Epsin and Sla2 form assemblies through phospholipid interfaces

Maria M. Garcia-Alai, Johannes Heidemann, Michal Skruzny, Anna Gieras, Haydyn D.T. Mertens, Dmitri I. Svergun, Marko Kaksonen, Charlotte Uetrecht & Rob Meijers

In clathrin-mediated endocytosis, adapter proteins assemble together with clathrin through interactions with specific lipids on the plasma membrane. However, the precise mechanism of adapter protein assembly at the cell membrane is still unknown. Here, we show that the membrane–proximal domains ENTH of epsin and ANTH of Sla2 form complexes through phosphatidylinositol 4,5-bisphosphate (PIP2) lipid interfaces. Native mass spectrometry reveals how ENTH and ANTH domains form assemblies by sharing PIP2 molecules. Furthermore, crystal structures of epsin Ent2 ENTH domain from *S. cerevisiae* in complex with PIP2 and Sla2 ANTH domain from *C. thermophilum* illustrate how allosteric phospholipid binding occurs. A comparison with human ENTH and ANTH domains reveal only the human ENTH domain can form a stable hexameric core in presence of PIP2, which could explain functional differences between fungal and human epsins. We propose a general phospholipid-driven multifaceted assembly mechanism tolerating different adapter protein compositions to induce endocytosis.

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Clathrin-mediated endocytosis is essential for protein retrieval during neurotransmission and receptor recycling. It is also involved in viral entry and the uptake of nutrients and hormones. During endocytosis, a cargo-containing vesicle is formed through the invagination of a plasma membrane patch, in a process that involves proteins and specific lipids. Accumulation of the adapter proteins at the plasma membrane initiates the endocytic event and contributes to membrane bending. The adapters recruit clathrin and associate with the actin cytoskeleton to accomplish vesicle budding. Finally, the vesicle coated with clathrin and adapter proteins is detached from the plasma membrane by dynamin.

The size and shape of the vesicle is determined by the interplay of adapter proteins and clathrin. Clathrin does not bind to the plasma membrane directly, but to a range of adapter proteins that interact with lipids on the plasma membrane. Many adapter proteins, such as epsin, AP180, and AP-2, contain positively charged patches that bind to the head groups of specific phospholipids. This binding is strong enough so that the adapter proteins remain attached to the membrane, and can undergo conformational changes to bind cargo. Adapter proteins containing an epsin N-terminal homology (ENTH) domain and some adapter proteins containing an AP180 N-terminal homology (ANTH) domain that belong to the subfamily of clathrin assembly lymphoid myeloid leukemia (CARM) proteins have an additional membrane-binding mode, inserting an N-terminal helix into the plasma membrane. It is proposed that there is a concerted mechanism where binding to the head group of phosphatidylinositol 4,5-bisphosphate (PIP2) triggers the N-terminal portion of these adapter proteins to fold into an α helix. The insertion of the α helix into the membrane contributes to its curvature.

It is unclear whether the clathrin-associated adapter proteins engage clathrin individually at the cell membrane, or whether they assemble in larger structures to drive membrane curvature and clathrin recruitment. It has also been proposed that membrane curvature is caused simply by a protein crowding mechanism, where the formation of the clathrin coat crowds adapter proteins together, leading to membrane curvature. Critically, the combination of membrane insertions and spherical scaffolds that curve the membrane have been observed, but their interactions are not understood. There are indications that adapter proteins themselves form larger oligomers, preceding the formation of the clathrin coat. Complex formation has been observed between the ENTH domain of epsin and the ANTH domain of Sla2, the yeast homolog of human huntingtin interacting protein 1 (HIP1R)12. The formation of this complex depends on the presence of PIP2 and has been observed in Saccharomyces cerevisiae. A pulldown experiment with full-length epsin-1 and Hip1R from Mus musculus suggested the same complex forms in mammals. Endocytosis is stalled in S. cerevisiae when the complex between the ENTH domain of epsin Ent1 and the ANTH domain of Sla2 is disrupted by mutations. Electron cryo-tomography studies on helical assemblies of epsin Ent1 ENTH and Sla2 ANTH domains obtained from giant unilamellar vesicles (GUVs) revealed that the co-assembly of ENTH/ANTH occurs. The ENTH/ANTH assembly may help organize the clathrin coat and could contribute to membrane curvature during the initial stages of endocytosis. It is of particular interest that epsin and Sla2 form a membrane-bound scaffold, because these adapter proteins have also been associated with actin recruitment to the coated pit. Actin polymerization is known to provide mechanical force for vesicle formation from membranes under tension.

The adapter assembly would harness singular weak interactions together to form an anchor that can withstand the strong forces generated by the actin cytoskeleton.

In this paper, we reveal the assembly process of epsin ENTH and Sla2/Hip1R ANTH domains through PIP2 interfaces by native MS. An X-ray crystal structure of the ENTH domain of epsin Ent2 from S. cerevisiae in complex with PIP2 reveals an allosteric mechanism underlying the assembly. The crystal structure of the Sla2 ANTH domain from C. thermophilum reveals how this subfamily of ANTH domains evolved to engage ENTH domains in assembly. We determine by native MS that some elements of the assembly process are evolutionarily conserved between the fungal and human adapter proteins. However, human epsin ENTH can autonomously form homo-oligomers, whereas fungal ENTH domains need the presence of Sla2 ANTH domains to create stable assemblies. Together, these data suggest that the ENTH/ANTH complex concentrates PIP2 locally on the plasma membrane to facilitate the formation of adapter complexes with cargo, clathrin, or other adapter proteins through PIP2-dependent interfaces.

## Results

### Cooperative binding of PIP2 to the ENTH domain of epsin.

To investigate how a lipid-dependent protein complex is formed between epsin ENTH domain and the Sla2/Hip1R ANTH domain, we first determined the binding between the PIP2 lipid (diC8-PIP2) and ENTH domains from S. cerevisiae alone using native MS. The Ent1 and Ent2 ENTH domains (ENTH1 and ENTH2) from S. cerevisiae as well as an ENTH domain of homologous epsin from C. thermophilum bind up to two PIP2 molecules per domain (Fig. 1a, b). Based on a direct MS approach, macroscopic dissociation constants were determined revealing dependence of the two PIP2-binding sites by positive cooperativity (Fig. 1c and Supplementary Fig. 1). Results were comparable for the MS optimized ammonium acetate concentration of 300 mM and the more physiological ionic strength of 160 mM ammonium acetate (Supplementary Table 1).

A crystal structure was determined of a complex between the ENTH domain of Ent2 (ENTH2) from S. cerevisiae and the PIP2 phospholipid to a resolution of 3.4 Å (Table 1). The structure shows two ENTH2 domains sandwiched around one PIP2 molecule (Fig. 2a and Supplementary Fig. 1). The inositol head group of PIP2 is bound to the previously identified phosphatidylinositol-binding pocket of one of the ENTH2 molecules. The N-terminal region of this ENTH2 molecule is folded into an α helix that corresponds to the helix (α0) that has been observed in the ENTH domain of rat epsin-1 in complex with the phosphoinositol (IP3) group, and this copy is labeled ENTH2α0. In the second ENTH2 molecule, the N-terminal α0 is unfolded (ENTH2Nα0), and the electron density suggests the N-terminal residues are extended. A superimposition of the ENTH1/IP3 domain complex structure of rat epsin-1 onto the ENTH2/PIP2 domain crystal structure from S. cerevisiae results in an RMSD of 1.1 Å for 147 residues showing distinct orientations of the α0 helix (Fig. 2b). In the ENTH1/IP3 structure, the α0 helix folds back onto the phosphoinositol group, so that Arg 8 and Lys 11 interact with an inositol head group. In the ENTH2/PIP2 structure, the α0 helix points away from the ENTH2 domain and there are no interactions with the phosphoinositol group. Instead, the evolutionarily conserved residue Tyr 16 of the ENTH2α0 molecule forms direct interactions with the inositol head group of PIP2.

As a result of α0 reorientation pointing away from the ENTH2α0 molecule, the second ENTH2Nα0 molecule can form interactions with the phosphatidylinositol head group. Interestingly, the ENTH2Nα0 domain is oriented toward the PIP2
molecule to involve residue Thr 104 on α6 in the ENTH2 dimer interface. This residue is crucial for the formation of the ENTH/ANTH complex. 3,12,14,21 Thr 104 of ENTH2α0 is in the periphery of the PIP2-binding site, and sits opposite Tyr 68 and Lys 69 of the ENTH2α0 molecule. Thr 104 is conserved in epsin ENTH domains and is essential for ENTH function, though independent from the canonical PIP2-binding site. 22,23 Native MS on a Thr104Glu mutant of ENTH1 from S. cerevisiae shows a reduction of PIP2 binding, where the binding of two PIP2 molecules is barely observed (Fig. 1b), indicating impairment of the binding site.

To analyze the contribution of the PIP2 molecule to complex formation, we calculated the buried surface area contributions for each molecule with PISA. 24 The total solvent accessible area of the head group is buried by the ENTH2 α0 molecule (306 Å², or 63% of the total available surface area of the PIP2 molecule), whereas the ENTH2α0 molecule covers 95 Å², or 20% of the available surface area. The buried solvent area between ENTH2α0 and ENTH2α0 is 1820 Å², with almost equal contributions from ENTH2α0 (955 Å²) and ENTH2α0 (865 Å²). However, most of the buried area (1020 Å²) contributed to the dimer interface comes from the α0 helix of the ENTHα0 domain, which is repositioned by the PIP2 molecule to facilitate dimer formation. The effect of PIP2 binding is therefore twofold; it attaches the ENTH domain to the membrane and displaces the α0 helix, so it can form a dimer with a second ENTH domain. As a further indication that the ENTH2 dimer, which sandwiched PIP2, is functionally relevant, a similar dimer interface is observed in a crystal structure of the epsin Ent1 ENTH domain (ENTH1) from S. cerevisiae at 2.9 Å resolution (Fig. 2c). Here, a 2-methyl-2,4-pentanediol (MPD) molecule from the crystallization solution has caused a displacement of the α0 helix to allow dimer formation between ENTH1 domains.

In the ENTH2/PIP2 structure, two α0 helices from symmetry-related ENTH2α0 molecules stack against each other to create a homotetramer containing two PIP2 molecules in the same plane, ready to insert into the membrane (Fig. 2d). In this planar orientation, four ENTH molecules line up so they could associate with the plasma membrane, whereas the C-termini are pointing away from the membrane. The α0 helices of the ENTH2α0 domains are not positioned to insert into the membrane. However, the unfolded N-terminal regions of ENTH2α0 as well as the neighboring positively charged PIP2-binding patch consisting of residues Lys 14, Arg 24, Arg 72, and His 72 are lying in the same plane as the lipid tail of PIP2 being aligned with the cell membrane. The N-terminus is therefore ideally positioned to bind another PIP2 molecule on the plasma membrane. We thus conclude that the PIP2/ENTH2 crystal structure shows an intermediate state, where the binding of one PIP2 dimerizes two ENTH2 domains and prepares the "empty" ENTH domain to bind another PIP2. This allosteric switch between PIP2 binding, dimerization, and the formation of α0 could potentially create larger epsin clusters on the cell membrane.

The Sla2 ANTH domain evolved to bind the ENTH/PIP2 complex. Native MS was also used to investigate PIP2 binding to ENTH domains. A For analyzing lipid binding to ENTH domains, proteins were measured in presence of PIP2 and cytochrome c as reference. Raw spectra show free cytochrome c and unspecific attachment of 1 PIP2 (gray), while ENTH domains (here C. thermophilum) bind 0–3 PIP2. B Signal intensities from MS were summed over all charge states (back) and corrected for unspecific PIP2 clustering based on the ratio of bound/unbound reference protein (front). Data of at least three independent measurements were normalized to the corrected signal of unbound ENTH and the averages of the relative signal intensities and their standard deviations were plotted. The signal for ENTH with three PIP2 observed in raw spectra disappears after correction. C Schematic illustration of microscopic and macroscopic dissociation constants of two PIP2 molecules (orange) binding independently to ENTH (blue). For the first binding event of PIP2, two pathways with the microscopic dissociation constants $k_{d,1}$ and $k_{d,2}$ are available, leading to one apparent species of ENTH+PIP2. Combined, they account for the macroscopic dissociation constant $K_{d,1}$. The second microscopic dissociation constant $K_{d,2}$ describes PIP2 binding to the thus far unoccupied binding site that yields the product ENTH+2PIP2. Again, this binding event can be partitioned into two pathways with the microscopic dissociation constants $k_{d,1}$ and $k_{d,2}$. If $k_{d,1}$ and $k_{d,2}$ are unaltered in the first and second binding event, binding sites are independent. Binding sites are represented by rounded rectangles.

![Diagram](image_url)
several ANTH domains, showing two PIP2 binding sites for all wild-type proteins (Fig. 3a and Supplementary Table 1). There is a striking difference in PIP2 binding between human CALM and the fungal Sla2 ANTH domains. The macroscopic dissociation constants for CALM suggest higher affinity for the first PIP2-binding site, and a lack of cooperative binding (Supplementary Table 1). Mutagenesis of the canonical PIP2-binding site on the Sla2 ANTH molecule from *S. cerevisiae* revealed the presence of a specific secondary binding site that has so far not been identified. Implementing the dissociation constant of the mutant into a mathematical model (Fig. 1c) revealed dependence and positive cooperativity of the two binding sites. In general, PIP2 dissociation constants are higher for Sla2 ANTH domains than for ENTH domains from *S. cerevisiae* showing $K_{D,2}$ of 100–250 µM (Supplementary Table 1).

To understand why the ANTH domain of Sla2/Hip1R is specifically suited to bind the ENTH domain of epsins through the lipid interface, we determined the crystal structure of the ANTH domain of homologous Sla2 from *C. thermophilum* to 1.8 Å resolution (Table 1). The overall ANTH fold consists of helices α1–α11 and is conserved between CALM and Sla2/Hip1R subfamilies (Fig. 3b). A structure-based sequence alignment of CALM subfamily members CALM and AP180 reveals two hitherto hidden, systematic and evolutionarily conserved features (Fig. 3b and Supplementary Fig. 2). Through insertion of a conserved Tyr 252 and a hydrophobic residue, an extra α helix (α12) uniquely occurs in Sla2 ANTH domains, providing rigidity. A loop connecting helices α8 and α9 has a five-residue extension in Sla2, although with non-conserved sequence. Based on the low-resolution electron microscopy tomogram of the Ent1 ENTH/ANTH Sla2 complex from *S. cerevisiae*, these insertions point at the epsin interface. Moreover, deleting the Sla2/Hip1R-specific α12 helix impaired the function of the Sla2 ANTH domain similarly to mutating Arg 29, which is essential for the ENTH/ANTH complex formation (Fig. 3c). In addition, the CALM ANTH domain has an N-terminal helix that is affecting membrane curvature and that is similar to oδ0 observed in epsins. Based on the *C. thermophilum* Sla2 ANTH crystal structure, ANTH domains of the Sla2/Hip1R subfamily do not contain an N-terminal α helix. This is further confirmed by circular dichroism experiments performed on several ANTH and ENTH domains (Supplementary Fig. 3). Sla2/Hip1R ANTH domains display a loss of secondary structure or aggregation at high PIP2 concentrations (Supplementary Fig. 3c, f). To address whether this is caused by PIP2 itself, or the presence of an amphipathic environment, we repeated the circular dichroism measurements in the presence of submicellar and micellar concentrations of the detergent n-Dodecyl-beta-Maltoside (DDM) with and without PIP2 (Supplementary Fig. 3g, h). Human Hip1R ANTH domain shows a loss of signal with DDM (Supplementary Fig. 3h). The protein seems to undergo unfolding in the presence of an amphipathic environment, which would not be expected for a protein that contains a helix inducible by lipid binding. Contrast, CALM displays a clear increase in signal in the presence of DDM and PIP2 compared to the protein in buffer alone or with PIP2. In addition, when only the detergent above micellar concentrations is added, we further observe an increase in the signal. This experiment shows that it is actually the presence of the micellar environment and not only the phospholipid per se that is inducing/stabilizing helical structures in the protein and is in agreement with what has been previously reported for CALM that contains an oδ0 amphipathic helix.

We therefore conclude that N-terminal amphipathic helices are not

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### Table 1 Data collection and refinement statistics

|                        | ENTH2 (S. cerevisiae) | ENTH2/PIP2 (S. cerevisiae) | ENTH1 (S. cerevisiae) | ANTH* (C. thermophilum) |
|------------------------|-----------------------|----------------------------|-----------------------|-------------------------|
| **Data collection**     |                       |                            |                       |                         |
| Space group            | $P_2_1$               | $F_{4}32$                  | $C_22_2_1$            | $P_2_1$                 |
| Unit cell dimensions   | 24.4, 65.7, 33.7       | a,b,c=211.4                | 68.9, 119.4, 130.0    | 47.8, 106.9, 51.6       |
| $\alpha$, $\beta$, $\gamma$ (°) | $\beta=97.3$         |                            |                       |                         |
| Resolution (Å)         | 66-180 (1.84-1.980)   | 120-335 (8.85-3.35)        | 27-2.75 (2.90-2.75)   | 47-1.84 (1.88-1.84)     |
| $R_{	ext{merge}}$     | 0.020 (0.353)         | 0.053 (1.04)               | 0.049 (0.49)          | 0.062 (0.752)           |
| $R_{	ext{free}}$      | 0.048 (0.580)         | 0.134 (1.62)               | 0.095 (0.80)          | 0.12 (1.42)             |
| Completeness (%)       | 99.2 (94.6)           | 98.6 (97.1)                | 99.1 (99.1)           | 99.5 (93.6)             |
| Redundancy             | 6.4 (3.5)             | 6.2 (3.0)                  | 5.3 (3.5)             | 4.6 (4.4)               |
| $CC_{1/2}$             | 0.76                  | 0.34                       | 0.71                  | 0.35                    |
| **Refinement**         |                       |                            |                       |                         |
| Resolution (Å)         | 33.5-1.90             | 75.0-3.35 (4.22-3.35)      | 27.1-2.75 (2.82-2.75) | 47.0-1.84 (1.89-1.84)   |
| No. reflections        | 7571                  | 5798                       | 13379                 | 41152                   |
| $R_{\text{work}}/R_{\text{free}}$ | 0.17/0.22             | 0.28/0.30                 | 0.24/0.29             | 0.18/0.23               |
| No. of atoms           | Protein: 1104         | 2378                       | 3578                  | 5038                    |
|                        | Ligand/ion: 36        | 8                          | 91                    |                         |
|                        | Water: 61             | N/A                        | N/A                   |                         |
| $B$-factors (Å²)       | Protein: 26           | 155                        | 135                   | 81                      |
|                        | Ligand/ion: 165       | 150                        | 101                   |                         |
|                        | Water: 35             | N/A                        | N/A                   |                         |
| RMS deviations         | Bond lengths (Å)      | 0.018                      | 0.004                 | 0.015                   |
|                        | Bond angles (°)       | 1.95                       | 0.83                  | 1.63                    |
| Ramachandran statistics| Favored (%)           | 100.0                      | 91.9                  | 91.4                    |
|                        | Disallowed (%)        | 0.0                        | 1.8                   | 0.5                     |

*Values in parentheses are for the highest-resolution shell.
characteristic of either ANTH subfamily, but are only present in a subset of CALM adapter proteins.

**Ordered assembly formation of fungal ENTH and Sla2 ANTH.**
To determine how universal PIP2-dependent interactions between ENTH and ANTH domains are, we analyzed PIP2-driven complex formation for a selection of ENTH and ANTH domains by native MS. We tested complex formation between the *S. cerevisiae* ENTH2 domain and the ANTH domain of Sla2 to verify the assembly relevant to the ENTH2/PIP2 crystal structure (Fig. 4). Indeed, an assembly with a stoichiometry of 8:8:24 ± 3 for ENTH2/ANTHSla2/PIP2 is formed, similar to the ENTH1 complex and ENTH/ANTH Sla2 complex of *C. thermophilum* (Fig. 4 and Supplementary Table 2). When equal amounts of *S. cerevisiae* ENTH1 and ENTH2 are mixed together with ANTHSla2 and measured by native MS, a complex containing equal amounts of ENTH1 and ENTH2 is formed, showing no preference for either isoform and highlighting the possibility to recruit different epsins into a complex with Sla2 (Supplementary Fig. 4a). Furthermore, complex formation was impaired for the ENTH1 Thr104 mutant (Supplementary Fig. 6), confirming previous ITC measurements.

Although the hetero 16-mer of the fungal ENTH/ANTH domain is most prominent, a smaller oligomer of ~330 kDa is also observed (Fig. 4) consisting of six ENTH and six ANTH domains (ENTH1/2/ANTHSla2/PIP2 is 6:6:19 ± 2). Further information on complex topology was obtained in collision-induced dissociation (CID) MS/MS, in which proteins unfold partially until small proteins from the periphery are ejected. Here, hetero 12-mers and 16-mers show identical dissociation pathways. The larger ANTH domain is ejected from the complexes while all PIP2 and ENTH molecules remain bound to the complexes, indicating localization of ANTH in the periphery around an ENTH/PIP2 core (Fig. 3a and Supplementary Fig. 4).

It seems that the ENTH/ANTH/PIP2 mixtures form transient complexes that can alter in size. To understand the relationship between the two oligomeric states, oligomeric composition of a mixture of ENTH, ANTHSla2 (both *S. cerevisiae*), and PIP2 was monitored by native MS over time. The changing signal intensities revealed a maturation process enriching the larger hetero 16-mer (Fig. 5a). Amounts of hetero 12-mer and 16-mer complexes are approximately equal 1 min after mixing. Within 2 min, the 12-mer signal fades, and the 16-mer gets predominant. These results and the equilibrium ratio of 12-mers and 16-mers show that the hetero 16-mer is the more stable ENTH/ANTH/PIP2 complex, suggesting that the 12-mer is an assembly intermediate. Furthermore, complex assembly is reversible upon depletion and subsequent replenishing of PIP2 (Supplementary Fig. 4c).

**Human ENTH forms a hexameric core upon PIP2 binding.** It was previously reported that full-length Hip1R can only be pulled down by murine epsin ENTH in the presence of PIP2, suggesting that the PIP2-dependent formation of ENTH/ANTH complexes

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**Fig. 2** Crystal structure of the ENTH2/PIP2 complex reveals an allosteric-binding mechanism. a Ribbon diagram of the ENTH2α0 (cyan) and ENTH2Noα0 dimer (blue) building block. N-terminal regions are colored (ENTH2α0, yellow; ENTH2Noα0, orange). PIP2 sitting in the interface between ENTH2 molecules is shown as spheres. b Superposition of the ENTH/IP3 epsin-1 complex (dark blue) and the ENTH2/PIP2 complex (yellow). The N-terminal α0 is colored (ENTH2/PIP2, yellow; ENTH/IP3, orange), the inositol head groups are shown as sticks. c Superimposition of the ENTH2/PIP2 dimer (blue/cyan) and the ENTH1 dimer (green/palegreen) shown as a ribbon diagram. The α0 helix of ENTH1 is oriented similarly to α0 of ENTH2. d Surface presentation of the ENTH2/PIP2 complex showing that unfolded N-termini of ENTH2 are in plane with the lipid tail of the PIP2 molecules (two ENTH2α0/ENTH2Noα0 building blocks, cyan/blue and magenta/violet; PIP2, spheres; N-termini for empty ENTH2, yellow and orange; Tyr 16, Arg 24, Arg 62 and His 72 of ENTH2Noα0 with empty PIP2-binding pocket, red).
Fig. 3  Evolutionarily conserved features in Hip1R subfamily of ANTH domains. a Native MS was used to investigate PIP2 binding to several members of the ANTH family: ANTH_Sla2, ANTH_Sla2_mut (four residues to Ala in the canonical PIP2-binding site, see Constructs) and CALM from indicated species. Signal intensities from mass spectra of at least three independent measurements were summed over all charge states, normalized to the corrected signal of the unbound protein and the average of the relative signal intensities and their standard deviations were plotted (back). After correction for unspecified clustering, relative signals of unbound and PIP2-bound ANTH domains are obtained (front). Signal for proteins with three attached PIP2 molecules observed in raw spectra disappear after correction. b Composite model of the ENTH and ANTH_Sla2 complex based on the low-resolution EM structure and the high-resolution X-ray crystal structures presented here (ENTH2/PIP2, yellow, and superimposed ANTH domains of Sla2 (violet), AP180 (cyan), and CALM (pale cyan). Several elements contributing to the interface are highlighted, including the inserted α helix in the ANTH_Sla2 domain, the proximate Arg 37 of ANTH_Sla2 and Thr 104 from ENTH, and the location of PIP2 from the secondary site of the ENTH2/PIP2 crystal structure. c Growth defects of Sla2(1–360) strains mutated in ANTH-specific features. Tenfold serial dilutions of sla2Δ strains expressing Sla2(1–360) wt, Sla2(1–360) NHL with α8-α9 loop replaced by residues NHL as occurring in Yap1802, Sla2(1–360) dYL deletion of conserved Tyr 252 and Leu 253 and Sla2(1–360) R29A were incubated on SC-Ura plates at 30, 34, and 37 °C for 2 days.
could be conserved in mammals. To verify evolutionary conservation of ENTH/ANTH/PIP2 complexes, a mixture of human epsin-1 ENTH domain with human Hip1R ANTH domain and PIP2 was analyzed by native MS, showing less homogeneity with a larger variety of oligomers (Fig. 5b). However, the hetero 16-meric complexes observed with S. cerevisiae domains were also present in samples with human domains. The majority of human ENTH/ANTH complexes consist of a combination of six epsin ENTH molecules and a range of ANTH Hip1R molecules. Contrary to the fungal samples, the ENTH hexamer is already observed in the presence of PIP2 alone (Fig. 5c). A minimum of six PIP2 molecules is required for human ENTH hexamer formation. The appearance of the hexameric ENTH core in the human homolog suggests higher stability compared to fungal homologs.

To confirm existence of the hexameric human ENTH core with PIP2 in solution and to gain further insight into its structure, small-angle X-ray scattering (SAXS) was performed on human ENTH (Supplementary Table 3). In the presence of PIP2, a uniform hexameric species is observed (Fig. 6a and Supplementary Table 3). Using the ENTH2/PIP2 dimeric interface from the S. cerevisiae ENTH2/PIP2 crystal structure as a rigid body, and representing the C-terminal parts missing in the crystal structure as dummy residue chains, a model for the hexamer was constructed yielding an excellent fit to the experimental data (Fig. 6b and Supplementary Fig. 4d). Three ENTH domains are oriented with α0 helices in plane of the cell membrane, in accordance with previous observations. For the three other ENTH domains, the PIP2 molecule is still oriented in plane with the cell membrane (Fig. 6c), but α0 is pointing away, available to interact with additional ENTH domains to form larger oligomers. Similarly, the interface involving Thr 104 is alternatively used for hexamer formation as well as further oligomerization through phospholipid interfaces.

Characterization of phospholipid-dependent complex formation. We used thermal denaturation aggregation assays by dynamic light scattering (DLS), to investigate stability differences between fungal and human ENTH/ANTH/PIP2 complexes. ENTH1 from S. cerevisiae in the presence of PIP2 shows a relatively low mid-aggregation temperature (T_agg) of 33 °C (Fig. 6d), which increases to 37 °C when ANTHSla2 is added. In contrast, the hexameric human ENTH core shows a T_agg of 45 °C in the presence of PIP2, which is more stable than the assembled fungal ENTH/ANTH complex. The optimal body temperature for H. sapiens is 37 °C, and the thermostability assay indicates that human ENTH domain alone will form a stable complex at that temperature, in contrast to ENTH domains from S. cerevisiae.

To test whether PIP2 binding is the driving force for complex formation, rather than direct protein–protein interactions, we looked for cross-species complexes from C. thermophilum and S. cerevisiae by native MS, ITC, and DLS (Supplementary Fig. 5). Indeed, such complexes can be formed and are surprisingly robust as measured by ITC (K_D = 450 nM ± 59 nM) (Supplementary Fig. 5c, d), despite low sequence identity. We also investigated whether the ANTHSla2 domains from S. cerevisiae and C. thermophilum bind to the human epsin ENTH core. The human-fungi cross-species assemblies show degenerated formation of PIP2-dependent complexes, with the 6:6 complex as the

Fig. 4 ENTH/ANTH/PIP2 complex formation in fungi. 6:6:18 and 8:8:25 ENTH/ANTH/PIP2 complexes are the observed stoichiometries in native MS measurements. A cartoon of the most prominent complex is shown with ANTH in green and ENTH in blue. Complexes from C. thermophilum (green) and S. cerevisiae, containing ENTH1 (orange) or ENTH2 (red), show the same stoichiometries, signal ratios and dissociation pathways in collision-induced dissociation (CID) MS/MS. Here, the dissociation of the +40 charged 8:8:25 complex into partially unfolded ANTHSla2 (top left, cartoon shows green ANTH domain) and a residual 8:7:23 complex (top right, showing also a cartoon of the remaining complex) is depicted. The annotation shows the stoichiometry (ENTH:ANTH:average PIP2 number), charge state of the main peaks, and average experimental masses. Ranges of PIP2 numbers, statistical errors, and an average FWHM value rating the MS resolution of all complexes can be found in Supplementary Table 2. ANTH dissociation in CID MS/MS measurements of 6:6:18 ± 1 ENTH2/ANTH/PIP2 complexes from S. cerevisiae is presented in Supplementary Fig. 4b.
These cross-species measurements confirm that human ENTH forms a stable hexameric core in the presence of PIP2, which can be decorated by ANTH domains through PIP2 interfaces. However, the fungal ANTH cannot open up the hexameric core to create larger assemblies as in the human ENTH/ANTH system.

**Discussion**

Clathrin-mediated endocytosis involves a diverse set of adapter proteins that help to curve the cell membrane and to recruit clathrin and cargo. The initial stages of vesicle coat assembly involve many adapter proteins and may involve many alternative assemblies\(^2^6\). Epsin and the phospholipid PIP2 are essential for clathrin-mediated endocytosis\(^2^2,2^7\), and we show how assemblies involving many adapter proteins and may involve many alternative assemblies\(^2^6\). Epsin and the phospholipid PIP2 are essential for clathrin-mediated endocytosis\(^2^2,2^7\), and we show how assemblies involving many adapter proteins and may involve many alternative assemblies\(^2^6\).

**Fig. 5** Dynamics of ENTH/ANTH/PIP2 complex formation in fungi and human assemblies. **a** Time course of ENTH2/ANTH\(_{Sla2}\)/PIP2 (\(S.\) *cerevisiae*) complex formation. Components were mixed, injected into the electrospray capillary and the spectra monitored over time. Relative signal intensities for 6:6 (green), 8:8 (dark blue), and dimers of 8:8 complexes (light blue) were determined and plotted against time (\(n = 3\)). The signal of the 6:6 complex drops within 2 min after mixing the complex components, while the signal of the 8:8 complex increases, suggesting a transition between these forms. The signal of the 8:8 dimer remains constant, ruling out aggregation effects.

**b** Native MS shows oligomerization of human epsin-1 ENTH and Hip1R ANTH in various stoichiometries, ranging from 6:0 to 8:8 with 6:6 being the main species. **c** Human epsin-1 ENTH domain forms hexamers (6:0) with at least six PIP2 molecules also in absence of Hip1R.
other and have been shown by mutagenesis to be essential for the formation of the ENTH/ANTH<sub>Sla2</sub> complex<sup>14</sup>. In addition, residue Arg 37 can toggle its side chain between the canonical PIP2-binding site of ANTH domains, and the ENTH/ANTH<sub>Sla2</sub> interface.

The biophysical experiments presented here have revealed that there is a difference in epsin and Sla2/Hip1R ENTH/ANTH complex assembly between fungal and human proteins. There is a general sequence of events revealed by native MS (Fig. 7). After PIP2 binding, ENTH domains form a core that is decorated on the outside by ANTH domains. Many different assemblies may occur, but in fungi the ENTH core alone is not stable enough to be maintained. The ENTH and ANTH domains cluster to yield a homogeneous 8:8 complex (Fig. 7a). In humans, the ENTH hexamer core is stable, but once it is decorated with ANTH, many different clusters may form (Fig. 7b).

**Fig. 6** Human ENTH forms a thermally stable hexamer with a predicted membrane-binding interface. a SAXS modeling of human ENTH in the presence of PIP2. SAXS data recorded for human ENTH in solution are shown (gray circles) along with a fit to the rigid-body model refined against the SAXS data with P3 symmetry using CORAL (orange solid line). Experimental errors are from counting statistics on the Pilatus 2M detector and propagated through the data reduction process as standard errors in the scattering intensities. The χ<sup>2</sup> for the fit is 1.05. The inset shows the dimensionless Kratky plot representation of the SAXS data and the same fit. b Backbone and surface representation of the SAXS refined rigid body model. C-terminal (Ct) residues not observed in the crystal structure of the tandem domains are modeled as dummy residues by CORAL. P3 symmetry was enforced and the tandem ENTH domains with bound PIP2 used as rigid bodies. c 90° rotation of the model, demonstrating that the bound PIP2 molecules are all located on one side of the protein, consistent with a membrane-binding interface. The Thr 104 residues involved in ENTH/ENTH homodimerization are colored pink, whereas the Thr 104 residues present on the surface of the hexamer are colored green. d Hydrodynamic radius as a function of temperature measured by DLS (ENTH, <i>S. cerevisiae</i>, orange; ENTH/ANTH complex, <i>S. cerevisiae</i>, green; hexameric ENTH core, <i>H. sapiens</i>, blue)
S. cerevisiae, Sla2 ANTH is needed for the stable binding of epsin ENTH to the endocytic site and both proteins are essential for actin-dependent endocytosis. In contrast, mammalian epsin ENTH binds to the endocytic site in the absence of Hip1R and is necessary for Hip1R recruitment to the endocytic site. Consequently, an absence of epsin has more impact on endocytosis than the lack of Hip1R, suggesting epsin ENTH is crucial in PIP2-driven adapter protein complex formation in mammalian endocytosis. The independent formation of higher oligomers of the human ENTH domain alone was also observed in giant unilamellar vesicles by fluorescence microscopy.

Methods

Chemicals. Phosphatidylinositol-4,5-biphosphate diC8 (PIP2) was purchased from Echelon. n-dodecyl β-D-maltoside (DDM) was purchased from Anatrace.

Constructs and plasmids. The coding sequence for the ANTH and ENTH homologs from Chaetomium thermophilum were amplified from an RNA library kindly provided by Peer Bork’s laboratory (see constructs in Supplementary Table 4); EMBL Heidelberg using the QIAGEN OneStep RT-PCR Kit. The reverse transcription was done at 50 °C for 30 min and the second round PCR was performed in the presence of solution Q and a touchdown procedure was introduced starting at 72 °C (see primers in Supplementary Table 5). The DNA coding sequence was cloned in pETM-11/LIC vector (EMBL). Human CALM coding sequence has been purchased from Addgene (Plasmid #27691) and the ANTH domain was cloned in pETM-11/LIC vector for expression with an N-terminal 6xHis-tag. Sequence coding for ANTH domain of human Hip1R (aa 1–300) was PCR amplified from human cDNA and cloned into the NcoI-XhoI sites of pETM30 (EMBL). Sequence coding for ENTH domain of human EPN1 (aa 1–158) was PCR amplified from human cDNA (with the additional codon for glycine after START codon) and cloned into the BamHI-XhoI sites of pETM30. Sequence coding for ANTH domain of Yap1802 (aa 1–272) was PCR amplified from yeast gDNA and cloned into the NcoI-XhoI sites of pETM30.
Protein production and purification. Recombinant human Hip1R ANTH, human ENTH and the yeast Yapl1802 ANTH domains were expressed in E. coli BL21 DE3 (Novagen) with a protein concentration of 4°C. Mammalian Hip1R human ENTH with no PiP2 added was used. Synchrotron radiation X-ray scattering data were collected (EMBL, P12, PETRA III, DESY, Germany)33 (Supplementary Table 3) with a PILATUS 2 M pixel detector (DECTRIS, Switzerland) (20 x 0.05 s frames). Solutions of human ENTH (0.4–1.5 mg/ml) were measured through a capillary (20°C). The sample-to-detector distance was 3.1 m, covering a range of momentum transfer 0.15 s ≤ 0.46 Å−1 (s = 4 × sin(β/2)). Frame comparison showed no detectable radiation damage. Data from the detector were normalized, averaged, buffer subtracted, and placed on an absolute scale that is relative to water, according to standard procedures. All data manipulations were performed using PRIMUSqt and the ATSAS software package42. The forward scattering I(0) and radius of gyration, Rg, were determined from Guinier analysis: I(0) = I0exp(−sRg2/2)). The indirect Fourier transform method was applied using the program GONOM43 to obtain the distance distribution function p(r) and the maximum particle dimensions Dmax. Molecular masses (MMs) of solutes were extracted from SAXS data by comparing the intraparticle forward scattering with that of a reference solution of glucose isomerase (Hampton), the hydrated particle/PoReO volume Vp, where molecular mass is estimated as 0.588 times Vp and from the excluded solvent volumes, Vex, is obtained from DAMMIF44 through ab initio modeling. Rigid body modeling was performed using the program CORAL53.

Natural mass spectrometry. Prior to native MS analysis45, purified proteins were buffer exchanged to 300 mM ammonium acetate (PN 431311, 99.99% purity, Sigma-Aldrich) and 1 mM DL-dithiothreitol (PN 43815, 99.5% purity, Sigma-Aldrich), pH 8.0, via centrifugal filter units (Vivaspin 500, MWCO 5000 and 10000, Sartorius) at 15,000 x g and 4°C. Complexes were assembled after buffer exchange by mixing 10 μM ENTH, 10 μM ANTH and 60 μM PiP2 (Phosphatidylinositol-4,5-bisphosphate diC8, Echelon). Thus, soluble PiP2 was used at a submicellar concentration that is in the range of its physiological concentration in the cell46.

ESI capillaries were produced in house. Therefore borosilicate capillaries (1.2 OD, 0.68 ID, World Precision Instruments) were processed in a two-step program with a microproppet puller (P-1000, Sutter instruments) using a squared box filament (2.5 x 2.5 mm, Sutter instruments) and subsequently gold-coated using a sputter coater (Q150T, 40 mA, 200 s, tooling factor of 2.3 and end bleed vacuum of 8 x 10−2 mbar, Argon, Quantum Technologies).

Native MS on protein complexes was performed on a QTof 2 (Waters and MS Vision) modified for high mass experiments27 with nanoESI source in positive ion mode. The gas pressures were set to ≈1.5 mtorr in the source region and 1.1 x 10−2 mbar xenon (purity 5.0) in the collision cell48,49. Mass spectra were recorded with applied voltages for capillary, cone, and collision of 1.35 kV, 120–150 V, and 30–60 V, respectively, optimized for good resolution and minimal complex dissociation.

Complexes were analyzed in MS/MS by ramping collision voltages from 10 to 400 V in order to eject protein subunits. Raw data was calibrated with 25 mg/ml cesium iodide spectra of the same day with the software MassLynx (Waters). MassLynx and MassLynx were used to assign peak series to protein species.

Lipid binding was studied on an LCT ESI-ToF system (Waters and MS Vision) using a 20 µM solution of the direct interactant PIP2 (M-1000, Sutter instruments) in 200 mM NaCl, supplemented with 200 mM P1P2.

Dynamic light scattering. Measurements have been performed using DynaPro Nanostar (serial no. 323-DPN, Wyatt Technology Corporation). Data have been processed using Dynamics v.7 software. 30 μM protein and PiP2 were mixed and incubated overnight at 4°C. Samples were filtered through 0.22 μm centrifugal filters (Millipore). The acquisition time was 3 s with a total of 20 acquisitions.
Yeast strain and plasmids. Yeast strain MKY0764 (MATa, his3Δ200, leu2-3,112, ura3-52, JEN2-801, sla2::NAT1) was transformed by pRS416-based centromeric plasmid expressing indicated variants of SlA2 aa 1–360 fragments (generated by overlapping PCR with mutagenic primers). The expression levels of protein constructs were assessed by immunoblotting with anti-HA antibodies (Covance MMS-101R) at 1:1000 dilution. The secondary antibody used was anti-mouse HRP conjugate (Bio-RAD, 1706516) at 1:3000 dilution.

Data availability. The atomic coordinates for the ENTH2/PiP2 complex (PDBID 5ON7), the ENTH1 structure (PDBID 5ONF), and the ENTH2 structure (PDBID 6ENR) from the S. cerevisiae as well as the Sla2 structure from C. thermophylum (PDBID 5SO0) are deposited at the Protein Data Bank. Other data are available from the corresponding authors upon reasonable request.

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Author contributions
R.M. conceived the work together with C.U. and M.K. M.G.-A. produced proteins and samples for crystallization, SAXS, and native mass spectrometry, determined the Sla2 ANTH crystal structure, designed and performed biophysical experiments, and interpreted the data together with M.S. and J.H. M.K. performed growth experiments in *S. cerevisiae*. A.G. produced proteins for native mass spectrometry and the ENTH1 and ENTH2/PIP2 crystals. H.D.T. M. performed SAXS analysis and interpreted the data together with D.I.S. R.M. wrote the manuscript with input from all authors.

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