Functional assignment of multiple ESCRT-III homologs in cell division and budding in Sulfolobus islandicus

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Summary

The archaea Sulfolobus utilizes the ESCRT-III-based machinery for cell division. This machinery comprises three proteins: CdvA, Eukaryotic-like ESCRT-III and Vps4. In addition to ESCRT-III, Sulfolobus cells also encode three other ESCRT-III homologs termed ESCRT-III-1, –2 and –3. Herein, we show that ESCRT-III-1 and –2 in S. islandicus REY15A are localized at midcell between segregating chromosomes, indicating that both are involved in cell division. Genetic analysis reveals that escrt-III-2 is indispensable for cell viability and cells with reduced overall level of ESCRT-III-1 exhibit growth retardation and cytokinesis defect with chain-like cell morphology. In contrast, escrt-III-3 is dispensable for cell division. We show that S. islandicus REY15A cells generate buds when infected with S. tengchongensis spindle shaped-virus 2 (STSV2) or when ESCRT-III-3 is over-expressed. Interestingly, Δescrt-III-3 cells infected with STSV2 do not produce buds. These results suggest that ESCRT-III-3 plays an important role in budding. In addition, cells over-expressing the C-terminal truncated mutants of ESCRT-III, ESCRT-III-1 and ESCRT-III-2 are maintained predominantly at the early, late, and membrane abscission stages of cell division respectively, suggesting a crucial role of the ESCRTs at different stages of membrane ingestion. Intriguingly, intercellular bridge and midbody-like structures are observed in cells over-expressing MIM2-truncated mutant of ESCRT-III-2.

Introduction

Cell division is a fundamental process in all cellular life forms. In archaea, it has been indicated that at least three distinct membrane remodelling systems exist: the FtsZ-based bacterial-type system in Euryarchaeota and Nanoarchaeota; the ESCRT-III based eukaryotic-like system in the majority of Crenarchaeota and all Thaumarchaeota, Aigarchaeota and Lokiarchaeota; and a putative novel Arcadin-2-based system in Thermoproteales of Crenarchaeota (Makarova et al., 2010; Ettema et al., 2011; Spang et al., 2015). In Sulfolobus, the ESCRT-III based system comprises CdvA as well as Eukaryotic-like ESCRT-III and Vps4 proteins (Lindās et al., 2008; Samson et al., 2008; Wollert et al., 2009).

In eukaryotes, the ESCRT-III machinery is the key driver of membrane deformation (Wollert et al., 2009; Hurley and Hanson, 2010). The core ESCRT-III complex in yeast consists of Vps20, Vps32, Vps24 and Vps2; and they appear to assemble in this order, while the ATPase Vps4 is recruited through a specific sequence (MIM1) within Vps2, where Vps4 acts to disassemble the ESCRT-III oligomers (Lata et al., 2009; Wollert et al., 2009; Hurley and Hanson, 2010). In the current model of cell division in Sulfolobus, the protein components involved include CdvA, ESCRT-III (CdVB) and Vps4 (CdVC) (Lindās et al., 2008; Samson et al., 2008). CdvA can bind both membrane and DNA and serves as a recruitment platform for ESCRT-III (Linda˚s et al., 2008; Samson et al., 2011; Dobro et al., 2013). CdvA interacts with the C-terminal WH (winged helix)-like motif of ESCRT-III, and in turn, ESCRT-III can also interact with the MIT domain of Vps4 through its MIM2 (MIT interaction motif 2) domain. Among the three ESCRT-III homologs, ESCRT-III-1 and ESCRT-III-2 also contain the predicted MIM2 domain, but ESCRT-III-3 does not. Vps4, an AAA+ ATPase, functions in efficient disassembly of ESCRT-III polymer to complete membrane ingestion.

Like eukaryotes, Sulfolobus cells have multiple ESCRT-III homologs. In addition to ESCRT-III,
**Sulfolobus** encode three other ESCRT-III homologs (Samson et al., 2008) termed ESCRT-III-1, –2 and –3 (CdvB1, CdvB2 and CdvB3) (Yang and Driessen, 2014). The observation of interactions between these proteins (Samson et al., 2008) suggests that, same as in eukaryotes, ESCRT-III family proteins in **Sulfolobus** can form homo- and hetero-oligomers. To date, only ESCRT-III has been shown to be involved in cell division. Whether the other three ESCRT-III homologs are involved in cell division and whether they are essential for cell viability remain obscure, and the detailed mechanism of the ESCRT-III-mediated cell division is unknown.

Herein, we provide evidences showing that ESCRT-III-1 and ESCRT-III-2 participate in cell division. In addition, by analysis of stains over-expressing C-terminal truncated mutants, we reveals that ESCRT-III, ESCRT-III-1 and ESCRT-III-2 play important roles at different stages of membrane ingestion. Interestingly, structures resembling eukaryotic intercellular bridge and midbody in shape are observed in cells over-expressing the ESCRT-III-2 mutant suggesting a novel common feature between eukaryotes and archaea.

**Results**

ESCRT-III-1 and ESCRT-III-2 are localized at midcell between segregating chromosomes

*S. islandicus* REY15A, like many other species of Sulfolobus, encodes four ESCRT-III-like proteins (Lindás et al., 2008; Samson et al., 2008; Guo et al., 2011) which we designate as ESCRT-III (SiRe_1174), ESCRT-III-1 (SiRe_1550), –2 (SiRe_1200) and –3 (SiRe_1388) (Supporting Information Table S1). Phylogeny analysis of these ESCRT-III proteins from four main Sulfolobus strains revealed that ESCRT-III, ESCRT-III-1 and –2, cluster closely together into a group from which ESCRT-III-3 is separated (Supporting Information Fig. S1). To understand whether the ESCRT-III-1, –2 and –3 are involved in cell division, immunofluorescence microscopy analysis of exponentially growing cells (OD<sub>600</sub> 0.2–0.4) of REY15A was performed. Specific peptides were used to prepare for the antibodies and there was no cross reaction among these homologs (Supporting Information Fig. S2). As shown in Fig. 1A, fluorescence belts of ESCRT-III-1 were observed at midcell between segregating as well as un-segregating nucleoids. The fluorescence belts of ESCRT-III-2 were also observed at midcell in the absence of visible nucleoid segregation as well as between segregating nucleoids (Fig. 1B). The belts constricted as membrane ingestion proceeded during cell division. By contrast, the fluorescence of ESCRT-III-3 dispersed evenly in the cells (Fig. 1C). These data provide direct evidence that, in addition to ESCRT-III, ESCRT-III-1 and –2 are also involved in cell division, while ESCRT-III-3 may not have a direct role in cell division.

ESCRT-III-2 is indispensable for cell viability in S. islandicus

To understand the importance of the ESCRT-III homologs in Sulfolobus cells, we next examined whether these genes could be deleted using allele replacement (AR) or mark insertion and target gene deletion (MID) methods (Deng et al., 2009; Zhang et al., 2010). Using AR method, we obtained a deletion mutant of *escrt-III-3* (Supporting Information Fig. S3A and S4). The deletion of *escrt-III-3* was confirmed by PCR and Southern blot analysis. The ESCRT-III-3 protein was undetectable in the cells by Western blot analysis (Supporting Information Fig. S4A–C). In contrast, we were unable to obtain a pure deletion mutant of *escrt-III-1* (Supporting Information Figs. S3B and S5). After six rounds of selection on uracil-free plate, about 20, 40 and 160 cells were spread on the plate lacking uracil to isolate single colonies. The colonies formed on the plates all turned blue with X-gal staining (Supporting Information Fig. S5A). The genotypes of the culture of the colonies were further analysed by PCR. Although PCR amplification using flanking primers could not obtain a fragment corresponding to the wild type, the *escrt-III-1* fragment could be amplified using the *escrt-III-1* internal primers (Supporting Information Fig. S5B). The wild type genotype was only present in a tiny fraction in the cells as indicated by semi-quantitative PCR analysis (Supporting Information Fig. S5C). The expression of ESCRT-III-1 in the culture could hardly be detected by Western blot (Supporting Information Fig. S5D). Thus we eventually obtained a cell culture with reduced overall level of ESCRT-III-1. For convenience, hereafter, we named the culture as ‘Δescrt-III-1’.

By MID method, we could not obtain a deletion mutant of *escrt-III-2* neither (Supporting Information Figs. S6–S8). Counter-selection of purified marker-integrated cells in the medium containing 5-FOA and uracil did not produce cells with a knockout genotype but only generated cells with spontaneous mutation at *pyrEF*, with a mutation rate of about 2 × 10<sup>−7</sup> (Supporting Information Fig. S7, S8 and Table S5). A mutant propagation assay was used to confirm the essentiality of the gene (Zhang et al., 2010) and the result showed that the deletion mutant was unable to propagate, indicating *escrt-III-2* is essential for cell viability (Supporting Information Fig. S8). These results indicated that *escrt-III-1* and *escrt-III-2* but not *escrt-III-3* were probably indispensable for cell viability in *S. islandicus*. The
essentiality of escrt-III-1 for cell viability needs further investigation.

‘Δescrt-III-1’ displays growth retardation and cell division defect with chain-like morphology

Interestingly, the culture of ‘Δescrt-III-1’ with reduced overall level of ESCRT-III-1 displayed growth retardation and cell division deficiency with chain-like morphology (Fig. 2). About 30–51% cells were in chain-like form, while the ratio of cells in aggregation was only 1.1–7% in REY15A (Supporting Information Table S6). The DNA content of the ‘Δescrt-III-1’ culture was further analysed by flow cytometry. As shown in Fig. 3A, only a small proportion of the cells contained DNA of one copy (1C) of chromosome in ‘Δescrt-III-1’ and a large proportion of the cells had more than two copies (>2C) of chromosomes compared with REY15A. The flow cytometry images of cells with >2C of chromosomes showed that the majority of the cells were linked with more than two cells (Fig. 3B). These results are in agreement with those observed by phase contrast and scanning electron microscopy (SEM) (Fig. 2C and E) and further support a role of ESCRT-III-1 in cell division.

Fig. 1. In situ immunofluorescence microscopy of S. islandicus REY15A cells. Cultures were sampled in exponential growth phase (OD₆₀₀ 0.2–0.4). Phase contrast, DAPI (4’,6-diamidino-2-phenylindole) staining of nucleoids, and fluorescent-labelled antibody staining are shown and the images are merged. (A), Anti-ESCRT-III-1. (B), Anti-ESCRT-III-2. (C), Anti-ESCRT-III-3. Scale bar, 1.0 μm.
ESCRT-III-3 plays a role in budding

In eukaryotes, ESCRT-III proteins also participate in membrane-enveloped virus budding in addition to their role in cell division (Hurley and Hanson, 2010; Hurley, 2015). Since Δescrt-III-3 exhibited neither growth nor cell division defect under normal cultivation conditions (Fig. 4A and B), we wanted to know whether ESCRT-III-3 has a role in budding. We tested the responses of wild type stain REY15A and Δescrt-III-3 to the S. tenchongensis spindle-shaped virus 2 (STSV2) infection. STSV2 is a lipid enveloped single-tailed fusiform double-stranded DNA virus that can be stably cultured over long periods with laboratory strains of Sulfolobus, and no evidence has been found that STSV2 can lyse host cells under different stress conditions (Erdmann et al., 2014). Both Δescrt-III-3 and REY15A displayed growth retardation on infection, and the growth of Δescrt-III-3 was slightly slower than REY15A (Fig. 4A). The experiment was repeated three times and the same results were obtained. Interestingly, the cells from STSV2-infected REY15A were enlarged after infection with STSV2 for 90 h, and buds began to appear at one end of the cell (Fig. 4C and D). The budding peaked between 120 and 150 h. At 144 h, 38.6% (193/500) of the cells showed budding morphology. In contrast, the cells from STSV2-infected Δescrt-III-3 did not show the budding morphology of REY15A (Fig. 4C). STSV2 virus was present during the whole infection process as shown by PCR analysis of stsv2_37 (encoding a virus coat protein) (data not shown). In addition, immunofluorescence microscopy analysis revealed that the virus coat protein STSV2_37 was localized at the buds (Fig. 4E). Importantly, ESCRT-III-3 formed foci in STSV2-infected cells (Fig. 4F), in contrast to the dispersed distribution of the protein in un-infected cells (Fig. 1C). These results strongly suggested that ESCRT-III-3 play a critical role in virus-induced budding.

To further analyse the role of ESCRT-III-3, we construct an ESCRT-III-3 over-expressing strain Sis/pSeSD-ESCRT-III-3. The expression of the protein was confirmed (Supporting Information Fig. S11D). Intriguingly, cells over-expressing ESCRT-III-3 showed a budding...
morphology (Fig. 5A), but cells over-expressing other ESCRTs did not generate buds (data not shown). Immunofluorescence microscopy analysis revealed that ESCRT-III-3 was localized at the budding site (Fig. 5B). In addition, ESCRT-III-1 and -2 were also localized at the budding sites (Fig. 5B). As far as we know, this is the first report showing that *S. islandicus* REY15 produces buds under certain conditions, and ESCRT-III-3 plays an important role in budding. The detailed mechanism of ESCRT-III-3 in budding needs further investigation.

**Cells with over-expression of negative-dominant mutants of the ESCRT-III’s are blocked at different stages of cell division**

To investigate the mechanism of cell division by ESCRT-III and its homologs, we constructed strains over-expressing mutants of CdvA lacking the ESCRT-III binding domain and ESCRT-III’s lacking the predicted C-terminal MIM2 domains (lacking both MIM2 and WH-like domain for ESCRT-III) (Supporting Information Figs. S9 and 10, Table S2), and analysed the growth and cell morphology of the mutants. *S. islandicus* REY15A (E233S) harbours the empty vector, and the vector containing WT genes were used as controls (Supporting Information Table S2). The expression of the proteins was confirmed by Western blot (Supporting Information Fig. S11). Although the growth of cells over-expressing CdvAΔC, ESCRT-IIIΔC and ESCRT-III-1ΔC did not show apparent differences (Supporting Information Fig. S12A–C), the cells exhibited distinct division deficiency (Fig. 6). The over-expression of CdvAΔC resulted in enlarged cells (about two-fold in diameter). It was calculated that 32.3% (224/696) of the cells over-expressing

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**Fig. 3.** Flow cytometry analysis of REY15A and ‘Δescrt-III-1’. The cells were cultured to early exponential phase (OD₆₀₀ 0.2) and sampled every 3 h as described in the Materials and Methods. (A) DNA content profiles. Cells having one, two and more than two copies of chromosomes are indicated as ‘1C’, ‘2C’ and ‘>2C’ with arrows. (B) Representative images of cells (dotted box in A) with more than two copies of chromosomes of ‘Δescrt-III-1’ in the flow cytometry analysis. Left, phase contrast. Right, PI staining. Scale bars, 2.0 μm.
Fig. 4. Growth, cell morphology and immunofluorescence microscopy of REY15A and Δescrt-III-3 in response to infection with S. tenchongensis spindle virus (STSV2).
A. Growth curves of REY15A and Δescrt-III-3 with (filled square and triangle) and without (empty square and triangle) infection of STSV2.
B and C. Phase contrast microscopy of cells with and without infection of STSV2.
D. Representative images of cells from STSV2_infected REY15A.
E and F. Immunofluorescence microscopy analysis of cells infected with STSV2. The cells were sampled after being infected with STSV2 for 2 (E) and 6 days (F), and treated for immunofluorescence microscopy analysis as described in the Materials and Methods. Scale bars, 2.0 μm.

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CdvAΔC exhibited enlarged cell morphology after induction for 12 h with arabinose. Intriguingly, over-expression of ESCRT-IIIΔC led to enlarged cells with ‘peanut-like’ morphology: two sister cells connected together. Approximately 33.7% (236/700) and 10.3% (72/700) of the cells over-expressing ESCRT-IIIΔC exhibited enlarged and peanut-like cell morphology respectively. This morphology suggested that the cells were held at the early or middle stage of cell division as the division furrow could obviously be observed. The over-expression strain of ESCRT-III-1ΔC exhibited chain-like morphology resembling that of ‘ΔESCRT-III-1’ and the cells were enlarged (about 1.5-fold in diameter), and 49.7% (326/656) of the cells over-expressing ESCRT-III-1ΔC exhibited chain-like cell morphology. By contrast, only less than 3% cells that harbour empty vector or vector carrying the wild type genes showed the deficient phenotypes.

Strikingly, over-expression of ESCRT-III-2ΔC resulted in obvious growth retardation (Supporting Information). This morphology suggested that the cells were held at the early or middle stage of cell division as the division furrow could obviously be observed. The over-expression strain of ESCRT-III-1ΔC exhibited chain-like morphology resembling that of ‘ΔESCRT-III-1’ and the cells were enlarged (about 1.5-fold in diameter), and 49.7% (326/656) of the cells over-expressing ESCRT-III-1ΔC exhibited chain-like cell morphology. By contrast, only less than 3% cells that harbour empty vector or vector carrying the wild type genes showed the deficient phenotypes.

Flow cytometry analysis of ESCRT-IIIΔC and ESCRT-III-2ΔC over-expression mutants

To confirm the specific functions of ESCRT-III and ESCRT-III-2 in cell division, the cells of ESCRT-IIIΔC and ESCRT-III-2ΔC over-expression strains were analysed by flow cytometry. As shown in Fig. 7, the proportion of cells with DNA content of one copy (1C) of chromosome decreased drastically in both over-expression strains (Sis/pSeSD-ESCRT-IIIΔC and Sis/pSeSD-ESCRT-III-2ΔC) after induction, while the proportion of cells with DNA content of four copies (4C) of chromosomes increased obviously, especially in Sis/pSeSD-ESCRT-III-2ΔC. In contrast, the profiles of the controls did not change after induction with 0.2% arabinose.
arabinose. The results are consistent with those observed by microscopy and indicate that overexpression of ESCRT-III-D and ESCRT-III-2-D resulted in severe cell division defects.

Phase contrast and scanning microscopy analysis of Sis/pSeSD-ESCRT-III-2 ΔC cells

To gain a deeper understanding of the formation of intercellular bridge and midbody-like structures, we further analysed the structures in cells over-expressing ESCRT-III-2ΔC by microscopy. The cell size gradually increased with the addition of arabinose. After 6 h, the structures that resembled the midbody-like structure at the telophase and abscission stage appeared (Fig. 8A). Specifically, at a putative early telophase, an intercellular bridge formed between the two sister cells and then a midbody-like structure gradually formed at the middle of the intercellular bridge. When cells entered into a putative late telophase, the midbody was located at one end of the bridge closer to one of the sister cells. In addition, at abscission stage, the midbody was abscised from one side of the intercellular bridge and the remaining structure was still adhered to one daughter cell (Fig. 8A). After induction for 24 h, the cell became apparently enlarged and the majority of the cells exhibited remarkable plasma membrane deformation (Fig. 8B). The structures were further confirmed by scanning electron microscopy (SEM) analysis (Fig. 8C and D).

ESCRT-III-1 and –2 are localized at the midbody-like structure

Furthermore, we examined the localization of the three ESCRT-III homologs in Sis/pSeSD-ESCRT-III-2ΔC. As shown in Fig. 9, both ESCRT-III-1 and ESCRT-III-2 were localized at the midbody-like structures. In addition, ESCRT-III-1 and –2 could still form a belt-like structure localizing at midcell between segregating chromosomes. However, we could not find similar localization of ESCRT-III-3 at the intercellular bridge and midbody-like structures (data not show). These results support that...
the midbody-like structures are related to cell division in which ESCRT-III-1 and -2 are involved.

**Discussion**

Of the four ESCRT-III homologs, only ESCRT-III (CdvB, Saci_1373) has been demonstrated to be involved in cell division in *S. acidocaldarius* until this investigation (Lindás *et al.*, 2008; Samson *et al.*, 2008). Here, we showed that the other two ESCRT-III homologs, ESCRT-III-1 (SiRe_1550), and ESCRT-III-2 (SiRe_1200), also play roles in cell division in *S. islandicus*. We observed that ESCRT-III-1 and -2 were localized at midcell between segregating chromosomes (Fig. 1). Cells over-expressing the C-terminal truncated mutant of ESCRT-III-2 exhibited severe division defects and obvious growth retardation. Consistent with its role in cell
division, escrt-III-2 is essential for cell variability and cannot be deleted. We were unable to get a pure deletion mutant of escrt-III-1 and only obtained a culture (named ‘Descrt-III-1’) with the majority of cells harbouring the deletion genotype. The level of ESCRT-III-1 seriously decreased compared with the wild type cells. Cells of ‘Δescrt-III-1’ and the over-expression strain of C-terminal truncated ESCRT-III-1 exhibited chain-like cell morphology, a defect likely in membrane ingression during cell division (Figs. 2 and 6).

For escrt-III-3, we obtained a gene deletion mutant (Supporting Information Figs. S3A and S4), and found that the gene inactivation had no effect on growth and cell morphology under normal cultivation conditions (Fig. 4A and B). In addition, we observed that ESCRT-III-3 is dispersed evenly in the cell during cell division (Fig. 1C), supporting that ESCRT-III-3 does not play a direct role in cell division. Previously, it was reported that, in response to UV irradiation, the transcription of all the three genes of the ESCRT-III machinery (CdvA, ESCRT-III and Vps4) in *S. solfataricus* were down-regulated, and the ESCRT-III homologs ESCRT-III-1 and ESCRT-III-2, but not ESCRT-III-3, were also down-regulated (Frols et al., 2007). Our results that ESCRT-III-1 and ESCRT-III-2, but not ESCRT-III-3, are involved in cell division are in agreement with the finding in the transcriptomic analysis. Nevertheless, our results are not in agreement with a study showing that all three ESCRT-III homologs in *S. acidocaldarius* could be deleted and that the deletion strain of escrt-III-3 exhibited the most severe growth defect (Yang and Driessen, 2014).

We demonstrated that ESCRT-III-3 is involved in budding in *S. islandicus* REY15A. Buds were generated when the cells of the wild type strain were infected with STSV2, a membrane-enveloped virus (Fig. 4), and our finding was consistent with a recent report of budding of archael fusiform virus SSV1 (Quemin et al., 2016). Budding was also observed in cells over-expressing ESCRT-III-3 but not ESCRT-III, ESCRT-III-1 and 2 (Fig. 5). We showed that ESCRT-III-3 as well as cell division proteins ESCTR-III-1, and ESCR-III-2 are localized at the budding site (Fig. 5), in agreement with the fact that cell division proteins also participate in membrane abscission in budding in Eukaryotes. Because ESCRT-III-3 is not required in cell division but is related to virus budding, it may be a protein that mediates cell budding. The working mechanism of ESCRT-III-3 in this process needs further study.

Previous reports showed that there exist inter-homologue interactions among the cell division proteins in *S. acidocaldarius* (Samson et al., 2008; Samson et al., 2011). Briefly, CdvA interact with the C-terminal winged helix-like domain of ESCRT-III. ESCRT-III also has interaction with its homolog ESCRT-III-1 and the three ESCRT-III homologs interact with each other. In addition, ESCRT-III interacts with the N-terminal MIT domain of Vps4 through the C-terminal MIM2 domain of ESCRT-III. In fact, MIM2 domains also exist in the other
two ESCRT-III homologs, ESCRT-III-1 and –2 in *Sulfolobus* (Supporting Information Fig. S9). Using yeast two hybrid assay, we showed that ESCRT-III (SiRe_1174), ESCRT-III-1 (SiRe_1550) and ESCRT-III-2 (SiRe_1200) all have interaction with Vps4 (SiRe_1175) and the C-terminal MIM2 domain of the ESCRT-III is important for the interaction, because deletion of the MIM2 domain abolished the interaction (Supporting Information Fig. S13). Conversely, ESCRT-III-3 (SiRe_1388) has no interaction with Vps4 (SiRe_1175) due to its lack of the C-terminal MIM2 domain. We found that the four ESCRT-III homologs have interaction with each other and the core fold of the ESCRT-III is vital to maintain the interaction (Supporting Information Figs. S9 and S13). The interaction network supports that ESCRT-III, ESCRT-III-1 and –2 can be disassembled through interaction with Vps4, while ESCRT-III-3 is connected to ESCRT-III, ESCRT-III-1 and –2 through their core folds.

In eukaryotes, the midbody is a compact structure composed of membrane vesicles, tubulin, actin and myosin, and is formed at the late stage of cell division (Steigemann and Gerlich, 2009). The ESCRT-I subunit TSG101 (tumour-susceptibility gene 101) and the ESCRT-III subunit CHMP4B (charged multivesicular body protein 4b) are sequentially recruited to the centre of the intercellular bridge, forming a series of cortical rings. Late in cytokinesis, CHMP4B is acutely recruited to the narrow constriction site of midbody where abscission occurs. The ESCRT disassembly factor VPS4 (vacular protein sorting 4) follows CHMP4B to this site, and cell division occurs immediately (Elia et al., 2011).

In Eukaryotic cells, dominant-negative ESCRT-III protein, CHMP31–179–GFP, localizes to the midbody and inhibits cytokinesis at the late stage (Dukes et al., 2008). We find that over-expression of ESCRT-III-2AC resulted in enlarged cells connected by intercellular bridge with a bulge in the middle (Figs. 5 and 7). This connection resembles in shape the intercellular bridge and midbody structures found in eukaryotes. However, the components of the intercellular bridge and midbody-like structures we observed are unknown. Since *Sulfolobus* cells lack the skeleton proteins in Eukaryotes, there must be many differences between the Eukaryotic midbody and the midbody-like structures observed in *Sulfolobus* cells. Here we showed that ESCRT-III-1 and –2 localized at the midbody-like structures by Immunofluorescence microscopy. Whether there are any other unknown proteins exist in the structure needs further study. However, whether there is such a stage of cell division in normal cells in *Sulfolobus* also needs to be further investigated. One possibility would be that the normal *Sulfolobus* cells undergo a stage of intercellular bridge and midbody formation during cytokinesis as in eukaryotes, but this process can hardly be observed because the mitotic phase is too short (about 5% of the cell cycle) (Lindás and Bernander, 2013). Over-expression of the mutant protein blocked cells at the membrane abscission stage and allowed the visibility of the structures.

In eukaryotes, the ESCRT-III complexes is required for membrane remodeling in many cellular processes, from abscission to viral budding and multi-vesicular body biogenesis; these processes need the forming of spiral polymer filaments of the proteins and relaxation of the filaments drives the membrane deformation (Carlson et al., 2015; Chiaruttini et al., 2015; McCullough et al., 2015). The phenotypes observed for these mutants in this study might be due to protein filament formation (Supporting Information Fig. S13B), and C-terminal deletion affected the interactions between ESCRT-III, ESCRT-III-1 and –2 proteins and Vps4, and the disassembly of the filaments. Yeast two hybrid assays showed that deletion of the C-terminal MIM2 domain of the ESCRTs abolished the interaction of them with Vps4 (Supporting Information Fig. S11). So, in *S. islandicus* cells, the membrane remodeling probably needs the formation of protein filaments and relaxation of the filaments drives membrane deformation (the belt-like structures observed by immunofluorescence microscope). Our study reinforces that *Sulfolobus* is an ideal model microorganism in studying basic cellular and molecular mechanism of archaea and eukaryotes, not only in nucleic acid metabolism and cell cycle, but also in cell division and host-virus interaction.

**Experimental procedures**

**Molecular phylogeny and secondary structure prediction of the proteins**

Protein sequences were retrieved from the National Centre of Biotechnology Information (NCBI) GenBank. The protein sequences were aligned for the generation of the phylogenetic tree by Molecular Evolutionary Genetics Analysis software Mega 6.0. The secondary structures were predicted by the server provided on the website (http://www.compbio.dundee.ac.uk/pred/index.html).

**Strains and growth conditions**

*Sulfolobus islandicus* strain REY15A(E233S)(ΔpyrEFΔlacS) (hereafter E233S) was grown aerobically at 75°C in STVU medium containing mineral salt, 0.2% (wt/vol) sucrose (S), 0.2% (wt/vol) tryptone (T), a mixed vitamin solution (V) and 0.01% (wt/vol) uracil (U), and the medium was adjusted to pH 3.5 with sulfuric acid, as described previously (Deng et al., 2009). SCV medium containing 0.2% (wt/vol) casamino acid (C) was used for cultivating uracil prototrophic transformants. ATV medium containing 0.2% (wt/vol)
arabinose (A) was used for protein expression. STVU medium supplemented with 5-fluoroorotic acid (5-FOA), STVUF, was used for counter-selection of the pyrEF auxotroph. Culture plates were prepared using phytagel (0.8%[wt/vol]). The strains constructed and used are listed in Supporting Information Table S2.

**Transformation and selection of strains**

*S. islandicus* E233S was transformed with linearized knockout plasmid (pMID or pK) or the over-expression plasmid DNA by electroporation, according to the method described (Deng et al., 2009). Transformed cells were selected and purified by several rounds of screening on solid MSCV plate. The transformant was confirmed by X-gal staining and PCR analysis. For knockout with MID (Marker Insertion and Target Gene Deletion) method, the purified marker (lacS-pyrEF)-inserted strain was subjected to several rounds of counter-selection with MTSV medium containing 5-FOA and uracil. The over-expression strains were confirmed by plasmid extraction and digestion with restriction enzymes and sequencing. The strains were also analysed by Western blot analysis.

**Gene knockout**

Allele replacement (AR) method was used for the gene-knockout of *SiRe_1388* (escrt-III-3) and *SiRe_1550* (escrt-III-1) (She et al., 2009; Huang, et al., 2015) (Supporting Information Fig. S3). The maker cassette (pyrEF-lacS) was inserted into the chromosome through a double-crossover between the linearized knock plasmid and the *S. islandicus* genome. The recombinant cells were selected on uracil-free solid medium.

The MID (Marker Insertion and Target Gene Deletion) method (Zhang et al., 2010) was utilized for *SiRe_1200* (escrt-III-2) deletion. The method consisted of two steps: Step 1, a double-crossover between linearized pMID-SiRe_1200 and *S. islandicus* genome occurred after transformation, yielding recombinant with a target gene allele that could grow on uracil-free medium. The transformants were identified as blue colonies when treated with X-gal. Step 2. a single-crossover recombination could occur between the two homologous arms on the chromosome of the transformant in the presence of 5-FOA, generating deletion mutants (1) (Supporting Information Fig. S6). Alternatively, spontaneous mutation could occur at pyrEF, generating colony with blue colour (2). The oligonucleotides used and the plasmids constructed and used are listed in Supporting Information Tables S3 and S4 respectively.

**Southern blot**

Standard protocol was used for Southern blot analysis. The selected restriction enzyme sites and the probes used are indicated in Supporting Information Fig. S4. Labelling of probe, hybridization and detection were performed using DIG High Prime labeling kit (Roche, Switzerland) according to the manufacturer’s instruction.

**Yeast two hybrid assays**

Standard protocol (Clontech, *Saccharomyces cerevisiae* Y2HGold and Y187 cells were transformed with the GAL4 DNA-binding domain (pGBKT7) and the GAL4 activation domain (pGADT7) fused plasmids respectively (Clontech Yeastmaker Yeast Transformation System 2). Transformants were selected by growth on SD-Trp agar and SD-Leu agar respectively at 30°C. After auto-activation detection, each type of the positive transformants was picked for mating and grown on SD-Trp-Leu agar at 30°C. Positives were spotted on SD-Leu-Trp (as a control) and on SD-Leu-Trp-His agar and SD-Leu-Trp-His-Ade agar and grow at 30°C. The mated Y2HGold [pGBKKT7–53] with Y187 [pGADT7-T] was used as the positive control and the mated Y2HGold [pGBKKT7-Lam] with Y187 [pGADT7-T] was used as the negative control.

**Protein expression and purification in vitro**

The plasmids used for protein purification were listed in Supporting Information Table S4 and were transformed into *E. coli* BL21-CodonPlus(DE3)-RIL for protein expression. For His6-tagged protein expression, cultures were grown in LB at 37°C, induced with 1 mM IPTG at OD600 = 0.4 for 4 h and harvested by centrifugation at 7000 rpm for 10 minutes. Cells were lysed into 50 mM Tris (pH 8.0), 500 mM NaCl by sonication. Cell extract was then heat treated for 20 minutes at 70°C and clarified by centrifugation at 10 000 rpm for 10 min, and the supernatant was used for purification by Ni-NTA – Agarose.

**Protein expression in vivo**

*S. islandicus* strains harbouring the expressing plasmids were inoculated into TSV medium to a final estimated OD600 of 0.05 and cultured to OD600 0.5 and this process was repeated at least three times. The cells were then inoculated into 30 ml TV medium to a final estimated OD600 of 0.05 and when the OD600 reached approximately 0.2, arabinose (0.2%, wt/vol) was added to induce protein expression.

**Western blot**

At the indicated times, 1 × 10⁹ cells (with or without induction) were collected by centrifugation at 1,643 g for 10 minutes and resuspended in 40 µl buffer A (50 mM Tris-HCl, pH8.0, 100 mM NaCl). After the addition of 10 µl 5X loading buffer, the samples were treated at 100°C for 10 minutes and analysed by SDS-PAGE. Antibodies against ESCRT-III-1, ESCRT-III-2, ESCRT-III-3 and TBP (tata-box binding protein) were produced using synthetic protein-specific peptides in rabbit and purified by HuaAn Biotechnology Co., (Hangzhou, Zhejiang, China). TBP was used as loading controls. The anti-His antibody was produced in mouse and IgG was purified by TIANGEN Biotechnology Co., (Beijing, China). The goat-anti-rabbit and goat-anti-
mouse second antibody were obtained from HuaAn and TIANGEN respectively.

Microscopy and immunomicroscopy

For microscopy, 5 μl of cell cultures were examined under an ECLIPSE 80i inverted fluorescence microscope (Nikon, Japan). Immunomicroscopic analyses were performed according to the method previously described (Bajorek et al., 2009). Briefly, the cells were cultivated to an OD_{600} of 0.2–0.4. About $6 \times 10^6$ cells were centrifuged at 6000 g for 3 min. The pellet was resuspended in 300 μl PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 12Na_2O, 2 mM KH_2PO_4, pH7.4). Cold ethanol was added to a final concentration of 70% (v/v). The cells were collected by centrifugation at 6000 g for 3 min and washed three times with PBST buffer (PBS plus 0.05% Tween-20). The cells were incubated at 4°C overnight with antibody diluted by 1:1000 and then washed with PBST buffer three times and incubated with Dylight 488 Affinipure Goat anti-Rabbit IgG (H + L) (EarthOx, USA) for 2 h at room temperature. For DAPI (4',6-diamidino-2-phenylindole) staining, the cells were washed with PBST buffer and resuspended in 30 μl PBS buffer containing 10 ng/ml DAPI. Samples (3–4 μl) were examined under a Carl Zeiss LSM780 microscope (Carl Zeiss, Germany).

Scanning electron microscopy

Approximately $1 \times 10^7$ cells were collected with a 0.45 μm filter. The filter was then placed in PBS buffer (pH3.3) containing 2.5% glutaraldehyde at room temperature overnight for cell fixation. After fixation, the samples were dehydrated with a gradient of 30, 50, 70 and 90% (v/v) ethanol for 20 min each. The cells were then treated with absolute ethanol three times for 20 min each. The samples were dried in a vacuum dryer after being treated with tert-butyl alcohol three times for 20 min each, with the final treatment performed at 4°C. The samples were then loaded onto SEM specimens stubs with double adhesive tape and sputter-coated with gold (Cressington Sputter Coater 108, Scientific Instruments Inc., USA). Microscopy analysis was performed under high vacuum mode with a low kV (5 kV) electron beam using a FEI Quanta FEG 250 environmental scanning electron microscope (ESEM).

Flow cytometry

Cell cultures (0.3 ml), with or without induction, were harvested at the indicated times and fixed in 70% cool ethanol overnight (more than 12 h). The fixed cells were then collected by centrifugation at 675 g for 20 min and resuspended in 1 ml of a buffer (10 mM Tris-HCl, pH 7.5, 10 mM MgCl_2). The cells were precipitated again and resuspended in 50 μl staining buffer containing 40 μg/ml propidium iodide (PI) and 100 μg/ml RNAase A. After treatment for at least 30 min, the samples were analysed for DNA content using an ImageStreamX MarkII Quantitative imaging analysis flow cytometry (Merck Millipore, Quantitative). The data from analysis of at least 100,000 cells were collected from each sample and analysed by IDEAS data analysis software. ModFit LT software (Verity Software House) was used to analyse the cell cycle.

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**Supporting information**

Additional supporting information may be found in the online version of this article at the publisher’s web-site.