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ARTICLE

Archaeal extracellular vesicles are produced in an ESCRT-dependent manner and promote gene transfer and nutrient cycling in extreme environments

Junfeng Liu[1,2] · Virginija Cvirkaite-Krupovic[2] · Pierre-Henri Commere[3] · Yunfeng Yang[1] · Fan Zhou[1] · Patrick Forterre[2] · Yulong Shen[1] · Mart Krupovic[2]

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Abstract

Membrane-bound extracellular vesicles (EVs), secreted by cells from all three domains of life, transport various molecules and act as agents of intercellular communication in diverse environments. Here we demonstrate that EVs produced by a hyperthermophilic and acidophilic archaeon *Sulfolobus islandicus* carry not only a diverse proteome, enriched in membrane proteins, but also chromosomal and plasmid DNA, and can transfer this DNA to recipient cells. Furthermore, we show that EVs can support the heterotrophic growth of *Sulfolobus* in minimal medium, implicating EVs in carbon and nitrogen fluxes in extreme environments. Finally, our results indicate that, similar to eukaryotes, production of EVs in *S. islandicus* depends on the archaeal ESCRT machinery. We find that all components of the ESCRT apparatus are encapsidated into EVs. Using synchronized *S. islandicus* cultures, we show that EV production is linked to cell division and appears to be triggered by increased expression of ESCRT proteins during this cell cycle phase. Using a CRISPR-based knockdown system, we show that archaeal ESCRT-III and AAA+ ATPase Vps4 are required for EV production, whereas archaea-specific component CdvA appears to be dispensable. In particular, the active EV production appears to coincide with the expression patterns of ESCRT-III-1 and ESCRT-III-2, rather than ESCRT-III, suggesting a prime role of these proteins in EV budding. Collectively, our results suggest that ESCRT-mediated EV biogenesis has deep evolutionary roots, likely predating the divergence of eukaryotes and archaea, and that EVs play an important role in horizontal gene transfer and nutrient cycling in extreme environments.

Introduction

Extracellular vesicles (EVs) are membrane-bound particles of variable diameter secreted by the cells into extracellular milieu. Although known for several decades, EVs were broadly regarded as cellular waste products, debris or artifacts of lipid aggregation [1]. However, the growing body of data shows that EVs play multiple, biologically important roles in all three domains of life [1–7]. During the past decade, it was discovered that EVs are responsible for intercellular shuttling of diverse cargoes, including proteins, DNA, RNA, lipids and various signaling molecules [5, 7–10], and may promote certain human pathologies [11, 12], including cancer [13, 14]. Furthermore, EVs hold great promise as vehicles for drug targeting and delivery [15, 16]. Finally, EVs may play an important ecological role, especially, in aquatic ecosystems [5]. It has been shown that DNA-carrying EVs produced by diverse bacteria, including *Prochlorococcus*, a numerically dominant marine cyanobacterium, are abundant in coastal and open-ocean seawater samples [7]. Importantly, *Prochlorococcus* EVs could support the growth of heterotrophic bacterial cultures, which implicates EVs in marine carbon flux [7]. Archaea of the phyla Euryarchaeota and Crenarchaeota are also known to produce EVs under laboratory conditions. *Thermococcus prieurii*, but not other *Thermococcus*

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✉ Mart Krupovic
mart.krupovic@pasteur.fr

[1] CRISPR and Archaea Biology Research Center, State Key Laboratory of Microbial Technology, Microbial Technology Institute, Shandong University, Qingdao, China

[2] Archaeal Virology Unit, Institut Pasteur, Paris, France

[3] Institut Pasteur, Flow Cytometry Platform, Paris, France

species, secrete EVs packed with elemental sulfur, presumably to prevent the accumulation of toxic levels of sulfur in the cytoplasm [17]. Furthermore, similar to bacteria, EVs produced by members of the phylum Euryarchaeota thriving in deep-sea hydrothermal vents (order Thermococcales) and saline lakes (order Halobacteriales) were shown to carry DNA [18–21]. Whether the same is true for EVs produced by crenarchaea of the order Sulfolobales, which represent major inhabitants of terrestrial acidic hot springs, remains unknown. It is also unclear whether archaeal EVs are secreted under natural growth conditions in the environment.

The mechanisms of EV biogenesis have been extensively studied in eukaryotes but remain poorly understood in bacteria and archaea [1, 3]. In eukaryotes, the most studied mechanism of EV formation relies on the endosomal sorting complex required for transport (ESCRT) machinery [22–24]. Many archaea also encode homologs of the ESCRT system but its involvement in EV biogenesis remains unclear. The ESCRT machinery is responsible for many key membrane remodeling processes in eukaryotic cells, including membrane abscission during cytokinesis, biogenesis of certain types of EVs and multivesicular bodies, and budding of enveloped viruses, such as HIV-1 and Ebola virus [22, 25, 26]. The ESCRT proteins assemble on the cytosolic face of the membrane and drive membrane bending and scission reaction [26]. The ESCRT machinery can be subdivided into several functionally distinct subcomplexes known as ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III as well as AAA+ ATPase Vps4. Among these, ESCRT-III and Vps4 are universally involved in ESCRT-dependent membrane remodeling processes, whereas ESCRT-0, ESCRT-I, and ESCRT-II are compartment-specific and facilitate recruitment of ESCRT-III to diverse membranes in different cellular contexts [25, 27]. ESCRT-III proteins form a ring-like filament at the membrane, whereas the Vps4 ATPase binds directly to ESCRT-III and dynamically disassembles the ESCRT-III complex in an ATP-dependent manner, thereby driving membrane-remodeling [28, 29].

Similar to eukaryotes, most archaea of the TACK (for Thaumarchaeota, Aigarchaeota, Crenarchaeota and Korarchaeota) and Asgard superphyla encode an ESCRT machinery [30–33]. Interestingly, the ESCRT machinery encoded by Asgard archaea is phylogenetically more closely related to the eukaryotic homologs compared to those from other archaea and Asgard Vps4 could efficiently complement the *vps4* null mutant of *Saccharomyces cerevisiae* [31, 34]. However, due to difficulties in cultivation and lack of genetic tools in Asgard archaea, the role of their ESCRT machinery in membrane remodeling remains to be investigated in this superphylum. The archaeal ESCRT system has been experimentally investigated in *Sulfolobus* and *Nitrosopumilus* species [33, 35–41], with *Sulfolobus* representing the model organism for elucidation of the role and functioning of the archaeal ESCRT machinery [30, 41, 42]. In hyperthermophilic archaea of the order Sulfolobales, the ESCRT machinery is the key component of cell division apparatus composed of AAA+ ATPase Vps4 (also known as CdvC), four ESCRT-III homologs (ESCRT-III [CdvB], ESCRT-III-1 [CdvB1], ESCRT-III-2 [CdvB2], ESCRT-III-3 [CdvB3]), and archaea-specific component CdvA. The latter protein binds to DNA [43, 44] and recruits ESCRT-III to the membrane [44]. CdvA is not homologous to the eukaryotic ESCRT-0, ESCRT-I, or ESCRT-II [45], and is missing in certain archaea, including some thaumarchaea [46] and aigarchaea [47]. Recently, it has been demonstrated that ESCRT machinery also mediates asymmetric cell division via budding in virus-infected *S. islandicus* cells [48]. It has been also proposed that the *Sulfolobus* ESCRT machinery is involved in viral assembly within the cytoplasm and in escape from the infected cell by using a unique lysis mechanism [49]. Whether the function of archaeal ESCRT machinery can be extended to other membrane remodeling processes, such as budding of enveloped viruses [50] and EVs [3], remains to be demonstrated. Notably, ESCRT-III-1, ESCRT-III-2, and Vps4 were identified among proteins present within EVs secreted by *Sulfolobus acidocaldarius*, *S. solfataricus* and *S. tokodaii* [51]. This finding suggested that ESCRT machinery is involved in EV biogenesis [51]. However, EVs are known to be produced by archaea which lack the functional ESCRT system and divide using the bacterial-like FtsZ-based cell division machinery, including halophilic archaea (class Halobacteria) and members of the order Thermococcales [18, 52].

Here we characterize the composition, role and biogenesis of EVs produced by a hyperthermophilic and acidophilic archaeon *Sulfolobus islandicus*. We demonstrate that besides proteins, *Sulfolobus* EVs carry chromosomal and plasmid DNA, and that EVs can transfer this DNA to recipient cells. We also investigate the role of the *Sulfolobus* ESCRT machinery in EV biogenesis and show that all four ESCRT-III homologs and Vps4 ATPase play an important role in this process, whereas CdvA appears to be dispensable. Using synchronized *S. islandicus* cultures, we demonstrate that EV production is linked to cell division and appears to follow the cell cycle-coordinated fluctuations in the expression of ESCRT proteins. Finally, we show that EVs similar to those produced by *Sulfolobus* cells under laboratory conditions can be also found in an environmental sample collected from a terrestrial hot spring. Collectively, our results suggest that the ESCRT-dependent mechanism of EV biogenesis is conserved in the archaeo-eukaryotic lineage and that EVs play an important role in gene transfer in extreme environments.

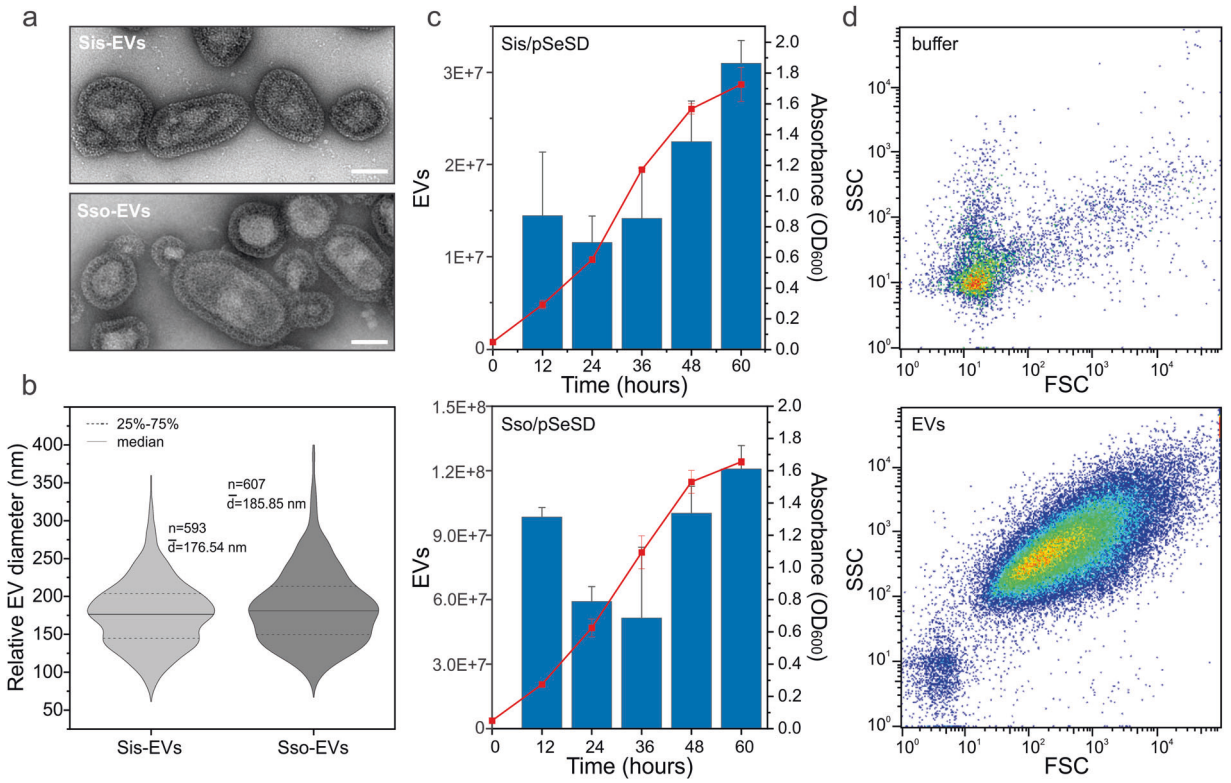

Fig. 1 Characterization of the *Sulfolobus islandicus* EVs. a Transmission electron micrographs of negatively stained Sis-EVs (top) and Sso-EVs (bottom). Scale bars, 100 nm. **b** Violin plots showing the size distributions of Sis-EVs ($n = 593$) and Sso-EVs ($n = 607$). The width of the distribution corresponds to the frequency of occurrence. **c** Growth curves of *S. islandicus* E233S and *S. solfataricus* PH1-16 harboring the vector pSeSD, and quantification of EVs released at indicated time points. Error bars represent standard deviation from three independent experiments. **d** Quantification of Sis-EVs by flow cytometry. Top panel shows the control with buffer only. SSC side scattering, FSC forward scattering.

Results and discussion

EV production and purification

To study the composition and function of archaeal EVs, and to investigate the role of ESCRT in their biogenesis, we established a procedure for purification and quantification of EVs from *Sulfolobus islandicus* REY15A and *Saccharolobus solfataricus* PH1 cells (Sis-EVs and Sso-EVs, respectively), and compared the EV production at different growth stages in both strains. Consistent with the observations made for EVs isolated from other *Sulfolobus* species [51], Sis-EVs and Sso-EVs were visibly coated with the proteinaceous surface (S-)layer (Fig. 1a) typical of *Sulfolobus* cells [53, 54] and displayed considerable variation in shape and diameter. The median diameters of Sis-EVs and Sso-EVs were 176.54 nm and 185.85 nm, respectively, with the Sso-EVs being slightly more variable in size (Fig. 1b). The EVs were collected at different stages of cell growth (Fig. 1c) and could be reproducibly quantified by flow cytometry using tubes containing a calibrated number of fluorescent beads (Fig. 1d and Fig. S1). With a notable exception of the 12-h time point, EV production by *S. islandicus* and *S. solfataricus* followed a similar pattern: EV titer increased throughout the growth of the cells (Fig. 1c). Given the similarities in EV production patterns in *S. islandicus* and *S. solfataricus*, for all subsequent experiments, we focused on EVs from *S. islandicus*, for which more advanced genetic tools are available [55]. We next tested whether there is a link between Sis-EV production and increase in the fraction of the dead cells in the growing *S. islandicus* population by performing the live/dead staining at different time points (see "Materials and Methods"). From 12 to 60 h (early exponential to stationary phase) the ratio of dead cells remained at around 1% and only when the cells progressed into the "death" phase, the ratio of dead cells increased sharply, with around 30% of dead cells at 72 h and more than 90% of dead cells at 84 h (Fig. S2). These results suggest that Sis-EV production is not a consequence of cell death. To verify that the EV preparations were devoid of cellular contaminants, we performed the following procedures: (i) flow cytometry profiles of the EV samples were compared with those containing *Sulfolobus* cells (Fig. S1); (ii) purified EV preparations were subjected to semi-quantitative transmission electron microscopy analysis (Fig. S3a); (iii) EV preparations were also analyzed by

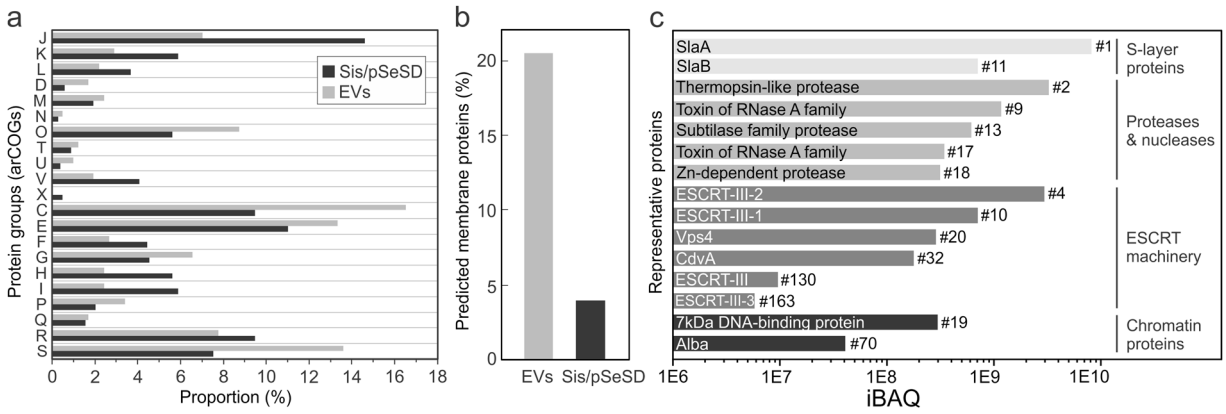

Fig. 2 Analysis of the Sis-EV protein content. The EVs were collected during the exponential growth phase (24 h) of Sis/pSeSD, purified on sucrose gradient, treated with DNase I and subjected to mass spectrometry analysis. **a** Functional classification of proteins identified in highly purified Sis-EVs using archaeal clusters of orthologous groups (arCOGs). arCOG categories are indicated with capital Roman letters, with the annotation provided in Table S1. **b** Fraction of proteins with predicted transmembrane domains in Sis-EV and cellular (Sis/pSeSD) proteomes. **c** Label-free intensity-based absolute quantification (iBAQ) of selected Sis-EV proteins (the corresponding functional categories are indicated on the right). Numbers next to each bar indicate the abundance rank.

fluorescence microscopy (Fig. S3b); and (iv) EV preparations were plated on solid medium supporting the growth of *Sulfolobus* cells (Fig. S4). None of these procedures revealed the presence of *Sulfolobus* cells, viable or otherwise, in the EV preparations.

Protein content of Sis-EVs

EVs are known to carry diverse cargo, including proteins [2–5]. Proteomics analysis of Sis-EVs and *S. islandicus* cells led to the identification of 413 and 1035 proteins, respectively (Supplementary Data 1). The number of proteins detected in Sis-EVs is considerably higher than that reported previously for EVs from *S. acidocaldarius*, *S. solfataricus* and *S. tokodaii* [51], possibly due to improved sensitivity of mass-spectrometry over the past decade. Notably, recent studies on the proteomics of EVs produced by diverse bacteria [56–59] as well as halophilic archaea [18] report the presence of hundreds of proteins in each type of EVs, consistent with our results. For instance, it has been shown that EVs produced by halophilic archaeon *Halorubrum lacusprofundi* contain 447 different proteins [18].

All but one functional protein categories found in *S. islandicus* proteome, as defined using the archaeal clusters of orthologous groups (arCOG; Table S1) [60], were represented in the Sis-EVs (Fig. 2a). Proteins of the arCOG category X (Mobilome: prophages, transposons) were not found in the Sis-EVs, likely due to the fact that only few proteins of this functional category are expressed in *S. islandicus* under normal growth conditions [61, 62]. The fractions of proteins of the categories J (Translation, ribosomal structure and biogenesis), K (Transcription), V (Defense mechanisms), H (Coenzyme transport and metabolism) and I (Lipid transport and metabolism) were more than twice smaller compared to their corresponding fractions in the total cellular proteome. By contrast, arCOG categories D (Cell cycle control, cell division, chromosome partitioning), N (Cell motility), O (Posttranslational modification, protein turnover, chaperones), U (Intracellular trafficking, secretion, and vesicular transport), C (Energy production and conversion), P (Inorganic ion transport and metabolism) and S (Function unknown) were enriched in Sis-EVs compared to the total cellular proteome (Fig. 2a). For instance, the D category proteins constitute only 0.6% of the total *S. islandicus* proteome, whereas in Sis-EVs, these proteins correspond to 1.7% of proteins (2.9-fold increase). There is also notable enrichment in Sis-EVs of proteins with predicted transmembrane domains compared to the cellular proteome (20% vs 4%; Fig. 2b). Of the top 100 most abundant proteins in the EVs, 65 have predicted transmembrane domains, whereas there are no such proteins among the top 100 most abundant cellular proteins. Thus, although Sis-EVs incorporate a considerable fraction of the total *S. islandicus* proteome, there is a strong enrichment for certain functional categories and, in particular, for membrane proteins. Presence in the Sis-EVs of proteins from nearly all functional categories suggests that many of these proteins are incorporated non-selectively, by entrapment of the cytosolic and membrane contents. It is possible, if not likely, that not all of the proteins are present within each Sis-EV, but are rather distributed across the EV population.

Sulfolobus EVs have been previously shown to carry toxins, dubbed sulfolobicins, active against closely related *Sulfolobus* species [63, 64]. However, homologs of these particular toxins are not encoded in the *S. islandicus* REY15A genome. Nevertheless, nearly one-third of proteins in the O category in Sis-EVs corresponded to diverse proteases and nucleases. Notably, we also detected two

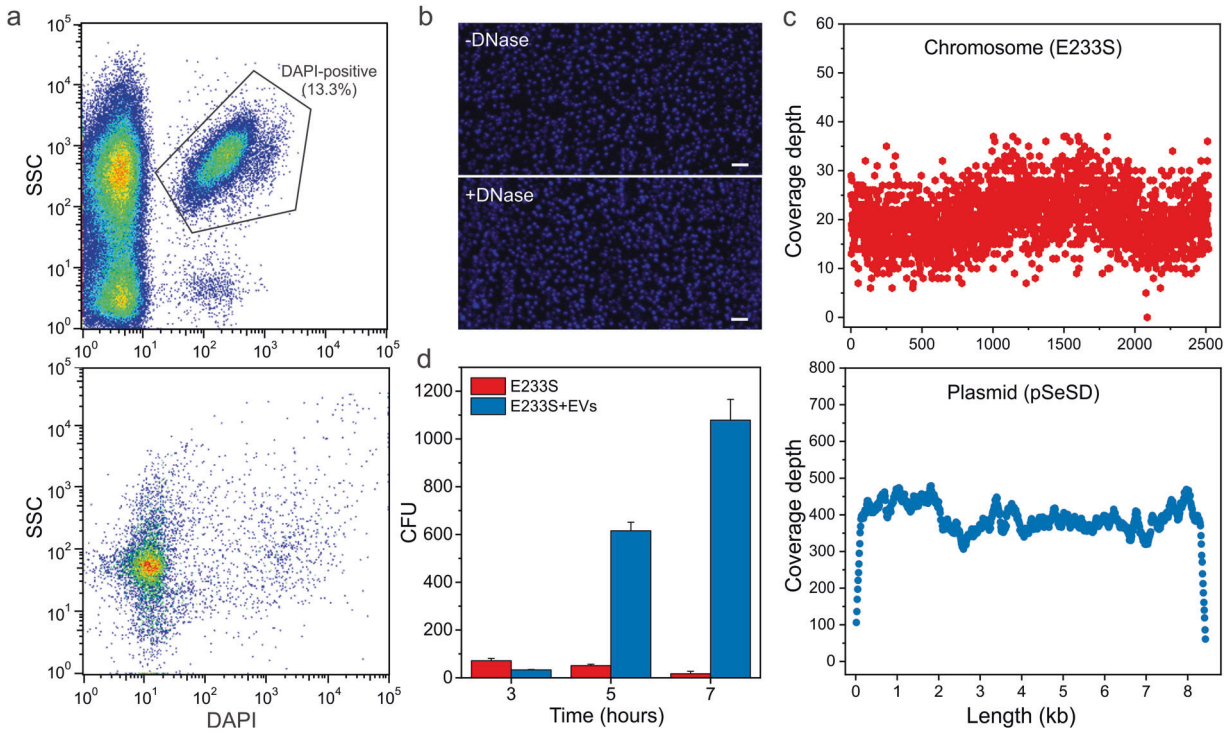

Fig. 3 Sis-EVs promote gene transfer. a Analysis of DAPI-stained Sis-EVs isolated from Sis/pSeSD (upper panel) by flow cytometry. All the events are shown, with the selected region indicating the DAPI-positive EVs harboring DNA. Note that most EVs are DAPI-negative. Bottom panel shows the control with the DAPI-containing buffer only. SSC, side scattering. **b** Fluorescence micrographs of DAPI-stained Sis-EVs prior (top) and after (bottom) DNase I treatment. Bar, 2 µm. **c** Sequencing depth across the chromosomal DNA (E233S) and plasmid (pSeSD). Each dot represents sequencing depth at the indicated position of the corresponding replicon. **d** Gene/plasmid transfer by Sis-EVs. Sis-EVs were treated with DNase I and then mixed with E233S cells, incubated for 3, 5, and 7 h and plated on selective plates. In the control experiment, E233S cells were mixed with the equal volume of PBS buffer. The number of obtained colony forming units (CFU) is plotted on the y-axis. Error bars represent standard deviation from three independent experiments.

putative toxins (WP_014512538 and WP_014512541) of the RNase A family and several hydrolases of diverse specificities (Fig. 2c; Supplementary Data 1). This finding suggested that deployment of the Sis-EV payload could be toxic to recipient cells lacking necessary immunity. Incubation of Sis-EVs with *Sulfolobus* cells for 3 h indeed resulted in modest, albeit significant, decrease in colony forming units for *S. solfataricus* cells, but not for the more distantly related *S. acidocaldarius* or *S. shibatae* (Fig. S5a). However, the inhibitory effect of Sis-EVs on *S. solfataricus* was temporary and was lifted when the incubation was prolonged to 5 h (Fig. S5b). Thus, Sis-EVs do not appear to participate in intermicrobial conflicts, at least, not among the tested *Sulfolobus* species.

Sis-EVs contained all six components of the *Sulfolobus* ESCRT machinery (arCOG category D; Fig. 2c), consistent with the possibility that ESCRT machinery is involved in EV biogenesis [51]. Label-free intensity-based absolute quantification (iBAQ) [65] of protein abundances showed that two of the ESCRT components, ESCRT-III-2 and ESCRT-III-1, were among the top-10 most abundant proteins in Sis-EVs (Fig. 2c). Western blot analysis has confirmed that both proteins were present and strongly enriched in Sis-EVs (Fig. S6). As expected, both S-layer proteins were found in the EVs, with SlaA being the most abundant protein in Sis-EVs (Fig. 2c).

Sis-EVs carry plasmid and genomic DNA

Sis-EVs carried the chromatin proteins Sac7d/Sso7d and Alba (Fig. 2c), responsible for compaction of the *Sulfolobus* chromosome [66, 67], suggesting that Sis-EVs contain DNA. Indeed, DNA has been previously observed in EVs from halophilic archaea and *Thermococcus* spp. (both in the phylum Euryarchaeota) [18, 19, 21, 68] but never reported in *Sulfolobus* EVs. To test if Sis-EVs carry DNA, purified EVs were treated with DNase I and stained with 4′,6-diamidino-2-phenylindole (DAPI). The DAPI-stained Sis-EVs could be detected by both flow cytometry (Fig. 3a) and fluorescence microscopy (Fig. 3b), consistent with the presence of DNA. Notably, only 13.3% of EVs detected by the flow cytometry were DAPI-positive, whereas the majority of EVs were DAPI-negative, indicating a heterogeneity of the EV content.

High-throughput sequencing of the DNA extracted from Sis-EVs yielded reads aligning to both the *S. islandicus*

chromosome and the resident extrachromosomal plasmid pSeSD. Both replicons were covered throughout their respective lengths (Fig. 3c), but the median sequencing depth of the plasmid was 19 times higher than that of the chromosome (386× versus 20× coverage, respectively). This difference cannot be explained by the higher copy number of the plasmid (3-5 copies per 1 chromosome copy) [69]. It is most probable that, as in the case of the *Thermococcus* [70] and bacterial [8] EVs, overlapping genomic fragments of variable sizes, rather than complete chromosome, are packed into the Sis-EVs.

To test the biological relevance of DNA incorporation into Sis-EVs, we investigated the ability of Sis-EVs to transfer the plasmid-borne *pyrEF* locus into a plasmid-free auxotrophic strain E233S of *S. islandicus* carrying a chromosomal deletion in the *pyrEF* operon responsible for uracil synthesis (Fig. S7) [69]. To this end, Sis-EVs produced by the pSeSD-carrying strain were purified, treated with DNase I, incubated with recipient E233S cells in liquid culture and plated on solid medium devoid of uracil. In the presence of EVs, strain E233S formed over 1000-fold more colonies than the control E233S cells (Fig. 3d), whereas plating of EVs alone did not yield colonies on either rich or uracil-deficient medium (Fig. S8a), further confirming that there were no cells contaminating EV preparation. Approximately half of the colonies obtained after incubation with Sis-EVs carried the pSeSD plasmid (Fig. S8b-e). To test if the plasmid-devoid strains carry the *pyrEF* cassette elsewhere on the chromosome, we performed PCR with the *pyrF*-specific primers (Fig. S7). However, *pyrF* gene was present exclusively in the pSeSD-carrying strains (Fig. S8f), indicating that there was no ectopic *pyrF* integration. Next, we verified if the ability of the plasmid-lacking strains to grow in the absence of uracil was inheritable. To this end, the plasmid-containing and plasmid-deficient cells from initial colonies were resuspended in the selective medium and spotted on the solid medium lacking uracil (Fig. S8g). Only plasmid-containing strains could grow, suggesting that initial growth of the colonies in the absence of uracil was supported by the nutrients provided by the EVs, whereas transfer of such colonies into fresh medium arrested the cell growth, unless the cells contained pSeSD. Collectively, these results demonstrate that Sis-EVs carry DNA and act as vehicles for gene transfer. The exact mechanism of EV-mediated gene transfer into recipient cells remains unclear but, presumably, it involves fusion between the EV and cell membranes.

Sis-EVs support heterotrophic growth of *Sulfolobus*

To further investigate if EVs can provide nutrients (other than uracil) to support heterotrophic cell growth, *Sulfolobus* cells were inoculated in media lacking nitrogen and/or carbon source. As expected, cells could not grow in a solution containing only mineral salts and a mix of vitamins (Fig. 4a), nor could they grow when only carbon source (sucrose) was added to this solution (Fig. 4b). Instead, slight decrease in optical density of the culture was observed, suggesting partial lysis. However, when either medium was supplemented with purified Sis-EVs, there was significant (two paired t test, $p < 0.05$), Sis-EV concentration-dependent increase in the optical density of *S. islandicus* culture, indicative of cell growth (Fig. 4). The same result was

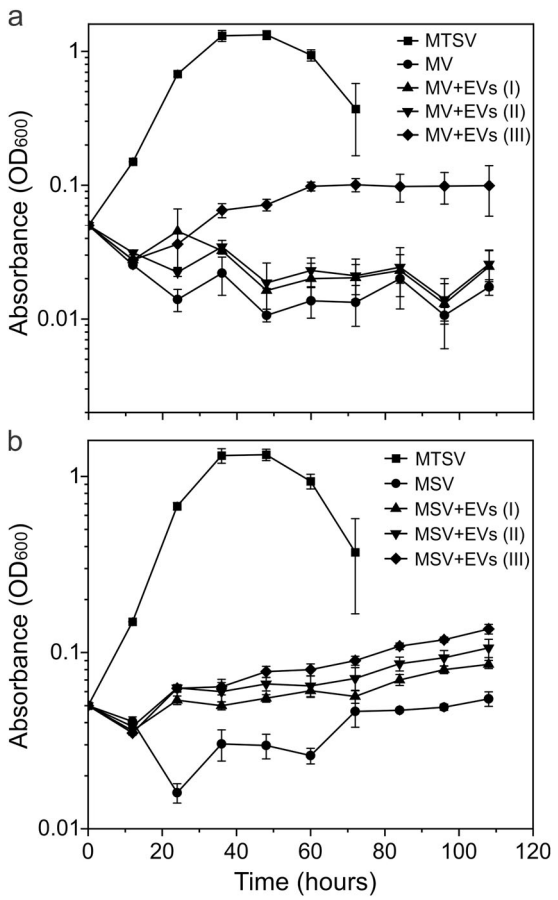

Fig. 4 Sis-EVs support heterotrophic growth of *Sulfolobus* cells. a Growth curves of *S. islandicus* REY15A in MV (M: mineral salts, V: vitamin mix) solution lacking organic carbon and nitrogen sources; MV solution supplemented with 1.5 ml preparations containing different amounts of EVs (MV + EVs): 24.8 µg for I, 49.6 µg for II, and 74.4 µg for III; and rich medium (MTSV), which in addition to MV solution contains 0.2% (wt/vol) sucrose (S) and 0.2% (wt/vol) tryptone (T). There was significant increase (two paired t test, $p < 0.05$) in optical density (OD_{600}) of REY15A cells in the MV solution supplemented with EVs (III), as compared to the control culture lacking EVs. **b** Growth curves of *S. islandicus* REY15A in MSV (M: mineral salts, S: sucrose, V: vitamin mix) medium lacking organic nitrogen source; MSV medium supplemented with different concentrations of EVs (MSV + EVs), and rich MTSV medium. There was significant increase (two paired t test, $p < 0.05$) in optical density (OD_{600}) of REY15A cells in the MSV medium supplemented with EVs, as compared to the control culture lacking EVs. Error bars represent standard deviations from three independent experiments.

obtained with EVs isolated from Sis/pSeSD-CdvA (see below). These results strongly suggest that EVs can serve as a source of both carbon and nitrogen, and hence play an important role in nutrient cycling in extreme environments. Similarly, it has been shown that EVs produced by cyanobacteria can support the growth of heterotrophic bacteria by serving as a carbon source [7].

To the best of our knowledge, production of EVs by different Sulfolobales strains or by any other archaeal strain has been reported only under laboratory cultivation conditions. To verify whether EVs are also produced in the environment, we analyzed a previously collected archaea-dominated hot spring sample [71] for the presence of EVs. The contents of the sample were directly concentrated by ultracentrifugation without prior cultivation in the laboratory and visualized by TEM. We observed multiple S-layer-coated EVs closely resembling those produced by Sulfolobales species (Fig. S9). The diameter of the observed EVs varied between 77 and 182 nm, which is considerably smaller than the size of the smallest known archaea, i.e., *Nanoarchaea* spp. with the diameter of ~400 nm [72, 73], confirming that these are subcellular structures. These results strongly suggest that Sis-EVs are not laboratory artifacts and are environmentally and biologically relevant.

Sis-EVs biogenesis is ESCRT-dependent

Sis-EV biogenesis occurs through budding from the cytoplasmic membrane (Fig. 5a) and ESCRT system is a prime suspect implicated in membrane constriction and scission. To investigate the involvement of ESCRT machinery in EV biogenesis, we constructed a collection of knockdown strains in which expression of each of the six ESCRT machinery components was depleted by the endogenous type III-B CRISPR-Cas system of *S. islandicus*. The utility of this strategy for gene knockdown has been recently demonstrated in *Sulfolobus* [54, 74, 75]. Quantitative reverse transcription PCR (RT-qPCR) analysis has shown that whereas expression of *escrt-III* was decreased by ~30%, expression of all other genes was down by 60–70% (Fig. 5b). Western blot analysis of *escrt-III-1* and *escrt-III-2* knockdown strains has shown that the levels of the corresponding proteins have been decreased by 99% and 60%, respectively (Fig. S6b). It has been previously shown that *escrt-III* and *vps4* are expressed from a bicistronic operon [44, 76] (Fig. S10a). Thus, we verified whether CRISPR targeting of the *escrt-III* gene has a polar effect on the expression of the *vps4*. There was no significant difference in the *vps4* transcript levels between the *escrt-III* knockdown and control cells (Fig. S10b). This is consistent with the previous results showing that cleavage of a transcript by type III-B CRISPR system in *S. islandicus* REY15A occurs within 20 bp of the CRISPR spacer targeting [77]; that is, the fragment of the transcript encoding Vps4 is unaffected by the cleavage within the ESCRT-III-encoding region (Fig. S10a).

Knockdown strains of *cdvA*, *vps4*, *escrt-III*, and *escrt-III-1* displayed considerable cell growth defects, whereas those of *escrt-III-2* and *escrt-III-3* showed nearly normal cell growth (Fig. S11a). The depletion of *cdvA*, *vps4*, *escrt-III* and *escrt-III-1* transcripts by CRISPR targeting resulted in obvious cell division defects (Fig. S11b, c), yielding cells 2–3 times larger than the control cells, and in slight increase (<7%) in the fraction of dead cells in the corresponding populations (Fig. S11d). Consistent with the growth dynamics (Fig. S11a), cell size of the *escrt-III-2* and *escrt-III-3* knockdown strains was similar to that of the control cells (Fig. S11b and S11c). The lack of growth retardation for the *escrt-III-2* knockdown strain is somewhat unexpected, given that all previous attempts to delete this gene in *S. islandicus* were unsuccessful, whereas *escrt-III-3* is known to be non-essential for normal growth [38, 78]. Notably, under the growth conditions used in this study, the expression of *escrt-III-3* was much lower than that of all other cell division genes (Fig. S12). By contrast, the transcription level of *escrt-III-2* was six and three times higher than those of *escrt-III* and *escrt-III-1*, respectively (Fig. S12). Thus, even with 60-70% decrease in transcript levels due to CRISPR targeting, the total level of *escrt-III-2* transcripts would be comparable to that of other ESCRT-III homologs. Presumably, these levels are sufficient for normal growth of *S. islandicus*. Notably, *escrt-III-2* is not essential in *S. acidocaldarius* [40]. Thus, we do not exclude the possibility that *escrt-III-2* is also not strictly required for the growth of *S. islandicus* and that previous attempts to delete this gene were hindered by other factors.

Quantification of Sis-EVs produced by the knockdown strains has shown that whereas depletion of *cdvA* had no significant effect on Sis-EV titer, all other knockdown strains, including the non-essential *escrt-III-3*, produced significantly less EVs compared to the control strain (Fig. 5c). The *vps4* knockdown strain displayed the strongest effect, with EV production being decreased by over 70%. Notably, it is possible that different ESCRT-III homologs can partially complement each other during EV biogenesis. Interestingly, overexpression of the ESCRT-III-1 and ESCRT-III-2 from a plasmid resulted in 200–250% increase in vesiculation (Fig. 5d) consistent with their role in Sis-EV budding. Unexpectedly, overexpression of CdvA resulted in hypervesiculation phenotype (Fig. 5d). However, the same effect was also observed when CdvA lacking the C-terminal domain (CdvAΔC) responsible for interaction with ESCRT-III [43, 44] was overexpressed (Fig. S13a), suggesting that excessive binding of CdvA to the membrane [44] precipitates the observed phenomenon.

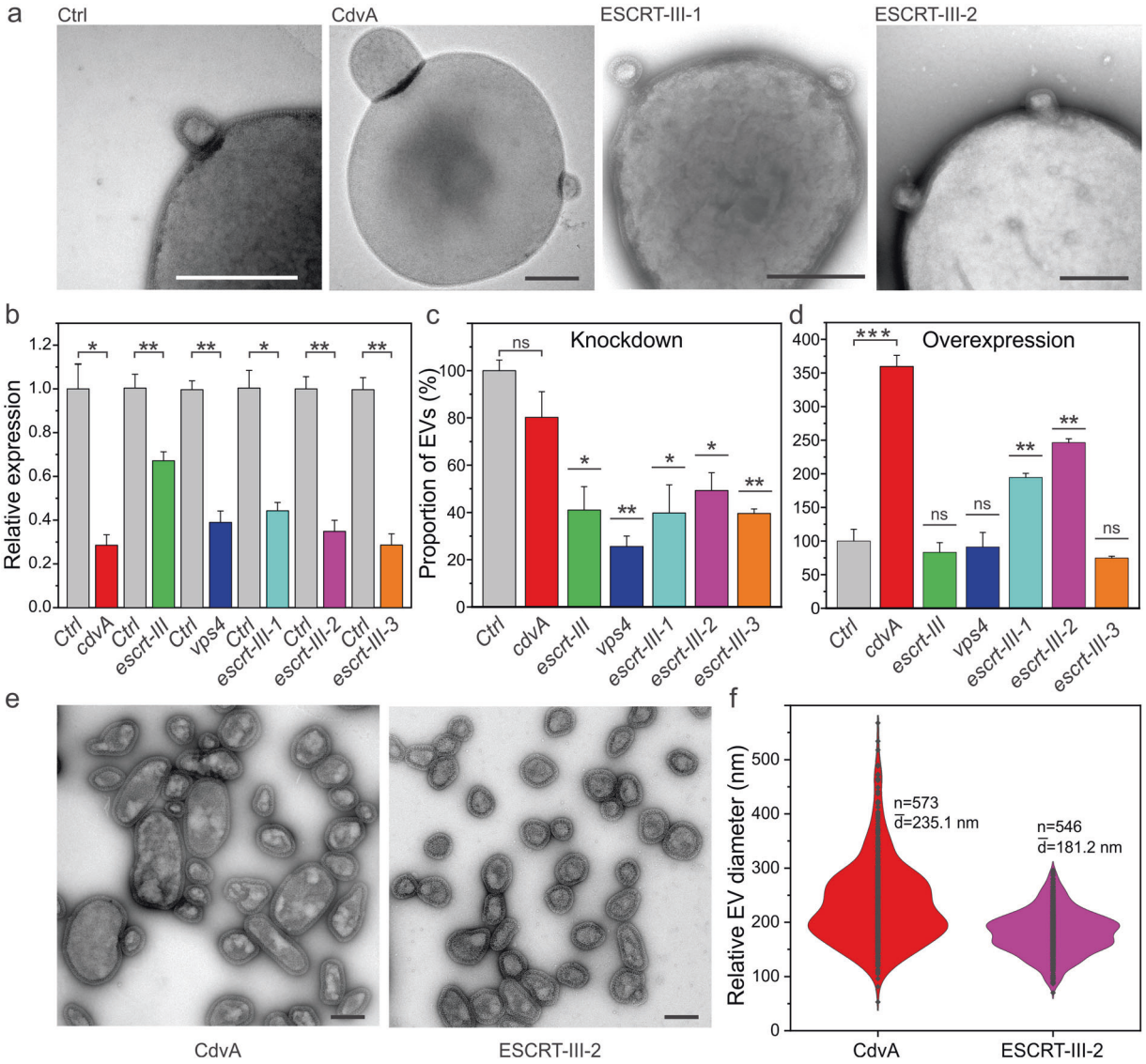

Fig. 5 ESCRT-dependent biogenesis of Sis-EVs. a Representative transmission electron micrographs showing EV budding from *S. islandicus* strains overexpressing indicated proteins. Ctrl: control E233S cells carrying empty vector pSeSD. Bars, 400 nm. **b** RT-qPCR analysis of the RNA interference efficiency. Stars indicate the significance levels based on the paired two-tailed *t* test. The *p* values are 0.01512, 0.00514, 0.00737, 0.0146, 0.00883, 0.00733, respectively. Error bars represent standard deviation from three independent experiments. **c** Quantification of Sis-EVs released from strains in which different ESCRT machinery components were depleted by CRISPR targeting. Stars indicate the significance levels based on the paired two-tailed *t* test. The *p* values are 0.01047, 0.00316, 0.02337, 0.01763, and 0.00177. ns, non-significant. Error bars represent standard deviation from three independent experiments. **d** Quantification of Sis-EVs released from strains overexpressing indicated ESCRT machinery components. Stars indicated the significance levels based on the paired two-tailed *t* test. The *p* values are 0.001, 0.0094, and 0.00435. ns, non-significant. Error bars represent standard deviation from three independent experiments. **e** Representative transmission electron micrographs of negatively stained Sis-EVs isolated from cells overexpressing CdvA and ESCRT-III-2. Bars, 200 nm. **f** Violin plots showing the size distributions of Sis-EVs isolated from cells overexpressing CdvA ($n = 573$) and ESCRT-III-2 ($n = 546$). The width of the distribution corresponds to the frequency of occurrence.

Overexpression of *cdvA* and *cdvAΔC* yielded cells with up to 2-5 fold larger diameters (Fig. S13b). By contrast, overexpression of ESCRT-III-1 and ESCRT-III-2 had no effect on cell size or cell viability (Fig. S13b and S13c). Taken together, the overexpression and knockdown results show that there is no apparent link between the cell size and EV biogenesis.

Budding of EVs from the control and overexpression strains was observed directly by TEM (Fig. 5a). Notably, EVs produced by the CdvA overexpression strain were considerably larger than those from the control (Fig. 1a) and ESCRT-III-2 overexpression (Fig. 5e) strains, with an average diameter of 235 nm versus 177 and 181 nm, respectively (Figs. 1b and 5f). To exclude the possibility

that the large EVs produced by the CdvA overexpression strain represent small cells, the EV-containing supernatant was filtered through 0.45 μm filter and plated on the solid medium. No colonies were formed (Fig. S4b). Our results strongly suggest that EV budding in *Sulfolobus* is dependent on the ESCRT machinery, including Vps4 ATPase and the ESCRT-III ensemble, whereas CdvA appears to be dispensable for this process.

Sis-EVs biogenesis is linked to cell division

The expression of ESCRT-III homologs in *S. acidocaldarius* is linked to the cell cycle [37, 79]. To verify whether the same is true for *S. islandicus* and if EV biogenesis is linked to the cell cycle, we synchronized the *S. islandicus* culture by adapting a protocol previously used for *S. acidcaldarius* [79, 80]. The cells were arrested at the G2 phase using acetic acid and could progress into cell division phase following the removal of the acid (see Materials and Methods for details). Analysis of the DNA content by flow cytometry has shown that the cells started transitioning from G2 into the cell division phase at 90 min following the removal of the acetic acid (Fig. 6a). Western blot analysis of the synchronized cells has shown that ESCRT-III, ESCRT-III-1, and ESCRT-III-2 proteins were undetectable during the G2 phase and became detectable at 90 min after the removal of acetic acid (Fig. 6b). Notably, however, whereas ESCRT-III was abundantly expressed at this time point, ESCRT-III-1 and ESCRT-III-2 were barely detectable. Conversely, at 150 min time point, when the expression of ESCRT-III-1 and ESCRT-III-2 peaked, the expression of ESCRT-III started to decline (Fig. 6b). This dynamics is consistent with the recent suggestion that ESCRT-III is the first to form a ring in the mid-cell during cell division, which serves a platform for subsequent recruitment of ESCRT-III-1 and ESCRT-III-2 [79]. We next analyzed the production of Sis-EVs in synchronized cultures at 60 (G2 phase), 90 (beginning of cell division) and 135 (advanced cell division) min after removal of acetic acid (Fig. 6c). There was a dramatic increase in EV production at the 135 min time point which coincides with active cell division (Fig. 6c, Fig. S14). These results strongly suggest that Sis-EV production is linked to the cell division and might be triggered by the natural, cell cycle-linked changes in the expression of ESCRT-III homologs. In particular, the active EV production appears to coincide with the expression pattern of ESCRT-III-1 and ESCRT-III-2, rather than ESCRT-III, suggesting a prime role of these proteins in EV budding. This conclusion is fully consistent with the observation that the two proteins are strongly enriched in EVs as well as with the fact that overexpression of ESCRT-III-1 and ESCRT-III-2, but not ESCRT-III, dramatically increases EV biogenesis.

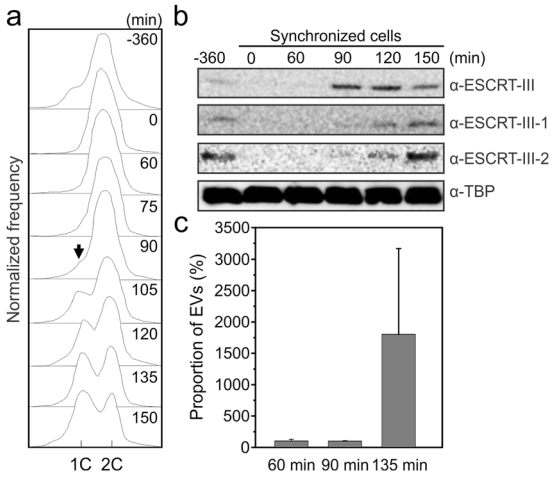

Fig. 6 EV biogenesis is linked to cell division. a Flow cytometry analysis of samples taken at the indicated time points during the progression of a synchronized culture of *S. islandicus*. The positions of peaks corresponding to one chromosome copy (1 C) and 2 C genome contents are indicated. Black arrow indicated the reappearance of the peak corresponding to the 1 C genome content, signifying cell division. **b** Western blot analysis of synchronized cells. Cells (~1×10^9) were collected at indicated time points and expression of ESCRT-III, ESCRT-III-1 and ESCRT-III-2 was analyzed using the corresponding antibodies. Tata-binding protein (TBP) was used as a loading control. **c** Flow cytometry analysis of Sis-EV production by synchronized Sis/pSeSD cells at different time points after removal of acetic acid: 60 min (prior to onset of cell division), 90 min (onset of cell division) and 135 min (active cell division). Error bars represent standard deviation from three independent experiments.

Concluding remarks

Here we have further characterized *Sulfolobus* EVs and showed that they carry DNA. Combined with the previous observation of DNA-containing EVs in euryarchaea (halobacteria and thermococci) [18, 19, 21, 52, 68, 81, 82], the finding that crenarchaeal EVs also contain DNA suggests that this property might be general across archaea. Horizontal gene transfer (HGT) is essential for the survival of microbial populations that otherwise deteriorate due to the Muller's ratchet [83, 84]. Some bacteria and archaea are naturally competent and can uptake DNA from the environment [85, 86]. However, in low-density populations residing in high-temperature, acidic environments, as is the case for Sulfolobales, extracellular DNA might be neither stable nor readily available. In bacteria, conjugative plasmids, transducing bacteriophages and phage-derived gene transfer agents are considered the main drivers of the HGT. Although conjugative plasmids are known in *Sulfolobus*, their role in HGT has not been assessed [87]. By contrast, transducing viruses or dedicated gene transfer agents have not been described in Sulfolobales. Full-length genomic DNA could not be detected in the agarose gel, suggesting

that only fragments of genomic DNA, which could represent byproducts of genome replication and repair, are incorporated into the Sis-EVs. Nevertheless, these DNA fragments collectively represented all genes present on the *S. islandicus* chromosome, as well as the resident plasmid. Importantly, Sis-EVs could successfully transfer the marker genes as well as the complete plasmid within the *S. islandicus* population. Furthermore, our data shows that *S. islandicus* can use EVs as carbon and nitrogen source, which is likely to be important in natural settings where nutrients are scarce. Collectively, these results indicate that EVs could play an important, yet overlooked role in gene transfer and nutrient flux in extreme environments. Indeed, we observed EVs resembling those produced by Sulfolobales directly in the environmental archaea-dominated sample, suggesting that properties of the EVs determined under laboratory conditions are biologically and environmentally relevant.

The mechanisms of EV biogenesis are poorly understood in prokaryotes [1]. Our results strongly suggest that *Sulfolobus* ESCRT machinery plays an important role in EVs formation. Importantly, EV budding appears to be specifically linked to cell division, when the expression of the ESCRT-III proteins is the highest. By contrast, CdvA appears to be dispensable for EV budding suggesting that there are mechanistic differences of the archaeal ESCRT functioning in different pathways of membrane remodeling. This would be similar to eukaryotes, where ESCRT-III complex is targeted to the membranes by different partner proteins [22]. We hypothesize that CdvA is substituted by a different targeting protein during EV budding. Notably, some archaea lack *cdvA* gene but encode ESCRT-III and Vps4 homologs [31, 45–47], suggesting that ESCRT-III targeting to the membrane in these organisms, similar to eukaryotes, is achieved by an unrelated protein or proteins. Alternatively, changes in membrane curvature at the EV budding sites might promote binding of ESCRT-III paralogs, without the necessary chaperoning of CdvA. Further in vitro experiments will be necessary to test this hypothesis. Regardless, our results show that the ESCRT-dependent mechanism of EV biogenesis is conserved in both archaea and eukaryotes, and likely represents one of the ancestral functions of the ESCRT system.

Materials and methods

Strains, growth conditions and transformation of *Sulfolobus*

Sulfolobus islandicus strains REY15A and E233S (REY15A*ΔpyrEFΔlacS*) [69], and *Sulfolobus solfataricus* PH1-16 (PH1 *pyrF* mutant) [88], hereafter PH1-16, were grown aerobically with shaking (145 rpm) at 75 °C in 30 ml of STVU medium containing mineral salts (M), 0.2% (wt/vol) sucrose (S), 0.2% (wt/vol) tryptone (T), a mixed vitamin solution (V) and 0.01% (wt/vol) uracil (U); the pH was adjusted to 3.5 with sulfuric acid, as described previously [69]. SCV medium containing 0.2% (wt/vol) casamino acids (C) was used for selection of uracil prototrophic transformants. ATV medium containing 0.2% (wt/vol) arabinose (A) was used to induce protein overexpression and RNA interference. The plasmids and strains constructed and used in this study are listed in Tables S2 and S3, respectively. *S. islandicus* cells were synchronized using acetic acid (final concentration, 6 mM) as previously described for *S. acidocaldarius* [79, 80] (see Supplementary Methods for further details).

Isolation and purification of EVs

EVs were isolated from liquid cultures of *S. islandicus* E233S or *S. solfataricus* PH1-16 strains carrying shuttle vector pSeSD. The cells were grown at 75 °C in appropriate medium and EVs were harvested at the indicated times. Cells were removed by centrifugation at 7000 rpm at 4 °C for 20 min. The supernatant was filtered with 0.45 μm filter and EVs collected by ultracentrifugation at 40,000 rpm (Type 45 Ti rotor) at 4 °C for 2 h, followed by 100,000 rpm (TLA 100.2 rotor) at 4 °C for 1 h, and then re-suspended in 500 μl of PBS.

For mass spectrometry (see Supplementary Methods) and DNA content sequencing, the EVs were collected during the exponential growth phase (24 h) and further purified by ultracentrifugation in sucrose gradient (50%, 45%, 40%, 35%, 30%, and 25%) at 25,000 rpm (SW 60 rotor) at 4 °C for 10 min. The EVs formed an opalescent band in the region of the gradient corresponding to 30-40% sucrose (Fig. S15). The band was collected and EVs pelleted by ultracentrifugation at 100,000 rpm (TLA100.2 rotor) at 4 °C for 1 h. The resulting pellet was re-suspended in 500 μl of PBS.

Transmission electron microscopy

For TEM analysis, EVs or cell cultures were absorbed to glow-discharged copper grids with carbon-coated Formvar film and negatively stained with 2.0% (w/v) uranyl acetate. The samples were observed under FEI Tecnai Spirit BioTwin 120 microscope (FEI, Einthoven, The Netherlands) operated at 120 kV.

Flow cytometry and quantification of EVs

EVs were isolated from 50 ml cultures of E233S and PH1-16 cells carrying pSeSD vector at the given time points of

cell growth, then 50 µl of the EV preparations were mixed with 250 µl PBS staining buffer containing 2.5 µg/ml DAPI (4', 6-diamidino-2-phenylindole; Thermo Fisher Scientific, USA) and kept at 4 °C for 30 min. The EVs were analyzed and sorted on the MoFlo Astrios cell sorter (Beckman Coulter) equipped with an EQ module specifically developed to detect nanoparticles and with 488 nm and 561 nm lasers at 200 mW. The calibration of the machine was carried out using FITC-labeled Megamix-Plus SSC beads from BioCytex (Fig. S1). The sheath-liquid 0.9% NaCl (Revol, France) was filtered through a 0.04 µm filter. The analysis was performed using the side-scattered (SSC) light parameter of laser 561, with threshold set to 0.012% in order to have maximum of 300 events per second. An M2 mask was added in front of the forward-scattered (FSC) light.

To count the EVs, we used Trucount™ Tubes (BD Biosciences, San Diego, CA), which contain a defined number of fluorescently labeled beads and have been specifically designed for reproducible counting of various biological nanoparticles, including EVs [89, 90]. For quantification, we added the same volume (300 µl) of EV preparations into the tubes that contained the constant number of beads. The EV number was calculated using the following formula: EV_{total} = (EV count/bead count) × total number of beads in the Trucount™ Tube. In each case, the samples were passed through the flow cytometer's detector until 2000 beads were recorded. All quantifications by flow cytometry were done in triplicate (Fig. S14 and Fig. S16). Further details on cell cycle and cell size analysis by flow cytometry can be found in Supplementary Methods.

DNA isolation from EVs and sequencing

To remove the traces of extravesicular nucleic acids, prior to DNA extraction, EVs were incubated with DNase I (15 U/ml) and RNase (100 µg/ml), in the presence of $MgCl_2$ (10 mM), at 37 °C for 30 min, followed by addition of EDTA (20 mM). EVs were disrupted by proteinase K (100 µg/ml) and SDS (0.5%) treatment at 55 °C for 30 min. The DNA was extracted by standard phenol/chloroform procedure, precipitated with 0.3 M sodium acetate (pH 5.3) and isopropanol. The resultant pellet was resuspended in DNase/RNase-free water and used for sequencing. Sequencing libraries were prepared from 100 ng of DNA with the TruSeq DNA PCR-Free library Prep Kit from Illumina and sequenced on Illumina MiSeq platform with 150-bp paired-end read lengths (Institut Pasteur, France). Raw sequence reads were processed with Trimmomatic v.0.3.6 and mapped to the reference genomes of REY15A and pSeSD plasmid using Bowtie2 [91] with default parameters and analyzed with Sequana [92].

EV-mediated gene transfer

EVs isolated and purified from 6 L of exponentially growing Sis/pSeSD culture (24 h) were used for gene transfer experiments as described previously [93], with some modification (see Supplementary Methods).

Live/Dead staining and fluorescence microscopy analysis

Live/Dead staining was carried out using the LIVE/DEAD *Bac*Light™ Bacterial Viability Kit (Invitrogen, US) [94, 95] according to the supplier's protocols. See Supplementary Methods for the detailed protocol.

Overexpression of ESCRT proteins

Plasmids expressing different ESCRT machinery components and their mutants were described previously [38] (Table S2). Briefly, cells harboring the plasmids were first inoculated into 30 ml of the MTSV medium and when the OD_{600} reached ~0.6–0.8, they were transferred into the ATV medium containing 0.2% (wt/vol) arabinose with an initial OD_{600} of 0.05 to induce protein expression. All plasmids are listed in Table S3.

Construction of the CRISPR type III-B-based RNA interference plasmids and RNA interference

The CRISPR type III-B-based RNA interference plasmids were constructed according to the methods described previously [74, 77]. The spacers selected and used in this study are listed in Table S4, whereas all other oligonucleotides are listed in Table S5. See Supplementary Methods for further details.

Western blot

ESCRT proteins were detected using antibodies against ESCRT-III, ESCRT-III-1 and ESCRT-III-2 (HuaAn Biotechnology Co., Hangzhou, Zhejiang, China), as described previously [38]. See Supplementary Methods for further details.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interest.

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