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Molecular view on the spatiotemporal organization of *Bacillus subtilis* subcellular compartments

vorgelegt von
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Wenn de jeck weeß, fängk et em Kopp aan.

Bekanntes kölsches Sprichwort

Content:

I SUMMARY

All living cells are highly organized and exhibit complex cellular machineries facilitating biochemical reactions. Compartmentalization is a prerequisite to allocate an appropriate environment for these processes. In this work, compartments that are involved in *Bacillus subtilis* membrane organization and cell division were studied. *B. subtilis* division site selection is dependent on the nucleoid occlusion and the Min system. The *B. subtilis* Min system consists of four components. MinC is the actual inhibitor of the tubulin homologue FtsZ that is a crucial component of the divisome, forming the so called Z-ring. MinC is bound to the ATPase MinD that is tethered via the adapter protein MinJ to DivIVA. DivIVA senses membrane curvature and was supposed to be stably tethered to the cell poles. Thereby a stable, static DivIVA / MinJDC gradient with minimum concentration at midcell is formed. Using advanced microscopy techniques like single cell time lapse microscopy, fluorescence recovery after photo bleaching and by utilization of photo-activatable / convertible fluorophores we could demonstrate that DivIVA is in vegetative cells recruited from the cell pole to mature septa. These data provide first evidence that the role of the *B. subtilis* Min system is not to define midcell, but prevents reinitiation of Z-ring constriction after fulfilled division. Utilizing single cell time lapse microscopy we could further demonstrate that proteins crucial to condense the chromosome are vital for correct chromosome segregation during cell division by influencing the replication fork velocity or resolution.

As a second compartment *B. subtilis* flotillin dependent membrane microdomains were studied. These domains are likely scaffolded by the membrane protein flotillin. This protein is pinned to the membrane via a hairpin loop as shown by SNAP–tag labelling experiments. Utilizing the anisotropic dye Laurdan we could further show spectroscopically and microscopically that flotillins prevent condensation of microdomains. Flotillin deletion strains also exhibit a generally more liquid ordered membrane compared to wild type cells. Using co–immunoprecipitation experiments several proteins interacting with flotillin were identified. These interactions were confirmed with microscopical co–localization analysis. *B. subtilis* flotillin was additionally heterologously purified via affinity chromatography. The purified protein creates large homo–oligomers likely in mega Dalton size. Using truncation mutants it could be shown that flotillin oligomerizes via a flotillin specific domain, namely the PHB domain. Though, contrary to eukaryotic cells, *B. subtilis* PHB domain does not contribute to lipid binding. However, several cellular machineries that interact with flotillins, as exemplary shown for the secretion machinery, are impaired in their functionality in absence of flotillins. These data provide first evidence that prokaryotic flotillins are elements that scaffold the plasma membrane and thereby provide a lipid environment that is vital for correct functionality of diverse cellular machineries.

II ZUSAMMENFASSUNG

Die räumliche und zeitliche Trennung von komplexen biologischen Prozessen durch Kompartimentalisierung bildet eine Grundlage für die Funktionalität von Zellen. In dieser Arbeit wurden verschiedene Kompartimente des Bakteriums *Bacillus subtilis* untersucht. Insbesondere Domänen die in der Membran und während der Zellteilung von *B. subtilis* vorzufinden sind wurden analysiert. Die Zellmitte wird in *B. subtilis* durch zwei unterschiedliche Systeme definiert, das Nucleoid Okklusions System und das Min System. Das Min System besteht aus vier Komponenten. MinC ist der Inhibitor des Tubulin Homologs FtsZ, welches ein zentraler Bestandteil des Divisoms ist und den Z-Ring bildet. MinC ist gebunden an die ATPase MinD die über das Adapterprotein MinJ an DivIVA gebunden ist. DivIVA bindet an gekrümmte Membranen und es wurde vermutet, dass es stabil an den Zellpol gebunden ist wodurch ein statischer DivIVA / MinJDC Gradient mit minimaler Konzentration in der Zellmitte entsteht. Durch die Verwendung fortgeschrittener Mikroskopietechniken, insbesondere durch die Verwendung von Foto–aktivierbaren / konvertierbaren Fluorophoren, konnte hier gezeigt werden, dass in vegetativen Zellen DivIVA vom Zellpol zum Septum rekrutiert wird. Diese Daten implizieren, dass das *B. subtilis* Min System nicht für die Findung der Zellmitte zuständig ist, sondern eine erneute Konstriktion des Z-Ringes nach vollzogener Zellteilung verhindert. Mittels Einzelzellenzeitreihenmikrosopie konnte außerdem gezeigt werden, dass Proteine, die bei der Chromosomkondensation involviert sind, auch für die korrekte Chromosomensegregation während der Zellteilung zuständig sind, da diese Proteine einen direkten Einfluss auf die Replikationsgabel haben.

Als ein weiteres Kompartiment wurden *B. subtilis* Membran-Mikrodomänen (Md) untersucht. Diese Md werden wahrscheinlich von dem Protein Flotillin gebildet. Flotillin bindet mittels einer Haarnadelschleife an die Membran, was mit Hilfe von SNAP–Markierungsexperimenten gezeigt wurde. Mit Hilfe des anisotropischen Farbstoffes Laurdan konnte spektroskopisch und mikroskopisch gezeigt werden, dass Flotilline ein Verschmelzen von Md verhindern. Flotilline halten somit eine Membranheterogenität aufrecht. Nach Deletion von Flotillinen war die Membran generell stärker kondensiert. Mittels Co-immunpräzipitationsexperimenten konnten zudem verschiedene Flotillin-Interaktionspartner identifiziert werden, die mikroskopisch mittels Kolokalisationsexerimenten bestätigt wurden. Die Abwesenheit von Flotillinen *in vivo* beeinflusst die Funktionalität verschiedener zellulärer Maschinen, was beispielhaft für das Sec-System gezeigt wurde. Des Weiteren wurde Flotillin heterolog exprimiert und gereinigt, wodurch gezeigt werden konnte, dass es große Oligomere in MDa Größe bildet. Durch die Reinigung von verkürzten Flotillin-Varianten konnte demonstriert werden, dass die Oligomerisierung über die PHB Domäne geschieht. Diese kann jedoch nicht an Lipide binden. Diese Daten implizieren das Flotilline nötig sind, um eine korrekte Lipidumgebung zu schaffen, die für die Funktionalität von verschiedenen Proteinen nötig ist.

III ABBREVIATIONS

3D-SIM = 3 dimensional - structured illumination microscopy

B. subtilis = *Bacillus subtilis*

Cl = cardiolipin

Dil = 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate

DNA = deoxyribonucleic acid

DPH = 1,6-diphenyl-1,3,5-hexatriene

DRMs = detergent resistant membranes

E. coli = *Escherichia coli*

FLIM = fluorescence life time imaging

FRAP = Fluorescence Recovery after Photobleaching

GFP = green fluorescent protein

GP = generalized polarisation

GUVs = giant unilamellar vesicles

Laurdan = 6-Dodecanoyl-2-Dimethylaminonaphthalene

ld = liquid disordered

lo = liquid ordered

LPG = Lysylphosphatidylglycerol

Md = Membran Mikrodomänen

NAO = 10-N-nonyl acridine orange

NAP = naphtho[2,3-a]pyrene

NBD-DPPE = 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[7-nitro-2-1,3-benzoxadiazol-4-yl]

noc = nucleoid occlusion

oriC = replication origin

PALM = photo-activated localization microscopy

PBP = penicillin binding proteins

PC = phosphatidylcholine

PE = phosphatidylethanolamine

PG = phosphatidylglycerol

PHB = prohibitin

PS = phosphatidylserine

PSF = point spread function

RIF = regions of increased fluidity

SCTLM = single cell time lapse microscopy

SPFH = stomatin/prohibitin/flotillin/HflKC

STED = stimulated emission depletion

STORM = stochastic optical reconstruction microscopy

TIRF = total internal fluorescence

TR-DPPE = Texas Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine

IV LIST OF PUBLICATIONS

Major parts of this thesis are published in the following publications:

[1] Gruber, S., Veening, J. W., **Bach, J**., Blettinger, M., Bramkamp, M., and Errington, J. (2014) Interlinked sister chromosomes arise in the absence of condensin during fast replication in *B. subtilis*, Curr Biol 24, 293-298.

[2] **Bach, J. N**., Albrecht, N., and Bramkamp, M. (2014) Imaging DivIVA dynamics using photo-convertible and activatable fluorophores in *Bacillus subtilis*, Front Microbiol 5, 59.

[3] **Bach, J. N**., and Bramkamp, M. (2013) Flotillins functionally organize the bacterial membrane, Mol Microbiol 88, 1205-1217.

[4] **Bach, J. N**., and Bramkamp, M. (2015). Dissecting the molecular properties of prokaryotic flotillins. PloS one 10, e0116750.

V DECLARATION OF INDIVIDUAL CONTRIBUTIONS

[1] Gruber, S., Veening, J. W., **Bach, J**., Blettinger, M., Bramkamp, M., and Errington, J. (2014) Interlinked sister chromosomes arise in the absence of condensin during fast replication in *B. subtilis*, Curr Biol 24, 293-298.

Juri Niño Bach has performed parts of the time-lapse experiments / microscopy presented in this publication and contributed to the figures 4 A/B; S4 A/B.

[2] **Bach, J. N**., Albrecht, N., and Bramkamp, M. (2014) Imaging DivIVA dynamics using photo-convertible and activatable fluorophores in *Bacillus subtilis*, Front Microbiol 5, 59.

Juri Niño Bach did all experiments shown in this publication with exception of cloning the plasmid pJPR1-DivIVA-Dendra. He co-wrote all parts of the paper and created all figures.

[3] **Bach, J. N**., and Bramkamp, M. (2013) Flotillins functionally organize the bacterial membrane, Mol Microbiol 88, 1205-1217.

Juri Niño Bach did all experiments shown in this publication, co-wrote all parts of the paper and created all figures.

[4] **Bach, J. N**., and Bramkamp, M. (2015). Dissecting the molecular properties of prokaryotic flotillins. PloS one 10, e0116750.

Juri Niño Bach did all experiments shown in this publication, co-wrote all parts of the paper and created all figures.

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1. INTRODUCTION

In this work the compartmentalization of the prokaryotic model organism *Bacillus subtilis* was studied. Compartmentalization is a phenomenon known from pro- and eukaryotes. It allows cells to allocate a specific environment for diverse cellular machineries. Compartmentalization makes it possible to spatially separate processes through borders created by lipid bilayers or by simply formed gradients and protein assemblies. Hence, this is an essential prerequisite for complex processes and the high degree of organisation in living organisms.

1.1 Compartmentalization

Compartmentalization is a long known phenomenon that goes back to the 19th century, but first ideas about the complex organization of live were already made in the 17th – 18th century with the invention of light microscopy. The first descriptions of cellular organization were already made by Athanasius Kircher 1658 and few decades later by Jan Swammerdam (Kirchner, 1658; Swammerdam, 1737; Mazzarello, 1999). But it was Robert Brown who mentioned 1833 the very first time the organization of distinct cellular components (Brown, 1833).

The actual definition of a compartment is that a distinct domain inside living cells is created. This might occur via diffusion barriers, gradients, by protein-protein interactions that create reaction cascades in close spatial proximity or by the physical separation of distinct spaces by protein or lipid layers (Ovadi and Saks, 2004; Diekmann and Pereira-Leal, 2013).

The most known compartments are organelles that have developed through symbiosis, what is also named the endosymbiotic theory (Sagan, 1967; Doolittle and Brown, 1994; Lopez-Garcia and Moreira, 1999). The endosymbiotic theory comprehends that a progenitor cell took up another cell, likely an early prokaryotic cell, and kept it as an endosymbiont. The genome of this endosymbiont was evolutionary reduced but preserved in the organelle (Sagan, 1967; Doolittle and Brown, 1994; Andersson et al., 1998; Sicheritz-Ponten et al., 1998; Kurland and Andersson, 2000; Dagan et al., 2013).

1.1.1 Eukaryotic compartmentalization

Some of the most prominent organelles are mitochondria and chloroplasts. Mitochondria developed through endosymbiosis of the alphaproteobacteria SAR11 clade and chloroplasts originated from cyanobacteria (Raven, 1970; Taylor, 1970; Thrash et al., 2011).

Beside typical organelles like the nucleus, mitochondria, endoplasmatic reticulum, golgi apparatus, vacuoles and chloroplasts many more structures that exhibit a remarkable degree of organisation exist. Organelles are compartments that are enclosed by a lipid bilayer. Inside this bilayer a specialized aqueous space exists that allocates the environment for complex biochemical processes (Alberts et al., 2003). Contrary to organelles compartments not necessary have to be surrounded by a lipid bilayer, but compartments are also able to separate reactions in space and time exemplary by protein assemblies separating the inner space of a assembly from the cytoplasm. Although the definition how to term these structures is still controversial, it is accepted that distinct compartments with a high degree of organisation exist. Among these compartments in eukaryotic cells are the proteasome, diverse cytoskeletal elements, ribosomes and others. The existence of compartments inside a cell has long been considered to be unique to eukaryotic cells, though it could be shown that many of these and other compartments also exist in bacteria (Diekmann and Pereira-Leal, 2013).

1.1.2 Prokaryotic compartmentalization

Contrary to early assumptions bacteria exhibit a high degree of molecular organisation (Figure 1). Among the most prominent compartments that exist in all prokaryotes are the membrane, ribosomes and the nucleoid. Exemplary the chromosome needs to be highly organized and compacted up to the 10^3 fold to fit into the limited size of the cell (Holmes and Cozzarelli, 2000; Wang et al., 2013). Chromosomal topological domains are created in an average size of 10 kb (Higgins et al., 1996; Postow et al., 2004). Further distinct protein cluster like Muk assembly, SMC and HU proteins maintain the chromosome in a condensed state (Rouviere-Yaniv et al., 1979; Larionov et al., 1985; Yamazoe et al., 1999; Cui et al., 2008). Condensin, that is a SMC – kleinsin complex, is essential in eukaryotes and deletion results in a severe growth phenotype in prokaryotes (Niki et al., 1991; Hirano and Mitchison, 1994; Britton et al., 1998). In *B. subtilis* condensing complexes are enriched on the chromosome near the origin of replication (*oriC*) by Spo0J (ParB) bound to *parS* sites (Gruber and Errington, 2009; Sullivan et al., 2009). Depletion of condensin in *B. subtilis* results in a defect in partitioning the chromosomes. This phenotype cannot be repressed by inhibiting transcription or translation but it can be by artificial reduction of the velocity of the replication fork (Gruber et al.,

2014). This indicates that condensin creates distinct functional domains at or behind the replication fork.

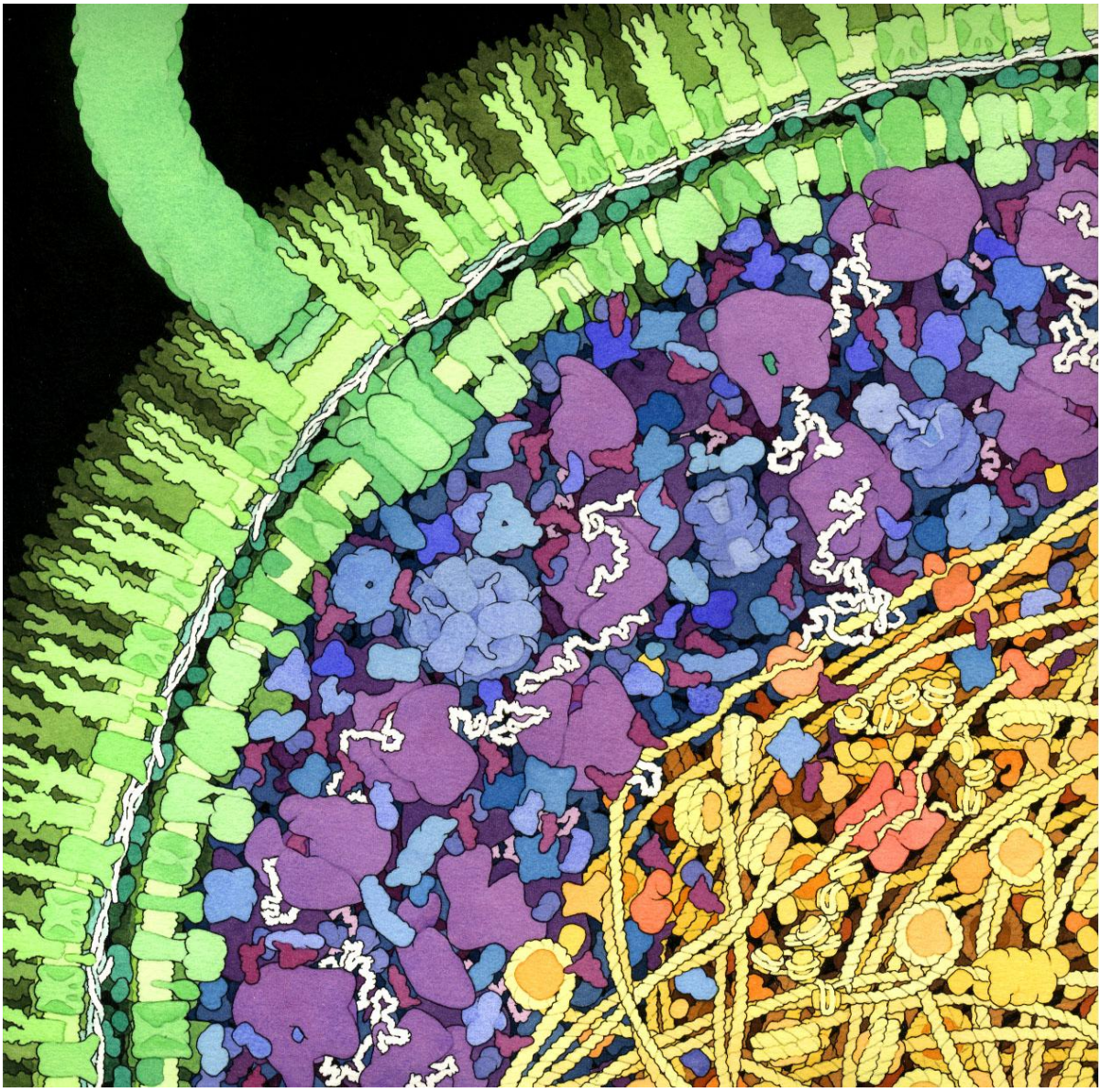


Figure 1: Compartmentalisation of an *E. coli* cell

Shown is a cross section of the molecular setup of an *E. coli* cell. The cell wall, the inner and the outer membrane are shown in green. A flagellum (also green) penetrates the cell wall and membranes ending in the cytoplasmic flagellar motor. Cytoplasmic machineries are shown in blue (various enzymes) and purple (ribosomes). mRNA is drawn as white strands and tRNA as L-shaped maroon molecules. The chromosomal region is shown in yellow (DNA, partially wrapped around HU proteins) and orange (replication fork with DNA polymerase)[1].

[1] Picture modified and used with kind permission of David S. Goodsell "David S. Goodsell, the Scripps Research Institute."

Also protein machineries like the transcription machinery directly act on the chromosome. Another domain that is formed by (deoxy) ribonucleic acid are plasmids that can be found as a distinct compartment in many bacterial phyla (Dubnau and Stocker, 1964; Richmond, 1965; Novick, 1967; Carattoli, 2013).

Many cellular machineries, as for example protease complexes, form compartments inside the prokaryotic cells similar to eukaryotic protease complexes. Notably, Clp proteases can also be considered as chaperone like structures. Beside classical functions of chaperones to support proper folding of proteins also proteolysis performed by Clp proteases in *B. subtilis* is a crucial mechanism to protect the cell against stress conditions that result in protein unfolding and protein aggregation (Moliere and Turgay, 2009).

In *B. subtilis* proteolysis is mostly performed by the so called Clp machinery. If the Clp protease complex turns active, a hexameric structure is formed creating a proteolytic chamber. The substrate is unfolded in an ATP – dependent manner, transported into the proteolytic chamber and finally degraded into small peptides (Dougan et al., 2002; Baker and Sauer, 2006). Notably, also chaperones form well defined oligomers that are responsible to disaggregate and refold protein complexes, in *B. subtilis* these are mainly GroEL/GroES, DnaK/DnaJ/GrpE, trigger factor and Hsp90/HtpG, (Weibezahn et al., 2005; Moliere and Turgay, 2009).

A large assembly that is formed in many bacteria is the flagellum that in gram-negativ bacteria crosses the cell wall, the periplasm, two membranes and originates from the cytoplasm. The flagella hook of *B. subtilis* alone exceeds a size of 70 nm (Kubori et al., 1997). More than 20 different proteins have to be assembled before a functional flagellum is formed (Erhardt et al., 2010). A question that could barely be solved so far is how it is possible that huge machineries like the flagellum can be assembled outside the cell. Recent investigations reveal that a transport mechanism is created that results in export of single flagella subunits that immediately incorporate into the extracellular flagellum (Evans et al., 2013). Even though, single subunits have to partially transported more than 20 µm before they are incorporated (Evans et al., 2014). All these findings indicate that flagella are complex, sophisticated machineries that require a high degree of organization.

A compartment that is crucial for mostly all prokaryotes is the cell division machinery and the cell wall synthetic machinery since they are essential for prokaryotic division.

1.2 *B. subtilis* cell division machinery

Cell division is one of the most crucial cellular events in all living organisms. During division diverse elements of the cytoskeleton assemble in large, hetero-oligomeric complexes that form distinct compartments facilitating cell division (Oliferenko et al., 2009). Also the spatiotemporal regulation of this event has to be tightly regulated with other cellular events as for example cell wall synthesis in fungi and prokaryotes (Oliferenko et al., 2009). In *B. subtilis* diverse components of the cell division machinery are recruited stepwise in a cascade of molecular events. One of the most important proteins for cell division in *B. subtilis* and *E. coli* is the essential tubulin-like protein FtsZ that assembles into a ring structure, the so called Z-ring, exactly at midcell (Bi and Lutkenhaus, 1991). The Z-ring also serves as a scaffolding element for other components downstream in the cell division machinery that together form the divisome (Bi and Lutkenhaus, 1991). FtsZ is structurally similar to tubulin and exhibits a self-activating GTPase activity (Adams and Errington, 2009), necessary for polymerisation of the Z-ring (Scheffers et al., 2002). The divisome itself is composed of various proteins that range from cytosolic proteins acting as scaffolds to integral membrane proteins that are mostly involved in cell wall synthesis (Carballido-Lopez and Formstone, 2007). Post polymerisation of FtsZ the membrane associated protein FtsA is recruited and tethers FtsZ to the membrane (Pichoff and Lutkenhaus, 2005). Notably, FtsA also recruits further downstream components of the *B. subtilis* divisome (Adams and Errington, 2009). The proteins SepF and ZapA promote polymerisation of FtsZ (Gueiros-Filho and Losick, 2002; Hamoen et al., 2006; Singh et al., 2008). After constriction of the Z-ring the trans-membrane protein EzrA is recruited (Haeusser et al., 2004). The exact function of EzrA is not known, but a deletion of EzrA leads to the formation of multiple Z-rings after complete cell division and mini cell formation (Levin et al., 1999). Hence, it seems likely that EzrA is involved in disassembly of the divisome or inhibits polymerisation of the Z-ring. After assembly of the divisome components further proteins like FtsL, penicillin binding proteins (PBP), DivIB, DivIC and DivIVA are recruited to the divisome (Gamba et al., 2009). The functions of DivIB and DivIC are mostly unknown, but it is known that DivIB, DivIC and FtsL interact with PBP2B and likely regulate the activity of PBPs (Wadsworth et al., 2008; Rowland et al., 2010). FtsL is one of the rate-limiting proteins during division and processed via regulated intramembrane proteolysis and protected by DivIC (Bramkamp et al., 2006; Wadenpohl and Bramkamp, 2010). DivIVA is recruited after fulfilled division to the divisome since it recognizes curved membrane that start to invaginate after Z–ring constriction. Importantly, DivIVA also belongs to the division site selection machinery in *B. subtilis*.

1.2.1 Division site selection

Cell division in bacteria has to be tightly regulated in space and time. It is vital for a cell to receive complete functional chromosomes; hence chromosome segregation has to take place before division is complete. Few is known about the actual timing of cell division in *B. subtilis*, but it is partially regulated by the protein UgtP that senses nutrient availability and directly interacts with the divisome. UgtP is therefore one component of the temporal regulation of cell division that directly links the nutritional state to the cell cycle (Weart et al., 2007; Chien et al., 2012). Contrary to the temporal components that regulate cell division the spatial components are well investigated. The two major regulatory mechanisms in *B. subtilis* regulating spatial cell division are the Min system and the nucleoid occlusion (noc) system (Monahan et al., 2014).

1.2.1.1 Nucleoid occlusion

The noc system is part of the division site selection mechanisms in *B. subtilis* and prevents division across the nucleoid. Initially it was proposed that DNA itself might prevent constriction of the Z-ring over the nucleoid (Mulder and Woldringh, 1989; Harry, 2001). Even though it is still a matter of debate if DNA is sufficient to inhibit constriction of the Z-ring (Moriya et al., 2010), proteins could be identified that bind DNA and inhibit Z-ring formation (Wu and Errington, 2004). Namely these are the proteins Noc in *B. subtilis* and the same function is fulfilled by SlmA in *E. coli* (Wu and Errington, 2004; Bernhardt and de Boer, 2005). Noc binds specific consensus DNA sequences (~74 binding sites per chromosome) mostly homogenously distributed over the chromosome, but absent from the terminus of the chromosome (Wu et al., 2009). Extra artificial binding sites of Noc at the terminus of the chromosome or over expression resulted in delayed division. Hence, also a temporal regulatory function of noc is assumed but not finally proven so far (Wu et al., 2009). Recent studies revealed that Z-ring formation preferentially takes place over unreplicated nucleoids with a bilobed morphology (in *B. subtilis* equivalent to low DNA concentration at midcell) (Moriya et al., 2010). However, no direct interaction of Noc and the divisome could be shown so far. Deletion of Noc together with parts of the Min system, as shown for double deletion *noc; minD*, resulted in altered formation of Z-rings over the segregated nucleoids (Wu and Errington, 2004).

1.2.1.2 The Min system

The second system required for spatial regulation of cell division is the Min system. Absence of the Min system leads to small, DNA-free mini cells (Adler et al., 1967). The best understood Min system is from *E. coli*. The *E. coli* Min system exists of the three proteins MinC, MinD and MinE (de Boer et

al., 1989). Deletion of one or more of these proteins results in the formation of mini cells due to constant reinitiation of FtsZ filament polymerisation after complete division (de Boer et al., 1990, 1992). MinC is the actual inhibitor of FtsZ and binds via two different domains to FtsZ. The N'-terminal domain of MinC binds independent of MinD to FtsZ and thereby inhibits the lateral interaction of FtsZ oligomers (Hu and Lutkenhaus, 2000; Scheffers, 2008). The second binding site of MinC to FtsZ is located in the C'-terminal domain. It binds to the same consensus sequence of FtsZ that also binds to FtsA. Hence, MinC directly competes with FtsA for FtsZ binding (Shen and Lutkenhaus, 2009). Via its C-terminal domain MinC interacts with itself and MinD (Hu and Lutkenhaus, 2000). For full functionality of MinC the presence of MinD is required. MinD belongs to the MinD/ParA family of Walker type ATPases. MinD dimerizes in the presence of ATP and binds in its ATP-bound state to the membrane (Hu et al., 2003; Lackner et al., 2003). After dimerization and membrane-binding of MinD, the actual inhibitor of Z-ring formation MinC is recruited and the functional MinCD complex is formed (Lackner et al., 2003). The MinCD complex dissociates after hydrolysis of ATP from the membrane and also the interaction of MinC and MinD disintegrates (Hu and Lutkenhaus, 2000; Hu et al., 2003). This process is mediated by MinE that oscillates between both cell poles and triggers ATP hydrolysis of MinD (Rowland et al., 2000; Fu et al., 2001). This results in an oscillating MinCD system with a stochastic minimum concentration of MinCD at midcell and highest concentration at the cell pole (Figure 2). Because of the minimum concentration of MinC at midcell this is the place where the Z-ring is formed (Halatek and Frey, 2012).

The Min system in *B. subtilis* is different organized compared to the *E. coli* Min system. It consists of four major components, namely DivIVA, MinJ, MinD and MinC. All of these proteins do self-interact and follow a distinct hierarchical interaction order (Bramkamp et al., 2008; Patrick and Kearns, 2008; van Baarle and Bramkamp, 2010). DivIVA interacts with MinJ, MinJ interacts with DivIVA and MinD, MinD interacts with MinJ and MinC. Similar to *E. coli*, in *B. subtilis* MinC is the actual inhibitor of Z-ring formation but N'- as well as the C'-terminal domain of MinC bind to different regions of FtsZ (Blasios et al., 2013). The architecture of *B. subtilis* MinD is similar to *E. coli* MinD, but till now it remains elusive what the function of the ATPase of *B. subtilis* MinD is. It is assumed that hydrolysis of ATP might control the oligomeric state of some Min components (Karoui and Errington, 2001). MinJ was found to be an adapter protein between DivIVA and MinD (Bramkamp et al., 2008; Patrick and Kearns, 2008). DivIVA is the topological factor of the *B. subtilis* Min system. It senses and binds negatively curved membranes and hence is accumulating at the cell pole and, in a ring like structure, to new forming septa (Edwards and Errington, 1997; Lenarcic et al., 2009; Eswaramoorthy et al., 2011). Notably, DivIVA is not sufficient to impose curvature (Lenarcic et al., 2009).

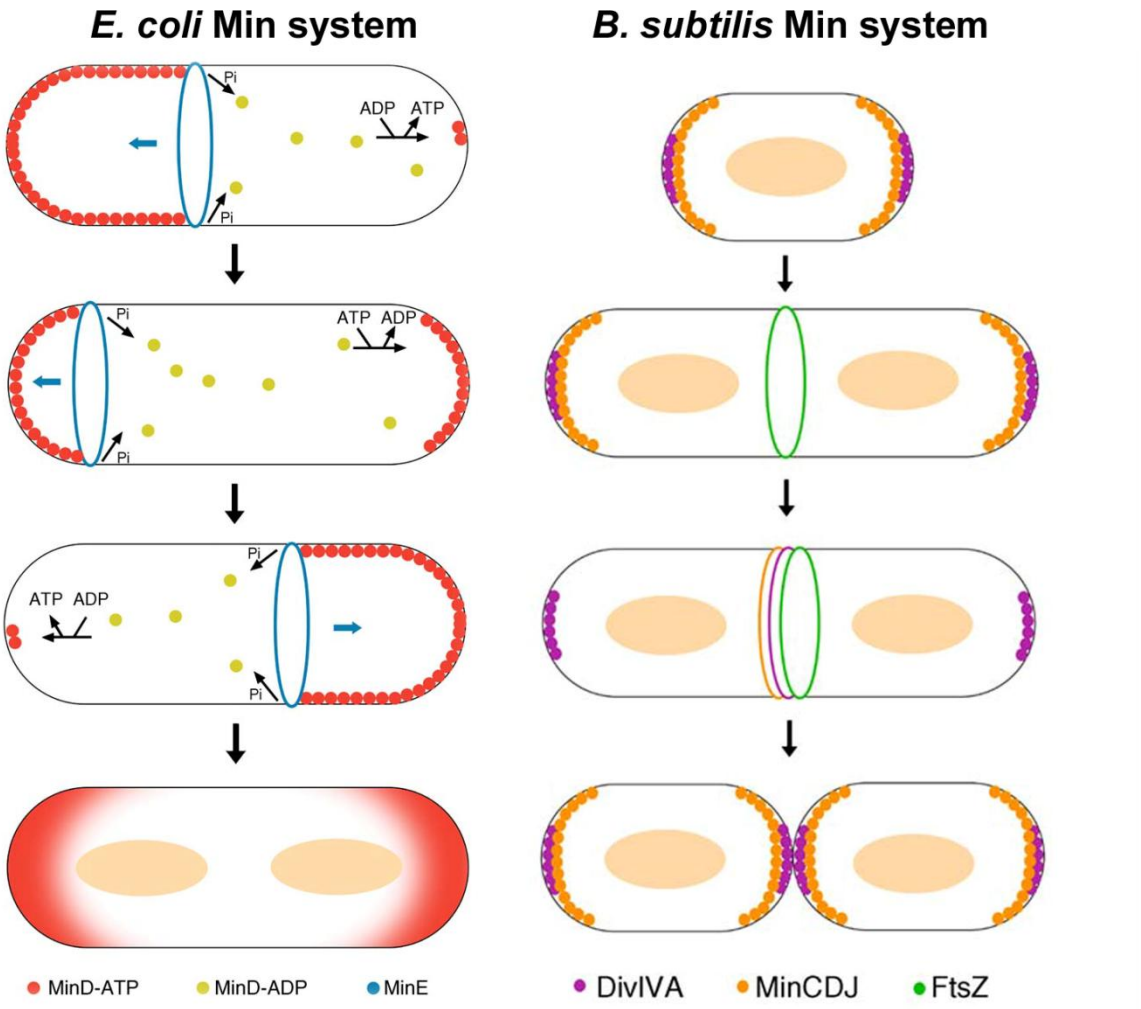


Figure 2: Comparison of the *E. coli* and *B. subtilis* Min system

On the left the *E. coli* Min system is shown. MinD in its ATP-bound form interacts with MinC and binds to the membrane. MinC dissociates from MinD after ATP hydrolysis and MinD dissociates from the membrane. MinE triggers this process by stimulating ATP hydrolysis. This oscillating system results in a stochastic minimum MinCD concentration at midcell. Contrary, the *B. subtilis* Min system is static and does not oscillate. In *B. subtilis* the topological factor DivIVA is stably attached to the cell pole and recruits the MinCDJ complex. This stable gradient results in a minimum concentration of MinCDJ at at midcell. Figure modified from Bramkamp and van Baarle, 2009.

Contrary to the *E. coli* Min system the *B. subtilis* Min system is attempt to be static and a simple gradient is formed with maximum concentration of DivIVA/MinCDJ at the cell poles and minimum concentration at midcell (Figure 2) (Errington et al., 2003; Adams and Errington, 2009). Though, some publications already indicate that parts of the *B. subtilis* Min system may not be as static as supposed and the Min system relocates after division (Gregory et al., 2008; Bramkamp and van Baarle, 2009; van Baarle and Bramkamp, 2010). To investigate if the Min system of *B. subtilis* is indeed somehow dynamic we wanted to investigate its behaviour by light microscopy. Hence it was crucial to setup a system that is suitable for *B. subtilis* live cell imaging. We setup and proved functionality of our life cell imaging system in the publication **"Interlinked sister chromosomes**

arise in the absence of condensin during fast replication in *B. subtilis*." (Gruber et al., 2014) (see above: 1.2 Prokaryotic compartmentalization).

To investigate the distribution of DivIVA in dividing cells we grew cells expressing DivIVA-GFP under its native promoter under the microscope and constantly followed the GFP signal. This revealed a dynamic behaviour of DivIVA after complete cell division. We further tested the dynamics of DivIVA-GFP with intact and inhibited protein biosynthesis machinery by classical fluorescence techniques like FRAP (Fluorescence Recovery after Photobleaching) that confirmed our results. Finally, we showed by utilizing photo-activatable and photo-convertible fluorophores that DivIVA is indeed recruited after completed division from the young cell pole to new forming septa. We used Dendra2 as a photoconvertable fluorophore. In its native state Dendra is green fluorescent but after illumination with a laser the fluorescence is converted to red. The shift of fluorescence is due to an irreversible chain break in the backbone of Dendra2 (Chudakov et al., 2007a, b). The "red"-state of Dendra2 is attempt to be photo stable over days (Gurskaya et al., 2006). Our data obtained here indicate that the true function of the *B. subtilis* Min system is rather to prevent reinitiation of division than defining a midcell position. These data have been published in **"Imaging DivIVA dynamics using photo-convertible and activatable fluorophores in *Bacillus subtilis*"** (Bach et al., 2014).

However, it could be shown that cell division or rather formation of the divisome is directly linked to membrane organization. The acyl-acyl carrier protein phosphate acyltransferase PlsX physically interacts with FtsA and FtsZ. That results in stabilization of the Z-ring (Takada et al., 2014). Deletion of PlsX results in aberrant Z-ring formation (Takada et al., 2014). It is speculated that PlsX is required to provide certain lipids with distinct bio-physical properties that are incorporated into the forming septum. This indicates that the organization of the membrane is vital for efficient cell division.

1.3 Membrane organization

Membranes are the border of a single cell and surround different intracellular compartments. They are set up by a variety of hydrophobic compounds, mostly represented by various phospholipids and proteins. The presence of lipids is an essential prerequisite for all living organisms (phospholipids for most bacteria and eukaryotes; ether lipids for most archea). The "ground" state of membranes is in a condensed state, though most biochemical reactions need to happen in aqueous state. Nevertheless, roughly 25% of all proteins are membrane proteins, hence they are directly dependent on the properties of the surrounding membrane (Goni, 2014). One of the first accepted models of the organization of the plasma membrane was the Singer and Nicolson fluid mosaic model (Singer and Nicolson, 1972).

1.3.1 The fluid mosaic model

The fluid mosaic model proposes very fundamental characteristics of biological membranes. The very first presumption that was made by the Singer and Nicolson model is that lipids are amphipathic. The head groups of lipids align to the outwards of the leaflet but the corresponding fatty acids are orientated to the inwards of the membrane and thereby the characteristic hydrophobic lipid bilayer is created (Danielli and Davson, 1935). Further, it was supposed that proteins are able to bind and insert into the plasma membrane, forming membrane associated and trans-membrane proteins. Singer and Nicolson also proposed that every protein and lipid can freely diffuse along the plasma membrane (Figure 3). Notably, this includes rotational movement in the range of $10^8 – 10^9$ s^{-1} for lipids; $10^3 – 10^5$ s^{-1} for trans membrane proteins and translational diffusion ($10^{-8} – 10^{-9}$ cm^2 s^{-1} for lipids; $10^{-9} – 10^{-11}$ cm^2 s^{-1} for trans membrane proteins) but transversional (transbilayer) movement is prohibited since the polar head groups cannot pass, in a reasonable time scale, the energy barrier created by the hydrophobic tails of the phospholipids (Kornberg and McConnell, 1971; McNamee and McConnell, 1973; Edidin, 1974; Goni, 2014). Interestingly, it was already observed that addition of sterols inhibits lateral movement of the membrane (Butler et al., 1970; Schreier-Muccillo et al., 1973). In later years several concepts of the fluid mosaic model have been proven and updates of the model were proposed (Figure 3).

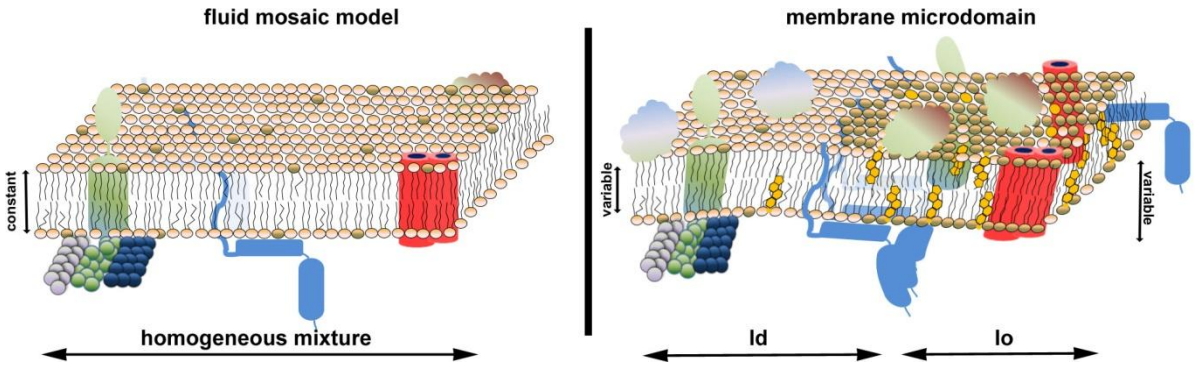


Figure 3: Models of biological membranes

The fluid mosaic model as supposed by Singer and Nicolsen is shown on the left. The membrane is a homogeneous mixture of proteins and lipids with a constant diameter between the two leaflets. On the right a more modern view of biological membranes, including the lipid raft model from Simons and Ikonen, is shown. Distinct membrane domains that likely have distinct biophysical properties (shown here as ld and lo) exist that restrict the translational and rotational freedom of proteins and lipids. Further it is accounted that the membrane exhibits a constant curvature/deformation, the leaflet can be variable thick and that the membrane is crowded with trans- and membrane associated proteins.

1.3.1.1 Membrane asymmetry and membrane curvature

The fluid mosaic model mostly neglects that membrane curvature or respectively membrane deformation is an overall phenomenon. Membrane curvature/deformation can be due to simple biophysical processes like interfacial effects or the membrane can be actively shaped by membrane processing proteins like dynamins, BAR proteins and others (Sens et al., 2008; Ferguson and De Camilli, 2012; Samaniuk and Vermant, 2014). Beside proteins that create membrane curvature also proteins that only sense membrane curvature exist. One prominent example is the *B. subtilis* protein DivIVA that is involved in *B. subtilis* division site selection (see above 0 1.2.1 Division site selection). However, membrane curvature can also occur due to protein asymmetry. When a hydrophilic protein, present only one side of the membrane, associates to the membrane this can induce membrane curvature (Kozlov et al., 2014).

It was assumed by Singer and Nicolson that biological membranes are asymmetric as consequence of the lack of transversional movement of lipids. Notably, this is in contrast to the biophysical behaviour of lipids that form bilayer *in vitro*. If bilayers are formed spontaneously in aqueous solution the composition of the inner and outer leaflet is similar, hence a biological reason for the observed asymmetry has been proposed (Singer and Nicolson, 1972).

The incorporation and hence the orientation of lipids, e.g. sphingomyelin, is based on the compartment where the corresponding lipid is synthesised (Bell et al., 1981; Cooper, 2000). Other lipids are synthesised in the cell and transported via flippases to the external leaflet (Chen et al., 1999; Hua et al., 2002; Pomorski et al., 2003). A remarkable example for asymmetry are phosphotidylcholine (PC) and sphingolipids that are highly enriched in the outer leaflet whereas phosphatidylserine (PS) and phosphatidylethanolamine (PE) mostly occur at the inner leaflet (Seigneuret and Devaux, 1984; Devaux et al., 1986; Xie et al., 1989; Pomorski et al., 2003). Strikingly, asymmetry of membranes has also direct biological functions. Exemplary, PS is flipped from the inner- to the outer leaflet during apoptosis what results in recognition of marcrophages (Fadok et al., 1992).

A nice example for protein asymmetry occurs during lysis of bacterial cells due to phage infection (Sturges and Rettger, 1922; Lewis, 2000). After infection through a bacteriophage the trans-membrane protein antiholin is flipped from the inner to the outer leaflet what leads to pore formation through oligomerization with holin (Wang et al., 2000). This finally results in release and activation of autolysins and depolarization of the membrane and hence cell lysis (Blasi and Young, 1996; Wang et al., 2000; Rice and Bayles, 2008). Though, the overall biological functions of asymmetry either on protein as well as on lipid levels are hardly understood till now (Sebastian et al., 2012).

1.3.1.2 Crowding of the membrane and membrane fluidity

Initially it was proposed that only a minor percentage of biological membranes is composed of proteins (Singer and Nicolson, 1972). Though, over the last decades it was demonstrated that the plasma membrane proteins occupy a major part (Engelman, 2005). In some bacteria even more than 25% of all putative genes encode for membrane proteins (Liu et al., 2002). Hence, almost every single lipid molecule is in contact with one or more proteins (Branton, 1971). The high abundance of membrane proteins also directly influences the fluidity of biological membranes (Saxton and Jacobson, 1997). Further, the occurrence of large protein domains due protein–protein interaction and various thicknesses of membranes due to different fatty acid compositions were not considered in the classical fluid mosaic model. The classical model has recently been replaced or rather updated by the lipid raft concept of Simons and Ikonen and others (Simons and Ikonen, 1997).

1.3.2 The lipid raft concept

The lipid model implies the existence of distinct membrane microdomains that initially have also been termed lipid rafts. These microdomains are enriched in certain lipids and proteins (Simons and Ikonen, 1997). One of the very first proofs, which also gave rise to the idea lipid microdomains, was to explain why the apical membrane of epithelial cells are enriched in glycolipids (Simons and van Meer, 1988). Further studies showed that membrane (micro)domains are also involved in processes like clathrin independent endocytosis, exocytosis, signalling, transport, protein translocation, cell division and others (Baumann et al., 2000; Lamaze et al., 2001; Nichols and Lippincott-Schwartz, 2001; Stuermer et al., 2001; Stuermer et al., 2004). Mass spectroscopy approaches revealed that eukaryotic lipid rafts are highly enriched in sphingolipids and cholesterol (Fridriksson et al., 1999; Pike et al., 2002). Hence, drugs that sequester or remove cholesterol results in disruption of lipid raft formation (Ohtani et al., 1989; Kilsdonk et al., 1995; Pike and Miller, 1998). Using *in vitro* systems it could be shown that addition of cholesterol can also be sufficient to induce lipid raft formation (de Almeida et al., 2003).

Generally, the lipid raft concept distinguishes between two different phases. The more hydrophobic liquid ordered (lo) and the more hydrophilic liquid disordered (ld) phase (Delmas et al., 2013). Likely, the driving force for formation of lo / ld domains is line tension (Kuzmin et al., 2005; Garcia-Saez et al., 2007). Notably, lipids and proteins in lo phase are more restricted in translational and rotational movement than in ld phase. Further, in ld phase water penetrates deeper between membrane headgroups due to the more hydrophilic properties compared to the lo phase. However, it was difficult to show if certain membrane domain exist in the lo or ld phase *in vivo*. Strikingly, domain formation and bilayer thickness seem to directly influence each other *in vitro*. With increasing incongruity of

membrane thickness the size of lo domains increase *in vitro* (Heberle et al., 2013). Increasing size of lo domains directly goes in hand with a decrease of lateral mobility of lipids and proteins (Heberle et al., 2013).

The keystone symposium defined lipid rafts as heterogeneous platforms that have a size between 10 – 200 nm (Pike, 2006). Though, theoretically lipid rafts can be stabilized and form larger transient platforms since a decrease in line tension goes directly in hand by the formation of larger lo domains (Karnovsky et al., 1982; Pike, 2009). However, membrane rafts are also considered to be highly dynamic and have a life time that can range from relatively short, transient structures with a half live time of 100 ms or less up to long lived stable structures (Pike, 2006).

Another definition, although it is controversial, of lipid rafts is that they can be isolated in detergent resistant membranes (DRMs). It has to be mentioned here that it is now mostly accepted that DRMs may, but not necessarily have to, contribute to lipid rafts since it could not been shown so far that DRMs truly contribute to domain formation *in vivo* and the preparation of DRMs may easily result in artificial results (Pike, 2006). DRMs cannot be solubilized with non-ionic detergents like Triton-X100 at 4°C but can be solubilized at 30°C (Brown and Rose, 1992; Bickel et al., 1997; Lang et al., 1998). These fractions can be collected using sucrose gradient ultra-centrifugation. Proteins that can routinely be found in the solubilized floating fraction (the DRMs fraction) are GPI anchored proteins and flotillin that is named due to its floating properties (Bickel et al., 1997; Salzer and Prohaska, 2001).

1.4 Flotillins

Flotillins can be found in all kingdoms of life (Tavernarakis et al., 1999; Hinderhofer et al., 2009). However, flotillins evolved via convergent evolution (Hinderhofer et al., 2009). Hence, it is crucial to understand the molecular setup of flotillins to understand their function.

1.4.1 Topology of flotillins

Flotillins exhibit a conserved molecular setup and are N`-terminally tethered to the membrane by a hairpin loop, that penetrates, but not crosses the membrane, a trans–membrane helix or a attached to the membrane by post-translational modifications (Figure 4) (Salzer et al., 1993; Huang et al., 1995; Roselli et al., 2002). This region is followed by a PHB (prohibitin) domain that is also often termed SPFH (stomatin/prohibitin/flotillin/HflKC) domain. This domain is in eukaryotic cells often posttranslationally modified by myristoylation or palmitoylation, which can also contribute to

membrane tethering (Dietzen et al., 1995; Tavernarakis et al., 1999; Morrow and Parton, 2005). The PHB domain is followed by an EA rich coiled – coil region that is also termed flotillin domain (Figure 4) (Browman et al., 2007). The flotillin domain is in eukaryotic cells involved in homo–oligomerization of flotillins (Neumann-Giesen et al., 2004; Rivera-Milla et al., 2006; Solis et al., 2007). Notably, flotillins form homo- and hetero-oligomers (Tatsuta et al., 2005; Solis et al., 2007; Hoegg et al., 2009). Further, purified prohibitins create ring like structures *in vitro* (Tatsuta et al., 2005; Browman et al., 2007).

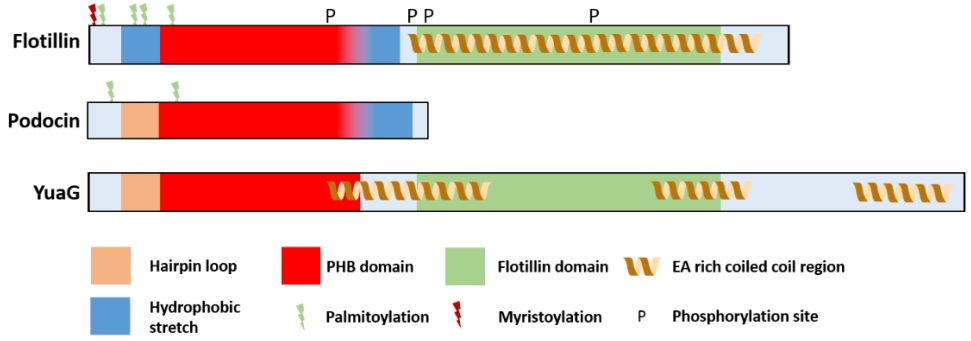


Figure 4: Structural domains in different classes of flotillins

A cartoon of the typical structural domains of flotillins, podocins and YuaG is drawn. All classes exhibit a similar setup. Membrane tethering in flotillins and podocins can also be achieved via palmitoylation and myristoylation. Cartoon was created according to our own data and the data presented in Rivera-Milla et al., 2006 and Browman et al., 2007.

The closest bacterial homologue to eukaryotic flotillins is the *B. subtilis* flotillin YuaG (FloT) with more than 35.4 % identity and 67.1 % homology to *Mus musculus* Flotillin2 (Donovan and Bramkamp, 2009; Lopez and Kolter, 2010). Notably, flotillins are not evolutionary conserved but emerged independently via covalent evolution, finally resulting in a similar domain structure what led to the conclusion that the molecular setup is a crucial and restricted feature for the functionality of flotillins (Hinderhofer et al., 2009).

1.4.2 Functions of flotillins

Flotillins are involved in various cellular processes. Flotillins were discovered as a factor involved in the regeneration of axons and were also termed Reggies (Schulte et al., 1997). Further studies revealed that flotillins are also involved in endocytosis (Gagescu et al., 2000; Huber et al., 2000), cell division

(Santamaria et al., 2003), diverse signalling processes (Baumann et al., 2000) and others (Banning et al., 2011).

The molecular function of flotillins is mostly unknown, though it is speculated that they may act as scaffolding proteins for membrane microdomains (Salzer and Prohaska, 2001; Stuermer, 2011). Even though, no direct function could be shown for flotillins so far. Many flotillins form hetero-oligomers with a second flotillin, hence two different flotillins exist in most organisms (Browman et al., 2007; Solis et al., 2007; Lopez and Kolter, 2010). Also the regulation of flotillins seems to be co-regulated. Down regulation of one flotillin also leads to down regulation of the second flotillin (Solis et al., 2007; Babuke et al., 2009; Zhao et al., 2011).

In *B. subtilis* the flotillin homologues YuaG (FloT) and YqfA (FloA) interact with each other (Lopez and Kolter, 2010). In wildtype cells YuaG and YqfA localize as highly dynamic foci at the cytoplasmatic membrane (Donovan and Bramkamp, 2009; Lopez and Kolter, 2010). Interestingly, in prokaryotes, flotillins are encoded together with a so called NfeD protein in an operon. Although, the function of NfeD proteins remains elusive (Green et al., 2009; Dempwolff et al., 2012a; Lee et al., 2012). However, YuaG is mostly expressed at late exponential or stationary phase (Donovan and Bramkamp, 2009). Both flotillins (YuaG and YqfA) localize as discrete foci at the cytoplasmatic membrane (Donovan and Bramkamp, 2009; Lopez and Kolter, 2010). Deletion of flotillins in *B. subtilis* results in a delay of the phosphorylation of the master regulator Spo0A (Donovan and Bramkamp, 2009; Lopez and Kolter, 2010). Phosphorylation of Spo0A is a crucial event for *B. subtilis* to differentiate into stationary phase behaviour like sporulation, cannibalism, biofilm formation and competence (Perego et al., 1989; Burbulys et al., 1991). In absence of flotillins the kinase KinC, that phosphorylates Spo0A due to specific environmental signals, becomes inactive (Lopez and Kolter, 2010). Hence, biofilm formation and sporulation is altered in flotillin null mutant strains (Donovan and Bramkamp, 2009; Lopez and Kolter, 2010). *B. subtilis* flotillins seem also to be involved in competence development. However, the reports are contradictory if and how flotillins are involved in competence development (Dempwolff et al., 2012a; Mann et al., 2013). Overproduction of both *B. subtilis* flotillins lead to a defect in cell differentiation and a shape defect due to a stabilization of the protease FtsH (Yepes et al., 2012; Mielich-Suss et al., 2013). Though, it remains elusive if *B. subtilis* flotillin directly influences the corresponding proteins or the plasma membrane. Previous studies revealed that distinct lipid domains exist in the *B. subtilis* membrane. Cardiolipin domains could be visualized by utilizing the dye NAO (10-N-nonyl acridine orange). NAO domains localize in heterogeneous patches at the plasma membrane in *B. subtilis* (Mileykovskaya and Dowhan, 2000; Kawai et al., 2004). Similar studies described a similar heterogeneous localization for PE and phosphatidylglycerol (PG) (Vanounou et al., 2002; Vanounou et al., 2003). In stationary phase the *B. subtilis* membrane consists of 12% CL, 30% PE, 36% PG and 22% Lysylphosphatidylglycerol (LPG)

(den Kamp et al., 1969). The fatty acid composition of *B. subtilis* is composed to ~90% of saturated fatty acids with a majority of 15:0, 16:0 and 17:0 chains. It further contains 10% unsaturated fatty acids with various acyl chain length (mainly 16:1). The *B. subtilis* membrane also exhibits hydrophobic compounds like hopanoids (Clejan et al., 1986; Bosak et al., 2008). Though, these are not involved in flotillin dependent microdomain formation (Lopez and Kolter, 2010). The presence of distinct membrane domains with certain biophysical properties *in vivo* also remained speculative.

A component that is enriched in eukaryotic lipid rafts is cholesterol and treatment with drugs that sequester cholesterol lead to disintegration of membrane rafts (Ohtani et al., 1989; Kilsdonk et al., 1995; Pike and Miller, 1998; Simons and Sampaio, 2011). Since the *B. subtilis* membrane does not contain sterols the function of cholesterol is likely fulfilled by other membrane components. A candidate that was proposed to match the function of cholesterol in *B. subtilis* is squalene or a squalene derivate. Strains lacking YisP, that was assumed to be a squalene synthetase, do not contain any DRMs anymore and the localisation of YuaG and YqfA is altered, so Lopez and Kolter (2010) assumed that squalene or its derivate is crucial for membrane microdomain formation in *B. subtilis* (Lopez and Kolter, 2010). Though, recent experiments demonstrated that YisP is a phosphatase catalysing the dephosphorylation of farnesyl diphosphate to farnesol (Feng et al., 2014). Hence, it is supposed that either farnesol or a still unknown lipid component might fulfil the role of cholesterol in *B. subtilis* membranes.

In the publication **"Flotillins functionally organize the bacterial membrane"** we elucidated the molecular function of the *B. subtilis* flotillin YuaG in membranes (Bach and Bramkamp, 2013). Pike already supposed at the keystone symposium that "in cell membranes, which are dynamic systems not in thermodynamic equilibrium, the underlying propensity of the lipids to phase separate is likely modulated by the presence of proteins and their state of aggregation as well as the continuous trafficking of lipids to and from the plasma membrane" (Pike, 2009). Using the anisotropic dye Laurdan (6-Dodecanoyl-2-Dimethylaminonaphthalene) we could demonstrate spectroscopically and microscopically that lo domains coalesce in absence of flotillins. This was to our knowledge the first direct influence of flotillins on membrane dynamics that could be shown experimentally.

It was also demonstrated that coalescence of these lo domains have a direct influence on various cellular machineries that are interacting with flotillins. Using pull down experiments of YuaG we could identify the second flotillin YqfA, proteins involved in cell wall metabolism, transport, energy metabolism, signalling and the Sec machinery to co-elute with YuaG. Indeed proteins of the mentioned processes also co-localize with YuaG (Lopez and Kolter, 2010; Yepes et al., 2012; Bach and Bramkamp, 2013). Finally, we demonstrated that absence of flotillins also directly impair the functionality of these machineries as shown exemplary for the Sec machinery. Likely these

machineries depend on the correct lipid or rather fatty acid environment that is provided by flotillins. We further wanted to proof if the oligomeric behaviour of prokaryotic flotillins is similar to eukaryotic flotillins. Indeed we could show by purification of YuaG and various analytical *in vitro* techniques that dynamic flotillin oligomers, properly in MDa size, exist. Likely these oligomeric structures formed by flotillins, as hetero- or homo-oligomers, organize specific lipid / fatty acids by simple protein–protein and protein-lipid interactions and thereby orchestrate the bacterial membrane.

In the publication: **"Dissecting the molecular properties of prokaryotic flotillins"** (Bach and Bramkamp, 2015) we addressed the question if prokaryotic flotillins indeed share the same molecular setup with eukaryotic flotillins (Figure 4). Here, we could show by SNAP-labelling experiments that the *B. subtilis* flotillin YuaG is not tethered to the membrane by a trans-membrane helix, but penetrates the membrane similar to eukaryotic flotillins via a hairpin loop. We further purified the PHB domain of YuaG and demonstrate by sedimentation assays that the PHB domain is contrary to eukaryotic flotillins not involved in YuaG lipid binding but is, also contrary to eukaryotic flotillins, sufficient to oligomerize. Strikingly, it is assumed that flotillins influence membrane domains via their PHB domain (Morrow et al., 2002). Since the YuaG-PHB domain seem not bind lipids it is unlikely that prokaryotic flotillins can influence membrane domains via their PHB domain. This opened a new perspective of the functionality of flotillins since similar molecular functions of both, eukaryotic and prokaryotic flotillins are predicted and shown, though this is achieved via different molecular configuration.

2. RESULTS / ABSTRACTS OF PUBLICATIONS

For copyright reasons, full text versions of the publications are not included in this thesis. Please follow the links to the publications directly from the publisher's website.

2.1 Publication I:

Interlinked sister chromosomes arise in the absence of condensin during fast replication in *B. subtilis*

Gruber, S., Veening, J. W., **Bach, J**., Blettinger, M., Bramkamp, M., and Errington, J.

Current Biology, 24, 293-298. (2014)

Link to PDF

Abstract:

Condensin-an SMC-kleisin complex is essential for efficient segregation of sister chromatids in eukaryotes [1-4]. In *Escherichia coli* and *Bacillus subtilis*, deletion of condensin subunits results in severe growth phenotypes and the accumulation of cells lacking nucleoids [5, 6]. In many other bacteria and under slow growth conditions, however, the reported phenotypes are much milder or virtually absent [7-10]. This raises the question of what role prokaryotic condensin might play during chromosome segregation under various growth conditions. In *B. subtilis* and *Streptococcus pneumoniae*, condensin complexes are enriched on the circular chromosome near the single origin of replication by ParB proteins bound to *parS* sequences [11, 12]. Using conditional alleles of condensin in B. subtilis, we demonstrate that depletion of its activity results in an immediate and severe defect in the partitioning of replication origins. Multiple copies of the chromosome remain unsegregated at or near the origin of replication. Surprisingly, the growth and chromosome segregation defects in rich medium are suppressed by a reduction of replication fork velocity but not by partial inhibition of translation or transcription. Prokaryotic condensin likely prevents the formation of sister DNA interconnections at the replication fork or promotes their resolution behind the fork.

2.2 Publication II:

Imaging DivIVA dynamics using photo-convertible and activatable fluorophores in *Bacillus subtilis*

Bach, J. N., Albrecht, N., and Bramkamp, M.

Frontiers in Microbiology, 5, 59. (2014)

Link to article

Abstract:

Most rod-shape model organisms such as *Escherichia coli* or *Bacillus subtilis* utilize two inhibitory systems for correct positioning of the cell division apparatus. While the nucleoid occlusion system acts in vicinity of the nucleoid, the Min system was thought to protect the cell poles from futile division leading to DNA-free miniature cells. The Min system is composed of an inhibitory protein, MinC, which acts at the level of the FtsZ ring formation. MinC is recruited to the membrane by MinD, a member of the MinD/ParA family of Walker-ATPases. Topological positioning of the MinCD complex depends on MinE in *E. coli* and MinJ/DivIVA in *B. subtilis*. While MinE drives an oscillation of MinCD in the *E. coli* cell with a time-dependent minimal concentration at midcell, the *B. subtilis* system was thought to be stably tethered to the cell poles by MinJ/DivIVA. Recent developments revealed that the Min system in *B. subtilis* mainly acts at the site of division, where it seems to prevent reinitiation of the division machinery. Thus, MinCD describe a dynamic behavior in *B. subtilis*. This is somewhat inconsistent with a stable localization of DivIVA at the cell poles. High resolution imaging of ongoing divisions show that DivIVA also enriches at the site of division. Here we analyze whether polar localized DivIVA is partially mobile and can contribute to septal DivIVA and vice versa. For this purpose we use fusions with green to red photoconvertible fluorophores, Dendra2 and photoactivatable PA-GFP. These techniques have proven very powerful to discriminate protein relocalization *in vivo*. Our results show that *B. subtilis* DivIVA is indeed dynamic and moves from the poles to the new septum.

2.3 Publication III:

Flotillins functionally organize the bacterial membrane

Bach, J. N., and Bramkamp, M.

Molecular Microbiology, 88, 1205-1217. (2013)

Link to PDF

Abstract:

Proteins and lipids are heterogeneously distributed in biological membranes. The correct function of membrane proteins depends on spatiotemporal organization into defined membrane areas, called lipid domains or rafts. Lipid microdomains are therefore thought to assist compartmentalization of membranes. However, how lipid and protein assemblies are organized and whether proteins are actively involved in these processes remains poorly understood. We now have identified flotillins to be responsible for lateral segregation of defined membrane domains in the model organism *Bacillus subtilis*. We show that flotillins form large, dynamic assemblies that are able to influence membrane fluidity and prevent condensation of Laurdan stained membrane regions. Absence of flotillins *in vivo* leads to coalescence of distinct domains of high membrane order and, hence, loss of flotillins in the bacterial plasma-membrane reduces membrane heterogeneity. We show that flotillins interact with various proteins involved in protein secretion, cell wall metabolism, transport and membrane-related signalling processes. Importantly, maintenance of membrane heterogeneity is critical for vital cellular processes such as protein secretion.

2.4 Publication IV:

Dissecting the molecular properties of prokaryotic flotillins

Bach, J. N., and Bramkamp, M.

PloS one *10*, e0116750. (2015)

Link to PDF

Abstract:

Flotillins are universally conserved proteins that are present in all kingdoms of life. Recently it was demonstrated that the *B. subtilis* flotillin YuaG (FloT) has a direct influence on membrane domain formation by orchestrating lipid domains. Thereby it allocates a proper environment for diverse cellular machineries. YuaG creates platforms for signal transduction, processes crucial for biofilm formation, sporulation, competence, secretion, and others. Even though, flotillins are an emerging topic of research in the field of microbiology little is known about the molecular architecture of prokaryotic flotillins. All flotillins share common structural elements and are tethered to the membrane N'- terminally, followed by a so called PHB domain and a flotillin domain. We show here that prokaryotic flotillins are, similarly to eukaryotic flotillins, tethered to the membrane via a hairpin loop. Further it is demonstrated by sedimentation assays that *B. subtilis* flotillins do not bind to the membrane via their PHB domain contrary to eukaryotic flotillins. Size exclusion chromatography experiments, blue native PAGE and cross linking experiments revealed that *B. subtilis* YuaG can oligomerize into large clusters via the PHB domain. This illustrates an important difference in the setup of prokaryotic flotillins compared to the organization of eukaryotic flotillins.

3. DISCUSSION

In this work different compartments of the model organism *Bacillus subtilis* were studied. The plasma membrane is one compartment that allocates a specific environment for diverse cellular processes. Flotillins were examined that act as a membrane scaffolding elements forming distinct domains in the plasma membrane. This revealed that flotillins are a crucial element to maintain membrane domain dynamics. As a second compartment the membrane curvature recognizing protein DivIVA was analysed that is involved in *B. subtilis* division site selection.

3.1 Dynamics of DivIVA in vegetative cells

We utilized life single cell time lapse microscopy (SCTLM), FRAP experiments with intact and inhibited protein biosynthesis and photo–activatable / convertible fluorophores, namely PA-GFP and Dendra2 to analyze dynamics of DivIVA. To perform SCTLM a system was established that ensures cell viability and allows investigations of proteins in live cells over long time periods. This was also true for FRAP, photo-activation / conversion experiments. Using these advanced microscopy techniques mentioned above we could show that DivIVA is, contrary to previous assumptions, not stably tethered to the cell poles but is recruited from the cell pole to new forming septa. This indicates a dynamic behaviour of DivIVA in vegetative cells. Strikingly, a dynamic behaviour of DivIVA indicates a different function of the complete Min system. The *B. subtilis* Min system was supposed to be static, contrary to the *E. coli* Min system that oscillates. In *B. subtilis* the topological factor DivIVA senses and binds curved membranes and interacts with MinJ (Bramkamp and van Baarle, 2009; Lenarcic et al., 2009). The transmembrane protein MinJ acts as an adapter and binds MinDC (van Baarle and Bramkamp, 2010). Hence, a DivIVA / MinJDC gradient is formed with a minimum concentration at midcell (Bramkamp and van Baarle, 2009). The topological factor DivIVA was supposed to be always stably attached to the cell pole. Further, it was known that DivIVA is also located in a ring like fashion to new forming septa. Since a new formed septum has curved membranes and becomes the new cell pole after fulfilled division this is not really contradictory to previous assumptions. New synthesis of DivIVA was supposed to be responsible for this behaviour (Eswaramoorthy et al., 2011). However, we could show that also DivIVA from the cell pole is recruited to the new formed septum. Recent investigations revealed that DivIVA is also recruited to the asymmetric septum that is created during sporulation in a SpoIIE dependent manner (Eswaramoorthy et al., 2014). This indicates that DivIVA dynamics is not only restricted to cell division.

The dynamics of DivIVA in vegetative cells implicate that the role of the Min system is rather to protect the cell poles after fulfilled cell division than to define midcell. This idea is supported by the fact that cells with a deleted Min and noc system still divide at midcell (neglecting the high occurrence of mini cells) (Rodrigues and Harry, 2012). This also indicates that another unknown system might exist in *B. subtilis* defining midcell (Barak, 2013). Likely, this is also true for *E. coli*. A recent paper from Bailey et al., 2014 revealed that despite the Min and SlmA system (equivalent to *B. subtilis* noc system) a third division site selection system in *E. coli* exists. In a strain lacking both *min* and *slmA* Z-ring formation still occurs mostly at midcell but contrary to wild type over the center of the nucleoid. SCTLM further revealed that Z-ring formation is initialized shortly after arrival of the replication terminus at the nucleoid center. In deletion strains lacking proteins that link the replication terminus to the divisome, e.g. MatP, mid cell division is less precise. Stunningly, SCTLM showed that MatP is present at midcell before divisome assembly in absence of *min* and *slmA*. Although, it remains elusive how it is recruited to midcell. In later stages of cell division MatP is stabilized by the divisome. Division site selection by the replication terminus or rather the decorating proteins is the first positive regulatory mechanism defining midcell known in *E. coli* (Bailey et al., 2014). The Min and the SlmA system are negative regulators of division site selection. Though, a high percentage of cells lacking *min*, *slmA* and *matP* still divided at midcell indicating that even a fourth division site selection system exists in *E. coli* (Bailey et al., 2014). A similar mechanism might be present in *B. subtilis*, though different localization properties of the replication terminus have been observed in *B. subtilis* compared to *E. coli* (Rodrigues and Harry, 2012; Bailey et al., 2014). However, the basic principle defining midcell might be similar.

Generally, it is speculated that a transcriptional / translational, negative linkage of the Z-ring to the nucleoid center exists (Norris, 1995). One idea is that protein crowding due to new synthesis over the nucleoid prevents Z-ring formation (Zaritsky and Woldringh, 2003). Another idea would be that chromosomal segregation creates mechanical forces that trigger divisome assembly to midcell (Rabinovitch et al., 2003). However, both hypotheses could not be proven so far.

The true role of different components of the *B. subtilis* Min system still remains elusive. This is especially true for MinJ and MinD. It could be shown for MinJ that it does interact with FtsA (Bramkamp et al., 2008). Furthermore, MinJ is recruited to midcell before DivIVA as dual colour SCTLM revealed (data not shown). This could indicate that the postulated hierarchy for the *B. subtilis* Min system is wrong. In stationary phase DivIVA might be the topological factor only binding to curved membranes and MinJ is bound to DivIVA. During ongoing cell division MinJ might dissociate from DivIVA and be recruited to FtsA, thereby promoting stabilization of the divisome. This could have a dual function. The first function might be recruitment of MinCD and hence prevention of further Z-ring constriction after fulfilled division (prevent mini cell formation). DivIVA could be

recruited in a second step to tether MinJDC to the new formed cell pole to allow future division at midcell. What still remains elusive is why single and multiple null mutants of DivIVA / MinJ/D/C also have a cell elongation phenotype (Karoui and Errington, 2001; Bramkamp et al., 2008; Patrick and Kearns, 2008; Gamba et al., 2009; van Baarle and Bramkamp, 2010; Eswaramoorthy et al., 2011). It might be possible that MinD is directly responsible for cell elongation. MinD has an ATPase domain with unknown cellular functions (Karoui and Errington, 2001). Potentially, MinD ATPase activity is crucial to dissociate MinC from FtsZ. If FtsZ might be continuously bound to MinC, Z-ring formation might be delayed. This might result in cell elongation. Another possibility could be that MinD ATPase function is involved in oligomerization of different Min components or MinD activates / inactivates MinC (Adams and Errington, 2009). MinD could also be required for the dissociation of MinJ from DivIVA. Though, dynamics of MinJ / DivIVA might also be dependent on simple biophysical association / dissociation processes. Future SCTLM experiments with multiple parts of the Min system and *in vitro* approaches in minimal systems might be required to elucidate the true role of the *B. subtilis* Min system.

Notably, our developed SCTLM was also utilized in Gruber et al., (2014). Here, SCTLM revealed that depletion of SMC results in a defect in the partitioning of replication origins (*oriC*). The partitioning defect of *oriC* was only present in rich medium, but not in minimal media. Since this phenotype can be rescued by using the drug hydroxyurea that reduces replication fork velocity we proposed two putative mechanisms how condesin might act on chromosome segregation. One possibility might be that prokaryotic condensin prevents interconnection of sister DNAs at the replication fork. Another possibility would be that condensin increases DNA resolution behind the replication fork (Gruber et al., 2014).

All these processes are somehow related to the plasma membrane which creates the border of the cell and provides a sophisticated network of specialized regions with distinct biophysical properties. A good example for a cellular process that is directly dependent on the correct membrane environment is the Min system. DivIVA localizes to membrane regions of strong curvature (Lenarcic et al., 2009). Regions of strong curvature are typically enriched with cardiolipin (Cl) due to its biophysical properties / cylindrical shape (Renner and Weibel, 2011). Also MinD is known to follow Cl domains in the *B. subtilis* membrane (Barák et al., 2008). Alterations in the membrane composition directly disturb functionality of the *B. subtilis* Min system (Barák et al., 2008; Barak, 2013). Despite a direct link of the Min system to certain membrane domains other processes like electron transport, solute transport, DNA replication and also cell division are directly linked to distinct membrane processes in bacteria (Zhang and Rock, 2008). To investigate the influence of the membrane on these molecular processes in *B. subtilis* its membrane organization was investigated.

3.2 Flotillin dependent membrane microdomains

Flotillins are proteins that are routinely used as markers for membrane rafts. In this study we could show that flotillins organize the bacterial membrane and likely are responsible to prevent coalescence of lo regions in the *B. subtilis* membrane. It has already been assumed that flotillins might act as scaffolding elements for membrane microdomains (Salzer and Prohaska, 2001; Langhorst et al., 2005; Stuermer, 2011; Zhao et al., 2011). Beside lo domains also scaffolding elements for regions of increased fluidity (RIF), equivalent to ld domains, exist in *B. subtilis*. A recent work revealed that RIF are organized by the cytoskeleton actin homologue MreB in *B. subtilis* (Strahl et al., 2014). This is contrary to the organization to eukaryotic lo regions that are supposed to be organized via actin that interact with flotillins (Langhorst et al., 2007; Ludwig et al., 2010). Hence, a direct functional link between cytoskeleton elements and membrane (lo) microdomains is assumed in eukaryotic cells (Head et al., 2014). Though, it seems to be different in *B. subtilis*. Strahl et al., 2014 utilized in a similar manner as we did the anisotropic dye Laurdan and could show that MreB co-localizes with low generalized polarisation (GP) regions / RIF (Strahl et al., 2014). Strikingly, proteins that were found in our work to interact with flotillins and are likely present in lo regions, as exemplary proteins involved in energy metabolism, were not influenced by disrupting MreB or RIF. This indicates that in *B. subtilis* different classes of proteins exist that are scaffolding distinct membrane domains. Till now this could be shown for ld regions that are influenced by MreB and lo regions that seem to be scaffolded by flotillins (Bach and Bramkamp, 2013; Strahl et al., 2014).

A putative mechanism how flotillins could scaffold the membrane might be facilitated via the hairpin loop. We could show that YuaG is tethered to the membrane via a hairpin loop and not by a trans–membrane helix as predicted by bioinformatical approaches (Bach and Bramkamp, 2015). Generally, hairpin loops are rare occurring phenomena in biological systems and are characterized by a helix–break–helix motive (Batenburg et al., 1988; Chupin et al., 1995; Baumgärtner, 1996). Theoretically any protein that penetrates the membrane can also directly influence its properties. Exemplary, membrane thickness and order can be influenced by trans–membrane helices (de Planque and Killian, 2003; de Jesus and Allen, 2013). It could be speculated that also hairpin loops are capable to directly influence the membrane, especially since the PHB domain of YuaG does not bind to lipids (Bach & Bramkamp, 2015). This is supported by the fact that the sequence of the hairpin loop of eukaryotic flotillins is conserved (Morrow and Parton, 2005), what is also partially true for prokaryotic flotillins (Bach and Bramkamp, 2015). Notably, the second flotillin homologue in *B. subtilis* YqfA is predicted to exhibit two helices that cross the membrane (amino acid 6-25 and 29-51) (Figure 5). Though, the putative extraplasmatic loop would contain only four amino acids and one of these is a proline that typically acts as a helix breaker. It might be possible that also YqfA does not cross the membrane but forms a hairpin loop. Though, it is also possible that multiple isoforms of the same protein may exist

that exhibit different topologies. An example for this is stomatin that can be tethered to the membrane via a hairpin loop but the same helix can also cross the membrane (Kadurin et al., 2009). The nature of this phenomenon remains elusive (Kadurin et al., 2009). It is also not fully understood how hairpin loops are integrated into the membrane (Baumgärtner, 1996). It is hypothesized that the formation of hairpin loops is dependent on positively charged amino acids at the N`- and C`- terminus of the loop that might stabilize this structure according to the "positive inside rule" (de Vrije et al., 1990). This postulates that mostly negatively charged lipids are located at the extracellular side of the bacterial membrane and positively charged amino acids at the cytoplasmic side of the membrane (von Heijne and Gavel, 1988). Hence, it is speculated that the lipid environment might directly influence the topology of proteins as exemplary known for LacY whose topology is directly dependent on the presence / absence of phosphatidylethanolamine (Vitrac et al., 2013). It could be possible that hairpin loops integrate into the membrane spontaneously due to the more biophysically favourable (hydrophobic) environment or the insertion is driven by the proton motive force that might support insertion (Van Voorst and De Kruijff, 2000).

1 - MDPSTLMILA IVAVAIIVLA VFFTFVPVML WISALAAGVK ISIFTLVGMR LRRVIPNRVV - 51

Figure 5: Sequence of the predicted trans-membrane helices of *B. subtilis* YqfA

The first 50 amino acids of *B. subtilis* YqfA are shown. The predicted trans - membrane helices are highlighted in yellow, the putative extraplasmatic loop in green and a central proline residue in red.

However, the majority of trans–membrane proteins integrate into the membrane via the twin arginine translocation pathway and the Sec machinery. Briefly, protein substrates in *B. subtilis* that should be secreted or integrated into the membrane via the Sec machinery harbour an N' – terminal signal peptidase targeting sequence and the substrate is transported via chaperone-like proteins to the Sec machinery. SecYEG forms a channel inside the membrane and the substrate is pushed via the ATPase SecA through the channel. Extracellular, the substrate is folded by chaperone-like proteins specific for soluble and membrane proteins (Campo et al., 2004). We could also identify the Sec machinery as an interactor of YuaG (Bach and Bramkamp, 2013). It could be supposed that this interaction might be artificial simply due to YuaG stalled during the translocation processes. Though, in our work we could demonstrate that the Sec machinery co-localizes with YuaG and is directly dependent on the presence of flotillins. In absence of flotillins the amount of secreted proteins is ~30% reduced compared to wild type (Bach and Bramkamp, 2013). This is in agreement with previous publications supporting the idea of a direct functional link between flotillins and the Sec machinery (Lopez and Kolter, 2010; Bach and Bramkamp, 2013). Strikingly, the Sec machinery is highly dependent on its lipid environment. It could be shown that the activity of the *B. subtilis* Sec machinery is directly proportional on the amount of

phosphatidylglycerol and phosphatidylethanolamine in the membrane *in vitro* and *in vivo* (van der Does et al., 2000; Van Voorst and De Kruijff, 2000). Also the insertion of the specific translocated substrate, exemplary via YidC, into the membrane is dependent on the lipid environment (Beck et al., 2001; Wang and Dalbey, 2011). Potentially, the specificity of the Sec machinery can also be altered by the lipid environment that might be allocated via flotillins and other proteins / mechanisms.

Further studies revealed that also other cellular machineries are directly dependent on flotillins. Namely, the protease FtsH that is involved in biofilm formation, sporulation and cell wall synthesis is miss-localized and partially down regulated in absence of flotillins. When both flotillins are highly over-expressed this also results in a defect in cell differentiation and cell shape (Yepes et al., 2012; Mielich-Suss et al., 2013). In agreement with these studies we also identified FtsH as an interactor of YuaG. However, Dempwolff et al., (2012ab) proposed that deletion of flotillins directly results in defects in cell shape, motility and competence (Dempwolff et al., 2012ab). This defect is even more dramatic in absence of bacterial dynamins (Dempwolff et al., 2012b). Curiously, these phenotypes cannot be reproduced in our hands (data not shown). A recent study by Mann et al., (2013) also disagrees with the postulations by Dempwolff et al, (2012a) that flotillin deletion(s) become incapable to take up DNA, which would go in hand with altered competence development and transformation efficiency (Dempwolff et al., 2012ab; Mann et al., 2013). Contrary, Mann et al., (2013) achieved even higher transformation efficiency and increased DNA uptake in flotillin deletion strains (Mann et al., 2013). The origin of these contradictory results remains elusive but it might be possible that this is due to different strain backgrounds used. In the studies by Dempwolff et al., (2012a,b) *B. subtilis* laboratory wild type PY79 was used whereat in the studies of Mann et al., (2013) the lab wild type 168 was used, which is also used in our lab. Strikingly, the strain PY79 used by Dempwolff et al., (2012a,b) has a dramatically reduced genome compared to *B. subtilis* 168 (Kunst et al., 1997; Schroeder and Simmons, 2013). It might be possible that some proteins are encoded in the 168 genome that rescue the phenotype upon flotillin and dynamin deletion. However, it is likely that flotillins act on various cellular processes as a membrane microdomain scaffolding element.

It has to be mentioned here, that it remains elusive what the true structure of a membrane microdomain is and how many different classes of microdomains exist. Membrane domain formation goes directly in hand with changes in the fluidity with respect to diffusion velocity. Notably, the bacterial cytoplasm is fluidized by metabolic activity (Parry et al., 2014). This might also be true for (bacterial) membranes. We could show that in stationary phase GP phase separation (not to mix with lipid phase separation) occurs in the absence of flotillins. The metabolic activity with respect to the new synthesis of membrane proteins and lipids decreases at stationary phase. Hence, it might be possible that flotillins are the elements that keep the membrane in a fluidized state with decreasing metabolic activity and thereby prevent phase separation as known from minimal *in vitro* systems.

Directed phase separation that occurs in *in vitro* systems could not be observed *in vivo* so far (Kusumi et al., 2005; Lagerholm et al., 2005). It might be possible that only two different classes, exemplary lo and ld, exist *in vivo*. Though, this is rather unlikely. It is more likely that numerous different membrane domains exist that all exhibit a certain composition of proteins, lipids, fatty acids and maybe more elements that are all required for proper functionality of diverse cellular machineries. A single eukaryotic cell can contain thousands different classes of lipids (hundreds of different classes are postulated for prokaryotic cells) and in theory myriads more of further different lipids could exist (Simons and Sampaio, 2011). Also roughly 5 % of all genes encoded are involved in lipid synthesis and the lipid mixture is highly diverse in mostly all organelles and membrane domains (van Meer et al., 2008; Lingwood et al., 2009). Since synthesis of lipids costs a high amount of energy it is highly improbable that the high diversity of lipids has no cellular functions. Certainly one role of the diverse lipid composition is to stabilize the membrane against mechanical stress and some lipids are involved in signalling processes (Schink et al., 2013). It is also known that certain proteins require a distinct lipid environment for full functionality. Despite classical membrane raft marker proteins, as GPI anchored proteins and flotillins that likely occur in distinct phases, several more proteins are known that prefer a definite lipid environment (Shogomori et al., 2005; Brown, 2006; Epand et al., 2006; Shaw et al., 2006). Also, the protein activity of numerous trans-membrane proteins is directly dependent on the surrounding lipids *in vitro* and *in vivo* (Seddon et al., 2004) what supports the idea that also distinct membrane domains exist in biological systems.

Another idea is that all proteins and lipids create domains in biological membranes. Thus, Spira et al., (2012) combined a microscopically total internal fluorescence (TIRF) approach with mathematic modelling. They could demonstrate that all (studied) proteins create domains along the plasma membrane. Accordingly, a "patchwork" organization of biological membranes with distinct but overlaying domains is predicted (Spira et al., 2012). Clearly the behaviour of proteins in biological membranes of living cells is way more complicated than in simplified *in vitro* systems. If a membrane is below its phase transition temperature (in an *in vitro* system) the translational and rotational movements of an embedded trans-membrane protein decreases compared to temperatures above phase transition (Aisenbrey and Bechinger, 2004; Tominaga et al., 2004). Surprisingly this is different in living cells. FRAP and TIRF experiments revealed that the viscosity of proteins *in vivo* is independent of the temperature. To show this GFP was fused to a trans-membrane helix and the diffusion coefficients of this construct were determined at different temperatures in *E. coli* (Nenninger et al., 2014). It is assumed that a decrease in temperature may result in clustering of proteins into mobile phases with similar mobility compared to temperatures above phase transition (Nenninger et al., 2014). Although the function or origin of this effect remains elusive, this example nicely demonstrates

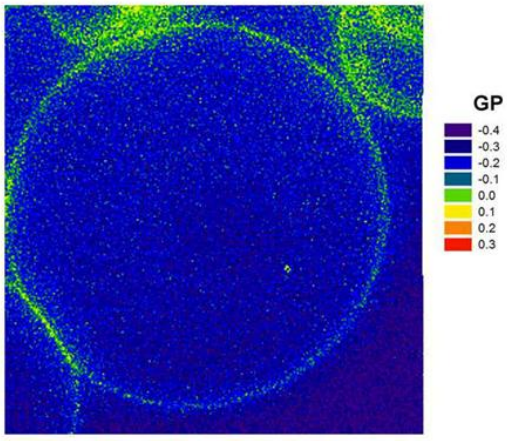
the complexity of biological membranes with respect to comparable simple physical processes observed in more controlled *in vitro* systems.

However, numerous approaches have been performed so far to understand membrane heterogeneity in minimal systems. An example for observed membrane domain formation *in vitro* is ring-raft formation in synthetic membrane budding processes. It could be shown that during budding of vesicles specific membrane compounds like cholesterol and sphingomyelin are recruited and form a ring like raft structure at the bud-neck facing the donor membrane (Ryu et al., 2014). This indicates that certain biophysical processes that also occur *in vivo* prefer a distinct lipid environment. This might simply be energetically more favourable (Ryu et al., 2014).

The likely most promising systems to create and investigate membrane (micro)domains *in vitro* are supported bilayers, nanodiscs and giant unilamellar vesicles (GUVs) (Chan and Boxer, 2007). These are often combined with atomic force microscopy or by using specific dyes that preferentially go into lo or ld phase. An example of GUVs stained with Laurdan and subsequent determination of GP can be found in Figure 6.

Figure 6: GUVs stained with Laurdan

GUVs made from *E. coli* total lipids stained with Laurdan. After fluorescence imaging using a standard widefield microscope fluorescent intensities were analyzed using ImageJ and SigmaPlot. No background subtraction was applied. The calculated GP is blotted according to the formula GP = ($Blue_{Intesity}$ - $Green_{Intesity}$) / ($Blue_{Intesity}$ + $Green_{Intesity}$). The higher the GP is, the more liquid ordered the membrane is.



Prominent examples for dyes that exhibit a certain fluorescence shift in different phases *in vitro* and *in vivo* are DPH (1,6-diphenyl-1,3,5-hexatriene) and Laurdan. Notably, also dyes that preferentially only go into only one phase like TR-DPPE (Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine), Dil (1,1′-dioctadecyl-3,3,3′,3′-tetra-methylindo-carbocyanine perchlorate) (with various acyl chain length from 12-16), NAP (naphtho[2,3-a]pyrene) and NBD-DPPE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[7-nitro-2-1,3-benzoxadiazol-4-yl]) exist. Of these examples only the last two prefer the lo phase whereat the others normally all occur in ld phase (Juhasz et al., 2010, 2012; Bach and Bramkamp, 2013). Importantly, lo/ld phase specific dyes in *in vitro* systems do not necessarily have the same phase preference in living systems. If phase specific

dyes are used in living systems the phase preference has always to be tested. Also fluorescent lipid analogues like NR12S that exhibit phase shift characteristics might be utilized. NR12S is a cholesterol analogue coupled to nile red, that clearly preferentially goes into lo domains (Kucherak et al., 2010; Oncul et al., 2010; Saxena et al., 2014). Using this dye in bacteria causes cell lysis even at low concentrations and to altered membrane dynamics (data not shown). However, using *in vitro* co-localization studies with these dyes with truncated or mutated variants of YuaG it might be possible to test if a certain domain in YuaG is required to localize into a specific phase. A good candidate for this might be the hairpin loop since YuaG with truncated hairpin loop does not localize to the membrane anymore *in vivo* (Donovan and Bramkamp, 2009) and the isolated PHB domain do not bind to lipids at all *in vitro* (Bach and Bramkamp, 2015).

However, if handled carefully the usage of membrane order or phase specific dyes in artificial systems can provide sophisticated data to elucidate membrane dynamics dependent on proteins. A change in phase separation might be followed by specific dyes after reconstitution of proteins. Exemplary, it might be possible that after reconstitution of a membrane scaffolding element, flotillin might be a good candidate here, an altered phase separation can be observed. Instead of separation in one lo and one ld phase it might be possible to identify several distinct phases. Thereby it would be possible to elucidate the function of several proteins that are assumed to be membrane scaffolding elements as expected for GPI anchored proteins, caveolins and flotillins (Langhorst et al., 2005; Frick et al., 2007; Hansen and Nichols, 2009; Head et al., 2014). Also physical approaches might be utilized to investigate the impact of proteins on membranes. An approach using X-ray reflectivity on supported bilayers with bound Annexin A2 (a lipid binding protein involved in exocytosis) demonstrated that the inner leaflet of the supported bilayer is directly deformed by the bound Annexin A2. After binding of Annexin A2 the lipid density increases (Fritz et al., 2010). Physical *in vitro* approaches as X-ray reflectivity or also neutron duality measurements that are capable to directly visualize the impact of proteins on membranes might be utilized to further elucidate scaffolding functions of flotillins.

Membrane microdomains can also be considered as “the non-random mixing of dissimilar molecules within a single phase” (van Meer et al., 2008). To visualize these domains different microscopy techniques can be applied that might solve the question if and when membrane microdomains are created, in what structure membrane microdomains exist in artificial and biological membranes and how they are created.

3.2.1 Microscopical approaches to visualize microdomains

Light microscopy is one of the most used techniques in biological sciences. The signal detected after illuminating a spot is called the point spread function (PSF) and can be described by a Gaussian

distribution. The size of the PSF and hence the resolution limit is directly dependent on the refraction index of light. Abbe (1873) was the first one to define the resolution limit of conventional light microscopy using the formula d = λ / 2*NA, where d is the resolution limit, λ is the wavelength of the light and NA is the numerical aperture of the objective used. The Rayleigh / Sparrow limit (the full width at half maximum of the PSF) determines the "diffraction limit of resolution" (Rayleigh, 1896; Schermelleh et al., 2010; Coltharp and Xiao, 2012). Hence, the resolution limit in x/y for most biological fluorophores is > 250 nm (Abbe, 1873; Coltharp and Xiao, 2012). This means that no spots in closer proximity than the resolution limit can be distinguished. The definition of membrane rafts is that they are smaller than 200 nm (Pike, 2006). Thus, conventional light microscopy is not suited to examine membrane rafts.

A putative way to visualize microdomains is electron microscopy. Though, electron microscopy has the disadvantage that labelling of specific proteins, exemplary by immunogold labelling, often requires membrane permeabilizing agents, dehydration of the sample, massive sample manipulation (e.g. thin sectioning) and hence is hardly suited for (membrane)- protein localization studies (Lagerholm et al., 2005). Several new light microscopy techniques that are able to circumvent the diffraction limit of light and hence increase the resolution have been developed during the last years. These techniques can and were utilized to elucidate the structure of membrane microdomains. The most prominent of these techniques are 3D-SIM (3 dimensional - structured illumination microscopy), STED (stimulated emission depletion) microscopy and PALM / STORM (photo-activated localization microscopy / stochastic optical reconstruction microscopy). The very basic principle of 3D-SIM is that interference patterns (normally 3 – 5 patterns are used) are applied to the fluorescent samples. The resulting image is also called moiré fringes (Heintzmann and Cremer, 1999). These moiré fringes are Fourier transformed and from the final interference pattern the final image is calculated and deconvolved (Gustafsson, 2000; Gustafsson et al., 2008). This results in a maximum resolution of ~100 nm in x/y and 250 nm in z axis (Schermelleh et al., 2010). Though, the increase in resolution is hardly sufficient to investigate membrane rafts dynamics. A different technique is used for STED microscopy. STED is mostly similar to confocal microscopy but STED microscopes are additionally equipped with a depletion laser. This laser is in a donut shape around the excitation laser and stimulates molecules to fall back from an excited state to the ground state (Hell and Wichmann, 1994). Hence, only molecules in an area within 20 - 80 nm x/y - axis and 100 nm z - axis are detected (Coltharp and Xiao, 2012; Blom and Widengren, 2014). Notably, an advantage of STED microscopy is that it can also be combined with life cell microscopy. That is partially also true for 3D-SIM but hardly possible for PALM (Lukinavicius et al., 2014). Recently, STED was coupled with FCS (fluorescence correlation spectroscopy) to investigate membrane dynamics (Mueller et al., 2011). Using these combined techniques the dynamics and size of various (micro)domains could be shown either *in vitro* as well as

in vivo (Mueller et al., 2011; Sezgin et al., 2012). Exemplary, it could be visualized, using supported bilayers labelled with specific ld / lo dyes, that actin directly affects lipid domain structure. Actin binding was sufficient to inhibit lateral diffusion of lipids and to prevent formation of macroscopic domain formation (Honigmann et al., 2014). Notably, in this approach actin was attached to the membrane by different pinning sites that either prefer the ld or lo phase but in both cases actin co-localized with the ld phase. This might be explained by physical process like membrane curvature that directly interferes with protein induced phase separation (Schick, 2012; Honigmann et al., 2014). Nevertheless, ld phase organization by actin is in contrast to previous assumptions. It was assumed that actin might organize lo domains *in vivo* (Langhorst et al., 2007; Ludwig et al., 2010). However, ld organization by actin might be similar to the ld organization mediated by the *B. subtilis* actin homologue MreB (Strahl et al., 2014).

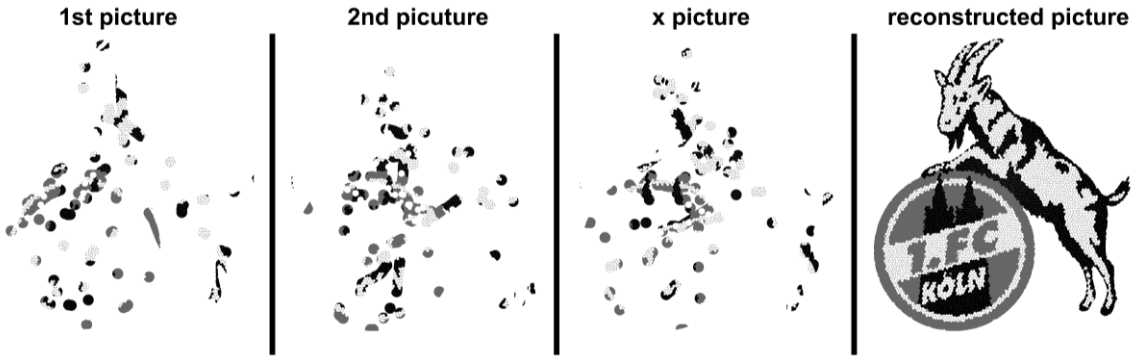


Figure 7: Principle of PALM microscopy

Single blinking events are detected in a sequence of imaging events. Finally, the full image is reconstructed.

Neither 3D-SIM nor STED microscopy could provide the resolution or light sensitivity to resolve bacterial flotillin microdomains so far (data not shown). Contrary to 3D-SIM or STED microscopy it is possible to track single molecules using PALM / STORM. The principle of PALM / STORM is based on the blinking properties of photo-activatable, convertible and switchable fluorophores and dyes. The basics of PALM and STORM are similar with the exception that PALM is used in biological systems with genetically expressed fluorophores whereat STORM requires blinking or organic fluorophores (Requejo-Isidro, 2013). However, single fluorophores / dyes are stochastically activated by a short laser pulse that results in blinking of the fluorophore. The blinking of the fluorophore / dye is monitored and the spot bleached (Betzig et al., 2006; Rust et al., 2006). This process is repeated till every molecule was activated and recorded (Figure 7). The exact localization precision can be determined by calculating back the recorded PSFs of single molecules and finally the localized precision is blotted. Therefore in theory a resolution limit of 2 nm in x/y – axis can be

achieved by PALM / STORM. That is also approximately the size of a single fluorophore (Yildiz et al., 2003). In modern PALM / STORM systems the z – axis resolution is limited to 50 nm (Huang et al., 2008).

In Bach et al., (2014) we already proved the functionality of the photo-convertible fluorophore Dendra2 by monitoring protein dynamics (Bach et al., 2014). This fluorophore can also be utilized for PALM in *B. subtilis* (Lippincott-Schwartz and Patterson, 2009). PALM might be a suitable system to elucidate the structure of flotillin microdomains in *B. subtilis*. Using this technique it might be possible to detect single molecules present in one flotillin domain in the normally diffraction limited spots. An example of PALM with blotted localized precision of single YuaG-Dendra2 molecules monitored from cells expressing YuaG-Dendra2 as a chromosomal replacement can be found in Figure 8.

Exemplary, the number of YuaG-Dendra2 molecules (15) detected in a flotillin ring–like structure in Figure 8 is in the range of to the calculated size of the oligomers detected biochemically for the full length protein (hexadecamer) and the truncated PHB domain (14.8 mer) indicating that these oligomers also exist in the *B. subtilis* membrane (Bach and Bramkamp, 2013; Bach and Bramkamp, 2015). It would be expedient to track the exact oligomerization sites of YuaG. So, PALM microscopy could be controlled and it could also be tested if homo-oligomerization is required for YuaG domain formation or if this is, at least partially, dependent on the presence of the second flotillin homologue YqfA. Another possibility might be that cluster formation of YuaG is only dependent on the presence of certain lipids. It would also be interesting to know if a strain expressing only YuaG with incapable oligomerization domain phenocopies a *yuaG* deletion strain. Strikingly, also two colour PALM could be performed to elucidate the interaction of both flotillins (Shroff et al., 2007).

Likely the size present inside a flotillin raft region is also sufficient to comprehend diverse cellular machineries. Exemplary, the diameter of the ring – like structure monitored by PALM (Figure 8) has a size of 80 nm. Assuming that the parts of the membrane covered by a flotillin domain would fit the mathematic criteria of a rotational paraboloid it is possible to calculate the space (A_0) that is available inside a flotillin microdomain using the formula:

$$A_0 = (\pi r/6h^2)*[(r^2 + 4h^2)^{3/2} - r^3]$$

Where h (height) of a flotillin domain is 30 nm and r (radius) is 40 nm (the values used for h and r were the measured distances of a flotillin domain by PALM).

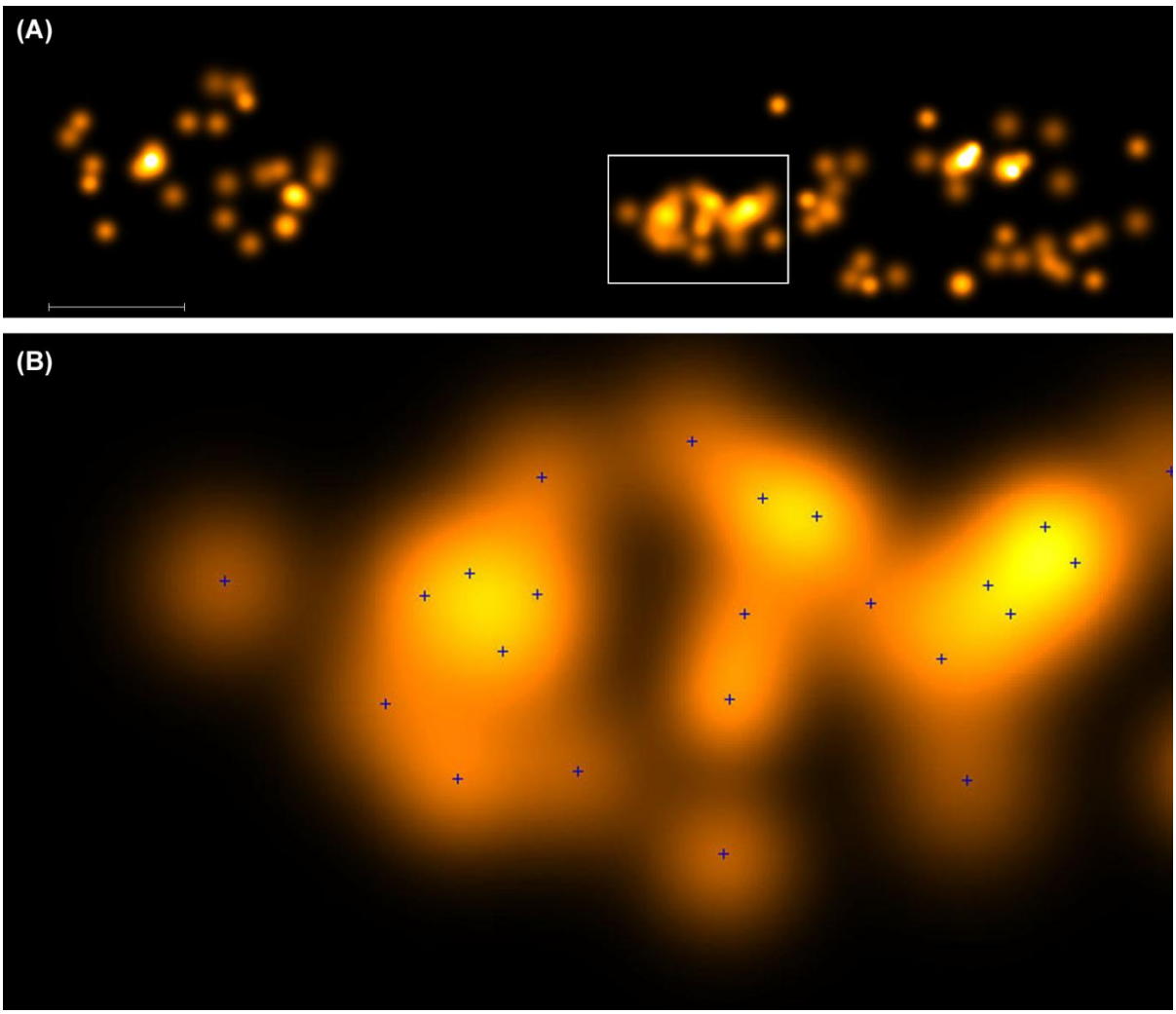


Figure 8: Single molecule tracking of YuaG-Dendra2

PALM image of YuaG-Dendra2. In **A** the image of the full cell is shown (all z stages are included). Scale bar is 100 nm. The part of the picture indicated with a white square is increased in **B**. The localized precision in x/y is 20 nm, in z 50 nm. Single molecule centres are indicated with blue **+**. Note that in **B** the calculated z localization centre of all molecules plotted is in a distance of ± 15 nm.

Hence, a two dimensional space of 7236 nm^2 would be created in a single flotillin domain. The number of molecules that might fit in this space can be calculated. Measuring the distance from the outer edges of the most distant trans-membrane helices of bacteriorhodopsin, simply used here as a standard protein with 7 trans-membrane helices (protein database template 1C3W), revealed a distance of ~30 Å. Assuming that these helices arrange in a ring like structure and calculating the corresponding space these helices would occupy, these helices would theoretically fit ~1021 times into a single flotillin domain. Obviously, this calculation is very simplified and many (likely critical) criteria are neglected, but certainly the space created by a flotillin domain would be sufficient to fully accommodate diverse cellular machineries.

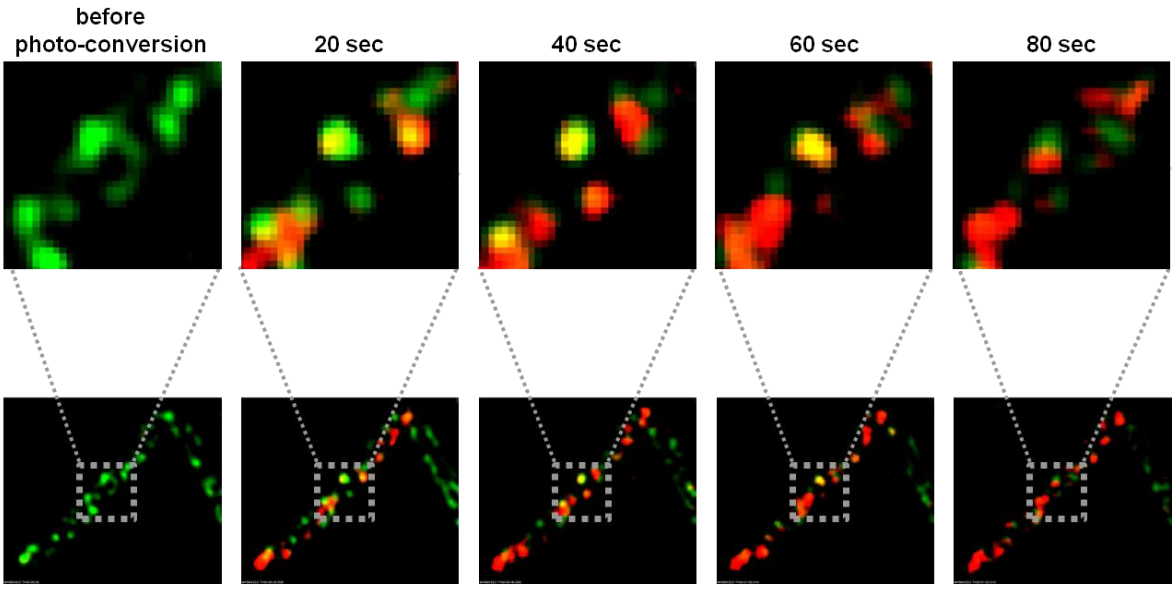


Figure 9: Dynamics of YuaG-Dendra2

Shown are cells expressing YuaG-Dendra2. After photo-conversion of Dendra2 the fluorescence of non-converted (green) and converted protein (red) were followed over time.

Strikingly, also the life time of these microdomains could be determined using sophisticated microscopy techniques. FLIM (fluorescence life time imaging), FRAP and TIRF experiments are powerful tools to provide data considering the dynamics of proteins. Though, using these systems it is hardly possible to determine the stability of membrane microdomains. Photo-convertible / activatable fluorophores can provide a mighty system to elucidate dynamics and life time of membrane rafts. The half-life time of membrane rafts is assumed to range from short lived structures with a half time in ms range to long lived mostly stable structures (Pike, 2006). Using photo-convertible proteins it is possible to perform pulse chase experiments (Figure 9). This means, that a certain region of the cell might be photo-converted and the fluorescence of the non-converted and converted fluorophores could be followed over time as we did in Bach et al., 2014. If the fluorescence of converted / non-converted fluorophores does overlay but did not overlay directly after conversion, this would indicate that flotillin domains have fused. If the fluorescence of the converted fluorophores split into multiple foci this would indicate that a region has disintegrated. An example for this experiment is shown in Figure 9. Assuming that a micro domain consist of two different flotillins (YuaG and YqfA) that are required as scaffolding elements these proteins could also individually be labelled, exemplary using PA-GFP and PA-mCherry (Subach et al., 2009; Baker et al., 2010; Subach et al., 2010). After photo-activation the fluorescence of these two fluorophores could be followed over time and hence the half-life time of the activated complexes directly determined.

The overall occurrence of microdomains is still a phenomena that is highly controversial and under intensive investigation. Future techniques provide the possibility to elucidate these processes. Also the overall organisation of the plasma membrane remains a subject of research. Likely, many new findings will be made and hence several updates on the theory how membranes are organized be applied in the future.

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