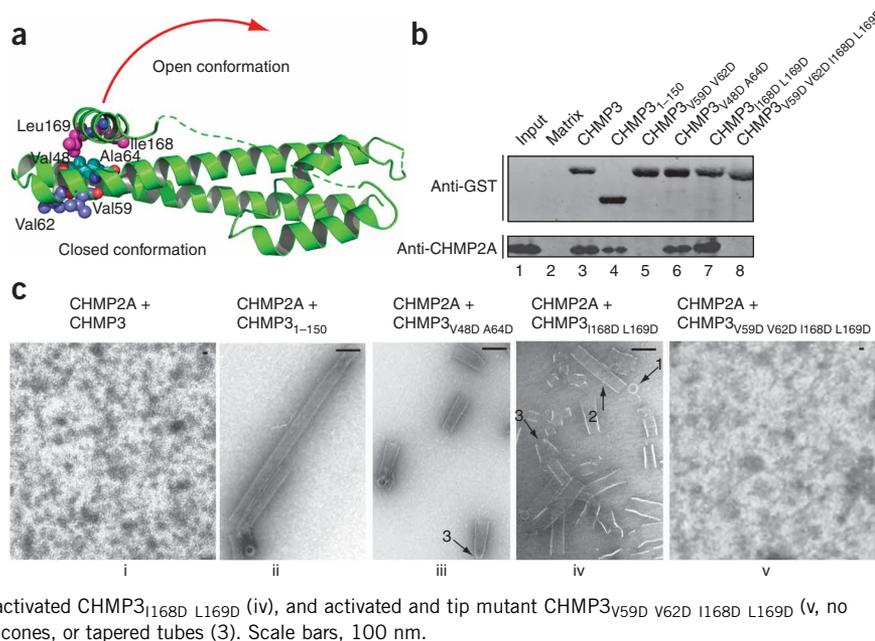
and **Fig. 5b**, below left). In contrast, three weakly binding IST1 mutants failed to rescue midbody arrest (18–21%; **Fig. 5a**, lanes 7–9, and **Fig. 5b**, images below middle and below right). Point mutations within the $\alpha 5$ -core interface that blocked CHMP1B binding also blocked rescue of the midbody arrest defect. However, these mutant proteins partitioned almost exclusively into the insoluble, membrane-bound cellular fractions, and their failure to support abscission may therefore reflect either aberrant CHMP1 binding or IST1 protein mislocalization (**Supplementary Fig. 4 and Supplementary Discussion**). Finally, two IST1 mutants with intermediate (I) CHMP1B binding affinities rescued midbody arrest to an intermediate extent (12–13%; **Fig. 5a**, lanes 5 and 6). Hence, there was an excellent correlation between the ability of different IST1 constructs to bind CHMP1B *in vitro* and to function in abscission, indicating that CHMP1 binding is required for IST1 abscission function(s).

IST1_{NTD} and CHMP1B form homopolymeric helices *in vitro*

CHMP3 can co-polymerize with truncated CHMP2A proteins to form helical tubes 50 nm in diameter *in vitro*⁶. As noted above, both IST1 and IST1_{NTD} became insoluble under low-ionic-strength conditions, and we therefore tested whether these proteins assembled into regular structures. We initially surveyed assembly conditions using light scattering to follow complex formation. Monomeric IST1_{NTD} spontaneously polymerized into high-molecular-weight complexes that strongly scattered light at 330 nm when the protein was diluted from a high-salt buffer (350 mM NaCl) into a low-salt buffer (100 mM NaCl) (**Fig. 6a**). In contrast, IST1_{NTD} did not scatter light when diluted into an otherwise equivalent high-salt buffer (350 mM NaCl).

EM analyses revealed that the light-scattering IST1_{NTD} assemblies were large tubular structures with diameters of ~ 700 nm (**Fig. 6b**, lower left images). The tubes seemed to be open along one edge and may therefore be curled sheets rather than fully closed

Figure 7 CHMP3 activation *in vitro*. (a) Ribbon diagram showing the locations of mutated CHMP3 residues at the tip of the $\alpha 1$ - $\alpha 2$ hairpin (purple) and activating mutations on either side of the interface between the core $\alpha 2$ helix (cyan) and the autoinhibitory $\alpha 5$ helix (magenta). The red arrow suggests how the closed CHMP3 conformation might convert into an open conformation by dissociation of the autoinhibitory $\alpha 5$ helix from the core. (b) GST pull-down analyses of the binary CHMP3–CHMP2A interaction. Pure recombinant CHMP2A (below, anti-CHMP2A) was tested for binding to a glutathione-Sepharose matrix (lane 2, negative control) or to immobilized wild-type (lane 3) or mutant GST-CHMP3 proteins (lanes 4–8). Both proteins were detected by western blotting, and input CHMP2A (0.3%) is shown in lane 1 for reference. (c) EM analyses of helical CHMP3–CHMP2A assembly. Different panels show assemblies formed by 1:1 mixtures of CHMP2A with: full length, wild-type CHMP3 (i, negative control, no assembly), CHMP3_{1–150} core domain (ii, positive control), activated CHMP3_{V48D A64D} (iii), activated CHMP3_{I168D L169D} (iv), and activated and tip mutant CHMP3_{V59D V62D I168D L169D} (v, no assemblies). Arrows highlight rings (1), tubes (2) and cones, or tapered tubes (3). Scale bars, 100 nm.



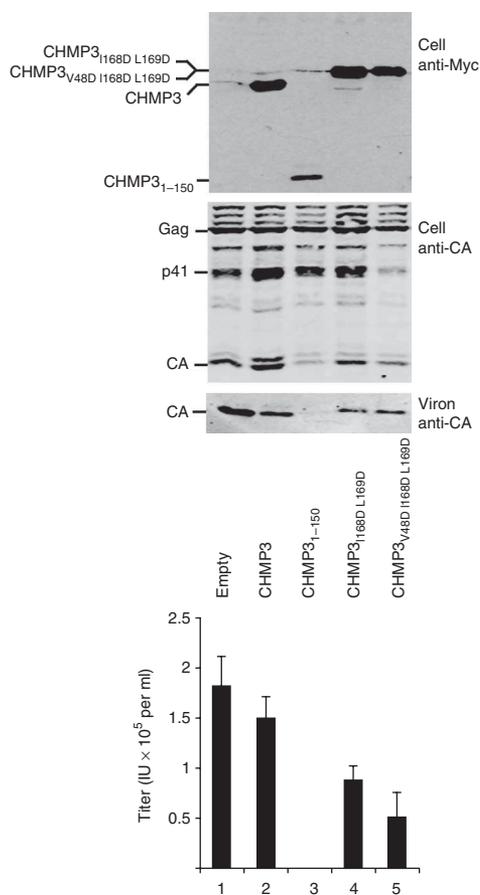


Figure 8 CHMP3 activation *in vivo*. HIV-1 vector expression and release upon co-expression with an empty vector (negative control, lane 1), or with vectors expressing wild-type CHMP3 (negative control, lane 2), a CHMP3₁₋₁₅₀ protein lacking the entire autoinhibitory region (positive control, lane 3), proteins with activating mutations in the $\alpha 5$ -core interface: CHMP3_{H168D L169D} (lane 4) and CHMP3_{V48D I168D L169D} (lane 5). The two uppermost western blots show cellular expression levels of CHMP3-Myc proteins (panel 1, anti-Myc) and of the viral Gag protein and its p41 and CA-processing products (panel 2, anti-CA). The western blot below shows levels of released, virion-associated CA proteins. The graph shows the infectious titers of HIV-1 vectors released under the different conditions (infectious units (IU) per ml; error bars show s.d. in multiple titer measurements, $n \geq 4$).

were not detectable (35 μ M CHMP2A, ~ 7 μ M GST-CHMP3). CHMP2A protein bound GST-CHMP3 (Fig. 7b, lane 3) but did not bind a matrix control (lane 2). CHMP2A also bound the truncated GST-CHMP3₁₋₁₅₀ protein (lane 4), indicating that CHMP2A interacted with the CHMP3 core and that the binary interaction was not strongly affected by removal of the terminal CHMP3 autoinhibitory sequences. In contrast, a double point mutation (V59D V62D) that disrupted the exposed hydrophobic surface on one side of the tip of the CHMP3 $\alpha 1$ - $\alpha 2$ hairpin eliminated CHMP2A binding (lane 5). These data suggest that CHMP2A and CHMP3 interact through a tip-to-tip interaction and demonstrate that the binary CHMP2A-CHMP3 and IST1-CHMP1B pairs interact via different binding surfaces, and therefore form structurally distinct complexes.

Autoinhibitory helix inhibits CHMP3 polymerization

Comparative analyses of the IST1_{NTD}, CHMP3₈₋₁₈₃ and CHMP3₈₋₂₂₂ structures suggested a model for ESCRT-III autoinhibition in which the $\alpha 5$ helix and flap elements fold back against the protein core to prevent higher-order assembly. We tested this idea by introducing destabilizing mutations within the $\alpha 5$ -core interface of the full-length CHMP3 protein and testing their effects on CHMP2A co-assembly. Although the full-length CHMP2A and CHMP3 proteins bind one another, these two proteins do not readily form higher-order helical assemblies⁶ (Fig. 7c,i). In contrast, a CHMP3₁₋₁₅₀ protein that lacked the flap and autoinhibitory helix readily co-polymerized with CHMP2A to form long, regular helical tubes (Fig. 7c,ii, positive control). As CHMP2A forms binary complexes with both CHMP3 and CHMP3₁₋₁₅₀, these experiments imply that CHMP3 residues 151-222 inhibit additional interaction(s) required for higher-order assembly.

To examine the mechanism of CHMP3 autoinhibition, we mixed full-length CHMP2A with full-length CHMP3 proteins that contained mutations on either side of the core- $\alpha 5$ interface (CHMP3_{V48D A64D} and CHMP3_{H168D L169D}; Fig. 7a). Both sets of mutations activated CHMP3 for assembly, as reflected in the formation of numerous rings, tubes and cones (Fig. 7c,iii,iv). The tubes were similar in diameter to those formed in the CHMP2A-CHMP3₁₋₁₅₀ control reaction (~ 50 nm) but were typically shorter, possibly because the point mutations activated CHMP3 proteins less fully than removal of the entire autoinhibitory region.

We also tested whether the binary CHMP2A-CHMP3 interface detected at low protein concentrations was required for higher-order assembly. As expected, mutations on either side of the CHMP3 autoinhibitory interface did not substantially affect CHMP2A binding to GST-CHMP3 (CHMP3_{V48D A64D} and CHMP3_{H168D L169D}; Fig. 7b, lanes 6 and 7). However, a CHMP3 protein carrying mutations in both the tip and autoinhibitory interfaces (CHMP3_{V59D V62D I168D L169D}) could neither bind CHMP2A in the GST pull-down assay (Fig. 7b, lane 8) nor assemble into helical tubes (Fig. 7c,v). These experiments

helices. Lattice lines were evident in some assemblies, and the clearest lattice spacing was ~ 440 Å, which presumably represents a supermolecular spacing because the maximal length of a single IST1_{NTD} molecule is only ~ 60 Å. As noted above, CHMP3 interactions can be mediated by various different kinds of tip-to-tip interactions^{6,7} (Fig. 3). A series of three point mutations in the analogous IST1 surface (Y43D, R51D and I54D) also inhibited IST1_{NTD} polymerization (Fig. 6a,b, right) (see Supplementary Fig. 5 for ESCRT-III protein alignments). IST1_{NTD} assembly therefore probably also requires some type of tip-to-tip interaction.

We also attempted to test whether IST1_{NTD} and CHMP1B could co-polymerize in a manner similar to the CHMP2A₉₋₁₆₁-CHMP3 pair⁶. However, controls for these experiments revealed that pure recombinant CHMP1B alone polymerized into helical tubes when diluted into low-salt buffers, even in the absence of IST1_{NTD} (Fig. 6c). CHMP1B tubes were of intermediate size (~ 230 nm in diameter), seemed to be fully closed and had visible lattice spacing of ~ 420 Å, similar to the lattice spacing in the IST1_{NTD} tubes. Thus, both IST1_{NTD} and CHMP1B, as for CHMP2A-CHMP3 (ref. 6), form tubular structures *in vitro*, and the ability to assemble into helical tubes therefore seems to be a property common to many ESCRT-III core domains. However, the IST1 and CHMP1B tubes were homopolymeric, and they were substantially larger than those formed by CHMP2A-CHMP3 heteropolymers⁶.

CHMP3 $\alpha 1$ - $\alpha 2$ hairpin tip binds CHMP2A

As with the IST1-CHMP1 pair, CHMP2A and CHMP3 bind one another preferentially. To investigate this interaction further, we used GST pull-down assays to test for binary interactions between CHMP2A and GST-CHMP3 (Fig. 7a,b). We performed these experiments at low protein concentrations where higher-order assemblies

imply that tip-to-tip interactions contribute to CHMP2–CHMP3 co-assembly, in good agreement with a previous study⁶.

Mutations in the autoinhibitory helix activate CHMP3 *in vivo*

Although co-expression of full-length CHMP3 has only minimal effects on HIV-1 budding, truncated CHMP3 constructs lacking the C-terminal autoinhibitory elements potently inhibit virus budding, presumably because the activated protein sequesters itself and other ESCRT-III proteins on cellular membranes²⁰. Inhibition of HIV release therefore provides a sensitive assay for CHMP3 activation *in vivo*. We used this system to examine whether mutations in the α 5-core interface also activate CHMP3 *in vivo*. Overexpression of full-length CHMP3 together with an HIV-1 vector system in 293T cells had little effect on the release of viral particles, as measured by the levels of virion-associated CA proteins released (Fig. 8, image 3, compare lanes 1 and 2) or by viral titers (Fig. 8, image 4, compare lanes 1 and 2). In contrast, HIV release and infectivity were potently inhibited by co-expression of a CHMP3 protein that lacked the autoinhibitory region entirely (CHMP3_{1–150}, positive control, compare lanes 2 and 3, showing a >250-fold infectivity reduction). In the actual experiment, two different CHMP3 proteins with point mutations within the α 5-core interface (CHMP3_{I168D L169D} and CHMP3_{V48D I168D L169D}) reduced HIV vector release and infectivity by 1.7-fold and 3-fold, respectively (compare lanes 4 and 5 to lane 2). Western blots confirmed that the full-length CHMP3 proteins and viral Gag proteins and proteolytic processing products were expressed at similar levels (images 1 and 2, respectively). Thus, point mutations in the α 5-core interface enhanced the ability of CHMP3 proteins to inhibit HIV-1 budding *in vivo*, although this enhancement was much weaker than that seen upon deletion of the entire autoinhibitory region. We presume that the weaker effects seen for the point mutants reflects either incomplete activation and/or their ability to interact with VPS4, which will reduce their propensity to become ‘trapped’ on membranes, as compared to CHMP3_{1–150} (which lacks a terminal VPS4 binding site).

DISCUSSION

Our studies have unexpectedly revealed that the N-terminal domain of IST1 contains an asymmetric four-helix bundle that closely resembles the core of the ESCRT-III protein CHMP3 (Fig. 2). As is true for other ESCRT-III proteins, IST1 (i) localizes to midbodies and functions in cytokinesis^{11,14} and MVB vesicle sorting^{12,13}, (ii) binds preferentially to another subset of ESCRT-III proteins (the CHMP1 proteins)^{11–14}, (iii) contains MIM elements near its C terminus that can bind VPS4 and LIP5 MIT domains^{11,14} and (iv) can self-assemble into tubular structures *in vitro* (Fig. 6). Thus, despite its larger size and primary sequence divergence, IST1 is an ESCRT-III protein family member.

Despite these similarities, there are also important differences between IST1 and CHMP3, particularly in the binary interactions that they make with their CHMP1 and CHMP2 binding partners. Our mutational analyses mapped the CHMP1 binding site to a groove formed by the autoinhibitory α 5 helix and the core α 2 helix of IST1_{NTD} (Fig. 4). CHMP1B binding was also inhibited by mutations expected to favor the (hypothetical) open IST1_{NTD} conformation, implying that CHMP1 binds the closed conformation seen in our IST1_{NTD} crystal structures (Fig. 2). IST1 mutations that blocked CHMP1B binding also prevented IST1 from functioning in abscission, implying that these two proteins must act together during this final stage of cytokinesis (Fig. 5). In contrast, binary interactions between CHMP2 and CHMP3 were not inhibited by mutations that displaced or even removed the α 5 helix entirely (Fig. 7). This interaction was

sensitive to mutations at the tip of the α 1– α 2 hairpin, however, suggesting that, unlike IST1 and CHMP1B, CHMP2 and CHMP3 probably interact through some sort of a tip-to-tip interaction (Fig. 7).

In vitro assemblies formed by ESCRT-III proteins include filaments, rings, cones, curled sheets and tubes^{6,7} (Figs. 6b,c and 7c). At present, we do not know which (if any) of these different *in vitro* assemblies mimic different ESCRT-III assemblies formed *in vivo*. As noted previously^{5–7}, however, filaments, rings and/or tubes could correspond to ‘collars’ within the necks of budding particles, and tapered tubes or spiraling cones could provide a mechanism for closing the neck during budding⁶ (Fig. 7c, structure type 3). Although the precise molecular interactions that mediate higher-order ESCRT-III assembly remain to be determined, both IST1_{NTD} and CHMP2–CHMP3 assemblies are inhibited by point mutations within the conserved, exposed hydrophobic surface located at the tip of the α 1– α 2 hairpin (Figs. 6b and 7c), suggesting that some type of tip-mediated interface forms within both assemblies. Two distinct types of homopolymeric ESCRT-III tip-to-tip interactions have now been seen crystallographically¹⁶ (Fig. 3), and a third type was inferred from cryo-EM studies of helical yeast Vps24 (CHMP3) assemblies⁷. Thus, the conserved hydrophobic surface at the tip of ESCRT-III proteins is a preferred interaction site, although it is not yet clear which, if any, of the tip-to-tip interactions characterized to date represent biologically relevant interactions. We also note that, although the IST1_{R51D} tip mutation inhibits IST1 assembly (Fig. 6a,b) it does not block the ability of IST1 to function in cytokinesis (Fig. 5), implying that there are different requirements for IST1 assembly *in vitro* and abscission function *in vivo*. Finally, in addition to tip contacts, at least one more protein-protein interface is required to create two-dimensional surfaces in helices or sheets. These secondary interaction(s) are disfavored by C-terminal autoinhibitory interactions (ref. 6 and this work) and may correspond to one of the several different kinds of higher-order packing interactions that have been proposed on the basis of cryo-EM analyses of helical ESCRT-III protein assemblies^{6,7}.

Our IST1_{NTD} structure revealed that the downstream flap and autoinhibitory helix can fold back against the ESCRT-III core, and this topology was unambiguously established by high-quality electron density throughout the flap region (Fig. 2 and Supplementary Fig. 1). Analogous models can be built for the CHMP3_{8–183} and CHMP3_{8–222} structures, although in those cases the connectivity has not been established unambiguously. Nevertheless, we suggest that all of these structures probably correspond to the closed, autoinhibited ESCRT-III conformation, for several reasons: (i) both IST1_{NTD} and CHMP3_{8–222} are monomers in solution under the conditions used for crystallization (although IST1_{NTD} polymerizes under low-salt conditions) (Fig. 1); (ii) IST1 is redistributed into the insoluble membrane-bound, assembled fractions of cellular extracts by mutations that disrupt core packing of the autoinhibitory elements (Supplementary Fig. 4); (iii) CHMP3 and CHMP2A can be activated for co-assembly by deletions that remove the autoinhibitory helices from CHMP2A⁶ or CHMP3, or by point mutations that destabilize packing of the CHMP3 autoinhibitory helix against the core (Fig. 7); (iv) mutations that remove the autoinhibitory helix or destabilize its core packing interactions render CHMP3 a dominant inhibitor of HIV-1 budding^{17,20} (Fig. 8). Nevertheless, there is also good evidence that additional contacts beyond those visualized in our IST1_{NTD} or CHMP3 structures help to stabilize the closed CHMP3 conformation even further, because point mutations in the autoinhibitory helix-core interface do not activate CHMP3 to the same extent as does deletion of the entire autoinhibitory region (Figs. 7c and 8), and because short

C-terminal deletions downstream of the autoinhibitory $\alpha 5$ helix can also enhance ESCRT-III membrane binding and inhibition of HIV-1 budding, albeit to a lesser extent than deletions that include $\alpha 5$ (refs. 17,20). These observations indicate that the terminal ESCRT-III helix 6 (and possibly also flap sequences) make energetically important contacts that favor the closed ESCRT-III conformation.

In summary, many ESCRT-III proteins can adopt two distinct conformational states^{6,16–21}, and our studies provide a molecular model for the closed, monomeric state adopted by ESCRT-III proteins when they are distributed throughout the cytoplasm (or loosely associated with nonspecific membranes)³⁴. Primary sequence and secondary-structure analyses indicate that all ESCRT-III proteins have analogous $\alpha 5$ helices in equivalent positions, and the mechanism of autoinhibition described here is probably quite general (**Supplementary Fig. 5a**). Surrounding elements can vary substantially in sequence, length and secondary structure, however, and they probably mediate various protein-protein interactions³³. Notably, these different protein-protein interactions can be enhanced, inhibited or unchanged by mutations that favor the open states of various ESCRT-III proteins (for example, refs. 21,22,28,33). Thus, changes in protein-protein and protein-membrane interactions that accompany ESCRT-III activation and assembly are likely to reflect the conformational changes described here, coupled with avidity effects created by the assembly of oligomeric ESCRT-III protein arrays.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/nsmb/>.

Accession Codes. Protein Data Bank: Coordinates and structure factor amplitudes have been deposited for the two IST1_{NTD} crystal forms (3FRR and 3FRS), CHMP_{8–222} (3FRT) and CHMP_{31–150} (3FRV).

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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ONLINE METHODS

Expression vectors and antibodies. Vectors and antibodies are summarized in **Supplementary Tables 1 and 2**.

Protein expression and purification. We expressed IST1 and CHMP proteins in *Escherichia coli* with N-terminal GST-affinity tags. Protein expression, purification and tag removal are described in the **Supplementary Methods**.

Equilibrium sedimentation analyses. We centrifuged purified CHMP3, CHMP3₈₋₂₂₂ and IST1_{NTD} in 10 mM Tris-HCl pH 8.0, 100 mM NaCl (CHMP3 proteins) or 50 mM Tris-HCl pH 7.0, 350 mM NaCl and 1 mM DTT (IST1_{NTD}) at 4 °C in an Optima XL-A centrifuge (Beckman). Initial protein concentrations are given in **Figure 1a–c**. Data were collected after equilibrium was reached at two speeds (20,000 r.p.m. and 24,000 r.p.m.) in a Beckman An-60 Ti rotor, and the molecular masses were determined from the resulting six data sets for each protein by globally fitting to single ideal species models using the nonlinear least-squares algorithms in the Heteroanalysis software³⁶. Protein partial specific volumes and solvent densities were calculated with the program SEDNTERP (version 1.09)³⁷.

Protein crystallization and data collection. We crystallized IST1_{NTD}, CHMP3₈₋₂₂₂ and CHMP3₁₋₁₅₀ by vapor diffusion at 13 °C. IST1_{NTD} crystals grew from a 12 mg ml⁻¹ solution in gel filtration buffer. Crystal form I (diamond-shaped morphology, *P*₄3212, 1 molecule per asymmetric unit) grew in 1–3 d by simple dehydration of a 2- μ l drop. Crystals were cryoprotected by passage through silica oil, suspended in a nylon loop and plunged into liquid nitrogen. Crystal form II (rods and plates, *P*₂₁, 1 molecule per asymmetric unit) grew from a 1:1 (v/v) mixture of protein and well solution (180 mM KCl, 50 mM HEPES pH 7.6, 35% pentaerythritol propexylate and 7% (v/v) ethylene glycol). Crystals were cryo-cooled directly without solution modification. Native data for both crystal forms were collected on a Rigaku Micromax 007HF rotating anode generator equipped with VeriMax-HR optics.

CHMP3₈₋₂₂₂ crystals (flat plates, *P*₂₁, 2 molecules per asymmetric unit) grew from a 1:2 (v/v) mixture of protein (30 mg ml⁻¹ in gel filtration buffer) and well solution (10–22% PEG 6000 and 0.1M Na-HEPES pH 7.0–8.0). Data were collected at SSRL beamline 11-1. CHMP3₁₋₁₅₀ crystals (hexagonal rods, *P*₆₁) grew from a 1:1 (v/v) mixture of protein (22 mg ml⁻¹ in gel filtration buffer) and well solution (16% (v/v) PEG 3350, 0.1 M Na HEPES pH 7.0–8.0, 200 mM proline). Data were collected on a rotating anode generator. Despite extensive effort, we were unable to collect better data at the synchrotron. Data were indexed and scaled with the program HKL2000 (ref. 38) (**Table 1**).

Structure determinations. We collected three-wavelength MAD data from selenomethionine-substituted IST1_{NTD} at SSRL beamline 9–1 to 3.4 Å. Three of the six potential selenium sites were identified using SOLVE³⁹, and the resulting 4.0-Å phases were solvent flattened and extended to 2.6-Å resolution using the native data and the program DM⁴⁰. The four core helices (α ₁– α ₄) were built using O⁴¹, and this partial model was used for molecular replacement determination of the higher-resolution form II crystal structure using PHASER⁴². The models were completed with COOT⁴³, refined with REFMAC5 (ref. 44) and analyzed using programs within the CCP4 suite⁴⁵. The similar IST1_{NTD} structures in the two crystal forms overlapped with an r.m.s. deviation of 1.25 Å on C α atoms 12–170.

We determined both CHMP3 structures by molecular replacement using the published CHMP3₈₋₁₈₃ structure (PDB 2GD5)¹⁶ as the search model. The correctness of the solutions is supported by PHASER⁴² Z-scores of 9.1 (CHMP3₈₋₂₂₂) and 8.6 (CHMP3₁₋₁₅₀) and by the appearance of electron density when portions of the search model were omitted from the molecular replacement calculation (**Supplementary Fig. 2**). Both structures are hampered by low resolution and a correspondingly low number of unique reflections. *R*_{free} sets of 5% contain too few reflections (~120) to be statistically useful. An optimal strategy was therefore determined by considering *R*_{free} values from multiple refinement runs, each starting from the same molecular replacement solution and with a different small number (~200) of reflections in the test set. This indicated that the best results were obtained using rigid-body refinement in PHENIX⁴⁶ with each helix treated as a rigid unit. This approach was

therefore applied to the starting molecular replacement solution but using all of the reflections in the calculation (no test set).

Biosensor binding assays. We used BIACORE2000 and BIACORE3000 instruments (GE Healthcare) to measure the binding of pure recombinant CHMP1B to immobilized GST-IST1_{NTD} proteins captured from *E. coli* extracts²⁸, as described in **Supplementary Methods**.

IST1 depletion and rescue experiments. We transfected HeLa-M cells with IST1 expression vectors (1 μ g per well, using Lipofectamine 2000, Invitrogen) and siRNA (20nM, Lipofectamin RNAi MAX, Invitrogen) following the time course: *t* = 0, seed cells in DMEM and 10% (v/v) FCS (six-well plates, 4 × 10⁵ cells per well); *t* = 24 h, add IST1 expression vector; *t* = 32 h, change media and add IST1 vector; *t* = 44 h, trypsinize and reseed cells on 18-mm glass cover slips for immunofluorescence analyses (3 × 10⁴ cells per cover slip) and in six-well plates for western blotting (3 × 10⁵ cells per well); *t* = 56 h, change media and add siRNA; *t* = 68 h, change media and add siRNA; *t* = 94 h, fix or harvest cells and analyze.

Direct visualization of cytokinesis defects. We fixed transfected HeLa-M cells with 4% (v/v) paraformaldehyde and stained with SYTOX Green (Invitrogen) and with mouse anti- α -tubulin and a secondary Alexa 594-conjugated goat anti-mouse antibody. We scored midbody arrest manually for \geq 3 blinded sets of 200 cells by direct visualization using fluorescence microscopy.

ESCRT-III assembly reactions. Assembly conditions for CHMP2A and CHMP3, IST1_{NTD}, and CHMP1B proteins are provided in the **Supplementary Methods**.

Transmission electron microscopy. We analyzed assembly reactions using TEM with negative staining. Carbon-coated grids were placed on aliquots of each assembly reaction (7 μ l, 90 s), washed with 3–4 drops of 0.1 M KCl, stained with 3–4 drops of 4% (v/v) uranyl acetate, air dried and imaged (Hitachi 7100 TEM).

GST pull-down assays. We tested the binding of pure recombinant CHMP2A proteins to various GST-CHMP3 proteins as described in the **Supplementary Methods**.

HIV-1 vector release and infectivity. We co-transfected 293T cells with vectors expressing CHMP3-Myc and HIV-1 and harvested supernatants and cells after 24 h. Western blotting was used to analyze cellular proteins and virions pelleted through 20% sucrose cushion and HeLa-M cell transduction to measure vector titers. Full details are provided in **Supplementary Methods**.

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