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Molecular and structural basis of ESCRT-III recruitment to membranes during archaeal cell division

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Summary

Members of the crenarchaeal kingdom, such as *Sulfolobus*, divide by binary fission yet lack genes for the otherwise near-ubiquitous tubulin and actin superfamilies of cytoskeletal proteins. Recent work has established that *Sulfolobus* homologs of the eukaryotic ESCRT-III and Vps4 components of the ESCRT machinery play an important role in *Sulfolobus* cell division. In eukaryotes, several pathways recruit ESCRT-III proteins to their sites of action. However, the positioning determinants for archaeal ESCRT-III are not known. Here, we identify a protein, CdvA, that is responsible for recruiting *Sulfolobus* ESCRT-III to membranes. Overexpression of the isolated ESCRT-III domain that interacts with CdvA results in the generation of nucleoid-free cells. Furthermore, CdvA and ESCRT-III synergise to deform archaeal membranes *in vitro*. The structure of the CdvA/ESCRT-III interface gives insight into the evolution of the more complex and modular eukaryotic ESCRT complex.

Introduction

Archaea of the genus *Sulfolobus* such as *Sulfolobus acidocaldarius* (Sac) grow in acidic hot pools with optimal growth at 80°C and at a pH between 2 and 4. The organisms are single celled and divide by binary fission. During logarithmic growth, *S. acidocaldarius* has a doubling time of about 180 minutes and has a cell cycle with two distinct gap phases (Bernander, 2007; Duggin et al., 2008). There is a short G1 period (less than 5 minutes) where cells have a single copy of their chromosome followed by an S-phase, lasting about 90 minutes. Approximately half of the cell cycle is spent in a state where cells have two copies of the chromosome. For much of this time, there is no visible separation of sister nucleoids. Furthermore, sister chromatids remain paired following replication for a significant period of the G2 phase (Robinson et al., 2007). During cell division, nucleoids segregate prior to the detection of invagination of the cell membrane and subsequent cell division by binary fission (Lindas et al., 2008; Samson et al., 2008). Until recently it was unclear how *Sulfolobus* cells effect division, as they, in common with other Crenarchaea, lack homologs of the tubulin and actin superfamilies of cytoskeletal proteins. However,

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examination of *Sulfolobus* genomes revealed the presence of genes encoding homologs of the ESCRT-III and Vps4 components of the well-characterized eukaryotic ESCRT apparatus (Hobel et al., 2008; Lindas et al., 2008; Obita et al., 2007; Samson et al., 2008). First identified for their role in endosome sorting, eukaryotic ESCRT proteins also play pivotal roles in additional membrane manipulation processes, including viral egress from infected cells and membrane abscission in the final stages of cytokinesis in human cells (reviewed in Teis et al., 2009; Wollert et al., 2009b; Carlton and Martin-Serrano, 2009). Studies in *Sulfolobus* have revealed that the genes encoding ESCRT-III and Vps4 homologs undergo transcriptional regulation during the cell cycle, with mRNA levels highest in dividing cell populations (Lindas et al., 2008; Samson et al., 2008). Furthermore, the ESCRT-III and Vps4 proteins localize between segregated nucleoids where they have been shown to form ring-shaped structures. The belt of ESCRT-III and Vps4 reduces in diameter concomitant with membrane ingression during cell division. Finally, overexpression of a *trans*-dominant negative allele of Vps4 ATPase in *Sulfolobus* led to aberrant cell morphologies indicative of failed cell division. More specifically, induction of this allele led to an accumulation of cells with elevated, non-integral DNA contents and also the appearance of cells lacking nucleoids (Samson et al., 2008). Thus, as in human cells (Carlton and Martin-Serrano, 2007; Morita et al., 2007), the ESCRT apparatus in archaea plays a key role in cell division.

In eukaryotes, complex networks of interactions lead to the appropriate spatial and temporal localization of the ESCRT-III machinery. However, while Crenarchaea clearly possess homologs of ESCRT-III and Vps4, they lack detectable sequence homologs of components of ESCRT-0, ESCRT-I and ESCRT-II complexes or Alix and Cep55 proteins that play roles in ESCRT-III positioning in endosome sorting, viral budding and midbody localization in eukaryotes. Furthermore, in eukaryotes, a number of components of the ESCRT machinery show binding preferences for lipids containing 3-phosphoinositides, moieties not found in archaeal lipids (Slagsvold et al., 2005; Gaullier et al., 1998; Kutateladze et al., 1999; Teo et al., 2006). Indeed, archaeal phospholipids are distinct from those in eukaryotes (Chong, 2010; Michell, 2008). They possess ether linkages between glycerol and hydrocarbon chains rather than the ester linkage found in bacteria and eukarya. In addition, in organisms such as *Sulfolobus*, greater than 95% of the lipids are membrane spanning tetraether lipids. Thus, how the archaeal ESCRT proteins interact with these unusual membranes has not yet been resolved.

Previous work from Lindas and colleagues proposed that a third open-reading frame, termed CdvA (Saci_1374), was co-transcribed and thus co-regulated with the ESCRT-III and Vps4 genes (Saci_1373 and Saci_1372). Furthermore, CdvA was shown to form structures at mid-cell between segregated nucleoids (Lindas et al., 2008). However, in the following, we reveal that the gene for CdvA shows distinct temporal regulation from the adjacent ESCRT-III and Vps4 genes. Furthermore, CdvA structures appear in the cell prior to nucleoid segregation. Our analyses reveal that CdvA interacts with a *Sulfolobus* ESCRT-III homolog. The crystal structure of the CdvA/ESCRT-III interface shows a novel protein-protein interaction based on a winged-helix-like fold. Additionally, we find that CdvA is a membrane-interacting protein that recruits ESCRT-III to liposomes made of archaeal lipids. Finally, we demonstrate that CdvA and a single ESCRT-III paralog can act synergistically to induce extensive membrane deformation in a reconstituted system.

Results

Interaction of CdvA (Saci_1374) with ESCRT-III (Saci_1373)

Examination of the Sac genome sequence reveals that the ESCRT-III homolog (Saci_1373) and Vps4 (Saci_1372) lie downstream of an open reading frame for CdvA (Saci_1374) that is conserved in the *Sulfolobales* and other Crenarchaea that possess the ESCRT machinery.

In addition, homologs of this gene are found in a second archaeal phylum, the Thaumarchaea, species that encode both ESCRT-III and FtsZ proteins. The protein sequence of CdvA suggests that the N-terminal 70 residues might form a β -barrel with similarity to the PRC-barrel, a widespread domain implicated in a number of cellular processes (Kanehisa and Goto, 2000; Anantharaman and Aravind, 2002). This is followed by an α -helix-rich region and finally a C-terminal tail of low sequence complexity but which has a highly conserved motif at the C-terminus of the protein (Fig. 1A and Fig. S1). All Crenarchaea that have the ESCRT machinery have a single ESCRT-III paralog with a C-terminal winged-helix (wH)-like domain (Fig. S2). The *cdvA* gene and that for the wH-like domain-containing ESCRT-III protein are adjacent in the Sulfolobales and some Desulfurococcales but these genes are separated in the chromosomes of other species (Fig. S3). Nevertheless, given the juxtaposition of the gene for CdvA and those for Vps4 and an ESCRT-III homolog in *Sulfolobus* species, we tested whether these proteins might interact. While we could not observe any interaction between CdvA and Vps4 by either GST-pull down or yeast 2-hybrid assays (data not shown), we could readily detect interaction between CdvA (Saci_1374) and the ESCRT-III homolog (Saci_1373). Deletion analysis coupled with GST-pull downs revealed that the wH-like C-terminal extension of the ESCRT-III protein was necessary and sufficient for interaction with residues 69-238 of CdvA (Fig. 1B). Next, we sought to delimit the minimal interaction interface. Initially, band shift analysis on native PAGE showed that a construct consisting of CdvA residues 208-238 was necessary and sufficient for interaction with the ESCRT-III wH-like C-terminal extension (data not shown). Fluorescence anisotropy measurements showed a K_d of about 6 μ M (Fig. 1C). Further yeast 2-hybrid analyses revealed that the C-terminal 18 residues of CdvA (hereafter referred to as the E3B – ESCRT-III binding - peptide) mediated the interaction with the wH-like domain of the ESCRT-III homolog (Fig. 1D).

Examination of the sequence of CdvA revealed that two valines, Val232 and Val234, are highly conserved between crenarchaeal CdvA homologs (Fig. 1A). We therefore mutated both of these residues to aspartate; this resulted in a loss of interaction with the wH-domain (Fig. 1D, “DRD”). In contrast, mutation of two nearby, but non-conserved, valines (Val226 and Val228) to aspartate had no detectable effect on the interaction (Fig. 1D, “DSD”). The impact of the V232D and V234D double mutant was confirmed using GST pull down assays (Fig. 1E). As also seen in Fig. 1B, GST fused to full length ESCRT-III (Saci_1373) efficiently pulls down CdvA (69-238) (Fig. 1E, left panel). In contrast, no interaction between CdvA containing the DRD mutations and ESCRT-III was detectable (Fig. 1E, right panel).

Sulfolobus species encode four ESCRT-III homologs, however, only the one proximal to CdvA has the wH-like extension. In agreement with the above data revealing the wH-like domain to be the CdvA-binding site, no interaction could be detected between the other three ESCRT-III homologs and CdvA (data not shown).

Overexpression of the wH domain generates nucleoid-free cells

Next, we wished to determine whether the CdvA-ESCRT-III interaction was important for ESCRT-III function in vivo. Our previous work revealed that overexpression of a Walker B mutant allele of Vps4 in *S. solfataricus* PH1-16 led to the accumulation of cells with elevated DNA content and also the generation of nucleoid-free cells. Accordingly, we expressed the wH domain of the orthologous *S. solfataricus* ESCRT-III (SSO0910) from an episome containing an arabinose inducible promoter in this strain. As can be seen in Fig. 2A, induction of the wH domain results in a three-fold increase in the generation of nucleoid-free cells that stain very brightly with the membrane-binding dye FM4-64X (Fig. 2B). Interestingly, and in contrast to the phenotype observed with the Vps4 mutant allele, we

did not detect significantly elevated levels of cells with increased DNA content (Samson et al., 2008). Nevertheless, the generation of nucleoid-free cells suggests that normal cell division processes have been perturbed by overexpression of the wH-like domain.

Structure of the CdvA E3B peptide in complex with the wH-like domain

In order to understand how the wH-like domain of ESCRT-III interacts with CdvA, we co-crystallised *S. solfataricus* ESCRT-III (residues 210-259) with the E3B peptide (residues 251-265) from *S. solfataricus* CdvA. The E3B boundaries used for the crystallization construct omitted flanking flexible regions identified by NMR measurements (see Experimental procedures). The two proteins engage each other to form a single domain with a novel winged-helix-related architecture (Fig. 3A, Fig. S4A). The classic wH fold consists of a β -sheet mounted on a three-helix tripod (Aravind et al., 2005). In the three-stranded wH domains, the hairpin connecting the final two strands of the β -sheet constitutes the eponymous “wing”. In the ESCRT-III/CdvA complex the ESCRT-III wH domain has what we will refer to as a “broken wing”: the ESCRT-III forms only the two outer strands of the β -sheet with a prominent gap between them. A segment of CdvA inserts into this gap to form the middle strand of the β -sheet thereby “healing” the “broken-wing” (Fig. 3A-C). In addition to this novel broken-wing architecture, the ESCRT-III/CdvA wH domain has a unique parallel β -sheet, whereas all other three-stranded wH domains have an anti-parallel β -sheet (Fig. 3A and Fig. S4).

The E3B region that heals the broken wing fold is conserved in other Crenarchaea (Fig. 1A), and its shape is closely complementary to the shape of the slot in the broken wing (Fig. 3D). Mutations of *S. acidocaldarius* E3B residues involved in this interface reduce or eliminate interaction with ESCRT-III (Fig. 1 and Fig. 4). The hydrophobic side chains of the *S. solfataricus* CdvA, Ile257, Val259 and Val261, are oriented into the interior of the broken wing (Fig. 3A), so we mutated the homologous residues in the *S. acidocaldarius* CdvA (Ile230, Val232 and Val234). The V232D and V234D mutations resulted in affinities that were too weak to measure. The I230N mutation is at the edge of the binding site and has only a slight affect on binding to the broken wing (Fig. 4). We also mutated a basic residue in the E3B region (Arg233 in *S. acidocaldarius*) that the structure suggests could form a salt link with an acidic residue in the wH domain (*S. solfataricus* Lys260 in the E3B with Glu258 in the wH). The R233D and R233A mutations reduce binding to the broken wing, while the R233K mutation has no effect.

The CdvA/ESCRT-III interaction expands the range of types of interaction formed by the versatile wH fold. Although wH domains can bind a range of macromolecules, including DNA, RNA and proteins, each of these uses distinct surfaces of the wH domain (Fig. S4) (Gajiwala and Burley, 2000; Soler, 2007; Haering et al., 2004; Hierro et al., 2004; Teo et al., 2004). Interestingly, in eukaryotes, the wH domain is a fundamental unit that builds up the ESCRT-II complex. Each of the four ESCRT-II subunits consists of two tandem repeats of a wH domain, which make extensive interactions to form the heterotetrameric complex (Hierro et al., 2004; Teo et al., 2004). Furthermore, a wH domain from the Vps25 subunit of ESCRT-II makes a direct link to the ESCRT-III subunit Vps20 (Fig. S4D) (Im et al., 2009).

Sub-cellular localization of CdvA

A previous study revealed that CdvA forms structures between segregated nucleoids (Lindas et al., 2008). Using synchronised cell populations, we also observed CdvA at mid-cell between segregated nucleoids (Fig. 5A-D). Further, the size of the structure decreased concomitant with membrane ingression in dividing cells (Fig. 5A-D), until small foci are observed in both daughter cells at the final stages of separation (Fig. 5C and D). Z-stack imaging, deconvolution and 3D reconstruction revealed that these structures formed both

open and closed ring shapes (Movies S1-S3). However, in addition to mid-cell structures, we also observed a significant number of cells where CdvA structures were not at mid-cell. In some cases, CdvA structures could be observed in cells where no detectable nucleoid segregation had occurred (Fig. 5E-G). We also detected CdvA structures lying perpendicular to the eventual plane of division between segregated nucleoids. For example in Fig. 5H, the cell indicated by an arrow shows a left-right segregation of nucleoids and the CdvA structure also lies in this plane. These latter two classes of CdvA structures differ significantly from the behavior of ESCRT-III and Vps4, both of which only form ring-structures between segregated nucleoids (Samson et al, 2008; Lindas et al, 2008).

Previous work has proposed that CdvA and the adjacent genes for ESCRT-III and Vps4 are co-regulated and form an operon (Lindas et al., 2008). However, a recent high-resolution analysis of transcripts in *S. solfataricus* has revealed distinct 5' ends for transcripts of the CdvA gene and for a bicistronic mRNA encoding both ESCRT-III and Vps4 (Wurtzel et al., 2010). We therefore profiled mRNA (Fig. 6A) and protein (Fig. 6B) abundance for the Sac CdvA and adjacent ESCRT-III gene following cell birth in a population of cells synchronized using a "baby machine" (Duggin et al., 2008). In agreement with our previous study, the abundance of ESCRT-III (Saci_1373) transcript showed cell cycle regulation with levels peaking at 180 minutes after establishment of synchrony in G1 (Samson et al., 2008). The CdvA transcript also showed cell cycle dependent regulation, however, the wave of expression rises 30 minutes before that of the ESCRT-III transcript and peak mRNA abundance was observed at 150 minutes. Western blotting of whole cell extracts revealed that the protein levels of ESCRT-III are very tightly modulated during the cell cycle, with levels highest in dividing cell populations. While CdvA abundance also showed some modulation at the protein level, this was less marked than in the case of the ESCRT-III protein, with CdvA protein detectable at all cell cycle stages. Taken together, these data suggest that CdvA may form structures in cells prior to ESCRT-III ring formation. We therefore performed co-localisation studies with anti-CdvA and anti-ESCRT-III antisera (Fig. 6C). While CdvA structures can be readily detected in a range of cells, both with and without segregated nucleoids, we only detect discrete ESCRT-III structures in a subset of CdvA-positive cells in which nucleoids have already segregated.

CdvA recruits ESCRT-III (Saci_1373) to liposomes

In light of the observations above, we speculated that CdvA might play a role in recruitment of ESCRT-III to the site of cell division. To test this, we prepared liposomes from purified *S. acidocaldarius* tetraether lipids and performed liposome-binding assays with purified recombinant proteins. We do not detect significant membrane binding by the N-terminal putative β -barrel domain of CdvA (Fig. 7A, left panel). However, as can be seen in the right hand panel of Fig. 7A, we observe strong liposome binding by the C-terminal half of CdvA (containing the α -helix rich region and the E3B peptide). Next, we tested whether the ESCRT-III (Saci_1373) protein could bind to the liposomes. We could not detect interactions between the ESCRT-III protein and the liposomes in this assay (Figure 7B, lanes 7 and 8). This was initially somewhat surprising in light of the ability of purified eukaryotic ESCRT-III to bind membranes directly. Comparison of the sequence and predicted structure of the *Sulfolobus* protein with that of human CHMP3 revealed that a basic patch on the human protein, known to be important for membrane interaction (Muziol et al., 2006), is not well conserved in the archaeal homolog (Fig. S7). Importantly, however, although the archaeal ESCRT-III does not bind to liposomes on its own, in the presence of CdvA, it is recruited to liposomes (Fig. 7B, lane 11). We repeated these assays replacing full-length ESCRT-III with the isolated wH-like domain and observed CdvA-dependent recruitment of the wH-like domain to the liposomes (Fig. 7C, lane 11). Importantly, performing the assays with the V232D/V234D double mutant CdvA that cannot bind the

ESCRT-III wH domain abrogated the recruitment (Fig. 7D, lanes 11 and 12) but did not affect CdvA's ability to associate with the liposomes (Fig. 7D, lanes 9 and 10). Thus, CdvA is a membrane interacting protein that recruits the ESCRT-III paralog to membranes via the wH domain interaction.

CdvA and ESCRT-III synergize to deform liposomes

Given that eukaryotic ESCRT-III subunits have a profound role in deforming membranes to generate multivesicular bodies (Saksena et al., 2009; Wollert and Hurley, 2010; Wollert et al., 2009a), we wished to test whether liposome morphology was affected by either CdvA and/or ESCRT-III (Saci_1373) proteins. We therefore incubated the proteins with liposomes and visualized the result by negative stain electron microscopy. We used CdvA at 10 μ M and ESCRT-III at 1 μ M, as in the liposome binding assays described above (Fig. 7B). At these concentrations, in the liposome binding assays we see recruitment of ESCRT-III to liposomes by CdvA protein but, importantly, we do not detect any sedimentation of the two proteins in the absence of liposomes (Fig. 7B, lanes 5 and 6). As can be seen in Fig. 7E (and Figure S8), the addition of CdvA results in the introduction of regular rugosities on the surface of the liposome. These features have a diameter of 10-15 nm. In contrast, when ESCRT-III is added on its own, no substantial deformation of the liposomes is observed. However, when a combination of both CdvA and ESCRT-III was added then intact liposomes were no longer detectable. Instead, the combination of CdvA and ESCRT-III had the dramatic effect of inducing the formation of an extensive network of inter-connected tubular structures of diameter varying between 10 and 20 nm.

Discussion

We and others have recently described a role for archaeal homologs of the eukaryotic ESCRT-III and Vps4 proteins in cell division in *Sulfolobus* (Samson et al., 2008; Lindas et al., 2008). In addition, *Sulfolobus* ESCRT-III and Vps4 proteins are associated with secreted vesicles (Ellen et al., 2009) and an ESCRT-III paralog is packaged in purified *Sulfolobus* Turretted Icosahedral Virus (Maaty et al., 2006). However, while archaeal ESCRT-III family members and Vps4 can be readily detected by bioinformatic approaches, searches for homologs of the upstream components of the eukaryotic ESCRT apparatus have been fruitless. This has raised the question of whether adaptor proteins are required for archaeal ESCRT-III function (Samson and Bell, 2009). In this regard, it has been possible to effect in vitro reconstitution of intraluminal vesicle formation with purified eukaryotic ESCRT-III proteins in isolation (Wollert et al., 2009a). However, more recent studies with yeast ESCRT components have revealed that ESCRT-II nucleates ESCRT-III assembly (Teis et al., 2010) and that the presence of ESCRT-I and ESCRT-II drive formation of invaginated buds, the necks of which are then cleaved by ESCRT-III (Wollert and Hurley, 2010). Two key differences are observed in the presence and absence of ESCRT-I and ESCRT-II. First, in the presence of these additional complexes, 40-fold lower concentrations of ESCRT-III components are required. Second, ESCRT-I and ESCRT-II apparently prevent ESCRT-III entry into vesicles and thus facilitate their recycling. These in vitro observations recapitulate the observed lack of ESCRT components in the intraluminal vesicles of multivesicular bodies and suggest a possible mechanistic difference from archaea, where ESCRT-III and Vps4 have been detected in secreted vesicles (Ellen et al., 2009).

In the current work, we reveal that the CdvA protein serves as a recruitment platform for the ESCRT-III protein, Saci_1373. Our discovery that a wH-like domain present in the ESCRT-III paralog interacts with CdvA might be relevant to the evolution of the eukaryotic ESCRT-II machinery. While in eukaryotes an ESCRT-III subunit interacts with a wH domain (from ESCRT-II), in archaea, an ESCRT-III subunit has a wH domain directly fused to its C-terminus. Previously, we have shown that this same archaeal ESCRT-III subunit interacts

with the MIT domain of the archaeal Vps4, in a manner similar to the way in which the C-terminal region (MIM2) of the ESCRT-III CHMP6 interacts with the mammalian Vps4 AAA-ATPase (Samson et al., 2008; Kieffer et al., 2008). This means that the single archaeal ESCRT-III subunit combines an ESCRT-III-like function (interaction with archaeal Vps4) with a wH module that is fundamental to ESCRT-II structure and function.

It is possible that the archaeal wH-containing ESCRT-III protein may represent an ancestral molecule with “hard-wiring” of three important domains: 1) the ESCRT-III core fold; 2) the MIT-interacting motif (MIM2, found in the eukaryal ESCRT-III proteins Vps20 and Vps24) and 3) the wH domain, prefiguring that of present day Vps25. Separation of these domains during evolution would allow for increased modularity of the ESCRT apparatus and would thus build in greater regulatory potential. We have demonstrated that the α -helical region of CdvA is responsible for membrane interaction and that CdvA and the ESCRT-III paralog Saci_1373 act synergistically to induce catastrophic deformation of liposomes. This again, draws parallels with the eukaryotic ESCRT machinery where ESCRT-I and ESCRT-II facilitate membrane cleavage at physiologically relevant concentrations of ESCRT-III proteins (Wollert and Hurley, 2010). Our electron microscopy studies reveal that CdvA and the ESCRT-III paralog Saci_1373 combine to bring about extreme deformation of liposomes *in vitro*, resulting in the formation of tubular structures between 10 - 20 nm in diameter. Interestingly, the yeast ESCRT-III protein Vps24 has been shown to form homomeric filaments of ~15 nm diameter (Ghazi-Tabatabai et al., 2008). However, studies with the human ESCRT-III paralogs CHMP2A and CHMP3 have revealed that these proteins form regular tubular structures of ~40 nm diameter (Lata et al., 2008). In this regard, it may be significant that *Sulfolobus* species encode three additional ESCRT-III paralogs and these additional paralogs have the potential to form higher order structures with the Saci_1373 ESCRT-III protein (Samson et al., 2008). It is plausible that once Saci_1373 has been recruited to its site of action by CdvA, it then facilitates the assembly of the additional ESCRT-III paralogs into a higher order structure, perhaps analogous to the 40 nm tubules. Such a model has precedent in the sequential assembly of the core yeast ESCRT-III components, Vps20, Snf7 and Vps24 and Vps2 (Saksena et al., 2009; Wollert and Hurley, 2010).

Our analysis of the timing of transcription and measurements of the protein abundance of CdvA and the adjacent ESCRT-III homolog contradict the previous proposal that these genes are co-regulated (Lindas et al., 2008). This discrepancy may be due to the use of an acetic acid treatment of cells to cause an arrest in G2 in the previous work. Following release of the acetate-induced block, the population of cells gradually re-enter cycle. The gradual and relatively asynchronous re-entry of cells into cycle after acetate treatment may have obscured finer details of the kinetics of expression of these genes. Recently, a high-resolution transcript mapping study has revealed that the transcripts for CdvA and for ESCRT-III and Vps4 have distinct 5' ends, suggesting they are independent transcription units (Wurtzel et al., 2010). Furthermore, in a number of the Desulfurococcales, the gene for CdvA is not adjacent to ESCRT-III paralogs or Vps4 (Fig. S3). In agreement with the differential expression of the genes, we observe CdvA structures in cells that have not yet segregated their nucleoids. These CdvA structures precede the appearance of ESCRT-III rings between nucleoids. One enticing possibility is that the CdvA structures actually facilitate nucleoid segregation, perhaps by acting as a brace point in cells for assembly of the as-yet unidentified segregation apparatus. Such a model would result in eventual positioning of CdvA at mid-cell between segregated nucleoids. Once nucleoids have segregated, the CdvA ring could then facilitate assembly of the ESCRT-III ring, mediated by the E3B-wH domain interaction. The ESCRT-III assembly, acting in conjunction with Vps4, would then drive membrane ingression and eventual abscission.

Experimental procedures

Strains and Growth Conditions

Sulfolobus strains were grown as previously (Duggin et al., 2008; Samson et al., 2009). Details are presented in Supplemental Data.

Protein Purification, Antibody Production and Western Blotting

The plasmids used for protein purification were generated by PCR-mediated cloning using the primers listed in Table S1 and were transformed into *E. coli* Rosetta cells (Novagen) for protein expression (Supplemental Data). Oligonucleotide-mediated site-directed mutagenesis was performed according to the QuickChange protocol (Stratagene). Polyclonal antisera were raised against purified *S. acidocaldarius* CdvA (residues 69-238) in two rabbits (Covalab). Antisera were affinity purified as described (Samson et al., 2008).

Quantitative RT-PCR

Total RNA was purified from *S. acidocaldarius* and analyzed as described in (Samson et al., 2008) and Supplemental Data.

Immunofluorescence Microscopy

For samples enriched in dividing cells, 2 ml were collected from synchronized *S. acidocaldarius* cultures at 180 minutes of grow-out. For *S. solfataricus* cells overexpressing the Saci1373 wH domain, 500 μ l were collected from cultures 9.5 hours post-induction. Preparation of the cells, immunodetection, image capture and processing were performed as described (Samson et al., 2008). Movies displaying three-dimensional images were created using AutoQuant X software, version X2.2.0 (Media Cybernetics, Inc., Bethesda, MD, USA). Three-dimensional representations were constructed using the blind 3D deconvolution function of the software from 50 Z-stacked images taken at 0.1 μ m increments.

Crystallisation conditions for the complex

The structure was determined using the following constructs: the ESCRT-III wH domain (His₆-SSO0910, residues 210-259) (plasmid TO-149) and CdvA E3B (SSO0911, residues 251-265) synthetic peptide (made by Cambridge Peptides Ltd). The LMB nanolitre crystallisation robotic facility was used for a broad initial screen of 1440 crystallisation conditions. Optimal crystals for the complex were obtained at 17°C by vapour diffusion. The ESCRT-III wH domain at 31 mg/ml was first mixed with the E3B peptide (stock at 21.4 mg/ml) at 1:1.2 molar ratio of ESCRT-III:E3B and 0.7 μ l of the protein mixture was added to 0.7 μ l of a reservoir solution containing 12% PEG4000, 5mM CdCl₂ and 0.1M sodium acetate pH 4.0. Crystals formed immediately, but then spontaneously dissolved after 1-2 days. Replacement of the reservoir with 30% PEG4000, 5mM CdCl₂ and 0.1M sodium acetate pH 4.0 solution resulted in reappearance of stable crystals. Crystals were frozen by dunking in liquid nitrogen.

Phasing, refinement and model building

A 2.15 Å resolution data set for the wH/E3B complex was collected at 100 K with Diamond beamline I02 using a 1.54 Å wavelength. Data were integrated with IMOSFLM (Leslie, 2006) and scaled with SCALA from the CCP4 interface (CCP4, 1994). SHELXD (Uson et al., 2003) was used to locate the two Cd²⁺ sites in the asymmetric unit using the single-wavelength anomalous dispersion (SAD) and the phases were refined with the programme AutoSharp (Vonnrhein et al., 2007). The anomalous phasing power was 2.5 overall and the figure of merit for the SAD refinement was 0.47. Density modification was

carried out with an optimized solvent content of 64.3%. A model was automatically built using ArpWarp (Perrakis et al., 1999) with Refmac (Murshudov et al., 1997). The structure was manually re-built with Coot (Emsley and Cowtan, 2004) and refined with Refmac. The final model has 96.6% of residues in the core areas of the Ramachandran plot with no residues in disallowed regions (Laskowski et al., 1993). The data collection and refinement statistics are given in Table S2. Figures depicting the structure were prepared with the program PYMOL (Delano Scientific).

Fluorescence anisotropy binding measurements

Analyte protein was titrated into a cuvette containing 15 nM ESCRT-III (Saci_1373, residues 194-261) N-terminally labelled with Lumio Green (FLAsH-Saci_1373) in 1.1 ml binding buffer (20mM Tris (pH 7.4, 20 °C), 100 mM NaCl, and 5 mM 2-mercaptoethanol) – see Supplemental Data for Lumio green labeling procedure. Fluorescence was measured using a Perkin-Elmer LS-55 spectrophotometer with an excitation wavelength of 490 nm and an emission wavelength of 530 nm. Excitation and emission slits were 10 nm. A 1.0 mM protein analyte was titrated into a cuvette using a Hamilton-MicroLab titrator. The K_d values were calculated from direct fitting of the titration data to a single-site model. At least two independent experiments were conducted to determine K_d values. The plasmids used for the binding experiments are listed in Table S3.

GST Pulldowns and Yeast 2 hybrid analyses

These procedures were performed essentially as described previously (Samson et al, 2008), see Supplemental Data for detail.

Electron microscopy

For negative staining, aliquots of liposomes and protein were loaded onto glow-discharged formvar-coated 400-mesh copper grids and negatively stained with 1% (wt/v) aqueous uranyl acetate. Excess stain was removed by blotting with a filter paper, and the grids were air-dried before being examined in an FEI Tecnai 12 electron microscope.

Sedimentation assay for binding to archaeal liposomes

Polar lipid fraction E (PLFE) tetraether lipids were isolated from *S. acidocaldarius* cells as previously described (Lo and Chang, 1990). To make PLFE liposomes, an appropriate amount of PLFE in chloroform/methanol/water (65/25/10, v/v/v) was pipetted into a container and the solvents were evaporated first by a stream of nitrogen gas and then under high vacuum for > 12 h. The dried lipids were dispersed in pre-warmed buffer (65°C) and the dispersions were vigorously vortexed at 65°C for several minutes to yield multilamellar vesicles. Unilamellar vesicles were generated from multilamellar vesicles by extrusion 10 times using a lipid extruder (Lipex, Vancouver, BC, Canada) at 65°C through two stacked polycarbonate membranes (pore size = 200 nm) under nitrogen gas pressure. Liposome pelleting assays contained 10 μ M CdvA (69-238) or V232D V234D mutant protein, 5 μ M ESCRT-III wH-like domain or 1 μ M full length ESCRT-III in 100 μ l reactions containing 20 mM Tris pH 7.4, 100 mM NaCl and 400 μ g/ml archaeal liposomes as indicated. Reactions were incubated at 25°C for 15 minutes before centrifugation at 140,000 $\times$ g in a Beckman TLA100 rotor for 15 min at 20°C. The supernatants and the pellets (resuspended in an equal volume of buffer) were analyzed by SDS-PAGE and stained with Coomassie.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- A conserved protein, CdvA, recruits ESCRT-III to membranes during cell division
- Recruitment is mediated by a peptide-winged helix domain interaction
- We have determined the structure of this complex
- CdvA and a single ESCRT-III protein can drive membrane deformation in vitro

Figure 1.

The CdvA C-terminal region binds to the wH domain of an ESCRT-III paralog

A Cartoon of the domain organisation of ESCRT-III (Saci_1373) and CdvA. The sequence of the conserved C-terminal tail of CdvA is shown for a range of Crenarchaea (Species key: Ape = *Aeropyrum pernix*; Iho = *Ignicoccus hospitalis*; Mse = *Metallosphaera sedula*; Hbu = *Hyperthermus butylicus*; Dka = *Desulfurococcus kamchatkensis*; Sma = *Staphylothermus marinus*; Sto = *Sulfolobus tokodaii*; Sis = *Sulfolobus islandicus* strain L.S.2.15; Sso = *Sulfolobus solfataricus* P2; Sac = *Sulfolobus acidocaldarius*). The positions of the valine residues mutated to aspartate (panel 1D) are shown below the alignment.

B GST pull downs showing the C-terminal half of CdvA (residues 69-238) interacts with the wH-like domain of the ESCRT-III.

C Binding of FIAH-tagged ESCRT-III (Saci_1373) wH domain to GST-tagged CdvA constructs. The fluorescence anisotropy of the wH domain was measured as a function of

CdvA concentration. The CdvA construct 69-238 aggregates at higher concentration, so the K_d was not determined. Two additional CdvA constructs consisting of residues 1-208 or 69-208 were well expressed, but during concentration became gelatinous, making anisotropy measurements impossible.

D Yeast 2-hybrid analysis showing interaction of the C-terminal 18 amino acid residues of CdvA with the ESCRT-III wH-like domain. While the DSD mutant (V226D, V228D) of CdvA interacts with the wH domain, the DRD mutant (V232D, V234D) of CdvA does not.

E GST pulldowns showing that while CdvA (69-238) interacts with full length ESCRT-III (Saci_1373) fused to GST, the “DRD” CdvA (69-238) (V232D, V234D) mutant protein does not.

Figure 2. Overexpression of ESCRT-III wH domain generates nucleoid-free cells

A Representative images of populations of cells following arabinose induction of wH-domain (wH) or empty vector control (Vector). Nucleoid-free cells with elevated membrane staining are outlined and indicated with arrows.

B Quantification of the appearance of these nucleoid-free, membrane-dense cells in repressing (Gal) or inducing conditions (Ara). n = number of cells counted.

Figure 3. Structure of the CdvA/ESCRT-III interface

A Ribbon representation of the *S. solfataricus* CdvA E3B/ESCRT-III wH domain structure. The wH domain is green and the E3B from CdvA is red. Residue numbers are given first for *S. solfataricus* and, where different, for *S. acidocaldarius* in parenthesis.

B A view down on the E3B binding cleft on the wH domain. The wH domain is shown in a molecular surface representation in green and the E3B peptide shown as backbone with the side chains of important residues shown in stick form.

C Representation of the complex showing surface views of the wH (green) and E3B peptide (red).

D A cross-section through the molecular surface of the E3B/ESCRT-III wH complex seen in panel C, to emphasize the complementarity of E3B to the slot in the wH domain.

Figure 4. Mutation of E3B/wH interface residues reduces binding

Quantitative analysis of the CdvA (Saci_1374) /ESCRT-III (Saci_1373) wH interaction as measured by change in fluorescence anisotropy of FIAsh-tagged ESCRT-III wH domain upon titration of GST-E3B peptides. The K_d s listed in the lower panel were derived based on a single-site binding model. The mean and standard deviations are based on three independent titrations.

Figure 5. Cellular localization and cell-cycle expression of CdvA

A-H Immunolocalization of CdvA in cells. Panels show FM4-64X staining of cells (Membrane), DAPI staining (DNA), immunolocalization with anti-CdvA antibodies (CdvA) and merged images.

A-D The CdvA structure constricts as membranes constrict during division.

E-G The cell has a belt of CdvA traversing the middle of the cell, yet nucleoids have not segregated.

H The cell indicated with the arrow has a CdvA structure lying orthogonal to where the plane of division will form between the segregated nucleoids.

Figure 6. CdvA structures precede ESCRT-III recruitment

A Transcript abundance of CdvA and ESCRT-III (Saci_1373) measured by RT_qPCR of RNA isolated from synchronised cells (see Fig. S6 for the flow cytometry profile of the synchronised cells). Transcript abundance is expressed relative to levels at 0 minutes.

B Western blotting of whole cell extracts prepared from synchronised cells with anti-CdvA, anti-ESCRT-III (Saci_1373) and TBP – loading control).

C Co-immunolocalization of ESCRT-III (green) and CdvA (red) in cells. Cells were visualized with phase contrast and fluorescence microscopy (DAPI shows the position of nucleoids, anti-CdvA antisera are visualized in red and anti ESCRT-III (Saci_1373) in

green. Arrows indicate the position of cells with CdvA structures. A merged image is shown in the bottom panel with co-localization shown in yellow.

Figure 7. Liposome binding and deformation by CdvA and ESCRT-III (Saci_1373)

A Liposome binding assays with N- or C-terminal domains of CdvA (10 μ M) reveal that CdvA (69-238) binds liposomes. Liposome-bound proteins in pellets (P) were separated by ultracentrifugation from non-bound proteins in the supernatants (S).

B CdvA (69-238) recruits full length ESCRT-III to liposomes. CdvA (69-238) was present at 10 μ M, ESCRT-III at 1 μ M where indicated.

C CdvA (69-238) recruits the wH-like domain of ESCRT-III to liposomes. CdvA (69-238) was present at 10 μ M, ESCRT-III wH domain at 5 μ M where indicated.

D CdvA (69-238, V232D V234D) fails to recruit the wH-like domain of ESCRT-III to liposomes. CdvA (69-238, V232D V234D) was present at 10 μ M, ESCRT-III wH domain at 5 μ M where indicated

E Negative stain electron microscopy reveals extensive deformation of liposomes in the presence of both CdvA and ESCRT-III but not with individual proteins. CdvA was added at 10 μ M, ESCRT-III at 1 μ M. The scale bar represents 100 nm.