Looking Back To Move Forward

Studying the Ancient Archaeal Cdv Cell Division Machinery for Synthetic Cells

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by

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A mi familia.
Gracias.

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GENERAL INTRODUCTION

Finding out what is the minimum form of life, is an exciting challenge that in recent years has gotten quite some attention from the scientific community. Reconstituting a minimal cell in a bottom-up way means putting all the different components that are essential to life together in an in vitro setting, such that through the interactions of these parts, complexity starts arising and we can better understand it. One key component of such a synthetic cell is a divisome that ensures its faithful division. In this chapter, we review different approaches that have been studied for establishing a synthetic divisome, mainly by in vitro reconstitution of the bacterial FtsZ and the eukaryotic ESCRT. I describe why it is worthwhile to explore the Cdv system for such an endeavor, which is the focus of this thesis, as it is composed of few components and can drive the cell division from the initial stage until scission, characteristics that other systems lack.

1

1.1. What is life?

Since the beginning of rational thinking, humans have tried to come up with answers to one of the most difficult questions: "what is life"? Life is an abstract concept, that lacks a commonly accepted definition, but whose existence is yet acknowledged by everyone. From the classical Greek to modern philosophers, descriptions of life were always found in intellectual and metaphysical debates. Scientific and empirical advancements in recent centuries have strived to push towards a standardized definition of what is alive, and what is not. It seems now accepted that life cannot be defined with a sharp line from which a clear distinction can be made between alive or lifeless. Instead, life is described as a series of acquired properties of matter that add up and build complexity, until some traits are met, and an organism can be considered alive.

As such, one can then see life as an emergent property of chemical reactions. All the complex regulatory processes that enzymes do in a living organism, are individually not considered alive. It is not until all these proteins work together, in a coordinated fashion (cell cycle), in a physically separated container (cell membrane), acquiring energy from the surroundings (nutrients or light), and with the ability of reproducing and generating new cells (cell division), that we do consider that conglomerate alive. Single biological cells are the simplest forms of life that we know of, and yet, they enclose a staggering level of complexity that can be very hard to fully comprehend.

1.2. Synthetic cells

Seeing life as an emergent property, it is conceivable that the appearance of a minimal form of complexity that we can consider life, is a process that can be reproduced in the lab. When understanding all the individual reactions and processes that constitute the minimal complexity required to be alive, those can in principle be put together in an artificial setting and create, what could be considered, a synthetic cell. Indeed, in recent years, efforts have been started to realize such synthetic cell from the bottom up (*1–4*).

One hope of such synthetic-cell research is that understanding how a minimal cell works and seeing what are the minimal biological processes needed, can tell us a lot about what the definition of life is and how its complexity arises. Successful attempts at "simplifying life" have come from the generation of minimal cells in a top-down approach (*5*), where the genome of the most simple organism that we know, *Mycoplasma genitalium*, was reduced to the bare minimum amount of genes that is needed to produce a viable cell. With this newly formed minimal organism, which still needs 473 protein-coding genes, we can identify which genes are indispensable for life. Yet, we still miss some important information about how the complexity in the cell arises, and perhaps most importantly, we don't know if, with a different set of genes, we could find even simpler life. There is, however, another approach to understand what a minimum cell is, that gives us a different view by reconstituting a minimal form of life in the opposite way: from the bottom up.

The bottom-up approach to a synthetic cell seeks to put together well-understood biological building blocks that are key to life, coordinate them, and slowly build up the complexity needed to establish a minimal form of life (Figure 1.1) (*6*). The first thing that a cell needs is a container, a barrier that physically separates it from the environment and contains all the components. Inside of this envelope, there will be some DNA that stores the biological information, with all the replication machinery and ribosomes to copy and read the DNA. In this DNA, a series of key processes will be coded to guarantee the viability of the organism, like the cell division and the metabolic enzymes needed to provide energy to the cell. All the different genes that encode for all these different processes will be put together from scratch, inside of an artificial container, trying to regulate them in a coordinated fashion. In this way, we not only hope to unveil what are the minimal requirements to life, but also understand how each of the individual processes in the cell work on their own.

From the conceptual point of view, this approach seems simple and straightforward. However, when facing how to do it, it is a humongous challenge that involves many tens, if not hundreds of components that need to be put together in a coordinated fashion. And more fundamentally: considering that there is not yet an accepted unified definition of what life is, there is also not a clear consensus on how a minimal cell should look like. However, more or less all working definitions function as a list of criteria that a living entity has to comply with: it autonomously interacts with its surroundings to take up nutrients, it is capable of replicating and giving rise to progeny, it encodes its biological information in some stable polymer, etc. When taking the most crucial criteria into consideration, the most common approach to defining a minimal cell usually revolves around it being a "simple cellular system that is both autonomously replicating and subject to Darwinian evolution" (*7*). Indeed, the ability to autonomously replicate is always regarded as one of the most important

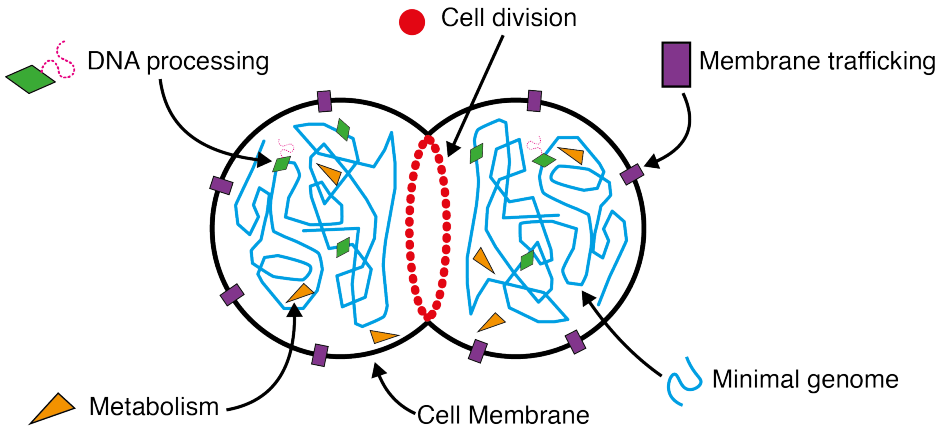

Figure 1.1. Schematic representation of a bottom up approach to build a synthetic cell

features of any living organism. In nature, there is a wide variety of systems that can, in principle, be used to build a machinery for the division of a synthetic cell.

1.3. Synthetic cell division

Cell division is a key process of the cell cycle of any biological cell that ensures the faithful division of a mother cell into newly formed daughter cells. This is also the way that unicellular organisms reproduce, and thus generate new members of the species to ensure its continuity. Finding a way to reproducibly split a cell into two is thus one of the most important tasks needed to build a synthetic cell.

One of the simplest forms of realizing cell division is by the manipulation of the cell membrane (*8*). When we think of a synthetic cell, we envision it enclosed in a phospholipid membrane, which, without any exo- or cytoskeleton to keep it in a defined shape, would adopt the shape of a sphere. In other words, the chassis of a synthetic cell would be a liposome (*9*). Liposomes can easily be shape-manipulated by means of changing lipid compositions, osmolarity, temperature, and other parameters that give rise to various different shapes (*10*). By using specific lipid mixtures, phase-separation phenomena can occur in the same vesicles that can promote dramatic shape deformations (*11*). These deformations can even lead to a complete scission of the original heterogeneous vesicle into two smaller vesicles of distinct lipid compositions when manipulating the vesicles near the transition temperatures of the different lipids (*12*, *13*). However, the control one can have over parameters like the size of the daughter cells or the time when division happens, is rather limited with these techniques.

A larger interest lies on the controlled mechanical manipulation of a liposome membrane, where it can be deformed until it is split into two. A well-reproducible way of approaching such a mechanical deformation, is by running the liposome through a microfluidic channel into a wedge, that will split it into two (*14*). This is however still heavily dependent on external inputs. The main interest of the synthetic cell field lies instead in aiming for a synthetic cell with a biological machinery that can be synthetized by the cell and that autonomously deforms and splits the membrane mechanically, just like living organisms do. The best way to do so, is a biomimetic approach where we learn how the simplest cells do divide, and then try to reproduce that process inside of a liposome.

1.4. In vitro reconstitution of divisome proteins

When looking into the division mechanisms of bacteria, the major cell-division system is the FtsZ-based machinery. FtsZ is a tubulin-like protein (*15*) that organizes a range of other proteins to orchestrate the cell division at the center of rod-shaped bacteria. While treadmilling along a circumferential ring at the center of the cell, FtsZ drives the peptidoglycan-synthesis machinery and promotes an inwards deformation of the membrane until it partitions the cell compartment into two (*16*). The FtsZ machinery is found across most bacteria (*17*), and has been the primary focus of attention for the development of

synthetic cell division. FtsZ has been found to form bundles of protein when reconstituted inside of liposomes (*18*), and when acting together with the membrane anchor ZipA and hydrolyzing GTP, it can also exert forces (*19*) and cause the constriction of the membranes (*20*) (Figure 1.2A).

It is furthermore interesting to combine the FtsZ machinery with the Min protein system that acts to organize the FtsZ location to mid center position. The Min system is a set of 3 proteins: MinC, MinD, and MinE (*21*). These proteins arrange themselves at the inner side of the bacterial membrane, and through the MinD/MinE interactions, their density oscillates dynamically from one pole to the other of the cell and back (*22*). *In vivo,* this oscillation regulates the correct placement of the division ring, as MinC blocks the membrane binding of FtsZ (*23*), hence, the ring will place itself at the center of the cell, where the concentration of Min proteins remains the lowest due to the oscillations. This elegant simple system to control the placement of the division ring is therefore of great interest for using it in synthetic cells. When reconstituting the Min system on a supported lipid bilayer (SLB), it forms characteristic wave and spiraling patterns (Figure 1.2B) (*24*), and when reconstituted in liposomes, it produces the same pole to pole oscillations as found *in vivo*, which can also deform the liposome shape (*25*, *26*). Combining the Min system *in vitro* together with FtsZ on SLBs led to waves of FtsZ propagating together with the Min waves (*27*).

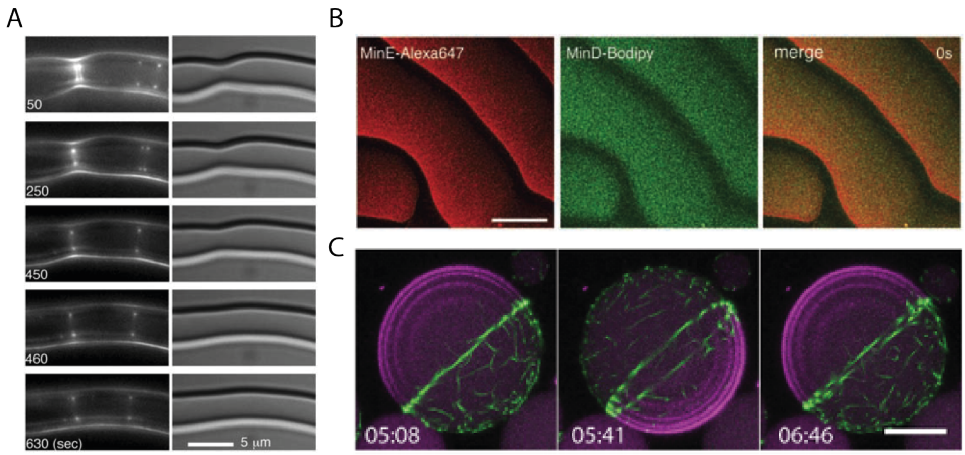

Figure 1.2. Examples of the in vitro reconstitution of the FtsZ and Min systems

A. Reconstitution of FtsZ inside of liposomes, showing how it forms bundles of protein that can deform the membrane. Reproduced from (18). **B**. Propagating wave patterns formed in vitro by Min proteins on a lipid membrane surface. Scale bar 50 μm. Reproduced from (24). **C**. In vitro reconstitution of an FtsZ ring (green) located at the centre of a liposome by the action of the pole-to-pole oscillations of Min proteins (purple). Scale bar 15 μm. Reproduced from (28).

Recently, reconstitution of an FtsZ ring was achieved at the center of a liposome, through control of the Min oscillations, and some contraction of the membrane was achieved (*28*), which represents a major step forward the construction of an autonomously dividing synthetic cell (Figure 1.2C). All in all, there is significant knowledge about the reconstitution of the FtsZ system *in vitro*, advancements on the control of its placement in liposomes have been made, and there seems to be a relation between the hydrolysis of GTP by FtsZ and its ability to constrict lipid membranes. However, it is still a big question whether this minimal system could indeed divide a synthetic minimal cell. It is not clear whether FtsZ can generate enough force to drive a liposome from spherical to dumbbell shaped, and even if that is the case, it is furthermore unknown if FtsZ can perform the last step of division, and promote membrane scission. The growing consensus portrays FtsZ not as a membrane constrictor, but mainly as the recruiter and guide for the cell-wall synthesis machinery, which is the actual responsible for the generation of a constricting force (*29*). Hence, it seems difficult that a minimal system with only a ring of FtsZ attached to the membrane, could successfully divide a cell.

Different kingdoms of life also present different mechanisms of cell division. While we have explored how bacteria divide, eukaryotes present a completely different machinery for their cytokinesis, which is the Endosomal Sorting Complex Required for Transport (ESCRT) (*30*). This protein system was first described in eukaryotic cells for its involvement in the creation of intraluminal vesicles (*31*). However, with time, its involvement was discovered in many other processes like cell division (*32*), vesicle trafficking (*33*), virus release (*34*), and basically any process in the cell that requires the scission of a membrane in any topology. During the cytokinesis of mammalian cells, the actomyosin skeleton of the cell remodels the membrane of the mother cell until there are two daughter cells connected by a membrane tube that bridges them together, called the midbody (*35*). Then the ESCRT machinery gets recruited at this structure, and finishes the abscission process by pinching the membrane and separating the two daughter cells (*36*). The ESCRT-III proteins are the ones that, once assembled at the midbody, are remodeling the membrane with energy provided by the ATPase Vps4, thus leading to the actual process of membrane scission (*37*).

There is extensive literature about the *in vitro* reconstitution of this machinery. When imaged on flat membranes, the ESCRT-III filaments from yeast assemble into spirals (*38*) that can exert forces on the membrane and that are depolymerized by Vps4 (Figure 1.3A). The human ESCRT-III proteins are known to form protein tubes both on their own or wrapping around lipid membranes (*39*, *40*) (Figure 1.3B). Their reconstitution inside of liposomes, has shown that these proteins have a preference to bind on membrane necks (*41*), and that they have a tendency to deform lipid tubes (*42*) into helical structures. Perhaps most interestingly, successful reconstitution of the ESCRT-III machinery has been reported inside of a GUV where the proteins would locate at the neck of the tube when pulling a membrane tube out of the GUV. When subsequently uncaging ATP, Vps4 did promote the cutting of the tube (*43*)

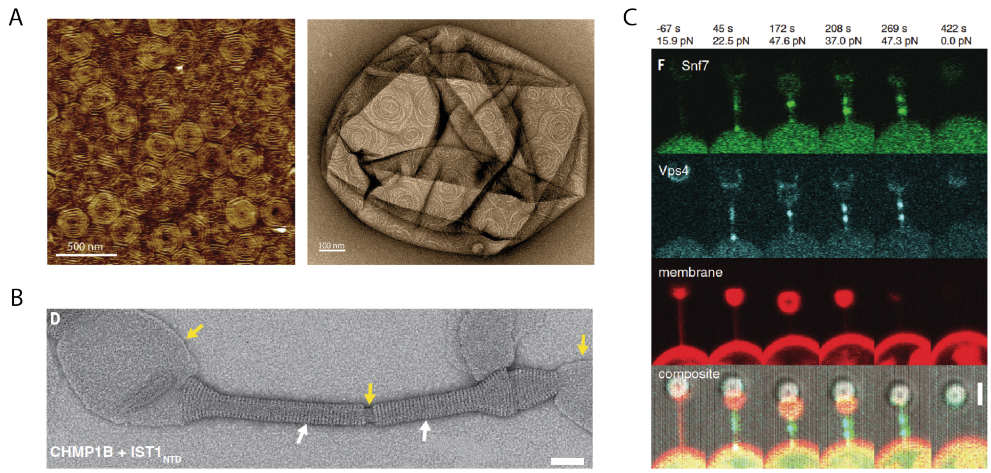

Figure 1.3. Examples of in vitro reconstitution of the eukaryotic ESCRT-III proteins

A. Spirals of the ESCRT-III protein Snf7 forming on a supported lipid bilayer imaged with AFM (left image), or imaged by TEM on the surface of liposomes (right image). Reproduced from (38). **B.** TEM images of the human ESCRT-III proteins CHMP1B + IST1 forming a spiralling tube (white arrows) around liposomes (yellow arrows). Scale bar 50 nm. Reproduced from (40). **C.** In vitro scission of a membrane tube that is pulled out of a GUV, promoted by Snf7 and Vps4 from within the vesicle. Scale bar 1.5 µm. Reproduced from (43)

(Figure 1.3C). This shows that *in vitro* protein-induced membrane scission from within the container can be obtained. However, this system faces a major drawback, because while being capable of cutting membranes, it can only do so on small membrane necks. The ESCRT protein machinery is not capable of large-scale deformations of the membrane on itself, but it is rather recruited at the last step of the cytokinesis, just to cut the membrane. Ideally, for a minimal cell, one would want a division machinery that is capable of arranging itself at the center of the cell, deforming the membrane down to a state where the two daughter cells are connected by a neck, and finally cleaving it. Other division machineries should therefore be explored, and this is why the Cdv system becomes an interesting option.

1.5. The Cdv system for synthetic cells

The Cdv system is the protein machinery responsible for the cell division in the crenarchaea phylum of the archaea (*44*, *45*). This system is composed of CdvA, CdvB and its paralogs, and CdvC (*44*), and it has the peculiarity that the CdvB paralogs (CdvB, CdvB1, CdvB2 and CdvB3) and CdvC are homologous to the eukaryotic ESCRT-III and Vps4 proteins respectively (*45*). It is one of the many protein families that are shared between archaea and eukaryotes, and that point towards a common evolutionary ancestor (*46*). The Cdv system is thus often portrayed as a simplified version of the ESCRT machinery, as it can divide the cells while having less components (*47*).

How the system works is – as we partly deduced in this thesis work of the past 4 years – that CdvA acts as an anchor that recruits CdvB to the membrane (*45*, *48*, *49*), and these in turn recruit CdvB1 and CdvB2 that are responsible for the constriction and scission of the membrane (*50*). CdvC is then thought to provide the disassembly of the membrane-bound components, needed to ensure a protein turnover that can deform the membrane, likely in the same way that it happens in eukaryotes (*51*, *52*). This means that this system alone appears to be responsible for all the process of cell division, from the initial ring formation at the center of the cell, inducing and propagating a membrane ingression all the way down to the final event of scission. Hence, it has been proposed as a valuable candidate for a division machinery of a synthetic cell (*8*, *53*). Nonetheless, many questions still remain unanswered about its functioning, and our knowledge of the proteins *in vitro* is currently very limited, with no successful functional reconstitutions of the system *in vitro* having been ever published.

While we know that CdvA can form filaments and bind lipid membranes while we know that CdvB does not (*48*, *49*), there has been no previous *in vitro* work done on any of the other CdvB paralogs. And while we know how CdvC structurally arranges *in vitro* (*54*), we don't have any idea if it really has a depolymerizing function like its eukaryotic homolog. Additionally, an important role has been reported recently for CdvB1 and CdvB2 *in vivo* (*55*), but we know nothing about their interactions and their recruitments to the membrane.

1.6. This thesis

In the research described in this thesis, we aimed to reconstitute the proteins of the Cdv system *in vitro*. The broader aim of the research is to better understand the Cdv system, as well as explore its potential use in the bottom-up building of a synthetic cell. We have made biochemical and biophysics studies of proteins that had already been reported to be purified *in vitro* such as CdvA, CdvB and CdvC, as well as we describe for the first time the *in vitro* characterization of CdvB1 and CdvB2.

The first description of this Cdv machinery now 15 years ago, was met with sudden spike of interest that was reflected in many publications describing the new machinery. This early enthusiasm decayed as no new discoveries were published, until very recently, where our view and understanding of the system was greatly altered with a number of new findings. For this reason, we review in **chapter 2**, this understudied mechanism in depth and summarize the most relevant information about it, from its discovery until the present day. We explore all the different functions that this protein machinery develops in the cells, as well as we describe all the different proteins that compose the system and the roles they play, both *in vivo* and *in vitro*.

In **chapter 3** we describe for the first time the *in vitro* characterization of CdvB1. This protein was identified to be a main player in the constricting ring of the cell. We find that it can self-

assemble into filaments that are depolymerized by the action of CdvC. Additionally, we show how CdvB1 can bind to lipid membranes and how CdvC can also resolubilize it from the liposomes into the solution. Interestingly, this membrane-binding capability is mutually exclusive with the formation of filaments, as these do not bind the membranes.

In **chapter 4** we explore the interactions between the different components of the Cdv machinery, and how these interactions govern their recruitment to membranes. We see that full-length CdvA does not bind to lipid membranes unless it is interacting with CdvB. We describe purified CdvB2 for the first time, and show how it needs to have its C-terminus domain removed for it to polymerize. When interacting with CdvB1, CdvB2 blocks its ability to bind lipid membranes, and we further explore these interactions inside of liposomes where we observe that Cdv proteins prefer the location to lipid necks.

Finally, in **chapter 5**, we provide a brief outlook on the future of the Cdv system research, the key major questions that should be addressed, and the perspective of its use in a synthetic cell.

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2

THE ARCHAEAL CDV CELL DIVISION SYSTEM

The Cdv system is the protein machinery that performs cell division and other membrane-deforming processes in a subset of archaea. Evolutionarily, the system is closely related to the eukaryotic ESCRT machinery, with which it shares many structural and functional similarities. Since its first description 15 years ago, the understanding of the Cdv system progressed rather slowly, but recent discoveries sparked renewed interest and insights. The emerging physical picture appears to be that CdvA acts as a membrane anchor, CdvB as a scaffold that localizes division to the mid-cell position, CdvB1 and CvdB2 as the actual constriction machinery, and CdvC as the ATPase that detaches Cdv proteins from the membrane. This chapter provides a comprehensive overview of the research done on Cdv and explains how this relatively understudied machinery acts to perform its cell-division function. Understanding of the Cdv system helps to better grasp the biophysics and evolution of archaea, and furthermore provides new opportunities for the bottom up building of a divisome for synthetic cells.

This chapter is under review at *Trends in Microbiology* as A. Blanch Jover, C. Dekker, The Archaeal Cdv Cell Division System.

2.1. Introduction

Cell division is a key process in the cell cycle of any living organism. A dedicated set of proteins and complex cellular signaling processes are needed to ensure a faithful splitting of a mother cell into two daughter cells. Eukaryotes make use of the Endosomal Sorting Complex Required for Transport (ESCRT) for their final stage of cell division, a complex machinery with a large set of proteins that are capable of severing membrane necks from the inside of the cell membrane (*1–3*). In virtually all bacteria, the process of cell division is driven by the tubulin-like protein FtsZ (*4–6*) which guides the cell-wall-synthesis machinery in building an inwards ingression of the cell wall and membrane until the cell splits in two.

Archaea, despite being prokaryotes like bacteria, present a broad heterogeneity in their cell-division machineries. Most archaeal phyla present different tubulin family proteins that govern shape control (*7*), including FtsZ homologs that are responsible for the cell division (*8*). The archaeal FtsZ presents several differences from the bacterial one, as many archaea use 2 different FtsZ proteins that play different roles during the division process (*9*), and archaea present a unique membrane anchor called SepF (*10*). However, some members of the TACK and Asgard superphyla of archaea present an interesting and less-studied membrane-remodeling machinery, the Cdv proteins (Figure 2.1A) – which are the subject of this review.

The Cdv system is a protein machinery responsible for cell division and vesicle formation in members of the TACK (Thamuarchaeota, Aigarchaeota, Crenarchaeota and Korarchaeota) superphylum or archaea, and it is also present in the Asgard archaea (*11*, *12*). These archaeal phyla are evolutionarily close to eukaryotes, and some protein families of these archaea are homologous to those found in eukaryotes, like the different DNA-processing machineries, ribosomal proteins, ubiquitination systems, and cell-division proteins (*13*). These similarities underlie the widely accepted idea of a common evolutionary ancestor between eukaryotes and archaea, and the notion that these two domains of life relate more closely to each other than to bacteria (*14*). One of the archaeal protein machineries that exhibits such a homology to eukaryotes is presented in the Cdv system.

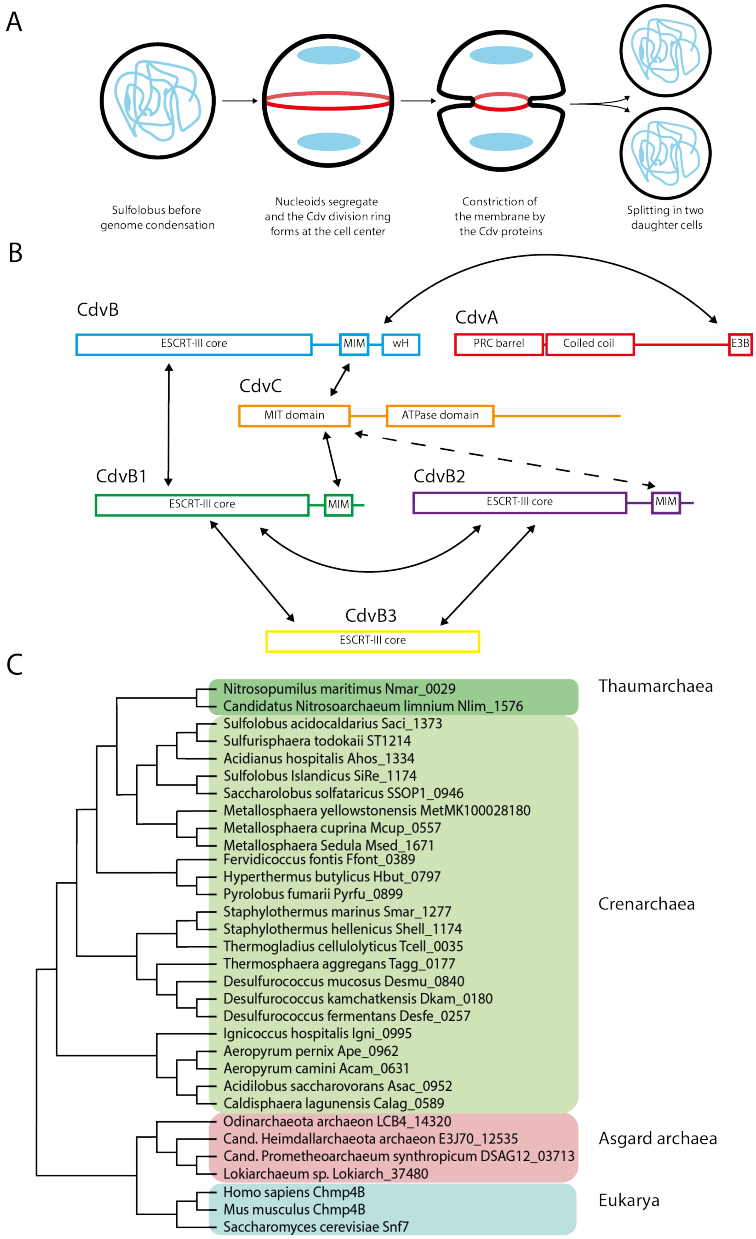

Figure 2.1. Overview of the Cdv system.

A. Schematic of the cell cycle of the archaeon model organism *Sulfolobus acidocaldarius*. **B.** Schematic representation of the protein domains of Cdv proteins from Sulfolobales and the interactions between them. Arrows show domains that interact with each other; dashed arrows show speculative interactions that have not yet been experimentally shown. **C.** Phylogenetic tree of the evolutionary relationship of the CdvB (ESCRT-III) proteins in different organisms. It shows how CdvB from the Asgard archaea is closer to the ESCRT-III of eukaryotes than the Crenarchaeal CdvB, as well as how the evolutionary divide of the Cdv machinery between the Asgard and the TACK happened before the appearance of eukaryotes.

A growing interest in the mechanistic understanding of the Cdv proteins in recent years has led to a larger body of published research. In this brief review, we summarize the most relevant research that has been done on the Cdv system since its first description almost 15 years ago (*11*, *15*). We describe the Cdv system, its evolutionary relationship to the eukaryotic ESCRT system, and its functional role in cell division and vesicle formation. While most knowledge has been obtained from genetic and cell-biology studies, more recently, modelling and *in vitro* studies began to disentangle the biophysical mechanism of the various components of the Cdv system. We finish the paper with an outlook that sketches a path forward, as well as discusses the potential use of the Cdv system for the bottom up building of a divisome for synthetic cells.

2.2. The Cdv system in the Sulfolobales

The Cdv system was first described in *Sulfolobus acidocaldarius* (*11*, *15*), an extremophile archaeon that inhabits volcanic areas with temperatures of about 80 °C and pH as low as 2-3 (*16*). Since its discovery, the Cdv system has generally been described as consisting of 3 classes of proteins: CdvA, CdvB (and multiple paralogs), and CdvC. CdvA is an exclusively archaeal protein that finds no homologs in eukaryotes. By contrast, all the CdvB paralogs display homology to the eukaryotic ESCRT-III proteins, while CdvC is an AAA ATPase that is homologous to the eukaryotic Vps4 (*17*). It is worth noting that it was recently described that the bacterial proteins Vipp1 and PspA belong to the same superfamily as the ESCRT proteins, as they present a similar protein sequence and structure (*18–20*). While the exact functions of these proteins in the cell still remains unclear, this presented the first evidence that proteins belonging to the ESCRT-III superfamily are present across all domains of life, thus posing exciting questions about their evolutionary relation. Although Cdv-homologs can be found in many different genus of archaea, most published experimental work has been done with organisms of the TACK superphylum. In this review, we focus on describing the reported experimental findings – mostly from the Sulfolobales– and the picture that they portray of the Cdv system.

The core proteins of the Cdv system of the Sulfolobales, CdvA, CdvB and CdvC, are encoded in an operon that is essential for the viability of the organisms (*21*), while the other CdvB paralogs are found in different parts of the genome (*11*). CdvA is a purely archaeal protein that has no structural homolog in eukaryotes, and it can interact with CdvB (*22*) through its ESCRT-III binding region (E3B) (Figure 2.1B). This E3B region of CdvA interacts with the winged Helix (wH) region present in CdvB (*23*), which is the only CdvB paralog that contains such region. Since CdvA only interacts with CdvB, and CdvA interacts with the cell membrane (while CdvB does not), it was suggested that the role of CdvA is the recruitment of CdvB to the membrane (*22*). Yeast 2 hybrid assays have shown that CdvB is also capable of interacting with CdvB1, while CdvB1 furthermore interacts with CdvB2 and CdvB3 (*15*).

In eukaryotes, ESCRT-III proteins are recruited to the membrane through a series of interactions with the ESCRT-0, ESCRT-I and ESCRT-II complexes of the system (*24*). At the membrane, they are structured into filamentous polymers that tend to form spirals (*25*, *26*) that deform the membrane through a coordinated depolymerization and reshaping of the filaments into an tighter spiral (*27*). Similarly, CdvB paralogs of archaea have recently been suggested to deform the cell membrane and perform scission of the cell (*28*). Clear images of micron-sized CdvB rings at the division site have been obtained, although their nanoscopic structure has so far remained unclear.

CdvC is the only active Cdv protein with a described ATPase domain (*23*, *29*). The role of CdvC appears to be providing energy to the system to depolymerize the ESCRT-III filaments at the membrane, remodel their structure, and thus deform and cut the membrane, analogously to the eukaryotic machinery (*30*). Specifically, by hydrolyzing ATP, CdvC may detach monomers of the membrane-bound CdvB filaments into solution, allowing for a remodeling of the CdvB structures at the membrane that leads to a narrower neck structure until scission occurs (*30*, *31*). Such a scenario is also suggested by the presence of a Microtubule Interacting and Transport (MIT) domain in CdvC that is capable of interacting with the MIT-Interacting Moiety (MIM) (Figure 2.1B) present in the CdvB proteins (*32*). This interaction is well-known in eukaryotes, where Vps4 (CdvC homolog) interacts with the ESCRT-III proteins in the same way (*32*), reinforcing the idea of a similar mechanism of action in the two systems.

S. acidocaldarius has emerged as a model organism for studying the Cdv system, since it was the first to be described (*11*, *15*), and because well-established protocols exist for its growth and manipulation (*33*). After the discovery of the Cdv system in *S. acidocaldarius,* which belongs to the Crenarchaeaota phylum, other Cdv systems were found in different archaea, although presenting differences in their composition. Some members of the Euryarchaeota phylum present genes coding for CdvB and CdvC (*34*), but not much is known about their functional roles. Interestingly, members of the Thaumarchaeota phylum, which are members of the TACK superphylum like Crenarchaea (*35*), contain genes encoding for *both* the Cdv machinery and a tubulin homolog similar to FtsZ (*5*, *36*). The thaumarchaeon *Nitrosopumilus maritimus* was found to use the Cdv system for cell division (*37*). However, while the thaumarchaeal FtsZ is a member of tubulin family, it is actually distant from the FtsZ clade of proteins and it does not possess the catalytically active domain common of the FtsZs that drives cell division (*38*). It can therefore not perform many of the classically associated functions of FtsZ that require of the hydrolysis of GTP, and its function in the Thaumarchaeaota remains unknown.

More recently, interest emerged in the Cdv proteins from the Asgard archaea. This newly described superphylum of archaea is evolutionarily closest to eukaryotes (*39*), and their CdvB proteins are the closest to the eukaryotic ESCRT-III as can be seen in the evolutionary

tree in Figure 2.1C. This evolutionary tree that we built for CdvB proteins displays interesting relations between the different archaea and the connection of Asgard archaea to Eukarya. This is in line with recent comparative phylogenetic studies that suggest that part of the complexity and characteristics that are commonly associated with eukaryotes, started appearing in the Asgard archaea before the formation of eukaryotes (*13*). More specifically, it has been suggested that eukaryotes arose from the formation of a symbiont between a Loki archaeon (part of the superphylum Asgard) and a bacterium, which slowly integrated their genes and functions with each other until forming the first eukaryote (*40*). While the Asgards possess Cdv proteins, they as well present 2 FtsZ homologs, like most of the Euryarchaeaota (*41*), which raises the question about the roles of each of these systems in these organisms. The Cdv proteins in Asgards have been found to be much more similar to the eukaryotic ESCRT machinery than the TACK archaea. Quite remarkably, they present homologs of the ESCRT-I and ESCRT-II machineries, and these interact with the ubiquitylation machinery of the organism, just like in eukaryotes (*42*). On the other hand, the Asgards lack the CdvA protein, while CdvB possesses an ANCHR motif similar to that of the eukaryotic ESCRT-III, which could allow it to directly bind the membrane (*43*). There are also some significant structural differences of the CdvB paralogs, which indicates that the protein interactions, and by extent the mechanism of their action, will also be different in the different phyla (*43*). This leads to us speculate that, while the Asgard Cdv machinery likely is involved in some membrane-deforming processes in the cell, the process of cell division may be carried out by the FtsZ homologs, just like in Euryarchaeaota. So far, however, there is no experimental proof for this, mostly due to difficulties in culturing and growing Asgard archaea in the lab (*44*).

As described above, different components of the Cdv protein system share a strong similarity to the eukaryotic ESCRT proteins, and for this reason Cdv is often portrayed as a simpler and more antique version of the ESCRT system (*15*). This parallelism between the two domains of life was used for many years to hypothesize how the archaeal machinery would work, given the extensive literature on the ESCRT and the limited knowledge and understanding of the Cdv proteins.

2.3. The role of Cdv proteins in the cell division of the Sulfolobales

Immunostaining images of dividing *S. acidocaldarius* cells showed a band of CdvA and CdvB forming at the center of the cell, between the two segregated nucleoids (Figure 2.2A), with a band of CdvC proteins observed at the same location (Figure 2.2B). These were some of the first clear indications that the Cdv operon proteins were directly involved in the process of cell division. Cdv protein concentrations also vary during the cell cycle of the organism, with expression of the genes encoded on the Cdv operon occurring before onset of cell division (*11*). Gene inactivation experiments in *S. acidocaldarius* showed that removal of the individual CdvB paralogs greatly affected cell growth and generated aberrant cells (*45*). This provided the first indications that the CdvB paralogs were involved in the process of cell

division as well, although they appeared to not be strictly essential since cells could still survive without them.

Recent super-resolution imaging techniques provided further detail (Figure 2.2C): at an early stage of cell division, first a CdvB ring forms at the middle of the cell (*28*). Then, CdvB1 and CdvB2 get recruited at the same ring location. Subsequently, the proteasome digests the initial ring of CdvB, leaving just a CdvB1 and CdvB2 ring (Figure 2.2C). This second ring appears to be responsible for the constriction process of membrane ingression and fission. These data showed that the CdvB paralogs play a key role in the cell division process.

The development of high-temperature (75°C) live-cell imaging microscopy of these thermophilic archaea, allowed to further investigate the role of these CdvB paralogs in archaeal division (*46*). Mutants of *S. acidocaldarius* that lacked CdvB1, presented abnormal division (Figure 2.2D), where some cells would suddenly arrest their division process. These results were somewhat similar to SEM observations for *Sulfolobus islandicus* cells (*47*) on mutants lacking CdvB1 (Figure 2.2E). These mutants presented a pearl-collar appearance, where chains of cells were kept attached to each other, while a genome-number analysis showed many cells containing multiple genomes (*47*). These data suggest that CdvB1 is implicated in the process of faithful completion of the fission process.

The CdvB2 protein appears to play a different role. Live imaging of *S. acidocaldarius* cells lacking CdvB2 showed that the division plane in cytokinesis was not located at the middle of the cell, yielding asymmetric division with differently sized daughter cells (Figure 2.2F). Immunostaining imaging provided a more detailed view of the localization of the division ring (Figure 2.2G). Normal cells presented an initial CdvB ring, followed by a recruitment of CdvB1 and CdvB2 to the site. While the initial-ring formation was unaffected by CdvB2, CdvB2-deficient cells presented an abnormal localization of the CdvB1 rings after the initial CdvB ring disassembled, and hence asymmetric divisions did occur (*46*). The data indicate that the role of CdvB2 is to keep the constricting ring at the correct mid-cell position, and hence ensure symmetric fission.

In contrast to CdvB1 and CdvB2, very little is known about the role of CdvB3 in the cell division. In *S. acidocaldarius*, CdvB3 did not form any kind of ring-like structure at the cell center during constriction (*45*). Mutants without CdvB3 were still able to survive, although giving rise to fewer and much larger cells. In addition, deletion of the protein led to an aberrant localization of CdvA throughout the cell, and prevented CdvB to form a defined structure (*45*). It thus seems that CdvB3 does not participate directly in the scission process, but instead it may coordinate the correct positioning of the initial CdvA:CdvB ring.

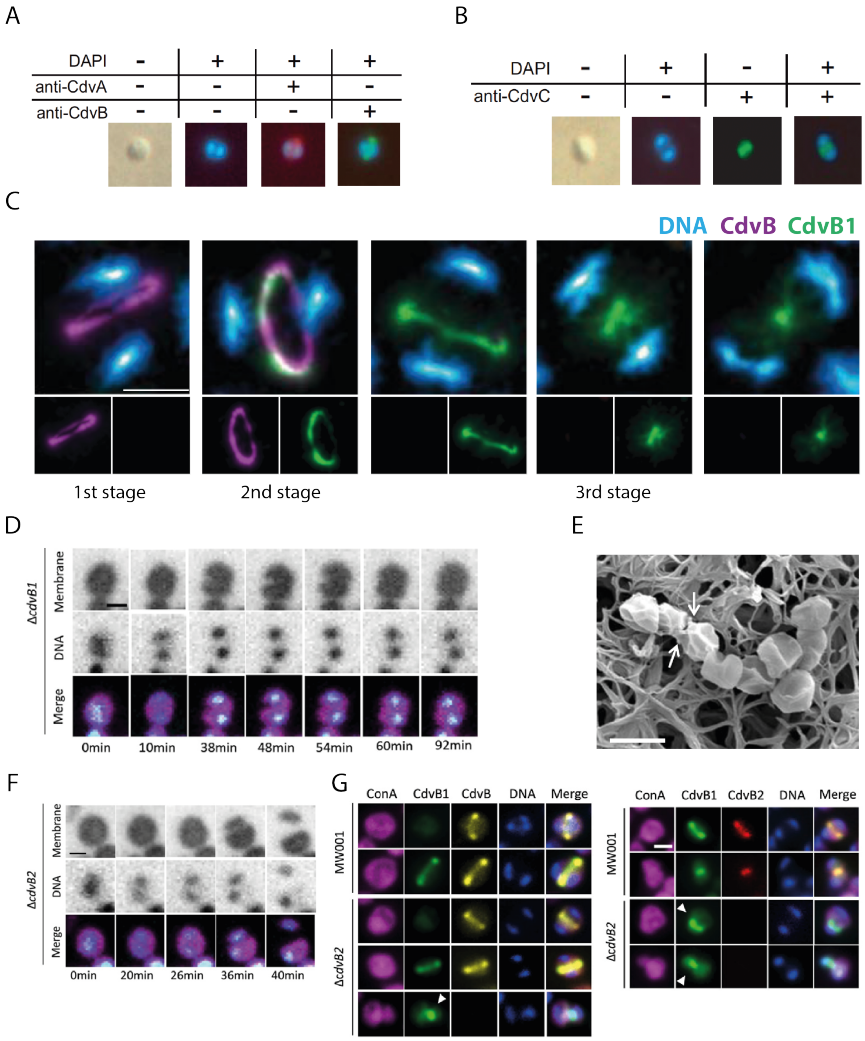

Figure 2. Images of the Cdv system in *Sulfolobus* cells during cell division.

A. Immunostaining images of fixed cells that show how a ring of CdvA and CdvB is formed between the two segregated nucleoids during cell division. **B.** The AAA ATPase CdvC also localizes in a ring between the nucleoids right before the cell division. **C.** High-resolution immunostaining images of *S. acidocaldarius* during cell division, showing how a first ring of CdvB is formed at the division site, then CdvB1 is recruited, CdvB is removed, and finally only CdvB1 remains for the constriction of the cell. Scale bar 0.5 µm. **D.** Live cell images of CdvB1-lacking mutants of *S. acidocaldarius*, showing a cell-division process that is arrested and where scission of the membrane does not occur. Scale bar 1 µm. **E.** SEM images of *S. islandicus* CdvB1- lacking mutants, showing chains of connected cells that were unable to realize full scission. Scale bar 2 µm. **F.** Live cell images of CdvB2- lacking mutants of *S. acidocaldarius* showing asymmetric division for some cells. Scale bar 1 µm. **G.** Immunostaining images comparing CdvB2-lacking mutants of *S. acidocaldarius* to a background strain. It shows how, in absence of CdvB2, the non-contractile CdvB ring forms normally, but the constriction of the cell after the removal of CdvB can be misplaced. In the background cells, CdvB2 is observed at the division site during the constriction of the membrane. 1 µm Panels A and B are adapted from Ref. (*11*). Panel C is adapted from Ref. (*28*).Panels D, F and G are adapted from Ref. (*46*).Panel E is adapted from Ref.(*47*).

While the above data describe the most well-studied Cdv proteins from members of the Sulfolobales, it is worth noting that a large variability in sequence and functional regions exists across the different archaeal species that possess the Cdv machinery (*43*). For example, many species of Asgard archaea lack some or all of the CdvB paralogs, as well as present different functional domains in the commonly shared proteins. This likely indicates an as of yet undiscovered set of protein interactions and mechanisms for the different Cdv machineries.

It also remains a question how the process of cell division is coordinated within the cell cycle of the organisms. In eukaryotes, the cell cycle is regulated through the action of cyclin-dependent kinases (*48*), but no clear homologs of these proteins are found in members of the TACK phylum of archaea. The crenarchaea present a differentiated cell cycle, with growth and chromosome segregation phases clearly differentiated from the cell-division phase (*49*). The expression of the genes of the Cdv system is regulated during the cell cycle right before cell division (*50*). Microscopy images of *S. acidocaldarius* and *N. maritimus* showed how the formation of Cdv rings at the mid cell was coordinated with the segregation of the chromosome prior to cell division (*11*, *37*). Some authors have speculated about a double role for CdvA, implicating it both in cell division and chromosome organization, since it has DNA-binding abilities (*51*). There is, however, no direct proof of that, and so far, little is known about the coordination of the Cdv proteins in the overall cell cycle of crenarchaea.

2.4. The role of Cdv proteins in vesicle formation

In eukaryotes, the ESCRT complex is involved in many different cellular processes that concern membrane deformation (*1*). It was in fact first described and characterized for its main role in the formation of vesicles in the multivesicular body pathway (MVB) (*52*), and its involvement in other membrane-deforming processes was subsequently revealed, e.g. in cytokinesis, nuclear envelope reformation, and viral budding (*53–55*). Basically, the ESCRT system is involved in all processes that require the deformation and pinching of small membrane necks in a reverse topology in cells. Interestingly, such a diversity of functions appears to be conserved in archaea, as the Cdv system is not only involved in cell division, but also in the formation of membrane vesicles and viral membrane budding (*56*).

The release of extravesicular bodies is a widespread phenomenon amongst the *Sulfolobus* genus (*56*). These extracellular vesicles (EVs) are made of the same lipid composition as the archaeal membrane lipids and covered by an S-layer, showing that they are an outwards protrusion of the membrane of the cells. The roles of these EVs are in general diverse, although its particular purpose for the Sulfolobales is unclear. EVs from *S. islandicus* were found to contain plasmid DNA, suggesting that they can be used for transfer of genetic material (*57*). These EVs in *S. islandicus* have furthermore been associated with the release of “sulfolobicins”, protein toxins that inhibit the growth of other Sulfolobales (*58*). Mass-spectrometry analysis of EVs showed that they contain several Cdv proteins, suggesting that

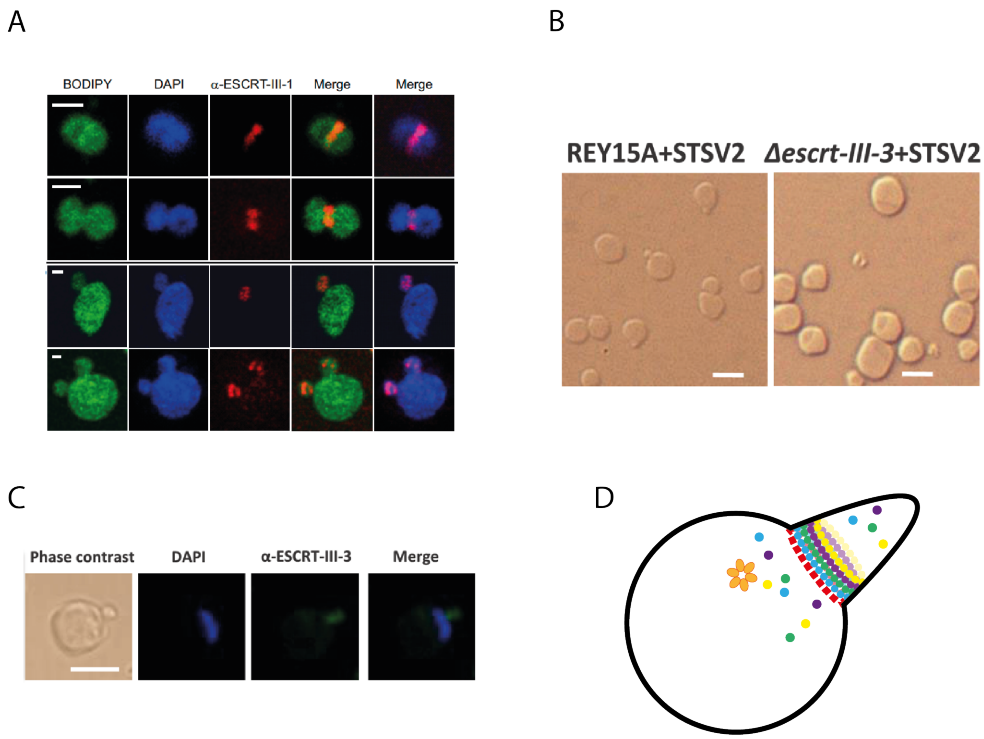

Figure 3. Cdv-mediated vesicle budding for viral release.

A. Immunostaining of healthy (upper 2 rows) and STSV2-infected (lower 2 rows) cells of *S. islandicus*. Healthy cells present CdvB1 at the centre of the cell where the membrane shrinks, whereas infected cells present CdvB1 at the neck of a bud performing an asymmetric division. Scale bar 1 μm **B.** Phase contrast imaging of *S. islandicus* cells (left) which have been infected with the STSV2 virus, showing protrusions coming out of them. Infected cells lacking CdvB3 (right) did not present such protrusions. Scale bar 2 μm **C.** Immunostaining of the infected cells did show that CdvB3 is found at the neck of the budding vesicle Scale bar 2 μm **D.** Schematic of the vesicle budding function of the Cdv system. Panel A is adapted from Ref. (*62*). Panels B and C are adapted from Ref.(*47*).

the Cdv system is involved in the formation of these EVs (*56*, *57*). When knocking out any of the different CdvB paralogs or CdvC using CRSPR (*57*), cells produced much lower amounts of EVs. Deletion of CdvA, however, largely left the production of vesicles unaffected, indicating that CdvA is dispensable for the process of EV formation.

Viruses also use of the ESCRT system, as newly formed virus particles inside infected cells escape the cell encapsulated inside EVs (*59*). A similar process was found in *Sulfolobus*, where archaeal viruses induced overexpression or repression of the expression of the Cdv proteins (*60*, *61*). In *Saccharolobus solfataricus*, cells infected with the STIV archaeal virus were observed to overexpress the Cdv proteins (*61*), and Cdv proteins of their host organisms were involved during the infection cycle (*61*).

Virus-infected cells have been useful to determine the role of different Cdv proteins in vesicle budding. The fact that CdvB3 appears not to be involved in the constriction during cell division in *S. islandicus*, raised the idea that it might be involved in the vesicle-budding process. Infection of *S. islandicus* cells with the archaeal virus STSV2 promoted the formation of buds coming out of the cells (Figure 2.3B). For mutants lacking CdvB3, however, these buds did not appear (Figure 2.3B). Immunostaining of the infected cells revealed a clear localization of CdvB3 inside of the buds (Figure 2.3C), providing direct evidence for the implication of CdvB3 in vesicle budding. In a more recent study of the STSV2 virus in *S. islandicus* (*62*), all Cdv genes were downregulated upon viral infection, and the cells suffered from gigantism, reaching a size up to 20 times larger than normal. It was found that spirals of CdvB1 were formed inside of the newly formed buds (Figure 2.3A), as opposed to the normally occurring mid-cell ring of healthy cells (Figure 2.3A).

2.5. *In vitro* studies of the *Sulfolobus* Cdv system

Studying the higher-order structures that these ESCRT proteins form *in vitro* provided valuable information about how they may work inside cells. Most eukaryotic ESCRT-III proteins present a duality between a soluble cytosolic state and a membrane-bound polymer form (*63*). Interestingly, many of these proteins stay monomeric when purified in their full length version, but polymerize into filaments when removing the last 40 amino acids of their sequence (*64*, *65*). The human ESCRT-III proteins CHMP2A and CHMP3 form helical straight tubes in solution that can be disassembled by the ATPase Vps4 (*66*). When incubated with lipid membranes, they polymerize forming straight tubes and cones around lipid tubes (*27*). Yeast ESCRT-III was shown to form 2D spirals on the surface of lipid bilayers (*25*), while Vps4 dynamically modified these structures (*67*). Furthermore, both human and yeast proteins were shown to assemble into filament bundles along membrane tubes (*68*, *69*).

ESCRT-III has been reported to form spirals that deform the membranes and bend them into domes or tubes (*27*), acting as springs that can exert forces (*25*). Similar membrane-bound protein complexes in archaea has not yet been reported, but first studies have been conducted to assess the interactions of the proteins with membranes. CdvA was found to be capable of binding the polar lipid fraction E (PLFE) in *Sulfolobus acidocaldarius* (*22*), whereas CdvB was not. A later study (*70*) reported that the truncated CdvA(69-238) was unable to polymerize into filaments on its own. However, when the protein was incubated with liposomes of PLFE, CdvA(69-238) assembled into filaments that wrapped around liposomes (Figure 2.4D). These images suggest that CdvA may arrange itself at the center of the cell in a spiral shape that serves as a template that accommodates other divisome components.

CdvA full-length from *Metallosphaera sedula* was found to polymerize *in vitro,* forming double-helical filaments (*23*) (Figure 2.4A). Much like the human ESCRT-III proteins that do not polymerize *in vitro* when they are full length, full-length CdvB from *M. sedula* did not polymerize (*23*). However, upon removal of the C-terminus domain of its sequence, it

polymerized into elongated filaments (Fig. 2.4B). By contrast, CdvB1 was found to self-assemble into filaments already in its full-length form (*71*) (Figure 2.4C). These filaments of CdvB1 were shown to be depolymerized by the action of CdvC (*71*), in the same way that the polymers of ESCRT-III proteins in eukaryotes are depolymerized by Vps4 (*66*). This is so far the only evidence of a depolymerization of a CdvB paralog polymer by the action of CdvC, and supports the idea that CdvC is responsible *in vivo* of generating a turnover of monomers at the division ring, supporting its sequential assembly and disassembly. In the same study (*71*), it was reported that the filaments that CdvB1 form *in vitro* (Figure 2.4C) cannot bind the lipid membrane, but that monomeric CdvB1 proteins can bind. This indicates that the membrane binding patch of the protein is not accessible anymore upon polymerization, and hence that, *in vivo,* polymerization of the protein likely occurs directly at the membrane.

The AAA ATPase CdvC presents many similarities with the eukaryotic Vps4. Both CdvC and Vps4 are composed of a MIT region, which is used to interact with the MIM domain of

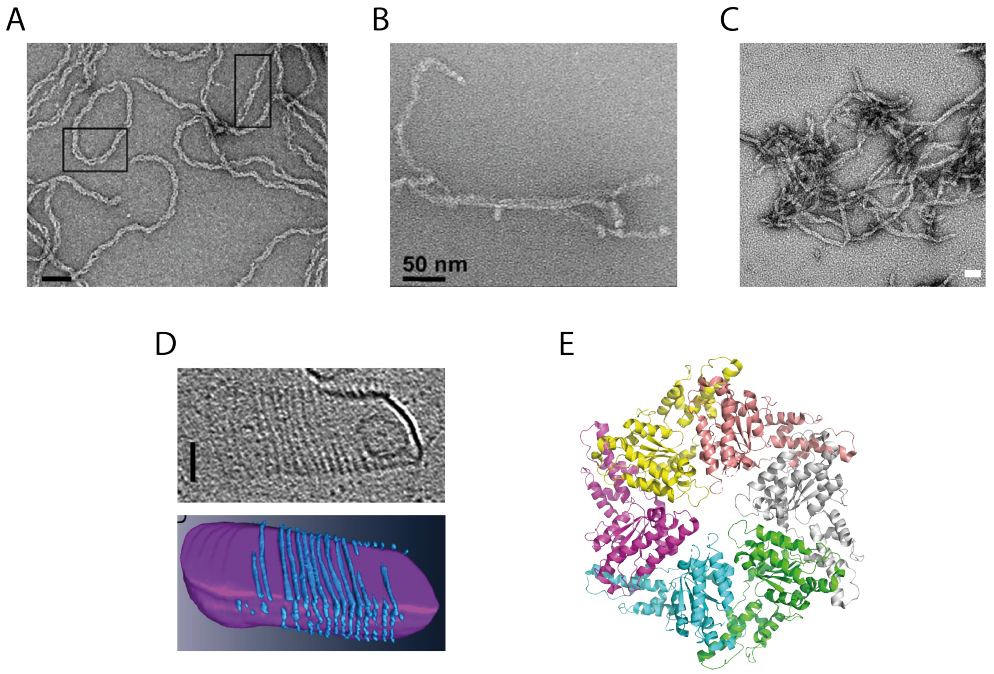

Figure 4. *In vitro* characterization of Cdv filaments.

A. CdvA spontaneously assembles into double-helical elongated filaments *in vitro*. Scale bar 50 nm **B.** Negative staining image of CdvBΔC, which shows *in vitro* polymerization into filaments upon removal of the C-terminus domain. **C.** Negative staining TEM image of CdvB1 filaments. Scale bar 50 nm **D.** Filaments of CdvA(69-264) that are wrapped around liposomes of purified archaeal lipids (Lipid Fraction E from *S. solfataricus*) Scale bar 50 nm. **E.** Crystal structure of CdvC from *M. sedula* obtained in (*29*) (PDB #4D80). Panels A, B and E are adapted from Ref.(*23*). Panel C is adapted from Ref. (*71*)

the ESCRT-III proteins, followed by an ATPase region, and a final C-terminal helix (Figure 2.1D) (*23*, *72*). The monomeric version of the proteins is inactive, whereas the proteins become catalytically active upon oligomerization into hexamers (*72*) (Figure 2.4E). The hexamers formed by CdvC and Vps4 are actually not closed rings, but instead form an open and flexible structure that can wrap itself around the ESCRT-III filaments through their MIM-MIT interactions (*73*). The hydrolysis of ATP generates conformational changes in the structure of this hexamer (*29*), which makes it probably 'walk' along the ESCRT-III filament as it depolymerizes it (*31*). Additionally, in yeast the Vps4 could be deleted and substituted by an Asgard CdvC, and the yeast cells were still viable, further showing the close evolutionary proximity between the Asgard and the eukaryotes (*74*).

2.6. Towards a physical mechanism for the archaeal Cdv divisome

Based on the current knowledge of the Cdv proteins of the Sulfolobales, one can attempt to sketch a picture that best summarizes how the Cdv system acts during the process of cell division (Figure 2.5I-V). Initial formation of a non-contractile ring involves CdvA and CdvB (Figure 2.5I). This ring, is located between two segregated chromosomes, and is anchored to the membrane by CdvA which recruits CdvB to the membrane at the mid-cell position. Subsequently, CdvB recruits CdvB1 and CdvB2 (Figure 2.5II), thus forming an extended ring at the center of the dividing cell (*28*, *46*). After recruitment of CdvB1 and CdvB2, CdvB gets digested by the proteasome (Figure 2.5III). After removal of the initial CdvB ring, only a CdvB1:CdvB2 contractile ring is left (Figure 2.5IV), and these two proteins appear to be responsible for the constriction and scission of the cell (*28*, *46*) (Figure 2.5V).

Once the CdvB1:CdvB2 constricting ring is formed, it needs to shrink and go from the low-curvature state in the fully spherical cell to a high-curvature state, while remodeling the membrane all the way down until scission of the cell. Coarse-grained MD simulations have shown that CdvB1:CdvB2 copolymers formed at the center of the cell can deform the membrane by merely transforming from a low-curvature to a high-curvature conformation (*28*). This implies that deforming the membrane, almost until the point of division, can be realized by shape changes of polymers without changing their length. However, these polymers would not be able to perform the final scission, as towards the end of the membrane-deformation process, steric hindrance at the neck of the dividing cell impedes the division into two daughter cells. Hence, the cell-division process requires the controlled removal of proteins from the ring. Since it has been shown that CdvC can depolymerize CdvB1 filaments and detach it from lipid membranes (*71*), it seems reasonable to hypothesize that CdvC is responsible for this turnover generation at the constricting neck, thus finalizing the process of cell division (Figure 2.5E).

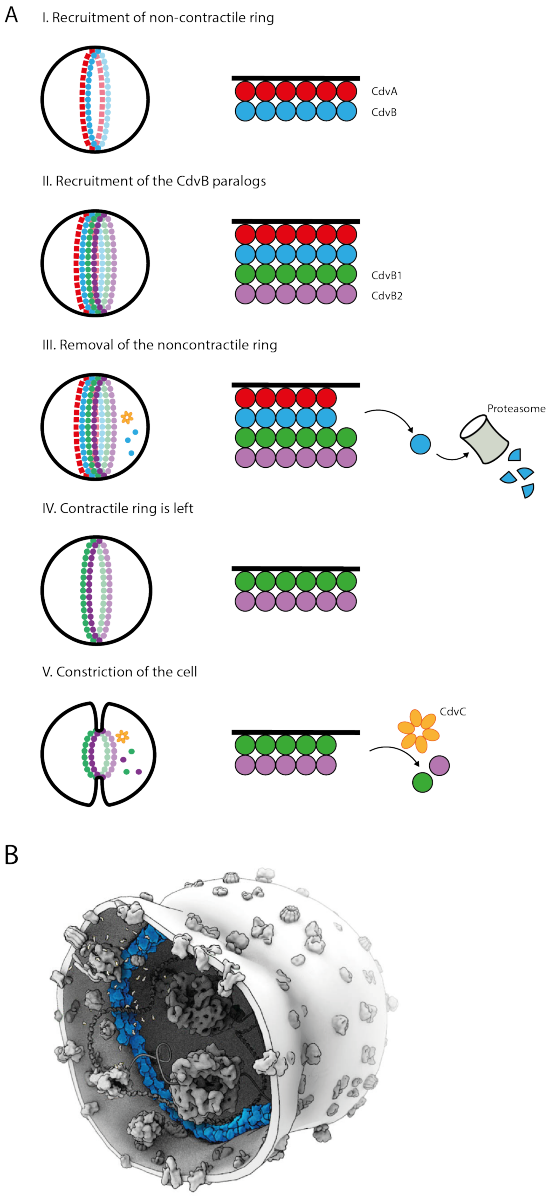

Figure 5. Schematic representation of Cdv-mediated cell division, and its potential for synthetic cells.
A. I. Recruitment of CdvA and CdvB at the centre of the cell in between the two segregated nucleoids. Images on the left in A-E show the shape deformation of the cell membrane of the dividing cell. Cartoons on the right portray a zoom of the arrangement of the proteins at the membrane. **II.** The CdvA:CdvB ring recruits both CdvB1 and CdvB2. **III.** The initial noncontractile ring gets removed from the membrane and CdvB gets digested by the proteasome. **IV.** Only the contractile ring formed by CdvB1 and CdvB2 is left at the division site. **V.** Constriction of the cell mediated by CdvB1:CdvB2, where CdvC removes the proteins from the membrane. **B.** Cartoon of a putative 'synthetic cell', a liposome that is filled with purified proteins that sustain metabolism, a synthetic genome, a divisome, etc. The blue polymer represents a Cdv filament that potentially could serve as a divisome machinery for synthetic cells.

In recent MD simulations (*75*), the CdvB1:CdvB2 copolymers were modelled and tested for different conditions of disassembly. A surprising suggestion from this work was that the Cdv polymers may not, like eukaryotic ESCRT, constrict the membrane with a single helix that spirals all around the membrane. Instead, filaments initially form a ring spanning the full cell circumference, which breaks up into a series of small and highly curved CdvB1:CdvB2 filaments, called hemihelices, that jointly constitute the division ring. These hemihelices effectively reduce the ring circumference, and a controlled unwinding of ring proteins then deforms more and more membrane until a narrow neck is left. At this stage, the hemihelices disappear and the membrane neck may spontaneously break, thus realizing abscission. This model proposes a new mechanism for the Cdv divisome, and it will be interesting to see if future experimental work will be able to verify it.

2.7. Outlook and concluding remarks

The Cdv machinery has proven to be an interesting cell-division system that differs from the more common FtsZ and ESCRT divisomes. Its partial homologies to the eukaryotic ESCRT system facilitate a better understanding of the evolutionary relationship between archaea and eukaryotes. In this review, we summarized the findings for the Cdv system from the Sulfolobales, which is by far the best studied.

Moving forward, a major aim is obtaining a more complete picture of the Cdv functionality across the different species that possess this machinery. The work of Frohn et al. (*43*) is a good example of computational work that portrays possible differences across the different species. However, as they point out, experimental work is needed to confirm their hypothesis, especially establishing new model organisms in the Asgard phylum. A first successful lab culturing of a member of the Asgard archaea *Candidatus Prometheoarchaeum synthrophicum* (*44*) was recently reported. Although its slow growth rate and the lack of genetic tools still pose challenges, this may open up studies of the Cdv system in this phylum of organisms. Understanding the function of the Cdv system in various species will also help to better grasp the evolution of archaea and eukaryotes.

Another open question is to obtain a better understanding of the molecular gymnastics of the various Cdv proteins in cell division and vesicles budding. While a certain sequence of events has emerged (Figure 2.5I-V), many mechanistic questions are still wide open, for example, on the mechanism of divisome positioning, the regulation of the CdvB versus CdvB1:CvdB2 ring formation, the distinct mechanistic roles of CdvB1 and CdvB2, the mechanism of constriction, the final stage of abscission, etc. The recently reported advances in high-temperature live cell imaging (*46*, *76*) open a new window to further cell-biology studies. Beyond that, it will be of interest to image the structural arrangements of the proteins in vitro and to observe the induced effects of the protein interactions with lipid membranes. And once an understanding of the Cdv divisome is obtained, new questions will follow, such as its link to other cellular functionalities, specifically chromosome segregation.

Finally, the Cdv system is of interest in an entirely different context, viz., for the bottom up building of a divisome for synthetic cells (Figure 2.5B). In the past decade, various efforts have been started to assemble in vitro protein modules for the various cellular functionalities, with the aim to eventually reconstitute a cell from components (*77–79*). The goal of such research is to disentangle the complexity of the cell by separately reconstituting the different cellular functionalities and subsequently putting such modules together, to hopefully build a cell from the bottom up. Obviously, such a synthetic cell will also need a division machinery. Efforts so far have largely concentrated on using the bacterial FtsZ (*80*), but success with this approach has been only modest, since, unlike optimistic early ideas (*81*), FtsZ does not appear to be able to apply much of a constriction force on liposomes (*82*). Instead, it appears that FtsZ acts more like a central organizing protein that attracts and coordinates cell-wall-generating proteins that establish the constriction (*83*), which however make the system complex and less attractive as a minimal divisome for synthetic cells. One could envision to instead use the eukaryotic ESCRT system, but its complexity is staggering as well. Here is where the archaeal Cdv system may come in useful, as this is at least somewhat simpler than its eukaryotic counterpart. The Cdv machinery may provide an elegant solution for a synthetic cell-division mechanism, as it does not require as many components as the eukaryotic ESCRT (6 proteins in the Sulfolobales vs ~25 in humans (*1*)).

Indeed, we find it useful to study the Cdv system more closely with this aim in mind. Reconstituting the system *in vitro* not only may take us one step closer to the development of synthetic cells, but will also provide valuable information about what minimal sets of the Cdv proteins will be sufficient for function. The idea to use Cdv as a divisome for synthetic cells was first mentioned a decade ago (*84*), but the understanding of the system was still very basic back then. New developments in recent years, especially the discovery of the Asgard archaea and new insights of the important role of the CdvB paralogs in the Sulfolobales, now provide a much better starting ground to build this minimal divisome, which opens up an exciting path forward.

2.8. Methods

For the phylogenetic tree assembly, the sequences of the CdvB homologs found in the eggNOG (*85*) database were used (entry COG5491), additionally adding sequences of the Thaumarchaean CdvB proteins, the eukaryotic ESCRT-III, and the Asgard CdvB described in (*43*). The tree was built using the MEGA 11: Molecular Evolutionary Genetics Analysis version 11 (*86*). All the protein FASTA sequences were aligned using MUSCLE alignment, and a Neighbor Joining Tree was generated using Bootsrap method (1000 bootstrap replications) and Poisson substitution model.

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3

THE ARCHAEAL DIVISION PROTEIN CDVB1 ASSEMBLES INTO POLYMERS THAT ARE DEPOLYMERIZED BY CDVC

The Cdv proteins constitute the cell-division system of the Crenarchaea, a machinery closely related to the ESCRT system of eukaryotes. Using a combination of TEM imaging and biochemical assays, we here present an in vitro study of M. sedula CdvB1, the Cdv protein that is believed to play a major role in the constricting ring that drives cell division in the Crenarchaea. We show that CdvB1 self-assembles into filaments that are depolymerized by the Vps4-homolog ATPase CdvC. Furthermore, we find that CdvB1 binds to negatively charged lipid membranes and can be detached from the membrane by the action of CdvC. Our findings provide novel insight into one of the main components of the archaeal cell division machinery.

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3.1. Introduction

The Cdv system is the protein machinery responsible for cell division in the archaeal phylum of the Crenarchaeaota (*1*). Many components of this cell division machinery share a high degree of homology with the eukaryotic ESCRT machinery (*1*, *2*) that is responsible for the cell division, vesicle budding, and multiple membrane-deforming processes in humans and yeast (*3*). This has led to the suggestion that the Cdv system is an evolutionary antique and simplified precursor of the eukaryotic ESCRT machinery (*4*), that may share the same mechanism at its core. While the eukaryotic protein complex is well studied, the complications of imaging live thermophilic cells such as the Crenarchaeaota, that live at ~85 °C, has long hindered a similarly fast growth in our understanding of the Cdv system.

Up until recently, most of our knowledge of the Cdv system was limited to CdvA, CdvB (an ESCRT-III homolog) and the AAA ATPase CdvC (Vps4 homolog), which are all found in the same operon (*1*). Basically, the CdvA protein was found to form a ring at the center of the cell together with CdvB (*1*). CdvA binds to the membrane in a spiral-like fashion (*5*) and acts as a membrane anchor for CdvB (*6*), as CdvB cannot bind to the membrane by itself (*7*). Furthermore, CdvC is also found at the ring at the middle of the cell during cytokinesis, (*2*). In eukaryotes, several proteins of the ESCRT-III complex interact with the AAA ATPase Vps4 through a MIM-MIT interaction (*8*), and the role of the ATPase is to detach the ESCRT-III proteins from the membrane and allow for the remodeling of the constricting filaments during the cytokinesis (*9*). Prompted by the homologies between the archaeal and eukaryotic proteins, and the evidence for the presence of CdvC at the division ring during cytokinesis (*2*), it was thus believed that the role of CdvC in archaea is that of Vps4 in eukaryotes, namely providing energy to the system by removing ring proteins from the membrane for its remodeling during the constriction.

For a long time, the role of the CdvB paralogs (CdvB1, CdvB2 and CdvB3) was unclear. It was shown that *Sulfolobus* cells lacking CdvB were unable to grow, while cells lacking the CdvB paralogs were still viable, albeit with a lower growth rate or aberrant daughter cells (*10*), and therefore the CdvB1-3 paralogs were deemed non-essential for cell division. Recently however, developments in high-temperature microscopy techniques (*11*, *12*) together with high-resolution imaging of fixed cells (*13*) shed valuable light onto how the CdvB paralogs participate in the cell division. It was shown that CdvB1 and B2 are recruited to the CdvB ring right before the cytokinesis, whereupon CdvB appeared to detach from the membrane as it was digested by the proteasome, while CdvB1 and CdvB2 carry out the deformation of the membrane needed for the division of the cell (*11*, *13*). Additionally, it was shown that *Sulfolobus* mutants without any CdvB2 undergo asymmetric cell divisions yielding differently sized daughter cells, while cells without CdvB1 occasionally failed to divide, yielding multiploid cells with 2 genomes (*11*). All this changed the view of the basic mechanism of the Cdv system. Now the initial CdvA:CdvB ring at the center of the cell appears to be viewed as a non-contractile assembly ring that recruits CdvB1 and CdvB2 to

the division site. In this picture, the initial ring gets digested by the proteasome and CdvB1 and CdvB2 are left to deform the membrane and perform the division of the cell.

While this renewed model for the Cdv system arises, many questions remain. The constriction of the membrane requires a continuous and controlled disassembly of the contractile ring during the membrane deformation for successful scission and division of the cell (*13*). The division ring starts from a low-curvature conformation at the beginning of the division, and needs to proceed to an invagination of the membrane all the way down to the final step of scission. In this process, molecules that initially form the ring need to be removed to allow the final scission of the membrane to occur and avoid steric hindrance at the neck of the division site. It, however, remains unclear what drives these processes of depolymerization and constriction.

Since CdvB1 contains a MIM domain at the C-terminus, it has been hypothesized that CdvC may interact with CdvB1 through the MIT-MIM interaction the same way that in eukaryotes Vps4 interacts with the ESCRT-III, and CdvC may thus be responsible for the disassembly of the contractile ring (*13*, *14*). In *S. islandicus*, yeast two-hybrid screenings showed interaction between CdvC and the CdvB paralogs (*15*), supporting the idea of its role in the disassembly of the contractile ring. However, there has so far been no experimental evidence that shows that this interaction leads to the depolymerization of structures formed by any of the CdvB paralogs. It is also unclear how the contractile ring stays bound to the cell membrane as the CdvB paralogs lack the wH domain that allows for the interaction with CdvA (*6*). Therefore, CdvB is presumed to act as a link between the membrane anchor CdvA, and CdvB1, which may start the recruitment of the contractile ring, but this raises the question of what links CdvB1 to the membrane after CdvB is gone. It has been proposed that the CdvA:CdvB ring does not fully disappear from the division site, but instead gets largely depolymerized, with a few proteins left behind which may be enough to hold the contractile ring in place (*14*). However, it has also been suggested that, in contrast to CdvB, the CdvB paralogs have a protein patch homologous to the membrane-binding domain of the human ESCRT-III CHMP3, with a certain degree of basicity to it, which could allow them to bind the membrane directly (*4*).

Here, we show how the CdvB paralog CdvB1 is able to polymerize on its own. We observe that heterologously expressed and purified CdvB1 proteins spontaneously self-assemble *in vitro* into filamentous structures. Furthermore, we show that CdvB1 filaments can get depolymerized by the action of CdvC, directly demonstrating both the presence of an interaction between these two proteins and the filament-remodeling activity of CdvC for the first time. Finally, we investigate the lipid-binding properties of CdvB1, and demonstrate that the ATPase activity of CdvC can detach CdvB1 from the lipid membrane.

3.1. Results

3.1.1. CdvB1 self assembles into polymeric filaments

CdvB1 and CdvC proteins from the archaeon *Metallosphaera sedula* (Figure 3.1A) were heterologously expressed in *E. coli* and purified (see Methods). To improve the handling and solubility of the protein, CdvB1 was fused to an MBP tag at the N-terminus of the protein, with an HRV 3C protease cleavage site in between the two. After the purification, the MBP was initially left on the protein, which largely impeded the polymerization of the protein, as can be seen by negative-staining TEM images (Figure 3.1B).

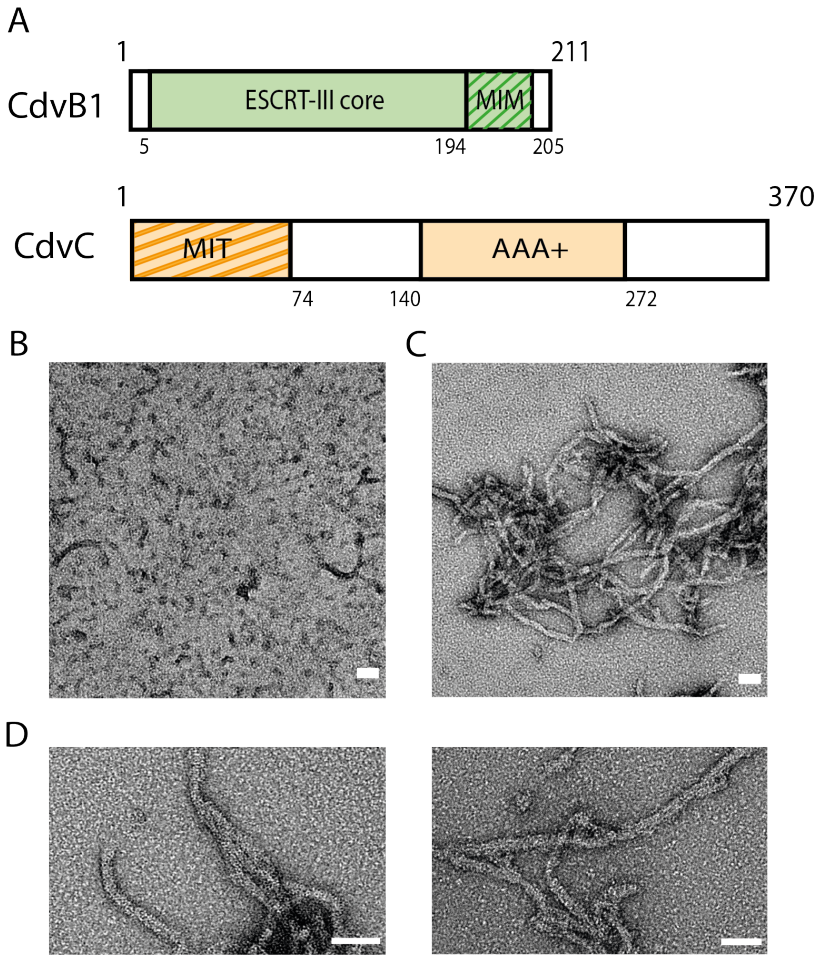


Figure 3.4. CdvB1 can polymerize into filamentous polymers

A. Schematic representation of CdvB1 and CdvC. **B.** Negative staining EM image of MBP-CdvB1 monomers and some short polymers. **C.** Negative staining EM image of the filamentous polymers formed by CdvB1 upon cleavage of the fused MBP. **D.** Closeup image of the CdvB1 polymers. All scale bars 50 nm.

It has been previously reported that CdvB, like many other ESCRT-III proteins, switches between an active and an inactive state when it comes to polymerization (*6*, *16*). More specifically, CdvB contains a self-inhibiting domain that prevents it from polymerizing, while it spontaneously forms filaments when this domain is removed. We observed that this is not the case for CdvB1. Upon cleavage of the MBP by the 3C protease, CdvB1 spontaneously self-assembled into elongated filamentous polymers (Figure 3.1C), without the need of any other protein or the removal of any domain. The filaments have a defined width of about 14 ± 2 nm and a variety of different lengths, presenting an average length of 320 ± 140 nm (Figure 3.1D). The filaments tended to stick to each other and thus form filamentous aggregates, making it difficult to properly measure the length when exceeding the 500 nm.

3.1.2. CdvB1 polymers are disassembled by CdvC

Next, we studied whether these polymers of CdvB1 can get depolymerized by the action of CdvC. It has been hypothesized that CdvB1 can interact with CdvC, as some components of the ESCRT-III complex possess a MIM domain (MIT-interacting domain) at the C-terminus of their sequence, which interacts with the MIT domain of the CdvC/Vps4 ATPase. The sequences of these two interacting domains are highly conserved among species, and the CdvB1 of various different crenarchaea exhibit, at the C-terminus of their sequence, a high degree of homology with the MIM2 domains of human's CHMP6 or the yeast's Snf7 (Figure 3.2A). The MIM2 domain of *Metallosphaera sedula* is practically identical to that of *Sulfolobus islandicus* (Figure 3.2A), organism in which CdvB1 and CdvC have been shown to interact through yeast 2 hybrid assays (*15*). Furthermore, the MIM2 consensus sequence shows that prolines and hydrophobic amino acids are highly conserved at specific locations (*17*), and thus CdvB1 has a high degree of sequence similarity to the human and yeast proteins.

The high temperature where the Crenarchaea live in their natural habitat posed an experimental challenge for testing the CdvB1-CdvC interaction. It had been previously reported that, *in vitro,* CdvC is enzymatically active at temperatures above 60°C (*6*). In our *in vitro* experiments, the CdvB1 polymers were broken down when incubated for prolonged times at temperatures above the 40°C, so we decided to verify whether a compromise between the ATPase activity of CdvC and the thermal stability of the CdvB1 polymers could be found. As shown in Figure 3.2B, CdvC did, as expected, not show any activity at temperatures up to 40°C. However, the protein did show a significant activity already at 50°C. At that temperature, together with the addition of Ficoll crowder, the CdvB1 polymers remained stable (Supplementary Figure 1), and hence, we chose this as our working temperature in the experiments.

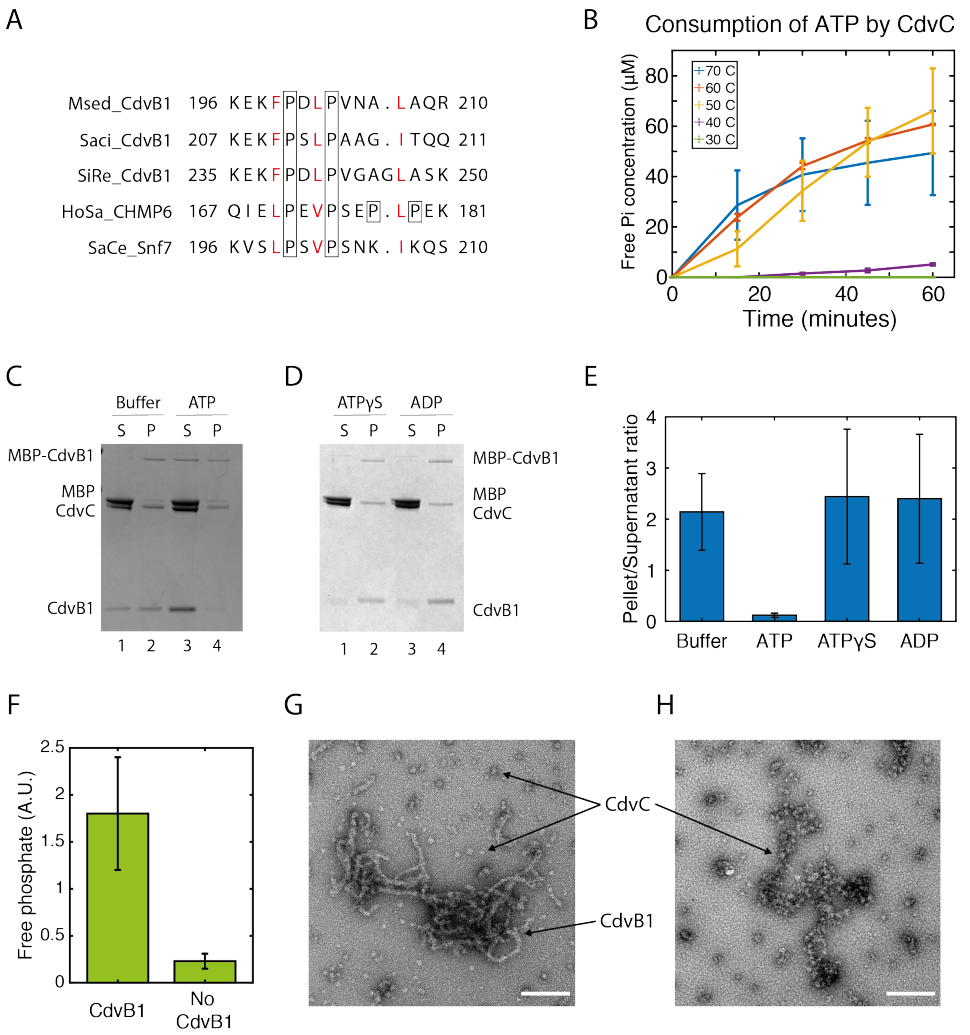


Figure 3.5 Filaments of CdvB1 are depolymerized by CdvB**.**

A. Sequence alignment of regions of CdvB1 from *Metallosphaera sedula* (Msed2179), *Sulfolobus islandicus* (SiRe1200), and *Sulfolobus acidocaldarius* (Saci_0451), with the MIM2 domain of human ESCRT-III protein CHMP6 and yeast *Saccharomyces cerevisiae* Snf7. Conserved hydrophobic residues (red) and the prolines (boxed) are highlighted. **B.** ATP consumption by CdvC at different temperatures. **C.** SDS page analysis of a sedimentation assay where the depolymerization of CdvB1 filaments is assessed. **D.** SDS page analysis of a sedimentation assay where ATPγS and ADP were added to the sample instead of ATP. No depolymerization of CdvB1 is observed. **E.** Quantitative analysis of the Pellet-to-Supernatant ratios shown in the sedimentation assays (n=3). **F.** Comparison of the ATP consumed by CdvC in 25 minutes at 50°C in the presence or absence of CdvB1 **G.** Filaments of CdvB1 with CdvC after incubation at 50°C but without ATP. **H.** EM image showing the disappearance of the CdvB1 filaments when mixed with CdvC, ATP and incubating at 50°C, leaving only aggregates of CdvC behind. All scale bars 50 nm.

To investigate if this interaction causes CdvC to disassemble CdvB1 filaments, we first formed CdvB1 polymers by cleaving off the MBP tag and allow them to polymerize. CdvC was then added to the samples, together with either ATP, ADP, non-hydrolysable ATP (ATPγS), or buffer without nucleotides (Buffer). These samples were incubated for 2 minutes at 50°C, to allow for the ATPase activity of CdvC, samples were centrifuged down at high speed, and pellet and supernatant were separately run on a gel. As can be seen from Figure 3.2C, polymerized CdvB1 was forming pellets at the bottom during the centrifugation, while monomeric CdvB1 remained in the supernatant (Figure 3.2C, lines 1 and 2). However, when adding ATP and therefore allowing CdvC to act, the pelleted fraction virtually disappeared, and most of the protein was found to be in the monomeric state (Figure 3.2C, lines 3 and 4). The CdvB1 pellet was also still present when, instead of ATP, non-hydrolysable nucleotides were provided to the reaction (Figure 3.2D). The filament-to-monomer ratio of ~2 was the same for the samples where no hydrolysable nucleotides were added (ADP or ATPγS), whereas that ratio was drastically lowered to a value of 0.1 when CdvC could consume ATP (Figure 3.2E). Several independent experiments were performed to corroborate this (Supplementary Figure 3.1). The depolymerization of CdvB1 did not occur when there was no CdvC present in the reaction (Supplementary Figure 3.2), indicating that the heating step did not disrupt the CdvB1 polymers, and it was indeed caused by CdvC.

To see if the depolymerization of the CdvB1 filaments is indeed the result of a specific interaction between CdvC and the CdvB1 filaments, we checked the level of ATPase activity of CdvC in the presence or absence of CdvB1 filaments. We observed a very clear increase in the ATPase activity in the presence of CdvB1, showing a consumption of ATP that was 8 times larger than for CdvC alone (Figure 3.2F). This suggests that the MIM domain of CdvB1 acts as a substrate for CdvC, which can bind it and perform its activity of depolymerizing the CdvB1 polymers, which consumes additional ATP.

We visualized the depolymerized filaments through negative staining EM. CdvB1 filaments were formed, CdvC was added and incubated at 50°C, as described above, and samples were imaged. When no hydrolysable nucleotide was added, CdvB1 filaments were observed in the sample, together with CdvC oligomers around them (Figure 3.2G). However, in samples where ATP had been added, the filaments of CdvB1 had vanished and only CdvC aggregates were observed (Figure 3.2H). Together with the evidences observed in the previous sedimentation assays, we thus conclude that the action of CdvC was responsible for the depolymerization of the CdvB1 filaments.

3.1.3. CdvB1 binds negatively charged lipid membranes and can be detached by CdvC

Since the Cdv proteins are involved in remodeling membranes, it is of interest to study their membrane-binding properties. First, we studied if CdvB1 was able to directly bind lipid membranes. We used a liposome flotation assay, which, through a gradient of different

concentrations of sucrose, allows distinguishing between the membrane-bound protein (that colocalizes with the liposomes), CdvB1 monomers (that stay in solution) and CdvB1 filaments (that precipitate to the bottom) (Figure 3.3A). We mixed MBP-CdvB1-Alexa488 with liposomes in a solution that contained the 3C protease. After incubation, we deposited the sample at the bottom of a 3-step sucrose gradient that we centrifuged at high speed. This resulted in 3 different fractions (Figure 3.3A): a top one where the liposomes were found (1), a middle one with monomeric protein (2) and a bottom one containing the filamentous CdvB1 (3). We tested liposomes made of DOPC + 0.1% Rhodamine-PE and a mixture of 70% DOPC + 30% DOPG + 0.1% Rhodamine-PE (percentages denote molar fractions) to examine the effect of the negative charges of the DOPG against the neutrality of DOPC. All the fractions of the gradient were analyzed by SDS PAGE where we imaged the fluorescence of both the lipids (red) and the CdvB1-Alexa488 (green). What we observed was that, as hypothesized, CdvB1 never bound to liposomes that were exclusively made of DOPC. However, when DOPG was present in the mixture, the CdvB1 protein showed clear binding to the liposomes (Figure 3.3B). This shows that it is not only CdvA that can bind lipid membranes, but that other components of the Cdv system can do so as well, similar to the way that different proteins of the ESCRT machinery in eukaryotes present different membrane-binding capabilities (*18*).

In view of this, and the depolymerization of CdvB1 by CdvC that was described above, we tested whether CdvC was able to detach CdvB1 from the lipid membrane. For this we used the same liposome flotation assay where we bound the protein to negatively charged liposomes, subsequently added CdvC to the sample, incubated with ATP and Mg^{2+} for 10 minutes at 50°C, and then deposited the sample in the 3-step sucrose gradient. As can be seen in Figure 3.3C, we observed that the CdvB1 protein remained bound to the membrane in samples with no ATP. By contrast, samples containing ATP showed a big portion of the protein that disassembled from the liposomes to go into the soluble fraction (Figure 3.3C). On average, about half of the protein that was bound to the membrane depolymerized in solution in our experimental conditions (Figure 3.3D). When no CdvC was added to the reaction, this detachment was not observed (Supplementary Figure 3.3).

This lipid-binding behavior was additionally tested for conditions where first CdvB1 filaments were allowed to form in the absence of liposomes, gently centrifuged to separate them from leftover monomers, then mixed with the liposomes, left to incubate for 1 hour at room temperature, and finally deposited and centrifuged in the 3-step sucrose gradient. Interestingly, we observed that, for these conditions, all of the CdvB1 was found in the bottom layer of the sucrose gradient, corresponding to the filamentous state, while no CdvB1 was found in the liposome fraction (Figure 3.3E). This shows that CdvB1 filaments, once formed, did not bind the membrane.

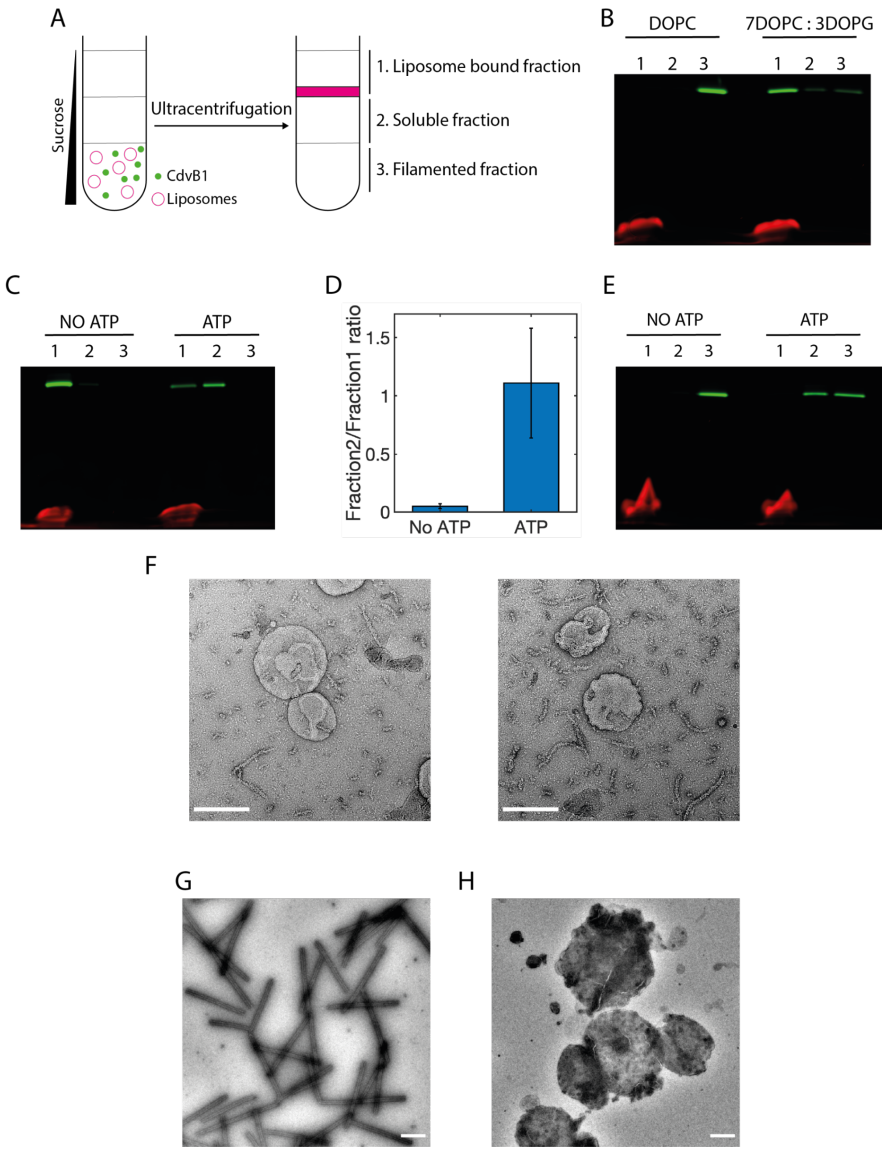


Figure 3.6. CdvB1 monomers bind to negatively charged lipid membranes but CdvB1 polymers do not.

A. Schematic of the liposome flotation assay. **B.** SDS page gel of the liposome flotation assay showing CdvB1-Alexa488 in the liposome fraction only when the assay is performed in the presence of negatively charged lipids (30% DOPG). **C.** Liposome flotation showing that CdvB1 protein bound to liposomes is detached from the lipid membrane by CdvC. **D.** Quantitative analysis of the protein detachment from the membrane, showing the soluble-to-lipid-bound ratio (n=3). **E.** Liposome flotation assay showing that CdvB1 protein filaments do not bind to liposomes. The monomers resulting from their depolymerization do not bind either. **F.** Negative-staining EM image showing that the CdvB1 protein filaments do not interact with the membrane of the liposomes. Scale bars 200 nm. **G.** Negative-staining EM image of human ESCRT III proteins CHMP2A and CHMP3 co-polymerizing into tube-like structures. Scale bar 500 nm. **H.** Negative-staining EM image showing that CHMP3 does not form tube-like polymers when incubated with liposomes. Scale bar 500 nm.

We then tried to see if depolymerization of the CdvB1 filaments with CdvC, would allow the newly solubilized CdvB1 to bind the lipids. For this, we formed CdvB1 filaments in the absence of lipids, mixed them with 7:3 DOPC:DOPG liposomes, CdvC, and ATP with Mg^{2+}, and incubated for 10 minutes at 50°C. After the depolymerization reaction, we left the proteins with the liposomes rest at room temperature to interact for 1 hour to allow for enough time for binding, and then performed the 3-step sucrose gradient. We observed that neither the filaments nor the depolymerized CdvB1 showed binding to the liposomes (Figure 3.3E). This may suggest that CdvC is actually unfolding the monomers of CdvB1 in the process of depolymerizing the filaments, and that the resulting depolymerized proteins lack a functional folded structure. Negative-staining TEM images of MBP-CdvB1 incubated with vesicles that contained the 3C protease in solution showed filaments of CdvB1 that were lying next to the vesicles (Figure 3.3F). Hardly ever were these filaments found on top of the vesicles or attached to them, consistent with the results from the liposome flotation assay.

Given the similarities of the archaeal Cdv proteins and the eukaryotic ESCRT, we wanted to see if this duality between polymerization and membrane binding was present in both systems. Interestingly, a similar behavior was indeed observed for the CHMP2A and CHMP3, which are human homologs of CdvB1 (*4*). These proteins from the human ESCRT-III machinery are well known for their *in vitro* co-polymerization into large helical structures that can be disassembled by the ATPase Vps4 (*19*, *20*). These tube-like structures easily form when mixing MBP-CHMP2AΔC and CHMP3 at a molar ratio of 10:1 (Figure 3.3G). However, when trying to polymerize these tubes in the presence of liposomes (9:1 DOPC:PIP2), we found no tubular polymerization, as seen in Figure 3.3H. Instead, we observed that the protein remained bound to the surface of the liposomes, but it would never polymerize into helical tubes and bind to liposomes at the same time.

3.2. Discussion

In this paper, we clarified a number of characteristics of the important but so far understudied Cdv protein CdvB1. We found that, *in vitro*, CdvB1 self assembles into filaments without the need of removing any inhibiting domain, like in many of its ESCRT-III homologs. Fusion to an MBP impeded activation and filament formation, which is a convenient way of controlling the polymerization when needed, facilitating its study in *in vitro* assays. We also showed how CdvB1 polymers are disassembled by CdvC, showing for the first time a direct proof of depolymerization of an archaeal ESCRT-III polymer by the action of the AAA ATPase CdvC. As ATPγS and ADP did not promote filament depolymerization, it appears that hydrolysis of ATP is needed to perform the task. Interestingly, hydrolysis of ATP by CdvC greatly increased in presence of CdvB1, suggesting that there is a specific MIM-MIT interaction between the 2 proteins, which stimulates the activity of CdvC, much in the same way that the MIM domain proteins stimulate Vps4 in eukaryotes (*21*). These findings strengthen the idea that the Cdv system can be considered as a relatively simpler version of

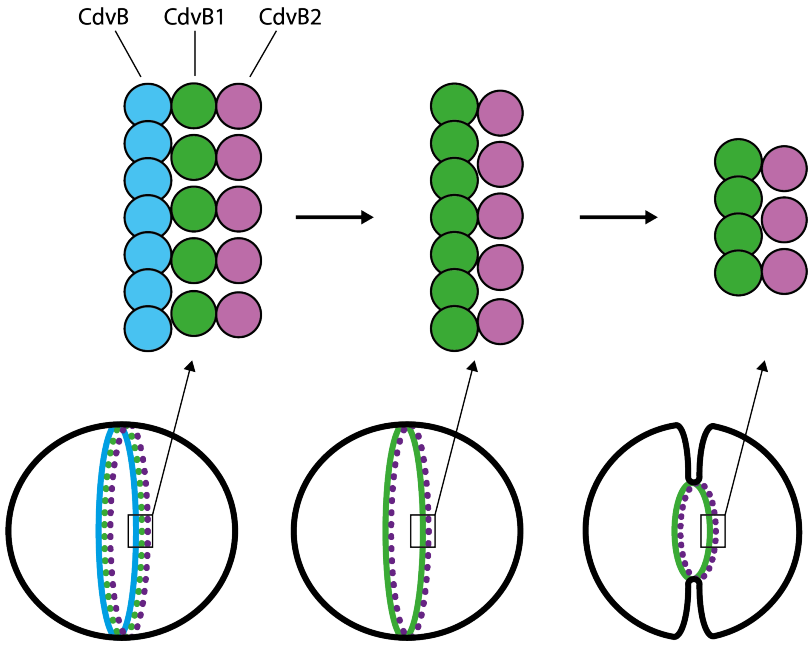


Figure 3.4. Proposed filament structure in crenarchaeal cell division.

First CdvB forms a non-contractile ring, whereupon it recruits monomeric CdvB1 and CdvB2. Upon removal of CdvB, CdvB1 can polymerize while remaining in the right place thanks to the action of CdvB2, and jointly they constrict the membrane

the homologous eukaryotic ESCRT machinery, thus reinforcing evidence for a mechanistic common ground between the archaeal and eukaryotic cell division systems.

Our data show that CdvB1 has the ability to directly bind membranes with negatively charged lipids without the need of any anchoring proteins (such as CdvA), which contrasts previous findings for CdvB. Furthermore, CdvC was found to remove the protein from the membrane. This may explain how CdvB1 can stay attached to the membrane during the cell division after the removal of CdvB. This is consistent with the view that the initial CdvA:CdvB ring merely serves as a scaffold for the recruitment of CdvB1 to the division site, whereupon the contractile ring can stay bound to the membrane by its own interaction with the lipids after the initial ring is removed. It was reported that the initial ring gets digested by the proteasome before the constriction of the cell (*13*), leaving only a contractile ring of CdvB1 and CdvB2 to perform the division. Our data suggest that CdvC is responsible for inducing the depolymerization of the contractile ring that is needed for the division of the cell to occur (*13*, *14*).

We also reported an interesting duality for CdvB1, as the polymerization of the protein and its membrane-binding capabilities were found to be mutually exclusive phenomena. After cleavage of the MBP tag, i.e., at the point where the protein is in its fully native conformation, one of two things can happen: either a CdvB1 protein finds the lipid membrane and binds to it, or it binds other CdvB1 proteins to polymerize into filaments that subsequently are not able to bind the lipid membrane. Interestingly, we showed a very similar behavior for the human ESCRT-III CHMP2A and CHMP3, that co-polymerize into large helical structures in the absence of lipids, but fail to do so when surrounded by liposomes. These data might be taken to suggest that upon polymerization, the membrane-binding patch of the proteins does not face the outside anymore, and thus loses its lipid-binding ability. However, this seems counter-intuitive regarding the function of the ESCRT-III proteins that form filamentous polymers that can be reshaped to deform the membrane. We suggest that instead, *in vivo*, CdvB1 is recruited in a monomeric state to the lipid membrane at the division site by CdvB, whereupon it may form filaments (see Figure 3.4). This would allow CdvB1 to bind to the membrane by its membrane-binding domain, where it may also recruit CdvB2 to the division site. There is a very high degree of conservation of positively charged amino acids between CdvB1 and CdvB2, which leads us to think that CdvB2 will likely bind to negatively charged lipid membranes as well. Therefore, we speculate that upon removal of CdvB from the membrane, with CdvB1 and CdvB2 left to constrict the membrane, CdvB1 may polymerize into filaments that will be stabilized and kept bound to the membrane by CdvB2. The action of CdvC may then remodel the CdvB1 filaments whereupon the membrane shrinks, while CdvB2 keeps the ring in place. This idea is in line with previous findings (*11*) where mutants lacking CdvB1 were less able to perform the fission of the membrane, indicating its role in membrane deformation, whereas mutants without CdvB2 would lead to asymmetric divisions, suggesting its guiding role.

An evolutionary correlation of these phenomena seems to be implied in the clear *in vitro* polymerization versus membrane binding that we observed in both the human and archaeal ESCRT-III proteins. Previous studies showed the importance of conformational changes of ESCRT proteins bound to a lipid membrane, and how the different protein states, either bound or unbound, facilitated different interactions (*22*). A similar scenario likely applies to the Cdv proteins where, depending on the interaction with the membrane, different polymerization states may occur. Future high-resolution studies of the proteins *in vivo* will help to better understand how the proteins arrange themselves at the division site to facilitate faithful cell division in the Crenarchaeaota.

3.3. Materials and Methods

3.3.1. Plasmids

The gene for CdvB1 from *M. sedula* (Msed_2179, UniProtID A4YIR6) was obtained from the Gen Bank data base, and was reverse translated using the EMBOSS Backtranseq tool, optimized for *E. coli* codon usage. To the resulting DNA sequence, a codon of a cysteine for fluorescent labelling was added at the N terminal of the protein, as well as Tobacco Etch Virus (TEV) and an HRV 3C proteases cutting sites (see Supplementary Table1 for full sequences). The whole gene construct was ordered as a synthetic gene already inserted in a pMAL-c5x vector from Biomatik, using BamHI and EcoRI cutting sites. The plasmid for CdvC from *M. sedula* (Msed_1672, UniProtID A4YHC5) was kindly provided by Patricia Renesto's lab.

3.3.2. Protein purification

MBP-CdvB1 was produced in a BL-21 *E. coli* strain. Cells were grown at 37°C in LB^{amp} medium to an OD of around 0.5. Expression was induced with a final concentration of 0.1 mM of IPTG for 4 hours. Cells were then harvested by centrifugation at 4500x g at 4°C for 12 minutes. The pellet was resuspended again in lysis buffer (50 mM Tris pH8.8, 50 mM NaCl, 50 μM TCEP, cOmplete™ Protease Inhibitor Cocktail (Roche)). Cells where lysed by French press, and the lysate was then centrifuged for 30 minutes at 45000 rpm in a Ti45 rotor (Beckman Coulter). Supernatant was then incubated rotating with 1ml of amylose resin (NEB) at 4°C for 2 hours. In a 4°C room, the lysate was then poured through a gravity chromatography column, then washed twice with 1 column volume of purification buffer (50mM Tris pH 8.8, 50mM NaCl, 50μM TCEP). The washed resin was incubated for 5 minutes with elution buffer (50mM Tris pH 8.8, 50mM NaCl, 50μM TCEP, 10mM maltose), and finally eluted the protein out using the same column. The protein was then concentrated down to a volume of 0.5 ml, and run through a Superdex™ 75 increase 10/300 GL size exclusion chromatography column mounted in an ÄKTA™ Pure system. Sample ran with purification buffer, and purity of the eluted peaks was evaluated by a 12% SDS PAGE gel stained with Coomassie blue. The resulting MBP-CdvB1 was snap frozen in liquid nitrogen and stored at -80 °C.

A fraction of the protein was separated after the amylose resin column elution, and dialyzed into the same purification buffer but at a pH of 7.4. A maleimide-cysteine conjugation reaction was then performed with Alexa488-maleimide to link it to the cysteine added to CdvB1, thus obtaining fluorescently labelled CdvB1. The rest of the purification stayed the same, and excess label was removed from the protein through the gel filtration column. The resulting MBP-CdvB1-Alexa488 was snap frozen in liquid nitrogen and stored at -80 °C. Whenever needed for an experiment, samples were thawed at room temperature and the MBP tag was cleaved off with a 3C protease right before use.

CdvC was produced in a C41(DE3) *E. coli* strain. Cells were grown at 37°C in LB^{amp} medium to an OD of around 0.5. Expression was induced with a final concentration of 0.1 mM of IPTG for 4 hours. Cells were then harvested by centrifugation at 4500x g at 4°C for 12 minutes. The pellet was resuspended in lysis buffer (50 mM Tris pH8.8, 50 mM NaCl, 1% CHAPS, 5 mM TCEP, cOmplete™ Protease Inhibitor Cocktail (Roche)). Additional Lysozyme at 1mg/ml was added to the resuspended cell pellet and left incubating for 1 hour at 30°C shaking. Then cells were lysed by sonication, and the lysate was centrifuged for 30 minutes at 45,000 rpm in a Ti45 rotor (Beckman Coulter). Supernatant was loaded into a HisTrap™ HP Ni^{+2}-NTA mounted in an ÄKTA™ Pure system column for affinity purification with the His-Tag on the CdvC. The eluted protein was then further concentrated and ran through a HiPrep Sephacryl S-300 HR size exclusion chromatography column, using a buffer of 50 mM Tris pH8.8, 50mM NaCl, 5mM TCEP. Purity of the eluted peaks was evaluated by SDS PAGE stained with Coomassie blue. The resulting CdvC was snap frozen in liquid nitrogen and stored at -80 °C.

3.3.3. TEM imaging

MBP-CdvB1 at a final concentration of 1 μM was mixed with 0.1 μM of the 3C protease in buffer containing 50mM Tris pH 7.4, 50mM NaCl and, to allow filaments for form for at least 1 hour. The measurements of width and length of the filaments were extracted from 3 independent experiments. For samples with liposomes, lipids used were DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), DOPG (1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)), PIP2 (1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-4',5'-bisphosphate)) and Rhodamine-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)) and they were all purchased from Avanti Polar Lipids. LUVs of 400nm in diameter of 7:3 DOPC:DOPG were prepared by extruding the lipid mixture at a concentration of 5 mg/ml through a polycarbonate filter. LUVs were then diluted down to 0,5 mg/ml, mixed with 0.1μM of 3C protease first, and then MBP-CdvB1 was added to a final concentration of 1 μM. It was all left to incubate at room temperature for at least 1 hour. Samples were absorbed on glow-discharged carbon-coated 400-meshh copper grid purchased from Quantifoil and stained with 2% uranyl acetate. They were then imaged on a JEOL JEM-1400plus TEM at 120kV of accelerating voltage with a TVIPS f416 camera.

MBP-CHMP2AΔC and CHMP3 were purified as previously described (16). MBP-CHMP2AΔC at 10uM and CHMP3 at 1uM were mixed in 50mM Tris pH 7.4, 150mM NaCl, 1mM TCEP in the presence or absence of liposomes and incubated overnight. Liposomes were made with a mixture 9:1 of DOPC:PIP2. Samples were deposited on carbon-coated grids and stained with 2% uranyl acetate. Images were obtained on a Tecnai 12 microscope at 120kV of accelerating voltage with a Gatan Orius SC1000 camera.

3.3.4. ATPase activity assay

The ATPase activity assay was done using the Phosphate Assay Kit – PiColorLock™ from Abcam and performed according to the manufacturer's guidelines. A final concentration of CdvC of 0.1 μM in buffer 50mM HEPES pH 7.5, 50mM NaCl and 5mM $MgCl_2$ was tested with a concentration of 0.1 mM ATP at different temperatures (Room temperature, 30, 40, 50, 60, 70°C). The reaction was stopped at various time points (15, 30, 45 and 60 minutes) by submerging the samples in liquid nitrogen. Afterwards they were thawed, added the reagent of the kit and measured the absorbance at 630nm in a 96 well plate reader. A free phosphate standard curve was plotted to calculate the amount of phosphate released by the protease during the reaction. All experimental conditions were performed in triplicates and results were normalized to buffer with ATP under the same conditions without the ATPase.

For the comparison of CdvC ATPase activity with or without CdvB1, we mixed CdvB1 filaments at a final concentration of 1 μM with CdvC at a final concentration of 0.1 μM, in a buffer containing 50mM HEPES pH 7.5, 50mM NaCl, 2mM MgCl2 and Ficoll 41.25 mg/ml. A sample was also similarly prepared without CdvB1. Right before incubation, ATP at a final concentration of 0.1 mM was added. The samples were then incubated at 50°C for 25 minutes, and the reaction was quenched by putting it on ice. ATP consumption was measured using the same reaction kit and protocol as mention above. All experimental conditions were performed in triplicates and results were normalized to buffer with ATP under the same conditions without the ATPase.

3.3.5. Sedimentation analysis of filament depolymerization

For the filament formation, 1μM of MBP-CdvB1 was mixed with 0.1 μM of 3C protease in buffer 50mM HEPES pH 7.4, 50mM NaCl, 5mM $MgCl_2$, Ficoll 41.25 mg/ml. Higher concentrations of Ficoll slightly diminished the activity of CdvC (Supplementary Figure 3.4) so a final concentration of 41.25 mg/ml was chosen, as this provided enough thermal stability but did not greatly reduce the CdvC ATPase activity. Incubated overnight at 4°C to guarantee full formation of the CdvB1 filaments. The next day, mix in 0.6 μM of CdvC and incubate for 30 minutes. For the depolymerization of the filaments, ATP was added to a final concentration of 1mM and then incubated at 50°C for 2 minutes in a thermocycler. The same reactions with either ATPγS or ADP instead of ATP were performed in parallel as control experiments. After incubation, the sample was transferred to an ultracentrifuge tube and spun down in a Ti 42.2 rotor at 140,000 xg for 30 minutes at 4°C. After centrifugation, the supernatant was collected, the pellet was resuspended in the same volume, and they were analysed by SDS-PAGE stained with Coomassie.

3.3.6. Liposome flotation assay for membrane binding

Protocol adapted from (*23*). Lipids were mixed to final ratios (mol:mol) of 99.9 DOPC : 0.1Rhodamine-PE or 69.9 DOPC : 30DOPC : 0.1Rhodamine-PE and evaporated in a glass

vial to a final amount of 500 µg. They were later resuspended in 100 µl of buffer containing 50mM HEPES pH 7.5, 50 mM NaCl and 300 mM sucrose. The lipid film was hydrated for 1 hour and thoroughly vortexed to form small lipid vesicles. In an ultracentrifuge tube, 300 µg of the lipid vesicles were mixed with 3C protease and MBP-CdvB1-Alexa488, to a final concentration of 1,5 µM. Lipids and protein were left to incubate for 45 minutes, and then buffer with sucrose was mixed to obtain a bottom layer of 80 µl of 30% sucrose solution. Carefully, on top of it, a layer of the same volume of buffer with 25% sucrose was deposited, and another layer with 0% sucrose buffer on top of all. Then it was centrifuged at 200,000 xg at 21 °C for 30 minutes in a Ti 42.2 rotor. Finally, the lipid and middle layers (fractions 1 and 2) were pipetted out, the remaining buffer was removed and the pellet at the bottom of the tube was resuspended in fresh buffer (fraction 3). All different fractions were analysed by SDS-PAGE. The acrylamide gel was imaged with a GE Amersham™ Typhoon gel imager to observe the fluorescence of protein and lipids.

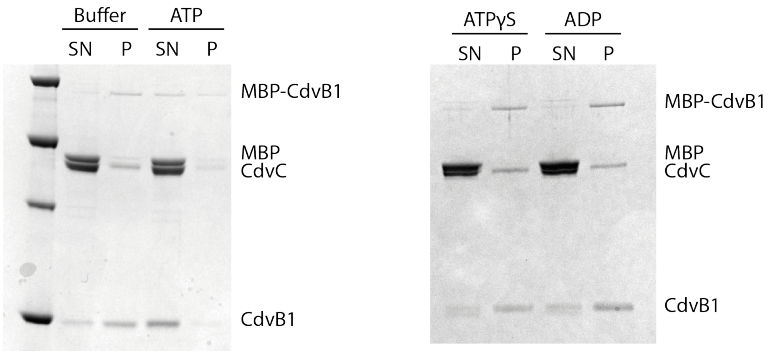
3.3.7. Detachment of CdvB1 from the membrane by CdvC

Binding of CdvB1 to the membrane was performed as described for the "Liposome Flotation Assay" but in a PCR tube instead of the ultracentrifuge tube. CdvC was added to the CdvB1 at a final concentration of 0.8µM, and the result was added to either ATP at a final concentration of 1mM, or simply to the storage buffer (50mM HEPES pH 7.5, 50mM NaCl, and 20mM $MgCl_2$). Samples were incubated for 10 minutes at 50°C in a thermocycler. After incubation, samples were moved to ultracentrifuge tubes and the sucrose gradient and subsequent centrifugation were performed as described in the liposome flotation assay for membrane binding.

3.3.8. Assessment of the binding of pre-formed CdvB1 polymers

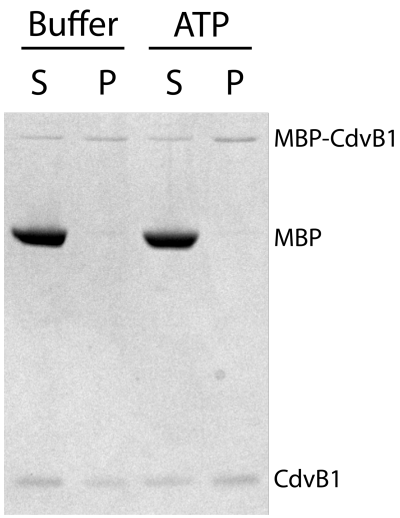
Polymers of CdvB1 were formed by cleavage of the MBP in absence of any lipids. MBP-CdvB1 at 14 µM was mixed with 1µM of 3C protease, and left incubating at RT. After 1 hour, the filaments were spun down at 70,000xg for 20 minutes to separate the filaments from remaining monomers. Then, the protein was mixed with the same amount of liposomes and CdvC as for the previous CdvB1 depolymerization experiments, and the depolymerization reaction was carried out by adding ATP at 50°C for 10 minutes. After that, the sample was left at room temperature to allow any binding of the proteins for 45 minutes, and then the sucrose gradient and subsequent centrifugation were performed as previously described. Samples were then analyzed by SDS page the same way as for the previous experiment of membrane detachment.

Appendix Chapter 3: Supplementary Information



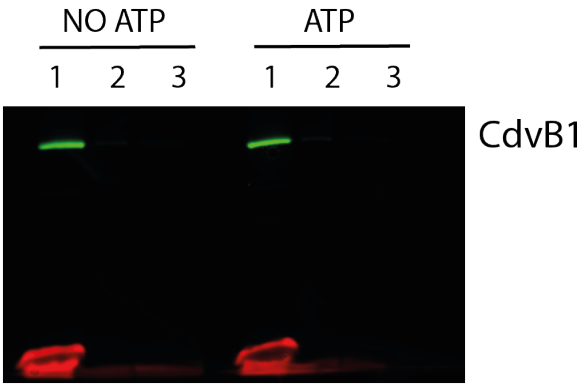
Supplementary Figure 3.1.

Examples of other independent experiments of depolymerization of CdvB1 by CdvC that were analysed in Fig.3.2 CD.



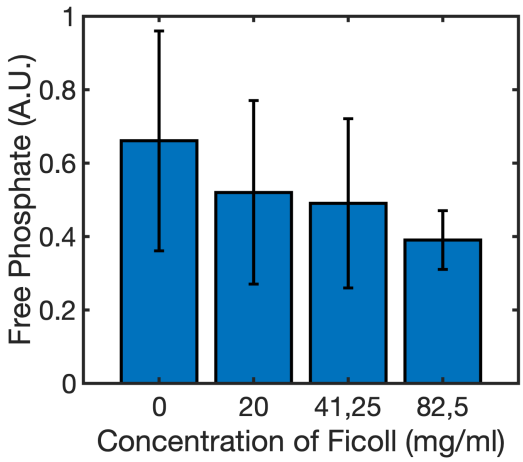
Supplementary Figure 3.2

Pelleting assay performed to samples containing only CdvB1 incubated at 50 °C, where no depolymerization of CdvB1 filaments was visible. The addition of ATP to the CdvB1 samples increased the aggregating trend of filaments, which can be seen in the more intense pellet when ATP was added with respect to the CdvB1 only sample.



Supplementary Figure 3.3

Membrane depolymerization control without any CdvC, where no depolymerization is visible in any case after the incubation at 50 °C with and without ATP. Fraction 1 shows liposome bound proteins, fraction 2 shows proteins in solution and 3 filamented proteins.



Supplementary Figure 3.4

Consumption of ATP by CdvC after 25 minutes at 50°C with different Ficoll concentrations. A lower activity of the protein is observed at higher Ficoll concentrations.

Supplementary Table 1

Insert into the plasmid

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GGAACAGCGCATGAAAGAAAAATTTCCGGATCTGCCGGTGAACGCGCTGGCGCAGCGCGCGTGAGAATTC

Yellow: TEV cutting site
Green: HRV 3C cutting site
Cyan: CdvB1

pMAL-c5x containing CdvB1 from *M.sedula*, HRC and TEV cutting sites between MBP and CdvB1 and additional cysteine at the N-terminus of CdvB1

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4

CDVA-CDVB PROTEIN INTERACTIONS DRIVE THE ASSEMBLY OF THE CDV DIVISION RING IN ARCHAEA

Cell division in the crenarchaea is accomplished by the Cdv system. In Sulfolobus, it was observed that an initial non-contractile ring of CdvA and CdvB forms at the center of the cell, which is followed by a second ring of CdvB1 and CdvB2 that appear to drive the constriction of the cell. Here, we explore in vitro how protein interactions among these Cdv proteins govern their recruitment to the membrane. We show that CdvA does not bind the membrane unless together with CdvB. We find that CdvB2 can polymerize if its self-inhibitory domain is removed, whereas interaction with CdvB1 blocks its ability to bind the lipid membrane. However, CdvB1:CdvB2 co-polymers can be recruited to the membrane by CdvA:CdvB. By visualizing proteins in dumbbell-shaped liposomes, we show that the Cdv proteins have a strong preference to localize at membrane necks of high curvature. Our findings clarify many of the mutual protein interactions of the Cdv system and their interaction with the membrane, and thus help build a mechanistic understanding of cell division in archaeal cells.

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4.1. Introduction

Cell division in the archaeal phylum of the crenarchaea is performed by the Cdv system (*1*, *2*). While this protein machinery is unique to archaea, some of its components are homologous to the ESCRT-III proteins that are responsible for the cell division, vesicle budding, and many other reverse-topology membrane-scission processes in eukaryotes (*3*, *4*). This homology is one of the many similarities that eukaryotes share with archaea, which reinforces the widespread idea that these two kingdoms of life share an evolutionary root (*5*).

Since the Cdv system was first described 15 years ago in *Sulfolobus sulfataricus*, it has been shown to be present in many other species of the TACK superphylum of archaea (*6*). It is composed of CdvA, the CdvB paralogs (homologous to ESCRT-III in eukaryotes), and CdvC (homologous to the eukaryotic Vps4) (*2*). The first cell imaging of the Cdv system showed a band of CdvA and CdvB forming at the center of the cell between 2 segregated nucleoids during division, which over time colocalized with a band of CdvC in the same position (*1*). All these 3 proteins are located in the same operon (*1*), while paralogs of CdvB (namely CdvB1, CdvB2 and CdvB3) are located in other parts of the genome. Recently, it was reported that these paralogs also play a crucial role in cell division (*7*). A recent model for cell division in crenarchaea is that CdvA and CdvB initially form a ring at the center of the cell, where it subsequently recruits CdvB1 and CdvB2 (*7*). At this point, CdvB is digested by the proteasome and the initial CdvA:CdvB ring gets removed from the membrane, while CdvB1 and CdvB2 are left to perform the constriction of the membrane, presumably through the interaction of CdvB1 directly with the membrane (*8*), until the final step of scission (*7*). CdvC is an ATPase that has been suggested to remove monomers of CdvB1 and CdvB2 from the ring, generating a turnover that ensures cellular constriction while avoiding steric hindrance at the final neck (*7*, *9*). This model of action of the Cdv proteins, is supported by some experimental findings on live cells. When generating mutant cells of *S. sulfataricus* lacking CdvB1, these presented a normal constriction of the membrane, but some cells failed to perform the last step of scission, leaving them with two full copies of the genome (*10*). Furthermore, cells lacking CdvB2 were able to perform the scission normally, but tended to present a misplacement of the constricting ring, resulting in aberrant daughter cells that were not equally sized (*10*). This indicates that the two paralogs responsible for the constriction actually perform different roles during the division process. However, it also shows how any of the two proteins, although with difficulties, can still perform the full process of constriction and scission on their own.

While a global picture has thus been emerging, many of the underlying mechanistic interactions remain unclear. The various Cdv proteins play distinct roles. CdvA has an E3B (ESCRT-III binding region; see Figure 4.1A) through which it is capable of interacting with the wH (winged helix) region of CdvB (*11*). CdvA can only interact with CdvB, as none of the other paralogs present such a wH domain (*12*), while additionally, CdvA can bind to lipid membranes (*13*), which CdvB is not able to do. Therefore, CdvA is seen as the membrane

recruiter of CdvB to the membrane. In turn, CdvB is known to interact with CdvB1, which then interacts with CdvB2 (*2*), suggesting that CdvB is the recruiter of the CdvB1:CdvB2 constricting ring. Finally, during the constriction of the membrane, the AAA ATPase CdvC is presumed to disassemble the filaments of the CdvB1:CdvB2 polymer to generate a turnover of protein and thus supply energy to the system to deform the membrane (*9*). This ATPase features a major structural similarity to the eukaryotic Vps4 (*14*), which is known to be responsible for the depolymerization of ESCRT-III filaments (*15*) and to create a turnover of ESCRT-III components at the membrane (*16*, *17*). In archaea, this similarity is further strengthened by *in vitro* experiments where CdvC was able to depolymerize CdvB1 filaments and detach CdvB1 from lipid membranes into solution (*8*).

Little is yet known about how the Cdv proteins are hierarchically recruited to the membrane, and how their mutual interactions affect this process. Especially, the knowledge about the recruitment and arrangement of the constricting ring has been limited. Here, we explore these questions. We find that CdvA can bind to lipid membranes only when it is interacting with CdvB, but not on its own; we observe that CdvB2 prevents CdvB1 from binding the lipid membranes, but jointly, they can be recruited to the membrane by CdvB; and finally, we show that Cdv proteins exhibit a preferential binding for highly curved membranes and thus preferentially localize at the necks of dumbbell-shaped vesicles.

4.2. Results

4.2.1. Full length CdvA binds lipid membranes only together with CdvB

Previous *in vitro* studies of purified CdvA were done with either a full-length version of the protein from *M. sedula* (*18*) or with an N-terminus-truncated CdvA from *S. acidocaldarius* that was missing the initial PRC barrel (Figure 4.1A) (*13*, *19*). The phenotype of these two versions differed in that the full-length protein formed double helical filaments that were reported to be stabilized by the binding of DNA, whereas the 69-238CdvA did not polymerize. At the same time, the 69-238CdvA clearly was shown to be able to bind to lipid membranes and recruit CdvB to the membrane along with it. For our study, we purified full length CdvA from *M. sedula*, following the protocol published by Moriscot et al. (*18*), and we obtained the same type of filaments as described in their work (Supplementary Figure 4.1). However, we never observed the protein binding to lipid membranes.

We then decided to fuse the full length CdvA to an MBP-tag. The resulting purified MBP-CdvA did not form long filaments, but rather short and thick polymers (Figure 4.1B), which had an average length of 90 ± 40 nm (mean ± SD, N=195) and a width of 12 ± 3 nm (mean ± SD, N=156). When treating the protein with a TEV protease that cleaved the MBP from the protein, CdvA polymerized into long and thin filaments (Figure 4.1C) with an average length of 220 ± 100 nm (mean ± SD, N=102) and a width of 5 ± 1 nm (mean ± SD, N=106). Formation of these filaments did not require addition of DNA. The presence of the MBP tag

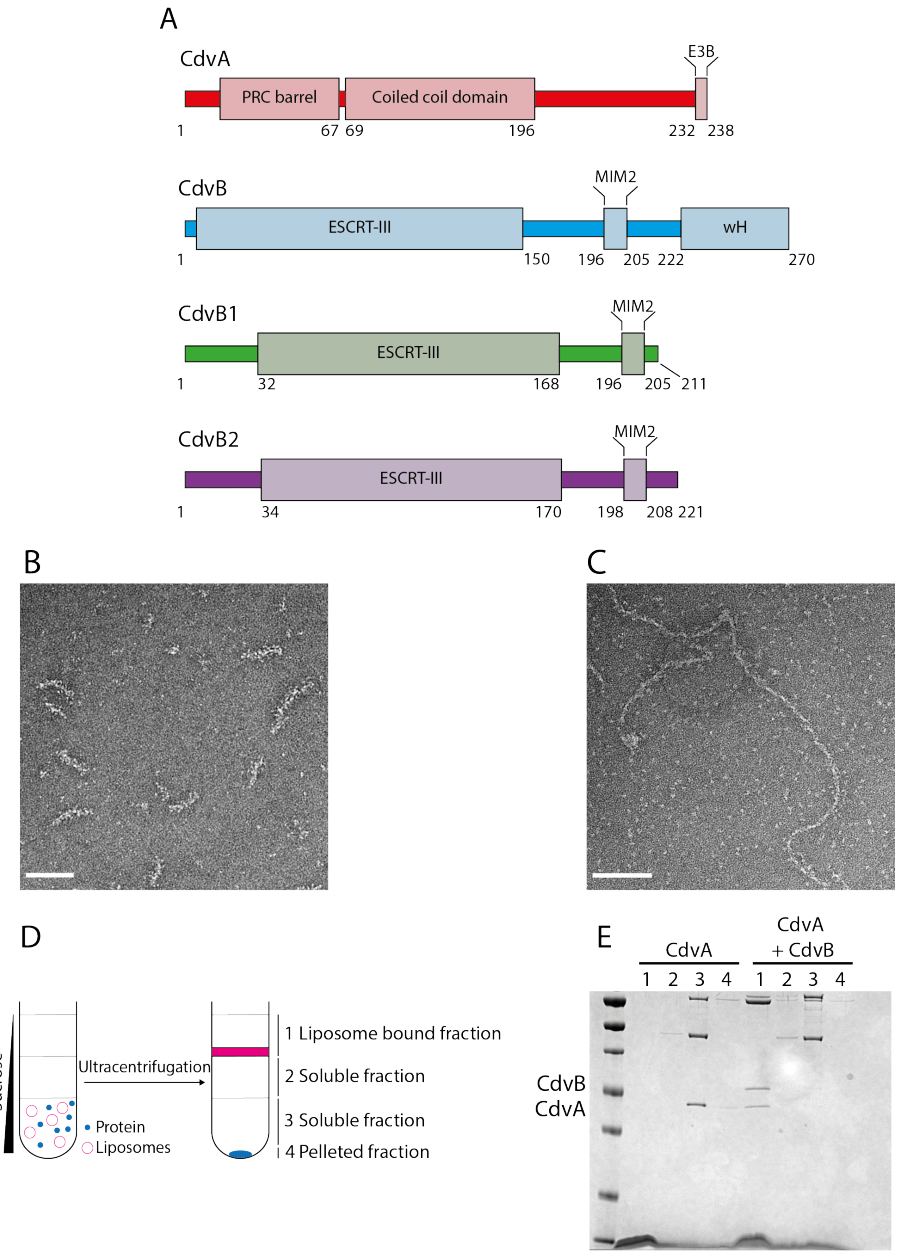


Figure 4.1. CdvA polymerizes only binds lipid membranes in collaboration with CdvB

A. Schematic of the relevant domains of the Cdv proteins explored in this chapter. **B.** MBP-CdvA forms short and thick polymers. **C.** Upon removal of the MBP from CdvA, it self-assembles into elongated filaments, without the need of DNA. Scale bar 80 nm **D.** Sketch of the liposome flotation assay used. **E.** Gel analysis of the flotation assay. CdvA is found to not bind lipid membranes on its own (i.e. lane 1 is empty). However, in presence of CdvB, both proteins are found binding the liposomes. All scale bars are 80 nm.

thus, allowed to control the polymerization of CdvA. Next, we sought to investigate the lipid-binding capabilities of the full-length CdvA. To address this, we used a liposome flotation assay, where protein was mixed with 7DOPC:3DOPG liposomes (see Methods), added to a sucrose gradient, and spun at high speed. This yielded multiple fractions, namely liposome fraction (1), soluble fractions (2 and 3), and a filament sediment at the bottom (4), see Figure 4.1D. In these assays, MBP-CdvA was mixed with the lipids together with TEV protease, in order to cleave the MBP tag.

When tested for the binding of CdvA to lipid membrane, we observed that the protein was always found spread between the soluble and filamented fractions, but never bound to lipids (Figure 4.1E). We then purified CdvB fused to MBP, which – as previously shown by Moriscot et al. (*18*) – presented no filamentation, regardless of having the MBP-tag or not. However, when CdvA was mixed with CdvB, both proteins were found primarily in the liposome-bound fraction (Figure 4.1E). This indicates that the binding of full-length CdvA to the lipid membrane occurs only when in complex with CdvB.

4.2.2. CdvB2 forms filaments when removing the C-terminal domain

Furthermore, we purified CdvB2 from *M. sedula* fused to an MBP-tag. The resulting protein was not presenting any spontaneous filamentation either with or without the MBP tag (Supplementary Figure 4.2). ESCRT-III proteins commonly feature an inactive soluble state and an active membrane-bound state (*20*). Indeed, *in vitro,* these proteins remain inactive soluble monomers while they may get activated and able to polymerize by deleting their C-terminus part (*21*). The same has been previously shown for purified CdvB (*18*), which did not present any *in vitro* polymerization, but spontaneously assembled into filaments upon removal of its C-terminus. Hence, we decided to explore if the same was true for CdvB2, and made a mutant version that contained amino acids 1-170 of CdvB2 (henceforth denoted as CdvB2ΔC).

TEM imaging of the fusion protein MBP-CdvB2ΔC showed that it was polymerizing into a characteristic shape of very stiff and well-defined short filaments (Figure 4.2C). The filaments had an average length of 166 ± 63 nm (mean ± SD; N=161) and a width of 20 ± 5 nm (mean ± SD; N=129). When instead cleaving the MBP tag from the protein, CdvB2ΔC assembled into longer and thinner filaments (Figure 4.2D), of average length 245 ± 95 nm (mean ± SD; N=36) and width of 14 ± 3 nm (mean ± SD; N=64). These data show that CdvB2 also presents a self-inhibiting domain, likely in the same way that CdvB does, and that filament formation can be triggered when removing it.

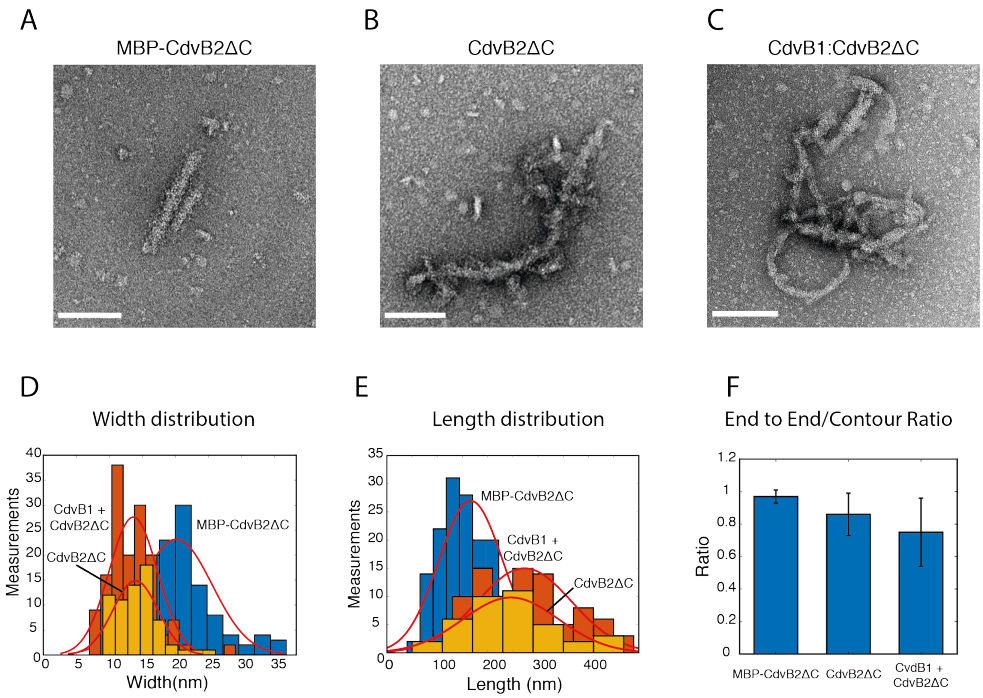


Figure 4.2. CdvB2 self-assembles into filaments when removing its self-inhibiting domain.

A. Negative staining TEM images of MBP-CdvB2ΔC, showing short and rigid filaments **B.** Upon removal of the MBP, CdvB2ΔC still presents filaments that are longer and thinner **C.** CdvB1 and CdvB2ΔC form a co-polymer that is more flexible than the filaments of CdvB2ΔC. **D.** Width distribution of the different polymers. MBP-CdvB2ΔC is seen to present clearly thicker filaments. **E.** Length distribution of the different polymers. CdvB2ΔC filaments get much longer once their MBP-tag is removed. **F.** Ratio of the end-to-end distance to contour length. The ratio is almost 1 for MBP-CdvB2ΔC, indicating that these are stiff and straight filaments, whereas the other samples present a lower ratio, indicating more flexible filaments. All scale bars are 100 nm

Since CdvB2 forms part of the constricting ring together with CdvB1, we explored the effects of their interaction on filament formation. When mixing CdvB1 and CdvB2ΔC together (Figure 4.2E), filaments formed with a very similar length (272 ± 94 (mean ± SD; N=84)) and width (15 ± 6 (mean ± SD; N=184) to that of CdvB2ΔC alone (Figure 4.2F, G). However, these filaments appeared to be more curved. To quantify that, we measured the ratio of the end-to-end distance to the contour length of filaments, which equals 1 for perfectly straight filaments but is <1 for curved ones. For MBP-CdvB2ΔC filaments we measured a ratio of 0.96±0.04, close to 1 (Figure 4.2H), while for CdvB2ΔC, this was reduced to 0.86 ± 0.13, indicating that these filaments are slightly more flexible without the MBP than with it. Finally, the co-polymer of CdvB1:CdvB2ΔC yielded a ratio of 0.75 ± 0.21, showing that this copolymer is more flexible than the CdvB2-variant filaments (Figure 4.2H).

4.2.3. Membrane-binding properties of the CdvB1:CdvB2 proteins

When performing the liposome binding assays with the inactive CdvB2, we found that the protein was neither binding directly to the lipid membrane, nor that it was being recruited by the other components of the Cdv system (Supplementary Figure 4.3). Subsequently, we tested the ability of CdvB2ΔC to bind lipid membranes. When CdvB2ΔC was mixed with 7DOPC:3DOPG liposomes and spun on a sucrose gradient, the protein was never found in the liposome fraction, but instead always in the soluble and filamented fractions (Figure 4.3A), indicating that CdvB2ΔC does not bind lipid membranes. That contrasts CdvB1, which presented a clear membrane binding when mixed with vesicles on its own (Figure 4.3A).

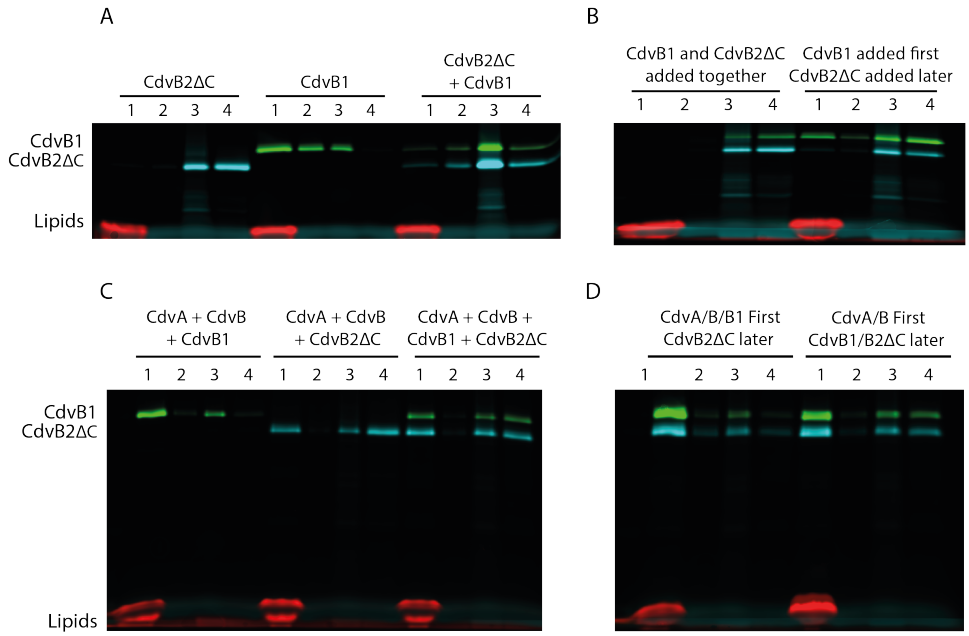


Figure 4.3. CdvB2ΔC blocks membrane binding of CdvB1 and gets recruited to the membrane by CdvA+CdvB.

A. Liposome flotation assays showing that CdvB2ΔC does not bind directly to lipid membranes (left), and how the interaction of CdvB1 with CdvB2ΔC, prevents CdvB1 to bind to lipid membranes anymore (right) Green: CdvB1, Cyan: CdvB2ΔC. Red: liposomes **B.** Liposome flotation assay where CdvB1 and CdvB2ΔC were added either together or sequentially. When CdvB1 was mixed with liposomes beforehand, it did bind, but if added together with CdvB2ΔC, both did not bind lipid membranes. **C.** Liposome flotation assay showing how CdvA+CdvB bound to the membrane and recruit CdvB2ΔC to it. When CdvA/CdvB/CdvB1/CdvB2ΔC were mixed together with liposomes, they all bound to lipid membranes **D.** Sequential addition of the different proteins to the membrane. No substantial difference was observed between the two protocols.

Interestingly, however, when mixing the two proteins at equimolar concentrations, and subsequently adding them to liposomes, none of the proteins were found to bind the liposomes, but they were rather observed in solution or sedimented as filaments (Figure

4.3A). CdvB2 was thus found to block membrane binding of CdvB1, presumably by forming CdvB1:CdvB2 filaments in solution. To see if this inhibition of membrane binding is dependent on the order in which the proteins are mixed with the lipids, we ran the same assay with two different conditions. In the first condition both proteins were first mixed with each other, and only subsequently added to the liposomes. Just like in the previous case, the copolymer of CdvB1:CdvB2ΔC was not found to get bound to the liposomes (Figure 4.3B). In the second condition, however, CdvB1 was mixed with the liposomes first, and only then CdvB2ΔC was added. In this case, interestingly, we observed that CdvB1 remained bound to the liposomes while not recruiting CdvB2ΔC to the membrane (Figure 4.3B).

Furthermore, we explored the interaction of CdvB2 with the CdvA:CdvB complex. In the liposome flotation assay, CdvA and CdvB (as well as CdvB1) can be found bound to the liposomes (Figure 4.3C, Supplementary 4.4). However, when adding CdvB2ΔC to CdvA, CdvB, and liposomes we observed that CdvB2ΔC was also found in the liposome fraction (Figure 4.3C, Supplementary 4.4), suggesting that it had been recruited to the membrane by the CdvA:CdvB copolymer. Finally, when mixing all 4 proteins together (CdvA, CdvB, CdvB1, and CdvB2ΔC) with liposomes, we observed that a fraction of CdvB1 and CdvB2ΔC bound to the liposomes, while an important part of the protein also filamented and remained soluble (Figure 4.3C, Supplementary 4.4). These data, interestingly, show that CdvB2:CdvB1 interactions lead to a polymer that does not bind the lipid membranes, whereas the interaction of CdvB2 with CdvA:CdvB, leads to the recruitment of CdvB2 to the membrane.

Seeing that the proteins bind to the lipids when all are mixed together at once, we sought to study the effect of adding the proteins to the lipids in a sequential manner. We tested two conditions, one in which CdvA, CdvB and CdvB1 are mixed together with the liposomes and only then CdvB2ΔC was added, and one where CdvA and CdvB we first bound to the liposomes, and then CdvB1 and CdvB2ΔC were added. In the first condition, all of the proteins were found in the liposome fraction, and there was almost no visible filamented fraction, indicating that most of the protein got bound to the lipids (Figure 4.3D, Supplementary 4.5). In the second condition where CdvB1:CdvB2ΔC were added together after the CdvA:CdvB had already bound the liposomes, no significant differences were appreciated as compared to the first condition, and again most of the CdvB1:CdvB2ΔC was found on the liposome fraction (Figure 4.3D, Supplementary 4.5). This indicates that the CdvB1:CdvB2 complex can be recruited to the membrane through the CdvA:CdvB, when CdvA:CdvB proteins are already bound to the membrane.

4.2.4. Cdv proteins localize at membrane necks of high curvature

The human ESCRT-III system preferentially locates at membrane necks that present high curvatures (*22*, *23*). We explored if a similar preference for high curvatures was observed for

Cdv proteins as well. For testing this, we used a recently developed assay that produces dumbbell shaped liposomes. A lipid mixture of 95DOPC:5DOPG in chloroform was dried out and resuspended in oil. In this oil, we formed water-in-oil droplets in which the proteins were encapsulated, and these droplets were then left to sedimented by gravity through a lipid interface into an outer water phase, thus obtaining unilamellar liposomes that contained the protein on the inside. The outer phase was made of buffer containing specific DNA origami structures, that acted as synthetic membrane shapers (SMS) (*24*). We thus obtain chains of dumbbell-shaped liposomes that are mutually connected through membrane necks. Protein that are encapsulated within these liposomes can be studied for their spatial binding properties in a topology that resembles cells that are dividing.

We observed a clear preference of the Cdv proteins to localize at the membrane necks (Figure 4.4A). Proteins (50-100 nM concentration) displayed a strong fluorescence signal at the membrane necks, while they showed only a residual weak homogeneous binding to other membrane regions of the dumbbell-shaped liposomes. Different protein combinations were tested : CdvA + CdvB-Alexa568 (Figure 4.4A); CdvA-Cy5 + CdvB (Supplementary Figure 4.6); CdvA + CdvB-Alexa568 + CdvB1-Alexa488, (Figure 4.4A); and CdvA + CdvB-Alexa568 + CdvB1-Alexa488 + CdvB2ΔC-Cy5, (Figure 4.4A). All of the Cdv proteins were observed to co-localize at the necks of the liposomes (Figure 4.4A). Consistent with the liposome flotation assay, we saw almost no binding of the proteins when mixing CdvB1-Alexa488 + CdvB2ΔC-Cy5, as well as no localization at necks (Supplementary Figure 4.7).

The fluorescence intensity of the Cdv proteins at the necks was about 7 times higher than that at the membrane away from the necks (Figure 4.4B), whereas the lipid fluorescence intensity at the neck was only ~2 times enhanced (Figure 4.4B). Control experiments with a protein that bound the membrane via a His-tag (ZipA from *E. coli*) in the same vesicles did not show any significant enrichment of the protein signal at the necks (Supplementary Figure 4.8), indicating that this preference for binding at highly curved membranes is a specific property of the Cdv proteins and not induced by the assay. FRAP experiments where we bleached membrane dyes in a liposome in a chain showed a fast recovery of the fluorescence (Supplementary Video 1), indicating that there is an open channel between the vesicles where the lipids can move freely, apparently unhindered by the Cdv protein at the necks.

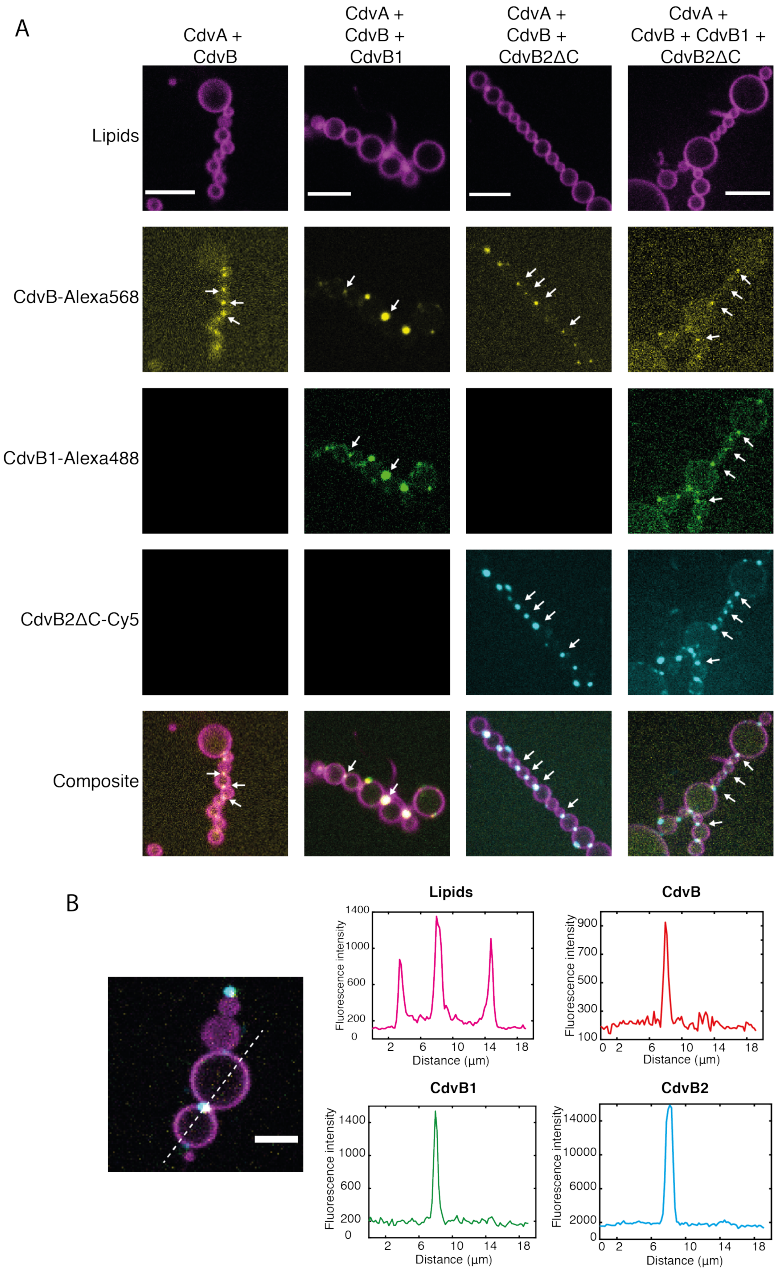


Figure 4.4. Cdv proteins have a preference to localize at membrane necks of high curvature.

A. Spinning disk confocal images of different combinations of interacting Cdv proteins inside of chains of dumbbell-shaped liposomes that are connected with narrow necks. The proteins preferentially co-localize at the necks. **B.** Fluorescence intensity along the dotted line shows that the intensity of the protein at the membrane neck is much larger than at the rest of the membrane. Scale bars 10 μm

4.3. Discussion

With these findings we can draw a more complete picture of how the interactions between the Cdv proteins govern their membrane recruitment. We showed that CdvA can form filaments without the need of DNA to stabilize them. Interestingly, we observed that CdvA in its full-length conformation does not bind lipid membranes, and it only does so upon interacting with CdvB. This suggests that inside cells, both proteins are likely recruited to the membrane jointly. It would be interesting to see crystal structures of interacting CdvA:CdvB proteins in order to study what kind of structures these two proteins arrange themselves into to allow for this behavior. Furthermore, we showed how CdvB2 does not polymerize in its full-length conformation, but it does so when removing the C-terminus domain. Previous work had shown the same for CdvB (*18*), but we previously showed that CdvB1, by contrast, could polymerize when it was full length (*8*). This is a relevant parallelism with the ESCRT machinery, where many of the proteins also only polymerize upon removal of the C-terminus. This parallelism may suggest that, in vivo, CdvB2 acts in a similar way as its eukaryotic counterparts and might assemble into similar structures at the cell.

We observed that CdvB2 cannot bind the membrane on itself. Furthermore, we observed that CvdB2 interacts with CdvB1 in solution, forming a polymer that no longer can bind the lipid membrane. We showed how CdvB2 as well as CdvB1:CdvB2 complexes can get recruited to the membrane by the CdvA:CdvB complex (Figure 4.5). These interesting *in vitro* observations allows to speculate about how the recruitment to the membrane may occur *in vivo*. We suggest that CdvB1 and CdvB2 are bound together in a state that does not allow them to bind the membrane, ensuring that the membrane binding of CdvB1:CdvB2 does not occur until they are recruited by CdvA:CdvB. This implies that CdvB1 and CdvB2 are recruited to the membrane at the same time. When recruited, they likely undergo a conformational change that allows them to directly bind the membrane. In this way, CdvB1 and CdvB2 can remain attached to the membrane when the initial non-contractile ring is disassembled, where they then can perform the constriction and scission of the membrane.

Our observations showed that the CdvB1:CdvB2 polymers form filaments that are more flexible than the CdvB2 filaments. This may relate to the constriction mechanism in division. Recent MD simulations suggested that the division ring formed by CdvB1 and CdvB2 is not a stiff continuous polymer that encircles the cell (*9*), but alternatively, consists of a series of short helices that meander through the cell midzone, overall forming a ring around the cell. This picture implies that these polymers are locally intrinsically curved and easily deformable – which is consistent with our observations.

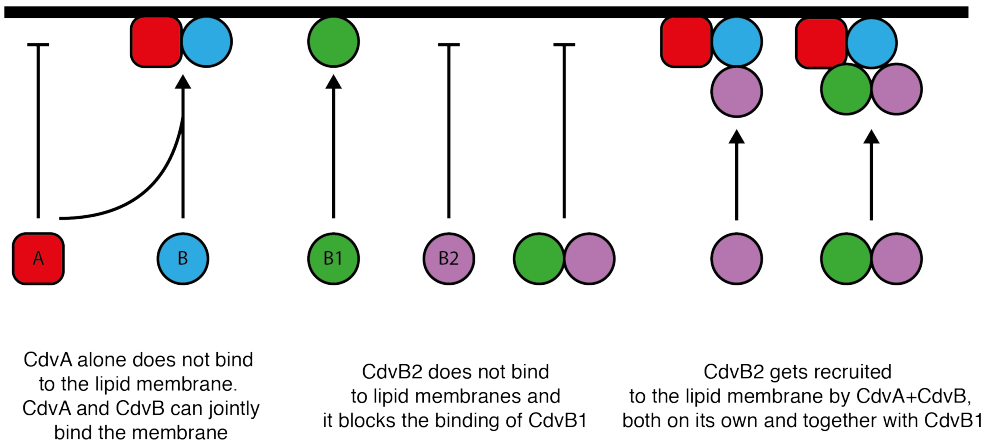


Figure 4.5. Schematic representation of how protein interactions govern membrane recruitment of the Cdv proteins.

Finally, we found that Cdv proteins primarily locate at membrane necks, providing the first indications of a preference of Cdv proteins for high-curvature membranes. This presents another common trait between the archaeal and eukaryotic systems, as many ESCRT proteins also preferentially bind highly curved membranes (*22*, *25*). Future experiments with higher resolution techniques, such as Cryo-EM, will help to better elucidate what kind of higher-order structures these proteins adopt on those necks. While our data provide valuable information on archaeal cell division by Cdv proteins, further research is necessary to fully clarify the mechanism of action of the scission machinery.

4.4. Materials and methods

4.4.1. Plasmids

All of the proteins that we used are from *Metallosphaera sedula*., The original plasmids for CdvA (Msed_1670, UniProtID A4YHC3) and CdvB (Msed_1671, UniProtID A4YHC4) were kindly provided to us by Patricia Renesto's lab. From those plasmids, the sequences of the proteins were copied and ordered as a synthetic gene already inserted in a pMAL-c5x from Biomatik, using BamHI and EcoRI cutting sites. Extra codons coding for cysteines were added at the N termini of the proteins for fluorescent labelling.

The plasmid for CdvB1 was the same as used in our previous work (*8*). The gene for CdvB2 (Msed_1695, UniProtID A4YHE8) was obtained from the Gen Bank data base, and was reverse translated using the EMBOSS Backtranseq tool, optimized for *E. coli* codon usage. To the resulting DNA sequence, a codon of a cysteine for fluorescent labelling was added at the N terminal of the protein, as well as Tobacco Etch Virus (TEV) and an HRV 3C proteases cutting sites. The whole gene construct was ordered as a synthetic gene already inserted in a pMAL-c5x vector from Biomatik, using BamHI and EcoRI cutting sites.

From the original plasmid for MBP-CdvB2, the whole plasmid except for the C-terminus of the protein was copied by PCR. The resulting linearized plasmid was checked on an agarose gel, and then treated with the KLD reaction mix (New England Biolabs, Ipswich, Massachusetts, USA) to digest the template, phosphorylate the ends of the linearized plasmid and ligate it all at once. The reaction mix was then transformed into NEB5alpha competent cells (New England Biolabs), some colonies were picked, and the plasmid was purified using the QIAprep Spin Miniprep Kit (QIAgen, Hilden, Germany) and sent for sequencing.

All primers and plasmids constructed in this work can be found in the Supplementary Table 1.

4.4.2. Protein purification

All proteins were produced in BL-21 *E. coli* strains. Cells were grown at 37°C in LB^{amp} medium to an OD of around 0.5, at which point expression was induced with IPTG and cells were left to express the protein for 4 hours. After that, cells were harvested by centrifuging at 4500x g at 4°C for 12 minutes.

For MBP-CdvA, the cell pellet was resuspended in lysis buffer (50mM Tris pH 8.8, 350 mM NaCl, 50 mM Glutamate, 50 mM Arginine, 0.05mM TCEP, cOmplete™ Protease Inhibitor Cocktail (Roche, Basel, Switzerland)), lysed by French press and centrifuged (150,000 g, 30 min, 4°C). The remaining supernatant was incubated with 1 ml of amylose resin (NEB, Ipswich, Massachusetts, USA) rotating for 2 hours at 4°C, after which it was poured through a gravity chromatography column and the protein was washed (50 mM Tris pH 8.8, 350 mM NaCl, 50 mM Glutamate, 50 mM Arginine, 0.05 mM TCEP) and eluted with elution buffer (50 mM Tris pH 8.8, 350 mM NaCl, 50 mM Glutamate, 50 mM Arginine, 0.05 mM TCEP, 10 mM maltose).

For MBP-CdvB, MBP-CdvB2 and MBP-CdvB2ΔC, the cell pellet was resuspended in lysis buffer (50mM Tris pH 8.8, 50 mM NaCl, 0.05 mM TCEP, cOmplete™ Protease Inhibitor Cocktail) lysed by French press and centrifuged (150,000 g, 30 min, 4°C). The remaining supernatant was incubated with 1ml of amylose resin rotating for 2 hours at 4°C, after which it was poured through a gravity chromatography column and the protein was washed (50 mM Tris pH 8.8, 50 mM NaCl, 0.0 5mM TCEP) eluted with elution buffer (50 mM Tris pH 8.8, 50 mM NaCl, 0.05 mM TCEP, 10 mM maltose). MBP-CdvB1 was purified just as described in (*8*).

After affinity chromatography, all proteins were run through a Superdex™ 75 increase 10/300 GL size exclusion chromatography column mounted in an ÄKTA™ Pure system. Samples were run with the same buffer as they were washed and stored by snap freeze in liquid nitrogen. Purity of the samples was evaluated by SDS PAGE stained with Coomassie blue. A fraction of all of the proteins was separated after the affinity chromatography and

dialyzed into the same buffer but with pH of 7.4 to perform a maleimide-cysteine conjugation reaction. MBP-CdvA was labelled with Cy5, MBP-CdvB with Alexa-568, MBP-CdvB2ΔC with Cy5 and CdvB1 with Alexa 488. The rest of the purification stayed the same, and excess label was removed from the protein through the gel filtration column.

4.4.3. TEM imaging

For imaging of MBP-CdvA, the protein was diluted down to 100 nM in buffer containing 50 mM Tris pH 7.4 and 50 mM NaCl (all samples were prepared using this buffer). For imaging the protein without MBP, 1μM of MBP-CdvA was mixed with 0.1 μM of TEV protease and left incubating at RT for 1 hour. The sample was then diluted 10 times before depositing it onto a carbon grid. MBP-CdvB2ΔC samples were diluted down to 100 nM in buffer, and samples without MBP were prepared by mixing 1 μM of MBP-CdvB2ΔC with 0.1 μM of TEV protease and left incubating at RT for 1 hour. Samples with CdvB1 and CdvB2ΔC were prepared by mixing MBP-CdvB1 and MBP-CdvB2ΔC both at 1μM concentration with 0.1 μM of TEV protease at RT for 1 hour. The samples were then diluted 10 times before depositing it onto a carbon grid. Samples were absorbed on glow-discharged carbon-coated 400-meshh copper grid purchased from Quantifoil (Großlöbichau, Germany) and stained with 2 % uranyl acetate. They were then imaged on a JEOL JEM-1400plus TEM (JEOL, Akishima, Tokyo, Japan) at 120 kV of accelerating voltage with a TVIPS f416 camera (TVIPS, Gauting, Germany).

4.4.4. Liposome flotation assay

Lipids used were DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), DOPG (1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)), and Rhodamine-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)), all of them purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). The lipids, dissolved in chloroform, were mixed to final ratios (mol:mol) of 69.9 DOPC : 30 DOPG : 0.1 Rhodamine-PE, and evaporated in a glass vial to obtain a thin lipid film. Lipids were resuspended in buffer containing 50 mM HEPES pH 7.5, 50 mM NaCl and 300 mM sucrose, at a final concentration of 5 mg/ml. The lipid film was hydrated for 1 hour and thoroughly vortexed to form small lipid vesicles. The lipids were then mixed with 0.1 μM of TEV protease and 1 μM of protein of interest. Lipids and protein were left to incubate for 1 hour at RT. The sample was then deposited at the bottom of an ultracentrifuge tube, and mixed with buffer containing sucrose to obtain a bottom layer of 30% of sucrose. Gently, another layer of buffer with 25% of sucrose was deposited, and a final layer of 0% of sucrose on top. Then it was centrifuged at 200,000 g at 4 °C for 30 minutes in a SW 60 Ti Swinging Bucket rotor. All the different fractions of the sucrose gradient were then pipetted out (Fraction 1, 2 and 3), and extra buffer was then added to resuspend the filamented pellet at the bottom (Fraction 4). The different fractions were then analysed by SDS PAGE, and stained with Coomassie blue. Experiments with CdvB2ΔC were done with a final concentration of all the proteins of 600nM, and gels

were imaged using a GE Amersham™ Typhoon gel imager to image the fluorescent label on the proteins and lipids.

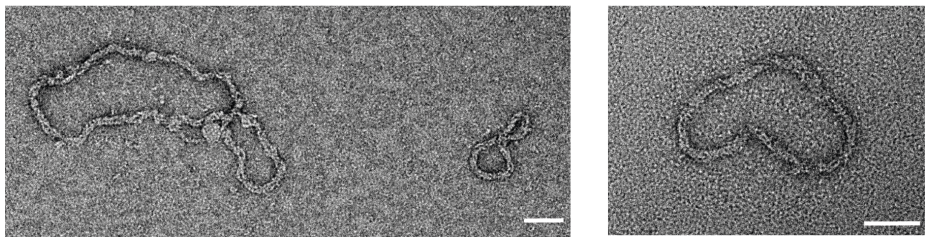
4.4.5. Preparation of lipid in oil suspension for dumbbell-shaped liposome preparations

DOPC, DOPE-PEG2000, DOPG and DOPE-Rhodamine (or DOPE-Atto390 for experiments with proteins with overlapping fluorescence) in chloroform were mixed in a ratio of 93:2:5:0.1 and evaporated in a glass vial under a blow of nitrogen. Lipid mixture was then resolubilized in chloroform to a final concentration of 0.2 mg/ml. A freshly prepared mixture of silicone and mineral oil that was added to the lipids in chloroform slowly dropwise while vortexing gently. After all oil is added to the chloroform, it was vortexed at max speed for 2 minutes and then sonicated for 15 minutes in an ice bath.

4.4.6. Preparation of dumbbell-shaped liposomes with the synthetic membrane shaper

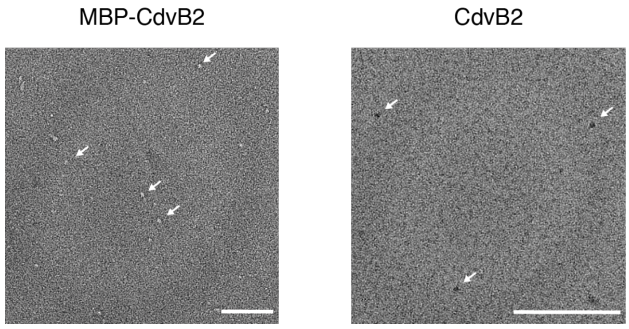
Cdv proteins were mixed in an inner buffer containing 50mM Tris pH 7.5 and 37 % w/v optiprep (Sigma Aldrich, St. Louis, Missouri, USA) to make the solution heavy. In parallel, an outer solution in buffer containing 50 mM Tris pH7.4, 5 mM $MgCl_2$ and glucose to match the osmolarity of the outer solution at 30 mOsm higher than in the inner solution. The DNA nanostars developed in Ref. (*24*) were then mixed into the outer solution and deposited at the bottom of an imaging chamber. Water in oil droplets of inner buffer containing protein were then formed by pipetting up and down 20 µl of inner solution into 400 µl of oil until a homogeneous droplet size was achieved. The droplets in oil were immediately deposited on top of the outer solution in the imaging chamber, and they were allowed to sediment by gravity through the oil-water interphase. The liposomes were imaged using spinning disk confocal laser microscopy (Olympus IXB1/BX61 microscope, 60× objective, iXon camera) with Andor iQ3 software. Analysis of the images was done with ImageJ (v.2.1.0).

Appendix Chapter 4



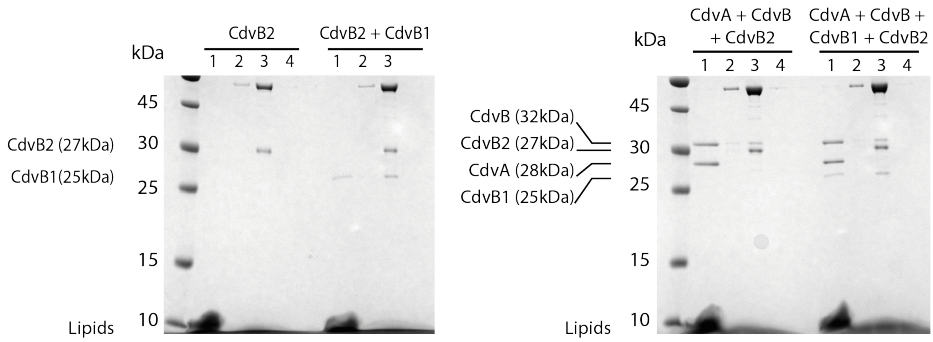
Supplementary Figure 4.1.

CdvA purified through His-Tag purification as described in Moriscot et al. 2011 PLOS One. Scale bar 50 nm



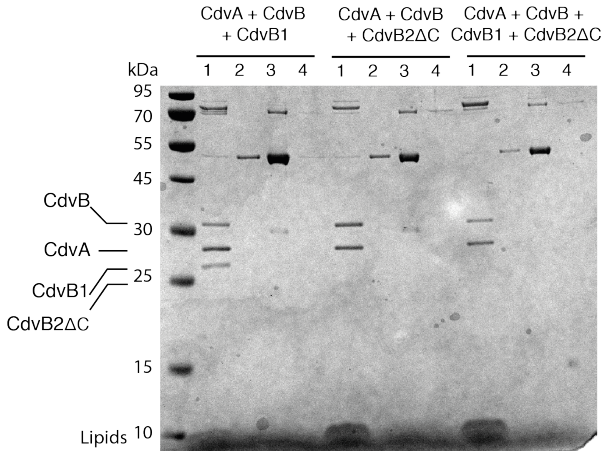
Supplementary Figure 4.2.

CdvB2 full length does not form filaments on its own. Scale bar 200 nm



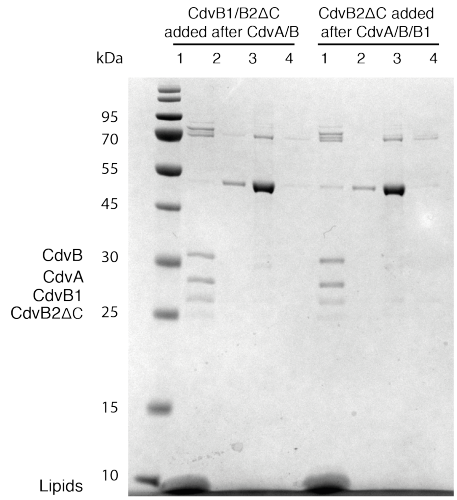
Supplementary Figure 4.3.

CdvB2 full length is never found in the liposome fraction (1), indicating that when it is in its inactive form, it does not bind to the membrane directly nor it gets recruited by the other components of the Cdv system. Although CdvB2 is almost the same size as CdvA, it can be slightly higher up in the gel, probably due to some incomplete denaturing of the protein and some remaining secondary structure



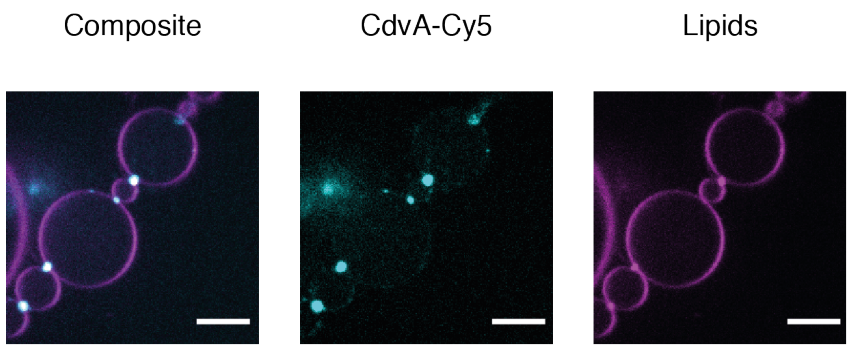
Supplementary Figure 4.4.

Coomassie staining of the gel where the different fractions of the sucrose gradient have been run. CdvB1 and CdvB2ΔC are too low concentration to be seen by Coomassie staining when they are spread through different fractions



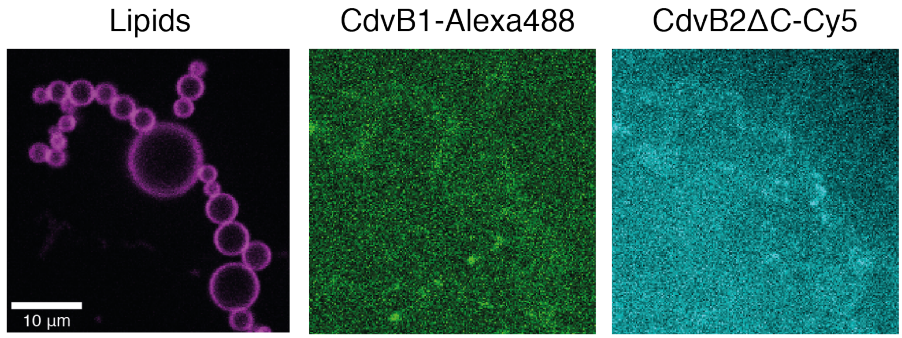
Supplementary Figure 4.5.

Coomassie staining of the gel where the different fractions of the sucrose gradient have been run.



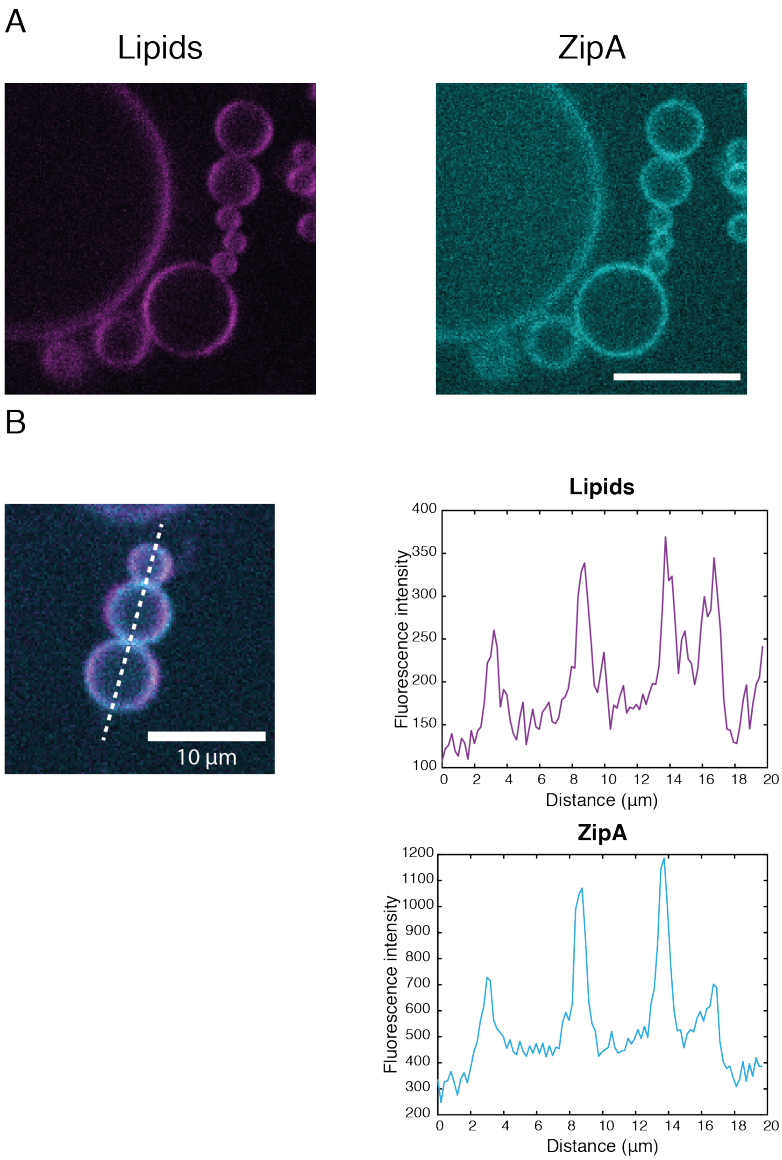
Supplementary Figure 4.6.

CdvA-Cy5 + CdvB showing preferential localization of protein at the vesicle neck Scale bar 10 μm



Supplementary Figure 4.7.

CdvB1:CdvB2 co-polymer presents no relevant membrane binding



Supplementary Figure 4.8.

A ZipA, a different membrane binding protein, binds the membrane of the vesicles in a homogenously distributed way, with no preference for the vesicle necks. **B** There is no significant difference between the pattern of membrane or protein intensity Scale Bar 10 µm

Supplementary Table 1

Sequence of pMAL-c5x-CdvA
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Purple: MBP
Green: TEV cutting site
Yellow: 3C cutting site
Red: Cys-Cys-CdvA

Sequence of pMAL-c5x-CdvB

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Purple: MBP
Green: TEV cutting site
Yellow: 3C cutting site
Cyan: Cys-CdvB

Sequence of pMAL-c5x-CdvB2

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Purple: MBP
Green: TEV cutting site
Yellow: 3C cutting site
Khaki: Cys-CdvB2

Forward primer CdvB2ΔC
5’ tgagaattccctgcaggtaat 3’
Reverse primer CdvB2ΔC
5’ gccggtaaaatcgcccgcttc 3’

Sequence of pMAL-c5x-CdvB2ΔC

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Purple: MBP
Green: TEV cutting site
Yellow: 3C cutting site
Blue: Cys-CdvB2ΔC

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5

Concluding Remarks

Fully understanding how cell division works is one of the major questions that biology is tackling at the moment. This also is a field where in vitro experiments have a lot to offer, which is why we have focused our efforts in this thesis to apply these in vitro studies to understand the Cdv proteins. In this final chapter, I reflect about the future of the Cdv research, the questions that remain unanswered and how those could be tackled. I also consider the current approaches to a synthetic cell divisome and whether the Cdv system truly is a promising candidate for this.

5.1. The future of the Cdv research

The last couple of years have seen a new surge of interest in the Cdv system, coming from newly published *in vivo* results which have been facilitated thanks to the development of new techniques such as super-resolution microscopy (*1*) or high-temperature live microscopy (*2*, *3*) of these thermophilic organisms. It would be beneficial to compliment these new advancements in the future with equally informative *in vitro* experiments.

In this thesis, we have seen how CdvB1 binds to lipid membranes and that the Cdv proteins have a preferential binding to membrane necks. However, we don't know what structures these proteins acquire when bound to the membrane. Recent developments in cryo-EM technologies and their 3D reconstitution of proteins, have given us a very clear idea of how the ESCRT proteins arrange themselves around vesicles (*4*, *5*). Conducting similar studies with the Cdv proteins will be of great interest to further understand the mechanics of the system, and better elucidate the evolutionary relationship between the two systems. Interestingly, the development of high-temperature fluorescent microscopes, also open the door to many promising *in vitro* experiments with the Cdv proteins, where a fully functional scission machinery could be studied just like in eukaryotes (*6*).

That being said, more *in vivo* studies will also provide us with a wider picture of how the system acts differently in the different types of archaea. There seems to be a high degree of variability on the phenotypes that the Cdv proteins present, and the involvement of certain proteins in the various processes is not always conserved amongst species (*7–9*). As we keep discovering new archaea that possess the Cdv system, such as the recent discovery of the Asgard archaea (*10*, *11*), the complexity and the variability of the system will keep increasing, which will help us to increasingly better understand the system as well as its origins.

Indeed, for now, multiple fundamental questions still remain unanswered, like how does the division ring position itself at the center of the dividing cell, or how is division of the cell coordinated with the genome separation. It has been speculated that CdvA might have some role to play in that, as the protein can also bind DNA (*12*), but much of this is speculation and our understanding of the Cdv system remains poor. In addition, we do not know how the division of the cell is coordinated with the synthesis of the external S-layer present in archaea, or if the Cdv proteins play any role in it.

5.2. The synthetic cell divisome

After having worked on this project, whose long-term goal was encompassed in building a synthetic divisome, it is worth reflecting on how that might look like in the future.

A lot of effort has been put into reconstituting the FtsZ ring of E.coli in an effort to have it constrict and divide liposomes, and it is probably one of the systems from which we have

more information (*13*). However, although some *in vitro* experiments have shown some membrane constriction caused by FtsZ, it seems more and more clear that, *in vivo*, it is not the main constriction generator (*14–16*). This leads to some questions regarding if FtsZ alone will be capable of performing a full division cycle. Hence, it is worth reflecting about other possible systems.

Many archaea for example also present an FtsZ machinery that is heavily understudied (*17*). Members of the Euryarchaea are known for containing 2 different types of FtsZ proteins (*18*), and they are both implicated differently in the process of cell division (*19*). Interestingly, it seems that FtsZ1 is initially recruited to the division site, whereupon SepF binds and recruits FtsZ2 (*20*). It seems that is FtsZ2 the responsible for exerting constricting force to the membrane at that point, as mutants with no or defective FtsZ2 would be viable but presented no division (*19*). If future in vivo experiments elucidate further how this system works, it could be a promising path to follow for the synthetic cell.

Exploring how much simpler organisms like Mycoplasmas divide might also open many possibilities for a synthetic divisome. Mycoplasma are extremely simple organisms that don't have a cell wall, but still present an FtsZ, which means that their division is not linked to the peptidoglycan synthesis. However, the FtsZ gene appears to not be crucial for the viability of the organism (*21*), so it still remains a bit uncertain what the role of this FtsZ might be. Nonetheless, reconstituting this system *in vitro* will help us better understand how FtsZ can work when it doesn't guide the PG synthesis machinery.

When looking into the division of eukaryotes and the use of the ESCRT system for a synthetic cell, it seems unlikely that this machinery could be a good fit for a minimal cell. Understanding eukaryotic complexity in a bottom-up way, and reconstituting the components of the cell *in vitro* are certainly interesting experiments that will provide us with valuable information about eukaryotic systems (*22*, *23*). Liposomes are excellent membrane compartments that allow for the reconstitution of cytoskeletal proteins such as actin, with great results and further potential in the understanding of these cell components (*24*, *25*). Likewise, a large part of the understanding that we have of the ESCRT system comes from its in vitro studies in situations that mimic the cell (*26*). Therefore, in vitro reconstitutions of eukaryotic systems are of high value. However, the amount of proteins and coordinated processes that are needed to perform cell division in eukaryotes, makes it very difficult to imagine that the ESCRT will be a good match for a minimal cell.

The reconstitution of naturally occurring division systems is of course of great interest on its own, as it is not only useful to build synthetic cells, but also gives us a lot of information about the system itself. However, as a complementary approach to building a synthetic cell, it will be of interest to pursue the design of artificial membrane shapers that help us manipulate the shape of the cell, with the flexibility of designing the properties of the

components (*27*). DNA origami is one of the most useful tools for such an application, as it is very robust and allows for a great level of precision in controlling the nanostructures that one wants to design (*28*). Accordingly, there have been recently reports of DNA origami structures capable of mimicking the action of membrane deforming proteins and capable of deforming liposomes into specific shapes (*29*, *30*). The use of DNA origami for membrane deformation is still on its infancy, and maybe a full divisome based on this technology might never be achievable. However, it seems a promising path worth exploring that will help with some of the problems that building a synthetic cell will face.

5.3. The Cdv system for a synthetic cell: is it worth it?

The initial scope for this thesis, was exploring the Cdv system for its potential use in building a divisome for a synthetic cell. Therefore, we must reflect on whether it is a viable system to divide a synthetic cell, and this question is not so simple.

On the one hand, this system presents a major drawback that we had to deal with for the entirety of the project, and that is the temperature where these proteins are active. The crenarchaea live at around 80°C, and thus all their enzymes have evolved to work optimally at those temperatures. We have shown that CdvC can still work, albeit less optimally, at temperatures of around 50°C, which is promising for the study of the Cdv proteins themselves. However, if one thinks of combining the Cdv with other protein systems of other organisms in a synthetic cell, then maintaining such a high temperature will just not be feasible. In this regard, however, the Cdv proteins from the recently discovered Asgard archaea may come as a great alternative that is worth exploring. They share the largest degree of evolutionary proximity to the ESCRT, and most importantly, it has been shown that the CdvC from the Asgards can hydrolyze ATP at 30°C (*11*). This is a very important trait that would make them compatible and potentially suitable for a synthetic cell. Unfortunately, however, as of now, we lack the very fundamentals of how these Asgard Cdv proteins work, both *in vivo* or *in vitro*.

Another key issue is the polymerization of the Cdv proteins. Contrary to FtsZ that forms bundles upon very well-defined conditions of crowding and presence of GTP, the Cdv proteins just spontaneously polymerize in vitro, without external control. In our work, we have solved this by fusing the proteins to an MBP tag, which has greatly helped the control over their polymerization and membrane binding. But in a self-replicating synthetic cell that produces its own proteins, this mechanism would be greatly inconvenient, as not only the synthesis of the MBP-fused protein would have to be controlled, but also the production of the protease that would cleave the tag.

Currently, the project of building a synthetic cell looks mostly into optimizing every individual process of a cell, with the aim to combine them together subsequently. These efforts are pursued regardless of the origin of the proteins. This will likely result in problems

when combining proteins from e.g. thermophilic organisms with those from mesophilic ones. The ease of use of mesophilic proteins, and the wider variety of organisms where to choose from, will likely always favor these to be chosen for this approach of synthetic cell construction. However, the drawbacks from the thermophilic systems can also be seen as opportunities. Archaeal lipids from thermophilic organisms are very stable and are very good at sustaining chemiosmotic gradients (*31*), which makes them very attractive for applications of drug delivery (*32*, *33*). Similarly, the stability of thermophilic enzymes makes them very attractive for a wide variety of industrial processes (*34*).

All in all, I find it unlikely that the Cdv system will win the race to be the first cell division mechanism in the first ever bottom-up reconstituted synthetic cell. However, I do foresee a future where a variety of different synthetic cells will be available to serve different purposes. When it comes to for example the biosynthesis of compounds that need high temperatures for favorable kinetics, or the bioremediation of highly sulphurated polluted waters, synthetic cells with thermally resistant and robust components will be the best solution. Therefore, pursuing the *in vitro* reconstitution of the Cdv system in a setting resembling a synthetic cell remains a path worth following, as I am confident that this will give us many more interesting discoveries and will help us better understand the system, as well as one day may open the door to new possibilities.

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SAMENVATTING

Alle levende organismen delen de behoefte om zich te voort te planten en te vermenigvuldigen om het voortbestaan van hun soort te verzekeren. Bij prokaryoten wordt dit over het algemeen gegarandeerd door een proces van celdeling waarbij een moedercel wordt gesplitst in twee dochtercellen van gelijke grootte, en over het algemeen is het een complex en heterogeen proces voor alle verschillende soorten. Als we kijken naar de Crenarchaea-stam van de archaea, vinden we een heel specifieke reeks eiwitten die verantwoordelijk zijn voor het orkestreren van dit proces van celdeling: het Cdv-systeem. Dit systeem is nauw verwant aan de ESCRT-machinerie, die ook verantwoordelijk is voor celdeling en vele andere membraanvervormingsprocessen in eukaryoten. Deze nauwe overeenkomst is een van de vele gemeenschappelijke kenmerken die wijzen op een gemeenschappelijke oorsprong tussen archaea en eukaryoten. Hoewel de eukaryote machinerie grondig en uitgebreid is bestudeerd, is er zeer weinig bekend over het archaeale delingssysteem. Om deze reden hebben we in dit werk gestreefd naar een beter begrip van deze archaeale eiwitten, gebruikmakend van in vitro technieken, met de langetermijnvisie om ze te gebruiken om met een bottom-up benadering een synthetische cel te bouwen.

In **Hoofdstuk 1** presenteren we wat minimale cellen zijn, en leggen we uit wat de aard is van een bottom-up benadering om ze te bouwen, en waarom dat belangrijk is om het leven in de kern beter te begrijpen. Meer specifiek gaan we dieper in op de verschillende benaderingen die eerder zijn gebruikt om celdelingsmachines uit bacteriën en eukaryoten in vitro te reconstrueren, en bekijken we de voor- en nadelen van deze systemen. Ten slotte onderzoeken we waarom het Cdv-systeem eigenlijk een interessant alternatief is voor een synthetisch celdelingssysteem.

In **Hoofdstuk 2** bespreken we alle eerder gepubliceerde werken over het Cdv-systeem. We bekijken het systeem als geheel, beginnend met te begrijpen welke archaea een dergelijk systeem vormen en welke relatie ze hebben met eukaryoten. Het Cdv-systeem wordt gevonden in veel verschillende soorten archaea, maar in de Crenarchaea is het geslacht Sulfolobus naar voren gekomen als het model om het beter te bestuderen. We lichten daarom alle relevante werken toe die met deze modellen zijn gepubliceerd en gaan dieper in op alle verschillende rollen die het Cdv-systeem daarin speelt, van celdeling tot membraanbelletjesafscheiding. Ten slotte beschrijven we hoe het momenteel wordt voorgesteld dat deze eiwitten werken, en proberen we het samen te vatten met een goed model voor de mechanistische werking ervan.

In **Hoofdstuk 3** karakteriseren we voor het eerst in vitro de CdvB1 van Metallosphaera sedula. Onlangs werd gemeld dat dit eiwit cruciaal is tijdens het proces van membraanvernauwing, en we beschrijven hoe het, in tegenstelling tot zijn paraloog CdvB, spontaan filamenten kan vormen. We hebben onderzocht hoe deze filamenten werden

gedepolymeriseerd door de werking van de AAA ATPase CdvC, en rapporteerden voor het eerst de depolymerisatie van een Cdv-eiwitfilament door CdvC. Tegelijkertijd hebben we ook beschreven hoe CdvB1-eiwit in staat was te binden aan negatief geladen liposomen, en hoe de werking van CdvC het eiwit van het membraan kon losmaken. Dit membraanbindend vermogen werd echter alleen vertoond door het eiwit in zijn monomere toestand. Eenmaal gepolymeriseerd, konden de eiwitfilamenten het membraan niet meer binden, en hiermee hebben we een parallel vastgesteld met de menselijke ESCRT-III-eiwitten CHMP2A en CHMP3, waar hetzelfde gedrag wordt waargenomen. We proberen dan alles samen te vatten in een hypothetisch model dat de observaties zou verklaren.

In **Hoofdstuk 4** probeerden we de verschillende interacties tussen de componenten van het Cdv-systeem te begrijpen, en hoe deze het gedrag van de eiwitten beïnvloeden. We realiseerden ons voor het eerst dat, hoewel eerder was gemeld dat CdvA een membraanbindend eiwit was, het eigenlijk een interactie met CdvB moet aangaan om dit te doen. Vervolgens hebben we in vitro de CdvB-paraloog CdvB2 gekarakteriseerd en we zagen dat het eiwit, net als CdvB, geen filamenten vormt in zijn volledige conformatie. Bij het verwijderen van het C-terminusdomein was het eiwit echter in staat te polymeriseren. Gezuiverd CdvB2 kan op zichzelf niet aan lipidemembranen binden en, interessant genoeg, terwijl CdvB1 membranen direct kan binden, kon het resulterende polymeer bij interactie met CdvB2 niet meer aan lipidemembranen binden. Zowel CdvB2 als de CdvB1:CdvB2-copolymeren worden echter naar het membraan gerekruteerd door het CdvA:CdvB-copolymeer. Hiermee kregen we een beter idee van het landschap van verschillende Cdv-eiwitinteracties, en hoe deze de rekrutering naar het membraan beïnvloeden. We besloten toen om naar deze interacties te kijken met de confocale microscoop en de eiwitten in te kapselen in liposomen die kunstmatig werden vervormd tot halters. Hierdoor ontstonden membraanhalzen die de verschillende halters met elkaar verbond, waardoor gelokaliseerde punten met een hoge kromming werden gecreëerd die ons in staat stelden te observeren dat de Cdv-eiwitten de neiging hebben om zich in deze gebieden met een hoge kromming te lokaliseren, op dezelfde manier als veel ESCRT-eiwitten.

In **Hoofdstuk 5** sluiten we af met na te denken over wat de toekomstige vragen zijn die vanuit het Cdv-systeem moeten worden opgelost en welke paden dit onderzoeksgebied waarschijnlijk zal inslaan. We vragen ons ook af of de Cdv-eiwitten inderdaad goede kandidaten lijken voor een synthetisch celdelingssysteem, en wat de realistische alternatieven daarvoor zijn.

Concluderend gaat dit proefschrift dieper in op het Cdv-systeem, dat lang onderbelicht is geweest en dat veel vragen heeft die beantwoord moeten worden. We maken een overzicht van de huidige kennis over het systeem en wijzen de ontbrekende vragen aan. We karakteriseren de vorming van CdvB1-filamenten en de depolymerisatie ervan door CdvC, die cruciale stappen zijn tijdens het celdelingsproces. We beschrijven beter de interacties

tussen de verschillende eiwitten van het systeem, en hoe deze de rekrutering van eiwitten naar het membraan beïnvloeden.

SUMMARY

All living organisms share the need to replicate and proliferate to ensure the survival of their species. In prokaryotes, this is generally guaranteed by a process of cell division where a mother cell is split into two equally sized daughter cells, and it is a complex and heterogeneous process across all the different species. When looking into the Crenarchaea phylum of the archaea, we find a very particular set of proteins that are responsible for orchestrating this process of cell division: the Cdv system. This system is closely related to the ESCRT machinery, which is also responsible for cell division and many other membrane deforming processes in eukaryotes. This close similarity is one of the many common traits that point towards a common origin between archaea and eukaryotes. Although the eukaryotic machinery has been thoroughly and extensively studied, very little is known about the archaeal division system. For this reason, in this work we aimed at better understanding these archaeal proteins, making use of *in vitro* techniques, with the long-term view of using them to build a synthetic cell form the bottom up.

In **Chapter 1**, we present what minimal cells are, explaining the nature of a bottom up approach to build them, and why that is important to better understand life at its core. More specifically, we take a closer look into the different approaches that have been previously made to reconstitute cell division machineries from bacteria and eukaryotes *in vitro*, and reviewing the advantages and weaknesses of these systems. Finally, we explore why is the Cdv system actually an interesting alternative for a synthetic cell division system.

In **Chapter 2**, we review all the previous published works about the Cdv system. We take a look at the system as a whole, starting by understanding which are the archaea that present such a system, and what relation they hold with eukaryotes. The Cdv system has evolved to be found in many different species of archaea, but in the Crenarchaea, the genus of Sulfolobus has emerged as the model for better studying it. We therefore explain all the relevant works that have been published with these models, and take a closer look at all the different roles the Cdv system play in them, from cell division to vesicle budding. Finally, we describe how it is currently envisioned that these proteins work, and try to summarize it with a good model for its mechanistic action.

In **Chapter 3**, we characterize *in vitro,* for the first time, the CdvB1 from *Metallosphaera sedula.* This protein was recently reported to be crucial during the process of membrane constriction, and we describe how, unlike its paralog CdvB, it can filament spontaneously. We studied how these filaments were depolymerized by the action of the AAA ATPase CdvC, reporting for the first time the depolymerization of a Cdv protein filament by CdvC. At the same time, we also described how CdvB1 protein was capable of binding to negatively

charged liposomes, and how the action of CdvC could detach the protein from the membrane. This membrane binding ability, however, was only presented by the protein in its monomeric state. Once polymerized, the protein filaments could not bind the membrane anymore, and we established a parallelism with the human ESCRT-III proteins CHMP2A and CHMP3, where the same behavior is observed. We finally then try to summarize everything into a possible model of action that would explain the observed behaviors.

In **Chapter 4**, we sought to understand the different interactions between the components of the Cdv system, and how these affect the behaviors of the proteins. We first realized that, while it had been previously reported that CdvA was a membrane binding protein, it actually needs to interact with CdvB in order to do so. We then characterized *in vitro* the CdvB paralog CdvB2, and we saw that just like CdvB, the protein does not filament in its full-size conformation. However, when removing the C-terminus domain, the protein was capable of polymerizing. Purified CdvB2 is not capable of binding to lipid membranes on its own and, interestingly, while CdvB1 can bind membranes directly, when interacting with CdvB2 the resulting polymer was uncapable of binding to lipid membranes anymore. However, both CdvB2 and the CdvB1:CdvB2 co-polymers are recruited to the membrane by the CdvA:CdvB copolymer. With this, we obtained a better idea of the landscape of different Cdv protein interactions, and how these affect the recruitment to the membrane. We then decided to look at these interactions with the confocal microscope, encapsulating them in liposomes that were artificially shaped into dumbbells. This created membrane necks that connected the different dumbbells, creating localized points of high curvature that allowed us to observe that the Cdv proteins present a tendency to localize in these high-curvature regions, the same way that many ESCRT proteins do.

In **Chapter 5**, we finalize by thinking about what are the future questions that are to be solved from the Cdv system, and which paths will this field of research likely take. We also ask ourselves if the Cdv proteins seem to be indeed good candidates for a synthetic cell divisome, and what are the realistic alternatives to it.

To conclude, this thesis looks deeper into the Cdv system, which has been long understudied and that has many questions to be answered. We make an overview of the current knowledge on the system, and point the missing questions. We characterize the CdvB1 filament formation and its depolymerization by CdvC, which are crucial steps during the cell division process. We describe better the interactions between the different proteins of the system, and how these affect the recruitment of proteins to the membrane.

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There is a famous Spanish poem by Antonio Machado, that roughly translates as “Traveler there is no path, you open the path as you walk. As you walk the path opens, and when you turn your view around, you see the path that shall never be walked again”. (*Caminante no hay camino, se hace camino al andar. Al andar se hace camino, y al volver la vista atrás, se ve la senda que nunca se ha de volver a pisar*). I have always felt it represents in the clearest way possible the journey of a PhD. There is no clear path forward when you start, you make it as you go, and now that I am finishing it, I can look back and see all of the steps I took along the way. And when I think about it, the one thing that I am the most grateful for, is that I never took one of those steps alone.

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LIST OF PUBLICATIONS

1. A. Blanch Jover, N. De Franceschi, C. Dekker CdvA-CdvB protein interactions drive assembly of the Cdv division ring in archaea, in preparation.

2. A. Blanch Jover, C. Dekker, The Archaeal Cell Division System, Trends in Microbiology, under review.

3. A. Blanch Jover, N. De Franceschi, D. Fenel, W. Weissenhorn, C. Dekker, The archaeal division protein CdvB1 assembles into polymers that are depolymerized by CdvC. FEBS Lett. (2022), doi:10.1002/1873-3468.14324