



**Machines in Motion:
Dynamics of Protein Transport, Folding, and
Membrane Insertion in Bacteria**

Inaugural-Dissertation

for the attainment of the title of doctor
in the Faculty of Mathematics and Natural Sciences

Heinrich Heine University Düsseldorf

presented by

Max Busch

From Solingen, Germany

Düsseldorf, August 2026

From Synthetic Membrane Systems, Institute of Biochemistry
at the Heinrich Heine University Düsseldorf

Published by permission of the
Faculty of Mathematics and Natural Sciences at
Heinrich Heine University Düsseldorf

Supervisor: Prof. Dr. Alexej Kedrov
Co-supervisor: Prof. Dr. Lutz Schmitt

Date of the oral examination: 25.09.2026

*... you know, like old lady science!
You know, she's a real, you gotta
hang on tight, you know, because
she bucks pretty hard.*

-Rick and Morty, S2 E6

Abstract

Correct folding at the right time and place is a prerequisite for proteins to perform their intended cellular function. This task, already nontrivial for soluble cytoplasmic proteins, becomes even more demanding for membrane and secretory proteins, which must navigate the additional barrier of the membrane itself. The Sec translocon is the main portal from the cytoplasm into and beyond the inner membrane. While some proteins reach their destination in the inner membrane, for others, translocation is just the first step of maturation, often assisted by various chaperones.

In the first part this thesis focuses on extracellular lipase LipA of *Pseudomonas aeruginosa* which requires its specific foldase LipH to obtain an active conformation. Here, combination of biochemical analysis and molecular dynamics simulations reveal that membrane-tethered chaperone is highly dynamic, and it interacts with its linker domain and the proximate membrane. Despite these constraints, LipH efficiently activates LipA in different membrane mimetics, albeit with reduced binding affinity in comparison to the isolated chaperoning domain. Mechanistically, we speculate that initial chaperone:client recognition is mediated by stable interactions of the C-terminal MD2 domain of LipH, while the opposing MD1 mediates folding of LipA. Furthermore, the negatively charged membrane environment promotes release of the folded client, enabling repeated chaperone cycles. Together, these findings demonstrate that membrane association and conformational flexibility allow LipH to balance substrate capture, folding, and release, thereby ensuring efficient LipA maturation and secretion.

The second part of the thesis focuses on membrane protein folding facilitated by the bacterial insertases SecYEG and YidC. Here, structural and biochemical analysis reveals that nascent membrane proteins can enter the membrane in a conserved back-of-Sec pathway, where YidC is recruited in a substrate-dependent manner. At this interface, YidC facilitates folding of transmembrane helices prior to their insertion into the membrane and engages extensively with the nascent chain. These findings unveil a gate-independent insertion pathway in bacteria and provide new insights into how coordinated action of the multi-subunit insertase machinery ensures efficient membrane protein biogenesis.

Although the bacterial membrane is widely recognized as a critical modulator of Sec-mediated transport, the mechanistic understanding of protein:lipid interactions within this system remains comparatively limited. By employing molecular dynamics simulations and *in vitro* transport assays, the last chapter examines the lipid dependence of the Sec translocon. The results show that the SecY lateral gate preferentially adopts an open conformation in di-oleoyl lipid environments, whereas it remains predominantly closed in palmitoyl-oleoyl lipids, caused by two aromatic residues at the periplasmic end of the transmembrane helix 7. These findings provide a molecular explanation for previously observed lipid-dependent transport deficiencies that cannot be attributed solely to impaired SecA binding, highlighting the critical role of membrane composition in regulating translocon dynamics and thereby function.

Zusammenfassung

Die korrekte Faltung zum richtigen Zeitpunkt und am richtigen Ort ist essentiell dafür, dass Proteine ihre vorgesehene zelluläre Funktion erfüllen können. Für Membran- und sekretierte Proteine ist diese Herausforderung noch größer. Das Sec-Translokon ist das zentrale Portal vom Cytoplasma in die innere Membran und darüber hinaus. Während manche Proteine ihr Ziel in der inneren Membran erreichen, ist für andere die Translokation lediglich der erste Schritt.

Der erste Teil dieser Thesis befasst sich mit der extrazelluläre Lipase LipA aus *Pseudomonas aeruginosa* welche eine spezifische Foldase, LipH benötigt, um eine aktive Konformation einzunehmen. Molekulardynamik-Simulationen und biochemische Analysen zeigen, dass membranverankertes LipH hochdynamisch ist – seine Chaperondomäne wird zeitweise durch Membran und eigene variable Domäne blockiert. Dennoch aktiviert LipH LipA effizient in verschiedenen Membranmimetiken, allerdings mit reduzierter Bindungsaffinität im Vergleich zur isolierten Chaperondomäne. Mechanistisch vermuten wir, dass die initiale Substraterkennung durch stabile Interaktionen der MD2-Domäne vermittelt wird, während MD1 mit transienten Kontakten zur Faltung beiträgt. Darüber hinaus begünstigt die negativ geladene Membran die Freisetzung des gefalteten Substrats und ermöglicht so wiederholte Faltungszyklen. Damit koordinieren Membranassoziation und konformationelle Flexibilität Substratbindung, Faltung und Freisetzung und gewährleisten effiziente Faltung und Sekretion.

Der zweite Teil befasst sich mit der Faltung von Membranproteinen, vermittelt durch die bakteriellen Insertasen SecYEG und YidC. Strukturelle und biochemische Analysen zeigen, dass entstehende Membranproteine über einen konservierten "Back-of-Sec"-Weg in die Membran gelangen können, wobei YidC substratabhängig rekrutiert wird. An dieser Stelle erleichtert YidC die Faltung von Transmembranhelices vor deren Insertion in die Membran und interagiert dabei umfassend mit dem neu entstehendem Membranprotein. Diese Ergebnisse decken einen Gate-unabhängigen Insertionsweg in Bakterien auf und liefern neue Erkenntnisse darüber, wie das koordinierte Zusammenwirken der mehrteiligen Insertase-Maschinerie eine effiziente Membranprotein-Biogenese sicherstellt.

Obwohl die bakterielle Membran als wichtiger Faktor des Sec-vermittelten Transports anerkannt ist, ist das mechanistische Verständnis von Protein-Lipid-Interaktionen begrenzt. Mittels Molekulardynamik-Simulationen und *In-vitro*-Transportassays untersucht der dritte Teil dieser Arbeit die Lipidabhängigkeit des Sec-Translokons. Die Ergebnisse zeigen, dass das "lateral gate" von SecY in Di-Oleoyl-Lipidumgebungen bevorzugt eine offene Konformation einnimmt, während es in Palmitoyl-Oleoyl-Lipiden überwiegend geschlossen bleibt, verursacht durch zwei aromatische Reste am periplasmatischen Ende der Helix 7. Diese Ergebnisse erklären zuvor beobachtete lipidabhängige Transportdefizite und unterstreichen die entscheidende Rolle der Membranzusammensetzung bei der Regulation der Translokon-Dynamik und damit der Funktion.

Table of Contents

1. Introduction	7
1.1 Protein Folding	7
1.2 <i>Pseudomonas aeruginosa</i> – a Critical Priority Pathogen	9
1.2.1 Protein Secretion in Bacteria	10
1.2.2 The Lipase A and its Steric Chaperone LipH	10
1.3 Biogenesis of Membrane Proteins in Bacteria	15
1.3.1 Sec Translocon SecYEG	17
1.3.2 YidC	19
1.4 The Two-Pathway System of Sec	22
1.4.1 Post-Translational Translocation	23
1.4.1.1 Targeting by Trigger Factor and SecB	23
1.4.1.2 The ATPase SecA	25
1.4.2 Co-Translational Insertion & SRP	26
1.4.2.1 SecYEG and SecYEG/SecA Insertion	28
1.4.2.2 YidC Insertion	28
1.4.2.3 SecY-YidC Cooperation	29
2. Aims and Objectives	31
3. Conformational dynamics of the membrane-anchored foldase LipH from <i>Pseudomonas aeruginosa</i> facilitates recognition and release of the client lipase	32
4. SecYEG/YidC Mediated Membrane Protein Biogenesis	76
4.1 Substrate-induced assembly and functional mechanism of the bacterial membrane protein insertase SecYEG-YidC	76
4.2 Probing for Lateral Gate Opening: LacY and the Translocon	119
5. Lipid structure modulates the dynamics of the bacterial translocon SecYEG	123
6. Conclusion	173
6.1 Conformational dynamics of the membrane-anchored foldase LipH from <i>Pseudomonas aeruginosa</i> governs recognition and release of its client lipase ..	174
6.2 SecYEG/YidC Mediated Membrane Protein Biogenesis	175
6.3 Lipid structure modulates the dynamics of the bacterial translocon SecYEG	177
7. References	178
8. List of Publications	192
9. Acknowledgement	193
10. Declaration	196

1. Introduction

1.1 Protein Folding

Protein folding is the process by which a linear polypeptide chain acquires its compact three-dimensional structure required for functional activity which may be seen as a large-scale conformational change driven by various intra- and intermolecular interactions. The amino acid composition determines the intended fold, which is essential to ensure the function of the protein and, consequently, the integrity of biological units from organelles to organisms. Despite decades of research, the fundamental question of what drives amino acid sequences to fold into specific three-dimensional structures remains a central problem in structural biology. The development of AlphaFold, awarded with the 2024 Nobel Prize in Chemistry, has revolutionized structure prediction, enabling highly accurate modeling of protein tertiary structure directly from sequence. Yet accurate prediction is not synonymous with mechanistic understanding: the physicochemical forces that actually drive the folding process remain incompletely understood.

The basis for our current understanding of protein folding was established by Christian Anfinsen and colleagues in 1954 and further refined in 1961. The foundational work provided initial insights into the structure, disulfide bridges and the terminal sequence of the RNase before in further experiments, published in 1961 the protein was denatured in the presence of urea and a reducing agent. Upon removal of both, the protein spontaneously refolded and fully regained its enzymatic activity. From these observations, Anfinsen concluded that "*...the native secondary and tertiary structure of a protein is determined by the amino acid sequence itself,*" a principle that later became known as Anfinsen's dogma [1]. While this may hold true for small, simple proteins or peptides, the *in vivo* reality is more complex. Moreover, if folding were determined solely by primary structure, protein misfolding diseases such as Creutzfeldt-Jakob disease, Alzheimer's disease, and Parkinson's disease would not exist [2].

Cyrus Levinthal's 1969 observation, known as Levinthal's paradox, revealed that if proteins folded by randomly sampling all possible conformations, the process would take longer than the age of the universe [3]. This insight implied that protein folding must follow specific pathways guided by folding intermediates rather than proceeding through random conformational searches. Subsequent studies confirmed this hypothesis, demonstrating that acquiring the correct fold is often kinetically controlled, with proteins following the fastest route rather than the most stable one [4].

Current models describing protein folding primarily include the nucleation-condensation model and the hydrophobic collapse model. The nucleation-condensation model proposes that secondary and tertiary structure formation occurs concurrently, with local and long-range interactions developing in a cooperative manner [5]. This contrasts with earlier studies suggesting a hierarchical folding process, in which secondary structure elements form first and subsequently assemble into tertiary and quaternary structures [6-8]. Complementary to this view, the

hydrophobic collapse model postulates that folding is initiated by a rapid compaction of the polypeptide chain driven by the hydrophobic effect of nonpolar side chains. This initial collapse is thought to generate a compact ensemble within which nucleation and condensation of native secondary and tertiary structures can subsequently occur, promoting the formation and stabilization of α -helices and β -sheets [2, 9].

One important piece in the bigger picture is still missing: while the Anfinsen dogma relates sequence to structure, the folding environment can vary considerably, adding another layer of complexity. For example, proteins may fold in an aqueous solution or within the anisotropic environment of a lipid bilayer and since numerous factors, including intra- and intermolecular interactions, environmental conditions, and physicochemical forces, can interfere with proper folding [10]. Given the critical nature of this process, cells employ a broad network of chaperones to ensure proteostasis and prevent the formation of toxic misfolded or aggregated species [11, 12]. Chaperones modulate folding kinetics primarily through hydrophobic, non-covalent interactions with folding intermediates. They serve multiple functions by mediating proper folding, preventing aggregation and misfolding, and directing protein targeting [13].

Even simple bacterial cells harbour broad networks of molecular chaperones, often upregulated under environmental stress associated with accumulation of aggregation-prone folding intermediates. Those chaperones are defined as proteins that assist other proteins in reaching their functionally active state without becoming part of the final structure [14, 15]. The most prominent chaperones belong to the heat shock protein (HSP) family, classified according to their molecular weight. These include HSP40, HSP60, HSP70, HSP90, and HSP100 (respectively: DnaJ, GroEL, DnaK, HtpG, and ClpB in *E. coli*), which can be subdivided into four major systems: GroEL/GroES, DnaK/DnaJ/GrpE, ClpB, and the small HSPs (sHSPs) IbpA and IbpB [16]. These classes of enzymes facilitate folding, and achieve high promiscuity through recognition of short hydrophobic sequences, while ATP-driven conformational dynamics enable cycles substrate binding and release [17].

The bacterial GroEL/GroES supercomplex consists of two heptameric GroEL rings capped by one or two heptameric GroES lid domains. They form a consolidated nanocage in which proteins can achieve their native fold [18, 19]. In the highly abundant DnaJ/DnaK/GrpE system, DnaK binds hydrophobic patches of client proteins and thereby prevents off-target interactions which allows interactions of the cochaperones DnaJ and GrpE with the substrate [15, 20, 21]. For ClpB a disaggregation function has been proposed, in which the hexameric complex uses ATP hydrolysis to remodel large aggregates into smaller, refolding-competent species, thereby facilitating their subsequent processing by the DnaJ/DnaK/GrpE system [22]. In contrast to these ATP-dependent systems, sHSPs act in an ATP-independent manner, binding a broad range of substrates, preventing irreversible aggregation, and maintaining them in a refolding-competent, non-native state that can later be processed by other chaperones [23-25]. The co-expression of those chaperones prior to recombinant protein production has been shown to enhance protein solubility by up to seven-fold making highlighting their role also in biotechnological applications [26].

Just like the ATP-independent sHSPs, bacterial periplasmic chaperones have evolved mechanisms to assist client proteins in the absence of ATP, since cofactors are restricted to the cytoplasm [27]. The periplasm contains a network of such chaperones, including the soluble SurA, FkpA, and Skp, which recognize and bind exposed hydrophobic regions of unfolded proteins and thus display a broad specificity range. These chaperones play key roles in the biogenesis of secretory proteins and outer membrane proteins by facilitating their transport across the periplasm to secretion systems or to the β -barrel assembly machinery [28-32]. In addition to these soluble chaperones, PpiD and YfgM, which are located at the periplasmic face of the inner membrane, act as auxiliary subunits of the Sec translocon, where they contribute to protein translocation [33, 34].

Besides the general chaperones discussed so far, a distinct class of steric chaperones exists, which are essential for guiding their individual client proteins toward the correct fold. Examples include lipase-specific foldases from organisms such as *Acinetobacter*, *Burkholderia*, *Pseudomonas*, and *Vibrio* as well as systems such as CpaA:CpaB from *Acinetobacter* and the FimC:FimH adhesin system from *E.coli* [35-39]. Those clients, often virulence factors, critically rely on their steric chaperones to “...imprint unique structural (steric) information onto their target proteins...” before being secreted to the extracellular space [40]. The chaperone mechanism and involvement of LipH in secretion is further discussed in chapter 1.2.2 and chapter 3.

1.2 *Pseudomonas aeruginosa* – a Critical Priority Pathogen

Pseudomonas aeruginosa is a Gram-negative bacterial species that belongs to the phylum of *Proteobacteria* and the class of *Gamma-proteobacteria* (NCBI: txid208964). It is a rod-shaped, motile, facultative aerobe microorganism with a heterotrophic lifestyle, notable for metabolic versatility and environmental adaptability [41, 42]. *P. aeruginosa* inhabits diverse niches, including soil, water, and moist environments, and can grow under various temperatures and conditions, enabling colonization of environmental and host-associated sites [41]. In humans, *P. aeruginosa* is an opportunistic pathogen causing hospital-acquired infections, particularly in immunocompromised individuals, burn patients, and those with chronic diseases such as cystic fibrosis [43, 44]. In pulmonary disease, it can dominate the lung microbiome, promoting inflammation, tissue damage, and chronic infection [45, 46]. Genome analyses identify multiple lineages broadly classified as environmental or clinical strains [41, 47]. Among these, *P. aeruginosa* PAO1, originally from a human wound, is a reference strain for molecular and genetic studies, while PA14, a burn wound isolate, is widely used to study pathogenicity and novel therapeutics especially, since it shows a higher virulence than PAO1 across plants, invertebrates, and vertebrates [41, 48-51].

P. aeruginosa genome of ~6.3 Mbp encodes extensive virulence, secretion, metabolic, and environment-sensing functions, which support colonization, tissue damage, and immune evasion [52-55]. It further also displays intrinsic and acquired resistance to

many antimicrobials, including β -lactams, via low outer membrane permeability, multidrug efflux pumps, and acquisition of resistance genes [52, 55, 56]. This combination of multidrug resistance, environmental persistence, and virulence, much of which relies on the coordinated secretion of virulence factors, places it among the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* species) pathogens, underscoring the need for a mechanistic understanding of the processes that drive its pathogenicity [57-60].

1.2.1 Protein Secretion in Bacteria

Protein secretion in Gram-negative bacteria is a fundamental biological process essential for nutrient acquisition, environmental adaptation, and pathogenesis. Unlike Gram-positive organisms, Gram-negative bacteria must organize a complex cell envelope consisting of an inner membrane, a periplasmic space, and an asymmetrical outer membrane. To overcome these physical barriers, they have evolved a specialized molecular armory of at least eleven distinct secretion systems (T1SS-T11SS). Those systems are categorized by whether they translocate substrates in a single step from the cytoplasm or utilize a two-step mechanism, where the conserved Sec or Tat pathways deliver the substrate into the periplasm to be exported via a specialized component [61, 62]. The cargo of these secretion systems includes toxins, adhesins, and enzymes that are essential for bacterial virulence, pathogenicity, immune evasion, and interbacterial competition [63]. Gaining a detailed understanding of their structural architecture and regulatory networks is therefore crucial for the development of novel antimicrobial strategies, as well as for improving the use of bacteria as platforms for recombinant protein production [64].

1.2.2 The Lipase A and its Steric Chaperone LipH

P. aeruginosa possesses a highly sophisticated repertoire of secreted molecules and surface-associated structures that collectively enable the pathogen to colonize host niches, evade immune surveillance, and induce tissue damage [65-67]. One of those enzymes is the highly abundant extracellular enzyme lipase A (LipA), which is known to contribute to lysis of host cells, thereby nutrient acquisition, is associated with biofilm formation and environmental adaptivity [68-70]. It is encoded in a single operon together with its specific foldase, LipH [71]. The lipase-foldase system is not exclusive to *Pseudomonas* but is also found in the genera *Burkholderia*, *Actinobacter*, and *Vibrio* [36, 37]. Following translation at the ribosome, the unfolded lipase precursor is translocated post-translationally into the periplasm via the SecYEG:SecA pathway [72].

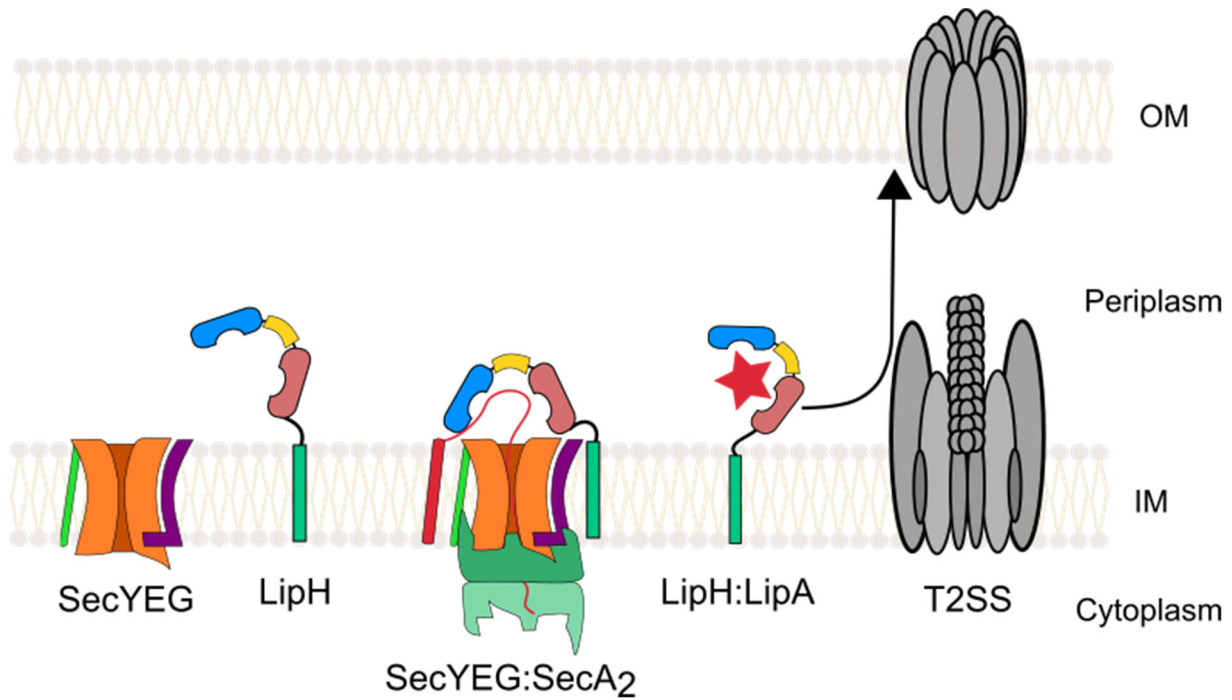


Figure 1: Scheme of the LipA secretion pathway in *Pseudomonas aeruginosa*. Translocation across the inner membrane (IM) via the SecYEG:SecA machinery is followed by recognition by the membrane-anchored steric chaperone LipH. LipH facilitates folding and activation of the lipase. Upon release, LipA becomes competent for secretion via the type II secretion system (T2SS).

Once in the periplasm, maturation of the lipase occurs through cleavage of the signal peptide by signal peptidase I and subsequent folding mediated by the membrane-bound foldase LipH [73]. Due to its high propensity for aggregation, a dedicated chaperone network facilitates proper folding and prevents aggregation within the periplasm [74, 75]. After reaching its active conformation, the mature enzyme is most likely recognized by the Xcp -Type II secretion system (Xcp = extracellular protein deficient [76]), primarily through interaction with one subunit, namely XcpP, and is subsequently secreted via the piston-like movement of the pseudopilus [77-79].

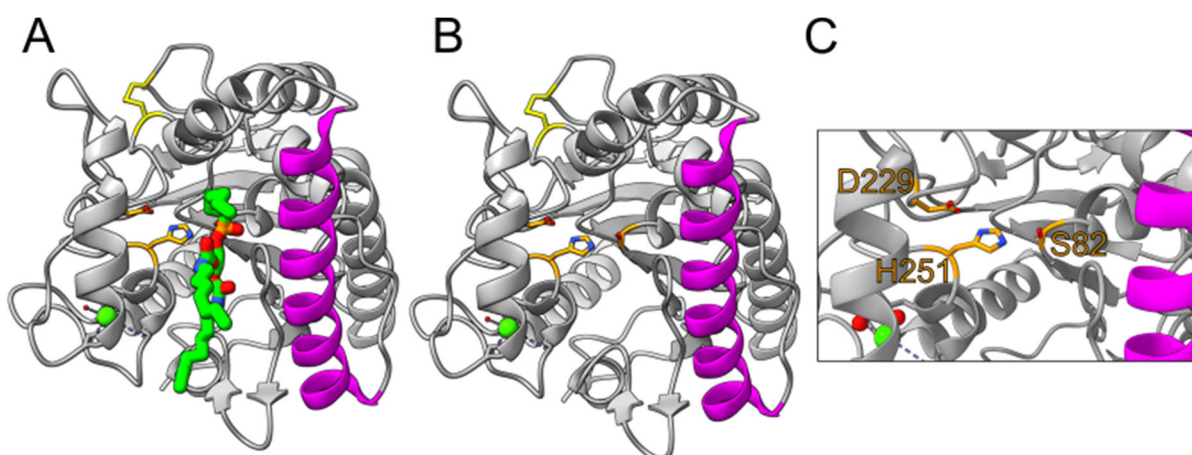


Figure 2: Crystall structure of the Lipase A. (A) Structure of Lipase A in the open conformation (PDB: 1EX9), with DCGOP (RC-(RP,SP)-1,2-dioctylcarbamoyl-glycero-3-O-octylphosphonate; green) covalently bound to the catalytic nucleophile Ser 82 in the active site. Helix 5, which undergoes displacement in the open conformation, is highlighted in magenta. The disulfide bond formed between Cys 183 and Cys 253 is shown in yellow, and the stabilizing calcium ion is indicated in green. (B) View of Lipase A in the open conformation with the substrate removed to allow clearer visualization of the catalytic triad. (C) Highlighted catalytic triad (orange) formed by Ser (S) 82, Asp (D) 229, and His (H) 251.

Structurally, LipA adopts the canonical α/β -hydrolase fold, consisting of five central β -sheets surrounded by five α -helices (Figure 2) [80]. The active site is formed by a conserved catalytic triad comprising Ser 82, Asp 229, and His 251, which is a characteristic of lipases within this family (Figure 2C) [80, 81]. Access to the active site is regulated by α -helical lid domain that undergoes conformational changes between closed and open states, thereby controlling substrate accessibility and contributing to enzyme stability [74, 82]. Additional structural features include an intramolecular disulfide bond between Cys 183 and Cys 235, which enhances protein stability, and a calcium-binding site formed by Asp 209 and Asp 253 that stabilizes the active-site architecture [83]. Molecular dynamics simulations further suggest that stabilization of one β -sheet affects the lipase lid domain and thereby the activation (Figure 3 A-C) [84].

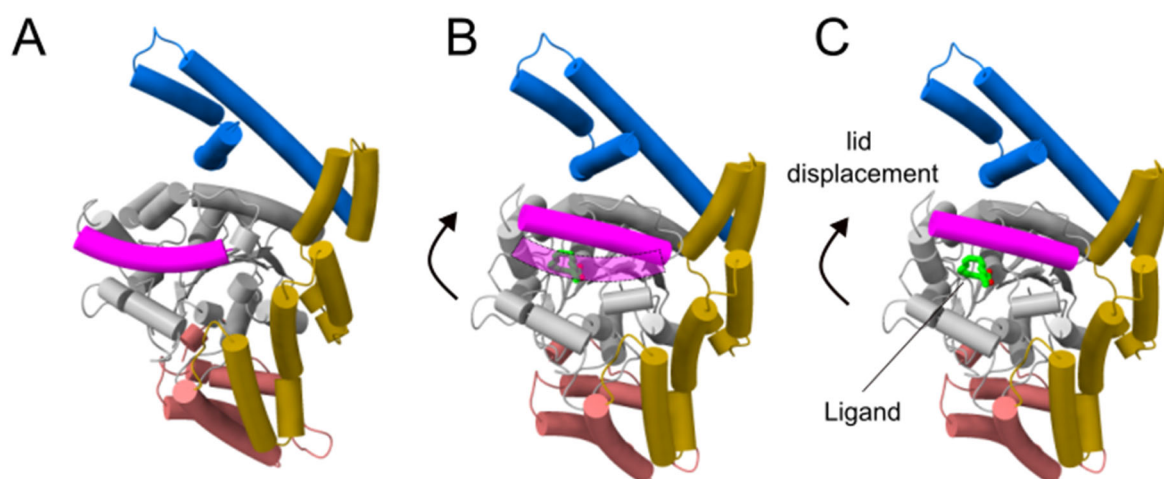


Figure 3: Gating mechanisms of Lipase A of *Pseudomonas aeruginosa*. (A) AlphaFold3-derived model of the LipH:LipA complex. The gating helix 5 of LipA (“lid domain”) is shown in pink, for clarity reasons only the chaperoning domain of LipH is shown. (B + C) AlphaFold3-derived model of the LipH:LipA complex in presence of an oleic acid molecule (green). Docking of the ligand is facilitated by a displacement of the lid domain, as indicated by the arrow. For clarity reasons, only the chaperoning domain of LipH is shown.

The biogenesis of LipA in *P. aeruginosa* critically depends on the lipase-specific foldase LipH/Lif, or historically LipB [85]. LipH is encoded in the same operon as LipA and functions as a steric chaperone or intermolecular folding catalyst that provides conformational information to its cognate client [10, 86]. The LipH:LipA system is conserved across several Gram-negative genera, including *Burkholderia*, *Acinetobacter*, and *Vibrio* [36, 37]. Structural analyses of foldase:lipase complexes from *Burkholderia glumae* and *Acinetobacter baumannii* revealed nearly identical architectures, underlining a conserved mechanism of lipase recognition and folding [87-89].

Structurally LipH can be divided into three major parts: TMH at the N-terminus, a long unstructured linker, and the chaperoning domain at the C-terminus (Figure 4 A). The region after the single TMH is the non-conserved variable domain (VD) linker that accounts for approximately 20% of the full-length LipH in *P. aeruginosa*. The VD of LipH is unusually enriched in proline residues and is predicted to lack defined secondary structure, suggesting intrinsic disorder [90]. Such features are typical for flexible tether regions and may allow the chaperoning domain to escape steric constraints imposed by the membrane, facilitating lipase capture, and interactions with the downstream secretion machinery [88, 91].

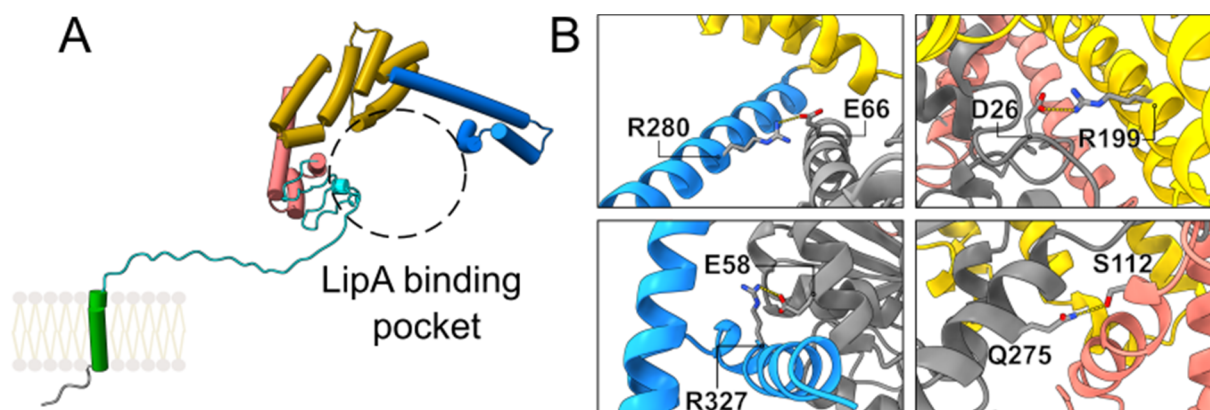


Figure 4: Structure of the foldase LipH. (A) Model of LipH based on all-atomistic MD simulations, with different regions color-coded as follows: transmembrane helix (TMH) in green, variable domain in cyan, mini-domain 1 (MD1) in salmon, extended helical domain (EHD) in gold, and mini-domain 2 (MD2) in blue. **(B)** Electrostatic interactions between LipH and LipA predicted based on the AlphaFold3-derived model. The color coding of the LipH domains correspond to Figure 9A, LipA is shown in grey.

The C-terminal chaperoning domain of LipH adopts a predominantly helical fold composed of two mini-domains (MD1 and MD2) connected by an extended helical domain (EHD), forming a “headphone”-like shape. MD1 contains a conserved signature motif (RXXFDY(F/C)L(S/T)A) that is characteristic of lipase-associated foldases [36]. Interactions within MD1 are further only marginally stable, since a single mutation within this region drastically affects the MD1:LipA assembly [92].

Comparative analysis of structural data from different organisms, together with AlphaFold3 predictions, reveals a conserved and extensive interaction interface between LipH and LipA. Key contact points within the *P. aeruginosa* LipA:LipH complex include helix 1 of LipH MD1, where Ser 112 is positioned near Gln 275 of the lipase; helix 5 (part of the EHD), where Arg 199 may interact with Asn 26 of LipA; and helix 11 (part of MD2), where Arg 280 and Arg 327 potentially build salt bridges with LipA Glu 66 and Glu 58, respectively (Figure 4B). Notably, this interaction interface does not involve the lipase lid domain that regulates access to the catalytic site. Instead, the lid domain is displaced into an open conformation while LipA remains bound to LipH, consistent with functional data indicating that substrate binding can occur in the foldase-bound state [93].

In addition to LipH, other periplasmic factors contribute to lipase maturation, including the chaperone Skp, which suppresses off-pathway aggregation, and therefore promotes stabilization of the folded enzyme [83]. These observations indicate that LipA biogenesis involves multiple transient protein:protein interactions within the periplasm before it can be released into the extracellular space by the T2SS (Figure 4) [75].

Although the architecture of the LipH:LipA complex has been resolved for closely related homologs, the molecular mechanism by which LipH recognizes, folds, and releases its client remains poorly understood. In particular, little is known about the contribution of the membrane-tethering segment of LipH, as previous studies have primarily focused on the isolated chaperoning domain. Moreover, a recently published

investigation of the conformational dynamics of this domain, including molecular dynamics simulations, suggest a highly unstable state that does not align with our previous experimental observations [75, 92]. Consequently, experimental characterization of the conformational dynamics and functional mechanism of full-length, membrane-anchored LipH is required to understand how LipA is recognized, folded, and released prior to secretion via T2SS.

1.3 Biogenesis of Membrane Proteins in Bacteria

Membranes are a key component of all living organisms, “...(they) enable life; they organize cells into protected compartments, generate and store energy, and defined cells structurally and phylogenetically”, as written by White, von Heijne and Engelmann in the opening chapter of “Cell boundaries – How Membranes and Their Proteins Work” [94]. Life would not be possible without the membrane interface formed by the delicate interplay between carbohydrates, lipids and proteins. The functionality of the cellular membranes is largely determined by integral membrane proteins (IMPs), which constitute up to 30 % of a genome content for each organism [95].

In contrast to soluble proteins, membrane proteins are characterized by extended stretches of hydrophobic residues that drive their partitioning into the apolar core of the lipid bilayer. However, this process is too inefficient to occur spontaneously, given the kinetic barrier of the aqueous cytoplasm and the need for precise topological orientation within the membrane. Consequently, cells rely on dedicated protein biogenesis machineries to catalyze and guide this process. Two distinct structural classes of membrane proteins are known, which both can be found in the cell envelope of Gram-negative bacteria. While the inner membrane is populated with α -helical proteins, the outer membrane is comprised nearly exclusively of β -barrels [96, 97]. In bacteria IMPs enter the inner membrane via the universally conserved Sec system, where the membrane protein insertases SecYEG and YidC play major roles [98]. In Gram-negative bacteria, membrane protein biogenesis extends beyond insertion into the inner membrane and ends in the assembly of proteins into the outer membrane. The insertion is catalysed by the β -barrel assembly machinery (BAM), a conserved complex composed of the core outer membrane protein BamA and four associated periplasmic lipoproteins, BamB, BamC, BamD, and BamE [99]. Biogenesis of α -helical membrane proteins is remarkably conserved across all domains of life, and despite extensive evolutionary divergence, the core components retain striking similarities in both structure and mechanism of action [100]. In eukaryotes, homologs of the bacterial SecYEG complex are found in the endoplasmic reticulum (ER) as the Sec61 translocon, where the Sec61 α , Sec61 β , and Sec61 γ subunits assemble into a heterotrimeric channel in a homologous manner to their bacterial counterparts. Studies in bacteria have demonstrated that SecYE constitutes the minimal functional unit required for membrane protein insertion and post-translational protein translocation (in combination with the cytoplasmic ATPase SecA), while SecG subunit is not essential [101]. In chloroplasts SecG is absent, the Sec machinery is streamlined to a minimal set of components, consisting of SecY, SecE, and SecA, together with the chloroplasts

SRP and its receptor FtsY [102, 103]. Although Sec61 β is not homologous to SecE, functional studies indicate that Sec61 β plays a major role in interactions with the signal recognition particle (SRP) and contributes to guanosine triphosphate (GTP) hydrolysis during targeting [104].

The translocon of *E. coli* is composed of the SecY, SecE, and SecG proteins which together form a central pore. The N- and C-terminal halves of the central subunit Sec can undergo lateral opening in a clamshell-like manner, thereby creating a lipid-exposed gap between the opposing TMHs 2b and 7. This gap, commonly referred to as the “lateral gate,” was recognized as the exclusive site where nascent TMHs partition into the lipid phase, both in the bacterial SecYEG complex and in the homologous Sec61 $\alpha\beta\gamma$ complex of eukaryotes [105]. Also, potential cooperation with additional insertion factors such as YidC, SecDF-YajC were predicted in close proximity to the lateral gate [106, 107]. The intuitive model was also supported by several crosslinking-based studies, which however nearly exclusively focused on the lateral gate as an interaction site between SecY and YidC [33, 106, 108].

In 2022, a novel model for IMP insertion was proposed. The groups of Ramanujan Hegde and Robert Keenan described an alternative pathway in which TMCO1, a member of the Oxa1 superfamily, is positioned at the back of Sec61 as part of the multi-subunit BOS-GEL-PAT complex, also referred to as the multipass membrane protein translocon (MPT) [109, 110]. In this pathway, IMPs are inserted not through the lateral gate of Sec61, but at the interface between Sec61 and the BOS complex, thereby challenging the previously established model in the field. Furthermore, global ribosome profiling indicated that the MPT is utilized by hundreds of eukaryotic multipass membrane proteins and is assembled in a substrate-dependent manner [111]. More recent studies have revealed the evolutionary conservation of this “back-of-Sec61” insertion route, demonstrating that it is not restricted to higher eukaryotes but is also present in yeast, thereby providing new perspectives on IMP biogenesis from the evolutionary perspective [112].

1.3.1 Sec Translocon SecYEG

The Sec pathway represents the principal route for protein export and membrane insertion in bacterial cytoplasmic membrane, where SecYEG translocon mediated both processes, which is enabled by its architecture [100, 113-115]. Despite being essential for bacteria and carrying out many modes of action, it has a relatively low abundance with approx. 500 copies per cell [116].

While the first high-resolution crystal structure of the homologous SecYE β of *Methanococcus jannaschii* captured the resting state of the channel, multiple structures of bacterial and eukaryotic translocons have been resolved since then in various functional states [115, 117-121]. Despite variations in amino acid sequence, the overall architecture of the Sec translocon is highly conserved, where the central subunit SecY is stabilized by SecE on one side and flanked by SecG on the other (in *E. coli* nomenclature). This structural conservation reflects the essential and ancient role in protein biogenesis and cellular homeostasis.

Bacterial SecY forms the main body of the channel and consists of ten TMHs arranged into two pseudosymmetric halves (TMH 1-5 and TMH 6-10) that create a clamshell-like, hourglass-shaped architecture (Figure5) [117, 118]. The cytosolic loops TMH 6/7 and TMH 8/9 of SecY serve as interaction platforms for the essential motor protein SecA, the FtsY receptor of the SRP pathway, and the ribosome [122-125]. A characteristic feature of SecY clamshell-fold is the long periplasmic loop connecting TMH 5 and TMH 6, called the hinge region, which allows the channel to undergo the conformational movements necessary for gating [125]. The movements can be categorized into quiescent, pre-open, and open states depending on the stage of substrate translocation [115, 117, 119, 121].

The internal cavity of SecY consists of two membrane-facing funnels separated by a narrow pore ring composed of hydrophobic and bulky amino acids, six isoleucine in *E. coli*, which form the main permeability barrier of the complex [117]. This pore ring ensures the ion-tight resting state of the channel [126]. On the periplasmic side, the channel is sealed by the plug domain (short TMH 2a). In the resting conformation, the plug prevents non-specific ion efflux, during translocation, it relocates to allow the passage of the polypeptide chain [117, 118, 127]. The lateral gate is formed between TMHs 2b and TMHs 7 of SecY. Upon engagement with SecA and the preprotein, SecY undergoes a series of conformational transitions that include opening of the lateral gate and displacement of the plug, resulting in formation of a stable open state that accommodates polypeptide chain [121, 128]. In its turn, dynamics of the lateral gate allows signal peptides and transmembrane helices of nascent IMPs to enter the surrounding lipid bilayer [115, 117].

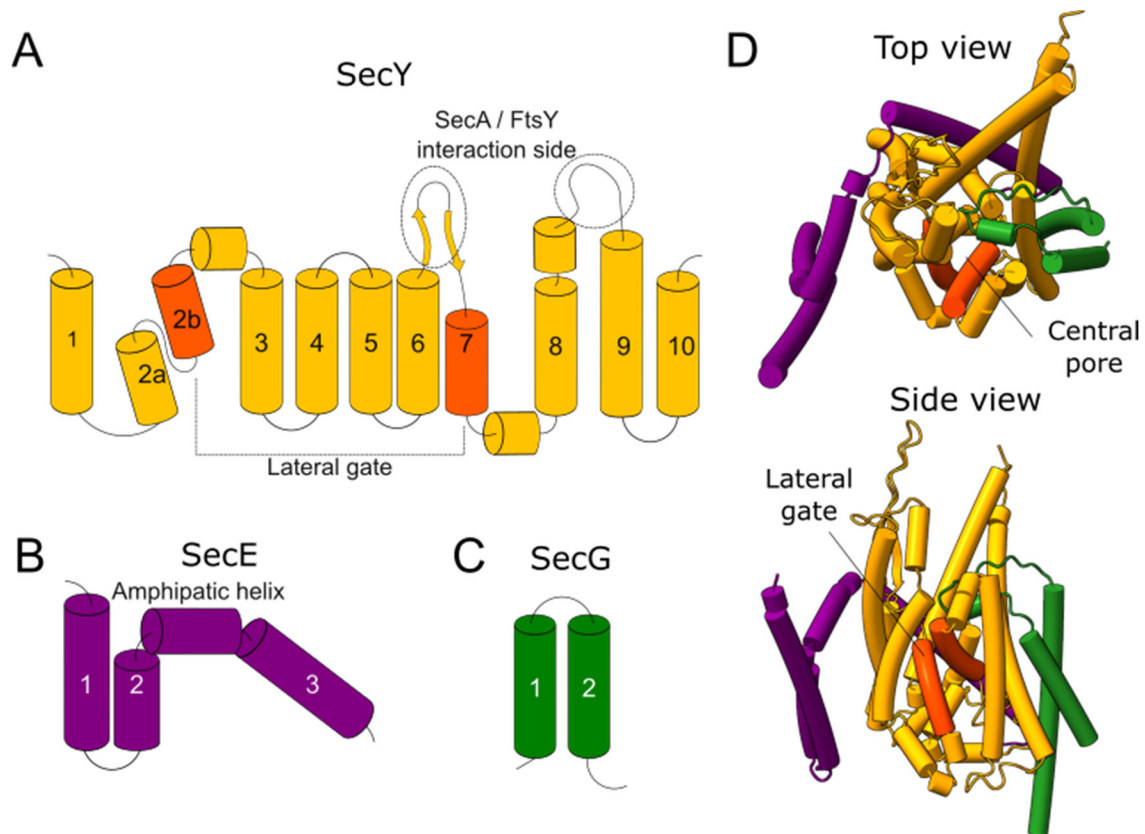


Figure 5: The SecYEG translocon. (A) Helical arrangement of the Sec translocon. SecY (yellow) is composed of ten transmembrane helices (TMHs). Key structural features are indicated, including the plug domain, which seals the channel against ion diffusion, the lateral gate (highlighted in orange), formed by TMHs 2b and 7, and the cytoplasmic loops 6/7 and 8/9, which provide binding sites for SecA, ribosomes, and FtsY. (B) SecE (purple) consists of three TMHs and an amphipathic helix that lies parallel to the membrane and stabilizes the two halves of SecY. (C) SecG (green) flanks the central pore. (D) Top view and side view of AlphaFold 3 model of the heterotrimeric *E. coli* SecYEG. Individual subunits are colored as indicated before. The central pore as well as the lateral gate are indicated. The long and unstructured C-terminus of SecG is left out for visualization purposes.

SecE is a key stabilizing component required for the structural integrity of SecY. In *E. coli*, SecE contains three TMHs and an amphipathic helix at the cytoplasmic interface, though only the third TMH and the amphipathic helix are essential for function. Positioned opposite the lateral gate, SecE clamps the two halves of SecY together and prevents its destabilization. The absence of SecE leads to rapid degradation of SecY by the membrane protease FtsH [117, 129, 130]. SecG, by contrast, is not strictly essential for cell viability or in vitro translocation, and is not evolutionary conserved [131]. In *E. coli*, it comprises two TMHs with a cytosolic loop that may contribute to sealing the cytoplasmic funnel of SecY [118]. Loss of SecG impairs protein transport efficiency and results in cold-sensitive growth defects, which can be suppressed by mutations affecting phospholipid biosynthesis [132-136]. SecG has also been implicated in modulating SecYEG:SecA interactions and becomes particularly important under conditions where the proton motive force (PMF) is diminished [123, 137, 138].

1.3.2 YidC

YidC is an essential membrane protein insertase in bacteria. It belongs to the widely conserved Oxa1/Alb3/YidC superfamily, whose members are found across all domains of life. Eukaryotic homologs include Oxa1 and Oxa2 (Cox18) in the mitochondrial inner membrane, Alb3 and Alb4 in the chloroplast thylakoid membrane, and the endoplasmic reticulum factors Get1, EMC3, and TMCO1, which function as insertases, chaperones, and assembly factors [139-141]. While both SecYEG and YidC can act as independent insertases, YidC also cooperates with SecYEG in the biogenesis of many membrane proteins [115, 142, 143]. Although mechanistic details vary between substrates, the conserved structural features of YidC and its homologs underpin their central role in membrane protein integration.

In *E. coli*, YidC is composed of six TMHs, with distinct periplasmic and cytoplasmic domains that contribute to its function. Closely related homologs have five TMHs (Oxa1, Alb3 and YidC from Gram-positive bacteria), while EMC3, Get1 and TMCO1 only have three TMHs, those are predicted to be the equivalents to TMHs 2, 3 and 6 highlighting the evolutionary conservation of the YidC fold [141, 144]. The high abundance of YidC with approx. 2500 copies per cell (in *E. coli*) further shows its importance for IMP insertion, as it can insert client proteins on its own or in concert with SecYEG [145, 146]. An additional driving factor is that the transmembrane helices of YidC are shorter than the thickness of the lipid bilayer. This hydrophobic mismatch induces local membrane thinning, which in turn lowers the energetic barrier for IMP insertion [147].

TMHs 2 to 6 form a globular transmembrane bundle, which defines the central structural element of YidC. This core contains several functionally significant features such as the hydrophobic groove in the inner leaflet of the membrane. It has been shown to be open towards the cytoplasm but closed on the periplasmic side and is thereby responsible for accommodation of hydrophilic stretches of client IMPs during their insertion [148]. TMHs 3 and 5 form a hydrophobic transmembrane gap that can clamp incoming clients, creating a “hydrophobic slide” mechanism for membrane integration (Figure 7). Hydrophobic residues on the outer sides of TMH 3 and 5 are positioned to make direct contact with entering TMHs [149].

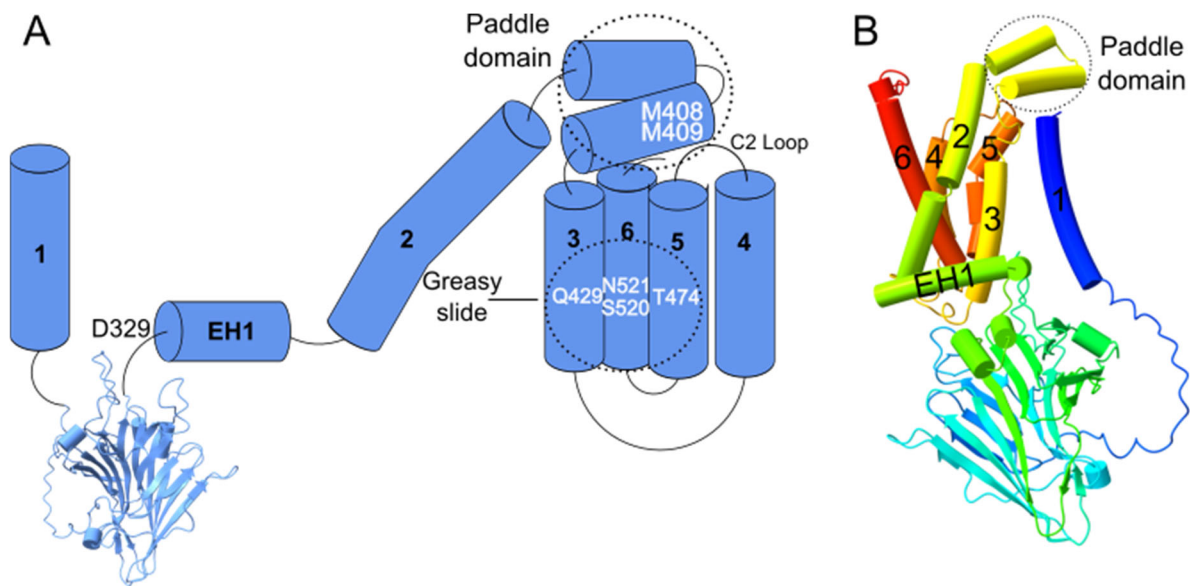


Figure 6: The membrane protein insertase YidC. (A) Helical arrangement of YidC and its six transmembrane helices (TMHs). Between TMH1 and the membrane-parallel EH1 lies the bulky, non-essential P1 domain. Following TMH2 on the cytoplasmic side is the paddle domain, which facilitates membrane insertion, followed by TMHs 3 to 6, where important residues and structural features are highlighted. **(B)** YidC structure displayed in a rainbow color scheme from the N-terminus (blue) to the C-terminus (red).

Between TMH 1 and TMH 2 lies a large periplasmic region known as the P1 domain (Figure 6). Although prominent in *E. coli*, this domain is non-essential, as many YidC homologs naturally lack it, and its deletion in *E. coli* does not impair viability or IMP insertion [150]. Just upstream of TMH 2, the P1 domain contains a conserved amphipathic helix (EH1) (Figure 7). Deletion of EH1 leads to a complete loss of YidC function both *in vivo* and *in vitro*, demonstrating its structural importance despite the dispensability of the larger P1 region (Figure 7) [150]. Between TMHs 2 and 3 is the cytoplasmic coiled-coil domain, referred to as the C1 region or paddle domain, formed by two α -helices that protrude into the cytoplasm (Figure 6B). Numerous studies have shown that this region is essential for YidC activity, though its precise mechanistic contribution remained long unresolved [151-153]. Moreover, YidC contains a C-terminal cytosolic extension, known as the C2 loop, which is rich in positively charged amino acids. This region can bind translating ribosomes and may contribute to substrate recruitment. However, deletion of the C2 loop does not impair insertion activity nor cell viability, suggesting that it modulates efficiency rather than forming an essential structural element [153-155].

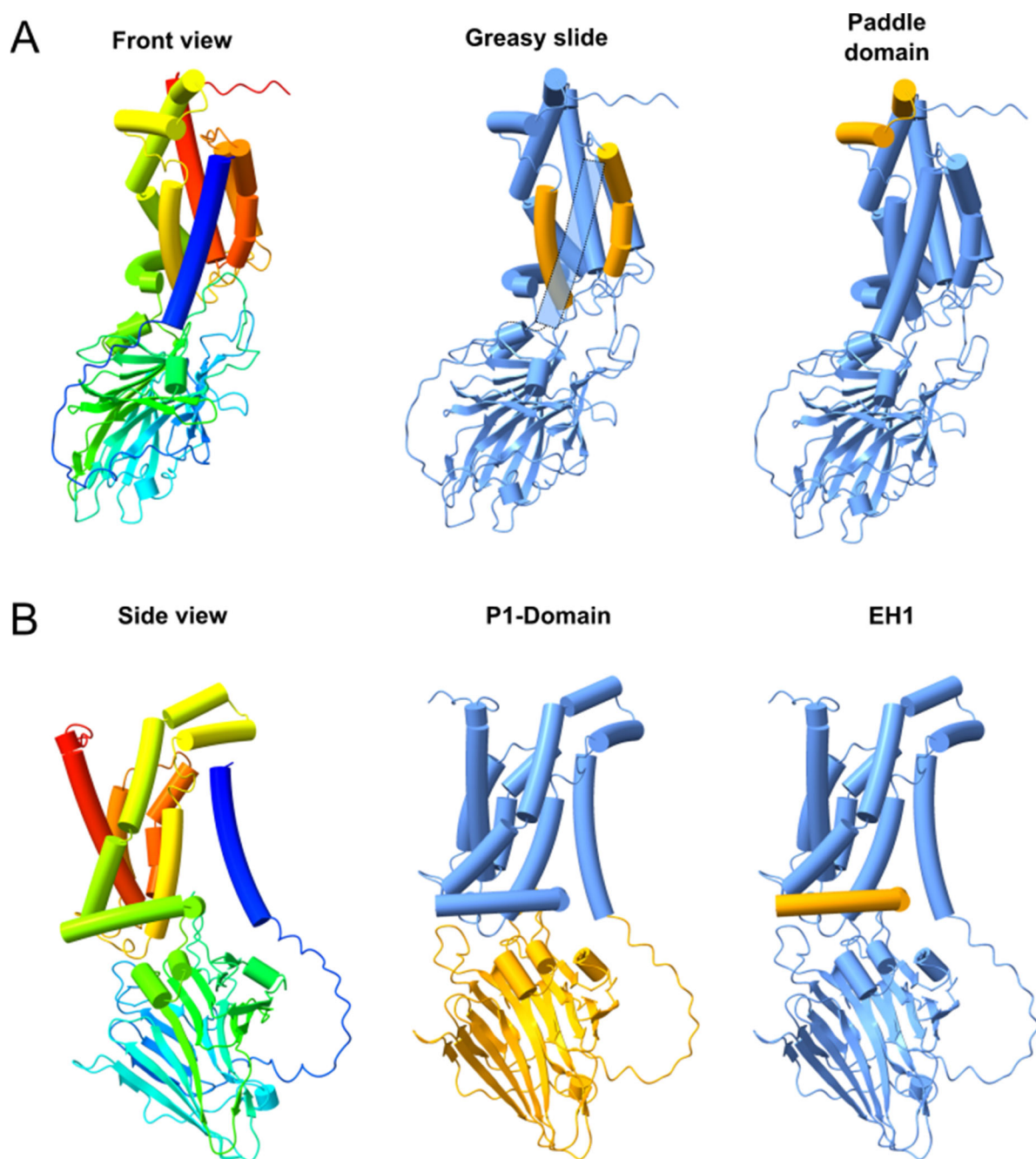


Figure 7: Features of YidC. Front (**A**) and side view (**B**) of the membrane protein insertase YidC, displayed in a rainbow color scheme from the N-terminus (blue) to the C-terminus (red). Key structural features are highlighted in yellow. For clarity, TMH1 in the highlighted view of the hydrophobic slide is shown as semi-transparent.

1.4 The Two-Pathway System of Sec

Most exported or membrane-integrated proteins interact with the universal Sec system, which sits in the cytoplasmic membrane of bacteria and archaea, and in the endoplasmic reticulum of eukaryotes. The bacterial Sec system carries out two major tasks: the translocation of secretory, periplasmic, and outer membrane proteins across the membrane, and the insertion of membrane proteins into the membrane (Figure 8) [125, 156]. Each functional mode is associated with a specific route of targeting and delivery of client proteins: The SecA-dependent post-translational pathway, largely employed for secretory, periplasmic and outer membrane proteins, and the SRP-dependent co-translational pathway followed mainly by IMPs [156-160]. Which path the nascent chain will take is commonly determined by the properties of its N-terminal part, such as signal peptide (SP) or the first TMH [161].

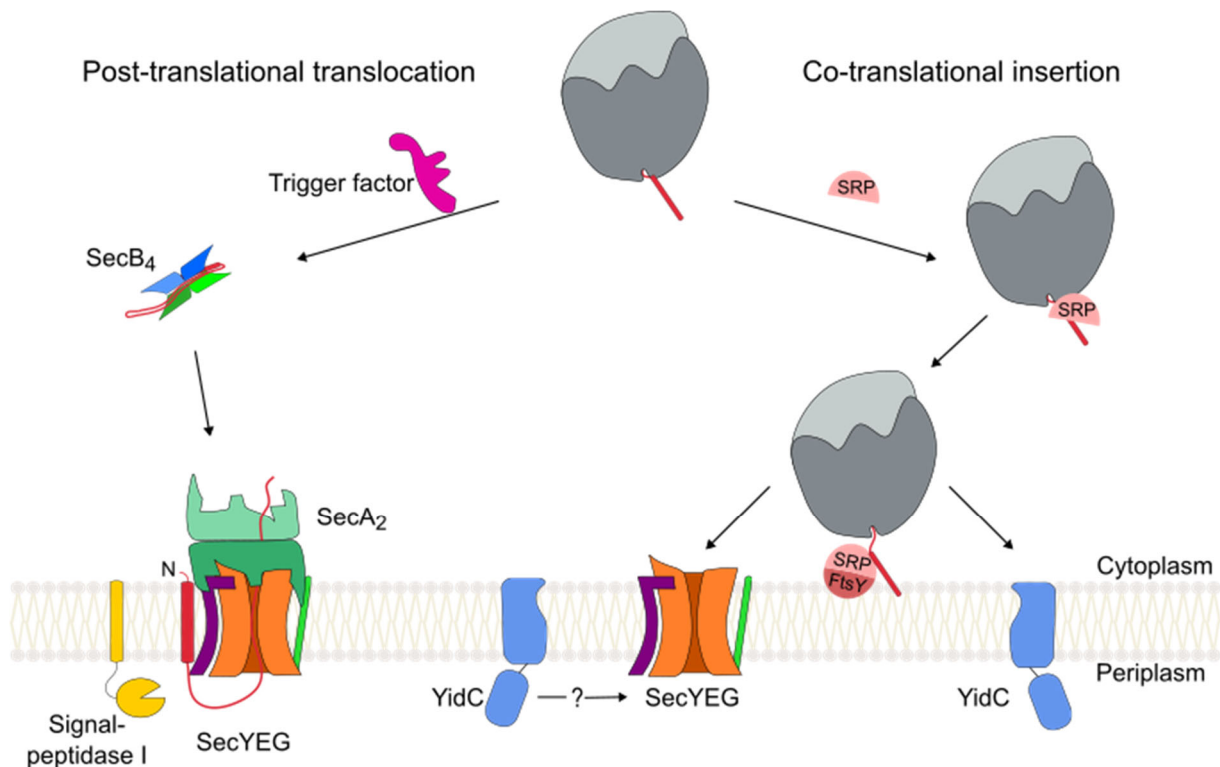


Figure 8: The general secretory pathway (Sec). The Sec system operates in two distinct modes: co-translational and post-translational. Proteins destined to cross the inner membrane are predominantly transported via the post-translational pathway. Following synthesis at the ribosome, these proteins may interact with cytoplasmic chaperones, such as Trigger Factor or SecB, before being translocated by SecA through the SecYEG complex. This process is completed by cleavage of the signal peptide by signal peptidase I. Proteins destined for membrane insertion are targeted by the signal recognition particle (SRP) to its receptor (FtsY) and subsequently transferred to the membrane protein insertase, which may consist of SecYEG and/or YidC. The insertion process is subsequently driven by the ribosome.

1.4.1 Post-Translational Translocation

In the post-translational translocation scenario, proteins are fully synthesized at the ribosome before they are delivered to the Sec machinery by cytoplasmic chaperones or targeting factors such as SecB and trigger factor (TF), which maintain nascent secretory proteins in an unfolded, translocation-competent state and hand them over to SecA [162-164]. SecA subsequently associates with SecYEG and drives polypeptide movement through the channel through cycles of ATP hydrolysis. Upon completion of translocation, the signal peptide is cleaved by a signal peptidase, releasing the mature protein into the periplasm, where further modifications can occur. The majority of secretory proteins follow this SecA-dependent, post-translational route [125, 159, 165].

1.4.1.1 Targeting by Trigger Factor and SecB

The delivery of secretory (pre)proteins to the bacterial translocon is the first step of post-translational translocation in bacteria. These preproteins are fully synthesized by the ribosome in the cytoplasm before their targeting to the membrane begins, whereupon engagement with the SecYEG translocon occurs. This pathway is, in most cases, supported by cytoplasmic chaperones, usually ATP-independent holdases that assist in keeping the preprotein unfolded, aggregation-free, and in a translocation-competent state [13, 166]. The function of these proteins goes beyond their holdase activity, with one prominent example being Trigger factor (TF), which is a known targeting factor as well and one of the first chaperones to interact with the emerging nascent chain at the ribosome [162, 167]. However, *in vitro* studies further suggest that SecA can directly interact with the ribosome and recognize preproteins, thereby targeting and translocating clients on its own [168].

Recognition at the ribosome, or shortly after synthesis of the first few amino acids, is therefore the first and most important step in post-translational translocation [169]. Signal peptides of proteins targeted to the Sec machinery are composed of three regions: a positively charged N-terminus, a hydrophobic region that later forms an α -helix in the membrane, and a rather polar C-terminus, where helix-breaking residues (prolines and glycine) and small amino acids are often found prior to the signal peptidase I cleavage site (Ala-X-Ala, where X can be any amino acid) [170-172]. Beyond serving as targeting signals, signal peptides influence chaperone interactions, delay folding of the mature domain, and modulate translocation efficiency [173, 174].

As the nascent chain emerges from the ribosomal exit tunnel, TF is recruited, thereby preventing SRP binding to the moderately hydrophobic peptide chain, except in the case of SRP-specific signal sequences and is thereby one of the first chaperones to interact with the ribosome and its nascent chain [162, 175, 176]. In complex with the ribosome, TF is monomeric, whereas in the cytoplasm and in complex with preproteins it has been observed in both dimeric and monomeric forms [177]. This, together with further reports, indicates a rapid monomer-dimer equilibrium [178]. However, TF is not only a targeting factor, as it is also involved in anti-aggregation of unfolded preproteins

by hydrophobic interactions [179]. It has the ability to keep OmpA in a translocation-competent form [180]. Multiple publications also suggest that TF is involved in folding assistance by the ATP-dependent chaperones DnaK/DnaJ and GroEL/GroES, which share overlapping substrate pools and can contribute to protein export, particularly under conditions where SecB is absent [13, 181-185]. TF and DnaK show functional redundancy, as both proteins interact with many proteins co-translationally, and as only a dual-knockout leads to aggregation and lethality above 30°C [181, 182, 186, 187]. Structurally, TF resembles a dragon, which can be divided into three parts: an N-terminal “tail” region, a “head” region, and a C-terminal region encoding the two “arms” of TF (Figure 9) [188]. The tail region is responsible for ribosome binding and also exhibits chaperone activity, with overall homology to the heat-shock protein Hsp33. The head region possesses peptidyl-prolyl isomerase activity as well as auxiliary chaperone activity. The C-terminal region mediates the main chaperone activity and shows homology to the *Mycoplasma* chaperone Mpn555 and the periplasmic chaperone SurA [189]. The cytoplasmic chaperones form a dynamic and interconnected network that supports preprotein targeting by preventing aggregation, stabilizing unfolded conformations, and promoting delivery to the SecA:SecYEG complex [13, 28]. Among these, SecB is the best-characterized holdase and fulfils a dual role in post-translational translocation by maintaining preproteins in a translocation-competent state and targeting them to SecA [190, 191]. SecB functions as a stable homotetramer, more specific a dimer of dimers, that binds extended regions of preproteins via hydrophobic grooves formed upon oligomerization, allowing substrates to wrap around the chaperone while being protected from misfolding (Figure 9) [192-194]. Binding of the substrate enhances the interaction between SecB and SecA, thereby promoting efficient targeting to the translocase [163, 195].

SecB is a cytoplasmic chaperone with a dual role in post-translational protein export, maintaining preproteins in a translocation competent state and targeting them to SecA. Initially, SecB client selection was explained by the kinetic partitioning hypothesis, which proposed that signal peptide mediated folding delays promote SecB binding [196]. However, multiple findings challenge this model. SecB interacts with long nascent chains prior to stable folding and binds its clients independently of their signal sequences [197, 198]. Furthermore, SecB:client interactions are rapid, reversible, and diffusion-limited, occurring at rates that exceed those of protein folding, indicating that folding behaviour alone cannot account for SecB specificity [199]. Repeated cycles of SecB binding maintain clients in an unfolded equilibrium that prevents stable folding [200, 201]. Certain proteins have been shown to depend on SecB in *E. coli*, using a combination of pulse-labeling protein secretion studies and proteomics approaches, including DegP, FhuA, FkpA, GBP, LamB, MBP, OmpA, OmpF, OmpT, OmpX, OppA, PhoE, TolB, TolC, YbgF, YgiW, and YncE [202]. More recent studies further suggest a role for SecB in inner membrane protein biogenesis, showing that SecB binds to membrane associated ribosome nascent chain complexes, engages nascent chains that have transiently dissociated from the translocon, and plays a critical role in membrane protein biogenesis [203].

Collectively, these findings underscore that efficient targeting in post-translational translocation emerges from the interplay between molecular chaperones and intrinsic preprotein features, shaping a translocation-competent secretome.

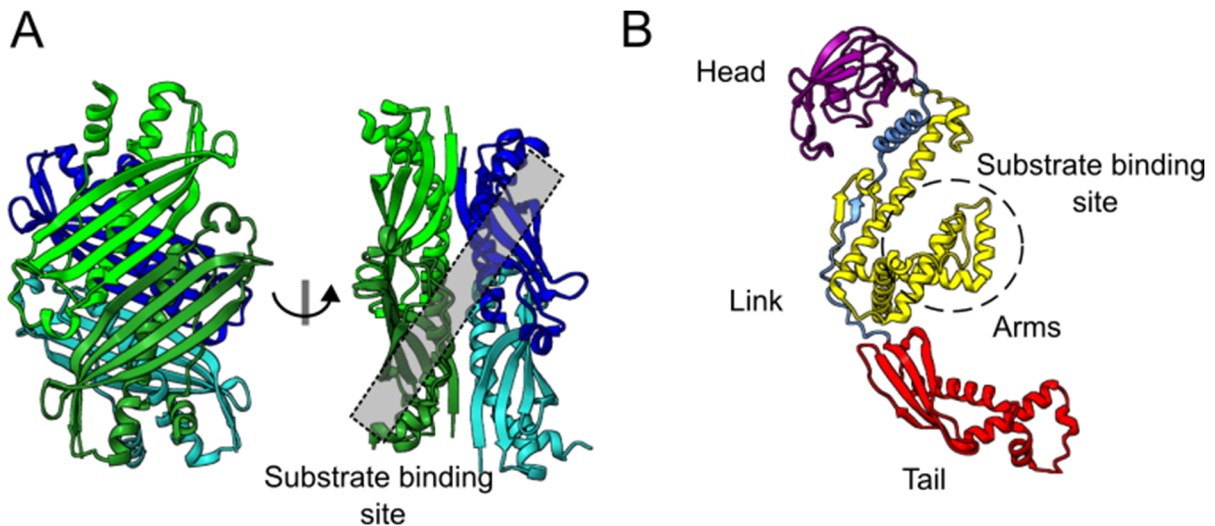


Figure 9: The cytoplasmic chaperones SecB and Trigger factor. (A) Crystal structure of *E. coli* SecB (PDB: 1QYN), showing the tetrameric chaperone arranged as a dimer of dimers. The top view highlights the antiparallel stacking of the two halves. The side view illustrates the sandwich-like organization, with a central hydrophobic cleft responsible for substrate binding located between the two dimer halves. (B) Crystal structure of *E. coli* Trigger factor (PDB: 1W26), with distinct regions highlighted in different colors. From N- to C-terminus: tail in red, link in blue head region in purple and arms in yellow.

1.4.1.2 The ATPase SecA

SecA is an essential peripheral membrane-associated ATPase that provides the energy required for bacterial protein translocation through SecYEG by coupling ATP hydrolysis to a mechanical force. It is a multidomain motor protein composed of an N-terminal amphipathic helix, two nucleotide binding domains (NBD), separated by a preprotein cross-linking domain (PPXD), a helical scaffold domain (HSD), a helical wing domain (HWD), a two-helix finger (THF) and the C-terminal linker domain (CTL) (Figure 10).

The N-terminal amphipathic helix is mainly responsible for lipid binding, since deletion of the N-terminus completely abolishes SecA binding to the membrane and preprotein translocation, which can be restored *in vitro* by artificially anchoring SecA to proteoliposomes [204]. Two nucleotide-binding domains, NBD1 and NBD2 (also known as intramolecular regulator of ATPase 2), are evolutionary related to DNA and RNA helicases form the conserved DEAD (Asp-Glu-Ala-Asp) motor responsible for chemical-mechanical coupling [205, 206]. PPXD serves for binding of the preprotein, as the preprotein can be crosslinked to, and the domain plays an important role in ATPase activity [207, 208]. PPXD is not the only SecA domain to interact with the

signal peptide: According to crosslinking studies, residues of HSD) and HWD are also involved [209]. While HSD has an interconnecting role within SecA, HWD is linked with the transfer of conformational changes upon ATP hydrolysis [210, 211]. Further towards the C-terminus is the two-helix finger, which is associated with pushing the substrate through SecY upon ATP hydrolysis or altering the conformation of the lateral gate, as the exact mechanism remains highly debated [128, 204, 212-216]. The C-terminus of SecA harbours a zinc finger domain that is important for the Interactions with SecB and the phospholipids [191, 217, 218].

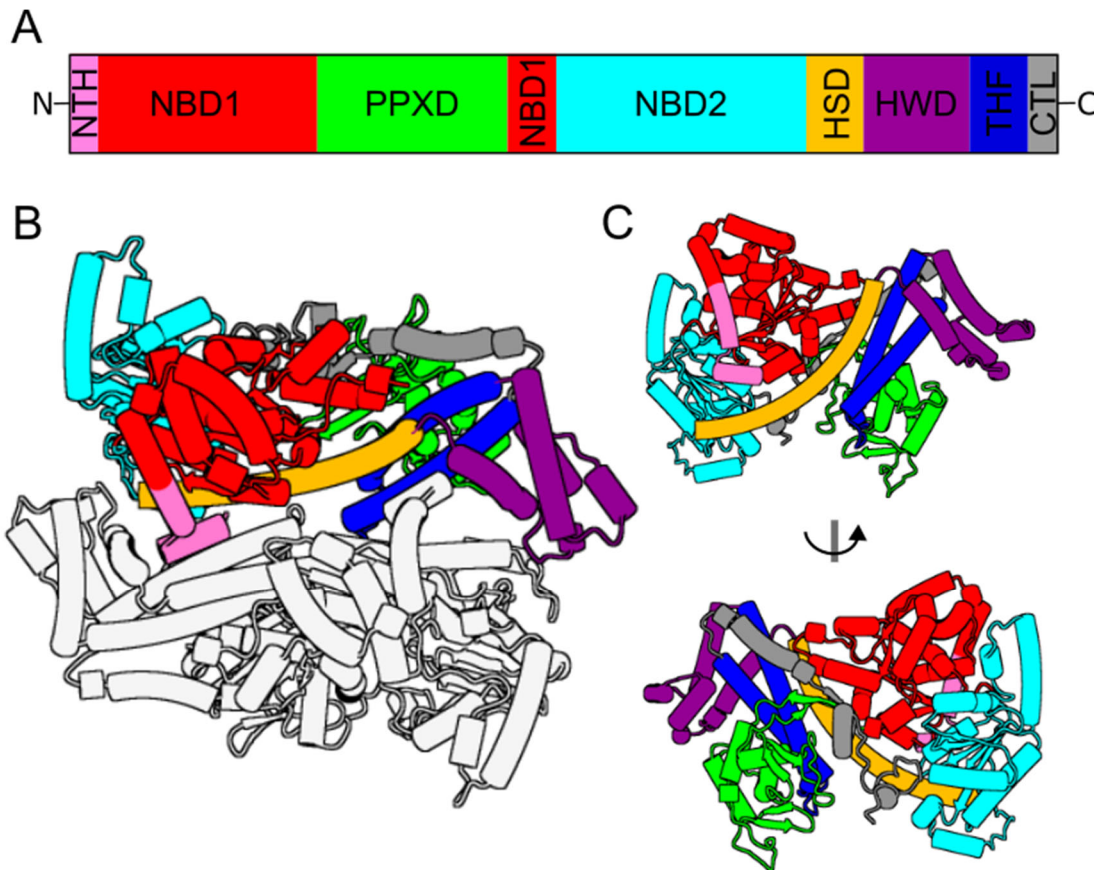


Figure 10: The structure of the motor protein SecA. (A) Organization of SecA. The N-terminus contains the amphipathic helix (pink), followed by two nucleotide-binding domains (NBDs; red and cyan), of which the first comprises the preprotein crosslinking domain (PPXD; green). The C-terminal half contains the helical scaffold domain (HSD; yellow) and the helical wing domain (HWD; purple), as well as the two-helix finger, preceding the C-terminal linker domain. (B) AlphaFold3 model of dimeric *E. coli* SecA. For clarity, only regions within one subunit are color-coded as described in (A). (C) Side and back views of monomeric SecA.

1.4.2 Co-Translational Insertion & SRP

Membrane proteins account for approximately 20-30% of the cellular proteome [95]. Biogenesis of α -helical IMPs in bacteria typically occurs in a co-translational manner: The nascent TMHs emerging from the ribosome are recognized by the SRP and

delivered to the universally conserved Sec translocon in the form of ribosome-nascent chain complexes (RNCs) that facilitates their insertion in the lipid bilayer [98, 219]. Those signal peptides are not conserved at the sequence level, but share common physicochemical properties, including high content of hydrophobic amino acids and a strong propensity to adopt α -helical structures [220, 221]. In contrast to signal peptides directing proteins to post-translational secretion pathways, SRP-dependent signal peptides often lack conserved positively charged residues at the N-terminus and display increased hydrophobicity [143, 222].

The SRP of *E. coli* is a ribonucleoprotein complex composed of a 4.5S RNA and the protein Ffh, a homolog of the eukaryotic SRP54 protein [223]. The 4.5S RNA primarily functions to stabilize the SRP-FtsY complex, with FtsY serving as the SRP receptor (Figure 11) [224, 225]. Ffh contains an N-terminal NG-domain that mediates GTP binding and hydrolysis as well as interaction with the receptor [226, 227]. Its C-terminal M-domain associates with the ribosomal exit tunnel, where it recognizes and binds hydrophobic signal sequences or transmembrane segments emerging from nascent membrane protein chains [228]. While being present only in a few hundred copies in the cell, SRP scans ribosomes quickly based on their filled ribosomal exit tunnels or exposed signal peptides, with a nanomolar to a sub-nanomolar affinity and switches to a targeting mode to its receptor [96, 229].

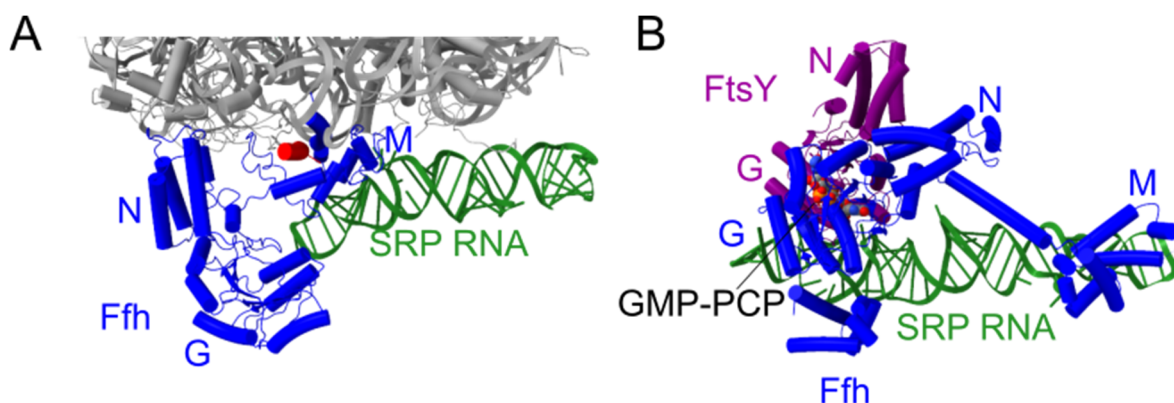


Figure 11: Signal recognition particle. (A) SRP (Ffh in blue, 4.5S RNA in green) in complex with the ribosome (only the ribosomal exit tunnel is shown in grey, RNC shown in red) (PDB:2J28) the N-, G- and M- region of Ffh are indicated. (B) SRP:FtsY complex (PDB: 2XXA) Ffh in blue, FtsY in purple. The GMP-PCPs between the two G-domains highlights their interaction interface.

Targeting proceeds through interaction of the SRP:RNC complex with the SRP receptor FtsY, a membrane-associated protein that interacts with the inner membrane and SecY via its A-domain and contains an NG-domain homologous to that of Ffh responsible for binding and hydrolysis of GTP [230]. Membrane association of FtsY depends on anionic lipids and formation of an amphipathic helix, although loss of the A-domain does not impair membrane protein targeting (Figure 11 B) [231-233]. The formation of the SRP:FtsY heterodimer subsequently stimulates mutual GTPase activity, driving conformational rearrangements that promote relocation of the complex

on the 4.5S RNA and facilitate handover of the RNC to the SecYEG translocon [228]. Finally, interactions with the distal end of 4.5S RNA trigger GTP hydrolysis, leading to dissociation of the SRP:FtsY complex and completion of co-translational targeting [229, 230, 234].

While most membrane proteins follow this canonical route via the SRP and its receptor, exceptions exist. For example, the membrane protein RodZ with a large cytosolic domain and a single TMH is targeted co-translationally via SecA [235, 236]. The periplasmic maltose-binding protein MalE, also referred as MBP, is another example of co-translational targeting mediated by SecA [237]. Another example of this non-canonical insertion route is the ability of the SRP to target certain substrates post-translationally, including several tail-anchored proteins [238, 239].

1.4.2.1 SecYEG and SecYEG/SecA Insertion

The SecYEG translocon is the central protein-conducting channel of the bacterial inner membrane and can function either as a standalone complex or in association with accessory factors, such as YidC, YajC, SecDF as well as the periplasmic chaperones PpiD and YfgM, to accommodate different classes of membrane proteins [33, 240]. In the most conserved and simple pathway, ribosome:nascent chain complexes (RNCs) are delivered to SecYEG in an SRP-dependent manner. SecYEG engages the ribosome at the exit tunnel through cytoplasmic loops located between TMHs 6/7 and TMHs 8/9 of SecY, and via the amphipathic helix of SecE [241, 242]. This interaction aligns the ribosomal exit tunnel with the translocon pore, forming a continuous conduit that enables direct transfer of the nascent polypeptide from the ribosome into the SecYEG channel [120]. Single-molecule analysis also suggests that the rate of TMH insertion is largely determined by ribosomal translation speed rather than translocon rearrangements [243].

In contrast, substrates with large periplasmic domains might require assistance of the SecA ATPase. In this hybrid SecYEG/SecA pathway, RNCs are initially targeted to SecYEG via SRP and the hydrophobic TMH is inserted co-translationally. Subsequent dissociation of the ribosome allows SecA to bind the translocon and drive post-translational translocation of extended periplasmic domains across the cytoplasmic membrane [244, 245]. The best described example of this pathway is the membrane protein FtsQ, which contains a single TMH followed by a long periplasmic tail [246, 247].

1.4.2.2 YidC Insertion

YidC can also work as an independent insertase and insert certain substrates independently of SecYEG. Those substrates are usually short, moderate hydrophobic and are rich in negatively charged residues. Proven examples are subunit c of the F₁ F_o ATP synthase, the mechanosensitive channel protein MscL, the phage proteins M13 procoat and Pf3 coat, and small membrane proteins such as YohP and YkgR,

which can be promiscuously be inserted either by the Sec translocon or YidC [238, 248-252]. Furthermore, YidC is also known to insert tail-anchored (TA) proteins such as TssL, SciP, DjlC, and Flk [239, 253-255]. In eukaryotic membranes, this role among Oxa1 superfamily members is fulfilled by GET1 of the guided entry of TA proteins (GET) complex in the yeast ER, and by EMC3 of the ER membrane protein complex (EMC) in mammalian cells [256-258]. A further eukaryotic protein involved in membrane protein biogenesis is TMCO1, though its precise function remains incompletely understood beyond an apparent role in the biogenesis of membrane proteins with more than four transmembrane helices (TMHs) [141, 259].

Members of the Oxa1 superfamily have also been linked to the insertion of C-terminal TMHs in multispanning proteins [260]. Geometrical considerations suggest that when translation is complete, 40-45 amino acids remain within the ribosomal tunnel and must therefore be inserted post-translationally, after the ribosome dissociates from the translocon [261]. In bacteria, this role is fulfilled by YidC, and in mammals, by EMC3 [260, 262].

Taken together, the fact that YidC alone accomplishes tasks distributed among several dedicated machineries in eukaryotes may point to an evolutionary diversification of the ancestral Oxa1 superfamily, driven by the greater complexity of eukaryotic membrane protein biogenesis.

1.4.2.3 SecY-YidC Cooperation

Many bacterial IMPs rely on the coordinated action of SecYEG and the membrane insertase YidC for efficient insertion and correct folding. Depending on the substrate, YidC can be either essential for membrane insertion itself or required at later stages to support proper folding of the protein within the lipid bilayer. These functional overlaps have led to the proposal that SecYEG and YidC form a transient complex [143, 263].

Known examples of this cooperation, proven by crosslinking and co-purification studies are substrates such as FtsQ, leader peptidase, and MtlA which interact with both SecYEG and YidC during their biogenesis [246, 264, 265]. FtsQ requires SecYEG for membrane insertion and translocation of the periplasmic domain, while YidC directly contacts its TMH during this process, indicating a supportive role during insertion and early folding [246]. For CyoA, a component of the respiratory chain of *E. coli*, it has been proposed that the signal peptide, as well as the first transmembrane helix is inserted by YidC, before the second TMH is inserted via SecYEG and potentially SecA for the long C-terminal periplasmic domain [266, 267]. Another example of cooperation between SecYEG and YidC is LacY, which can be inserted in absence of YidC by SecYEG only, however crosslinking studies suggest interactions leading to the assumption that YidC is important for obtaining the correct fold and supports insertion by acting as a chaperone [263, 268]. Similar observations were made for the MalF subunit, where targeting to the SecYEG complex happens via the SRP, but despite YidC being in close proximity, YidC is not essential, but it improves stability and supports folding into stable maltose transport complex [265].

At the molecular level, YidC has been shown to contact the lateral gate of SecY and to facilitate the release of nascent transmembrane helices from the translocon into the membrane, as evidenced by crosslinks between YidC and TMHs 2b, 3, 7, and 8 of SecY [106]. This paradigm has been lately challenged by the discovery of the eukaryotic insertase machinery multipass translocon that facilitates insertion of multipass inner membrane proteins at the back of the ribosome-bound Sec61. The discovery of the alternative “back-of-Sec” pathway in higher eukaryotes, first identified in human embryonic kidney cells (HEK cells) and its subsequent identification in yeast through the orthologous Sec61-SND3 machinery has raised important questions regarding its evolutionary conservation and origin [109, 111, 112, 269].

While SecYEG is the core of translocation in bacteria, it is also believed to function in a super-complex of seven proteins, the so-called holotranslocon [270]. Components are besides the SecYEG heterotrimer, the ancillary SecDF-YajC subcomplex, from which SecDF is believed to be responsible for proton motive force-driven translocation of preproteins, further supported by crosslinks of SecF to SecG and the P1 domain of YidC [271]. The holotranslocon has also been shown to have also a 6-fold higher affinity for the ribosome *in vitro* which might also explain the improved insertion efficiency of CyoA into proteoliposomes containing the holotranslocon compared to the ones just containing SecYEG [270]. The exact organization of this super-complex is still not fully elucidated, given the rather low resolution achieved in the accompanying cryo-EM analysis [107]. Therefore, multiple questions still arise: which path is the nascent chain taking from the ribosome into the membrane? What is the exact architecture of the SecYEG:YidC complex and the holotranslocon? How does the translocon differentiate between different insertion pathways?

2. Aims and Objectives

Protein transport, folding, and membrane insertion are tightly coordinated processes that rely on the interplay of multiple cellular factors. Elucidating the molecular mechanisms underlying these processes is essential for understanding bacterial protein biogenesis and may facilitate the development of novel antibacterial strategies.

The first part of this thesis focuses on the opportunistic human pathogen *P. aeruginosa* and the biogenesis of one of its most abundant virulence factors, the secreted lipase LipA. The maturation of LipA in the periplasm requires the specific foldase LipH. Previous studies have primarily focused on the chaperoning domain of LipH, whereas the function of the preceding N-terminal domain has remained unclear. This work therefore aims to elucidate the role of the LipH variable domain and the membrane in LipA biogenesis. In addition, the initial recognition of the LipA client protein by LipH will be investigated using biochemical and structural approaches.

In the following parts of this thesis, the focus shifts from maturation in the periplasm to either insertion into or transport across the inner membrane by the central component of the bacterial Sec machinery, the SecYEG translocon. The second part of this work focuses on how membrane proteins enter the membrane. Since the complete path from the ribosomal exit tunnel into the membrane has not been fully resolved yet, this chapter introduces an *in vitro* platform to study the co-translational assembly of the ribosome with the membrane protein insertase (complex). Building on this system, several key questions are addressed: Is there cooperativity between SecYEG and accessory proteins such as YidC? If so, where is YidC positioned? And finally, how does YidC facilitate the insertion of nascent membrane proteins on a molecular level?

The third and final part of this study focuses on the role of membrane lipid composition, in modulating Sec translocon activity. Recent studies have identified lipid packing as an underappreciated determinant of protein transport efficiency, with unsaturated acyl chains enhancing translocation, likely by promoting SecA membrane association. However, this prevailing SecA-centered model does not fully explain the magnitude of the observed effects. Therefore, this chapter aims to investigate how lipid packing influences the Sec translocon itself, particularly the dynamics of the lateral gate. A key objective is to determine whether SecY alone acts as the decisive factor in this process and to identify the translocon components responsible for lipid-dependent modulation of lateral gate dynamics.

3. Conformational dynamics of the membrane-anchored foldase LipH from *Pseudomonas aeruginosa* facilitates recognition and release of the client lipase

Authors: Max Busch, Jennifer Loschwitz, Athanasios Papadopoulos, Jens Reiners, Wieland Steinchen, Vincenzo Calvagna, Sander H.J. Smits, Karl-Erich Jaeger, Alexej Kedrov

Published: Journal of Biological Chemistry

Contribution: **Max Busch:** Writing – Review & Editing; Writing – Original Draft; Visualization; Methodology; Investigation; Formal Analysis; Data Curation

Jennifer Loschwitz: Writing – Review & Editing; Writing – Original Draft; Visualization; Methodology; Investigation; Formal Analysis; Data Curation

Athanasios Papadopoulos: Writing – Review & Editing; Methodology; Formal Analysis; Data Curation

Jens Reiners: Writing – Review & Editing; Writing – Original Draft; Visualization; Methodology; Investigation; Formal Analysis; Data Curation

Wieland Steinchen: Writing – Review & Editing; Writing – Original Draft; Visualization; Methodology; Investigation; Formal Analysis; Data Curation

Vincenzo Calvagna: Investigation; Formal Analysis

Sander H.J. Smits: Writing – Review & Editing; Supervision; Funding Acquisition

Karl-Erich Jaeger: Writing – Review & Editing; Project Administration; Conceptualization

Alexej Kedrov: Writing – Review & Editing; Writing – Original Draft; Formal Analysis; Data Curation; Supervision; Funding Acquisition; Project Administration; Conceptualization



Conformational dynamics of the membrane-anchored foldase LipH from *Pseudomonas aeruginosa* governs recognition and release of its client lipase

Received for publication, March 13, 2026, and in revised form, June 3, 2026 Published, Papers in Press, June 19, 2026
<https://doi.org/10.1016/j.jbc.2026.113266>

Max Busch^{1,†}, Jennifer Loschwitz^{1,†}, Athanasios Papadopoulos^{1,2,†}, Jens Reiners², Wieland Steinchen³, Vincenzo Calvagna¹, Sander H. J. Smits^{2,4,5}, Karl-Erich Jaeger^{6,7}, and Alexej Kedrov^{1,5,*}

From the ¹Heinrich Heine University Düsseldorf, Synthetic Membrane Systems, Institute of Biochemistry, Düsseldorf, Germany; ²Center for Structural Studies, Heinrich Heine University Düsseldorf, Düsseldorf, Germany; ³Center for Synthetic Microbiology & Department Chemistry, Marburg University, Marburg, Germany; ⁴Heinrich Heine University Düsseldorf, Structural Biochemistry, Institute of Biochemistry, Düsseldorf, Germany; ⁵Interfaculty Center for Membrane Research, Heinrich Heine University Düsseldorf, Düsseldorf, Germany; ⁶Institute of Molecular Enzyme Technology, Heinrich Heine University Düsseldorf, Düsseldorf, Germany; ⁷Forschungszentrum Jülich GmbH, Institute of Bio- and Geosciences IBG-1: Biotechnology, Jülich, Germany

Reviewed by members of the JBC Editorial Board. Edited by Chris Whitfield

The lipase LipA from *Pseudomonas aeruginosa* is an extracellular enzyme that plays an important role in bacterial infections. Prior to its export *via* the type II secretion system, LipA requires the cognate membrane-anchored foldase LipH for maturation in the periplasm. Though structural studies elucidated the architecture of the LipH:lipase complex, how the full-length, membrane-tethered foldase recognizes, folds, and releases its client has remained barely understood. Here, we combine *in silico* and *in vitro* analysis to resolve the conformational dynamics and function of the full-length LipH in a membrane context. Simulations reveal that the membrane-anchored LipH is highly dynamic, sampling a broad ensemble of conformations. The chaperoning cavity is transiently closed or occluded by both the proximal membrane and the linker polypeptide, which is further confirmed by structural analysis. Despite the steric hindrance, the full-length LipH reconstituted into lipid-based nanodiscs and amphipols efficiently activates LipA, though it displays substantially reduced affinity for the client. We propose that the negatively charged membrane promotes the release of the folded client, enabling multiple chaperoning cycles. Hydrogen/deuterium exchange analysis reveals that the MD2 domain of LipH is engaged in stable interactions with LipA, whereas MD1 contacts are transient. Consistently, LipH readily captures N-terminal fragments of LipA, indicating that initial recognition relies on local interactions *via* the MD2 domain. Together, our results show how membrane coupling and intrinsic conformational plasticity modulate the function of the steric chaperone, and suggest that the membrane-anchored LipH balances capture, folding, and release of LipA to enable its efficient secretion.

Protein secretion in bacteria plays a central role in colonization, invasion and host-pathogen interactions, as, among other clients, it enables transport of virulence factors across cellular membranes. In pathogenic bacteria, such as an opportunistic human pathogen *Pseudomonas aeruginosa*, specialized secretion systems facilitate the delivery of toxins and effector proteins, either into the environment or into host cells (1). The type II secretion system (T2SS), also referred to as the general secretion pathway due to its high conservation among Gram-negative bacteria and broad specificity, mediates export of folded client proteins from the periplasm into the extracellular space (2). In *P. aeruginosa*, the lipase A (LipA) is a highly abundant secretory protein, which is exported *via* Xcp-T2SS (for “extracellular protein deficient”) (3–5). Due to its association with biofilms and contribution to lysis of host cells, LipA is considered as a virulence factor (5), and understanding the route of LipA folding and secretion would be valuable for combating the pathogenesis of *P. aeruginosa*. Biogenesis and secretion of the lipase occur in distinct steps, starting with translation of the precursor protein and its translocation into the periplasm *via* the SecYEG:SecA route (Fig. 1A). There, maturation of LipA, *i.e.* cleavage of the signal sequence, takes place, followed by folding into its enzymatically active form, recognition by T2SS and secretion across the outer membrane (6). Notably, LipA secretion is essentially dependent on the specific chaperone LipH, also referred to as lipase-associated foldase or *Lif* (6, 7). Encoded in one operon with LipA, LipH is a membrane-anchored chaperone that recognizes its cognate client in the periplasm and facilitates its correct folding, so the lipase overcomes the energetic barrier towards the catalytically active conformation (6, 8–10). In a following step, the folded LipA should be released from LipH and recognized by the dedicated Xcp-T2SS, potentially *via* interactions with the XcpP protein in *P. aeruginosa* (11), and then it is expelled upon a piston-like movement of the T2SS pseudopilus (5).

[†] Those authors contributed equally to this work.

* For correspondence: Alexej Kedrov, Kedrov@hhu.de.



Lipase-specific foldase: dynamics and client interactions

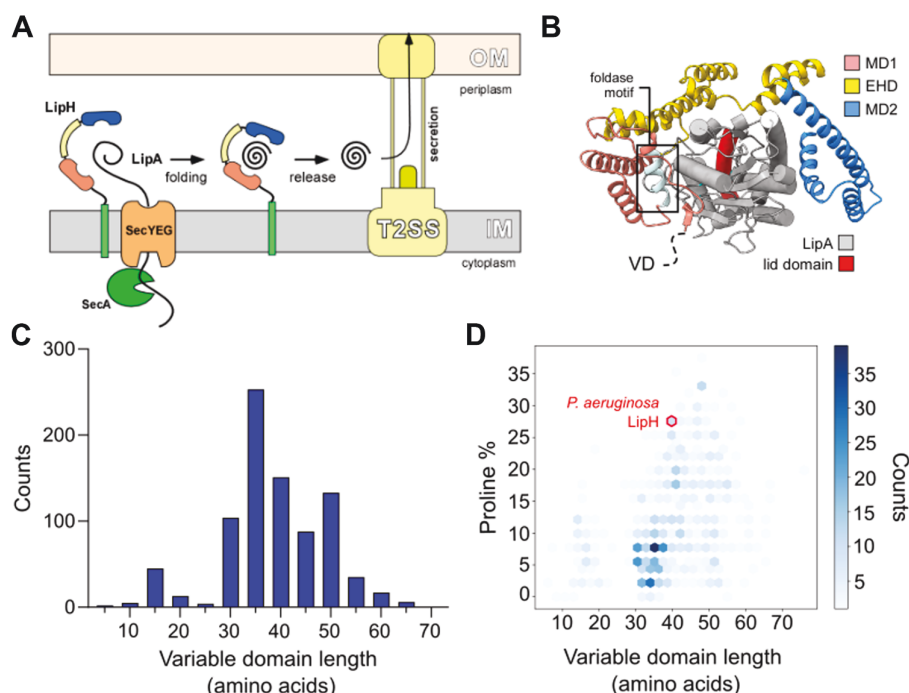


Figure 1. Architecture of the lipase-specific foldase LipH. A Scheme of LipA secretion route in *P. aeruginosa*. Translocation across the inner membrane (IM) via the SecYEG:SecA machinery is followed by recognition via the membrane-anchored steric chaperone LipH. LipH facilitates folding and activation of the lipase. After being released, LipA is competent for secretion via the type II secretion system (T2SS). B AlphaFold3-based model of *P. aeruginosa* LipH:LipA complex. The chaperoning domain of LipH (residues 63–340) is shown as a helical ribbon, with the structural elements color-coded. The N-terminal fragment (residues 1–62), including the variable domain (VD) is omitted for clarity. C Histogram of the variable domain length distribution among the identified lipase-specific foldases (in total, 858 sequences). D Distribution of the polypeptide length and the proline content of the variable domains among the identified lipase-specific foldases. Position of *P. aeruginosa* LipHVD is highlighted in red.

The lipase-foldase system is not limited to *Pseudomonas*, but also found in the genera *Burkholderia*, *Acinetobacter*, and *Vibrio* (6, 12). The crystallized foldase:lipase complexes from *Burkholderia glumae* and *Acinetobacter baumannii* reveal nearly identical structures (13, 14), and the earlier model of *P. aeruginosa* LipH:LipA (15), as well as the AlphaFold-based prediction manifest a highly similar organization (Figs. 1B and S1). Briefly, the chaperoning domain of LipH is helically folded and it consists of two mini-domains, MD1 and MD2, connected via an extended helical domain (EHD), forming together a crescent-like structure, also called “headphone” shape. MD1 contains the characteristic sequence R₉₄NLFDYFLSA₁₀₃ found among the foldases (consensus motif RXXFDY(F/C)L(S/T)A, where X can be any amino acid) (6). The available structures and the AlphaFold model reveal a large interaction interface between the chaperone and the lipase. Key contact points within the *P. aeruginosa* LipA:LipH complex include helix 1 of LipH MD1, where Ser-112 is positioned near Gln-275 of the lipase; helix 5 (part of the EHD), where Arg-199 may interact with Asn-26 of LipA; and helix 11 (part of MD2), where Arg-280 and Arg-327 potentially build salt bridges with LipA Glu-66 and Glu-58, respectively (the numbering corresponds to *P. aeruginosa* LipA without the signal sequence). Notably, the complex assembly does not involve helix 5 of the lipase, also referred as a

lid domain, that regulates access to the lipase catalytic site formed by residues Ser-82, Asp-229 and His-251 (15). The available structures, as well as the AlphaFold models, show a displacement of the lid domain (Fig. S2), and a fatty acid can be docked via AlphaFold within the open conformation of LipA, while still bound to LipH, in agreement with functional studies, where LipA is enzymatically active also when bound to LipH (16, 17).

Extensive functional and structural studies on LipH:LipA interactions have commonly focused on the chaperoning domain of LipH, leaving the preceding N-terminal region out of consideration, although it constitutes nearly 20% of the full-length LipH in *P. aeruginosa*. The region consists of an N-terminal transmembrane helix (TMH) followed by a non-conserved linker, referred as variable domain (VD). The region serves as a tether and prevents LipH co-secretion with the client lipase (6), so the membrane-anchored foldase may sequentially facilitate the fold and release of multiple LipA molecules. The VD linker of *P. aeruginosa* LipH is 41 residues long, of which 11 are prolines, and the AlphaFold-based model suggested lack of secondary structure in the region. The composition of the linker in *P. aeruginosa* LipH stands out among other lipase-related foldases: Analysis of 858 LipH homologs suggested that the linker length commonly occurs within 30 to 55 residues (Fig. 1C), but the proline content is

Lipase-specific foldase: dynamics and client interactions

typically below 10%, while it reaches 27% for *P. aeruginosa* LipH (Fig. 1D). Such high proline content is often observed in disordered polypeptide chains (18), which may enable movements of the chaperoning domain above the membrane to escape the steric hindrance, as it would be required for capturing the client lipase and/or for downstream interactions with T2SS (8, 19). However, no experimental studies addressing the dynamics and interactions of the full-length LipH are available, despite their relevance to folding and maturation processes of the client virulence factor.

Here, we combine molecular dynamics simulations, structural characterization, and biochemical analysis to probe the conformational dynamics of *P. aeruginosa* LipH, its interaction with the client lipase, and the effect of the membrane environment on the chaperoning activity. We show that LipH is highly flexible due to the unstructured linker VD and the intrinsically dynamic chaperoning domain, enabling it to

sample a large periplasmic space. We propose that the client LipA is initially recognized at its N-terminal fragment through interactions with the MD2 domain of LipH. Once the full-length LipA binds, the loosely bound LipH MD1 domain likely contributes to positioning, folding and eventual release of the folded lipase, which is assisted by the proximate membrane environment. Together, our work proposes a mechanistic model for the steric foldase function related to the pathogenicity of *P. aeruginosa*.

Results

Dynamics of the full-length LipH at the membrane interface

Aiming to resolve the dynamic interactions of LipH, we first set out to characterize the full-length protein ("LipH^{FL}": Fig. S3) at a membrane interface *via* all-atomistic molecular dynamics (MD) simulations (Fig. 2A). LipH^{FL} was anchored

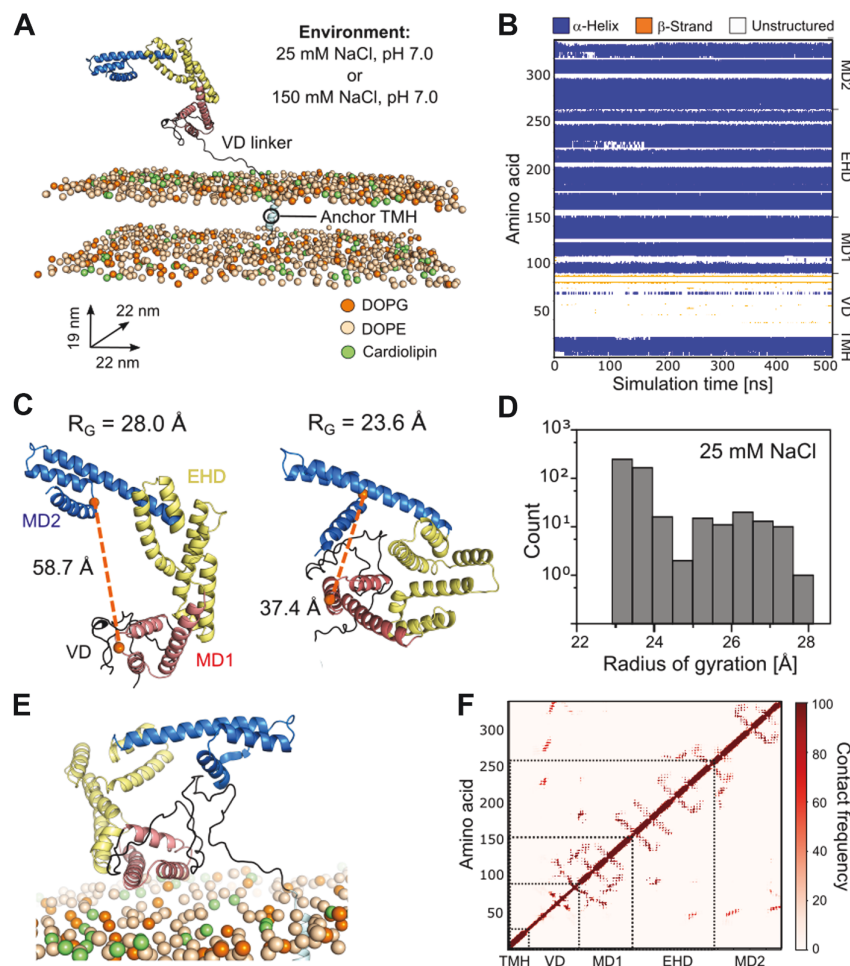


Figure 2. Molecular dynamics simulation of the full-length LipH at the lipid membrane interface. A, design of the molecular dynamics simulation system. LipHFL was anchored with its N-terminal helix (TMH, cyan) in a native-like lipid membrane. The dimensions of the designed simulation box are indicated. B, The color-coded secondary structure of LipHFL along a simulation course (25 mM NaCl) is plotted against the simulation time. C, dynamics of the chaperoning domain within LipHFL. Left: Conformation at the beginning of the simulation. Right: An exemplary conformation of a compact state acquired along the simulation. To analyse the dimensions of the domain, the distance between the C α atoms of Glu-107 (MD1) and Glu-321 (MD2) was monitored (indicated as the orange spheres and the dashed line). D, distribution of the radius of gyration of the LipH chaperoning domain along a simulation course (25 mM NaCl). E, A representative conformation of LipHFL in proximity to the lipid membrane shows extensive contacts of the unstructured VD polypeptide within the chaperoning domain. F, Map of intramolecular contacts within LipHFL along a simulation course indicates interactions of the VD linker with MD1, EHD and MD2 domains.

Lipase-specific foldase: dynamics and client interactions

with its N-terminal helix in the phospholipid bilayer composed of phosphatidylethanolamine (PE, 75 mol %), phosphatidylglycerol (PG, 20 mol %) and cardiolipin (CL, 5 mol %), which approximated the content of the major lipids found in the inner membrane of *P. aeruginosa* (20–22). The simulation box of $22 \times 22 \times 19 \text{ nm}^3$ was designed to be sufficiently large to trace the movements of LipH domains above the membrane interface, and the non-restricted atomistic simulations allowed to reconstruct the dynamics of the chaperoning domain, as well as the VD linker. Furthermore, two different environments were studied, containing NaCl at either 25 mM or 150 mM concentration. The low ionic strength approximated the periplasmic conditions of *P. aeruginosa* in the soil habitat, and the high ionic strength reflected the environment upon the tissue or lung invasion (23).

During all performed simulations, LipH^{FL} manifested little to no change in the secondary structure content, *i.e.* the chaperoning domain and the membrane anchor retained their α -helical fold, and the VD polypeptide remained largely unstructured, with transient formation of short β -strands (Figs. 2B and S4). However, the architecture of the chaperoning domain was not rigid, as the mini-domains MD1 and MD2 underwent extensive movements, causing transient widening and closing of the chaperoning cavity. Thus, the distance between the mini-domains measured for the chosen residues Glu-107 (MD1) and Glu-321 (MD2) varied from 32.93 to 68.12 Å, and the hydrodynamic radius of the chaperoning domain ranged from 23.07 to 29.77 Å in 25 mM

NaCl and from 23.13 to 29.17 Å in 150 mM NaCl (Figs. 2, C and D, S5 and S6). At the end of the simulations, the chaperoning domain commonly acquired more compact states, both at the low and high ionic strength. Notably, multiple conformations of LipH showed the VD polypeptide ingressing the chaperoning cavity, so the MD1 domain, and to a lesser extent, MD2 repeatedly interacted with the unstructured region (Figs. 2, E and F, S7). Together with the movements of the MD1/MD2 domains, this unexpected occlusion could sterically hinder specific LipH:LipA interactions and, therefore, affect folding and activation of the client lipase.

The proximate lipid membrane is another factor that potentially affects the conformational dynamics of the anchored LipH and its accessibility for LipA, as electrostatic interactions at the interface may attract or repel the solvent-exposed proteins. The chaperoning domain of LipH is highly polar, though the charged amino acids are distributed in a non-uniform manner over its surface: While the cationic residues are predominantly found within the LipA binding interface, the anionic residues are exposed at the outer surface, with pronounced clusters within MD1 and EHD on the same side of LipH (Fig. 3A). This negatively charged area should ensure electrostatic repulsion from the abundant anionic lipids, such as PG and CL, at the membrane. Indeed, when only the chaperoning domain (“LipH^{Chap}”, Fig. S3) was simulated in the absence of the TMH anchor and the VD polypeptide, it was found exclusively in the solvent, forming no contacts with the lipid leaflet (Figs. 3B and S8), and no

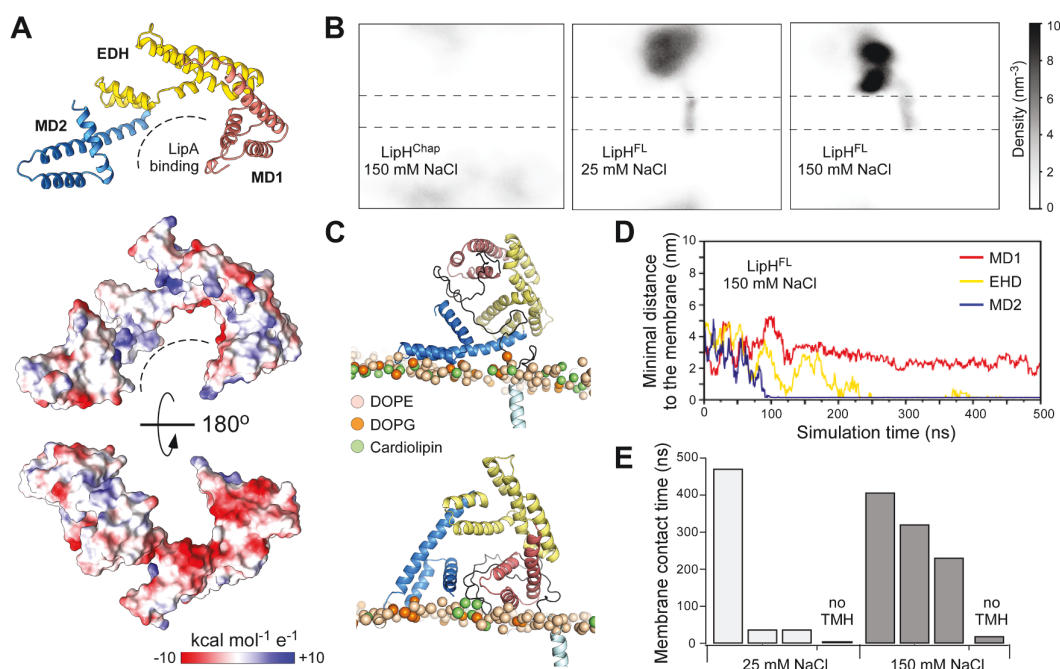


Figure 3. LipH interactions with the lipid membrane interface. A, AlphaFold-based model of *P. aeruginosa* LipH suggests presence of a negatively charged cluster of residues within the MD1 domain. Above: A ribbon visualization of the LipH chaperoning domain; below: the corresponding molecular surface coloured according to the local electrostatic potentials (blue = cationic; red = anionic; the scale bar shown below). B, localization of the soluble chaperoning domain LipHChap and the lipid-anchored LipHFL within the simulation box shown as density maps. The lipid bilayer borders are indicated as dashed lines. C, examples of conformations acquired by LipHFL upon contacting the membrane interface. D, Minimal distances of the individual LipH domains to the membrane over the course of a simulation. E, duration of LipH:membrane contacts in individual simulations at 25 and 150 mM NaCl.

interactions with liposomes were observed for the anchor-free LipH in the floatation assay (Fig. S9). In contrast, when examining the dynamics of LipH^{FL}, we observed multiple events, when the minimal protein-lipid distance measured for the chaperoning domain approached zero, reflecting a close contact with the membrane (Figs. 3B and S10). When comparing different ionic strengths, LipH docking at the membrane interface was observed in a single simulation at 25 mM NaCl, where both MD1 and MD2 domains formed a long-lasting contact with the lipid leaflet, thus blocking the access to the chaperoning cavity (Figs. 3, B–E and S10). Two other simulations at the low ionic strength showed LipH exposed to the solvent, being accessible for the client binding. In contrast, all three simulations at 150 mM NaCl indicated LipH:membrane interactions (Figs. 3E and S10, B and C). Here, the negatively charged MD1 repeatedly approached the surface, so the electrostatic repulsion was apparently compensated by the elevated salt concentration. Thus, LipH:LipA interactions may be hindered by the membrane interface at the elevated salt concentrations, and efficient secretion of LipA would require accessory holdase chaperones (17).

Dynamics of the LipH chaperoning domain

The MD simulations of the membrane-anchored LipH^{FL} suggested extensive dynamics within the chaperone in the absence of the client. Previously, single-molecule FRET studies and MD simulations on the isolated LipH^{Chap}

construct have led to qualitatively similar conclusions about the movements of the constituting domains (19). However, the N-terminal VD linker was omitted from consideration, so its putative interactions with the chaperoning domain have not been experimentally tested. To do so, we designed a soluble LipH variant consisting of the chaperoning domain with the preceding VD polypeptide (“LipH^{VD}”, Fig. S3) and carried out its biochemical and structural analysis. The purified protein was monomeric as tested by size-exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) (Fig. 4, A and B), and small-angle X-ray scattering (SAXS) was further performed to assess its conformational dynamics. SAXS recordings confirmed the monomeric state of LipH^{VD} (Table S1) but also provided estimates of its spatial dimensions and the conformational heterogeneity in the molecular ensemble. The pair-distance distribution function $p(r)$, *i.e.* the distance between each individual pair of atoms of the molecule, suggested the maximal dimensions of the molecule (r_{max}) of 12.61 nm a radius of gyration (R_g) of 3.41 nm (Figs. 4C and S11B). The broad width of Kratky plot reflected the typical behavior of a flexible and elongated molecule, in line with the $p(r)$ function (Fig. S11C). To investigate the flexibility of LipH^{VD} and to assess its possible conformations based on SAXS data, we employed the Ensemble Optimization Method (EOM), assuming independent movements of the individual sub-domains MD1, EHD and MD2, and the N-terminal VD polypeptide. The resulting R_g distribution of the molecular ensemble exhibited a bimodal profile with maxima at 2.8 nm and 3.8 nm (χ^2 value of 1.026,

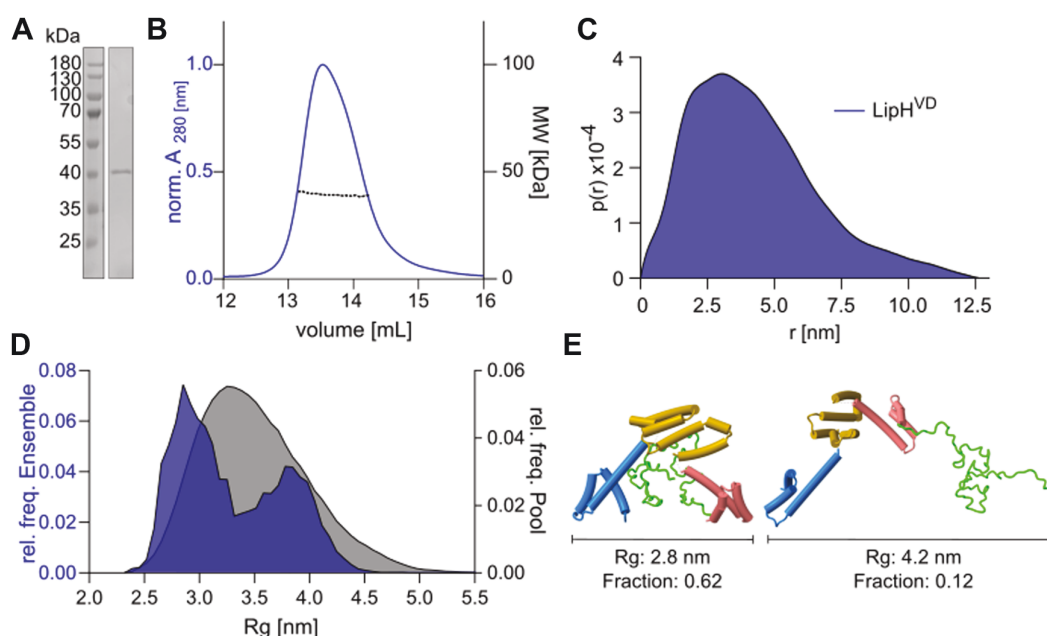


Figure 4. Conformational dynamics of LipH *in vitro*. A, SDS-PAGE of the isolated soluble LipHVD. B, SEC-MALS chromatograms of LipHVD. Blue line shows the normalized protein absorbance at 280 nm (left Y-axis). Dashed line indicates the molecular weights in kDa (right Y-axis). C, the pair distance distribution function $p(r)$ of LipHVD as determined by SAXS. D, gyration radii distribution (R_g) of LipHVD as determined by SAXS. The distribution of the pool models used for fitting is shown in grey; the ensemble of selected EOM models is shown in blue. E, representative AlphaFold3-based models of LipHVD used for EOM-fitting the SAXS data. The remodeled parts of the N-terminal polypeptide are shown in green loops. Gyration radii R_g and the occupancy of each fraction in the EOM pool are indicated. Further models are shown in Figure S10.

Lipase-specific foldase: dynamics and client interactions

CorMap p -value of 0.553) (Fig. S11, D and E). This indicated a distinct heterogeneity among LipH^{VD}, and the bimodal distribution could be described well with structural models containing the VD polypeptide within the chaperoning domain (the compact state, 60% of the conformational ensemble) or stretched away from the protein (the extended state) (Figs. 4E and S11F), in line with the MD simulations. Furthermore, the SAXS data and the EOM-based models also implied substantial movements of the sub-domains MD1, EHD and MD2, in agreement with the extensive flexibility of the chaperone seen in the MD simulations.

Biochemical characterization of the full-length LipH

MD simulations reveal that both the steric hindrance and electrostatic interactions with the membrane affect the conformation of LipH^{FL}, and so they support the idea that the proximate lipid bilayer influences LipH:LipA interactions. To scrutinize the effect of the membrane on the chaperoning properties, we set out to isolate the full-length foldase and study its interactions with LipA after reconstitution into membrane mimetics, also in comparison with the soluble construct LipHVD. The N-terminally tagged LipH^{FL} was

heterologously expressed in *Escherichia coli*, extracted with help of non-ionic detergents, DM, DDM, Cymal-6 or LMNG, and isolated *via* IMAC and subsequent SEC (Fig. 5A). With the calculated molecular mass of 39 kDa, the recombinant LipH^{FL} appeared as a band at ~43 kDa, so it demonstrated a slower migration upon SDS-PAGE (Fig. 5B), which is a common behaviour of single-spanning membrane proteins (24). While each LipH^{FL} isolation resulted in a sharp peak upon SEC, the elution volume was detergent-specific, varying from 10.5 to 12.5 ml (Fig. 5A). To examine whether the shift was caused by differences in LipH^{FL} oligomeric state or by the dimensions of the detergent micelles, we performed SEC-MALS in DDM and Cymal-6, two detergents with well-characterised micellar weights (70 kDa for DDM, 30 kDa for Cymal-6) (25–28). The protein mass calculated for both samples was in the range of 70 to 80 kDa (Fig. S12), indicating that LipH^{FL} was purified as a dimer. The dimerization was attributed to the membrane-anchoring helix, since the LipH^{VD} construct remained monomeric even at the elevated concentrations employed in SAXS measurements.

The DDM-solubilized LipH^{FL} was further used for reconstitution in membrane mimetics, *i.e.* polymer-based particles and lipid-based nanodiscs (29, 30). Upon removal of the

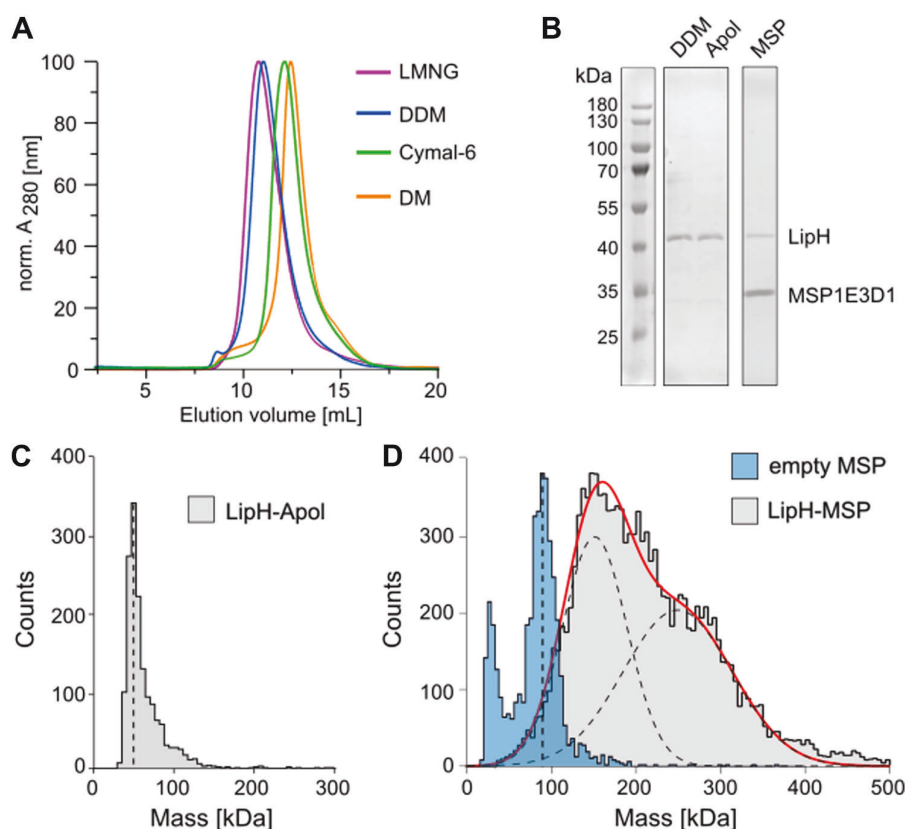


Figure 5. Isolation and characterization of the full-length LipH. A, size-exclusion chromatography profiles of LipH^{FL} purified in the indicated detergents. B, SDS-PAGE of LipHFL in the detergent micelles ("DDM"), amphipols ("Apol"), and nanodiscs ("MSP"). C, mass distribution of the amphipol-reconstituted LipH ("LipH-Apol") determined by single-molecule mass photometry. The dashed line indicates the distribution peak at approx. 50 kDa. D, mass distribution of the MSP1E3D1-based particles, with reconstituted LipHFL ("LipH-MSP") and without it ("empty MSP"). For the empty MSP, the dashed line indicates the distribution peak at approx. 90 kDa. Fitting the LipH-MSP distribution with two Gaussian peaks (dashed lines) suggests presence of two populations with mean masses of approx. 150 kDa and 250 kDa.

detergent, the amphipathic polymer (amphipol) A8-35 wraps over the hydrophobic surfaces of the target membrane protein (29), and the solvent-exposed carboxylate groups ensure solubility of the assembled particle, also in the absence of additional lipids. Differently, the nanodiscs consist of a lipid bilayer with an embedded target membrane protein, and the assembly is stabilized by a dimer of the membrane scaffold protein (MSP) bound at the periphery. The MSP1E3D1 scaffold employed here produces nanodiscs of ~12 nm diameter (31), which were loaded with DOPC:DOPG lipids (70:30 M ratio). LipH^{FL} reconstituted in amphipols ("LipH-Apol") and nanodiscs ("LipH-MSP") were analysed by single-molecule mass photometry to determine the molecular weight of the formed particles and to resolve the oligomeric state of the chaperone. The amphipol-based sample manifested a sharp peak at 50 kDa (Fig. 5C), so LipH^{FL} was embedded as a monomer (40 kDa), and the excess mass could be assigned to the polymer (32). Differently, LipH-MSP showed a broad mass distribution reflecting the heterogeneity within the ensemble (Fig. 5D). The distribution peaked at 150 kDa, and its tail could be fitted with a second Gaussian centred at 250 kDa. As the lipid-loaded nanodiscs in the absence of LipH^{FL} ("empty MSP") manifested a narrow distribution around 90 kDa, the excess masses measured for LipH-MSP likely corresponded to dimers of LipH^{FL} (peak at 150 kDa) and higher oligomers (peak at 250 kDa). Thus, the nanodiscs captured multiple copies of LipH^{FL}, possibly in alternating topologies. However, the individual chaperoning domains should be exposed to the solvent, being accessible for interaction with the client LipA.

Full-length LipH may conduct multiple chaperoning cycles

Previous studies showed that the isolated chaperoning domain LipH^{Chap} prevents aggregation of the urea-denatured lipase LipA and facilitates its correct folding and activation *in vitro* (17, 33). With the assembled set of LipH variants, *i.e.*, the soluble LipH^{VD}, and LipH-Apol and LipH-MSP containing the full-length protein, we set out to analyse the functionality of the chaperone and to identify potential effects of the N-terminal domain and the membrane environment. For example, both occlusion of the binding pocket by the VD polypeptide, as seen in SAXS and MD simulations, and interactions with the membrane surface may suppress the recognition of the client lipase and its chaperone-dependent folding, but may also favour release of the folded client followed by a next chaperoning cycle, with a non-trivial effect on the resulting enzymatic activity.

To test the chaperone:client interactions as a first step, the lipase was fluorescently labelled with CF647-maleimide, and the equilibrium assembly of the LipH:LipA complex with different foldase variants was monitored through the spectral shift in the dye fluorescence emission (34). The assay was conducted under lipase stabilizing conditions in 5 mM Tris-HCl pH 9.0, 5 mM glycine, 1 mM CaCl₂, and 5% glycerol (17, 35). To prevent aggregation in the capillaries, the conditions were further optimized by adding 0.5 mg/ml BSA,

0.05% (w/v) Tween-20, and, in the case of LipH-MSP, 0.05% FOM. The interaction was resolved for all tested constructs (Fig. 6A), though the affinities varied broadly. LipH^{VD} manifested high affinity to LipA, with K_D measured in low-nM range, in agreement with previous studies (19), so neither the fluorescent labelling of the client lipase, nor the presence of the VD polypeptide abolished the assembly. Thus, we concluded that the VD polypeptide only transiently interacts with the chaperoning pocket, or it is displaced upon binding of the client lipase. In a striking contrast, LipH^{FL} reconstituted in either amphipols or the nanodiscs showed the affinity in the range of 200 to 500 nM, thus being two orders of magnitude lower than that of the soluble LipH^{VD} (Fig. 6A). Thus, the proximate environment strongly influenced the chaperone:client interactions, which have not been previously recognized.

The differences in the affinity suggested that the environment of LipH^{FL} either suppressed the lipase recognition/binding, or promoted the dissociation of the complex and release of the lipase, potentially in a folded and enzymatically active state. To evaluate those scenarios, we set out to assess the LipH-dependent activity of LipA. As previously established in LipH:LipA *in vitro* studies (6, 8–10, 17), once the lipase has acquired its functional conformation, hydrolysis of a model substrate pNPB (para-nitrophenyl butyrate) to p-nitrophenolate can be monitored *via* a colorimetric reaction (Fig. S13), and the enzymatic activity does not require LipA release from the chaperone. In contrast, the lipase remains inactive/misfolded in absence of LipH, so only low signal associated with the substrate autohydrolysis can be observed. Hence, we implemented the enzymatic assay to compare the chaperoning properties of the soluble and the full-length LipH variants. Strikingly, both LipH-Apol and LipH-MSP constructs facilitated high activity of LipA, approx. two-fold higher than that of LipH^{VD}, despite the reduced affinity (Fig. 6A). If the membrane mimetics hindered the client recognition, a decline in folding and activity of LipA could be expected. Instead, another scenario seems plausible: The low affinity measured for LipH^{FL}:LipA might originate from enhanced dissociation of the complex, so the native-like LipH^{FL} variants were able to release their folded clients into solution and to facilitate the new chaperoning rounds. Thus, a single membrane-anchored foldase could assist in the folding of multiple LipA molecules, resembling the pathway taking place at the cellular membrane, while the membrane-less LipH^{VD} remained tightly bound to its clients.

LipH MD2 region mediates tight interactions with the client lipase

Release of the lipase towards its further secretion requires rupturing interactions within the LipH:LipA complex, learning the dynamics of the complex would be instrumental to resolve the dissociation mechanism. The available crystal structures of the homologous complexes (13, 14), as well as the model of LipH:LipA from *P. aeruginosa*, show nearly identical tight packing of the chaperone around the folded

Lipase-specific foldase: dynamics and client interactions

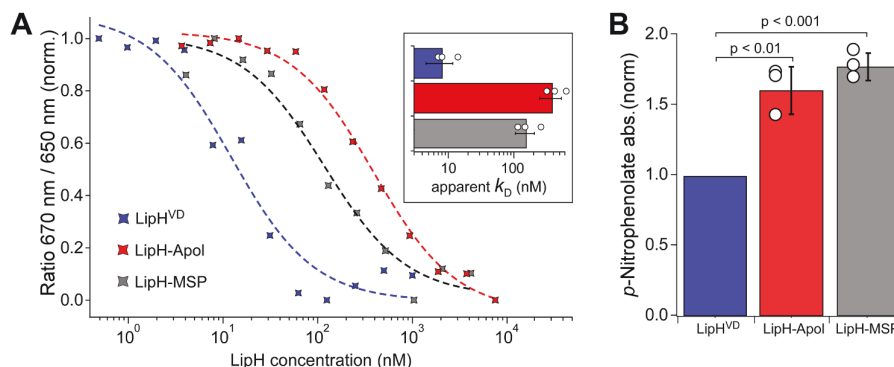


Figure 6. LipH:LipA assembly and activity *in vitro*. A, exemplary recordings of the spectral shift in LipA-CF647 fluorescence upon titrating the LipH variants. Normalized ratios of LipA-CF647 fluorescence intensity at 670 nm and 650 nm are plotted against the LipH concentration. The apparent dissociation constants K_D calculated for each interaction as an inflection point of the fitting function (dashed lines) are shown in the inset. The error bars correspond to the standard deviations, calculated from three technical replicates. The individual measurements are shown as white circles. B, lipase activity *in vitro* mediated by the LipH variants was determined with p-nitrophenylbutyrate as the substrate. The error bars correspond to the standard deviations, calculated from three technical replicates. The individual measurements are shown as white circles. *p*-Values based on one-way ANOVA test were 0.003 (LipH^{VD} vs LipH-Apol) and 0.00015 (LipH^{VD} vs LipH-MSP).

client (Fig. S1A). Interactions of LipH MD1 are centred around the C-terminal helix of the lipase, while the MD2 contacts at least three helices of the N-terminal part of LipA (Fig. 1B). To assess the potential protein dynamics within the complex, we implemented hydrogen/deuterium exchange mass spectrometry (HDX-MS) of LipH:LipA *versus* the individual LipH and LipA, while using the minimal functional foldase domain LipH^{Chap}, which allows for folding and activation of the lipase (15, 17). HDX-MS is a valuable tool to study conformational changes, incl. folding, and protein:protein interactions, as it allows to discriminate between the solvent-exposed and buried areas of a target protein based on the polypeptide deuteration levels (36). By comparing HDX levels within LipA, we expected to trace its chaperone-mediated folding. In its turn, analysis focused on LipH^{Chap} could reveal how binding of the client changes the solvent exposure of the chaperone, and which areas become shielded upon this interaction. For the free LipH^{Chap} one would expect higher exposure to the solvent and rapid HDX rates, while LipA binding should suppress the exchange at the involved interfaces. With that information we may elucidate what contacts within the complex remain stable over time, and which areas may be transiently exposed due to structural fluctuations.

HDX-MS experiments showed a decline in accessibility of LipA regions in the presence of LipH^{Chap}, (Fig. S14), in excellent agreement with the structural model of LipH:LipA complex, as the effect was most pronounced for the LipA areas covered by the chaperone. In particular, the N-terminal fragment of LipA (residues 28–125, Figs. S14, C and D, S15) exhibited rapid HDX indicative for the lack of secondary structure, which was decelerated in the presence of LipH^{Chap}. Folding-associated HDX reduction within the C-terminus of LipA was also apparent (e.g., residues 269–311, Figs. S14C and S15) albeit less pronounced. Thus, LipA indeed acquired its folded state and remained bound to LipH^{Chap}, while in absence of the chaperone it probably formed diverse aggregates. For the foldase, HDX-MS analysis allowed for nearly

complete coverage of the free LipH^{Chap} and the *in vitro* assembled complex LipH^{Chap}:LipA (Fig. S16). Expectedly, binding of the client lipase suppressed the HDX within the chaperone, but the effect was not uniformly distributed over the structure (Figs. 7A, S16C and S17): The reduction was most pronounced within the EHD and MD2 sub-domains suggesting stable chaperone:client interactions, such as putative salt bridges between conserved residues Arg-280 and Arg-327 of LipH, and Glu-58 and Glu-66 of LipA (Fig. 7B). In contrast, minor changes in the HDX levels were observed within MD1 despite the latter forming close contacts with LipA within the known structures (Fig. S1). Transient detachment of MD1 from the bound lipase or changing its position/orientation at the lipase surface would explain the high HDX rates within this region, primarily stabilized *via* LipH Ser-112 and LipA Gln-275.

The N-terminal fragment of LipA contains a recognition element for chaperoning

The available structures of LipH:LipA homologs and the AlphaFold-based model of the studied complex from *P. aeruginosa* suggest multiple interactions between LipH EHD and MD2 with the N-terminal domain of the lipase (Fig. 7B) (37). Thus, we questioned whether the chaperone:client interaction can be established with a fragment of LipA truncated at the C-terminal side, which would mimic a partially translocated lipase (Fig. S18). To this end, we designed and isolated a series of N-terminal fragments of LipA and examined whether those are competent to interact with the polyhistidine-tagged LipH^{VD} bound to IMAC resin. Strikingly, all tested fragments co-eluted with the chaperone, including the shortest fragment LipA^{Δ163}, consisting of only 122 amino acids (Fig. 7C). As the elution yield dropped by approx. 80% in the absence of LipH^{VD}, we concluded that LipA fragments were indeed recognized and caught by the chaperone. Notably, the urea-denatured outer membrane protein OmpA was not co-

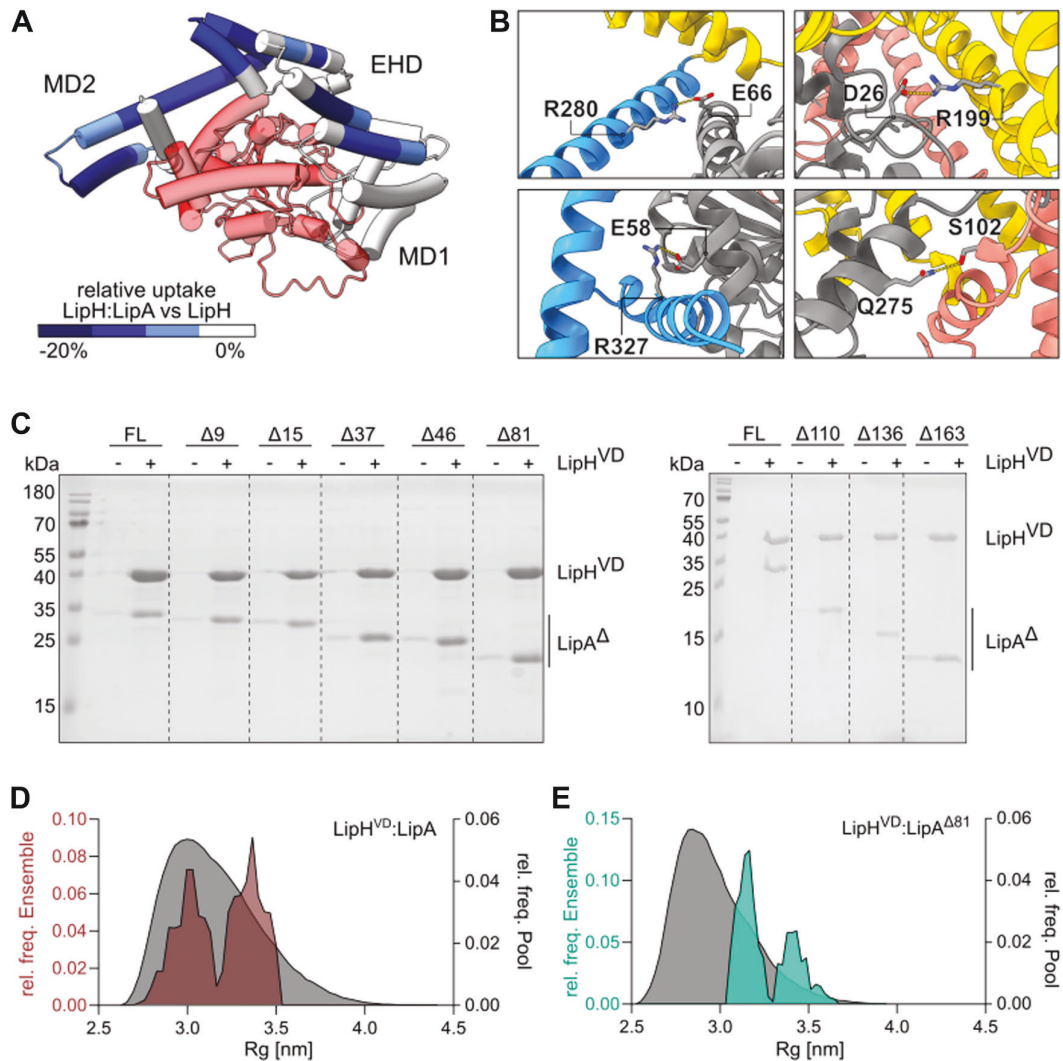


Figure 7. Mechanism of LipH:LipA recognition. *A*, changes in the hydrogen/deuterium exchange within LipH upon LipA binding plotted on the modelled LipH:LipA complex. *Blue* regions of LipH indicate lower deuterium incorporation of LipH in presence of LipA, and so shielding of the protein. LipA is shown in transparent *red*. *B*, electrostatic interactions between LipH and LipA predicted based on the AlphaFold3-derived model. The coloring of the LipH domains corresponds to [Figure 1B](#); LipA is shown in *grey*. *C*, co-elution assay of LipH^{VD} and either the full-length LipA ("FL"), or the lipase fragments ("LipA^Δ"). The number of residues deleted from the C-terminal end is indicated above each LipA fragment. For each LipA fragment, elution without and with LipH^{VD} is shown. *D* and *E*, Gyration radii distribution (R_g) from EOM of LipH^{VD}:LipA and LipH^{VD}:LipA^{Δ81} complexes as determined by SAXS. The distribution of the ensemble pool used for fitting is shown in *grey*; selected EOM models are shown in *brown* (LipH^{VD}:LipA) and *cyan* (LipH^{VD}:LipA^{Δ81}). Wider dimensions of LipH^{VD}:LipA^{Δ81} are attributed to the flexibility of the MD1 domain. Bi-modal distributions likely originate from the flexibility of the VD polypeptide.

eluted with LipH^{VD} ([Fig. S19](#)), in agreement with the specificity of the LipH:client interaction.

To validate that the interaction between LipH and LipA does not essentially require the full-length lipase, we analysed the assembly of the chaperone with the lipase fragment LipA^{Δ81}, as well as the full-length LipA, using SEC-SAXS ([Figs. S20](#) and [S21](#)). The measured molecular masses of LipHVD:LipA^{Δ81} (58.2 kDa) and LipH^{VD}:LipA (67.1 kDa) closely matched the calculated values of 59.6 kDa and 68.5 kDa, respectively. Thus, the chaperone:client complexes were indeed formed in 1:1 stoichiometry, and the N-terminal fragment of LipA was sufficient for binding ([Table S1](#)). For

both complexes, Kratky plots showed bell-shaped profiles indicating compact and folded molecules ([Figs. S20C](#) and [S21C](#)), which contrasted the broader profile seen for LipH^{VD} alone ([Fig. S11C](#)). Thus, the flexibility of the chaperone substantially decreased upon binding the client. Interestingly, even though the LipHVD:LipA^{Δ81} complex had a lower molecular weight, it manifested a larger radius of gyration than that of LipH^{VD}:LipA, 3.53 nm vs. 3.26 nm, as provided by the pair-distance distribution function $p(r)$ ([Table S1](#)). Thus, LipHVD:LipA^{Δ81} acquired more extended conformation than LipH^{VD}:LipA, which could be explained by missing interactions of LipH MD1 with the C-terminal part of LipA. In

Lipase-specific foldase: dynamics and client interactions

agreement, fitting the SAXS data with structural models of the chaperone:client complex *via* the EOM approach resulted in bimodal Rg distribution behaviour for both complexes (Fig. 7, D and E and Table S1), but a more relaxed distribution was observed for LipH^{VD}:LipA^{Δ81} in comparison to LipH^{VD}:LipA. Taken together, the results suggest that the N-terminal fragment of the lipase carries the recognition features that stabilise the complex in a more compact conformation.

Discussion

Chaperone-assisted protein folding has been a topic of extensive research over decades, which has offered detailed insights on the universally conserved families of ATP-dependent chaperones, such as Hsp70/40, or energy-independent bacterial chaperones, such as trigger factor, SurA, and Skp (38, 39). Those chaperones constitute essential parts of cellular proteostasis, as they participate in a global control of the protein content *via* balancing protein folding and degradation. To ensure their broad specificity, the recognition of clients is based on basic physico-chemical principles, such as exposure of hydrophobic polypeptide stretches or aromatic residues, which is characteristic of incomplete or aberrant folding states (40). Lack of specific fine-tuned interactions results in weak affinities, typically in the range of tens of μM , so one client protein may subsequently interact with several chaperones prior to being either correctly folded or routed for degradation (41).

Unlike those, several classes of chaperones with narrow specificity have been described in bacteria, with examples of type III secretion system adaptor components (42), and steric foldases. While the T3SS adaptors are widespread and diverse, only two classes of steric foldases have been identified so far, which facilitate folding of either bacterial lipases or type II proteases, both targeted then for secretion *via* T2SS (43, 44). The known steric foldases share a primary topology, as their periplasm-exposed chaperoning domains are anchored *via* transmembrane helices and unstructured linker polypeptides to the lipid bilayer, so the chaperones are not co-secreted together with their clients. On example of *P. aeruginosa* LipH, our MD simulations and SAXS analysis show here that the unstructured linker allows extensive motions of the chaperoning domain, so the membrane-anchored LipH is able to sample a large area within the periplasm to bind the client lipase, though the linker may transiently occlude the binding interface. Notably, the foldases with the unstructured linkers clearly differ from the well-studied membrane-anchored chaperones PpiD and YfgM that contain short and structured domains (Fig. S22) (45, 46). The PpiD-YfgM assembly interacts with the SecYEG translocon (47, 48) so the rigid linkers may optimally position the chaperoning moiety for screening and/or capturing the newly translocated clients. Interestingly, no realistic model could be generated for SecYEG-LipH by AlphaFold-3 (Fig. S23), suggesting that the translocon does not form a stable complex with the specialized chaperone, though the dynamic LipH chaperoning

domain may be transiently recruited, *e.g.* in response to the translocation of LipA.

Remaining in the proximity to the membrane, the chaperone domain of LipH can interact with the lipid leaflet, and the simulations suggest that the chaperone accessibility depends on the environment: An elevated ionic strength allows interactions of the chaperoning domain with the membrane interface, which may negatively affect the client capture. Interestingly, our previous work showed that at such conditions, the chaperone Skp efficiently rescues LipA from aggregation in solution and supports its secretion *in vivo*, so Skp can serve as a holdase chaperone for the lipase and provide additional timing for LipH to bind its specific client, thus minimizing the off-route misfolding pathways (17).

Both MD simulations of LipH^{FL} and SAXS analysis of the soluble LipH^{VD} confirm flexibility within the chaperoning domain, where the mini-domains MD1 and MD2 move at the opposing sides of the bridging EHD. These findings qualitatively corroborate the results of an earlier study, where simulations and single-molecule FRET experiments revealed a continuum of conformations explored by LipH^{Chap} (19). Those movements, together with the dynamics of the VD polypeptide, result in a wide ensemble of LipH configurations which differ from the defined conformation seen in the foldase:lipase structure, raising a question about the recognition mechanism. Our biochemical and structural analyses suggest that the recognition may not involve the complete LipA, but shorter fragments may be captured, first of all at the MD2-EHD interface of LipH, followed by loading of a complete lipase into the chaperoning cavity for folding and activation. Hypothetically, such recognition could occur already during LipA translocation *via* the SecA:SecYEG machinery, once the N-terminal tail emerges at the periplasmic side, followed by gradual loading coupled with the elongation of the LipA polypeptide chain.

While LipH remains flexible upon recognition of the LipA fragment, SAXS analysis shows that the LipH dynamics is suppressed once the chaperone binds the full-length LipA. Notably, the HDX-MS data reveal low to no shielding of the MD1 domain by the lipase, implying that the interactions formed by MD1 are marginally stable. Indeed, a single mutation within this region drastically affects the MD1:LipA interactions, but also the chaperoning properties of LipH (49, 50). Thus, it is tempting to speculate that the LipH MD2 domain plays a leading role in recognition and binding of the client, while MD1 contributes to the chaperoning activity, such as correct positioning of LipA helices, and, possibly, release of the correctly folded lipase. Mutagenesis at the chaperone:lipase interaction interface, first of all focused on the putative salt bridges, followed by biophysical and functional analysis, should reveal details in recognition, folding and activation of the lipase, and may also explain the high specificity of the foldase to its client. Furthermore, potential interactions of LipH with C-terminal fragments of LipA remain to be tested, as those may reveal alternative routes for the chaperone:client recognition.

The mechanism of the client release remains another open question in our understanding of the steric chaperones. Our data, as well as previous studies, demonstrate high, nanomolar affinity of the LipH chaperoning domain to the lipase, which may not be compatible with spontaneous release of LipA for secretion *via* Xcp-T2SS. Release of clients from the non-specific periplasmic chaperone Spy has been recently linked to its disordered N-terminal end: Electrostatic interactions of the polypeptide with the binding pocket of Spy serve to expel client proteins (51). In contrast, the non-conserved VD polypeptide of LipH^{VD} did not affect the affinity for the lipase, despite its transient interactions with the chaperoning domain. This finding speaks against its direct role in destabilization of the complex. However, our functional analysis of the full-length chaperone embedded in nanodiscs and amphipols suggested that the membrane mimetics may stimulate release of the client lipase: While LipH retained its chaperoning properties, the affinity to LipA dropped manifold upon reconstitution. We hypothesize that the anchored LipH^{FL} facilitated multiple chaperoning rounds associated with binding and release of the client lipase, thus resembling the pathway occurring in the cell. One potential stimulus for LipA release could be the electrostatic properties of the proximate environment. Both the phospholipid-based nanodiscs and the carboxyl-rich amphipol A8-35 are the highly polar and negatively charged “carriers”, which may either modulate dynamics of the LipH MD1 domain or may interact directly with the folded lipase, which exposes its cationic side to the solvent (Fig. S24). Interestingly, when LipH was incorporated into nanodiscs in absence of anionic lipids, we observed low-nM affinity to LipA and reduced hydrolysis of the substrate (Fig. S25). This observation corroborates the hypothesis of the charge-mediated release of the client lipase, but further targeted analysis of the protein:lipid interplay and kinetics of LipH:LipA interactions, *e.g.* *via* surface plasmon resonance, are required to validate the proposed model.

To summarize, our study offers the first comprehensive view on the conformational dynamics of the lipase-specific foldase LipH at the membrane interface, and suggests that

the foldase:lipase recognition is mediated by specific regions of both proteins, *i.e.* MD2 of LipH and the N-terminal fragment of LipA, while MD1 dynamics serves for the lipase folding and release (Fig. 8). It remains to be tested though, whether the molecular mechanism derived from *in vitro* and *in silico* analysis can be recapitulated in the living cells. Our results provide a solid ground for such studies, which may test potential effects of specific point mutations at the LipH:LipA interface on the lipase secretion *in vivo*. Once successful, the chaperone-dependent folding of the lipase may be modulated at various stages to suppress secretion of a virulence factor in *P. aeruginosa* and related species, but also to facilitate production of the functional lipase in biotechnological applications.

Experimental procedures

Bioinformatics analysis of the LipH variable domain

To identify the LipH homologs across bacterial families, the EFI - Enzyme Similarity Tool with default options (52) was used to find sequences similar to the one of *Pseudomonas aeruginosa* LipH (*pseudomonas.com*: PA2863). The obtained protein accession numbers were added to Uniprot ID mapping server to get the corresponding FASTA data, which were further analysed *via* InterPro (53) using the tools from Superfamily (54) and TMHMM (55). Proteins containing both the N-terminal hydrophobic anchor and the foldase-like domains were used for further analysis, with the final count of 858 sequences. The polypeptide chain between the predicted anchor and the foldase-like domain corresponded to the “variable domain” region of LipH, and the length and the amino acid composition was analysed. The identified sequences and the corresponding analysis are provided in the Supporting Dataset 1.

Expression and purification of LipH^{VD}

The gene encoding for the soluble LipH variants, which lack the residues five to 22 (LipH^{VD}) and 5 to 62 (LipH^{Chap}) of the full-length foldase, were cloned into pET21a vector as

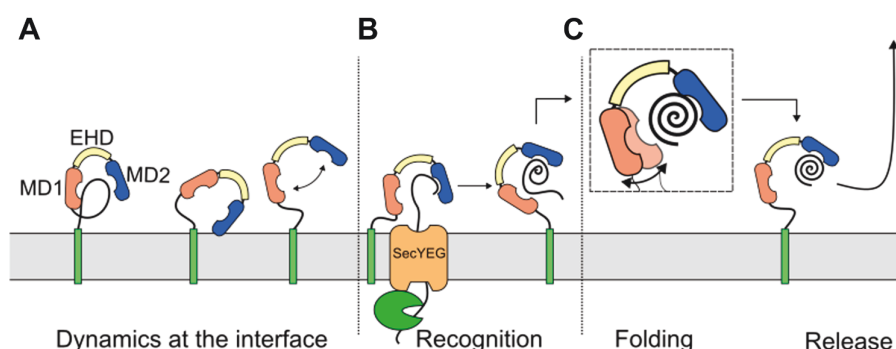


Figure 8. LipH conformational dynamics and interactions with the client lipase. A, the foldase LipH exhibits distinct dynamic behaviours at the membrane interface, ranging from transient contacts of the unstructured linker polypeptide to breathing motions of the chaperoning domain, including opening and closing transitions, and membrane interactions. B, recognition and binding of the N-terminal fragment of the client lipase is facilitated by the MD2 domain of LipH (blue), potentially at the translocation stage. C, MD1 (salmon) contributes predominantly to the chaperoning process, including proper positioning of LipA helices, and weak interactions in this region potentially facilitate the release of the folded lipase.

Lipase-specific foldase: dynamics and client interactions

previously described (17). *E. coli* BL21(DE3) cells transformed with the respective plasmid were grown in LB medium supplemented with 100 µg/ml ampicillin at 37 °C while shaking at 180 rpm. Upon reaching OD₆₀₀ of 0.6, LipH expression was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Merck/Sigma-Aldrich) and carried out for 2.5 h. Cells were harvested and lysed using a microfluidizer (M-110P, Microfluidics Corp.), and the debris and membranes were removed by ultracentrifugation at 42,000 rpm for 1 h (45Ti rotor, Beckman Coulter). For further purification *via* immobilized metal ion affinity chromatography (IMAC), the supernatants were incubated with Ni²⁺-NTA agarose resin (Protino, Macherey-Nagel GmbH & Co. KG) for 1 h at 4 °C. The resin was washed with 50 mM Hepes-KOH pH 7.4, 500 mM KOAc, 5% glycerol (v/v), 10 mM imidazole, and the target protein was eluted with 50 mM Hepes-KOH pH 7.4, 150 mM KOAc, 5% glycerol, 200 µM, 300 mM imidazole. The elution fractions were concentrated and subjected to size exclusion chromatography (SEC) in 50 mM Hepes-KOH pH 7.4, 150 mM KOAc, 5% glycerol, using Superdex 200 Increase GL 10/300 column with ÄKTA pure set-up (Cytiva). The protein concentration was determined spectrophotometrically (NeoDot, NeoBiotec) using the calculated extinction coefficient of 19,940 M⁻¹ cm⁻¹ for both variants.

Expression and purification of the full-length LipH

The gene encoding for the full-length foldase LipH^{FL} was cloned into pBAD vector *via* KpnI and HindIII restriction sites to contain an N-terminal octa-histidine tag. *E. coli* BL21(DE3) cells transformed with the respective plasmid were grown in LB medium supplemented with 100 µg/ml ampicillin at 37 °C while shaking at 180 rpm. Upon reaching OD₆₀₀ of 0.6, LipH^{FL} expression was induced by adding 0.2% arabinose and carried out for 2 h. The cells were harvested by centrifugation at 5000g for 20 min at 4 °C (rotor SLC-6000, Sorvall/Thermo Fisher) and subsequently resuspended in 20 mM Tris (hydroxymethyl)aminoethane-HCl pH 8.0, 100 mM NaCl, 1 mM DTT (Merck/Sigma-Aldrich), 5% glycerol and cComplete protease inhibitor cocktail (Roche). Cells were lysed using a Microfluidiser (M-110P, Microfluidics Inc.), and the cell debris was removed by centrifugation at 18,000g for 10 min at 4 °C (rotor SS34, Sorvall/Thermo Fisher). The supernatant was then centrifuged at 205,000g for 1 h at 4 °C (rotor 45 Ti, Beckman Coulter) to pellet crude membranes. The membranes were resuspended in the same buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM DTT, 5% glycerol, and cComplete protease inhibitor cocktail) and stored at -80 °C until further usage. To extract LipH^{FL}, n-dodecyl-β-D-maltopyranoside (DDM; Glycon Biochemicals GmbH), n-decyl-β-maltoside (DM; Glycon Biochemicals GmbH), lauryl maltose neopentyl glycol (LMNG; Anatrace) and 6-cyclohexyl-1-hexyl-β-D-maltoside (Cymal-6; Anatrace) were used. Solubilization was performed with 1% of each detergent in 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.2 mM TCEP, 5% glycerol, and cComplete protease inhibitor cocktail

for 1 h at 6 °C on a rolling table. After solubilization, the samples were centrifuged at 20,000g for 10 min at 4 °C (Hermle Z216M) to remove non-soluble material. The his-tagged LipH^{FL} were immobilized on Ni²⁺-NTA agarose resin equilibrated with the wash buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.2 mM TCEP, 20 mM imidazole, cComplete protease inhibitor cocktail) supplemented with the detergent of choice. The detergent concentrations for the washing and elution steps were ~5-fold above the specific critical micelle concentrations (CMC), resulting in 0.05% (w/v) for DDM, 0.4% for DM, 0.005% for LMNG and 0.1% for Cymal-6. The resin was washed extensively with the wash buffer to remove weakly bound impurities, and the protein was eluted with 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.2 mM TCEP, 300 mM imidazole, 10% glycerol, cComplete protease inhibitor cocktail. For size-exclusion chromatography, the protein was loaded on Superdex 200 Increase 10/300 GL column (Cytiva) in the desired buffer compositions (e.g., 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.2 mM TCEP, 10% glycerol, 0.005% LMNG). The concentration of the purified LipH^{FL} was determined spectrophotometrically and the purification yield was further analysed *via* SDS-PAGE.

LipH^{FL} reconstitution into membrane mimetics

For the amphipol reconstitution, 15 nmol LipH^{FL} in DDM was mixed with a threefold excess of amphipol A8-35 (Anatrace) and incubated for 2 h while rolling at 6 °C. To remove the detergent, 60 mg washed and dried Bio-Beads SM-2 sorbent (Bio-Rad Laboratories GmbH) was added to the mixture and incubated overnight while rolling at 6 °C. The reconstitution reaction was loaded on Superdex 200 Increase 10/300 GL, and the fractions were analysed *via* SDS-PAGE. For the nanodisc reconstitution, membrane scaffold protein MSP1E3D1 was purified as described before (30). Liposomes were prepared of 70% 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and 30% 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1-rac-glycerol) (POPG) (Avanti Polar Lipids, Inc). Lipids were mixed from chloroform stocks to achieve the target POPC:POPG ratio, and the solvent was evaporated at 200 mbar and 40 °C for 30 min. The film of dried lipids was resuspended in 50 mM Hepes/KOH pH 7.4, 50 mM KCl and extruded through a porous polycarbonate membrane (200 nm) using the Mini-Extruder set (Avanti Polar Lipids, Inc). Subsequently, the liposomes were dissolved using 0.5% Triton X-100. LipH^{FL} purified in DDM was reconstituted into nanodiscs at the protein:MSP:lipid molar ratio of 1:6:600. To remove the detergent, 60 mg washed and dried Bio-Beads SM-2 sorbent was added to the mixture and incubated overnight while rolling at 4°. The empty and loaded nanodiscs were separated by size exclusion chromatography using Superdex 200 10/300 Increase GL column, and the fractions were analysed *via* SDS-PAGE. Mass photometry analysis of the reconstituted LipH^{FL} samples was performed using the Two^{MP} instrument (Refeyn Ltd) and calibrated using NativeMark Unstained Protein Standard (Invitrogen/Thermo Fisher Scientific).

Expression and purification of LipA

The mature lipase LipA^{F144E} (*pseudomonas.com*: PA2862), lacking the signal peptide (residues 1–27), and its C-terminal truncations were expressed and isolated as previously described (17). The mutation F144E within the lid domain reduces the aggregation propensity of the lipase (17). For the C-terminal truncations, the amino acids 300 to 309 ($\Delta 9$), 294 to 309 ($\Delta 15$), 272 to 309 ($\Delta 37$), 263 to 309 ($\Delta 46$), 228 to 309 ($\Delta 81$), 199 to 309 ($\Delta 110$), 173 to 309 ($\Delta 136$) and 146 to 309 ($\Delta 163$) were removed *via* PCR with subsequent blunt-end ligation. *E. coli* BL21(DE3) cells were transformed with a corresponding pET22b-based plasmid and grown at 37 °C, shaking at 180 rpm. Overexpression was induced by the addition of 0.5 mM IPTG and conducted for 2 h. Afterwards, the cells were harvested by centrifugation at 5000g at 4 °C for 20 min. The cell pellet was resuspended in *E. coli* Lysis reagent (New England Biolabs GmbH) and incubated at 20 °C for 15 min. The inclusion bodies were pelleted upon centrifugation and repeatedly washed with 20 mM Tris-HCl pH 7.5. After three rounds of washing, the inclusion bodies were dissolved in 8 M urea, 50 mM Tris-HCl, pH 7.5. LipA constituted above 90% of the inclusion bodies' content, as determined *via* SDS-PAGE and following staining (Quick Coomassie stain, Serva).

In vitro activity of LipA

The hydrolytic activity of LipA was measured *in vitro* using the model substrate *para*-nitrophenyl butyrate (*p*NPB) as described elsewhere (17). For the measurement, 2 μ M of the urea-denatured LipA was mixed with an equimolar amount of the soluble chaperone variant LipH^{VD} or the full-length foldase LipH^{FL} embedded into nanodiscs or amphipols. Due to the intrinsic absorbance of MSP1E3D1, the concentration of LipH^{FL} in nanodiscs was estimated colourimetrically based on the band intensity on SDS-PAGE (Amersham 600 RGB imager, ImageQuant software, both Cytiva). The total volume of the reaction was 40 μ L, adjusted with TGCG buffer (5 mM Tris-HCl pH 9.0, 5 mM glycine, 1 mM CaCl₂, and 5% glycerol). The samples were incubated for 15 min at 37 °C to allow for LipH:LipA complex formation. Afterwards, 10 μ L sample were transferred into a 96-well plate containing 100 μ L TGCG. *p*NPB stock was diluted to 10 mM in acetonitrile, and it was further diluted 10-fold with 50 mM triethanolamine pH 7.4 prior the measurement. 100 μ L of the *p*NPB solution was pipetted to each well for measurement and the reaction was carried out at 37 °C up to 3 h, while monitoring the absorbance of the hydrolysis product, *p*-nitrophenolate, at 410 nm (Infinite 200 PRO plate reader, Tecan). Absorbance values within the exponential phase (900 s) were used to compare the hydrolysis levels at different conditions. Loading controls were performed for all checked conditions and the intensity of bands on the SDS-PAGE was quantified (ImageQuant, Cytiva). The obtained values were corrected for variations in LipA as well as the chaperone after deduction of autohydrolysis. The measurements were performed in technical triplicates (individual assays, same LipH preparations), each containing five measured samples.

LipH^{VD}:membrane interaction assay

To investigate LipH^{VD}:membrane interactions, we performed a flotation assay as described elsewhere (56). The dried lipid film composed of 70 mol % DOPC and 30 mol % DOPG was resuspended in 50 mM HEPES-KOH pH 7.4, and 50 mM NaCl prior to liposome formation. LipH^{VD} was added to the liposomes at a molar ratio 1:5.000, incubated for 15 min at 20 °C and then used to prepare the high-sucrose layer of the gradient (30% sucrose, w/v). The salt concentration within the gradient was either 25 mM or 150 mM NaCl. A reference sample in the absence of the liposomes was prepared at 150 mM NaCl. Following centrifugation, the gradients were fractionated into top (125 μ L), middle (250 μ L), and bottom (125 μ L) fractions, which were subsequently precipitated by the addition of 250 μ L 30% TCA. The material was used for SDS-PAGE to evaluate LipH^{VD} distribution.

Spectral shift measurements

The interactions of LipH variants with LipA were probed *via* the fluorescence spectral shift technique. LipA was labelled with the far-red dye CF647-maleimide (Biotium Inc.) *via* coupling to the endogenous cysteines. Prior to each measurement, the urea-denatured LipA-CF647 was diluted with buffer containing 5 mM Tris-HCl pH 9.0, 5 mM glycine, 1 mM CaCl₂, 5% glycerol, 0.5 mg/ml BSA, 0.05% (w/v) Tween-20. LipA-CF647 concentration was set to 20 nM in experiments with LipH^{VD} and LipH-MSP, and 10 nM for LipH-Apol. LipH concentrations ranged from 0.5 nM to 7.5 μ M. Fluorinated octyl maltoside (Anatrace) was added at a concentration of 0.05% to the reaction with LipH-MSP to prevent the sample aggregation at the capillary surface (Monolith premium Capillaries, MO-K025, Nanotemper Technologies GmbH). The experiments were performed at Monolith.X instrument (Nanotemper Technologies GmbH). The excitation power was set to 40%, with medium infra-red laser power, and the fluorescence spectral shift of the LipA-bound dye between wavelengths of 670 nm and 650 nm was measured. The LipH-dependent shift values were fitted using Hill equation, with a Hill coefficient of 1, and providing an estimate for the LipH: LipA dissociation constant. The measurements were performed in technical triplicate.

Co-elution interaction assay

To test interactions between the soluble foldase variant LipH^{VD} and LipA fragments, an IMAC-based co-elution assay was designed. 50 ml Ni²⁺-NTA agarose resin was loaded into low-binding reaction tubes (Sarstedt), and 30 μ M of the hexahistidine-tagged LipH^{VD} was added to the resin. Only buffer was added for reference reactions. The samples were mixed by vortexing on Vortex genie 2 (NeoLab) for 5 s. The reaction tubes were placed on a rolling bench for 5 min at 4 °C to let the chaperones bind to the resin. After this, 400 μ L of buffer consisting of 5 mM Tris, 5 mM glycine, 1 mM CaCl₂ was added, supplemented with 5 mM imidazole to reduce unspecific binding (wash buffer). Then an equimolar amount of LipA was added and left for 30 min on the rolling bench at 4 °C. Afterwards, the resin was

Lipase-specific foldase: dynamics and client interactions

pelleted for 30 s at 20,000g in a tabletop centrifuge (Hermle Z 216 M) and the supernatant was discarded. After two rounds of washing/pelleting steps, 100 μ l elution buffer containing 300 mM imidazole was added, the tubes were vortexed and incubated for 10 min on the rolling bench at 4 °C. The resin was pelleted, and the supernatant with the eluted material was carefully collected. 10 μ l of the elution samples were loaded on SDS-PAGE.

Small-angle X-ray scattering

SAXS data from LipH^{VD} were collected on a Xeuss 2.0 Q-Xoom system (Xenocs), equipped with a PILATUS 3 R 300K detector (Dectris) and a GENIX 3D CU Ultra Low Divergence X-ray beam delivery system. The chosen sample-to-detector distance for the experiment was 0.55 m, resulting in an achievable q-range of 0.05 to 5.5 nm⁻¹. The measurement was performed at 10 °C with a protein concentration range up to 11.8 mg/ml. The samples were injected into the Low Noise Flow Cell (Xenocs) *via* autosampler, and 24 frames were collected with an exposure time of 10 min/frame.

SAXS data from the LipH^{VD}:LipA complex were collected on the P12 beamline (PETRA III, DESY Hamburg (57)). The sample-to-detector distance of the P12 beamline was 3.00 m, resulting in an achievable q-range of 0.03 to 7.0 nm⁻¹. For the complex formation of LipH^{VD} and LipA, both proteins were mixed in the given ratio and preincubated for 30 min at 37 °C. Prior to injection, the samples were centrifuged for 15 min at 20,000g. SEC-SAXS of the LipH^{VD}:LipA complex was performed at 20 °C on a Superdex 200 Increase 10/300 G1 column (100 μ l inject, buffer: 5 mM Tris, 5 mM glycine, 1 mM CaCl₂, 5% glycerol, pH 9) with a flow-rate of 0.6 ml/min 2400 frames were collected with an exposure time of 0.995 s/frame. SEC-SAXS data from LipH^{VD}:LipA ^{Δ 81} complex was collected on beamline BM29 at the ESRF Grenoble (58). The BM29 beamline was equipped with a PILATUS 2M detector (Dectris) at a fixed distance of 2.827 m, resulting in an achievable q-range of 0.025 to 5.5 nm⁻¹. SEC-SAXS was performed at 20 °C on a Superdex 200 increase 10/300 G1 column (100 μ l inject, buffer: 5 mM Tris, 5 mM glycine, 1 mM CaCl₂, 5% glycerol, pH 9) with a flow-rate of 0.6 ml/min 1200 frames were collected with an exposure time of 2 s/frame. All collected data were scaled to absolute intensity against water.

Programs used for data processing were part of the ATSAS Software package ver. 3.0.5 (59). Primary data reduction was performed with the programs CHROMIXS (60) (for SEC-SAXS) and PRIMUS (61). With the Guinier approximation (62), we determine the forward scattering $I(0)$ and the radius of gyration (R_g). The program GNOM (63) was used to estimate the maximum particle dimension (D_{max}) with the pair-distribution function $p(r)$. Structural models of LipH in *apo*-state, as well as in complex with LipA variants were built *via* AlphaFold3 (64). For a better agreement with the experimental scattering data in *apo*-state, EOM was used to model the N-terminal polypeptide of LipH and to allow movement of the MD1, EHD and MD2 domains (65, 66). For the complexes formed with LipA and LipA ^{Δ 81}, only the flexibility of the LipH N-terminal end was allowed.

Hydrogen/deuterium exchange mass spectrometry

Protein sample stocks for HDX-MS contained 50 μ M of either individual LipA or LipH^{Chap}, or both proteins premixed to form the LipH:LipA complex, and preparation of individual samples was aided by a two-arm robotic autosampler (LEAP Technologies) essentially as previously described (67). In short, HDX reactions were initiated by 10-fold dilution of the protein stock solutions with TGC buffer (5 mM Tris-HCl pH 9.0, 5 mM glycine, 1 mM CaCl₂) prepared in D₂O and incubated for 10, 100, 1000 or 10,000 s at 25 °C. The HDX reaction was stopped upon adding an equal volume of quench buffer (400 mM KH₂PO₄/H₃PO₄, 2 M guanidine-HCl; pH 2.2) pre-dispensed and thermostated at 1 °C. Then, 100 μ l of the resulting mixture was injected into an ACQUITY UPLC M-Class System with HDX Technology (68). Non-deuterated samples were generated by the same procedure through 10-fold dilution with TGC buffer prepared with H₂O. The injected HDX samples were washed out of the injection loop (50 μ l) with water supplemented with 0.1% (v/v) formic acid at 100 μ l/min flow rate and guided to a column containing immobilized porcine pepsin thermostated at 12 °C. The resulting peptic peptides were collected on a trap column (2 mm $\times$ 2 cm), that was filled with POROS 20 R2 material (Thermo Scientific) and kept at 0.5 °C. Digestion and trapping was conducted for 3 min, the trap column was placed in line with an ACQUITY UPLC BEH C18 column (1.7 μ m, 1.0 $\times$ 100 mm; Waters), and peptides eluted with a gradient of water supplemented with 0.1% (v/v) formic acid (eluent A), and acetonitrile supplemented 0.1% (v/v) formic acid (eluent B) at the flow-rate of 30 μ l/min as follows: 0 to 7 min/95-65% A, 7 to 8 min/65-15% A, 8 to 10 min/15% A.

Eluting peptides were guided to a Synapt G2-Si mass spectrometer (Waters) and ionized with by electrospray ionization (capillary temperature 250 °C, spray voltage 3.0 kV). Mass spectra were acquired with the software MassLynX MS version 4.1 (Waters) over a range of 50 to 2000 m/z in enhanced high-definition MS (HDMS^E) (69, 70) or high-definition MS (HDMS) mode for non-deuterated and deuterated samples, respectively. Lock mass correction was conducted with (Glu 1)-Fibrinopeptide B standard (Waters). During separation of the peptides on the C18 column, the pepsin column was washed three times by injecting 80 μ l of 0.5 M guanidine hydrochloride in 4% (v/v) acetonitrile. Blank runs (injection of double-distilled water instead of protein sample) were performed between each sample. All measurements were carried out in triplicates.

Peptides were identified and evaluated for their deuterium incorporation with the software ProteinLynx Global SERVER 3.0.1 (PLGS) and DynamX 3.0 (both Waters) as previously described (67) employing the amino acid sequences of LipA, LipH^{Chap}, and porcine pepsin as database. Whenever possible, multiple charge states were utilized. All spectra were manually inspected and omitted if necessary, *e.g.* in case of low signal-to-noise ratio or the presence of overlapping peptides disallowing the correct assignment of the isotopic clusters. Raw data of deuterium uptake by the identified peptides and

residue-specific HDX is deposited at the ProteomeXchange Consortium *via* the PRIDE partner repository, the dataset ID PXD074746.

Molecular dynamics simulations

All molecular dynamics (MD) simulations carried out in this study are summarized in Table 1. The systems were built *via* CHARMM-GUI (71–73) by using the force field CHARMM36m and TIP3P (74) water model, with the temperature set at 298 K (25 °C), the pressure - at 1 bar, and pH 7.0. The membrane was composed of di-oleoyl-phosphatidylethanolamine (DOPE; 75 mol %), di-oleoyl-phosphatidylglycerol (DOPG; 20 mol %) and 1',3'-bis{18:1/18:1-sn-glycero-3-phospho]-glycerol (cardiolipin, CL; 5 mol %), thus mimicking the bacterial inner membrane at two different ion conditions (150 mM and 25 mM NaCl). To perform the simulations and analysis, we used MD software package GROMACS 2021/2022 (75).

Derived on the AlphaFold-based model of the full-length LipH, the N-terminal helix was inserted in the membrane *via* CHARMM-GUI (71–73). The lipid bilayer had lateral dimensions of $22 \times 22 \text{ nm}^2$ (x/y-plane), and it was placed in the middle of the simulation box with a total height of 19 nm (z-axis), resulting in a system size of $\sim 900,000$ atoms. The *mdp* scripts were used for energy minimization, equilibration in five steps and production run from CHARMM-GUI web server since they are optimized for protein-membrane simulations. The energy was minimized to $1000 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{nm}^{-1}$ using the steepest descent algorithm, followed by five-step equilibration to the desired temperature of 298 K (25 °C) and pressure of 1 bar. First, two *NVT* equilibration steps were applied to keep constant the number of atoms (*N*), the box volume (*V*), and temperature (*T*), followed by three-step *NpT* equilibration to adjust the pressure (*p*). The protein's and lipid's heavy atoms were restrained to allow the water molecules and ions to relax around the solute but they were decreased by every equilibration step. The Berendsen thermostat (76) was employed to regulate the temperature in the *NVT* simulations, while the Berendsen thermostat and the semi-isotropic Berendsen barostat (76) were employed for the *NpT* simulations. The PME method (77, 78) was applied to calculate long-range electrostatic interactions with periodic boundary conditions. van der Waals and Coulombic interaction cut-offs were set to 1.2 nm using the LINCS algorithm (79) to constrain all bond-lengths to hydrogens. MD runs were performed for 500 ns with a time step of 2 fs by recording the coordinates and velocities every 20 ps as well as

the Nosé-Hoover thermostat (80, 81) and the semi-isotropic Parrinello-Rahman barostat (82).

To follow possible changes in the secondary structure of LipH during the MD simulations, DSSP algorithm (for *Define Secondary Structure of Proteins*) was employed (83). The contact map shows the intramolecular interactions within the chaperone domain of LipH as percentage during the simulation. Here, a cut-off value under 6 Å was applied to consider as contact, which is depicted in a blue-white-red color scheme, where red colours represent higher contact percentage. The DSSP and contact maps were calculated *via* MDAnalysis 2.9.0 (84) and own python script. For DSSP analysis, the secondary structure was classified as helical (α -, 3_{10} and π), β -sheets/ β -strands, and coil that summarized other elements provided by the algorithm (coil, bend, turn). To assess the dynamics of the chaperoning domain of LipH, the radius of gyration and the minimal distance between residues 107 and 321 was analyzed.

To visualize positions of LipH in the simulation box during MD simulations, the density maps of ions and water were assembled *via* *gmx densmap*. Here, the direction of the y-axis was used to integrate the signal, the binning size was set to 0.08 and the unit was molecule per nm^3 . *DulvyTools* (<https://github.com/CharlesHahn/DulvyTools>) were used to convert the format from *xpm* to a matrix *dat* file (*dit xpm2dat*), as required for plotting the density maps *via* an own python script. The minimal distance between the membrane and LipH was computed for the whole protein, as well as the different domains to characterize LipH:membrane interactions. If not stated otherwise, all analyses were computed with GROMACS 2021/2022, and data was plotted with Gnuplot. PyMol (85) was used for rendering structural figures related to MD simulations.

Data availability

The SAXS data have been deposited to the Small Angle Scattering Biological Data Bank (86), with the accession codes SASDYM9 (LipH^{VD}), SASDYN9 (LipH^{VD}:LipA) and SASDYP9 (LipH^{VD}:LipA^{Δ81}). Collected SAXS raw data from the ESRF (proposal ID MX-2603) can be found under doi.org/10.1515/ESRF-DC-2396821007. HDX-MS data have been deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository (87) with the dataset identifier PXD074746.

Supporting information—This article contains supporting information (57–61, 63, 65, 66, 86, 88–93).

Acknowledgments—We would like to thank Lucas Kühn and Dr Florian Altegoer for the help with the analysis of LipH homologs and bioinformatics, Dr Cristian Rosales-Hernandez for the mass photometry measurements, and Dr Filip Kovacic for many discussions on the topic. We acknowledge the computational infrastructure and support provided by the Centre for Information and Media Technology (HHU Düsseldorf), and Prof. Dr Birgit Strodel to providing the computing time on the supercomputer JURECA (Forschungszentrum Jülich). We

Table 1
The list of simulations performed in this study

Systems	System size (atoms)	Length
Full-length LipH, 150 mM NaCl	897,978	3 × 500 ns
Full-length LipH, 25 mM NaCl	896,955	3 × 500 ns
LipH ^{Chap} , 150 mM NaCl	1,016,609	1 × 500 ns
LipH ^{Chap} , 25 mM NaCl	1,015,219	1 × 500 ns

Lipase-specific foldase: dynamics and client interactions

acknowledge DESY (Hamburg, Germany), a member of the Helmholtz Association HGF, for the provision of experimental facilities. Parts of this research were carried out at PETRA III, and we would like to thank Tobias Gräwert and Cy M. Jeffries (EMBL Hamburg) for their assistance in using the beamline P12. We acknowledge the European Synchrotron Radiation Facility (Grenoble, France) for provision of synchrotron radiation facilities under proposal ID MX-2603, and we would like to thank Mark Tully for his assistance in using beamline BM29.

Author contributions—J. R., A. P., J. L., M. B., A. K., W. S., S. H. J. S., and K-E. J. writing – review & editing; J. R., J. L., M. B., A. K., and W. S. writing – original draft; J. R., J. L., M. B., A. K., and W. S. visualization; J. R., A. P., J. L., M. B., and W. S. methodology; J. R., J. L., M. B., W. S., and V. C. investigation; J. R., A. P., J. L., M. B., A. K., W. S., and V. C. formal analysis; J. R., A. P., J. L., M. B., A. K., and W. S. data curation; S. H. J. S. and A. K. Supervision, A. K. and S. H. J. S. funding acquisition. A. K. and K-E. J. project administration; A. K. and K-E. J. conceptualization.

Funding and additional information—The study was funded by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) within the Collaborative Research Center 1208 “Identity and Dynamics of Membrane Systems” (grant number 267205415 to A. K. and K. E. J.) and supported through the Marburg core facility for Interaction, Dynamics and Assembly of Biomolecular Structures (DFG grant numbers 324652314 and 260989694 to Prof. Gert Bange, Marburg). The Center for Structural Studies is part of StrukturaLINK Rhein-Ruhr funded by the DFG (grant number 573727698 and 417919780) and INST 208/761-1 FUGG.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: EHD, extended helical domain; LipA, lipase A; MD, molecular dynamics; TMH, trans-membrane helix; POPG, 1-palmitoyl-2-oleoyl-sn-glycero- 3-phospho-(1-rac-glycerol); SAXS, small-angle X-ray scattering; T2SS, type II secretion system; VD, variable domain.

References

- Mulani, M. S., Kamble, E. E., Kumkar, S. N., Tawre, M. S., and Pardesi, K. R. (2019) Emerging strategies to combat ESKAPE pathogens in the era of antimicrobial resistance: a review. *Front. Microbiol.* **10**, 539
- Green, E. R., and Mecsas, J. (2016) Bacterial secretion systems: an overview. *Microbiol. Spectr.* **4**, VMBF-0012-2015
- Wretling, B., and Pavlovskis, O. R. (1984) Genetic mapping and characterization of *Pseudomonas aeruginosa* mutants defective in the formation of extracellular proteins. *J. Bacteriol.* **158**, 801–808
- König, B., Jaeger, K. E., Sage, A. E., Vasil, M. L., and König, W. (1996) Role of *Pseudomonas aeruginosa* lipase in inflammatory mediator release from human inflammatory effector cells (platelets, granulocytes, and monocytes). *Infect. Immun.* **64**, 3252–3258
- Douzi, B., Ball, G., Cambillau, C., Tegoni, M., and Voulhoux, R. (2011) Deciphering the Xcp *Pseudomonas aeruginosa* type II secretion machinery through multiple interactions with substrates. *J. Biol. Chem.* **286**, 40792–40801
- Rosenau, F., Tommassen, J., and Jaeger, K. E. (2004) Lipase-specific foldases. *ChemBiochem.* **5**, 152–161
- El Khattabi, M., Van Gelder, P., Bitter, W., and Tommassen, J. (2000) Role of the lipase-specific foldase of *Burkholderia glumae* as a steric chaperone. *J. Biol. Chem.* **275**, 26885–26891
- Pauwels, K., Lustig, A., Wyns, L., Tommassen, J., Savvides, S. N., and Van Gelder, P. (2006) Structure of a membrane-based steric chaperone in complex with its lipase substrate. *Nat. Struct. Mol. Biol.* **13**, 374–375
- Ellis, R. J. (1993) The general concept of molecular chaperones. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **339**, 257–261
- Pauwels, K., Van Molle, I., Tommassen, J., and Van Gelder, P. (2007) Chaperoning anfinen: the steric foldases. *Mol. Microbiol.* **64**, 917–922
- El Khattabi, M., Ockhuijsen, C., Bitter, W., Jaeger, K. E., and Tommassen, J. (1999) Specificity of the lipase-specific foldases of gram-negative bacteria and the role of the membrane anchor. *Mol. Gen. Genet.* **261**, 770–776
- Hobson, A. H., Buckley, C. M., Aamand, J. L., Jorgensen, S. T., Diderichsen, B., and McConnell, D. J. (1993) Activation of a bacterial lipase by its chaperone. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 5682–5686
- Pauwels, K., Loris, R., Vandenbussche, G., Ruyschaert, J. M., Wyns, L., and Van Gelder, P. (2005) Crystallization and crystal manipulation of a steric chaperone in complex with its lipase substrate. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **61**, 791–795
- de Oliveira Silva, Y. R., Contreras-Martel, C., Rodrigues de Melo, R., Zanthorlin, L. M., Trindade, D. M., and Dessen, A. (2025) Architecture of an embracing lipase-foldase complex of the type II secretion system of *Acinetobacter baumannii*. *Structure* **33**, 601–612.e604
- Verma, N., Dollinger, P., Kovacic, F., Jaeger, K. E., and Gohlke, H. (2020) The membrane-integrated steric chaperone lif facilitates active site opening of *Pseudomonas aeruginosa* lipase A. *J. Comput. Chem.* **41**, 500–512
- Jaeger, K. E., Ransac, S., Dijkstra, B. W., Colson, C., van Heuvel, M., and Misset, O. (1994) Bacterial lipases. *FEMS Microbiol. Rev.* **15**, 29–63
- Papadopoulos, A., Busch, M., Reiners, J., Hachani, E., Baeumers, M., Berger, J., et al. (2022) The periplasmic chaperone Skp prevents misfolding of the secretory lipase A from *Pseudomonas aeruginosa*. *Front. Mol. Biosci.* **9**, 1026724
- Williamson, M. P. (2023) Protein binding: a fuzzy concept. *Life (Basel)* **13**, 855
- Dittrich, J., Popara, M., Kubiak, J., Dimura, M., Schepers, B., Verma, N., et al. (2023) Resolution of maximum entropy method-derived posterior conformational ensembles of a flexible system probed by FRET and molecular dynamics simulations. *J. Chem. Theor. Comput.* **19**, 2389–2409
- Morein, S., Andersson, A., Rilfors, L., and Lindblom, G. (1996) Wild-type *Escherichia coli* cells regulate the membrane lipid composition in a “window” between gel and non-lamellar structures. *J. Biol. Chem.* **271**, 6801–6809
- Albelo, S. T., and Domenech, C. E. (1997) Carbons from choline present in the phospholipids of *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* **156**, 271–274
- Deschamps, E., Schaumann, A., Schmitz-Afonso, I., Afonso, C., De, E., Loutelier-Bourhis, C., et al. (2021) Membrane phospholipid composition of *Pseudomonas aeruginosa* grown in a cystic fibrosis mucus-mimicking medium. *Biochim. Biophys. Acta Biomembr.* **1863**, 183482
- Grandjean Lapierre, S., Phelippeau, M., Hakimi, C., Didier, Q., Reynaud-Gaubert, M., Dubus, J. C., et al. (2017) Cystic fibrosis respiratory tract salt concentration: an exploratory cohort study. *Medicine (Baltimore)* **96**, e8423
- Rath, A., and Deber, C. M. (2013) Correction factors for membrane protein molecular weight readouts on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.* **434**, 67–72
- Stetsenko, A., and Guskov, A. (2017) An overview of the top ten detergents used for membrane protein crystallization. *Crystals* **7**, 197
- Slotboom, D. J., Duurkens, R. H., Olieman, K., and Erkens, G. B. (2008) Static light scattering to characterize membrane proteins in detergent solution. *Methods* **46**, 73–82
- Strop, P., and Bronger, A. T. (2005) Refractive index-based determination of detergent concentration and its application to the study of membrane proteins. *Protein Sci.* **14**, 2207–2211

28. Oliver, R. C., Lipfert, J., Fox, D. A., Lo, R. H., Doniach, S., and Columbus, L. (2013) Dependence of micelle size and shape on detergent alkyl chain length and head group. *PLoS One* **8**, e62488
29. Popot, J. L., Althoff, T., Bagnard, D., Baneres, J. L., Bazzacco, P., Billon-Denis, E., *et al.* (2011) Amphipols from A to Z. *Annu. Rev. Biophys.* **40**, 379–408
30. Ritchie, T. K., Grinkova, Y. V., Bayburt, T. H., Denisov, I. G., Zolnerciks, J. K., Atkins, W. M., *et al.* (2009) Chapter 11 - Reconstitution of membrane proteins in phospholipid bilayer nanodiscs. *Methods Enzymol.* **464**, 211–231
31. Denisov, I. G., Grinkova, Y. V., Lazarides, A. A., and Sligar, S. G. (2004) Directed self-assembly of monodisperse phospholipid bilayer nanodiscs with controlled size. *J. Am. Chem. Soc.* **126**, 3477–3487
32. Gohon, Y., Giusti, F., Prata, C., Charvolin, D., Timmins, P., Ebel, C., *et al.* (2006) Well-defined nanoparticles formed by hydrophobic assembly of a short and polydisperse random terpolymer, amphipol A8-35. *Langmuir* **22**, 1281–1290
33. Rashno, F., Khajeh, K., Dabirmanesh, B., Sajedi, R. H., and Chiti, F. (2018) Insight into the aggregation of lipase from *Pseudomonas* sp. using mutagenesis: protection of aggregation prone region by adoption of alpha-helix structure. *Protein Eng. Des. Sel.* **31**, 419–426
34. Langer, A., Bartoschik, T., Cehlar, O., Duhr, S., Baaske, P., and Streicher, W. (2022) A new spectral shift-based method to characterize molecular interactions. *Assay Drug Dev. Technol.* **20**, 83–94
35. Jaeger, K. E., and Kovacic, F. (2014) Determination of lipolytic enzyme activities. *Methods Mol. Biol.* **1149**, 111–134
36. Hodge, E. A., Benhaim, M. A., and Lee, K. K. (2020) Bridging protein structure, dynamics, and function using hydrogen/deuterium-exchange mass spectrometry. *Protein Sci.* **29**, 843–855
37. Hioki, T. (2025) Prediction of lipase-specific foldase-dependence in bacterial lipase subfamilies I.1 and I.2. *BMC Genomics* **26**, 520
38. Castanie-Cornet, M. P., Bruel, N., and Genevaux, P. (2014) Chaperone networking facilitates protein targeting to the bacterial cytoplasmic membrane. *Biochim. Biophys. Acta* **1843**, 1442–1456
39. De Geyter, J., Tsigotaki, A., Orfanoudaki, G., Zorzini, V., Economou, A., and Karamanou, S. (2016) Protein folding in the cell envelope of *Escherichia coli*. *Nat. Microbiol.* **1**, 16107
40. Sucec, I., Bersch, B., and Schanda, P. (2021) How do chaperones bind (partly) unfolded client proteins? *Front. Mol. Biosci.* **8**, 762005
41. Arhar, T., Shkedi, A., Nadel, C. M., and Gestwicki, J. E. (2021) The interactions of molecular chaperones with client proteins: why are they so weak? *J. Biol. Chem.* **297**, 101282
42. Parsot, C., Hamiaux, C., and Page, A. L. (2003) The various and varying roles of specific chaperones in type III secretion systems. *Curr. Opin. Microbiol.* **6**, 7–14
43. Urusova, D. V., Kinsella, R. L., Salinas, N. D., Haurat, M. F., Feldman, M. F., and Tolia, N. H. (2019) The structure of acinetobacter-secreted protease CpaA complexed with its chaperone CpaB reveals a novel mode of a T2SS chaperone-substrate interaction. *J. Biol. Chem.* **294**, 13344–13354
44. Kinsella, R. L., Lopez, J., Palmer, L. D., Salinas, N. D., Skaar, E. P., Tolia, N. H., *et al.* (2017) Defining the interaction of the protease CpaA with its type II secretion chaperone CpaB and its contribution to virulence in acinetobacter species. *J. Biol. Chem.* **292**, 19628–19638
45. Gotzke, H., Palombo, I., Muheim, C., Perrody, E., Genevaux, P., Kudva, R., *et al.* (2014) YfgM is an ancillary subunit of the SecYEG translocon in *Escherichia coli*. *J. Biol. Chem.* **289**, 19089–19097
46. Weininger, U., Jakob, R. P., Kovermann, M., Balbach, J., and Schmid, F. X. (2010) The prolyl isomerase domain of PpiD from *Escherichia coli* shows a parvulin fold but is devoid of catalytic activity. *Protein Sci.* **19**, 6–18
47. Miyazaki, R., Ai, M., Tanaka, N., Suzuki, T., Dhoma, N., Tsukazaki, T., *et al.* (2022) Inner membrane YfgM-PpiD heterodimer acts as a functional unit that associates with the SecY/E/G translocon and promotes protein translocation. *J. Biol. Chem.* **298**, 102572
48. Jauss, B., Petriman, N. A., Drepper, F., Franz, L., Sachelar, I., Welte, T., *et al.* (2019) Noncompetitive binding of PpiD and YidC to the SecYEG translocon expands the global view on the SecYEG interactome in *Escherichia coli*. *J. Biol. Chem.* **294**, 19167–19183
49. Shibata, H., Kato, H., and Oda, J. (1998) Random mutagenesis on the *Pseudomonas* lipase activator protein, LipB: exploring amino acid residues required for its function. *Protein Eng.* **11**, 467–472
50. Viegas, A., Dollinger, P., Verma, N., Kubiak, J., Viennet, T., Seidel, C. A. M., *et al.* (2020) Structural and dynamic insights revealing how lipase binding domain MD1 of *Pseudomonas aeruginosa* foldase affects lipase activation. *Sci. Rep.* **10**, 3578
51. He, W., Li, X., Xue, H., Yang, Y., Mencius, J., Bai, L., *et al.* (2022) Insights into the client protein release mechanism of the ATP-independent chaperone spy. *Nat. Commun.* **13**, 2818
52. Oberg, N., Zallot, R., and Gerlt, J. A. (2023) EFI-EST, EFI-GNT, and EFI-CGFP: enzyme Function Initiative (EFI) web resource for genomic enzymology tools. *J. Mol. Biol.* **435**, 168018
53. Blum, M., Andreeva, A., Florentino, L. C., Chuguransky, S. R., Grego, T., Hobbs, E., *et al.* (2025) InterPro: the protein sequence classification resource in 2025. *Nucleic Acids Res.* **53**, D444–D456
54. Gough, J., Karplus, K., Hughey, R., and Chothia, C. (2001) Assignment of homology to genome sequences using a library of hidden Markov models that represent all proteins of known structure. *J. Mol. Biol.* **313**, 903–919
55. [Preprint] Hallgren, J., Tsigotaki, K. D., Pedersen, M. D., Almagro Armenteros, J. J., Marcanti, P., Nielsen, H., *et al.* (2022) DeepTMHMM predicts alpha and beta transmembrane proteins using deep neural networks. *BioRxiv*. <https://doi.org/10.1101/2022.04.08.487609>
56. Kamel, M., Lowe, M., Schott-Verdugo, S., Gohlke, H., and Kedrov, A. (2022) Unsaturated fatty acids augment protein transport via the SecA: SecYEG translocon. *FEBS J.* **289**, 140–162
57. Blanchet, C. E., Spilotros, A., Schwemmer, F., Graewert, M. A., Kikhney, A., Jeffries, C. M., *et al.* (2015) Versatile sample environments and automation for biological solution X-ray scattering experiments at the P12 beamline (PETRA III, DESY). *J. Appl. Crystallogr.* **48**, 431–443
58. Tully, M. D., Kieffer, J., Brennich, M. E., Cohen, R., Aberdam, R., Florial, J. B., Hutin, S., *et al.* (2023) BioSAXS at European synchrotron radiation facility - extremely brilliant source: BM29 with an upgraded source, detector, robot, sample environment, data collection and analysis software. *J. Synchrotron Radiat.* **30**, 258–266
59. Manalastas-Cantos, K., Konarev, P. V., Hajizadeh, N. R., Kikhney, A. G., Petoukhov, M. V., Molodenskiy, D. S., *et al.* (2021) ATSAS 3.0: expanded functionality and new tools for small-angle scattering data analysis. *J. Appl. Crystallogr.* **54**, 343–355
60. Panjkovich, A., and Svergun, D. I. (2018) CHROMIXS: automatic and interactive analysis of chromatography-coupled small-angle X-ray scattering data. *Bioinformatics* **34**, 1944–1946
61. Konarev, P. V., Volkov, V. V., Sokolova, A. V., Koch, M. H. J., and Svergun, D. I. (2003) A windows PC-based system for small-angle scattering data analysis. *J. Appl. Crystallogr.* **36**, 1277–1282
62. Guinier, A. (1939) La diffraction des rayons X aux très petits angles : application à l'étude de phénomènes ultramicroscopiques. *Ann. Phys.* **11**, 161–237
63. Svergun, D. I. (1992) Determination of the regularization parameter in indirect-transform methods using perceptual criteria. *J. Appl. Crystallogr.* **25**, 495–503
64. Abramson, J., Adler, J., Dunger, J., Evans, R., Green, T., Pritzel, A., *et al.* (2024) Accurate structure prediction of biomolecular interactions with AlphaFold 3. *Nature* **630**, 493–500
65. Bernado, P., Mylonas, E., Petoukhov, M. V., Blackledge, M., and Svergun, D. I. (2007) Structural characterization of flexible proteins using small-angle X-ray scattering. *J. Am. Chem. Soc.* **129**, 5656–5664
66. Tria, G., Mertens, H. D., Kachala, M., and Svergun, D. I. (2015) Advanced ensemble modelling of flexible macromolecules using X-ray solution scattering. *IUCr* **2**, 207–217
67. Osorio-Valeriano, M., Altegoer, F., Steinchen, W., Urban, S., Liu, Y., Bange, G., *et al.* (2019) ParB-type DNA segregation proteins are CTP-dependent molecular switches. *Cell* **179**, 1512–1524.e1515
68. Wales, T. E., Fadgen, K. E., Gerhardt, G. C., and Engen, J. R. (2008) High-speed and high-resolution UPLC separation at zero degrees celsius. *Anal. Chem.* **80**, 6815–6820
69. Geromanos, S. J., Vissers, J. P., Silva, J. C., Dorschel, C. A., Li, G. Z., Gorenstein, M. V., *et al.* (2009) The detection, correlation, and

Lipase-specific foldase: dynamics and client interactions

- comparison of peptide precursor and product ions from data independent LC-MS with data dependant LC-MS/MS. *Proteomics* **9**, 1683–1695
70. Li, G. Z., Vissers, J. P., Silva, J. C., Golick, D., Gorenstein, M. V., and Geromanos, S. J. (2009) Database searching and accounting of multiplexed precursor and product ion spectra from the data independent analysis of simple and complex peptide mixtures. *Proteomics* **9**, 1696–1719
 71. Jo, S., Kim, T., Iyer, V. G., and Im, W. (2008) CHARMM-GUI: a web-based graphical user interface for CHARMM. *J. Comput. Chem.* **29**, 1859–1865
 72. Wu, E. L., Cheng, X., Jo, S., Rui, H., Song, K. C., Davila-Contreras, E. M., et al. (2014) CHARMM-GUI membrane builder toward realistic biological membrane simulations. *J. Comput. Chem.* **35**, 1997–2004
 73. Lee, J., Patel, D. S., Stahle, J., Park, S. J., Kern, N. R., Kim, S., et al. (2019) CHARMM-GUI membrane builder for complex biological membrane simulations with glycolipids and lipoglycans. *J. Chem. Theory Comput.* **15**, 775–786
 74. Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W., and Klein, M. L. (1983) Comparison of simple potential functions for simulating liquid water. *J. chem. phys.* **79**, 926–935
 75. Abraham, M. J., Murtola, T., Schulz, R., Páll, S., Smith, J. C., Hess, B., et al. (2015) GROMACS: high performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX* **1-2**, 19–25
 76. Berendsen, H. J. C., Postma, J. P. M., Vangunsteren, W. F., Dinola, A., and Haak, J. R. (1984) Molecular-dynamics with coupling to an external bath. *J. Chem. Phys.* **81**, 3684–3690
 77. Essmann, U., Perera, L., Berkowitz, M. L., Darden, T., Lee, H., and Pedersen, L. G. (1995) A smooth particle mesh Ewald method. *J. Chem. Phys.* **103**, 8577–8593
 78. Darden, T., York, D., and Pedersen, L. (1993) Particle mesh Ewald - an N. Log(N) method for Ewald sums in large systems. *J. Chem. Phys.* **98**, 10089–10092
 79. Hess, B., Bekker, H., Berendsen, H. J. C., and Fraaije, J. G. E. M. (1997) LINCS: a linear constraint solver for molecular simulations. *J. Comput. Chem.* **18**, 1463–1472
 80. Nose, S. (1984) A molecular-dynamics method for simulations in the canonical ensemble. *Mol. Phys.* **52**, 255–268
 81. Hoover, W. G. (1985) Canonical dynamics: equilibrium phase-space distributions. *Phys. Rev. A. Gen. Phys.* **31**, 1695–1697
 82. Parrinello, M., and Rahman, A. (1981) Polymorphic transitions in single-crystals - a new molecular-dynamics method. *J. Appl. Phys.* **52**, 7182–7190
 83. Kabsch, W., and Sander, C. (1983) Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* **22**, 2577–2637
 84. Michaud-Agrawal, N., Denning, E. J., Woolf, T. B., and Beckstein, O. (2011) MDAnalysis: a toolkit for the analysis of molecular dynamics simulations. *J. Comput. Chem.* **32**, 2319–2327
 85. Schrodinger, L. L. C. (2011) *The PyMOL Molecular Graphics System*
 86. Kikhney, A. G., Borges, C. R., Molodenskiy, D. S., Jeffries, C. M., and Svergun, D. I. (2020) SASBDB: Towards an automatically curated and validated repository for biological scattering data. *Protein Sci.* **29**, 66–75
 87. Perez-Riverol, Y., Bai, J., Bandla, C., Garcia-Seisdedos, D., Hewapathirana, S., Kamatchinathan, S., et al. (2022) The PRIDE database resources in 2022: a hub for mass spectrometry-based proteomics evidences. *Nucleic Acids Res.* **50**, D543–D552
 88. Fischer, H., Neto, M. D., Napolitano, H. B., and Polikarpov, I. (2010) Determination of the molecular weight of proteins in solution from a single small-angle X-ray scattering measurement on a relative scale. *J. Appl. Cryst* **43**, 101–109
 89. Franke, D., and Jeffries, C. M. (2015) Correlation Map, a goodness-of-fit test for one-dimensional X-ray scattering spectra. *Nat. Methods* **12**, 419–422
 90. Goddard, T. D., Huang, C. C., Meng, E. C., Pettersen, E. F., Couch, G. S., Morris, J. H., and Ferrin, T. E. (2018) UCSF ChimeraX: Meeting modern challenges in visualization and analysis. *Protein Sci.* **27**, 14–25
 91. Hajizadeh, N. R., Franke, D., and Jeffries, C. M. (2018) Consensus Bayesian assessment of protein molecular mass from solution X-ray scattering data. *Sci. Rep.* **8**, 7204
 92. Porod, G. (1951) Die Röntgenkleinwinkelstreuung Von Dichtgepackten Kolloiden Systemen .1. *Kolloid-Zeitschrift and Zeitschrift Fur Polymere* **124**, 83–114
 93. Rambo, R. P. (2013) Accurate assessment of mass, models and resolution by small-angle scattering. *Nature* **496**, 477–481

Conformational dynamics of the membrane-anchored foldase LipH from *Pseudomonas aeruginosa* governs recognition and release of its client lipase

Max Busch, Jennifer Loschwitz, Athanasios Papadopoulos, Jens Reiners,
Wieland Steinchen, Vincenzo Calvagna, Sander H.J. Smits,
Karl-Erich Jaeger, Alexej Kedrov

Supporting information

Table of Contents

Figure S1. Conserved organization of the foldase:lipase complex.	S2
Figure S2. Gating-associated dynamics of <i>P. aeruginosa</i> LipA.	S2
Figure S3. LipH architecture and design of the studied constructs.	S3
Figure S4. LipH retains its secondary structure over the course of molecular dynamics simulations. .	S4
Figure S5. Conformational dynamics of LipH at 150 mM NaCl.	S5
Figure S6. Conformational dynamics of LipH at 25 mM NaCl.	S6
Figure S7. Maps of intramolecular contacts within LipH ^{FL}	S7
Figure S8. LipH ^{Chap} localization along molecular dynamics simulations.	S7
Figure S9. LipH ^{VD} :membrane interactions at different ionic strengths.	S8
Figure S10. LipH interactions with the membrane interface.	S9
Figure S11. Small-angle X-ray scattering analysis of LipH ^{VD}	S10
Figure S12. SEC-MALS of the full-length LipH in DDM and Cymal-6 micelles.	S11
Figure S13: <i>In vitro</i> analysis of LipH-mediated enzymatic activity of LipA.	S11
Figure S14. Hydrogen/deuterium exchange mass spectrometry of LipA.	S12
Figure S15. HDX behaviour of representative LipA peptides.	S13
Figure S16. Hydrogen/deuterium exchange mass spectrometry of LipH ^{Chap}	S14
Figure S17. HDX behaviour of representative LipH ^{Chap} peptides.	S15
Figure S18. Design of LipA fragments for studying interactions with LipH.	S16
Figure S19. Co-elution assay to probe non-specific interactions of LipH.	S16
Figure S20. Small-angle X-ray scattering analysis of the LipH ^{VD} :LipA complex.	S17
Figure S21. Small-angle X-ray scattering analysis of the LipH ^{VD} :LipA ^{Δ81} complex.	S18
Figure S22. AlphaFold3-based models of the membrane-anchored chaperones.	S19
Figure S23. AlphaFold 3 analysis of a potential SecYEG:LipH ^{FL} complex.	S19
Figure S24. The electrostatic potential at the surface of the folded LipA.	S20
Figure S25. Lipid-dependent activity of LipH ^{FL}	S20
Table S1. Summary of SAXS data acquisition and analysis.	S22
Table S2. Overview of data obtained by hydrogen/deuterium exchange mass spectrometry.	S24
Supporting references.	S24

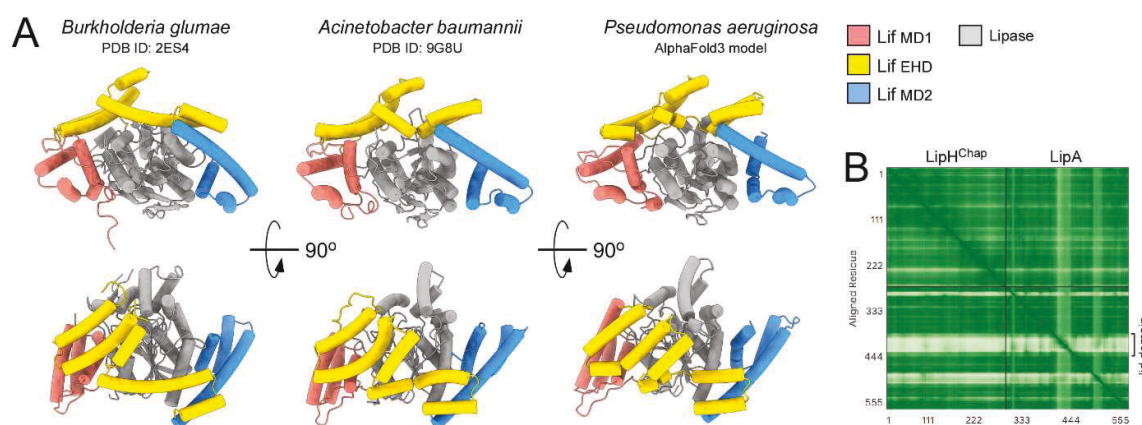


Figure S1. Conserved organization of the foldase:lipase complex.

(A) Crystal structures of lipases from *B. glumae* and *A. baumannii* bound to their cognate foldase chaperones (Lif's), in comparison to the AlphaFold3-derived model for the LipH^{Chap}:LipA complex of *P. aeruginosa* (excluding the N-terminal anchor/linker region; ipTM score 0.81).

(B) Predicted aligned error plot for the LipH^{Chap}:LipA complex of *P. aeruginosa*. The low-confidence region corresponds to the dynamic lid domain of LipA.

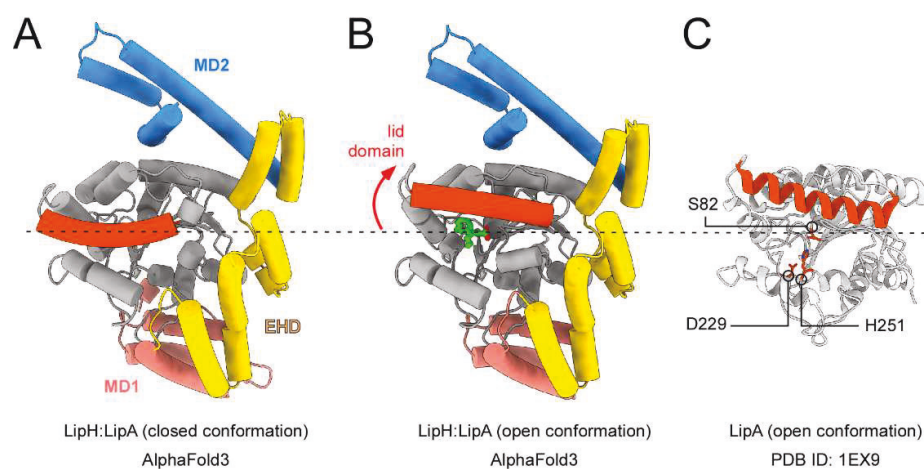


Figure S2. Gating-associated dynamics of *P. aeruginosa* LipA.

(A) AlphaFold3-derived model of the LipH:LipA complex. The gating helix 5 of LipA ("lid domain") is shown in red.

(B) AlphaFold3-derived model of the LipH:LipA complex in presence of an oleic acid molecule (green). Docking of the ligand is facilitated by a displacement of the lid domain, as indicated by the arrow.

(C) Crystal structure of *P. aeruginosa* LipA shows a displaced lid domain. The residues forming the catalytic triad are indicated.

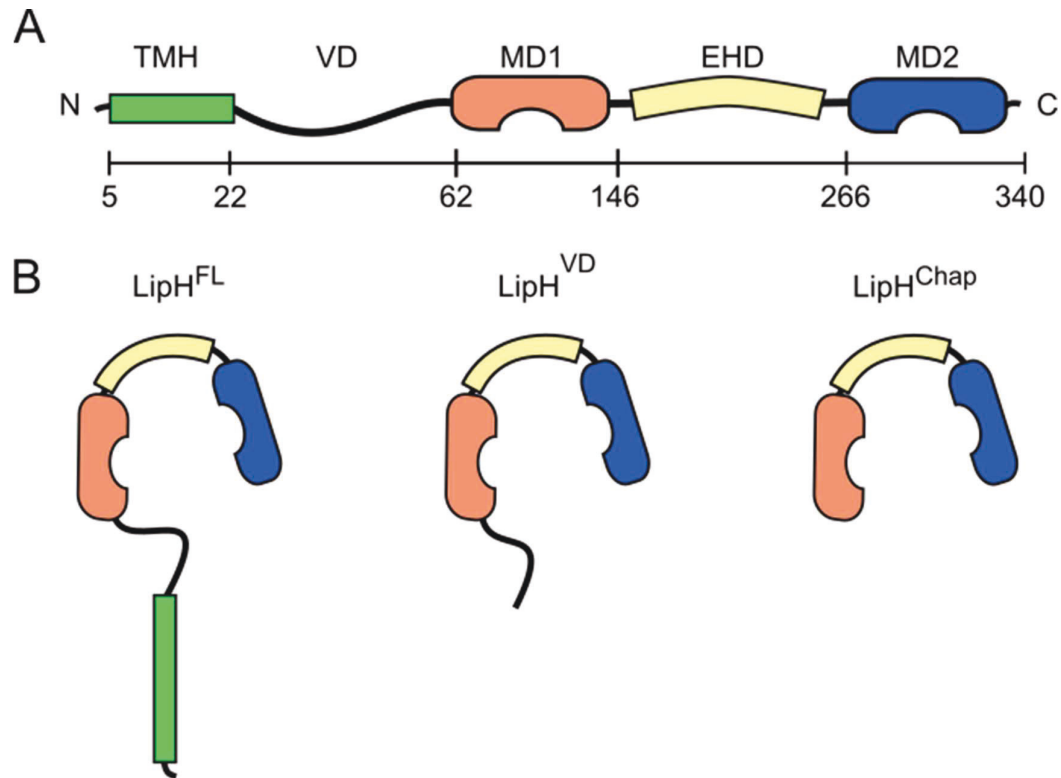


Figure S3. LipH architecture and design of the studied constructs.

(A) Organization of the full-length foldase LipH. The structural elements and their positions within the polypeptide chain (numbers in amino acids) are indicated.

(B) Schematic overview of the constructs employed in the study

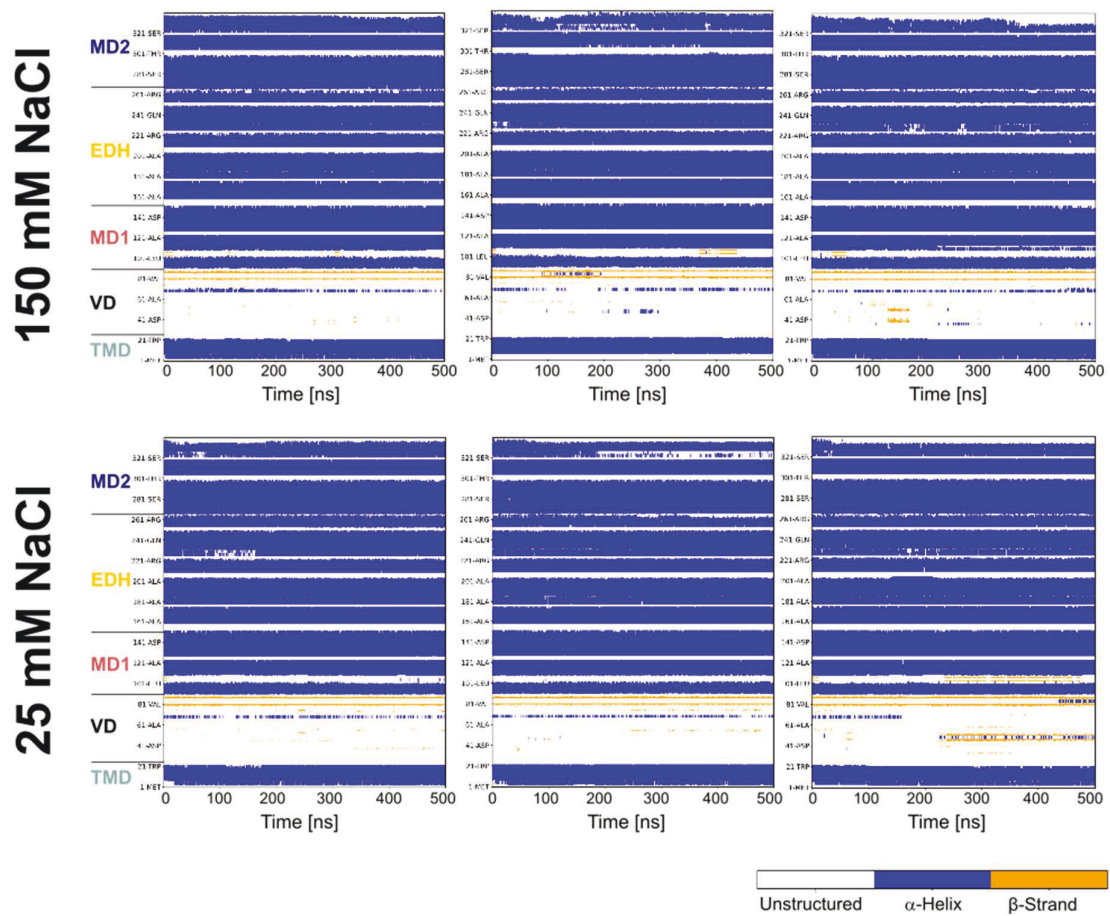


Figure S4. LipH retains its secondary structure over the course of molecular dynamics simulations. The color-coded secondary structure of LipH^{FL} along individual simulation courses at 150mM and 25 mM NaCl is plotted against the simulation time. The structural domains of LipH are indicated on the left.

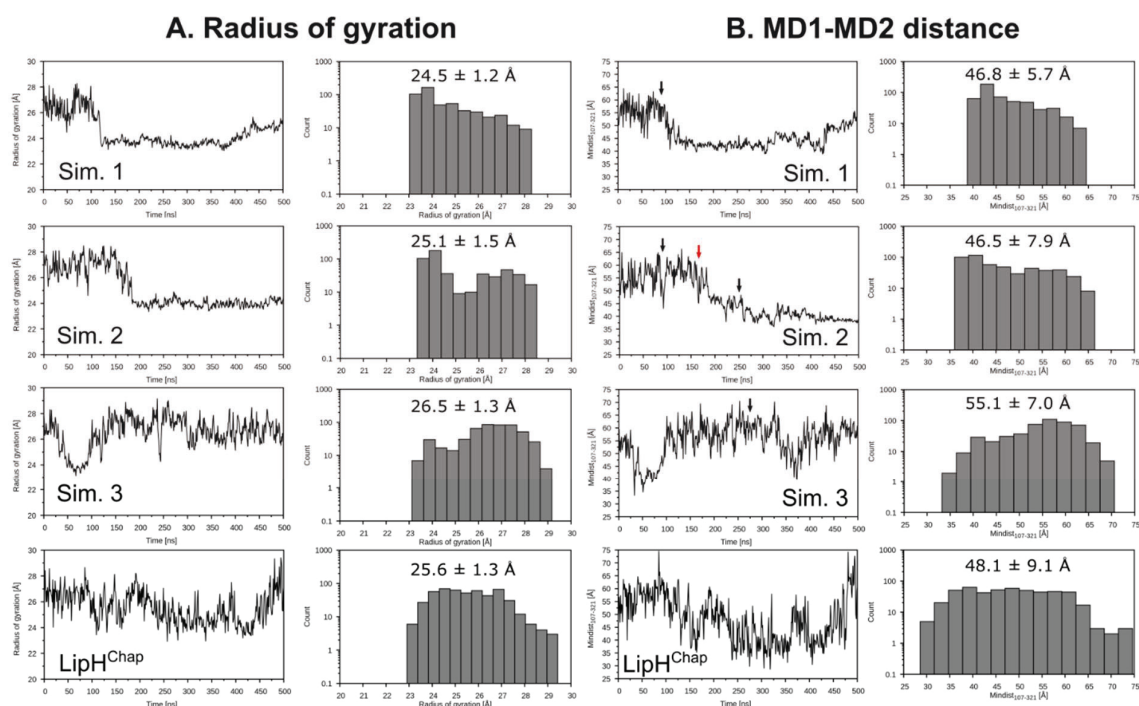


Figure S5. Conformational dynamics of LipH at 150 mM NaCl.

(A) Variations in the radius of gyration of the chaperoning domain within LipH^{FL} over the time course of individual simulations (left) and the corresponding distributions of the radius of gyration (right). The mean value $\pm$ standard deviations are indicated for each simulation. “LipH^{Chap}” corresponds to the LipH chaperoning domain simulated without the membrane anchor and VD.

(B) Variations in the distance between MD1 and MD2 domains within LipH^{FL} over the time course of individual simulations (left) and the corresponding distributions of the distance (right), with the mean value $\pm$ standard deviations indicated.

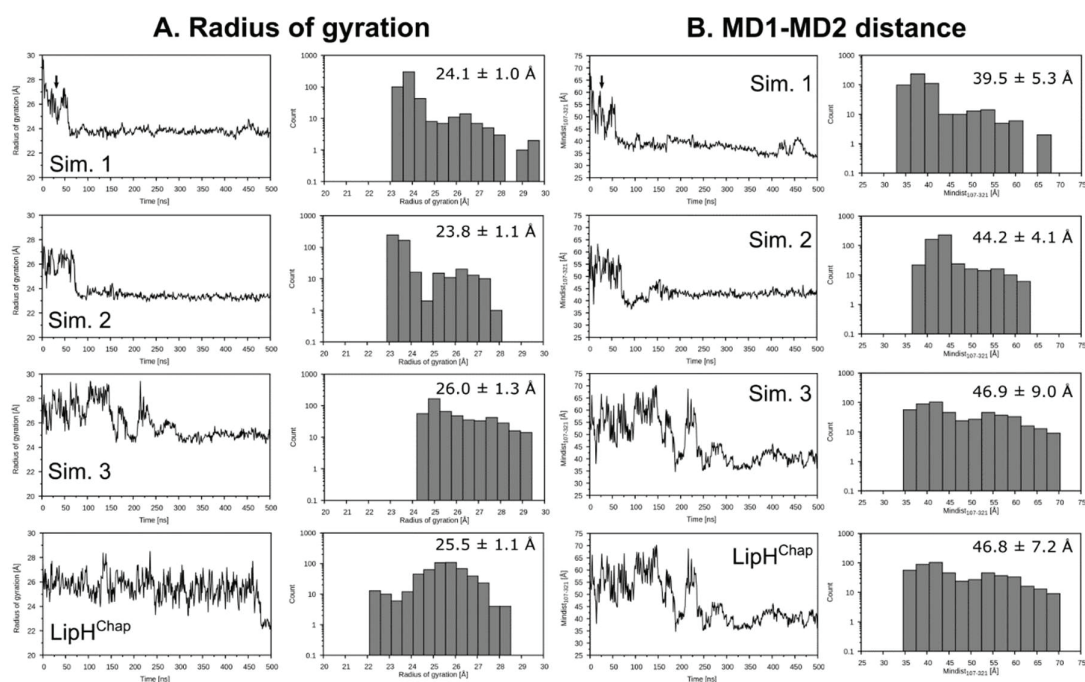


Figure S6. Conformational dynamics of LipH at 25 mM NaCl.

(A) Variations in the radius of gyration of the chaperoning domain within LipH^{FL} over the time course of individual simulations (left) and the corresponding distributions of the radius of gyration (right). The mean value $\pm$ standard deviations are indicated for each simulation. “LipH^{Chap}” corresponds to the LipH chaperoning domain simulated without the membrane anchor and VD.

(B) Variations in the distance between MD1 and MD2 domains within LipH^{FL} over the time course of individual simulations (left) and the corresponding distributions of the distance (right), with the mean value $\pm$ standard deviations indicated.

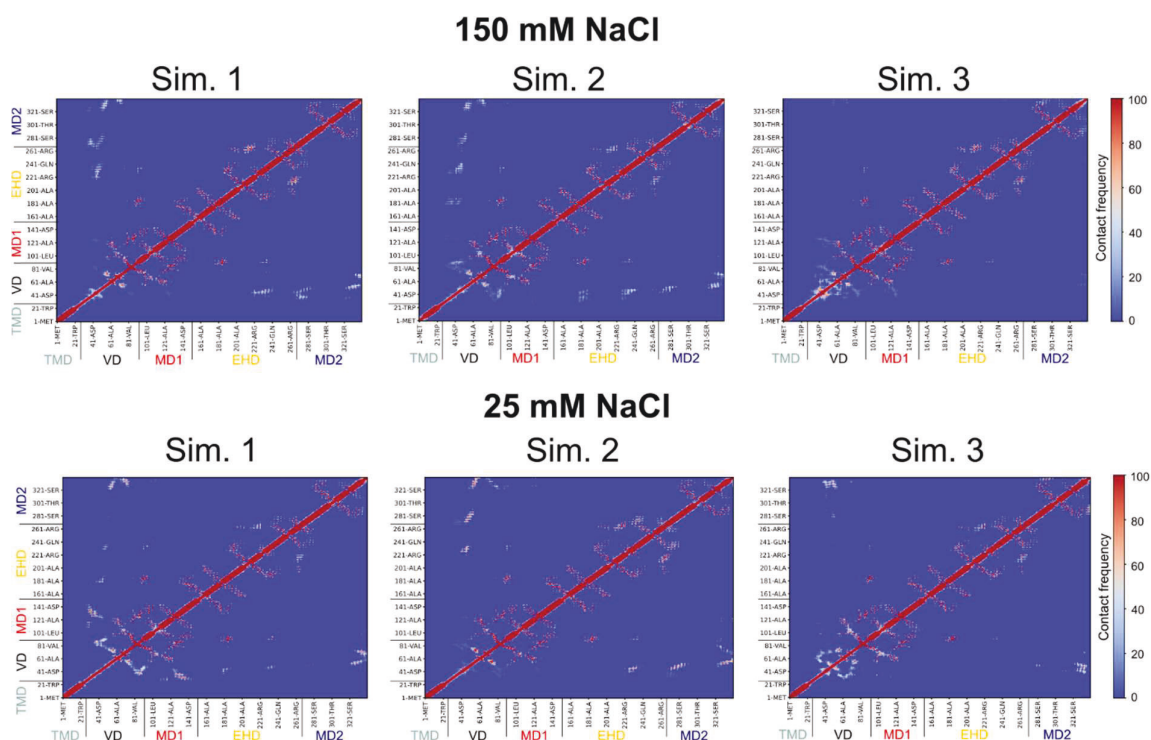


Figure S7. Maps of intramolecular contacts within LipH^{FL}. Over the course of individual simulations at 150 mM and 25 mM NaCl. The structural domains of LipH are indicated.

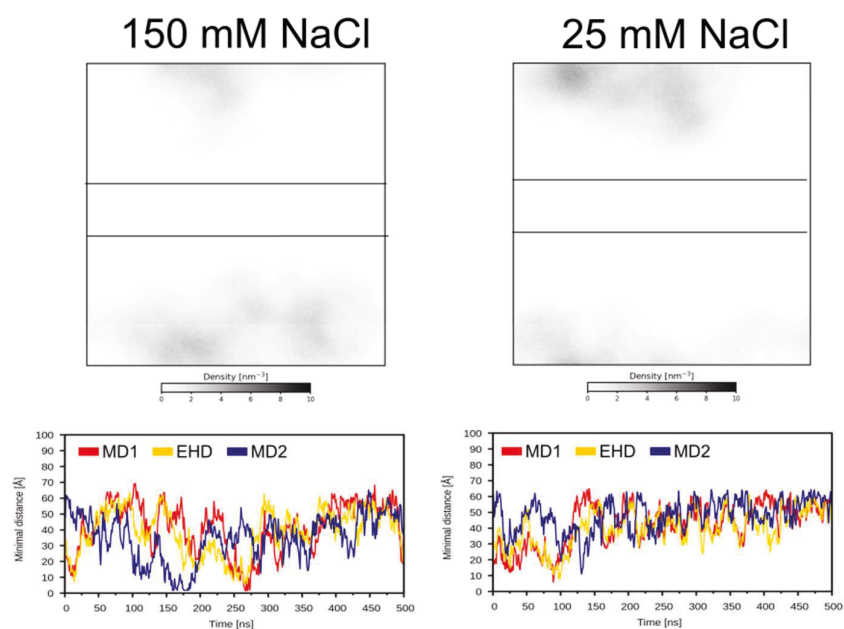


Figure S8. LipH^{Chap} localization along molecular dynamics simulations.

Top: Density maps of LipH^{Chap} localization within the simulation box calculated for 150 mM and 25 mM NaCl.

Bottom: Corresponding minimal distances of the individual LipH domains to the membrane over the course of each simulation.

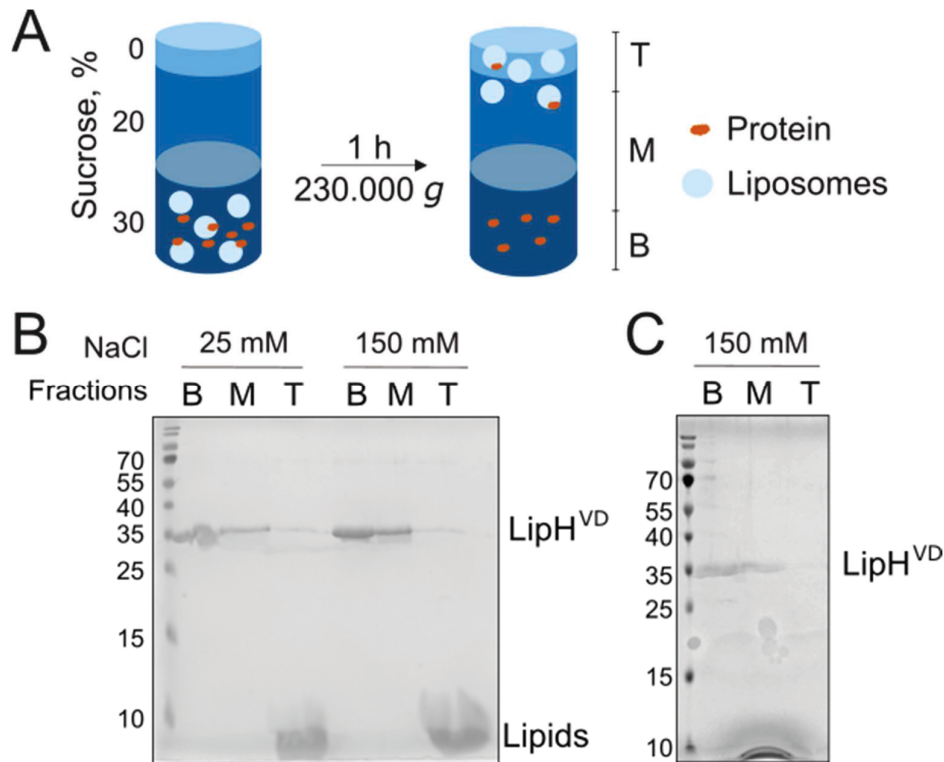


Figure S9. LipH^{VD}:membrane interactions at different ionic strengths.

(A) Schematics of the flotation assay to probe protein:membrane interactions. During centrifugation, the liposomes filled with a sucrose-free buffer migrate to the top of the sucrose density gradient. Liposome-bound proteins can afterwards be found in the top fraction (T) while the unbound and dissociated proteins predominantly stay in the bottom (B) and the middle fractions (M).

(B) SDS-PAGE of fractionated sucrose gradients at 25 mM and 150 mM mM NaCl in presence of liposomes composed of DOPC:DOPG. LipH^{VD} could be detected only in bottom and middle fractions, and the lipids could be seen in the top fraction.

(C) SDS-PAGE of the fractionated sucrose gradients 150 mM mM NaCl performed in absence of liposomes. LipH^{VD} could be detected only in bottom and middle fraction.

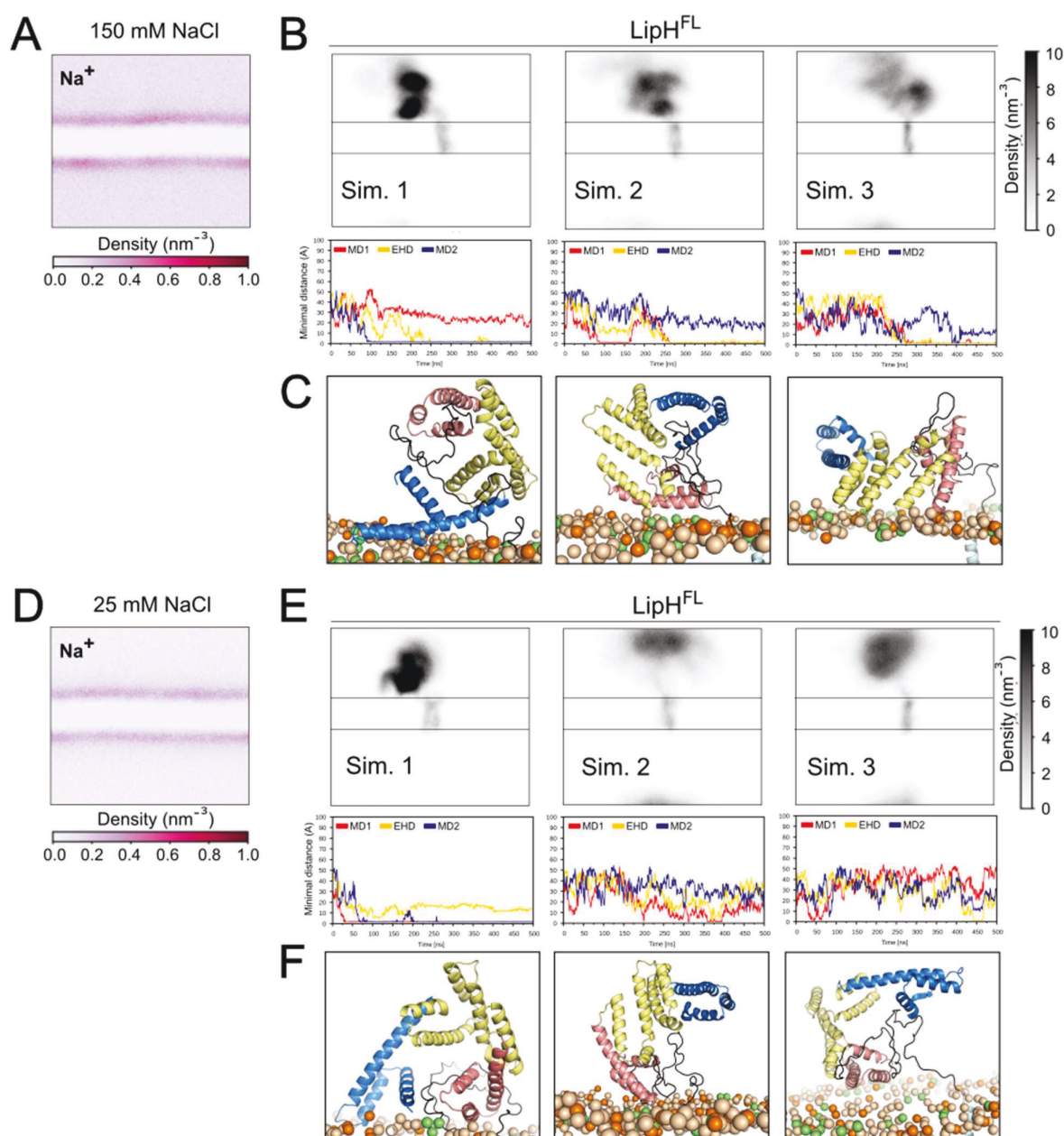


Figure S10. LipH interactions with the membrane interface.

(A) Exemplary density map of sodium ions at 150 mM NaCl (derived from simulation #1) highlights the lipid membrane interface.

(B) Density maps of LipH^{FL} localization within the simulation box calculated for individual simulations at 150 mM NaCl. The lipid bilayer borders are indicated as lines. Below: Corresponding minimal distances of the individual LipH domains to the membrane over the course of each simulation.

(C) A selection of poses taken by LipH at the membrane interface in simulations at 150 mM NaCl.

(D-F) Same as **(A-C)** upon simulations at 25 mM NaCl.

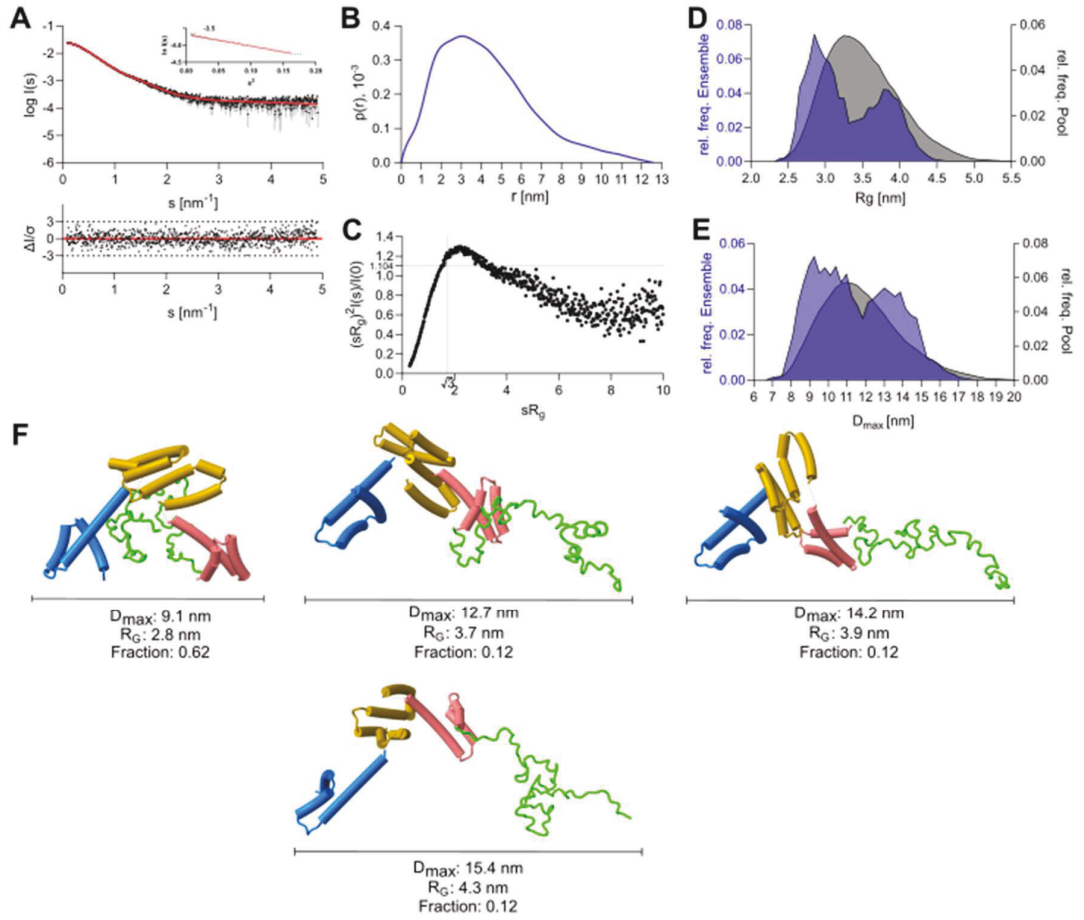


Figure S11. Small-angle X-ray scattering analysis of LipH^{VD}.

(A) Experimental data are shown in black dots, with grey error bars. The EOM model fit is shown as red line; below is the residual plot of the data. The Guinier plot of LipH^{VD} is shown in the inset.

(B) The pair distance distribution function $p(r)$ of LipH^{VD} as determined by SAXS.

(C) Dimensionless Kratky plots of LipH^{VD}.

(D) Relative frequency distribution against the R_g of the EOM model. The selected ensemble is shown in blue, while the relative frequency of the initial pool is shown in grey.

(E) Relative frequency distribution of the D_{max} among the EOM model. The selected ensemble is shown in blue, while the relative frequency of the initial pool is shown in grey.

(F) Selected EOM models with their corresponding D_{max} and R_g values and the fractions within the ensemble.

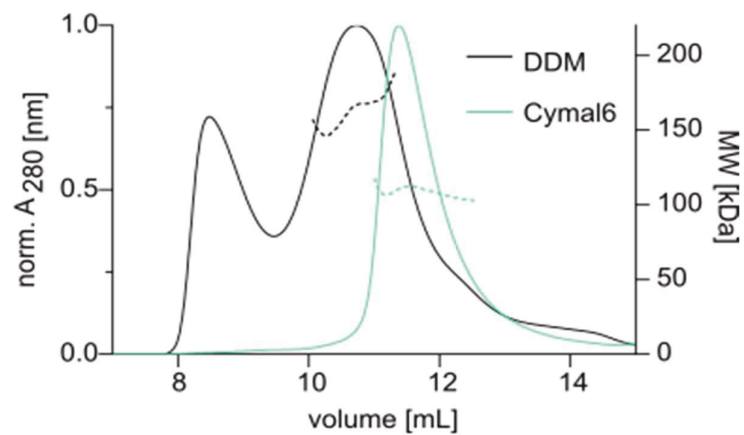


Figure S12. SEC-MALS of the full-length LipH in DDM and Cymal-6 micelles. Normalized UV absorbance of LipH^{FL} in DDM (black) and Cymal-6 (petrol) is shown as solid lines. Dashed lines show the molecular weights in kDa determined by MALS (right Y-axis). Peak at ~8.5 mL for DDM-based sample likely reflected partial aggregation of the protein absence of glycerol, and it was excluded from analysis by fitting the chromatogram within the range of 10 to 12 mL.

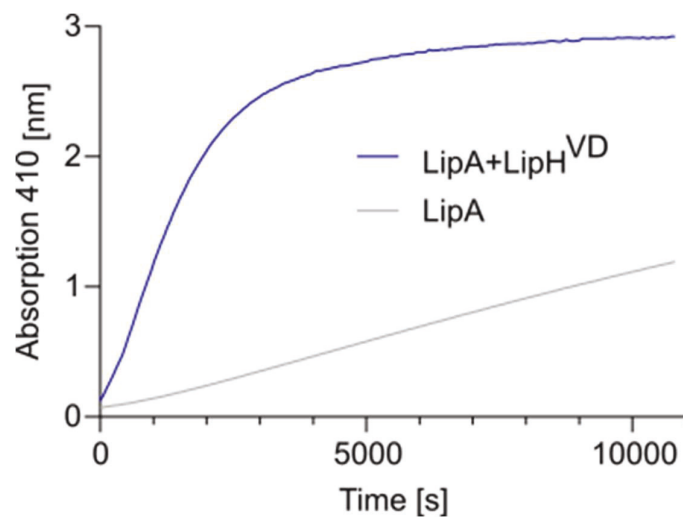


Figure S13: *In vitro* analysis of LipH-mediated enzymatic activity of LipA.

The lipase activity induced by LipH was monitored via hydrolysis of *p*-nitrophenyl butyrate to *p*-nitrophenolate and butyric acid, measured as an increase in absorbance at 410 nm. The substrate hydrolysis is shown in the presence of the chaperone LipH^{VD} (blue) and in its absence (grey).

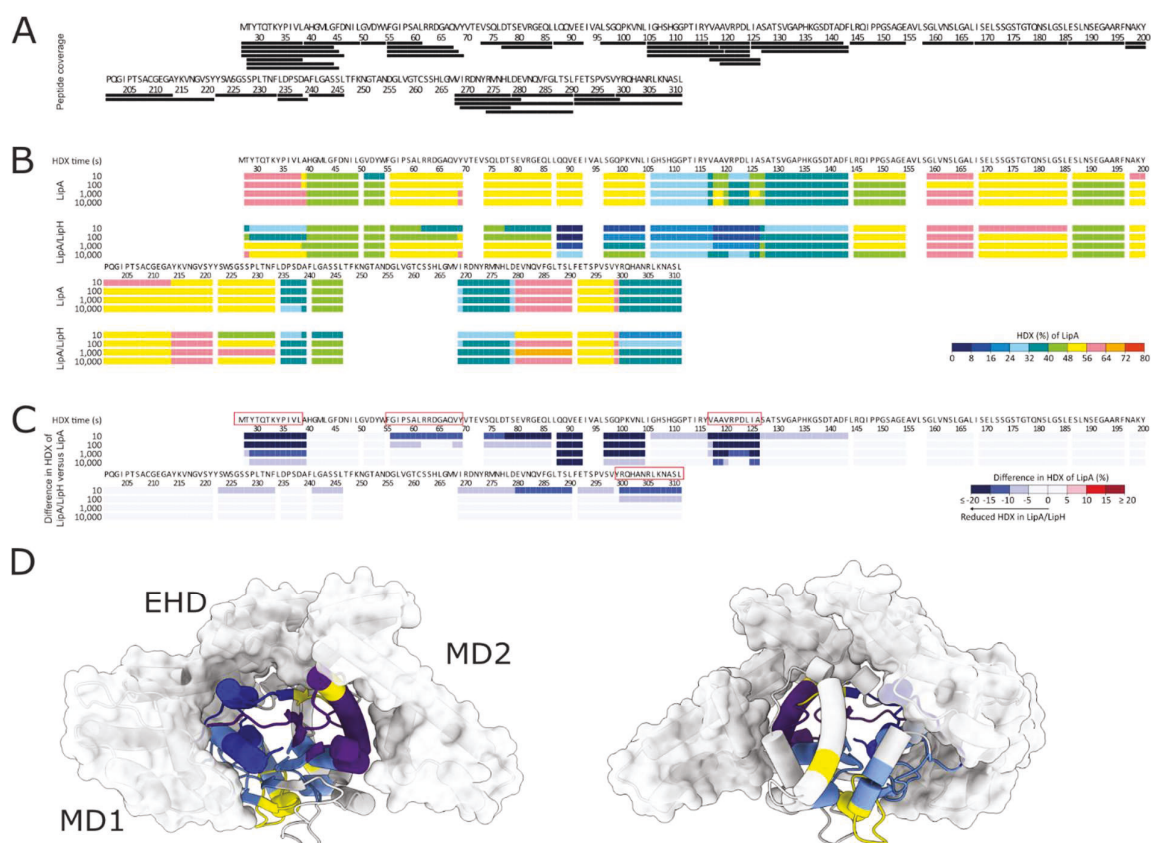


Figure S14. Hydrogen/deuterium exchange mass spectrometry of LipA.

(A) Overview of LipA peptides identified in HDX-MS experiments. Each black bar represents an identified peptide. In total, 49 peptides spanning 89.5% of the LipA^{F144E} amino acid sequence were analyzed for their H/D exchange.

(B) The residue-specific HDX of individual LipA and of LipA in presence of LipH^{Chap}, color-coded from 0% (blue) to 80% (red).

(C) The difference in the residue-specific HDX of LipA in presence of LipH^{Chap} and individual LipA is color-coded from ≤-20% (blue) to ≥20% (red). Red boxes indicate areas of bimodality in HDX (see also Suppl. Figure 15).

(D) Differences in the residue-specific HDX levels plotted on LipA structure in complex with LipH^{Chap}. The major HDX decrease is observed within the N-terminal fragment of LipA which interacts with MD2 of the chaperone.

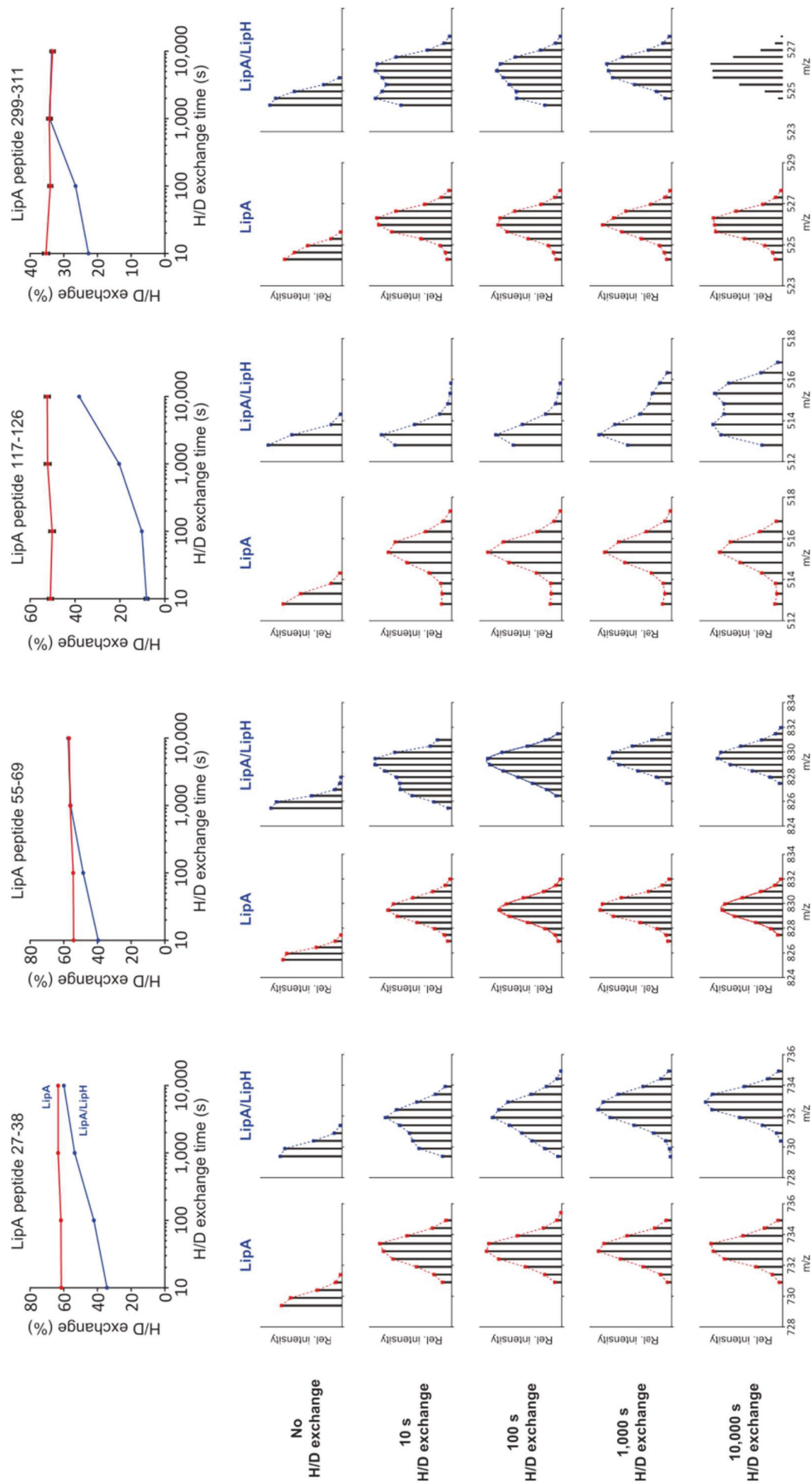


Figure S15. HDX behaviour of representative LipA peptides. *Top:* HDX over time observed for the indicated peptides of samples containing either LipA in isolation (red) or the LipA/LipH complex (blue). Data represent mean $\pm$ s.d. of $n=3$ technical replicates. *Bottom:* Mass spectra (depicted as ion sticks) of those representative LipA peptides over the HDX time course for either sample.

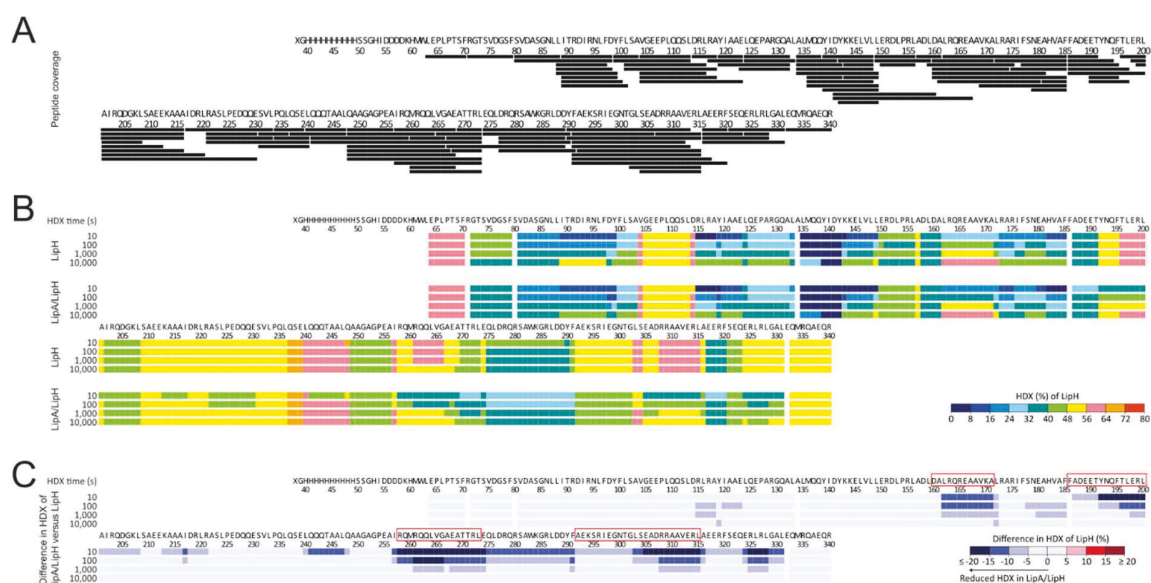


Figure S16. Hydrogen/deuterium exchange mass spectrometry of LipH^{Chap}.

(A) Overview of LipH^{Chap} peptides identified in HDX-MS experiments. Each black bar represents a peptide of LipH^{Chap}. In total, 126 peptides spanning 91.7% of the LipH^{Chap} amino acid sequence were analyzed for their H/D exchange.

(B) The residue-specific HDX of individual LipH^{Chap} and of LipH^{Chap} in presence of LipA is color-coded from 0% (blue) to 80% (red).

(C) The difference in residue-specific HDX of LipH^{Chap} in presence of LipA and individual LipH^{Chap} is color-coded from ≤-20% (blue) to ≥20% (red). Red boxes indicate areas of bimodality in HDX (see also Suppl. Figure 17).

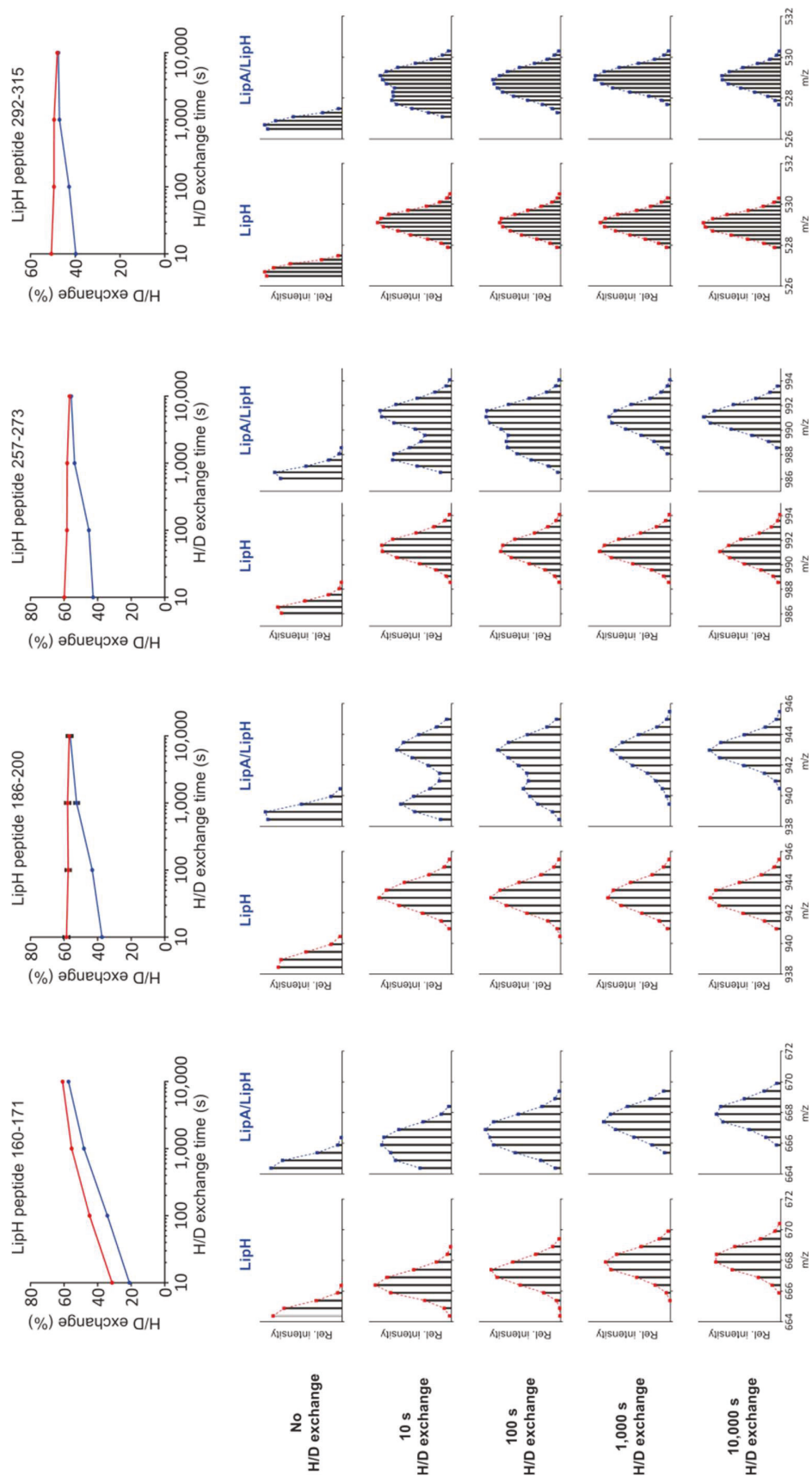


Figure S17. HDX behaviour of representative LipH^{Chap} peptides. *Top:* HDX over time observed for the indicated peptides of samples containing either LipH in isolation (red) or the LipA/LipH complex (blue). Data represent mean $\pm$ s.d. of $n=3$ technical replicates. *Bottom:* Mass spectra (depicted as ion sticks) of those representative LipH peptides over the HDX time course for either sample. For both LipA and LipH proteins two distinct species are apparent in the LipA/LipH complex samples from the mass spectra (**B**) likely due to an equilibrium between unbound and complex-bound LipA or LipH, respectively.

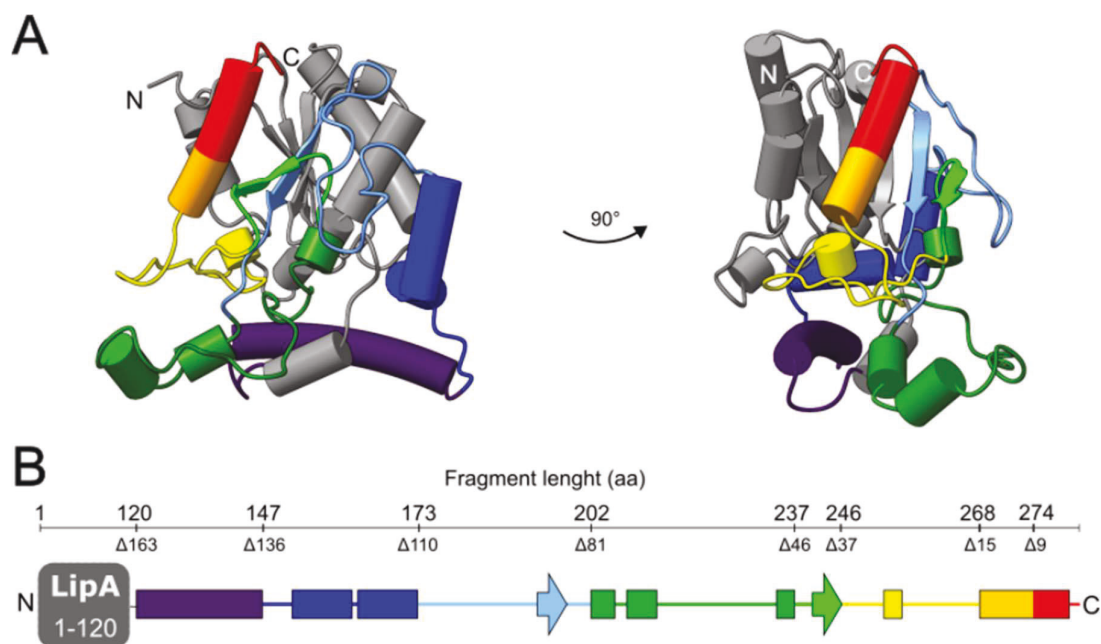


Figure S18. Design of LipA fragments for studying interactions with LipH.

(A) AlphaFold3 model of *P. aeruginosa* LipA with individual deletions highlighted using a rainbow color gradient (red to purple).

(B) Individual deletions were designed based on the secondary structure of LipA. α -Helices are indicated as bars, β -strands as arrows. The lengths of the designed fragments are indicated on the upper axis, the sizes of the corresponding C-terminal deletions are indicated below. The color-coding of the deleted fragments corresponds to that in the panel **(A)**.

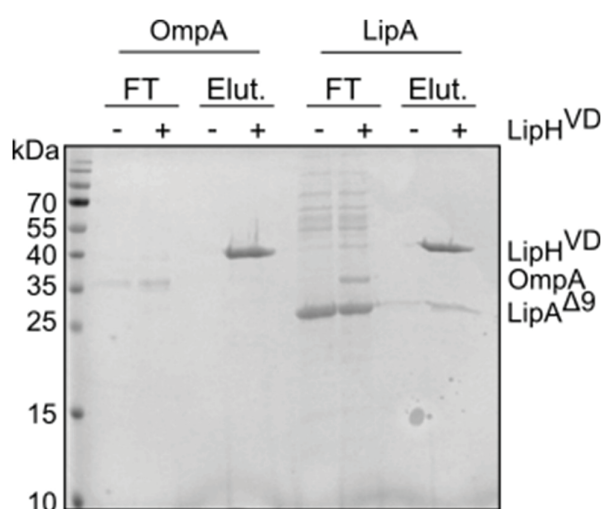


Figure S19. Co-elution assay to probe non-specific interactions of LipH.

SDS-PAGE of the assay to test unspecific interactions of LipH^{VD} using the outer membrane protein A (OmpA) of *E. coli* as a client. Elution fractions do not show a band for OmpA in presence or absence of LipH^{VD}, suggesting absence of interactions. The lipase fragment LipA^{Δ9} is used as a positive reference.

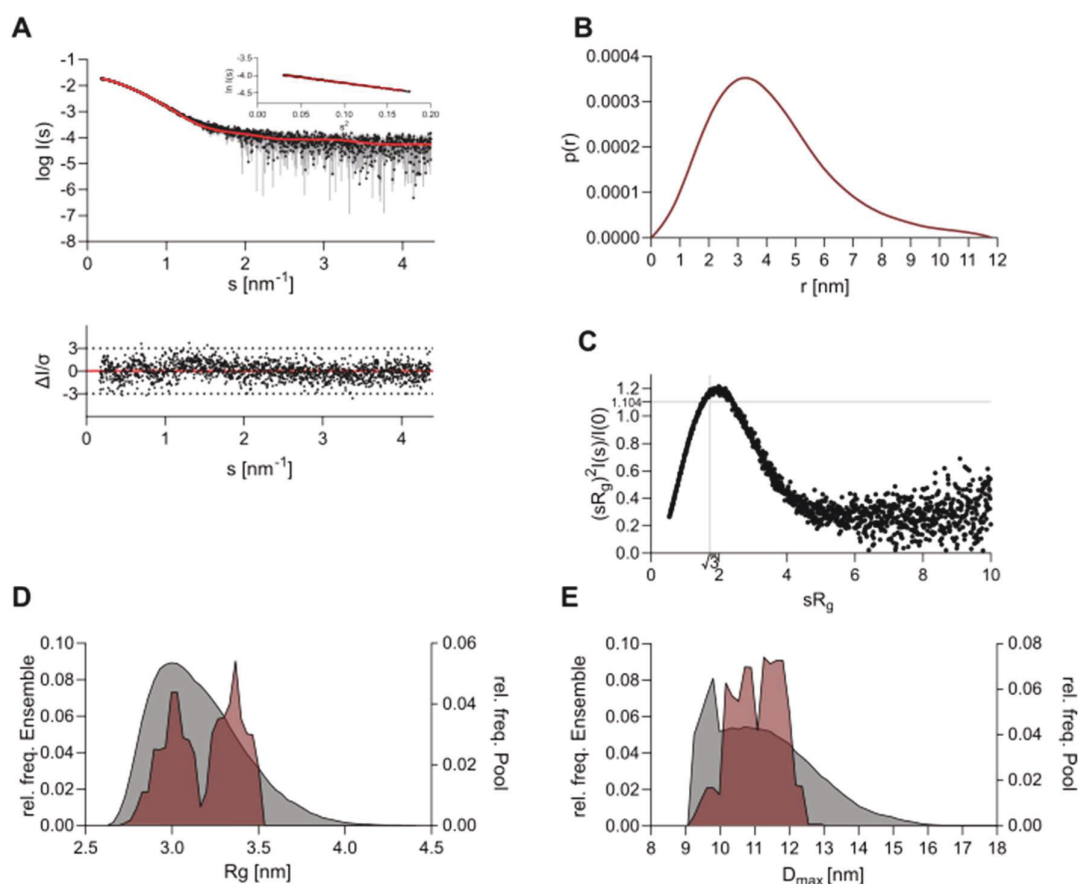


Figure S20. Small-angle X-ray scattering analysis of the LipH^{VD}:LipA complex.

(A) Experimental data are shown in black dots, with grey error bars. The EOM ensemble fit is shown as red line; below is the residual plot of the data. The Guinier plot of LipH^{VD}:LipA is shown in the inset.

(B) The pair distance distribution function $p(r)$ of LipH^{VD}:LipA complex as determined by SAXS.

(C) Dimensionless Kratky plots of LipH^{VD}:LipA complex.

(D) Relative frequency distribution against the R_g of the EOM model. The ensemble is shown in brown, while the relative frequency of the pool is shown in grey.

(E) Relative frequency distribution against the D_{max} of the EOM model. The ensemble is shown in brown, while the relative frequency of the pool is shown in grey.

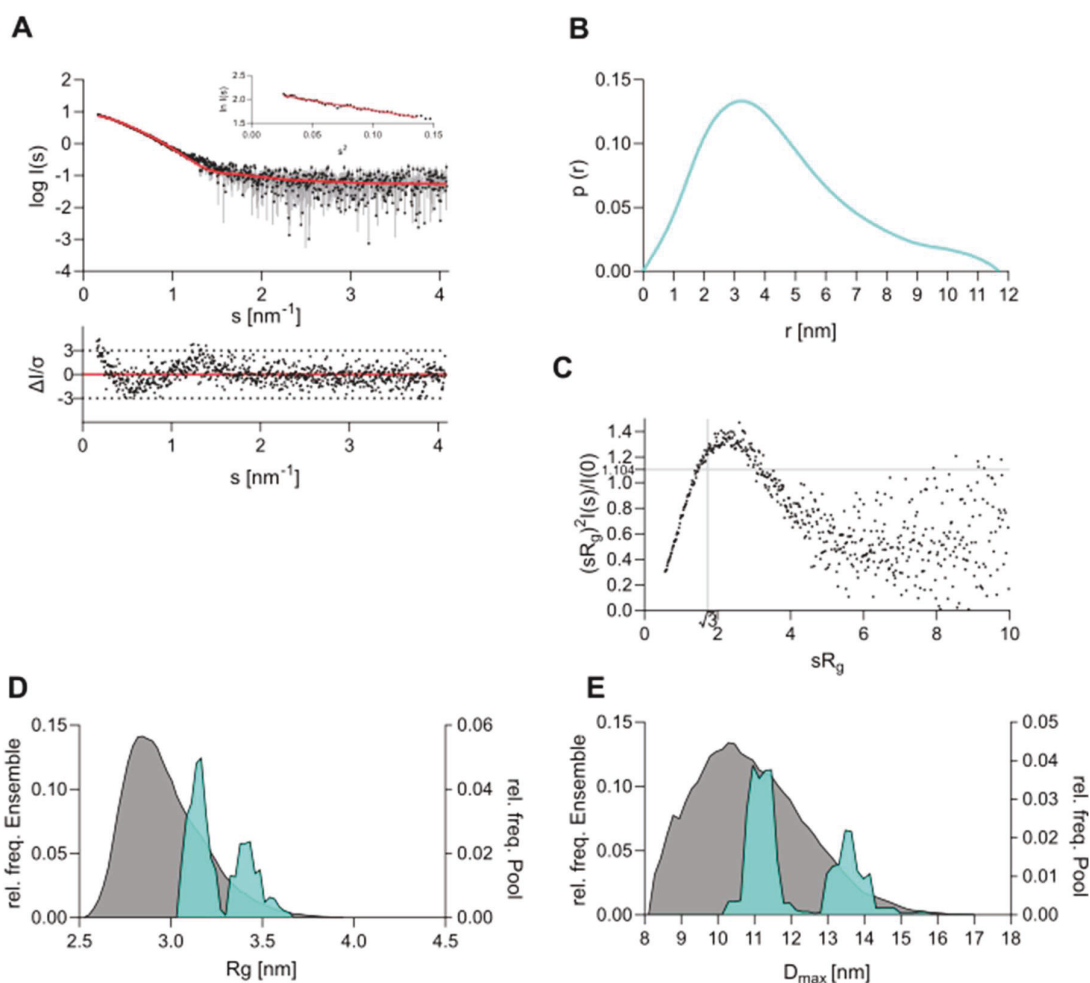


Figure S21. Small-angle X-ray scattering analysis of the LipH^{VD}:LipA^{Δ81} complex.

(A) Experimental data are shown in black dots, with grey error bars. The EOM ensemble fit is shown as red line; below is the residual plot of the data. The Guinier plot of LipH^{VD}:LipA^{Δ81} is shown in the inset.

(B) The pair distance distribution function $p(r)$ of LipH^{VD}:LipA^{Δ81} complex as determined by SAXS.

(C) Dimensionless Kratky plots of LipH^{VD}:LipA^{Δ81} complex.

(D) Relative frequency distribution against the R_g in nm of the EOM model. The ensemble is shown in cyan, while the relative frequency of the pool is shown in grey.

(E) Relative frequency distribution against the D_{max} in nm of the EOM model. The ensemble is shown in cyan, while the relative frequency of the pool is shown in grey.

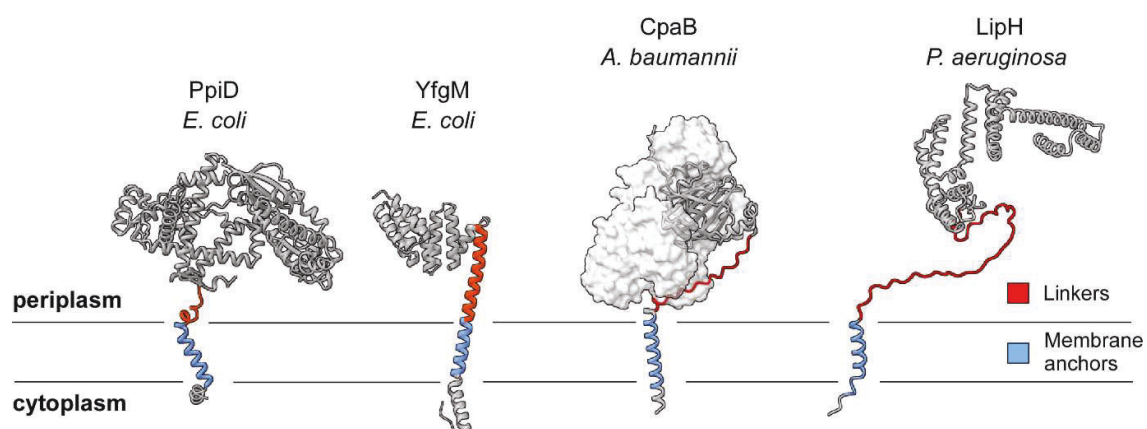


Figure S22. AlphaFold3-based models of the membrane-anchored chaperones. Membrane anchors are predicted based on the hydrophobicity via TMHMM. For *A. baumannii* CpaB chaperone, its cognate client protease CpaB is shown (surface visualization).

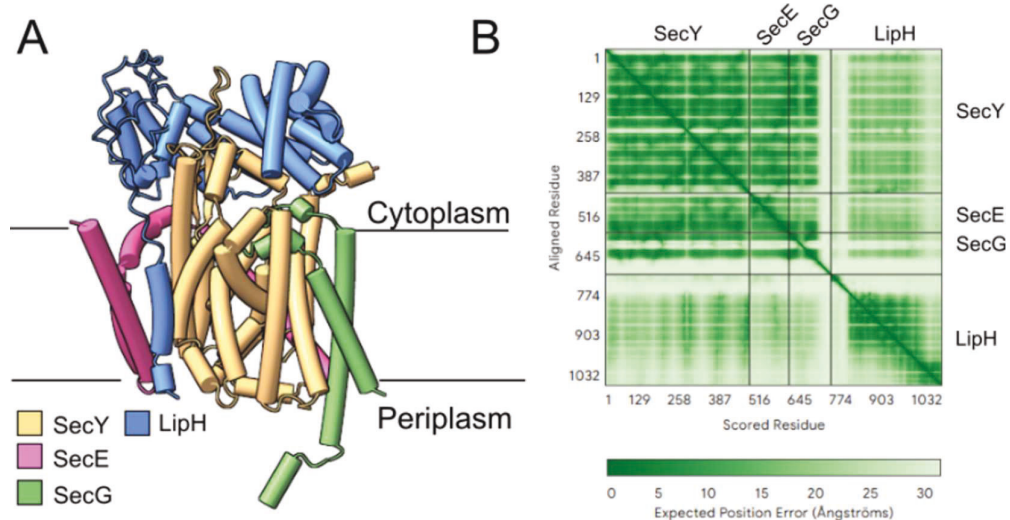


Figure S23. AlphaFold 3 analysis of a potential SecYEG:LipH^{FL} complex.

(A) AlphaFold3 model for the SecYEG:LipH^{FL} complex of *P. aeruginosa* (ipTM score 0.59).

(B) Predicted aligned error plot for the SecYEG:LipH^{FL} complex of *P. aeruginosa*.

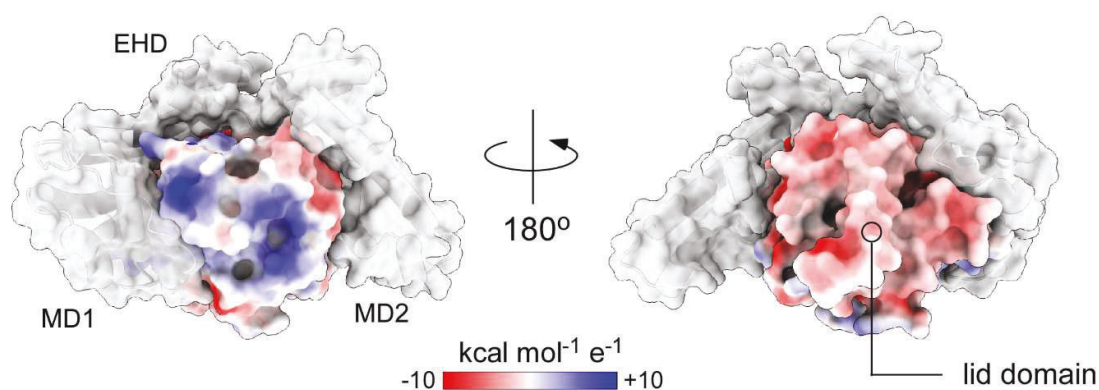


Figure S24. The electrostatic potential at the surface of the folded LipA. The molecular surface of LipA is coloured according to the local electrostatic potentials (blue = cationic; red = anionic; the scale bar shown below). The chaperoning domain of LipH is shown as a semi-transparent grey surface, with positions of the structural sub-domains indicated.

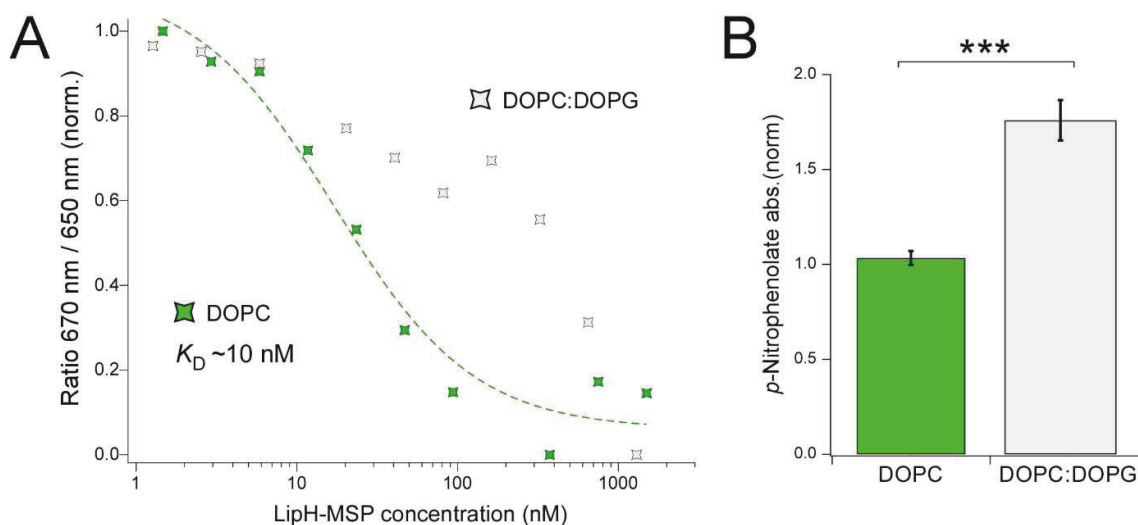


Figure S25. Lipid-dependent activity of LipH^{FL} in nanodiscs.

(A) Exemplary recordings of the spectral shift in LipA-CF647 fluorescence upon titrating LipH^{FL} reconstituted into nanodiscs with either DOPC or DOPC:DOPG lipids. Normalized ratios of LipA-CF647 fluorescence intensity at 670 nm and 650 nm are plotted against the LipH-MSP concentration. The apparent dissociation constant could be estimated for DOPC-based sample, but not for DOPC:DOPG sample due to the low affinity.

(B) Normalized lipase activity *in vitro* mediated by LipH^{FL} in presence of different lipids. Mean values are shown as bars, the error bars correspond to the standard deviations, calculated from three technical replicates. p-Value determined via one-way ANOVA test is 0.00036.

Table S1. Summary of SAXS data acquisition and analysis.

Data collection parameters			
SAXS Device	Xenocs Xeuss 2.0 with Q-Xoom	P12, PETRA III, DESY Hamburg (Blanchet et al. 2015)	BM29, ESRF Grenoble (Tully et al. 2023)
Detector	PILATUS 3 R 300K windowless	PILATUS 6 M	PILATUS3 x 2 M
Detector distance (m)	0.550	3.0	2.827
Beam size	0.8 mm x 0.8 mm	120 μm x 200 μm	200 μm x 100 μm
Wavelength (nm)	0.154	0.124	0.099
Sample environment	Low Noise Flow Cell, 1 mm ø	Quartz glass capillary, 1 mm ø	
Absolute scaling method	Comparison with scattering from pure H ₂ O		
Normalization	To transmitted intensity by beam-stop counter or direct beam		
Scattering intensity scale	Absolute scale, cm ⁻¹		
s range (nm ⁻¹) [†]	0.05 – 5.5	0.03 – 7.0	0.025–5.5
Sample	LipH ^{VD}	LipH ^{VD} :LipA complex	LipH ^{VD} :LipA ^{Δ81} complex
Organism	<i>Pseudomonas aeruginosa</i> PAO1		
UniProt ID	Q01725	LipH: Q01725 LipA: P26876	LipH: Q01725 LipA: P26876
Mode of measurement	batch	Online SEC-SAXS	
SEC-Column	-	Superdex 200 Increase 10/300 GL	
Flowrate (mL/min)	-	0.6	0.6
Injection volume (μL)	-	100	100
Temperature (°C)	10	20	20
Exposure time s (# frames)	600 (24)	0.995 (2400)	2 (1200)
# frames used for averaging	20	24	18
Protein buffer	50 mM Tris, 100 mM NaCl, 100 μM TCEP, 5% glycerol, pH 8.0	5 mM Tris, 5 mM glycine, 1 mM CaCl ₂ , 5% glycerol, pH 9.0	5 mM Tris, 5 mM glycine, 1 mM CaCl ₂ , 5% glycerol, pH 9.0
Protein concentration [mg/mL]	11.82	8 mg/mL LipH ^{VD} + 0.8 mg/mL LipA ^{FL}	1 mg/mL LipH ^{VD} + LipA ^{Δ81}
Structural parameters			
Guinier Analysis (PRIMUS)			
I(0) ± s (cm ⁻¹)	0.025 ± 0.0001	0.021 ± 0.00004	8.834 ± 0.072
R _g ± s (nm)	3.22 ± 0.022	3.14 ± 0.001	3.51 ± 0.043
s-range (nm ⁻¹)	0.088 – 0.403	0.173 – 0.411	0.163 – 0.369
min < sR _g < max limit	0.282 – 1.297	0.544 – 1.289	0.572 – 1.294
Data point range	1 – 55	1 – 85	1 – 43
Linear fit assessment (R ²)	0.990	0.996	0.972

<i>PDDF/P® Analysis (GNOM)</i>			
$I(0) \pm s \text{ (cm}^{-1}\text{)}$	0.026 ± 0.0001	0.021 ± 0.00003	8.874 ± 0.058
$R_g \pm s \text{ (nm)}$	3.41 ± 0.026	3.26 ± 0.0095	3.53 ± 0.028
$r_{\max} \text{ (nm)}$	12.61	11.82	11.70
Porod volume (nm ³)	69.97	110.09	103.55
$s\text{-range (nm}^{-1}\text{)}$	0.088 – 4.895	0.173 – 4.360	0.163 – 4.081
$\chi^2 / \text{CorMap P-value}$	1.015 / 0.553	1.003 / 0.087	1.069 / 0.092
Molecular mass (kDa)			
From $I(0)$	34.62	n.d.	n.d.
From Qp (Porod 1951)	40.03	69.34	63.04
From MoW2 (Fischer et al. 2010)	34.20	68.07	51.40
From Vc (Rambo and Tainer 2013)	38.87	66.58	55.40
Bayesian Inference (Hajizadeh et al. 2018)	37.70	67.08	58.15
From sequence	38.32	68.46 (1:1 stoichiometry)	59.61 (1:1 stoichiometry)
Flexibility ensemble modelling			
EOM			
Symmetry	P1	P1	P1
$s\text{-range for fit (nm}^{-1}\text{)}$	0.088 – 4.895	0.173 – 4.360	0.163 – 4.081
χ^2 , CorMap $P\text{-value}$	1.026 / 0.553	1.136 / 0.166	1.528 / 0.000003
SASBDB accession codes (Kikhney et al. 2020)			
	SASDYM9	SASDYN9	SASDYP9
Software			
ATSAS Software Version (Manalastas-Cantos et al. 2021)	3.0.5		
Primary data reduction	CHROMIXS (Panjkovich and Svergun 2018)/ PRIMUS (Konarev et al. 2003)		
Data processing	GNOM (Svergun 1992)		
Flexibility ensemble modelling	EOM (Bernado et al. 2007; Tria et al. 2015)		
Statistic goodness-of-fit test	χ^2 , CorMap (Franke, Jeffries, and Svergun 2015)		
Model visualization	Chimera X 1.10 (Goddard et al. 2018)		

$s = 4\pi \sin(\theta)/\lambda$, 2θ – scattering angle, n.d. not determined

Table S2. Overview of data obtained by hydrogen/deuterium exchange mass spectrometry.

Protein	LipA ^{F144E}	LipH ^{Chap}
Conditions of H/D exchange	25 °C in 5 mM Tris-HCl pH 9.0, 5 mM glycine, 1 mM CaCl ₂ Final D ₂ O during HDX = 89.5% (v/v)	
Time course of H/D exchange	10/100/1,000/10,000 s	
Samples	1) 50 µM LipA ^{F144E} (5 µM during HDX) 2) 50 µM LipH ^{Chap} (5 µM during HDX) 3) 50 µM LipA ^{F144E} + 50 µM LipH ^{Chap} (5 µM during HDX)	
Replicates	3 technical replicates (separate H/D exchange reactions)	
Number of Peptides	49	126
Average peptide length (aa)	10.86	11.44
Sequence coverage (%)	89.5	91.7
Redundancy	2.47	6.09
Back-exchange	No correction for back-exchange	
Repeatability (average SD)	0.06 Da / 0.47%	0.07 Da / 0.62%
Significance criterium applied	5% difference in relative HDX	

Supplemental references

- Bernado, P., E. Mylonas, M. V. Petoukhov, M. Blackledge, and D. I. Svergun. 2007. 'Structural characterization of flexible proteins using small-angle X-ray scattering', *J Am Chem Soc*, 129: 5656-64.
- Blanchet, C. E., A. Spilotros, F. Schwemmer, M. A. Graewert, A. Kikhney, C. M. Jeffries, D. Franke, D. Mark, R. Zengerle, F. Cipriani, S. Fiedler, M. Roessle, and D. I. Svergun. 2015. 'Versatile sample environments and automation for biological solution X-ray scattering experiments at the P12 beamline (PETRA III, DESY)', *J Appl Crystallogr*, 48: 431-43.
- Fischer, H., M. D. Neto, H. B. Napolitano, I. Polikarpov, and A. F. Craievich. 2010. 'Determination of the molecular weight of proteins in solution from a single small-angle X-ray scattering measurement on a relative scale', *Journal of Applied Crystallography*, 43: 101-09.
- Franke, D., C. M. Jeffries, and D. I. Svergun. 2015. 'Correlation Map, a goodness-of-fit test for one-dimensional X-ray scattering spectra', *Nat Methods*, 12: 419-22.
- Goddard, T. D., C. C. Huang, E. C. Meng, E. F. Pettersen, G. S. Couch, J. H. Morris, and T. E. Ferrin. 2018. 'UCSF ChimeraX: Meeting modern challenges in visualization and analysis', *Protein Sci*, 27: 14-25.
- Hajizadeh, N. R., D. Franke, C. M. Jeffries, and D. I. Svergun. 2018. 'Consensus Bayesian assessment of protein molecular mass from solution X-ray scattering data', *Sci Rep*, 8: 7204.
- Kikhney, A. G., C. R. Borges, D. S. Molodenskiy, C. M. Jeffries, and D. I. Svergun. 2020. 'SASBDB: Towards an automatically curated and validated repository for biological scattering data', *Protein Sci*, 29: 66-75.
- Konarev, P. V., V. V. Volkov, A. V. Sokolova, M. H. J. Koch, and D. I. Svergun. 2003. 'a Windows PC-based system for small-angle scattering data analysis', *J Appl Crystallogr*, 36: 1277-82.
- Manalastas-Cantos, K., P. V. Konarev, N. R. Hajizadeh, A. G. Kikhney, M. V. Petoukhov, D. S. Molodenskiy, A. Panjkovich, H. D. T. Mertens, A. Gruzinov, C. Borges, C. M. Jeffries, D. I. Svergun, and D. Franke. 2021. 'ATSAS 3.0: expanded functionality and new tools for small-angle scattering data analysis', *J Appl Crystallogr*, 54: 343-55.
- Panjkovich, A., and D. I. Svergun. 2018. 'CHROMIXS: automatic and interactive analysis of chromatography-coupled small-angle X-ray scattering data', *Bioinformatics*, 34: 1944-46.
- Porod, G. 1951. 'Die Röntgenkleinwinkelstreuung Von Dichtgepackten Kolloiden Systemen .1.', *Kolloid-Zeitschrift and Zeitschrift Fur Polymere*, 124: 83-114.
- Rambo, R. P., and J. A. Tainer. 2013. 'Accurate assessment of mass, models and resolution by small-angle scattering', *Nature*, 496: 477-81.
- Svergun, D. I. 1992. 'Determination of the Regularization Parameter in Indirect-Transform Methods Using Perceptual Criteria', *J Appl Crystallogr*, 25: 495-503.
- Tria, G., H. D. Mertens, M. Kachala, and D. I. Svergun. 2015. 'Advanced ensemble modelling of flexible macromolecules using X-ray solution scattering', *Iucrj*, 2: 207-17.
- Tully, M. D., J. Kieffer, M. E. Brennich, R. Cohen Aberdam, J. B. Florial, S. Hutin, M. Oscarsson, A. Beteva, A. Popov, D. Moussaoui, P. Theveneau, G. Papp, J. Gigmes, F. Cipriani, A. McCarthy, C. Zubietta, C. Mueller-Dieckmann, G. Leonard, and P. Pernot. 2023. 'BioSAXS at European Synchrotron Radiation Facility - Extremely Brilliant Source: BM29 with an upgraded source, detector, robot, sample environment, data collection and analysis software', *J Synchrotron Radiat*, 30: 258-66.

4. SecYEG/YidC Mediated Membrane Protein Biogenesis

4.1 Substrate-induced assembly and functional mechanism of the bacterial membrane protein insertase SecYEG-YidC

Authors: Max Busch, Cristian Rosales Hernandez, Michael Kamel, Yulia Schaumkessel, Eli van der Sluis, Otto Berninghausen, Thomas Becker, Roland Beckmann, Alexej Kedrov

Published: The EMBO Journal

Contribution: **Max Busch:** Data curation; Formal analysis; Investigation; Visualization; Writing – original draft; Writing – review and editing

Cristian Rosales Hernandez: Data curation; Formal analysis; Investigation; Visualization; Methodology; Writing – original draft; Writing – review and editing

Michael Kamel: Conceptualization; Formal analysis; Investigation; Methodology; Writing – review and editing

Yulia Schaumkessel: Investigation; Methodology; Writing – review and editing

Eli van der Sluis: Resources; Supervision; Investigation; Methodology; Writing – review and editing

Otto Berninghausen: Data curation; Validation; Investigation; Writing – review and editing

Thomas Becker: Data curation; Formal analysis; Supervision; Visualization; Writing – review and editing

Roland Beckmann: Conceptualization; Funding acquisition; Project administration; Writing – review and editing

Alexej Kedrov: Conceptualization; Supervision; Funding acquisition; Visualization; Methodology; Writing – original draft; Project administration; Writing – review and editing

Substrate-induced assembly and functional mechanism of the membrane protein insertase SecYEG-YidC

Max Busch^{1,8}, Cristian Rosales-Hernandez^{2,4,8}, Michael Kamel^{1,5,8}, Yulia Schaumkessel^{1,6}, Eli O van der Sluis^{1,7}, Otto Berninghausen², Thomas Becker², Roland Beckmann²✉ & Alexej Kedrov^{1,3}✉

Abstract

The Sec translocon and the YidC/Oxa1-type insertases universally mediate biogenesis of α -helical membrane proteins, but the molecular basis of their cooperation has remained disputed. Recent discovery of multi-subunit insertases assembled at the back of the translocon in fungi and higher eukaryotes has raised questions about the architecture and mechanism of the putative bacterial ortholog SecYEG-YidC. Here, we combine cryogenic electron microscopy with cell-free protein synthesis to visualize biogenesis of the SecYEG/YidC-dependent multipass membrane protein Nuok. The nascent chain of Nuok does not enter the lateral gate of SecYEG but instead crosses the translocon towards its back side, where YidC is recruited in the nascent substrate-dependent manner. The SecY-YidC interface promotes folding of the transmembrane helices before insertion, consistent with thermodynamic principles of membrane protein folding. YidC forms extensive contacts with the nascent chain, suggesting its key role in the insertion event. These findings provide mechanistic insight into membrane protein insertases, support evolutionary conservation of a gate-independent insertion route, and expand current models of membrane protein biogenesis.

Subject Categories Membranes & Trafficking; Structural Biology; Translation & Protein Quality

<https://doi.org/10.1038/s44318-026-00837-6>

Received 24 January 2026; Revised 5 May 2026;

Accepted 22 May 2026

Published online: 26 June 2026

Introduction

Integral membrane proteins (IMPs) are encoded by ~30% protein-coding genes in each organism and they determine the functionality

of the cellular membranes (von Heijne, 2007). Biogenesis of α -helical IMPs at the cytoplasmic membrane of bacteria and the endoplasmic reticulum (ER) in eukaryotes typically occurs in a co-translational manner: The nascent transmembrane helices (TMHs) emerging from the ribosome are identified by the signal recognition particle (SRP) and delivered in the form of ribosome-nascent chain complexes (RNCs) to the universally conserved Sec translocon that facilitates their insertion in the lipid bilayer (Cymer et al, 2015b; Itskanov and Park, 2023). The translocon of *Escherichia coli* is a heterotrimeric complex composed of SecY, SecE and SecG proteins forming a central pore. N- and C-terminal halves of the central subunit SecY may open laterally in a clam-shell manner, resulting in a lipid-exposed gap between the opposing TMHs 2b and 7, located at the front side of the translocon. This gap, commonly referred as the “lateral gate”, has been seen as the exclusive site for the nascent TMHs to partition into the lipid phase, both for bacterial SecYEG and the homologous Sec61a β in eukaryotes. This model has been supported by extensive biochemical studies (du Plessis et al, 2009; Sachelararu et al, 2013) and via structural insights, commonly focused on signal peptides of secretory proteins and single-pass IMPs exposing their N-terminal ends into the cytoplasm (Jomaa et al, 2016; Kater et al, 2019; Voorhees and Hegde, 2016). However, multipass IMPs have their TMHs in alternating topology, suggesting higher complexity within the biogenesis pathways (Bischoff et al, 2014; Ou et al, 2025).

In bacteria, biogenesis of multiple IMPs relies on a cooperative action of SecYEG and the essential and ubiquitous membrane protein insertase YidC (Steinberg et al, 2018; Zhu et al, 2013). To facilitate folding and membrane insertion of the nascent IMPs, YidC forms a polar water-filled groove in the cytoplasmic lipid leaflet, and its unusually short TMHs 3, 4 and 5 may distort the lipid bilayer (Chen et al, 2017; McDowell et al, 2021). The groove between TMHs 3 and 5 in the core of the membrane serves for insertion of the client IMPs (Kedrov et al, 2016; Wickles et al, 2014). Extensive intermolecular cross-linking studies suggested that YidC is located in front of the lateral gate of SecYEG where it could access the nascent TMHs emerging from the lateral gate (Jaus et al,

¹Synthetic Membrane Systems, Institute of Biochemistry, Heinrich Heine University Düsseldorf, Düsseldorf 40225, Germany. ²Gene Center Munich, Ludwig Maximilian University Munich, Munich 81377, Germany. ³Interfaculty Center for Membrane Research, Heinrich Heine University Düsseldorf, Düsseldorf 40225, Germany. ⁴Present address: Max Planck Institute of Biophysics, Frankfurt/Main, Germany. ⁵Present address: Dept. Biology, Osnabrück University, Osnabrück, Germany. ⁶Present address: Institute of Biochemistry and Molecular Biology I, Heinrich Heine University Düsseldorf, Düsseldorf, Germany. ⁷Present address: Kavli Institute of Nanosciences, Delft University of Technology, Delft, the Netherlands. ⁸These authors contributed equally: Max Busch, Cristian Rosales-Hernandez, Michael Kamel. ✉E-mail: Beckmann@genzentrum.lmu.de; Kedrov@hhu.de

2019; Petriman et al, 2018; Sachelaru et al, 2013), and the model was further supported by low-resolution cryo-electron microscopy (cryo-EM) analysis (Botte et al, 2016). Remarkably, the AlphaFold algorithm confidently predicts a very different architecture for the machinery of *E. coli*, with YidC placed at the back of SecYEG, and the docking is barely affected by presence of other SecYEG-associated proteins, such as SecDF and PpiD-YfgM (Fig. EV1; Appendix Fig. S1) (Miyazaki et al, 2022; Tsukazaki, 2018). Nearly identical SecYEG-YidC assemblies are predicted for other γ -proteobacteria, including pathogens *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, while diverse architectures are proposed for species from other bacterial clades (Appendix Fig. S1D; Appendix Table S1, Dataset EV2). Notably, the confidence of the models beyond γ -proteobacteria is low, suggesting that other factors, such as accessory proteins, specific lipids, but also the bound ribosome and the nascent chain may be required for the complex assembly.

Recent biochemical and high-resolution cryo-EM studies of the eukaryotic insertase machinery revealed that the YidC homolog TMCO1 was located at the back of the Sec61 complex, being a component of the large multi-subunit complex BOS-GEL-PAT, also referred as multipass membrane protein translocon (MPT) (Smalinskaite et al, 2022; Sundaram et al, 2022). Furthermore, insertion of a nascent IMP opsin occurred not via the lateral gate, but between Sec61 and the BOS complex, challenging the long-standing paradigm in the field. Complementary, global ribosome profiling has suggested that the MPT is assembled in a substrate-dependent manner, and that hundreds of eukaryotic multipass IMPs utilize the “back-of-Sec” insertion route (Sundaram et al, 2025). The accumulated controversy between the insights from the eukaryotic homologs and the conventional model of the SecYEG-YidC complex has raised the question about the mechanism of IMP folding in bacteria and the architecture and dynamics of the insertion machinery (Smalinskaite and Hegde, 2023). To tackle this issue, here we employed nanodisc-reconstituted SecYEG-YidC and cell-free protein synthesis (CFPS) to analyze the biogenesis of the bacterial multipass IMP NuoK, the subunit K of the NADH-quinone oxidoreductase that requires both components for the membrane integration and folding in *E. coli* (Price and Driessen, 2010). By means of cryo-EM, we describe the detailed architecture of the RNC-bound SecYEG-YidC complex at different stages of NuoK insertion. YidC is recruited to the back of SecYEG in the substrate-dependent manner thus indeed resembling the settings at the eukaryotic insertase machinery. The nascent chain emerging from the ribosome folds within a hydrophobic pocket formed by SecY loops and then it is routed away from the lateral gate to be inserted between SecYEG and YidC. Overall, the data elucidate the functional architecture of the bacterial co-translational IMP insertion machinery and reveal the evolutionary conservation of the “back-of-Sec” insertion route, thereby offering a new perspective on membrane protein biogenesis in bacteria.

Results

YidC engages the nascent chain of NuoK at a late stage of insertion

Though multiple membrane proteins require both SecYEG and YidC for their insertion and folding, only for few of them the

biogenesis pathways have been investigated in detail. In particular, mass spectrometry of YidC-depleted *E. coli* membranes indicated that NuoK, a subunit of the respiratory complex I (Fig. 1A; Appendix Fig. S2A), is dependent on the insertase (Price and Driessen, 2008). In vitro experiments showed that YidC was required for insertion of NuoK TMH 2 and TMH 3 in proteoliposomes (Fig. 1B) (Price and Driessen, 2010). We questioned whether interactions of YidC with the nascent NuoK occurred in the cellular membrane. We assembled defined co-translational insertion intermediates of NuoK in vivo, where N-terminal fragments of NuoK containing either 48 or 86 residues (NuoK⁴⁸ and NuoK⁸⁶) were followed by a 30-aa-long linker, including haemeagglutinine (HA)-tag and a C-terminal ribosome stalling sequence derived from SecM (here referred to as SecM*; Fig. 1C; Appendix Fig. S1B) (Cymer et al, 2015a; Kempf et al, 2017). With this construct, the SecM* peptide and the linker/tag would occupy the exit tunnel of the stalled ribosome, while two or three NuoK TMHs would be exposed for NuoK⁴⁸ and NuoK⁸⁶, respectively, being accessible for the insertase complex. After expressing the constructs and lysing the cells, the isolated membranes were solubilized with detergent and subjected to centrifugation through sucrose cushion, so only large macromolecular complexes, such as ribosomes and the associated proteins, were expected to be sedimented. Immunodetection confirmed equal amounts of YidC in all membrane preparations, while presence of YidC in the cushion pellets was clearly specific to the nascent chain (Fig. 1D; Appendix Fig. S3D): Only faint signals were detected in absence of NuoK or upon expression of NuoK⁴⁸, while NuoK⁸⁶ strongly promoted recruitment of YidC. Thus, we concluded that YidC engages the nascent NuoK at rather late insertion stage in vivo, which can be recapitulated in a reconstituted system (Price and Driessen, 2010).

SecYEG and YidC-dependent insertion in nanodiscs

While our efforts to visualize the SecYEG-YidC complex in detergent by means of cryo-EM were not successful, possibly due to destabilized protein:protein interactions in absence of lipids, we set out to elucidate its architecture using lipid-based nanodiscs, a well-defined system for studying IMP insertion (Kater et al, 2019; Kedrov et al, 2013; Kedrov et al, 2016; Koch et al, 2018) (Fig. 2A). To co-reconstitute both SecYEG and YidC in the relevant topology and to avoid their stochastic distribution among the nanodiscs, YidC and SecE were genetically fused via a cleavable linker (Appendix Fig. S3A), while the non-essential cationic C-terminal end of YidC was removed to exclude spontaneous electrostatic interactions with the ribosome observed in vitro (Kedrov et al, 2013; Kohler et al, 2009). The fusion protein was expressed and isolated together with SecY and SecG subunits (Fig. 2B and Appendix Fig. S3B). The complex was reconstituted into a sufficiently large nanodisc formed by the scaffold protein MSP2N2 (expected diameter up to 17 nm) (Ritchie et al, 2009) and treated with the HRV-3C protease to release YidC from SecE (Fig. 2C; Appendix Fig. S3C–E). As a result, each nanodisc contained one copy of both SecYEG and YidC, presumably in the correct orientation, while the dimensions of the surrounding lipid bilayer would allow for lateral diffusion of the proteins, assembly of the functional complex and insertion of the nascent TMHs. Supporting this notion, the molecular weight distribution of the nanodisc-

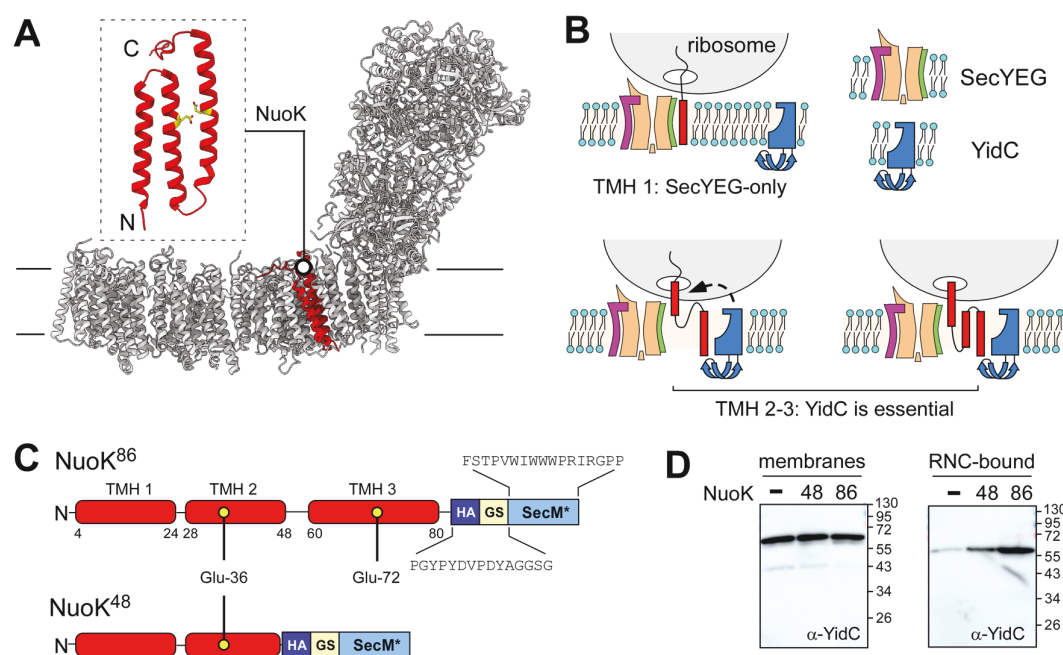


Figure 1. YidC insertase is involved in *E. coli* NuoK biogenesis.

(A) Structure of the *E. coli* NuoK subunit within the assembled respiratory complex I (PDB ID 7NYR). Glu-36 and Glu-72 residues are shown in yellow. (B) Presumable insertion pathway of NuoK. Insertion of NuoK TMH 1 is mediated by SecYEG, while YidC is required for NuoK TMH 2 and TMH 3. (C) Design of the nascent chains mimicking NuoK⁴⁸ and NuoK⁸⁶ insertion intermediates. NuoK TMHs are shown in red with the flanking residue positions indicated. Positions of the glutamate residues within TMHs 2 and 3 are shown as yellow circles. The sequence of the C-terminal linker consisting of the HA-tag (HA) and a glycine-serine linker (GS) is shown for NuoK⁸⁶. SecM* represents a SecM-based ribosome stalling sequence. (D) Immunodetection of YidC in *E. coli* membranes (left) and the ribosome-bound material (right) upon expression of the NuoK insertion intermediates. The NuoK length is indicated above. Source data are available online for this figure.

embedded SecYEG-YidC complexes measured by single-molecule mass photometry peaked at 290 kDa (Fig. 2D), and the difference from the calculated total protein weight of ~225 kDa likely reflected the presence of the co-reconstituted lipids. The lipid membrane consisted of 70 mol % POPC and 30 mol % POPG, thus mimicking the ratio of zwitterionic/anionic lipids and the degree of the acyl chain unsaturation found within the bacterial membrane (Sohlenkamp and Geiger, 2016).

Visualization of the active RNC:insertase complex

SecYEG-YidC nanodiscs were supplied to the CFPS reaction based on *E. coli* S30 extract. With that, the translating ribosomes were targeted to SecYEG-YidC, presumably by the endogenous SRP and the SRP receptor FtsY, to initiate nascent IMP insertion. The CFPS approach was chosen to ensure sequential incorporation of nascent NuoK TMHs into the membrane and reduce the risk of erroneous insertase:substrate complexes. We chose NuoK⁸⁶ construct that engages with YidC in vivo (Fig. 1D) and requires both SecYEG and YidC for insertion in proteoliposomes (Price and Driessen, 2010). Notably, synthesis of NuoK TMH 3 in vivo is followed by completion of the translation, so the membrane insertion of the helix occurs post-translationally. Presence of the SecM* stalling sequence at the C-terminal end of the nascent chain in the reconstituted system should trap NuoK TMH 3 exposed from the ribosome, with TMH 1 and TMH 2 already inserted in the membrane, thus enabling visualization of the late-stage folding intermediate and the insertase machinery by cryo-EM.

The NuoK⁸⁶-RNC:SecYEG-YidC complexes were sequentially purified via centrifugation in the sucrose gradient (to isolate 70S ribosomes) and affinity chromatography against poly-histidine-tagged MSP2N2 (to isolate the ribosome-bound nanodiscs), and presence of the stalled nascent chains was confirmed via immunoblotting (Appendix Fig. S2C). The samples were stabilized by rapid glutaraldehyde cross-linking before vitrification and cryo-EM analysis. 3D classification of 70S ribosomes followed by focused classification and local refinement of the exit tunnel region revealed two reconstructions containing extra density for SecYEG and YidC (YidC is recognized by its large periplasmic domain P1) and two reconstructions with only SecYEG (Fig. EV2A). From the best resolved SecYEG-YidC class we obtained a final density map with an average resolution of 2.4 Å (Fig. EV2B). The reconstruction showed a ribosome programmed with tRNAs in the A- and P-sites, a continuous nascent chain density ranging from the CCA-end of P-site tRNA through the exit tunnel, and a clear density below the tunnel accounting for SecYEG and YidC embedded into a nanodisc of approx. 15 nm in diameter (Fig. 2E). Local refinement of the nanodisc density resulted in a local resolution of 3 to 5 Å for SecYEG and 4 to 7 Å for most parts of YidC (Fig. EV2C) and allowed unambiguous docking of the modeled SecYEG-YidC complex with only minor adjustments (Appendix Figs. S4 and S5). The structure shows the RNC-bound SecYEG in the center of the nanodisc, while YidC is positioned at the back side of SecYEG near SecE TMH 3, with the large P1 domain of YidC exposed to the “periplasmic” side of the nanodisc. As anticipated, an additional

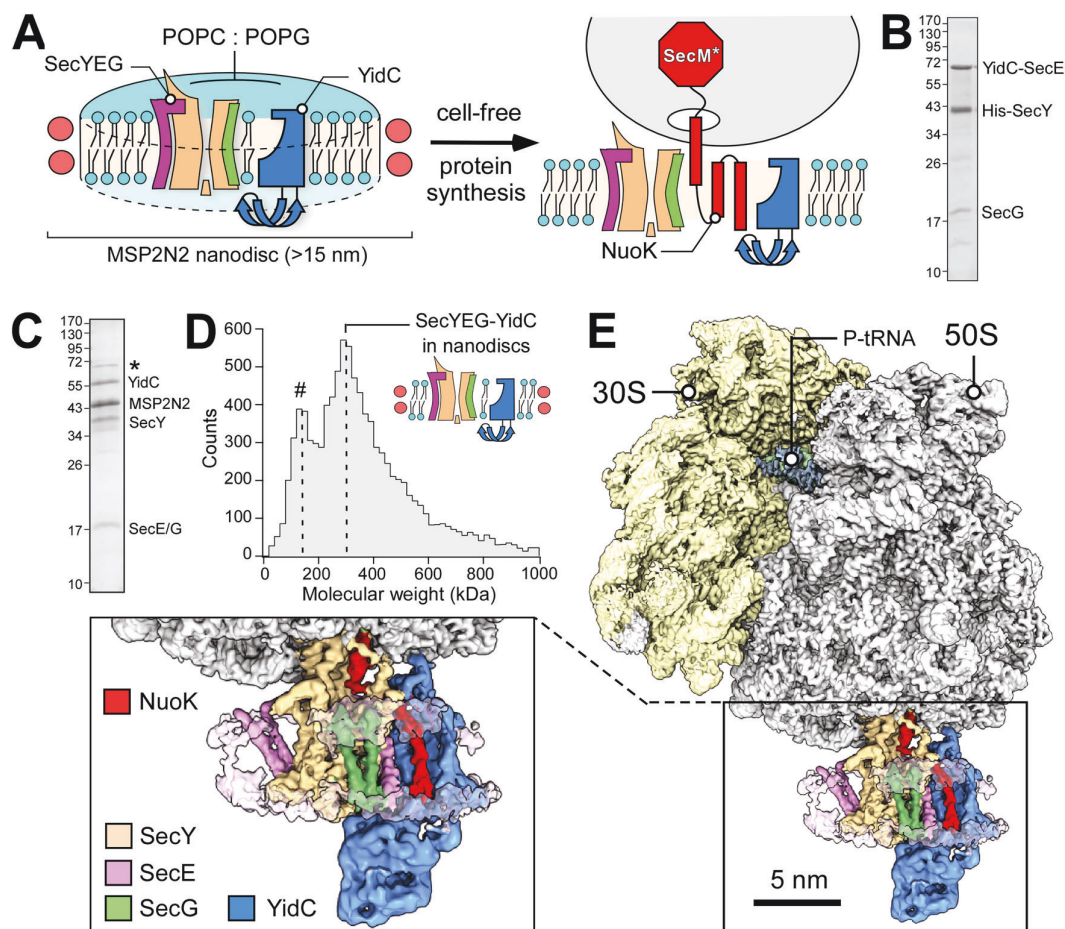


Figure 2. Substrate-induced assembly of the RNC:SecYEG-YidC complex.

(A) Scheme of the assay to study Nuok biogenesis. SecYEG and YidC are co-reconstituted into MSP2N2-based nanodisc in presence of POPC:POPG lipids and introduced into CFPS reaction. Synthesis of the substrate Nuok is interrupted by SecM* stalling sequence, so the stable insertion intermediate is formed for structural analysis. (B) SDS-PAGE of the affinity-purified SecYEG-YidC fusion insertase complex, with the subunits indicated. (C) SDS-PAGE of the nanodisc-reconstituted SecYEG-YidC complex after the protease treatment. The minor band at 70 kDa (*) corresponds to the residual YidC-SecE fusion protein. (D) Mass photometry recording of the isolated SecYEG-YidC nanodiscs manifests the main peak at 290 kDa. The minor peak at 130 kDa (#) corresponds to the nanodiscs loaded only with lipids. (E) Cryo-EM reconstruction of Nuok⁸⁶-RNC:SecYEG-YidC assembly. Shown is a composite map consisting of isolated densities of the ribosome subunits 30S and 50S, P-site peptidyl-tRNA ("P-tRNA") and locally refined SecYEG-YidC, with subunits and the substrate Nuok indicated. Source data are available online for this figure.

density accounting for TMH 2 and TMH 3 of Nuok is found between SecYEG and YidC, building extensive interactions with both proteins within the membrane and at the interface (Fig. 2E). At lower contour levels, an additional transmembrane density is visualized near Nuok TMH 2, which likely represents Nuok TMH 1 (Appendix Fig. S4F), though YidC TMH 1 could not be excluded. Notably, the membrane-inserted fragment appears in an inverted topology in comparison to the full-length Nuok within the assembled complex I, and the possible scenarios for the topology are provided in the Discussion chapter. Importantly, the visualized route of the nascent polypeptide via the SecYEG-YidC insertase may be taken by multiple other clients, so we believe that the mechanistic insights described below are of general importance for understanding the membrane protein biogenesis in bacteria.

As YidC was reconstituted into the nanodiscs as a fusion protein with SecE TMH 1, it likely was initially positioned near the lateral

gate of SecYEG. This arrangement is indeed predicted by AlphaFold for the fusion protein but with high error values for SecYEG:YidC interactions (Fig. EV3A,B), indicating that YidC positioning at the lateral gate is rather unlikely or unstable. After the linker was cleaved, YidC must have relocated substantially within the lipid-filled nanodisc to interact with the client Nuok at the back of SecYEG. Indeed, the observed dimensions of the nanodisc provide sufficient space for the lateral diffusion (Fig. EV3C). Though the confinement could potentially affect the embedded protein assembly, we observed no contacts of either YidC or SecYEG with the edges of the nanodisc, suggesting that the complex was not perturbed by the environment. To test whether the localization of YidC was not artificially induced by the chemical cross-linking, we carried out cryo-EM analysis of the non-cross-linked Nuok⁸⁶-RNC:SecYEG-YidC sample (Appendix Fig. S6). Though somewhat weaker and less resolved, the YidC density was

clearly present in the same position at the back of SecYEG, implying that the cross-linked sample reflects the undisturbed architecture of the substrate-bound SecYEG-YidC machinery.

Architecture of the SecYEG-YidC insertase

The clear density for all TMHs of SecY, SecE and SecG present in the locally refined map offered the by-now best-resolved view on the translocon structure in the lipid environment (Fig. 3A; Appendix Fig. S4). As observed previously for SecYEG-only assemblies, SecY is anchored via its cytoplasmic loops 6/7 and 8/9 to the exit tunnel of the large ribosomal subunit 50S (Fig. 3B,C). Interestingly, SecY loop 6/7 is somewhat remodeled compared to the previously reported RNC-FtsQ:SecYEG assembly (Kater et al, 2019). To avoid a clash with the NuoK nascent chain, the loop shifts away from the exit tunnel towards the RNA helix H6/7 and uL23 (Appendix Fig. S7A). Here, SecY Tyr-258 is tightly accommodated in a pocket formed by bases A91, U92 and G93 of the RNA helix H7, while Arg-256 stacks on A63 of H6, and Arg-255 interacts with the loop of uL23 (His-70/Gly-71/Gln-72) (Fig. 3B). In fact, this distortion in the SecY loop 6/7 leads to a re-positioning of SecYEG with respect to the ribosome, with the 50S subunit tilting by $\sim 10^\circ$ towards the N-terminal half of SecYEG (Appendix Fig. S7B). Upon tilting, Lys-348 of the SecY loop 8/9 established a contact with the flipped-out base C490 of 23S rRNA (helix H24), and Arg-357 formed a stacking interaction with A1392 (H53). The latter may be further stabilized by a salt bridge with Glu-18 of the proximate ribosomal protein uL23 (Fig. 3C). Further interactions include the backbone of SecY Lys-347 with U1318 (H50), and Arg-340 with Lys-364 approaching the flipped-out base A1535 of H59. The C-terminal cytoplasmic extension of SecY TMH 10 is bowed at Met-425/Ser-426 toward the SecY loop 8/9, so another contact is formed with the flipped-out base C490 of rRNA H24 (likely via Ala-436) (Fig. 3D). Shortening of the polypeptide and deletion of the proximate Lys-433 and Lys-434 appeared lethal for the cells (Chiba et al, 2002), highlighting the importance of this poorly studied element of SecYEG. As this SecY:ribosome contact was absent in earlier structures (Bischoff et al, 2014; Jomaa et al, 2016; Kater et al, 2019), we speculated that its formation is associated with the specific nascent chain routed for insertion. Notably, the lateral gate formed by TMHs 2b and 7 is in a tightly closed conformation (Appendix Fig. S4B,C) indicating that it is not employed by the NuoK nascent chain, and the central pore is sealed by the “plug” domain, TMH 2a of SecY.

The conformation of YidC within the RNC-bound complex clearly deviates from the structure of isolated YidC obtained via X-ray crystallography (Kumazaki et al, 2014b) (Fig. 3E; Appendix Fig. S4E). The paddle domain, i.e., the hairpin built of helices CH1 and CH2 at the cytoplasmic interface, shifts by 1 nm in direction of YidC TMH 5, and so to the periphery of the complex. There, it appears in close vicinity to the ribosomal protein uL24, but does not form a direct contact (Fig. 3A). The shift of the paddle domain allows the passage of the nascent chain (Fig. 3E). Furthermore, YidC TMH 6 is located at the periphery of the nanodisc, and its C-terminal extension is not required for the ribosome:SecYEG-YidC assembly, though this polypeptide is essential for binding to an RNC in absence of SecYEG in vitro (Kedrov et al, 2013), and it is also important for the homologous ribosome:Oxa1 interactions in mitochondria (Haque et al, 2010). The large density at the

periplasmic side matches the structure of the YidC P1 domain known from crystallography studies (Kumazaki et al, 2014b; Ravaud et al, 2008). With respect to SecYEG, YidC is oriented with its TMH 3 facing TMH 3 of SecE, and the loop of the paddle domain (residues 397–406) faces the N-terminal end of SecY. While the SecY N-terminal polypeptide (residues 1–15) is not resolved in the structure, it may reach the paddle domain, thus explaining the efficient cross-links observed between the residue 399 of YidC and SecY in *E. coli* membranes (Petriman et al, 2018). At the periplasmic side, the essential amphipathic helix EH1 and SecY TMH 5 (Fig. 3E) form the only pronounced contact between these two proteins (discussed below). Apart from this contact, SecYEG and YidC are held together mainly by the NuoK⁸⁶ nascent chain, i.e., TMH 2 within the membrane region and TMH 3 at the cytoplasmic interface, suggesting that YidC is likely recruited to the RNC-bound SecYEG in a substrate-dependent manner.

Routing and insertion of the nascent NuoK

The nascent chain could be unambiguously traced from the CCA-end of P-site tRNA to the tunnel exit site within the ribosome (Fig. 4A,B). All side chains of the SecM* stalling element (₁₁₁WWWPRIRGPP₁₂₀) were resolved with Pro-120 attached to the CCA-end of the A-site tRNA, while the preceding modified motif ₁₁₇RGP₁₁₉ (₁₆₃RAG₁₆₅ in SecM) was coupled to the CCA-end of the P-site tRNA (Fig. 4B). The conformation of the stalling peptide, tRNAs and critical bases in the peptidyl-transferase center were highly similar to the structure of SecM-stalled *E. coli* ribosome (Gersteuer et al, 2024), indicating the same trapping mechanism as described for the RAG/P motif and its modified versions. Further, residues Arg-115 and Arg-117 interacted with Ψ2504 and A2062, and the aromatic rings of the tryptophan motif (₁₁₂WWW₁₁₄) with bases A2059 and U2586 in the 23S rRNA (Fig. 4C). Less resolved density accounting for the linker and the HA-tag continues from the constriction by uL4 and uL22 towards the mouth of the tunnel (Fig. 4A).

At the mouth of the exit tunnel, the NuoK nascent chain acquires a helical fold accounting for three C-terminal turns of its TMH 3, which are docked between the SecY loops 6/7 and 8/9, the C-terminal extension of TMH 10 and the tip of the ribosomal uL24 (Fig. 4D). This newly formed arrangement of SecY creates a hydrophobic pocket in the otherwise hydrophilic environment of the exit tunnel and the solvent that allows early folding and accommodation of a short hydrophobic helix (Fig. 4E). As the C-terminal polypeptide of SecY sterically hinders the access to the lateral gate, the emerging nascent chain is not routed into the central channel. Instead, once approaching the cytoplasmic funnel of SecY, NuoK TMH 3 density is kinked by approx. 120° towards the back side of the SecYEG, and so it acquires an interfacial topology laying over TMH 3 of SecE and TMH 10 of SecY (Fig. 5A). These crossed TMHs form a sawhorse-like crevice at the membrane interface oriented toward YidC. In its turn, the paddle domain of YidC covers the nascent NuoK helix from the cytoplasmic side and completes a triangular window for the hydrophobic client. This observation is also consistent with previously proposed interactions of the evolutionary conserved paddle with substrates (McDowell et al, 2021). Like the SRP M-domain or Get3/TRC40, the YidC paddle contains a “methionine bristle” for client binding and, indeed, our structure now

shows how the membrane-facing Met-408 and Met-409 in CH2 interact with NuoK TMH 3 prior its insertion (Fig. 5A). Notably, replacing these methionine residues with charged residues (Lys, Arg, Asp) or asparagine, but not alanine or serine, rendered a cold-sensitive phenotype in *E. coli* upon depletion of the wild-type YidC (Fig. 5B; Appendix Fig. S8). We speculate that the effect originates from defects in IMP biogenesis, and that the apolar environment provided by the paddle domain is an important factor for the nascent IMP folding at the lipid membrane interface prior the insertion, in agreement with the physical considerations (White and Wimley, 1999).

The close SecYEG-YidC association ensures hand-over of the nascent chain to YidC, and the position of NuoK TMH 2 in our structure suggests the insertion route (Fig. 5C). The membrane-inserted NuoK TMH 2 is separated from TMH 3 by a kink, likely a short flexible loop, at the level of YidC Cys-423. NuoK TMH 2 occupies the wide groove between YidC TMHs 3 and 5, with Leu-426, Met-430 and Leu-434 as the closest residues (Fig. 5C), forming the functional site of the insertase described in previous studies (Kedrov et al, 2016; Yuan et al, 2007). As the groove narrows towards the periplasm, the N-terminal end of NuoK TMH 2 is expelled from the helical bundle of YidC, being fully exposed to the lipid environment. Notably, the visualized NuoK does not reach YidC TMH 2 and does not interact with Arg-366 located deeply in the YidC groove (Fig. 5C). This residue is shown to be important for YidC functionality, as it may destabilize the lipid membrane and ensure polarity and water access within the groove (Chen et al, 2017), but also interactions of the conserved arginine with charged/polar residues of client proteins have been reported (Kumazaki et al, 2014a; Tsukazaki, 2019). Although not observed in our structure, we cannot exclude that such interactions are formed at other stages of NuoK insertion, or it may be specific for particular nascent IMPs.

Determinants for the SecYEG-YidC assembly

The C-terminal linker and the stalling peptide of the designed nascent chain retain NuoK⁸⁶-RNC bound to the SecYEG-YidC complex, offering a view on a late insertion intermediate, with NuoK TMH 3 trapped at the interface prior its insertion. In absence of the C-terminal extension, the helix would be released from the ribosome and inserted post-translationally, while the ribosome could detach from SecYEG. To test whether the observed SecYEG-YidC assembly also occurs upon co-translational insertion, we focused on an earlier NuoK intermediate formed prior the nascent chain release (Appendix Fig. S9). The shorter construct NuoK⁷⁰ contained TMH 1 and TMH 2 fully exposed from the ribosome and the N-terminal part of TMH 3 emerging from the tunnel (Fig. 6A; Appendix Fig. S2). The length of the linker/stalling sequence within the ribosomal tunnel matched the length of the replaced C-terminal part of NuoK (30 residues), so the construct approximated the full-length NuoK. Cryo-EM analysis of the assembled NuoK⁷⁰-RNC:SecYEG-YidC complex revealed an architecture very similar to the NuoK⁸⁶-based sample (Fig. 6B,C): While the lateral gate of SecY was closed, YidC was located at the back side of the SecYEG complex, with a slight shift towards the N-terminal half of the translocon, as concluded based on a rigid body fit. Similar to NuoK⁸⁶, a rod-like density accounting for NuoK TMH 3 emerged from the ribosomal tunnel into the hydrophobic pocket formed by

the cytoplasmic loops and the C-terminal polypeptide of SecY. Another prominent though less defined density was located above the SecY-SecE crevice, most likely corresponding to the pre-inserted part of NuoK TMH 2. At low contour levels, a density for the membrane-embedded part of NuoK TMH 2 was found near the insertion groove of YidC (Fig. 6B). Despite the limited resolution achieved in presence of NuoK⁷⁰ nascent chain, both reconstructions for the “post-translational” event (NuoK⁸⁶-RNC) and “co-translational” event (NuoK⁷⁰-RNC) reveal nearly identical organization of the assembled SecYEG-YidC machinery. Importantly, YidC was recruited to NuoK⁷⁰ prior TMH 2 was fully embedded into the membrane, thus suggesting that YidC indeed is an insertase rather than a chaperone for the pre-inserted TMHs.

We next asked how specific elements of the nascent chain may facilitate recruitment of YidC and determine the complexes’ assembly. As YidC involvement in NuoK biogenesis was previously linked to Glu-36 and Glu-72 residues within TMH 2 and TMH 3 of the nascent IMP, respectively (Price and Driessen, 2010), we substituted both glutamates with lysines and performed cryo-EM analysis of the NuoK^{86mut}-RNC:SecYEG-YidC complexes (Fig. 6D; Appendix Fig. S10). Despite the charge inversion and without the glutaraldehyde stabilization, the characteristic YidC density was observed in the same position at the back of SecYEG as upon wild-type NuoK⁸⁶ insertion. Thus, the SecYEG-dependent nascent protein nevertheless promotes recruitment of YidC and the assembly of the insertase complex, though this dynamics could not be addressed in previous studies utilizing protease-protection assays (Price and Driessen, 2010). However, when the length of the nascent chain was reduced to 48 amino acids, so only TMH 1 and TMH 2 of NuoK were exposed from the ribosome (NuoK⁴⁸-RNC), only the RNC-bound SecYEG in its closed state was resolved within the nanodisc (Fig. 6E; Appendix Fig. S11). Notably, neither the C-terminal polypeptide of SecY, nor the helical density exiting the ribosomal tunnel were resolved, suggesting that the translocon conformation is tuned by the emerging client protein. The signal for YidC was lost, so the insertase remained mobile within the surrounding membrane, and the nanodisc manifested the characteristic tilt by ~10°. Thus, we concluded that the SecYEG-YidC complex is dynamically assembled to mediate insertion of a nascent IMPs emerging via the “back-of-Sec” route, and the YidC recruitment may not depend on specific charge within the substrate, but may rely on other determinants, such as the length and the folding status of the emerging chain.

Notably, the observed structure of the active SecYEG-YidC complex shows a very similar architecture to the sequence-based model provided by AlphaFold, despite the absence of the ribosome and the nascent chain (Appendix Fig. S5). The direct contacts between SecYEG and YidC are limited to those at the periplasmic side, involving the essential amphipathic helix EH1 of YidC (Fig. 3E) (Kedrov et al, 2016; Li et al, 2014). Here, SecY Arg-211 (TMH 5) and YidC Asp-329 (end of EH1) potentially form a salt bridge that would stabilize the interaction between both proteins (Figs. 6F and EV4A). Though not conserved through bacterial species, this pair of charged residues is commonly found in γ -proteobacteria, where AlphaFold confidently predicts the SecYEG-YidC association (Appendix Fig. S1). We speculated that disrupting this contact may be detrimental for IMP biogenesis and affect the cell viability. Accordingly, we replaced YidC Asp-329 with either alanine or arginine residues. Both YidC variants could be

recombinantly expressed and purified, suggesting that the mutated proteins were correctly folded (Fig. EV4C), and immunodetection of the polyhistidine-tagged variants confirmed expression in *E. coli* FTL 10 cells under permissive conditions (Fig. EV4D). When testing the functional complementation of these YidC variants in *E. coli* FTL10 strain, we observed that the plasmid-encoded YidC^{D329A} fully supported the cell growth under depletion of the wild-type YidC (Figs. 6G and EV4E). In contrast, introducing a positive charge was detrimental for the cellular viability, similar to the known effect of a minor deletion within the proximate EH1 (Kedrov et al, 2016). The effect may be explained by electrostatic repulsion between SecY and YidC, however, we cannot exclude other consequences of the mutation, such as inhibition of YidC-mediated insertion. Future work using a combination of complementary in vivo and in vitro methods, such as site-specific cross-linking, co-purification, and fluorescence imaging, but also molecular dynamics simulations should provide further insights on the SecYEG-YidC binding interface.

Comparison of bacterial and eukaryotic insertases

Guiding of the nascent chain to the back side of SecYEG for YidC-mediated insertion manifests an obvious resemblance to the route of multipass IMP biogenesis in the endoplasmic reticulum of eukaryotes (Fig. 7A,B) (Smalinskaite et al, 2022). Within the eukaryotic MPT machinery, the ribosome-bound Sec61 translocon builds a core of the substrate-dependent super-complex composed

of BOS (“back-of-Sec61”), GEL (“GET- and EMC-like”) and PAT (“proteins associated with the translocon”) complexes (Fig. 7B). We thus compared the arrangement of the *E. coli* SecYEG-YidC with the human insertase machinery trapped with a nascent chain of rhodopsin. The bacterial insertase forms a compact assembly, where YidC is tightly packed against SecYEG and the nascent chain, providing a simple solution to transfer a newly synthesized hydrophobic protein from the ribosome to the membrane-embedded groove of YidC. In contrast, the loosely packed eukaryotic MPT extends far from the Sec61 core and forms a lipid-filled “peninsula” for inserting nascent TMHs. The YidC homolog TMCO1 (a constituent of the GEL complex) is found more than 3 nm away from Sec61 and the nascent TMH, suggesting its role as a membrane chaperone within the MPT rather than an insertase. The position of YidC within the eukaryotic machinery is occupied by TMEM147, a subunit of the BOS complex, that bridges the core translocon with the MPT. Apart from its interactions with Sec61, TMEM147 is known to associate with the ER membrane protein complex (EMC) (Page et al, 2024), likely serving as an adaptor subunit in various routes of membrane protein biogenesis.

Most recently, a structure of the ribosome-bound Sec61aβγ translocon from the thermophilic fungus *Chaetomium thermophilum* has been visualized in complex with SND3, a membrane machinery involved in biogenesis of a subset of IMPs in eukaryotes (Fig. 7C) (Aviram et al, 2016; Yang et al, 2025). The observed assembly consists of the Snd3 protein and CCDC47-Asterix, i.e., PAT sub-complex, located at the back of Sec61, thus resembling the

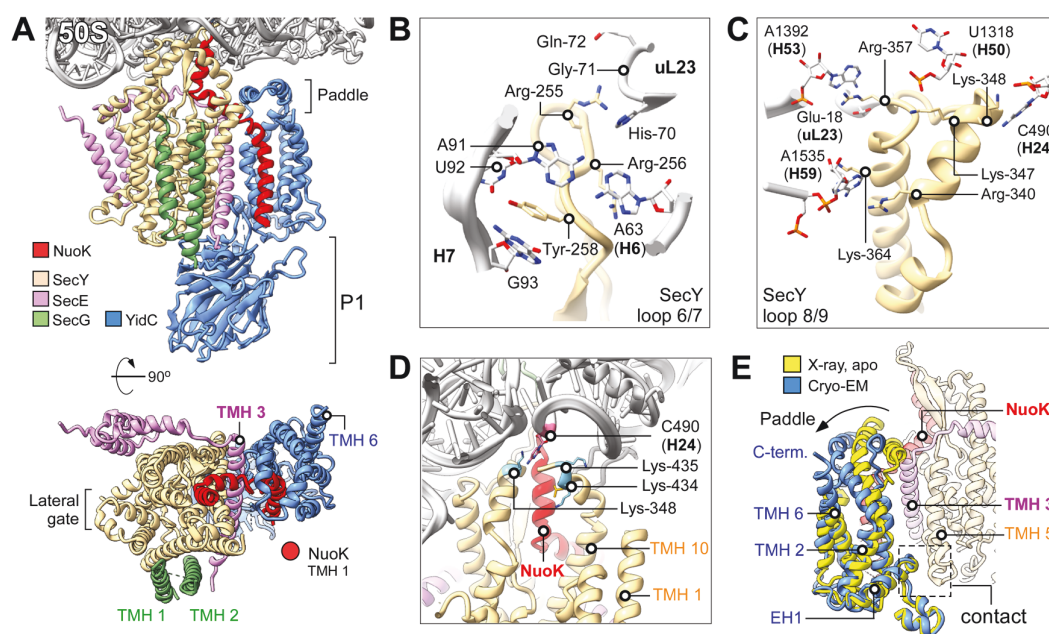


Figure 3. Architecture of the SecYEG-YidC insertase and its interactions with the RNC.

(A) Molecular model of the SecYEG-YidC insertase complex bound to Nuok^{R6}-RNC. The putative position of the membrane-inserted Nuok TMH 1 is indicated as a red circle in the cytoplasmic view (bottom). (B, C) The network of ribosome:SecYEG interactions mediated by SecY cytoplasmic loops 6/7 and 8/9. The ribosomal RNA and proteins are shown in gray. (D) The C-terminal extension of SecY contacts the flipped-out base C490 of 23S rRNA. This interaction is likely stabilized by Lys-435 close to the H24 rRNA backbone and Lys-348 stacking on the C490 from the opposite site. (E) Overlay of X-ray and cryo-EM structures of YidC highlight the conformational change within the insertase. The movement of the YidC paddle domain upon entry of the Nuok nascent chain is indicated with an arrow. The closest SecY-YidC contact at the periplasmic side is indicated with the dashed box. SecY, SecE and Nuok are shown as semi-transparent ribbons for clarity. The crystal structure of *E. coli* YidC resolved in lipidic cubic phase was used as a reference (PDB ID 3WVF).

architecture of the MPT and SecYEG-YidC. Both for the MPT and SND3, the extended helices of CCDC47 at the cytoplasmic interface reach the N-terminal half of Sec61 α and restrict its mobility required for opening the gate, so the nascent IMP is forced to follow the “back-of-Sec” route. In the fungal machinery (Fig. 7C), the entry to the gate is additionally blocked by a cytoplasmic extension of Sec61 β , resembling the barrier formed by the C-terminal extension of *E. coli* SecY. Though not evolutionary related to YidC/Oxa1 superfamily, the structure of the Snd3 protein manifests a number of features characteristic for insertases, such as short/tilted TMHs that distort the lipid bilayer, and a polar groove that may serve as the interface for IMP partitioning. While the observed Sec61-SND3 complex likely represents an idle state of the insertase in the absence of a client protein, it certainly highlights the complexity of IMP biogenesis routes beyond the partitioning via the lateral gate.

Discussion

Variations in TMH topologies and lengths, appearance of partially unfolded regions and charged residues within the membrane core, point to the complexity in IMP biogenesis across the kingdoms of life. Membrane insertion of hydrophobic TMHs via the lateral gate of the universally conserved SecYEG/Sec61 $\alpha\beta\gamma$ translocon has been described as a general route upon IMP folding. The paradigm has been lately challenged by the discovery of the eukaryotic insertase

machinery MPT that facilitates insertion of multipass IMPs at the back of the ribosome-bound Sec61. The discovery of the alternative “back-of-Sec” route in higher eukaryotes (Smalinskaite et al, 2022; Sundaram et al, 2025), lately substantiated by identifying the ortholog machinery Sec61-SND3 in yeasts (Yang et al, 2025), has immediately raised a question about its origins and the evolutionary conservation (Smalinskaite and Hegde, 2023). Here, we show that the minimal bacterial machinery composed of SecYEG and YidC can assemble within the lipid bilayer in the substrate-dependent manner to facilitate insertion of the multipass IMP NuoK via the “back-of-Sec” route. Differently to the lactose permease LacY (Ou et al, 2025), the NuoK nascent chain does not pass the lateral gate, but it folds within a hydrophobic pocket formed by SecY loops and leaves via the sawhorse-shaped crevice at the back, where it is encountered by YidC. Notably, folding of a nascent α -helix at the membrane interface appears in excellent agreement with the free energy considerations, as the hydrogen bonds should be established within the helical backbone to enable TMH insertion into the hydrophobic core of the lipid bilayer (White and Wimley, 1999). Once exposed from SecYEG, the pre-folded nascent chain is covered by the partially hydrophobic paddle domain of YidC that assists with handover to the insertion site. The SecYEG-YidC assembly is induced by the nascent chain of sufficient length, and YidC is recruited to assist in insertion/folding of NuoK TMH 2. Although different in the complexity level, the overall architecture of the active SecYEG-YidC insertase manifests a conceptual similarity to the eukaryotic MPT machinery

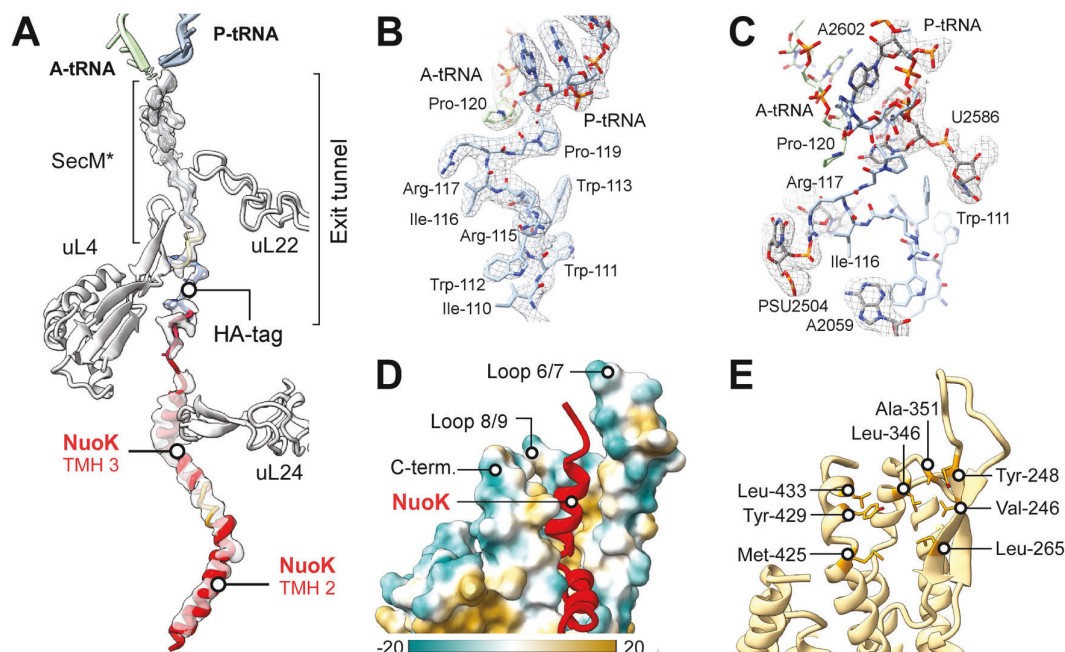


Figure 4. SecYEG and YidC jointly mediate delivery of the nascent chain to the membrane.

(A) Isolated density of the nascent chain NuoK (in transparent) with fitted model; tRNAs and the ribosomal proteins lining the ribosomal exit tunnel are indicated. (B) Isolated cryo-EM density (transparent mesh) of the SecM* stalling sequence in the ribosomal exit tunnel with the fitted model. The residues of SecM* peptide are indicated. (C) Model for the SecM*:ribosome interactions. Hallmark bases of the PTC and involved in SecM* binding are shown as in SecM-stalled ribosome (PDB ID 8QOA), fitted into the respective density in (transparent mesh). (D) Surface representation of the molecular model for SecY cytoplasmic loops 6/7, 8/9 and the C-terminal tail colored according to the molecular lipophilicity potential (scale bar shown). The emerging NuoK nascent chain (red ribbon) acquires α -helical fold within the hydrophobic pocket. (E) The indicated apolar residues form the hydrophobic pocket for the nascent chain folding, as shown in (D).

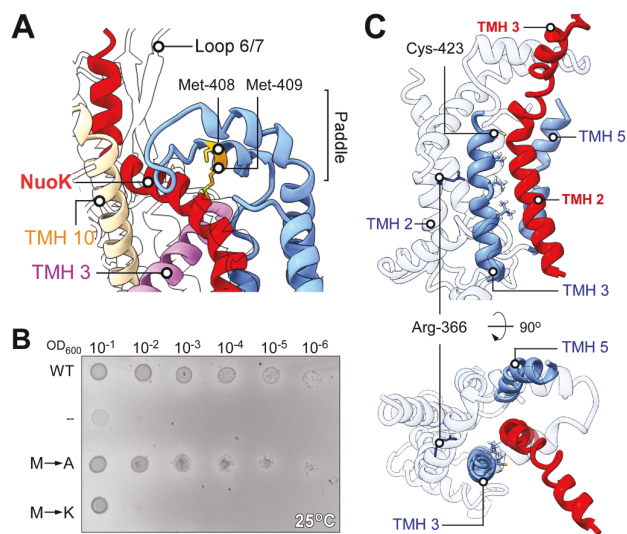


Figure 5. YidC-mediated insertion of the nascent membrane protein.

(A) The architecture of the nascent chain hand-over interface between SecYEG and YidC. The methionine residues within the YidC paddle domain are indicated. (B) Complementation test using wild-type YidC ("WT"), mutants YidC^{M408A, M409A} ("M → A") and YidC^{M408K, M409K} ("M → K") and empty vector ("–") in presence of glucose at 25 °C. (C) Inserted NuoK TMH 2 is docked at the groove of YidC between TMHs 3 and 5 with the accommodated NuoK TMH 2. The aliphatic residues within YidC TMH 3 of the insertion groove are shown as sticks (Cys-423, Leu-426, Met-430 and Leu-434). Conserved Arg-366 within YidC TMH 2 is indicated. The rest of YidC is shown as semi-transparent ribbon for clarity. Source data are available online for this figure.

and suggests an evolutionary conservation of the "back-of-Sec" route for IMP biogenesis.

Multiple studies have described interactions of nascent IMPs with the lateral gate of SecYEG and suggested that YidC is located in its proximity to access the emerging nascent TMHs (Ou et al, 2025; Petriman et al, 2018; Sachelaru et al, 2013). In this position, YidC may serve as a chaperone for the newly inserted IMPs, e.g., mediate correct partitioning of moderately hydrophobic helices and ensure assembly of multipass proteins (Fig. 8) (Jauss et al, 2019; Petriman et al, 2018; Sachelaru et al, 2013; Zhu et al, 2013). Focused on this route, the previous cross-linking studies extensively investigated the lateral gate region for SecYEG-YidC interactions, while other potential interfaces were barely explored (Fig. EV5). Together with AlphaFold-based modeling, our structural findings demonstrate that an alternative functionally relevant architecture is possible for *E. coli* SecYEG-YidC within the lipid membrane. Notably, the predicted models from other species manifest various, though low-confidence architectures (Appendix Fig. S1). While the insertion pathway is likely to be conserved amongst the organisms, further specie-specific membrane constituents, i.e., accessory proteins and/or the anisotropic and heterogeneous lipid environment lacking in the models, may be required to facilitate the assembly of the active insertase.

Similar to the eukaryotic MPT (Sundaram et al, 2022), assembly of the SecYEG-YidC insertase is dependent on the nascent chain, and the chain composition as well as the propensity to fold may dictate the insertion route. The "back-of-Sec" route in eukaryotes is taken by nascent IMPs lacking long polar/charged loops (Sundaram

et al, 2025), which otherwise require translocation via Sec61αβγ. The translocation is associated with opening of the translocon and widening of the lateral gate (du Plessis et al, 2009), so the primed gate can serve for insertion of a following TMH. Alike, the length and polarity of periplasmic loops dictate the dependence of bacterial IMPs on SecYEG (Soman et al, 2014). Biogenesis of a multipass protein, such as LacY, may require multiple translocation events mediated by the ATPase SecA and the proton-powered complex SecDF (Zhu et al, 2013), so insertion of the proximate TMHs likely occurs via the lateral gate (Fig. 8) (Ou et al, 2025). Importantly, interactions of SecYEG with SecA and a ribosome are mutually exclusive, as both utilize the same binding interface of the translocon (Wu et al, 2012). Thus, the ribosome would transiently break the contact with SecYEG upon synthesis of a polar polypeptide stretch, but the nascent chain would serve as a linker to enable re-binding in presence of a following TMH. In contrast, the short loops of NuoK should not depend on SecA-mediated translocation, so the protein manifests the features required for "back-of-Sec" insertion, as observed in the acquired structures. It remains to be explained though, what features of a nascent chain serve as signals for YidC recruitment either at the lateral gate or "back-of-Sec" interface, and whether switching between different routes may occur along insertion of a single IMP.

Notably, the involvement of YidC at the late stage of NuoK biogenesis correlates with the recent findings that both YidC and the evolutionary related EMC complex facilitate insertion of the C-terminal TMHs of polytopic IMPs (Kalinin et al, 2024; Wu et al, 2024). This final insertion event would occur post-translationally when the translation termination happens soon after the synthesis of the last TMH. NuoK would be an example of such IMP: In absence of the stalling, the nascent polypeptide chain would be released from the peptidyl transferase center, and the membrane insertion of NuoK TMH 3 complex would occur post-translationally. As both YidC and EMC ensure folding of post-translationally delivered clients (Anghel et al, 2017; Page et al, 2024), the C-terminal TMHs of multipass IMPs may utilize the same recognition and insertion routes, while the SecYEG-YidC assembly would facilitate the efficient substrate delivery (Fig. 8).

The topology acquired by NuoK in the nanodisc is another puzzling outcome of our study. The model based on the resolved TMHs suggests that the N-terminus of the insertion intermediate is oriented into the cytoplasm, thus being inverted in comparison to NuoK within the assembled complex I. The non-native topology could possibly originate from the experimental *in vitro* set-up, e.g., due to the chosen synthetic lipid composition, or absence of the physiological electrostatic potential across the membrane, an important factor for the IMP orientation. While NuoK insertion in proteoliposomes efficiently occurred in absence of the membrane potential (Price and Driessen, 2010), the employed protease-protection assay did not account for the resulting protein topology. However, if the electrostatic potential was a decisive factor, we would expect to find NuoK in two different topologies in cryo-EM reconstructions, possibly utilizing different insertion routes, while only one class was experimentally observed. As the observed topology is likely determined by the insertion of NuoK TMH 1, further analysis should assess this early event. However, another scenario should be considered upon the interpretation: Several IMPs, such as four-TMH EmrE, were shown to undergo partial or even complete topology inversion along their folding pathway, and

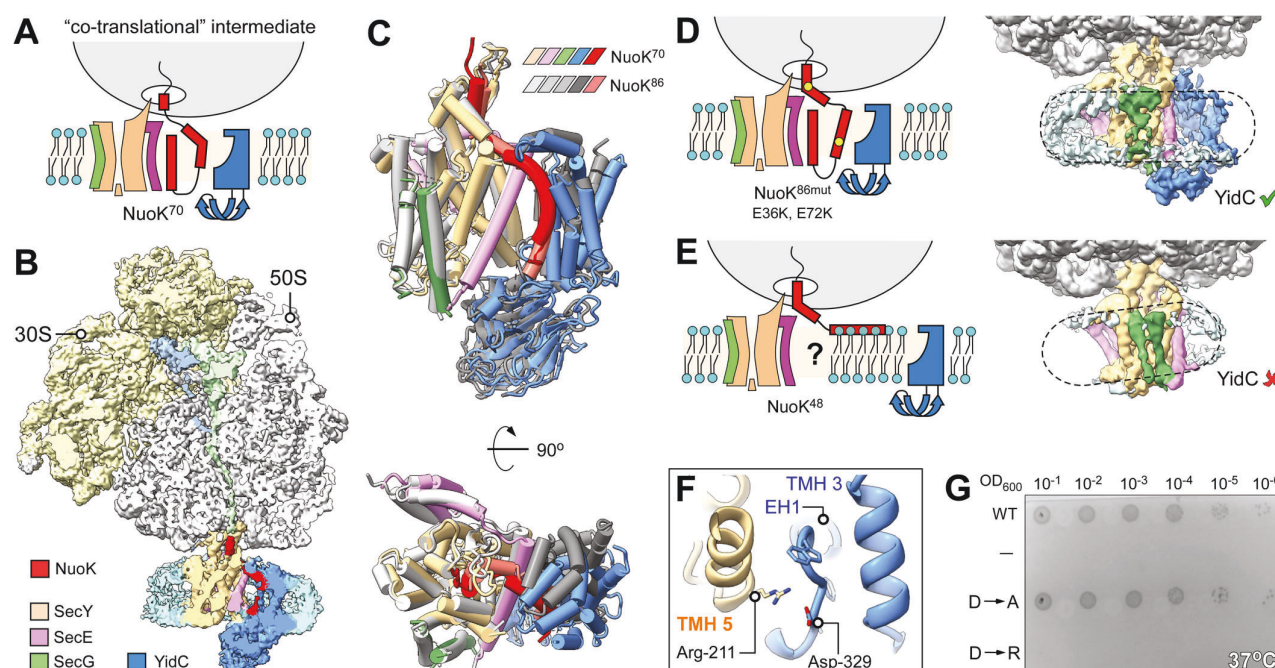


Figure 6. Substrate-induced assembly of the SecYEG-YidC insertase complex.

(A) Schematic of the Nuok⁷⁰ intermediate mimicking co-translational insertion via the SecYEG-YidC machinery. (B) Cryo-EM reconstruction of the Nuok⁷⁰-RNC:SecYEG-YidC assembly (central cross-section) with the individual densities indicated. (C) Molecular model of the SecYEG-YidC insertase complex bound to Nuok⁸⁶-RNC. The model of the complex bound to Nuok⁸⁶-RNC is shown in gray. (D) Schematic of the Nuok⁸⁶ insertion intermediate bearing E36K, E72K mutations (yellow circles), and the corresponding cryo-EM map (right). The outline of the nanodisc is shown with the dashed line. (E) Schematic of the Nuok⁴⁸ insertion intermediate and the corresponding cryo-EM map (right). The outline of the nanodisc is shown with the dashed line. (F) View on the molecular model of the SecY-YidC complex focusing on the closest contact site at the periplasmic interface. A putative salt bridge is formed between SecY Arg-211 and YidC Asp-329. (G) Complementation test using wild-type YidC ("WT"), mutants YidC^{D329A} ("D → A") and YidC^{D329R} ("D → R") and empty vector ("---") in presence of glucose at 37 °C. Source data are available online for this figure.

a single charged residue here can be decisive for the final orientation (Lu et al, 2000; Seppala et al, 2010; Seurig et al, 2019; Woodall et al, 2017). As Nuok does not contain long loops between the TMHs but contains a stretch of three arginine residues within its C-terminal end, the protein may acquire its correct topology in the cellular membrane once the complete polypeptide chain is released from the ribosome. The flip-flop of the inserted TMHs may be promoted by the proximate YidC, as the insertase would distort the lipid bilayer and so destabilize the Nuok intermediate that eventually results in the new topology, in accordance to a model suggested for the eukaryotic MPT (Smalinskaite and Hegde, 2023).

Our study delivers a new view on the organization of the bacterial SecYEG-YidC insertase machinery, and the revealed architecture is calling for detailed investigation on its dynamics, as well as re-evaluation of the earlier data. One current limitation of the employed nanodisc-based system is the pre-defined stoichiometry of SecYEG and YidC within the complex, while YidC is substantially more abundant in the bacterial membrane (Urbanus et al, 2002). Thus, even larger insertase complexes may be envisioned for the cellular membrane, where multiple YidC copies could manage biogenesis of polytopic IMPs (Fig. 7). Experiments on the native bacterial membranes, such as those by super-resolution fluorescent imaging and cryogenic electron tomography (Gemmer et al, 2023; Wirth et al, 2023), as well as reconstituted systems will be of the utmost importance to explore such modes of

interaction, but also to determine exact roles played by YidC along insertion of client IMPs, and so deliver detailed and possibly direct insights on the key process of membrane protein biogenesis.

Methods

Reagents and tools table

Reagent/Resource	Reference or Source	Identifier or Catalog Number
Experimental models		
<i>E.coli</i> C41(DE3) Δ <i>ompF</i> - Δ <i>acrAB</i>	https://doi.org/10.1186/s12934-019-1182-1	NA
<i>E.coli</i> FTL10	https://doi.org/10.1046/j.1365-2958.2003.03642.x	NA
Recombinant DNA		
pEM472	This study	NA
pRSET Nuok86	This study	NA
pRSET Nuok71	This study	NA
pRSET Nuok48	This study	NA
pET24a Nuok 86	This study	NA
pET24a Nuok 48	This study	NA

Reagent/Resource	Reference or Source	Identifier or Catalog Number
pTRC99A YidC wt	10.1016/j.jmb.2013.07.042	NA
pTRC99A YidC D329R	This study	NA
pTRC99A YidC D329A	This study	NA
pTRC99A YidC wt His	10.1016/j.jmb.2013.07.042	NA
pTRC99A YidC D329R His	This study	NA
pTRC99A YidC D329A His	This study	NA
pTRC 99A YidC M408/409A	This study	NA
pTRC 99A YidC M408/409K	This study	NA
pTRC 99A YidC M408/409D	This study	NA
pTRC 99A YidC M408/409N	This study	NA
pTRC 99A YidC M408/409R	This study	NA
pTRC 99A YidC M408/409S	This study	NA
pBAD YidC His wt	https://doi.org/10.1016/j.celrep.2016.11.059	NA
pBAD YidC His D329R	This study	NA
pBAD YidC His D329A	This study	NA
pMSP2N2	Addgene/Stephen Sligar	29520
TargeTron pAR1219	Sigma-Aldrich	T2076
Antibodies		
Anti His	Dianova	DIA-900-200
Anti HA	Santa Cruz Biotechnology	sc-7392
Anti YidC (rabbit polyclonal)	This study	NA
Anti-rabbit-HRP conjugate	Sigma-Aldrich	A0545
Anti-mouse-HRP conjugate	Dianova	SEC-183364
New materials (DNA, antibodies) are available upon request		
Chemicals, Enzymes and other reagents		
Phusion High-Fidelity DNA Polymerase	New England Biolabs	
(NEB)	M0530L	
Sall	NEB	R3138S
NheI	NEB	R3131S
HindIII	NEB	R3104S
T4DNA Ligase	NEB	M0202S
PNK	NEB	M0201S
DpnI	NEB	R0176S
Kanamycin	Sigma-Aldrich	60615
Ampicillin	Sigma-Aldrich	A0166
Arabinose	Roth	5118.2
Glucose	Fisher scientific	10335850
LB medium	Chemsolute	8822.5000
LB-Agar	Chemsolute	8843.0500
IPTG	Roth	2316.5
HEPES	Roth	6763.2
TRIS	Roth	4855.2
Potassium hydroxide	Fisher scientific	10509390

Reagent/Resource	Reference or Source	Identifier or Catalog Number
Potassium acetate	Grüssing GmbH	122401000
Magnesium acetate	Chemsolute	1405.1000
PMSF	Sigma-Aldrich	P7626
Glycerol	Roth	3783.3
TCEP	TCI	T1656
Imidazole	Sigma-Aldrich	56750
Bio-Beads SM-2	Bio-Rad	1523920
DDM	Glycon Biochemicals GmbH	D97002
Sucrose	Roth	4621.1
PVDF Membrane	Cytiva	10600023
Methanol	VWR	20847.307
Milk powder	Roth	T145.3
BSA	Biomol	01400.100
Clarity Western ECL substrate	Bio-Rad	1705061
POPC	Avanti Polar Lipids	850457
POPG	Avanti Polar Lipids	840457
HRV-3C Protease	Thermo Fisher	88947
Ammonium acetate	VWR	21200.297
Sodium Pyruvate	Sigma-Aldrich	P8574
Spermidine	Sigma-Aldrich	85578
Putrescine	Sigma-Aldrich	51799
Sodium oxalate	Sigma-Aldrich	71800-100 G
ATP, GTP, CTP, UTP	Sigma-Aldrich	A2383 G8877 C1506 94370
Folinic acid	Roth	T912.1
<i>E.coli</i> tRNA	Roche Diagnostics GmbH	MRE 600
NAD ⁺	Sigma-Aldrich	10127981
Coenzyme A	Sigma-Aldrich	C3144
Ni ²⁺ + -NTA agarose resin	Macherey-Nagel GmbH & Co. KG	745400.100
Glutaraldehyde	Roth	4157.1
1H, 1H, 2H, 2H perfluorooctyl)- β -D-maltopyranoside FOM	Anatrace	O310F
Software		
SnapGene v4.3.11	www.snapgene.com	
Prism v10.1	www.graphpad.com	
ChimeraX v1.10	www.cgl.ucsf.edu/chimerax/	
MotionCor2	www.emcore.ucsf.edu/ucsf-software	
Phenix v1.20	www.phenix-online.org/	
Coot v0.9.8.95	www2.mrc-lmb.cam.ac.uk/personal/pemsley/cool/	
cryoSPARC v4.4	cryosparc.com/	
GIMP 2.10	www.gimp.org/	

Reagent/Resource	Reference or Source	Identifier or Catalog Number
Other		
Microfluidizer M-110P	Microfluidics Ltd.	
Sonopuls GM2200	Bandelin	
SS34 rotor	Sorvall/Thermo Scientific	
45Ti rotor	Beckman Coulter	
FiberLite F8-6x1000y rotor	Piramoon Technologies Inc	
S120 AT6 rotor	Sorvall/Thermo Scientific	
SW 40 Ti rotor	Beckmann Coulter	
Gradient Station	BioComp Instruments	
ÄKTA pure	Cytiva	
Superdex 200 increase GL 10/300	Cytiva	
Superose 6 increase GL 10/300	Cytiva	
Electrophoresis cell	Bio-Rad	
PowerPac Basic Power Supply	Bio-Rad	
NeoDot	NeoBiotech	
Amersham Imager 680	GE Life Sciences	
TwoMP MassPhotometer	Refeyn	
Amicon Ultra-4 Ultracel 30 K centrifugal filters	Merck/Milipore	
Quantifoil Cu 300 mesh R3/3 grids	Quantifoil	
Vitrobot Mark IV	Thermo Fisher Scientific	
Titan Krios	Thermo Fisher Scientific	
Falcon 4i direct electron detector	Thermo Fisher Scientific	
SelectrisX imaging filter	Thermo Fisher Scientific	

Molecular cloning

The primers were synthesized by Eurofins Genomics and Merck/Sigma-Aldrich. The used restriction enzymes and cloning kits, incl. the Phusion High-Fidelity DNA polymerase and the Gibson assembly kits were purchased by New England Biolabs. Sequencing was conducted by Eurofins Genomics and Microsynth AG.

The genes encoding for SecYEG-YidC complex were cloned in a pBAD-TOPO (Thermo Fisher Scientific) derived vector. The gene encoded for *E. coli* YidC (1-535) was fused to the N-terminus of SecE with a sequence coding for HRV-3C protease cleavage site inserted in between; the construct also contained a N-terminally decahistidine-tagged SecY and unmodified SecG. The construct was created using conventional restriction-ligation cloning techniques resulting in the plasmid pEM472. To study the effects of point mutations within YidC in complementation assays, the YidC mutants were cloned into pTrc99A-based plasmid pKAD107 (Kedrov et al, 2013). For the expression of the YidC mutants, the corresponding genes were cloned into pBAD-based plasmid (Kedrov et al, 2016).

For the IMP substrate, the DNA encoding 86 amino acids of *E. coli* NuoK and the C-terminal extension containing the HA-tag for immunodetection, a linker (GGSG) and the SecM* stalling sequence (FSTPVIWWPPIRGPP) (Gersteuer et al, 2024) was synthesized (GenScript Biotech, Netherlands), and the construct was cloned into pRSET vector (Thermo Fisher Scientific) via Sali/HindIII restriction sites. The genes encoding shorter nascent chains were prepared by removing fragments of the initial plasmid corresponding to NuoK residues 71–86 for NuoK⁷⁰ and residues 49–86 for NuoK⁴⁸ via PCR, followed by blunt-end ligation. For the analysis in vivo, the sequences the genes encoding for the nascent chains were amplified by PCR, while introducing NheI and HindIII restriction sites, and the resulting PCR products were ligated into pET24a vector.

Isolation of ribosome-associated complexes from *E. coli* membranes

E. coli BL21(DE3) cells were transformed with the pET24a-based plasmids encoding for NuoK intermediates with SecM* stalling sequences, or empty pET24a vector. The cells were grown in LB medium supplemented with 50 µg/mL kanamycin at 37 °C while shaking at 180 rpm. Upon reaching OD₆₀₀ 0.8, protein expression was induced by 0.5 mM IPTG and carried out for 30 min. Cells were harvested, lysed using a microfluidizer (M-110P, Microfluidics Corp.), and the debris was removed by centrifugation at 18,000 × g for 10 min (SS34 rotor, Sorvall/Thermo Scientific). Membranes were isolated by ultracentrifugation at 42,000 rpm for 1 h (45 Ti rotor, Beckman Coulter). Membranes were then suspended in 50 mM Hepes-KOH pH 7.4, 150 mM KOAc, 25 mM Mg(OAc)₂, 5% glycerol, 200 µM tris(2-carboxyethyl) phosphine (TCEP), 1% n-dodecyl β-maltoside (DDM; Glycon Biochemicals GmbH) for 1 h at 4 °C. The solubilized material was loaded on sucrose cushion (50 mM Hepes-KOH pH 7.4, 150 mM KOAc, 25 mM Mg(OAc)₂, 0.05% DDM, 200 µM tris(2-carboxyethyl) phosphine (TCEP), 1.1 M sucrose) and centrifuged for 1 h at 110,000 g (AT3 rotor, Sorvall/Thermo Scientific). The supernatant was carefully removed, and the pellet resuspended in 50 mM Hepes-KOH pH 7.4, 150 mM KOAc, 25 mM Mg(OAc)₂, 5% glycerol, 200 µM tris(2-carboxyethyl) phosphine (TCEP), 0.05% DDM prior to loading on SDS-PAGE and subsequent Western blotting.

After SDS-PAGE and transfer onto a methanol-activated PVDF membrane, the membrane was blocked for 2 h at 20 °C in TBS-T buffer supplemented with 5% (w/v) milk powder. To detect YidC, polyclonal rabbit antibodies were raised against the YidC periplasmic domain (residues 26 to 331; Davids Biotechnologie GmbH). The serum containing the antibodies was diluted 1:1000 in TBS-T supplemented with 2% (w/v) bovine serum albumin (BSA), and incubated for 2 h at 20 °C. For detection, horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies was applied at 1:10,000 dilution in TBS-T complemented with 2% (w/v) BSA and incubated for an additional 2 h at 20 °C. Detection reaction was performed with the Clarity Western ECL substrate (Bio-Rad Laboratories) and imaged with the Amersham Imager 680 (GE Life Sciences).

Expression of the membrane insertases

E. coli C41(DE3)ΔompF-ΔacrAB strain (Kanonenberg et al, 2019) transformed with the pEM472 plasmid encoding for the SecYEG-

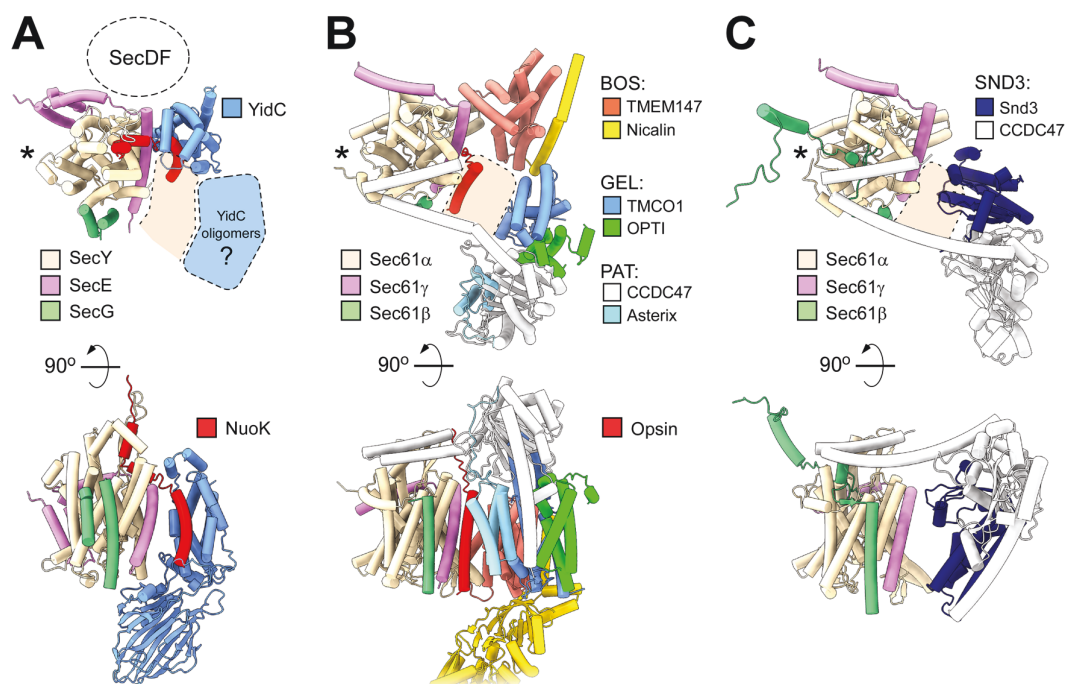


Figure 7. Comparison of the bacterial and eukaryotic IMP insertases.

(A) Cytoplasmic and side views on *E. coli* SecYEG-YidC assembly and the route of the NuoK nascent chain. The putative position of the accessory SecDF complex is indicated, based on a high-confidence AlphaFold3 prediction (Fig. EV1). A hypothetical position of YidC oligomers is shown in blue. The position of the lateral gate is indicated with an asterisk. (B) Cytoplasmic and side views on the eukaryotic multipass membrane protein translocon (MPT) with the subcomplexes and individual subunits indicated (PDB ID 7TUT). The partially resolved route of the opsin nascent chain is indicated. (C) Cytoplasmic and side views on the fungal Sec61-SND3 assembly formed in the absence of a client IMP (PDB ID 9I78).

YidC fusion construct was grown in LB medium supplemented with 100 µg/mL ampicillin at 37 °C while shaking at 180 rpm. Upon reaching OD₆₀₀ 0.6, expression of the SecYEG-YidC complex was induced by adding 0.5% arabinose (w/v) and carried out for 2.5 h. Cells were harvested and lysed using a microfluidizer (M-110P, Microfluidics Corp.), and the debris was removed by centrifugation at 18,000 × g for 10 min (SS34 rotor, Sorvall/Thermo Scientific). Membranes were isolated by ultracentrifugation at 42000 rpm for 1 h (45Ti rotor, Beckman Coulter). Membranes were then suspended in 50 mM Hepes-KOH pH 7.4, 150 mM KOAc, 5% glycerol, 200 µM tris(2-carboxyethyl)phosphine (TCEP), 1% DDM for 1 h at 4 °C. The solubilized material was centrifuged at 21,380 × g for 10 min at a tabletop centrifuge (Hermle Z 216 MK, Hermle Labortechnik GmbH) and the supernatant was incubated with Ni²⁺-NTA agarose resin (Macherey-Nagel GmbH & Co. KG) for 1 h at 4 °C. The resin was washed with 50 mM Hepes-KOH pH 7.4, 500 mM KOAc, 5% glycerol (v/v), 200 µM TCEP, 0.05% DDM, 10 mM imidazole and the target protein was eluted with 50 mM Hepes-KOH pH 7.4, 150 mM KOAc, 5% glycerol, 200 µM TCEP, 0.05% DDM, 300 mM imidazole. The elution fractions were concentrated and subjected to size exclusion chromatography (SEC) in 50 mM Hepes-KOH pH 7.4, 150 mM KOAc, 5% glycerol, 0.05% DDM using Superdex 200 Increase GL 10/300 column with ÄKTA Pure set-up (Cytiva). The protein concentration was determined spectrophotometrically (NeoDot, NeoBiotec) using the calculated extinction coefficient of 134,000 M⁻¹ cm⁻¹. Expression of YidC mutants, YidC^{D329A} and YidC^{D329R}, was achieved using

pBAD-based plasmid and the proteins were solubilized and purified in DDM according to the previously published protocol (Kedrov et al, 2016).

Assembly of SecYEG-YidC nanodiscs

To prepare the nanodiscs, liposomes were first formed using 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC; 70 mol %) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1-rac-glycerol) (POPG; 30 mol %) (Avanti Polar Lipids, Inc). POPC was chosen as a zwitterionic component to substitute phosphatidylethanolamine (PE) naturally occurring in *E. coli* membranes. Due to its cylindrical shape, POPC readily forms planar membranes, and so ensures higher homogeneity among formed nanodiscs. Furthermore, both POPC and POPG manifest order-disorder transition below 0 °C, in contrast to POPE (transition temperature at 25 °C), so the composite lipid bilayer should remain in the fluid state along nanodiscs formation. The lipids were mixed from chloroform stocks to achieve the desired ratio, the solvent was evaporated and the liposomes were prepared, as described (Kater et al, 2019). The detergent-purified SecYEG-YidC complexes were reconstituted in MSP2N2-based nanodiscs in a protein:MSP:lipid molar ratio of 1:4:400 following the published protocol (Kater et al, 2019). After forming the nanodiscs, the linker connecting YidC and SecE was cleaved by HRV-3C protease for 1 h at 4 °C and SEC was performed in 50 mM HEPES-KOH pH 7.4, 150 mM KOAc, 5% glycerol using Superose 6 Increase GL 10/300 column with ÄKTA Pure set-up.

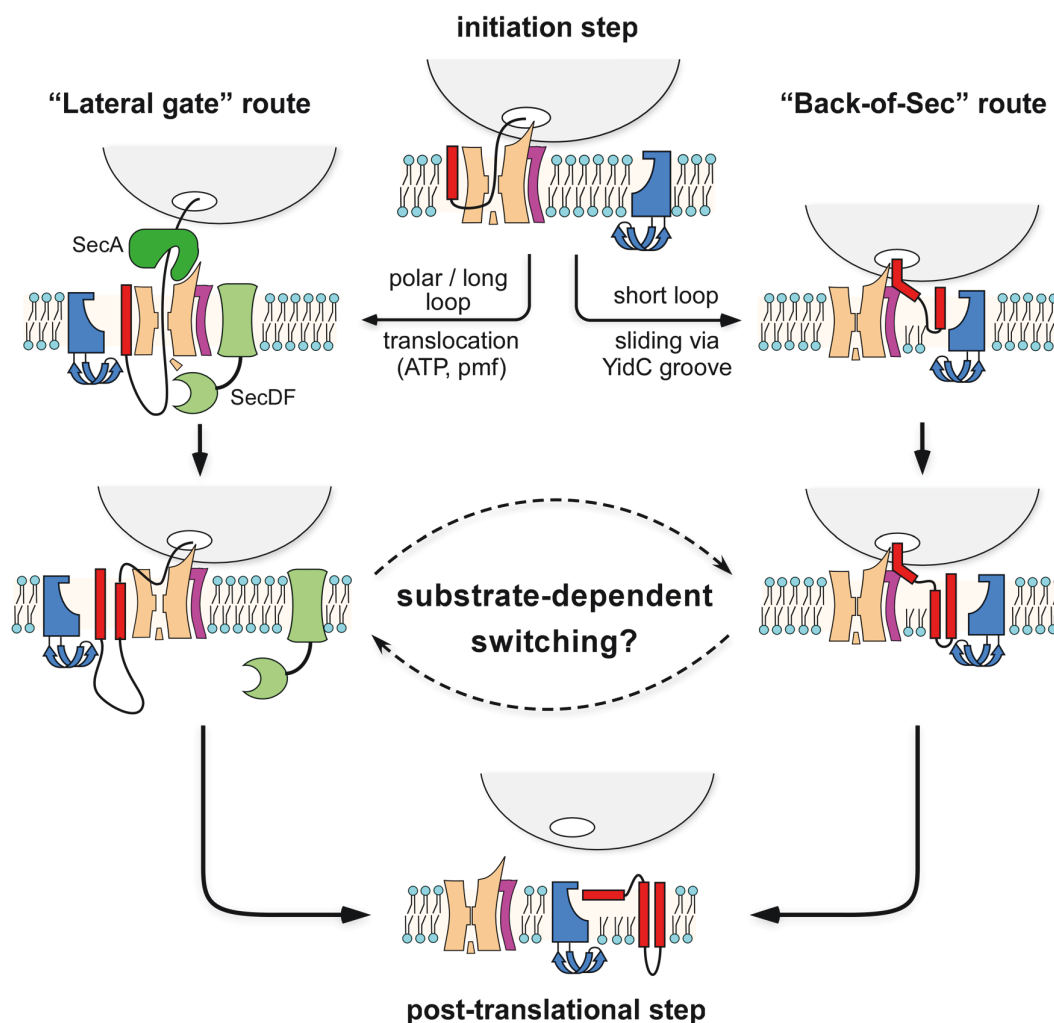


Figure 8. Routes for multipass membrane protein insertion via the bacterial SecYEG-YidC complex.

After the SRP-mediated targeting and insertion of the first TMH (here, shown for SecYEG only), membrane partitioning of the following TMHs may occur either via the lateral gate or via the "back-of-Sec" route. The length and the composition of the extramembrane domains determine the biogenesis pathway: SecA/SecDF-mediated translocation of the periplasmic loops requires widening of the lateral gate, and so likely favors the corresponding insertion route. YidC may interact with SecYEG at either of the interfaces to serve as a chaperone, but also assist in insertion of the nascent proteins. Once the translation is completed and the ribosome detaches from SecYEG, insertion of the last TMH occurs post-translationally, being assisted by YidC.

SEC fractions containing SecYEG-YidC nanodiscs were concentrated to $\sim 10 \mu\text{M}$ using Amicon Ultra-4, Ultracel 30 K centrifugal filters (Merck/Millipore). Mass photometry measurements were performed using Two^{MP} instrument (Refeyn Ltd.) calibrated using β -amylase present in various oligomeric states, with the molecular masses ranging from 56 kDa (monomer) to 224 kDa (tetramer).

Cell-free protein synthesis

E. coli S30 extract for CFPS was prepared based on previously published protocols (Zubay, 1973). Briefly, *E. coli* BL21(DE3) cells were transformed with TargoTron pAR1219 plasmid (Sigma-Aldrich) encoding for T7 RNA polymerase. 2 L of 2x YPTG media was inoculated with 100 mL overnight cultures, the cells were grown to OD_{600} 0.5, and the T7 RNA polymerase expression was

induced with 1 mM IPTG. The cells were further grown to reach OD_{600} 1.0 and then they were harvested at 7500 rpm for 15 min (FiberLite F8-6x1000y rotor, Piramoon Technologies Inc.). The cell pellet was washed three times with 10 mM Tris-acetate pH 8, 60 mM KOAc, 14 mM $\text{Mg}(\text{OAc})_2$, and 1 mM PMSF, and the pellet was resuspended in the same buffer at the ratio of 1 mL per 1 g pellet. Subsequently, cells were lysed by sonication (10 times, 15 s on, 30 s off, 50% power, 5 pulsed cycles) (Sonopuls GM2200, Bandelin). The lysate was cleared via two-steps centrifugation, $12,000 \times g$ for 15 min and $30,000 \times g$ for 30 min (S120 AT6 rotor, Sorvall/Thermo Fisher). The supernatant was aliquoted and stored at -75°C .

The CFPS reaction was composed of 40% S30 extract, the master mix (10 mM ammonium acetate, 130 mM KOAc, 33 mM sodium pyruvate, 1.5 mM spermidine, 1 mM putrescine, 4 mM sodium

oxalate, 1.2 mM ATP, 0.85 mM of GTP, CTP and UTP, 34 µg/ml folinic acid, 170.6 µg/mL of *E. coli* tRNA MRE 600 (Roche Diagnostics GmbH), 0.33 mM NAD⁺, 0.26 mM coenzyme A and 2 mM of each amino acid), and Mg(OAc)₂. The optimal concentration of Mg(OAc)₂ was identified for each new batch of the S30 extract upon screening within the range of 0 to 12 mM, while using synthesis of the yellow fluorescent protein as a read-out. For NuoK synthesis, at least 7 ng/µL plasmid DNA encoding the nascent chain was added to the reaction, as well as at least 100 nM of the nanodisc-reconstituted SecYEG-YidC. CFPS reactions were performed at 37 °C for 1 h while shaking at 450 rpm. The synthesis/stalling was evaluated via Western blotting using monoclonal antibodies against the HA-tag (sc-7392, Santa Cruz Biotechnology).

Isolation of ribosomes from CFPS reactions

To isolate the ribosomes, 10–40% linear sucrose gradients were formed in SW40 tubes (Beckman Coulter) using the Gradient Station (BioComp Instruments). CFPS reactions (100 µL) were loaded on top of the gradients and the samples were centrifuged for 16 h at 16,500 rpm (SW40 Ti rotor; Beckman Coulter). The gradients were fractionated using the Gradient Station while monitoring the absorbance at 280 nm (*A*₂₈₀). The peaks with the ribosomal fractions occurring in the sucrose concentration range of 20–25% were pooled together and incubated with Ni²⁺-NTA agarose resin (Macherey-Nagel GmbH & Co. KG) for 1 h at 4 °C. The resin was washed with 50 mM Hepes-KOH pH 7.4, 500 mM KOAc, 25 mM Mg(OAc)₂, 10 mM imidazole. The complexes were eluted with 50 mM Hepes-KOH pH 7.4, 150 mM KOAc, 25 mM Mg(OAc)₂ supplemented with 300 mM imidazole, and the eluate was concentrated using Amicon Ultra-4 Ultracel 30 K centrifugal filters (Merck/Millipore) while exchanging the buffer to 50 mM Hepes-KOH pH 7.4, 150 mM KOAc, 25 mM Mg(OAc)₂. The presence of the ribosome-bound nascent chain was confirmed by immunoblotting against the HA-tag. The concentration of ribosomes was then estimated by measuring the absorbance at 260 nm (*A*₂₆₀).

In order to improve the resolution of the cryo-EM data on NuoK⁸⁶-RNC:SecYEG-YidC and NuoK⁷⁰-RNC:SecYEG-YidC, glutaraldehyde was added to the purified samples. The sample was diluted 5-fold to prevent inter-particle cross-linking, and it was then incubated with 0.1% glutaraldehyde (v/v) for 15 min on ice and quenched by 100 mM Tris-HCl pH 7.5. Afterwards, the sample was concentrated using Amicon Ultra-4 Ultracel 30 K filters, flash-frozen and stored at –75 °C before grid preparation.

Cryo-EM sample preparation and data collection

Isolated NuoK-RNC:SecYEG-YidC samples were supplemented with (1H, 1H, 2H, 2H-perfluorooctyl)-β-D-maltopyranoside (FOM, Anatrace) to a final concentration of 0.03% to favor random orientation of the particles and plunge frozen. For each grid, 3.5 µL of the sample was applied onto glow-discharged Quantifoil Cu 300 mesh R3/3 grids with an additional 2 nm layer of carbon. After a waiting time of 45 s, the grids were blotted for 3 s and plunge frozen in liquid ethane at 4 °C and 100% humidity using a Vitrobot Mark IV (Thermo Fisher Scientific). Data collection for RNC-NuoK:SecYEG-YidC samples was performed at 300 keV using a Titan Krios

microscope equipped with a Falcon 4i direct electron detector and a SelectrisX imaging filter (all Thermo Fisher Scientific) at a pixel size of 0.727 Å. Dose-fractionated movies were collected in a defocus range from –0.5 to 3.5 µm and with a total dose of 60 e[–] per Å², fractionated in 60 frames to obtain a total dose of 1 e[–] per Å² per frame. Gain correction, movie alignment and summation of movie frames were performed using MotionCor2 (Zheng et al, 2017). Further processing, including CTF estimation, was carried out in cryoSPARC v4.4 (Punjani et al, 2017).

Data processing

For the cross-linked NuoK⁸⁶-RNC:SecYEG-YidC complex, 27,660 micrographs were selected. Blob Picker was used to pick 1,420,623 particles which were sorted by 2D classification, yielding a subset of 636,995 particles. An ab initio job with 2 classes was run to further clean the particle set. A consensus refinement of 582,663 particles resulted in a map of 70S with clear extra density below the exit tunnel. A soft mask covering this region (accounting for nanodisc, SecYEG-YidC and eventually the NuoK nascent chain) was used to sort the particles into 6 classes, using a 3D Classification job. Two of the classes, representing a total of 221,144 particles, displayed a strong SecYEG and YidC density. The class with best resolved nascent chain density containing 113,368 particles (19.5%) was selected and refined to a final resolution of 2.44 Å. Local refinement was performed on the tunnel exit region yielding a map with a final resolution of 3.76 Å. This map was used to build the atomic model for the NuoK:SecYEG-YidC assembly (PDB: 9RBF; EMDB:53892; EMDB:53893 (local refinement); EMDB:53894 (composite map)). Data processing for this dataset is summarized in Fig. EV2.

For the non-cross-linked NuoK⁸⁶-RNC:SecYEG-YidC assembly, 10,234 micrographs were selected and manually curated. A total of 693,102 particles was picked with Blob Picker and sorted by 2D classification to generate templates for the Template Picker job. From 2,300,222 template-picked particles, 406,978 were selected after 2D classification. An ab initio job (three classes) allowed further cleaning of the dataset. A consensus refinement with a set of 217,418 particles resulted in a map of 70S ribosome with clear extra density below the exit tunnel. Focused sorting below the exit tunnel region into five classes yielded a class of 33,912 particles showing density for the extramembrane P1 domain of YidC. These particles were used to train a TOPAZ picking model, which resulted in 360,610 particles. The particles were further curated and focused sorted into a final class of 70,670 particles. This class was refined to a final resolution of 2.73 Å (EMD-53587). A local refinement in the SecYEG-YidC region yielded a map with a final resolution of 3.19 Å (EMD-53589). Data processing for this dataset is summarized in Appendix Fig. S6.

For the NuoK⁷⁰-RNC:SecYEG-YidC complex, an initial set of 1,145,565 particles was picked using Blob Picker based on 31,572 micrographs. A final set of 956,488 particles was selected after 2D classification for homogeneous refinement, using an initial map of the 70S ribosomes obtained by an ab initio reconstruction with two classes. Next, 3D classification was performed using a mask for the nanodisc region, resulting in two classes that differed by the presence of YidC. The SecYEG-YidC class (529,825 particles) was further sorted into three classes using a 3D classification job with a mask in the nanodisc region. This resulted in a class of 113,106 particles (21.7%) with well-resolved YidC features. Using a mask

around YidC, those particles were further sub-sorted into a class containing only SecYEG and a class representing the SecYEG-YidC assembly (58,063 particles). The SecYEG-YidC class was refined yielding a final map with 2.85 Å resolution. Local refinement with a mask for the nanodisc region yielded a map with a final resolution of 2.90 Å. These maps were used to build an atomic model for the NuoK⁷⁰-RNC:SecYEG-YidC complex (PDB: 9T5X; EMDB:55570; EMDB:55571 (local refinement); EMDB:55598 (composite map)). Data processing for this dataset is summarized in Appendix Fig. S9.

For the NuoK^{86mut}-RNC:SecYEG-YidC complex assembled upon NuoK^{E36K, E72K} synthesis Blob Picker was used to pick an initial set of 586,721 particles from 9099 micrographs. The particles were curated and after 2D classification, a subset of 287,399 particles was used to generate an ab initio reconstruction (two classes) of 70S ribosomes with extra density for the insertase complex. The same set was further used to generate templates for template picking, which resulted in 2,057,403 particles, from which a final set of 56,980 particles was obtained after extensive 2D and 3D classification. The particles were fed into a TOPAZ training job, obtaining 433,249 particles after picking and extraction. These particles were used to generate a consensus refinement and focused sorted into eight classes. The class with the best resolved insertase region, comprising 48,952 particles, was further refined to generate a reconstruction with final resolution of 2.87 Å (EMD-53584). A local refinement with a mask in the translocon region generated a map of 3.20 Å (EMD-53585). Data processing for this dataset is summarized in Appendix Fig. S10.

For the NuoK⁴⁸-RNC:SecYEG-YidC complex assembled upon synthesis of the early NuoK intermediate, an initial set of 741,311 particles was picked using a Blob Picker job based on 21,440 micrographs. The particles were cleaned by 2D classification jobs to yield a final 511,016 particle set. An ab initio reconstruction (two classes) job was used to generate an initial map of the ribosome-bound SecYEG in the nanodisc. Successive, focused 3D-classifications from a consensus refinement were used to obtain a final class of 66,486 particles. These particles were refined to a final resolution of 2.62 Å (EMD-53560). A local refinement with a mask in the translocon region resulted in a reconstruction of 2.90 Å (EMD-53568). Data processing for this dataset is summarized in Appendix Fig. S11.

For visualization of all cryo-EM maps, consensus maps were generated consisting of isolated density for the 70S ribosome from the global refinements and isolated density for the nanodisc-embedded insertase from the local refinements. Statistics to cryo-EM data collection and model refinement is provided in Appendix Table S2.

Model building and refinement

The molecular model for the cross-linked NuoK⁸⁶-RNC:SecYEG-YidC was built as follows: For the 70S ribosome a previously released model based of high-resolution cryo-EM maps of a 70S ribosome and a SecM-stalled RNC were used as templates (PDB IDs 7K00, 8QOA) (Gersteuer et al, 2024; Watson et al, 2020). The model for the tRNA-Gly (in SecM) was changed to tRNA-Pro and the mRNA model was adjusted from GCU-GGC-CCU (Ala-Gly-Pro in SecM) to GGU-CCU-CCG (Gly-Pro-Pro in SecM*). A model for the SecYEG-YidC assembly was generated based on the rigid body fitting the AlphaFold3 prediction of this complex (see also Appendix Fig. S4) that was fitted into the locally refined cryo-EM density with only minor adjustments (see also

Fig. EV2). For the nascent chain, the modified SecM* sequence could be modeled de novo based on well-resolved density for this region. Less-resolved density was present to fit the backbone and a few bulky side chains for major parts of glycine-serine linker and the HA-tag. NuoK TMHs 2 and 3 were identified based on the rod-like shape of extra density present at the mouth of the exit tunnel and between SecY and YidC. While at the given resolution we cannot be sure about the exact register, we positioned residues 73–82 of NuoK THM 3 into the corresponding density supporting the predicted α -helical conformation. 70S ribosome and SecYEG-YidC atomic models were processed independently in multiple rounds of manual real-space refinement in Coot v0.9.8.95 (Emsley and Cowtan, 2004). The models were later merged and further refined in Phenix v1.20.1-4487 (Adams et al, 2010). The NuoK⁸⁶-RNC:SecYEG-YidC model was then used as a template to build a model for the cross-linked NuoK⁸⁶-RNC:SecYEG and the cross-linked NuoK⁷⁰-RNC:SecYEG-YidC structures. For the former, the YidC model as well as the section of NuoK not visible in the map was deleted from the template, for the latter only the NuoK sequence was adjusted accordingly. Both models were real-space refined in Coot and Phenix. The Molprobit tool was used for model validation. Visualization was done in UCSF ChimeraX v1.9 (Goddard et al, 2018).

Complementation assay

The complementation assay was prepared as described before (Kedrov et al, 2013). Briefly, a single colony of FTL10 cells (Hatzixanthos et al, 2003) transformed with either YidC-encoding plasmid or empty pTrc99A vector was grown in LB medium with 0.2% arabinose, 25 µg/mL kanamycin and 100 µg/mL ampicillin for 16 h at 37 °C at 180 rpm. The overnight cultures were diluted to OD₆₀₀ of 0.05 and grown until the early logarithmic phase before diluting them all to OD₆₀₀ of 0.1 and doing a subsequent serial dilution. 5 µL of each diluted culture were transferred on the plates (LB agar supplemented with 25 µg/mL kanamycin, 100 µg/mL ampicillin and either 0.2% arabinose or 0.2% glucose). The plates were incubated at 37 °C for 16 h or at 25 °C for 24 h and photographed with the Amersham Imager 680 (GE Life Sciences). For visualization purposes, brightness levels were adjusted with GIMP 2.10 software.

To test the expression of YidC mutants in *E. coli* FTL 10 strain under permissive conditions, the cells were transformed with pTrc99A-based plasmids encoding for His-tagged YidC variants (Kedrov et al, 2013) and grown overnight at 37 °C in LB medium supplemented with 25 µg/mL kanamycin, 100 µg/mL ampicillin and 0.2% arabinose. The cultures were adjusted to the same optical density, and the cells were lysed in SDS-PAGE sample buffer. Subsequent anti-His Western blot analysis (primary antibodies DIA-900-200, Dianova) confirmed expression of all tested YidC^{His} variants.

Data availability

The cryo-EM structural data have been deposited in the Electron Microscopy Data Base (EMDB) and the Protein Data Bank (PDB) repositories under the accession numbers EMD-53892, EMD-53893, EMD-53894 and PDB-9RBF (NuoK⁸⁶-RNC:SecYEG-YidC (cross-linked)); EMD-55570, EMD-55571, EMD-55598 and PDB-9T5X (NuoK⁷⁰-RNC:SecYEG-YidC (cross-linked)); EMD-53587

and EMD-53589 (NuoK⁸⁶-RNC:SecYEG-YidC); EMD-53584 and EMD-53585 (NuoK^{86mut}-RNC:SecYEG-YidC); EMD-53560 and EMD-53568 (NuoK⁴⁸-RNC:SecYEG-YidC).

The source data of this paper are collected in the following database record: [biostudies:S-SCDT-10_1038-S44318-026-00837-6](https://doi.org/10.1038/s44318-026-00837-6).

Expanded view data, supplementary information, appendices are available for this paper at <https://doi.org/10.1038/s44318-026-00837-6>.

Peer review information

A peer review file is available at <https://doi.org/10.1038/s44318-026-00837-6>

References

- Adams PD, Afonine PV, Bunkoczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung LW, Kapral GJ, Grosse-Kunstleve RW et al (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* 66:213–221
- Anghel SA, McGilvray PT, Hegde RS, Keenan RJ (2017) Identification of Oxa1 homologs operating in the eukaryotic endoplasmic reticulum. *Cell Rep* 21:3708–3716
- Aviram N, Ast T, Costa EA, Arakel EC, Chuartzman SG, Jan CH, Hassdenteufel S, Dudek J, Jung M, Schorr S et al (2016) The SND proteins constitute an alternative targeting route to the endoplasmic reticulum. *Nature* 540:134–138
- Bischoff L, Wickles S, Berninghausen O, van der Sluis EO, Beckmann R (2014) Visualization of a polytopic membrane protein during SecY-mediated membrane insertion. *Nat Commun* 5:4103
- Botte M, Zaccari NR, Nijeholt JL, Martin R, Knoop K, Papai G, Zou J, Deniaud A, Karuppusamy M, Jiang Q et al (2016) A central cavity within the holo-translocon suggests a mechanism for membrane protein insertion. *Sci Rep* 6:38399
- Chen Y, Capponi S, Zhu L, Gellenbeck P, Freitas JA, White SH, Dalbey RE (2017) YidC insertase of *Escherichia coli*: water accessibility and membrane shaping. *Structure* 25:1403–1414.e1403
- Chiba K, Mori H, Ito K (2002) Roles of the C-terminal end of SecY in protein translocation and viability of *Escherichia coli*. *J Bacteriol* 184:2243–2250
- Cymer F, Hedman R, Ismail N, von Heijne G (2015a) Exploration of the arrest peptide sequence space reveals arrest-enhanced variants. *J Biol Chem* 290:10208–10215
- Cymer F, von Heijne G, White SH (2015b) Mechanisms of integral membrane protein insertion and folding. *J Mol Biol* 427:999–1022
- du Plessis DJ, Berrelkamp G, Nouwen N, Driessen AJ (2009) The lateral gate of SecYEG opens during protein translocation. *J Biol Chem* 284:15805–15814
- Emsley P, Cowtan K (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 60:2126–2132
- Gemmer M, Chaillet ML, van Loenhout J, Cuevas Arenas R, Vismpas D, Grollers-Mulderij M, Koh FA, Albanese P, Scheltema RA, Howes SC et al (2023) Visualization of translation and protein biogenesis at the ER membrane. *Nature* 614:160–167
- Gerstner F, Morici M, Gabrielli S, Fujiwara K, Safdari HA, Paternoga H, Bock LV, Chiba S, Wilson DN (2024) The SecM arrest peptide traps a pre-peptide bond formation state of the ribosome. *Nat Commun* 15:2431
- Goddard TD, Huang CC, Meng EC, Pettersen EF, Couch GS, Morris JH, Ferrin TE (2018) UCSF ChimeraX: meeting modern challenges in visualization and analysis. *Protein Sci* 27:14–25
- Haque ME, Spremulli LL, Fecko CJ (2010) Identification of protein-protein and protein-ribosome interacting regions of the C-terminal tail of human mitochondrial inner membrane protein Oxa1L. *J Biol Chem* 285:34991–34998
- Hatzixanthis K, Palmer T, Sargent F (2003) A subset of bacterial inner membrane proteins integrated by the twin-arginine translocase. *Mol Microbiol* 49:1377–1390
- Itskanov S, Park E (2023) Mechanism of protein translocation by the Sec61 translocon complex. *Cold Spring Harb Perspect Biol* 15:a041250
- Jauss B, Petriman NA, Drepper F, Franz L, Sachelaru I, Welte T, Steinberg R, Warscheid B, Koch HG (2019) Noncompetitive binding of PpiD and YidC to the SecYEG translocon expands the global view on the SecYEG interactome in *Escherichia coli*. *J Biol Chem* 294:19167–19183
- Jomaa A, Boehringer D, Leibundgut M, Ban N (2016) Structures of the *E. coli* translating ribosome with SRP and its receptor and with the translocon. *Nat Commun* 7:10471
- Kalinin IA, Peled-Zehavi H, Barshap ABD, Tamari SA, Weiss Y, Nevo R, Fluman N (2024) Features of membrane protein sequence direct post-translational insertion. *Nat Commun* 15:10198
- Kanonenberg K, Royes J, Kedrov A, Poschmann G, Angius F, Solgadi A, Spitz O, Kleinschrodt D, Stuhler K, Miroux B et al (2019) Shaping the lipid composition of bacterial membranes for membrane protein production. *Micro Cell Fact* 18:131
- Kater L, Frieg B, Berninghausen O, Gohlke H, Beckmann R, Kedrov A (2019) Partially inserted nascent chain unzips the lateral gate of the Sec translocon. *EMBO Rep* 20:e48191
- Kedrov A, Sustarsic M, de Keyser J, Caumanns JJ, Wu ZC, Driessen AJ (2013) Elucidating the native architecture of the YidC: ribosome complex. *J Mol Biol* 425:4112–4124
- Kedrov A, Wickles S, Crevenna AH, van der Sluis EO, Buschauer R, Berninghausen O, Lamb DC, Beckmann R (2016) Structural dynamics of the YidC:ribosome complex during membrane protein biogenesis. *Cell Rep* 17:2943–2954
- Kempf N, Remes C, Ledesch R, Zuchner T, Hofig H, Ritter I, Katranidis A, Fitter J (2017) A novel method to evaluate ribosomal performance in cell-free protein synthesis systems. *Sci Rep* 7:46753
- Koch S, Driessen AJM, Kedrov A (2018) Biophysical analysis of Sec-mediated protein translocation in nanodiscs. *Adv Biomembr Lipid Self-Assem* 28:41–85
- Kohler R, Boehringer D, Greber B, Bingel-Erlenmeyer R, Collinson I, Schaffitzel C, Ban N (2009) YidC and Oxa1 form dimeric insertion pores on the translating ribosome. *Mol Cell* 34:344–353
- Kumazaki K, Chiba S, Takemoto M, Furukawa A, Nishiyama K, Sugano Y, Mori T, Dohmae N, Hirata K, Nakada-Nakura Y et al (2014a) Structural basis of Sec-independent membrane protein insertion by YidC. *Nature* 509:516–520
- Kumazaki K, Kishimoto T, Furukawa A, Mori H, Tanaka Y, Dohmae N, Ishitani R, Tsukazaki T, Nureki O (2014b) Crystal structure of *Escherichia coli* YidC, a membrane protein chaperone and insertase. *Sci Rep* 4:7299
- Li Z, Boyd D, Reindl M, Goldberg MB (2014) Identification of YidC residues that define interactions with the Sec Apparatus. *J Bacteriol* 196:367–377
- Lu Y, Turnbull IR, Bragin A, Carveth K, Verkman AS, Skach WR (2000) Reorientation of aquaporin-1 topology during maturation in the endoplasmic reticulum. *Mol Biol Cell* 11:2973–2985
- McDowell MA, Heimes M, Sinning I (2021) Structural and molecular mechanisms for membrane protein biogenesis by the Oxa1 superfamily. *Nat Struct Mol Biol* 28:234–239
- Miyazaki R, Ai M, Tanaka N, Suzuki T, Dohmae N, Tsukazaki T, Akiyama Y, Mori H (2022) Inner membrane YfgM-PpiD heterodimer acts as a functional unit that associates with the SecY/E/G translocon and promotes protein translocation. *J Biol Chem* 298:102572

- Ou X, Ma C, Sun D, Xu J, Wang Y, Wu X, Wang D, Yang S, Gao N, Song C et al (2025) SecY translocon chaperones protein folding during membrane protein insertion. *Cell* 188:1912–1924.e13
- Page KR, Nguyen VN, Pleiner T, Tomaleri GP, Wang ML, Guna A, Hazu M, Wang TY, Chou TF, Voorhees RM (2024) Role of a holo-insertase complex in the biogenesis of biophysically diverse ER membrane proteins. *Mol Cell* 84:3302–3319.e3311
- Petriman NA, Jauss B, Hufnagel A, Franz L, Sachelar I, Drepper F, Warscheid B, Koch HG (2018) The interaction network of the YidC insertase with the SecYEG translocon, SRP and the SRP receptor FtsY. *Sci Rep* 8:578
- Price CE, Driessen AJ (2008) YidC is involved in the biogenesis of anaerobic respiratory complexes in the inner membrane of *Escherichia coli*. *J Biol Chem* 283:26921–26927
- Price CE, Driessen AJM (2010) Conserved negative charges in the transmembrane segments of subunit K of the NADH:ubiquinone oxidoreductase determine its dependence on YidC for membrane insertion. *J Biol Chem* 285:3575–3581
- Punjani A, Rubinstein JL, Fleet DJ, Brubaker MA (2017) cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. *Nat Methods* 14:290–296
- Ravaud S, Stjepanovic G, Wild K, Sinning I (2008) The crystal structure of the periplasmic domain of the *Escherichia coli* membrane protein insertase YidC contains a substrate binding cleft. *J Biol Chem* 283:9350–9358
- Ritchie TK, Grinkova YV, Bayburt TH, Denisov IG, Zolnerchiks JK, Atkins WM, Sligar SG (2009) Chapter 11 - Reconstitution of membrane proteins in phospholipid bilayer nanodiscs. *Methods Enzymol* 464:211–231
- Sachelar I, Petriman NA, Kudva R, Kuhn P, Welte T, Knapp B, Drepper F, Warscheid B, Koch HG (2013) YidC occupies the lateral gate of the SecYEG translocon and is sequentially displaced by a nascent membrane protein. *J Biol Chem* 288:16295–16307
- Seppala S, Slusky JS, Lloris-Garcera P, Rapp M, von Heijne G (2010) Control of membrane protein topology by a single C-terminal residue. *Science* 328:1698–1700
- Seurig M, Ek M, von Heijne G, Fluman N (2019) Dynamic membrane topology in an unassembled membrane protein. *Nat Chem Biol* 15:945–948
- Smalinskaite L, Hegde RS (2023) The biogenesis of multipass membrane proteins. *Cold Spring Harb Perspect Biol* 15:a041251
- Smalinskaite L, Kim MK, Lewis AJO, Keenan RJ, Hegde RS (2022) Mechanism of an intramembrane chaperone for multipass membrane proteins. *Nature* 611:161–166
- Sohlenkamp C, Geiger O (2016) Bacterial membrane lipids: diversity in structures and pathways. *FEMS Microbiol Rev* 40:133–159
- Soman R, Yuan J, Kuhn A, Dalbey RE (2014) Polarity and charge of the periplasmic loop determine the YidC and sec translocase requirement for the M13 procoat lep protein. *J Biol Chem* 289:1023–1032
- Steinberg R, Knupffer L, Origi A, Asti R, Koch HG (2018) Co-translational protein targeting in bacteria. *FEMS Microbiol Lett* 365:fny095
- Sundaram A, Li Q, Wan Y, Tang J, Wu H, Smalinskaite L, Hegde RS, Ji Z, Keenan RJ (2025) Global analysis of translocon remodeling during protein synthesis at the ER. *Nat Struct Mol Biol* 32:2517–2525
- Sundaram A, Yamsek M, Zhong F, Hooda Y, Hegde RS, Keenan RJ (2022) Substrate-driven assembly of a translocon for multipass membrane proteins. *Nature* 611:167–172
- Tsukazaki T (2018) Structure-based working model of SecDF, a proton-driven bacterial protein translocation factor. *FEMS Microbiol Lett* 365:fny112
- Tsukazaki T (2019) Structural basis of the Sec translocon and yidC revealed through X-ray crystallography. *Protein J* 38:249–261
- Urbanus ML, Froderberg L, Drew D, Bjork P, de Gier JW, Brunner J, Oudega B, Luijck J (2002) Targeting, insertion, and localization of *Escherichia coli* YidC. *J Biol Chem* 277:12718–12723
- von Heijne G (2007) The membrane protein universe: what's out there and why bother? *J Intern Med* 261:543–557
- Voorhees RM, Hegde RS (2016) Structure of the Sec61 channel opened by a signal sequence. *Science* 351:88–91
- Watson ZL, Ward FR, Meheust R, Ad O, Schepartz A, Banfield JF, Cate JH (2020) Structure of the bacterial ribosome at 2 Å resolution. *Elife* 9:e60482
- White SH, Wimley WC (1999) Membrane protein folding and stability: physical principles. *Annu Rev Biophys Biomol Struct* 28:319–365
- Wickles S, Singharoy A, Andreani J, Seemayer S, Bischoff L, Berninghausen O, Soeding J, Schulten K, van der Sluis EO, Beckmann R (2014) A structural model of the active ribosome-bound membrane protein insertase YidC. *Elife* 3:e03035
- Wirth JO, Scheiderer L, Engelhardt T, Engelhardt J, Matthias J, Hell SW (2023) MINFLUX dissects the unimpeded walking of kinesin-1. *Science* 379:1004–1010
- Woodall NB, Hadley S, Yin Y, Bowie JU (2017) Complete topology inversion can be part of normal membrane protein biogenesis. *Protein Sci* 26:824–833
- Wu H, Smalinskaite L, Hegde RS (2024) EMC rectifies the topology of multipass membrane proteins. *Nat Struct Mol Biol* 31:32–41
- Wu ZC, de Keyser J, Kedrov A, Driessen AJ (2012) Competitive binding of the SecA ATPase and ribosomes to the SecYEG translocon. *J Biol Chem* 287:7885–7895
- Yang TJ, Mukherjee S, Langer JD, Hummer G, McDowell MA (2025) SND3 is the membrane insertase within a distinct SEC61 translocon complex. *Nat Commun* 16:9566
- Yuan J, Phillips GJ, Dalbey RE (2007) Isolation of cold-sensitive yidC mutants provides insights into the substrate profile of the YidC insertase and the importance of transmembrane 3 in YidC function. *J Bacteriol* 189:8961–8972
- Zheng SQ, Palovcak E, Armache JP, Verba KA, Cheng Y, Agard DA (2017) MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. *Nat Methods* 14:331–332
- Zhu L, Kaback HR, Dalbey RE (2013) YidC protein, a molecular chaperone for LacY protein folding via the SecYEG protein machinery. *J Biol Chem* 288:28180–28194
- Zubay G (1973) In vitro synthesis of protein in microbial systems. *Annu Rev Genet* 7:267–287

Acknowledgements

The authors would like to thank Hanna Kratzat, Susanne Rieder and Charlotte Ungewickl for the support with the cryo-EM collection and initial data analysis, Joanna Musial for assistance with cloning and biochemical characterization of the SecYEG-YidC construct, Laura Czech and Gert Bange for the support upon the development of CFPS procedure, and Arnold J.M. Driessen and Florian Altegoer for discussions and valuable advices. The work was funded by German Research Foundation (DFG, grants Ke1879/3 and Collaborative Research Center 1208, project A10 to AK) and European Research Council (Advanced Grant “CryoTranslation” to RB).

Author contributions

Max Busch: Data curation; Formal analysis; Investigation; Visualization; Writing—original draft; Writing—review and editing. **Cristian Rosales-Hernandez:** Data curation; Formal analysis; Investigation; Visualization; Methodology; Writing—original draft; Writing—review and editing. **Michael Kamel:** Conceptualization; Formal analysis; Investigation; Methodology; Writing—review and editing. **Yulia Schaumkessel:** Investigation; Methodology; Writing—review and editing. **Eli O van der Sluis:** Resources; Supervision; Investigation; Methodology; Writing—review and editing. **Otto Berninghausen:** Data curation; Validation; Investigation; Writing—review and editing. **Thomas Becker:** Data curation; Formal analysis; Supervision; Visualization; Writing—

review and editing. **Roland Beckmann:** Conceptualization; Funding acquisition; Project administration; Writing—review and editing. **Alexej Kedrov:** Conceptualization; Supervision; Funding acquisition; Visualization; Methodology; Writing—original draft; Project administration; Writing—review and editing.

Source data underlying figure panels in this paper may have individual authorship assigned. Where available, figure panel/source data authorship is listed in the following database record: [biostudies:S-SCDT-10_1038-S44318-026-00837-6](https://www.ebi.ac.uk/biostudies/studies/S-SCDT-10_1038-S44318-026-00837-6).

Funding

Open Access funding enabled and organized by Projekt DEAL.

Disclosure and competing interests statement

The authors declare no competing interests.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and

reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. Creative Commons Public Domain Dedication waiver <http://creativecommons.org/publicdomain/zero/1.0/> applies to the data associated with this article, unless otherwise stated in a credit line to the data, but does not extend to the graphical or creative elements of illustrations, charts, or figures. This waiver removes legal barriers to the re-use and mining of research data. According to standard scholarly practice, it is recommended to provide appropriate citation and attribution whenever technically possible.

© The Author(s) 2026

Expanded View Figures

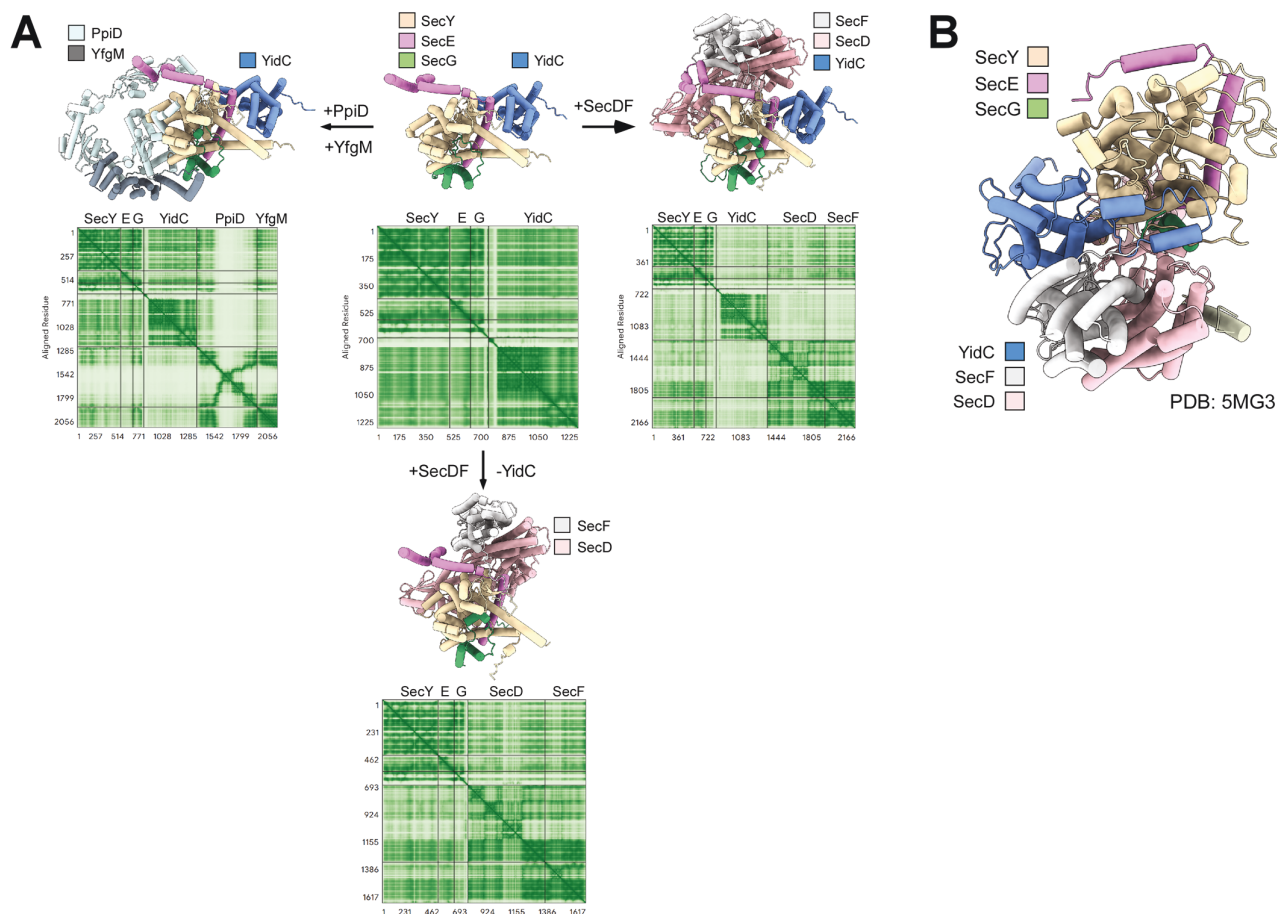


Figure EV1. Potential interaction network of *E. coli* SecYEG.

(A) AlphaFold3-based models and PAE plots of putative complexes formed by *E. coli* SecYEG with the accessory proteins. YidC is docked at the back of SecYEG with high confidence (low PAE levels), though co-assembly with SecDF negatively affects the model confidence for both YidC and SecDF. This may be explained by steric hindrance and partial overlap of the binding sites at SecYEG. Single-span protein YajC manifested random positions between the models, with high PAE scores, so it was excluded from analysis. The generated AlphaFold models are provided in the Dataset EV1. (B) Model of the SecYEG-YidC-SecDF assembly in detergent, based on the low-resolution cryo-EM reconstruction (PDB ID 5MG3).

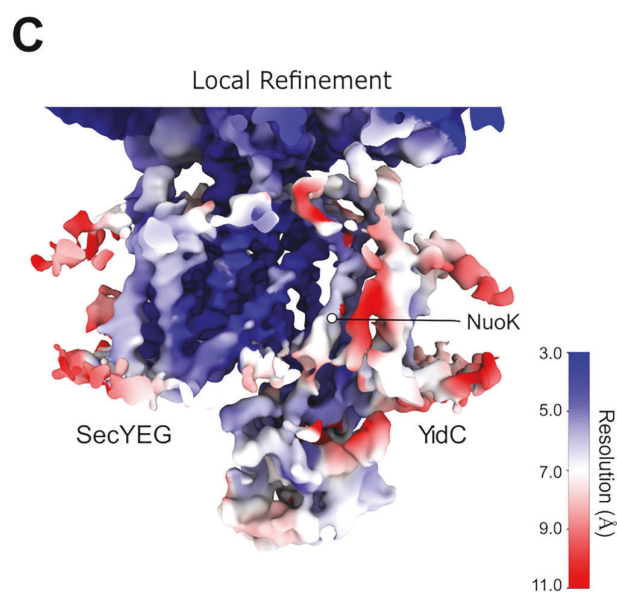
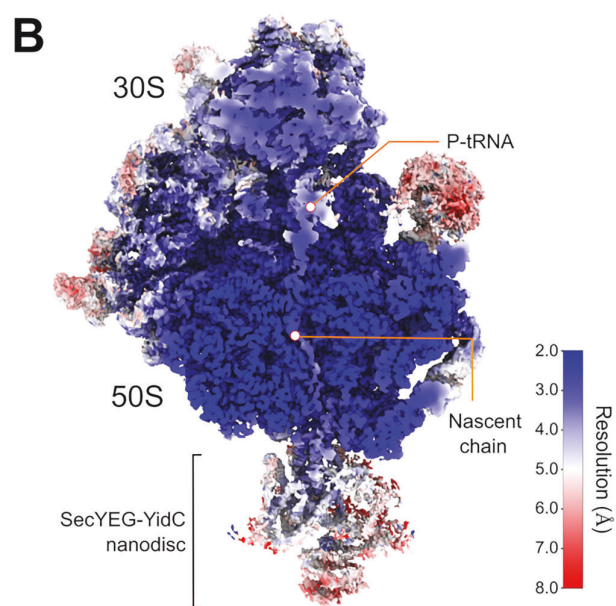
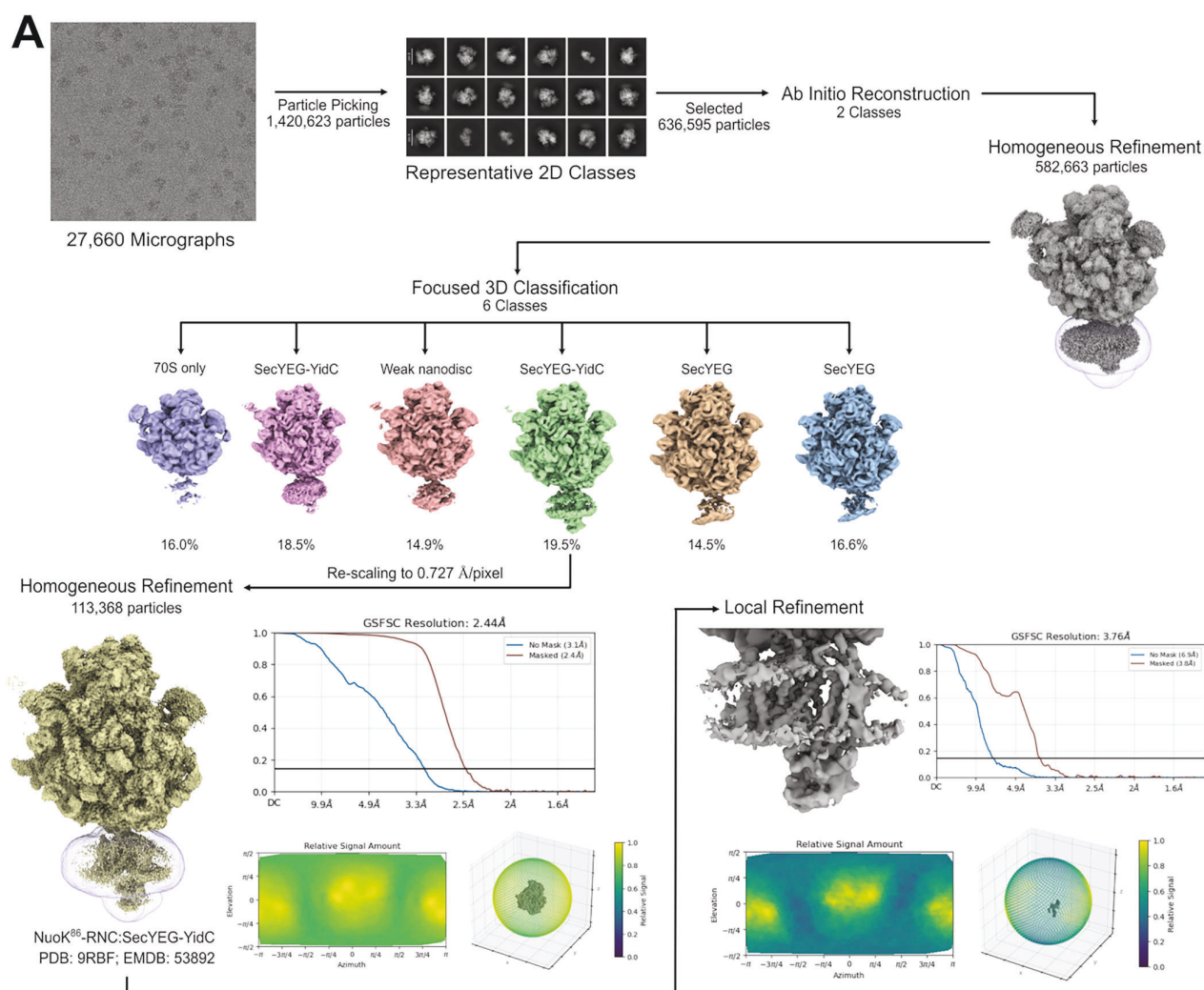


Figure EV2. Cryo-EM data analysis, classification and resolution of the cross-linked Nuok⁸⁶-RNC:SecYEG-YidC complex.

(A) Classification and refinement scheme employed for single-particle analysis. The structurally distinct classes, distribution of views and Fourier shell correlation plots are shown. (B) Globally refined cryo-EM map for the main SecYEG-YidC class, with the color-code according to the local resolution. Gold-standard Fourier shell correlation (GS-FSC) curve as well as angular distribution plot are shown. (C) Locally refined cryo-EM map of the nanodisc-embedded insertase with the color-code according to the local resolution.

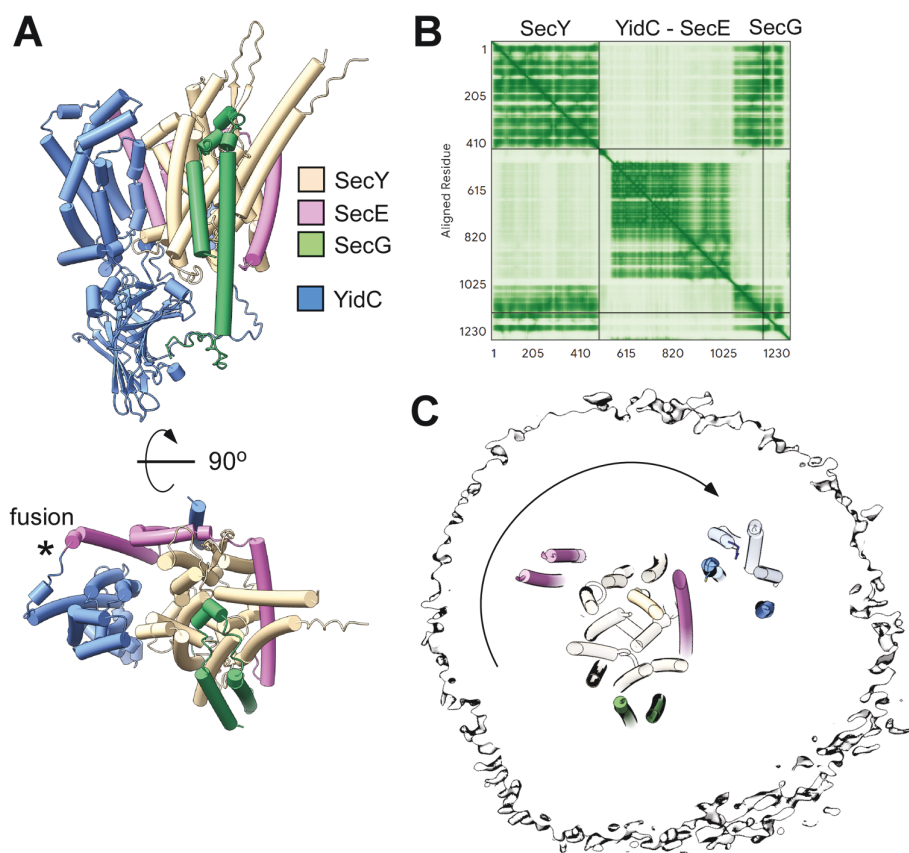


Figure EV3. Repositioning of YidC within the nanodisc upon the assembly with SecYEG.

(A) AlphaFold3-based model of SecYEG-YidC with the genetically fused YidC-SecE, as employed in reconstitution. YidC is positioned at the lateral gate. (B) PAE plot for the model shown in (A) suggests low confidence for SecY-YidC interactions. (C) Cross-section of MSP2N2 nanodisc shows the available lipid-filled space around SecYEG-YidC, allowing for YidC diffusion.

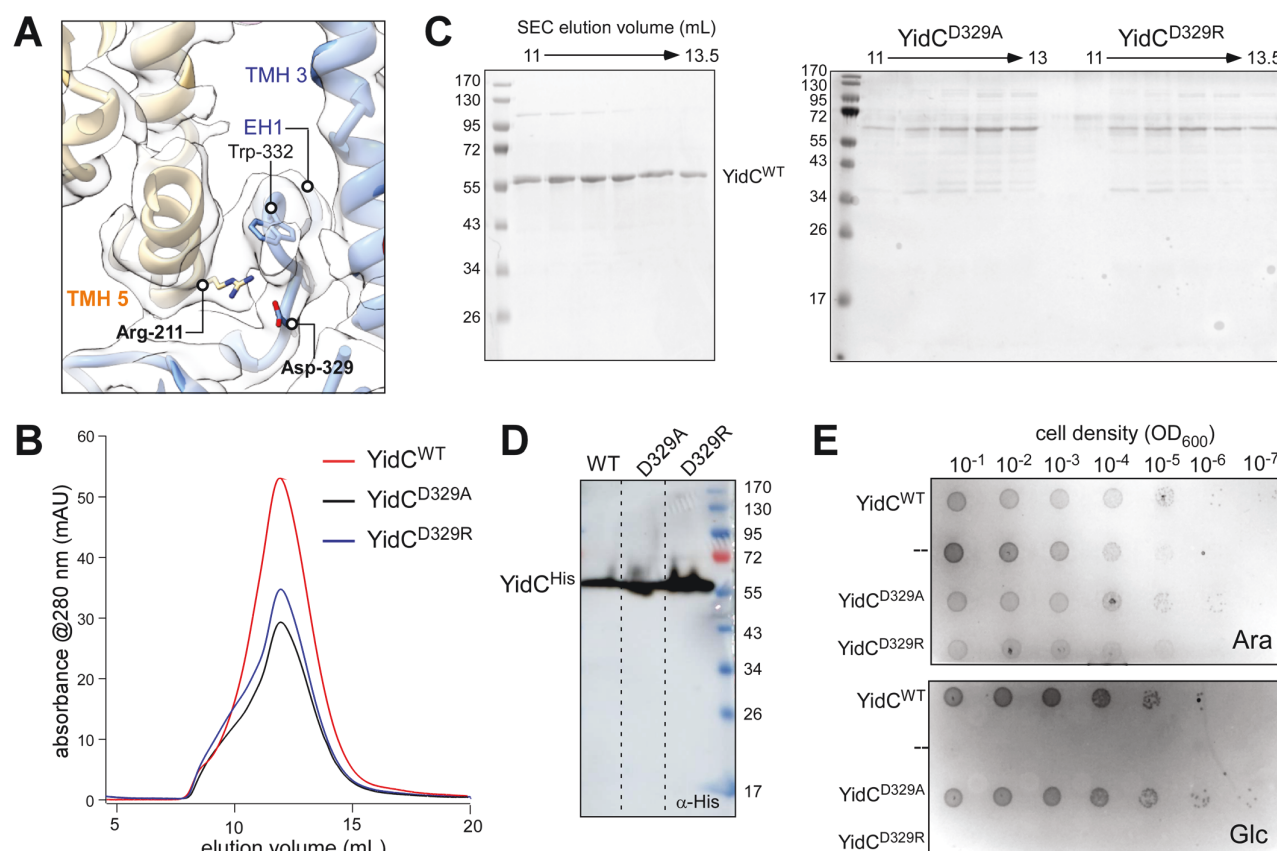


Figure EV4. Charge inversion at YidC position 329 is deleterious for the cells.

(A) View focusing on the periplasmic SecY-YidC interface displaying the molecular model of the SecY-YidC assembly fitted into the cryo-EM map (transparent). Proximate residues SecY Arg-211 and YidC Asp-329 are indicated in bold. (B) Size exclusion chromatography profiles of the DDM-solubilized and purified YidC variants. (C) SDS-PAGE of the size exclusion chromatography fractions shown in (B). (D) Immunodetection of the poly-histidine-tagged YidC variants expressed in *E. coli* FTL10 under permissive conditions (0.2% arabinose, 37 °C). (E) Complementation assay to test the effect of mutations at position 329 of YidC under permissive (0.2% arabinose, "Ara") and the complementation (0.2% glucose, "Glc") conditions. The assay was performed at 37 °C. Source data are available online for this figure.

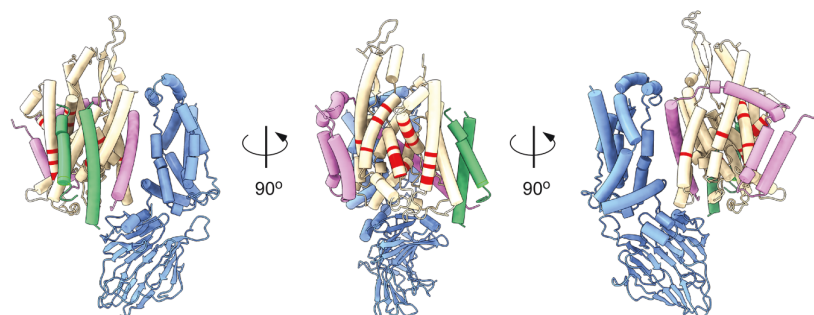


Figure EV5. Overview of the site-specific cross-linking positions to probe SecYEG-YidC interactions.

Positions employed to introduce photo-cross-linkers within SecYEG in earlier studies are shown in red (Jauss et al, 2019; Petriman et al, 2018; Sachelaru et al, 2013).

Appendix for:

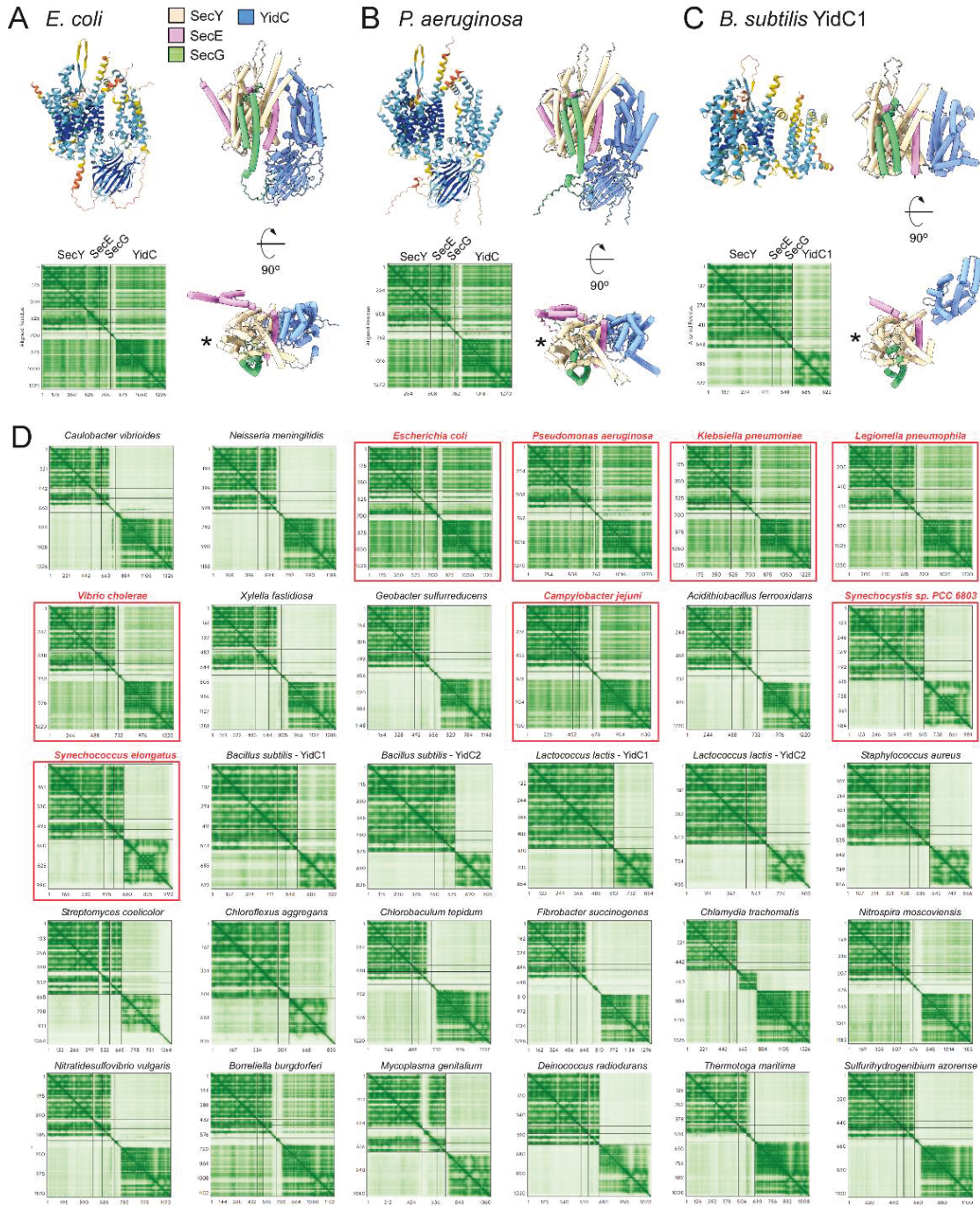
Substrate-induced assembly and functional mechanism of the membrane protein insertase SecYEG-YidC

Max Busch, Cristian Rosales-Hernandez, Michael Kamel,
Yulia Schaumkessel, Eli O. van der Sluis, Otto Berninghausen,
Thomas Becker, Roland Beckmann, Alexej Kedrov

Table of Contents

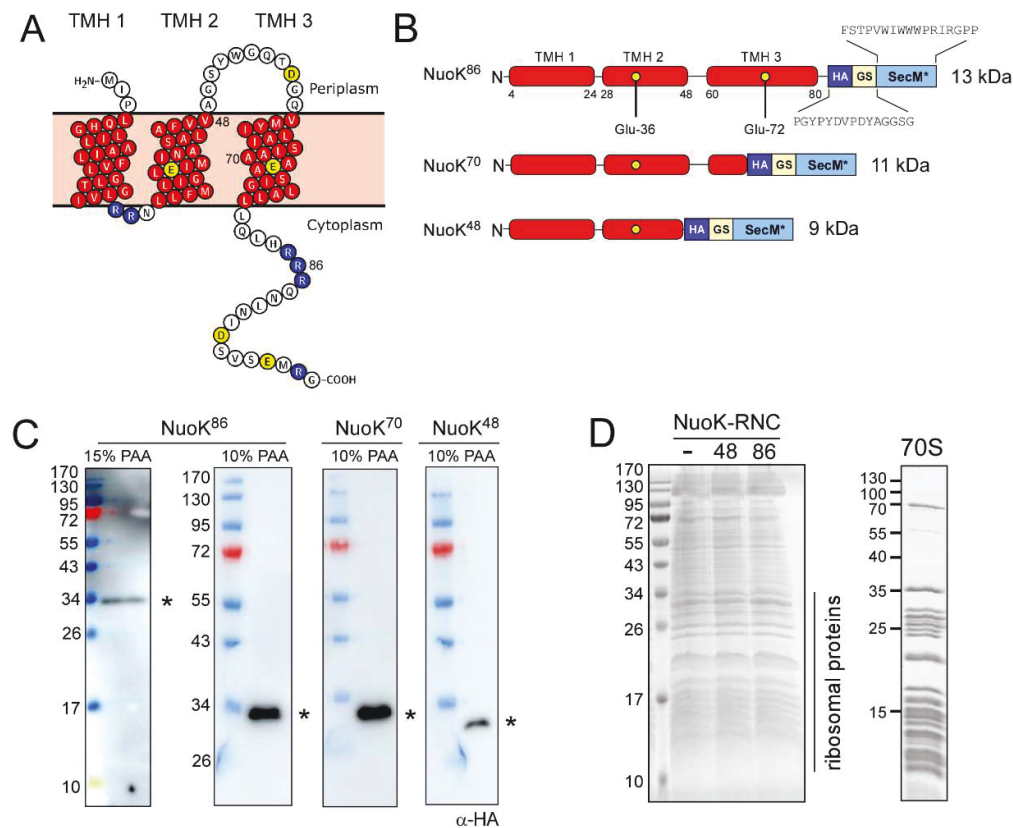
Appendix Figure S1. Diverse architectures of modelled SecYEG-YidC complexes.	2
Appendix Figure S2. Design of the NuoK nascent chain constructs.....	3
Appendix Figure S3. Preparation of the nanodisc-reconstituted SecYEG-YidC.	4
Appendix Figure S4. Architecture of the SecYEG-YidC insertase and the substrate NuoK....	5
Appendix Figure S5. Computational model vs resolved structure of SecYEG-YidC complex. 6	
Appendix Figure S6. Cryo-EM data analysis, classification and resolution of the non- crosslinked NuoK ⁸⁶ -RNC:SecYEG-YidC complex.....	7
Appendix Figure S7. Docking geometry of SecYEG on RNC depends on the nascent chain. 9	
Appendix Figure S8. Mutations in the YidC paddle domain affect <i>E. coli</i> viability at low temperature.....	9
Appendix Figure S9. Cryo-EM data analysis, classification and resolution of the NuoK ⁷⁰ - RNC:SecYEG-YidC complex.....	10
Appendix Figure S10. Cryo-EM data analysis, classification and resolution of the NuoK ^{86mut} - RNC:SecYEG-YidC complex.....	12
Appendix Figure S11. Cryo-EM data analysis, classification and resolution of the NuoK ⁴⁸ - RNC:SecYEG complex.....	14
Appendix Table S1. AlphaFold 3 predictions of SecYEG-YidC complexes of different bacterial clades	16
Appendix Table S2. Cryo-EM data collection, refinement and validation statistics.....	17

Appendix Figures



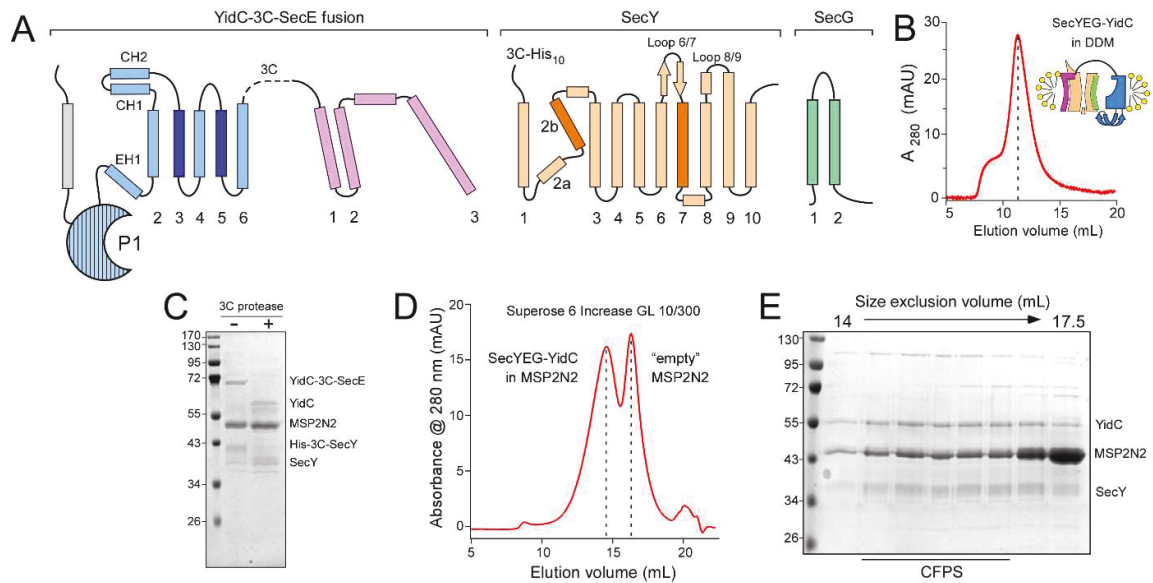
Appendix Figure S1. Diverse architectures of modelled SecYEG-YidC complexes.

AlphaFold3-based prediction for SecYEG-YidC assembly for *Escherichia coli* (A), *Pseudomonas aeruginosa* (B), and *Bacillus subtilis* with the primary YidC homolog (YidC1/SpolIJJ) (C). The uniform color coding of the proteins is indicated in (A). The position of the SecY lateral gate for each model is indicated with the asterisk. (D) Predicted aligned error (PAE) plots for SecYEG-YidC models from indicated bacterial species (see also Suppl. Table 1). Representatives from the major bacterial phyla were chosen based on a recent phylogeny analysis (Hug *et al.* 2016 *Nature Microbiol.*). The models matching the cryo-EM structure of *E. coli* SecYEG-YidC complex are indicated in red. The generated AlphaFold models are provided in the Dataset EV2.



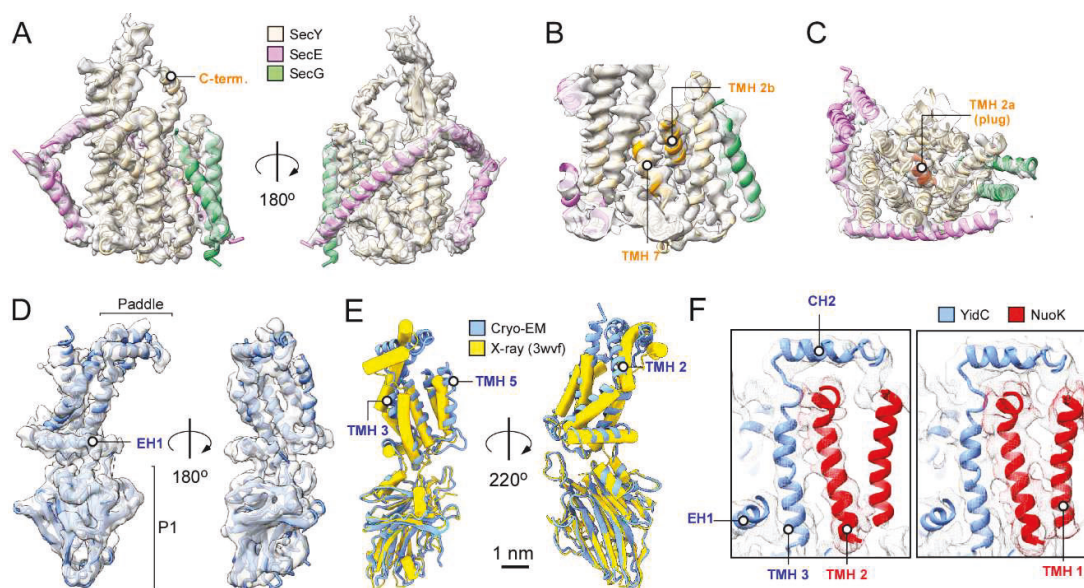
Appendix Figure S2. Design of the NuoK nascent chain constructs.

- (A) Primary structure of *E. coli* NuoK. Residues within TMHs are shown in red, charged residues are shown in blue (arginines) and yellow (aspartates, glutamates). Terminal residues of the tested nascent chains (Val-48, Ala-70 and Arg-86) are indicated by their numbers. The image is prepared via Protter service (wlab.ethz.ch/protter) and modified in Inkscape (Inkscape Project).
- (B) Design of the nascent chains mimicking NuoK⁴⁸ and NuoK⁸⁶ insertion intermediates. NuoK TMHs are shown in red with the flanking residue positions indicated. Positions of the glutamate residues within TMHs 2 and 3 are shown as yellow circles. The sequence of the C-terminal linker consisting of the HA-tag (HA) and a glycine-serine linker (GS) is shown for NuoK⁸⁶. SecM* represents a SecM-based ribosome stalling sequence.
- (C) Western blots against the HA-tag on the isolated NuoK-RNC:SecYEG-YidC complexes. The NuoK nascent chains coupled to tRNA (additional molecular mass ~25 kDa) are indicated with asterisks. For NuoK⁸⁶-RNC blots were performed using SDS-PAGE with either 15 or 10 % polyacrylamide (PAA), as indicated.
- (D) SDS-PAGE of *E. coli* membrane isolates after stalling NuoK translation *in vivo* showing equal amounts of the total protein content, incl. the abundant ribosomal proteins. A characteristic SDS-PAGE of purified 70S ribosomes is provided as a reference.



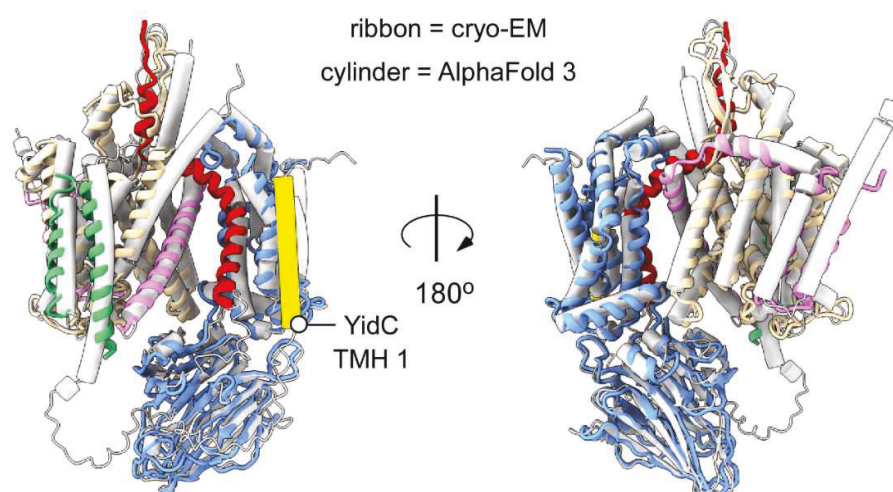
Appendix Figure S3. Preparation of the nanodisc-reconstituted SecYEG-YidC.

- (A) The architecture of the SecYEG-YidC fusion complex. YidC TMHs 3 and 5 forming the insertion site are shown in dark blue, SecY TMHs 2b and 7 forming the lateral gate are shown in dark orange. The non-essential YidC TMH 1 (not resolved in the cryo-EM map) is shown in grey.
- (B) Size exclusion chromatography profile of the detergent-isolated SecYEG-YidC complex.
- (C) SDS-PAGE of the nanodisc-reconstituted SecYEG-YidC complex before and after HRV-3C protease treatment.
- (D) Size exclusion chromatography profile of the nanodisc reconstitution reaction. Peaks corresponding to MSP2N2-nanodiscs containing SecYEG-YidC and those containing only lipids ("empty") are indicated with the dashed lines.
- (E) SDS-PAGE of the size exclusion chromatography fractions shown in (D).



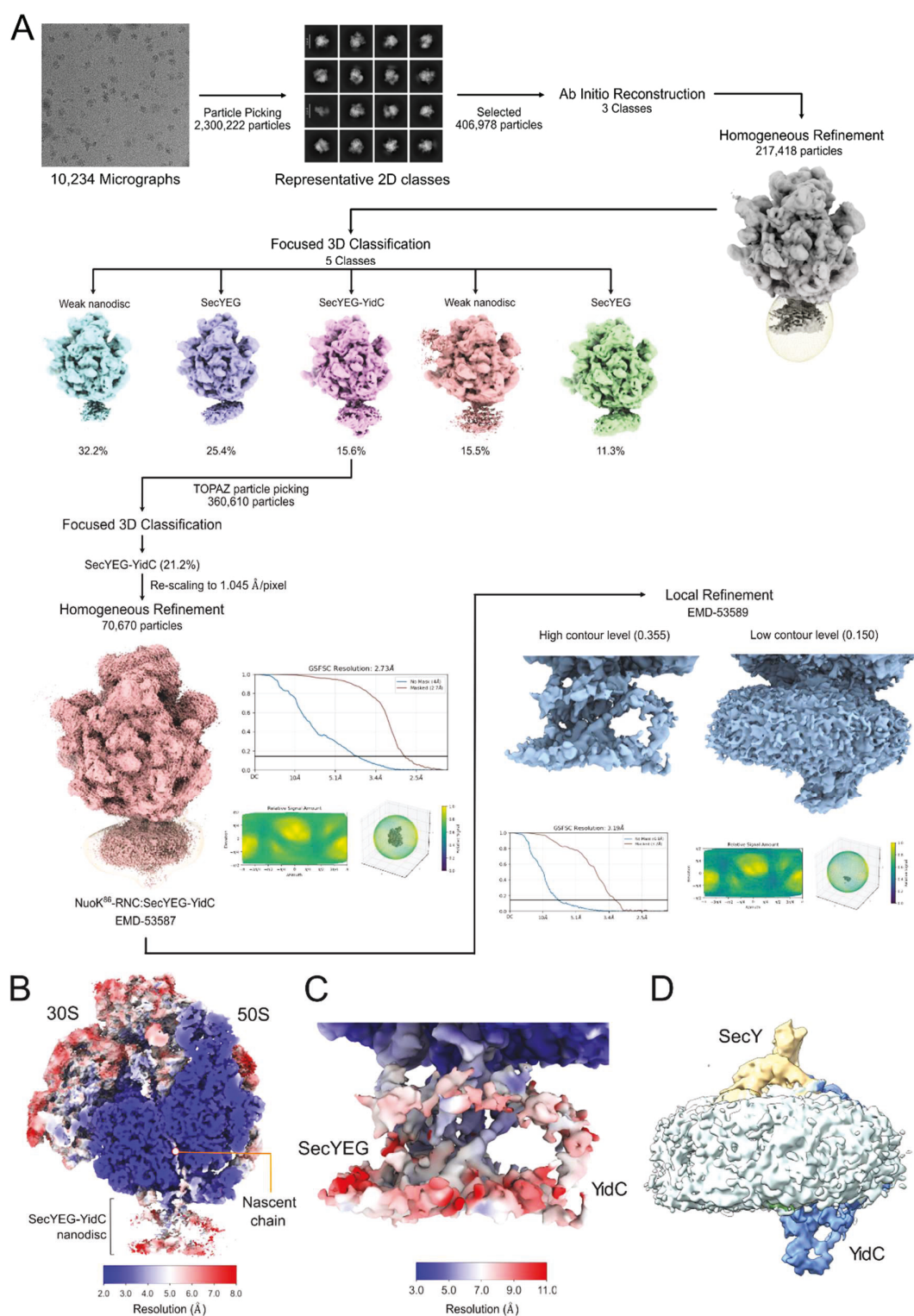
Appendix Figure S4. Architecture of the SecYEG-YidC insertase and the substrate NuoK.

- (A) Side views on the isolated cryo-EM density (transparent) of SecYEG with fitted model. The C-terminal extension of SecY is indicated (C-term).
- (B) View on the lateral gate of SecYEG formed by SecY TMHs 2b and 7.
- (C) View on SecYEG from the periplasmic side showing the central sealing position of the “plug” domain, TMH 2a.
- (D) Side views on the isolated cryo-EM density of YidC with the fitted model. Selected structural elements are indicated.
- (E) An overlay of the cryo-EM-based model of YidC within the active insertase complex (blue ribbons) with the X-ray structure of the idle YidC (PDB ID 3WVF; yellow cylinders). TMHs with changed positions are indicated.
- (F) Putative position of the membrane-inserted NuoK TMH 1. A rod-shaped density is revealed at a lower contour level (right).



Appendix Figure S5. Computational model vs resolved structure of SecYEG-YidC complex.

Comparison of the cryo-EM-derived model for the SecYEG-YidC complex in the presence of NuoK86-RNC (ribbon representation) and the AlphaFold 3-based model (cylinder representation).



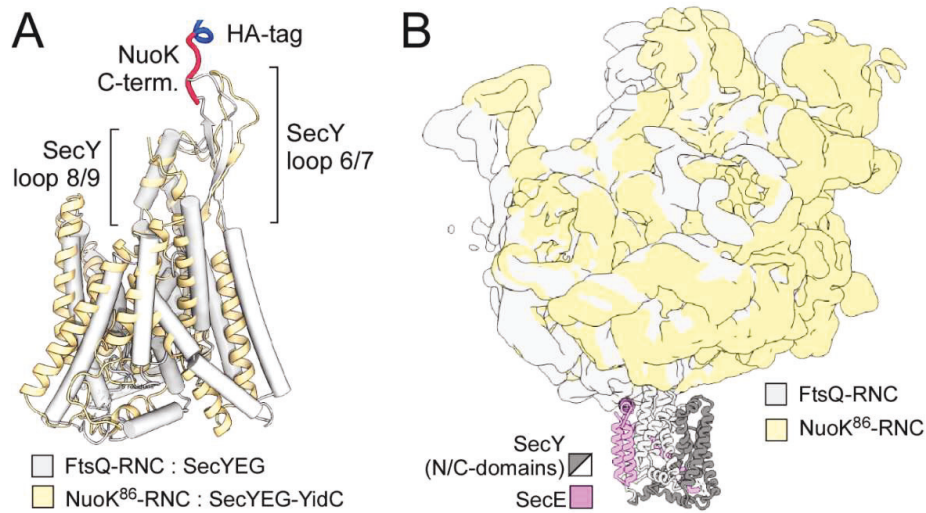
Appendix Figure S6. Cryo-EM data analysis, classification and resolution of the non-crosslinked Nuok⁸⁶-RNC:SecYEG-YidC complex.

(A) From a total of 10,234 micrographs, 2,300,222 particles were picked and used for 2D classification using cryoSPARC, which yielded a total of 408,798 ribosomal particles (70S) with an extra density

at the tunnel exit. After *ab-initio* reconstruction and homogenous refinement, 217,418 particles were selected for focused 3D classification with a mask covering the tunnel exit density (shown in transparent yellow). This yielded five classes, of which one class showed density for SecYEG and YidC including the P1 domain. This class was used to train a TOPAZ picking model. 360,610 particles were classified and refined again, yielding a final map at 2.73 Å average resolution from 70,670 particles. Local refinement on the SecYEG-YidC region resulted in map of 3.19 Å resolution that showed density for YidC at low contour levels. Gold-standard Fourier Shell Correlation (GS-FSC) curves as well as angular distribution plots are shown for globally and locally refined maps.

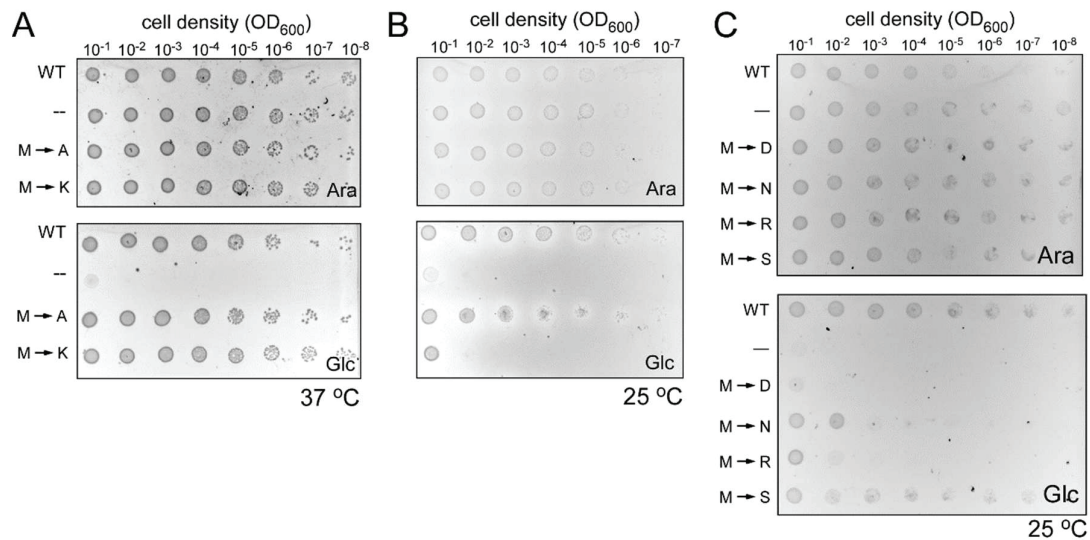
(B, C) Globally and locally refined cryo-EM maps are color-coded according to local resolution.

(D) Cryo-EM reconstruction of the non-crosslinked NuoK⁸⁶-RNC:SecYEG-YidC complex. The focused refined map was colored based on the molecular model of the crosslinked SecYEG-YidC complex (Figure 2A). The extra density for YidC was present at the “back-of-Sec” position suggesting that the architecture of SecYEG-YidC complex was not affected by the stabilizing crosslinking procedure.

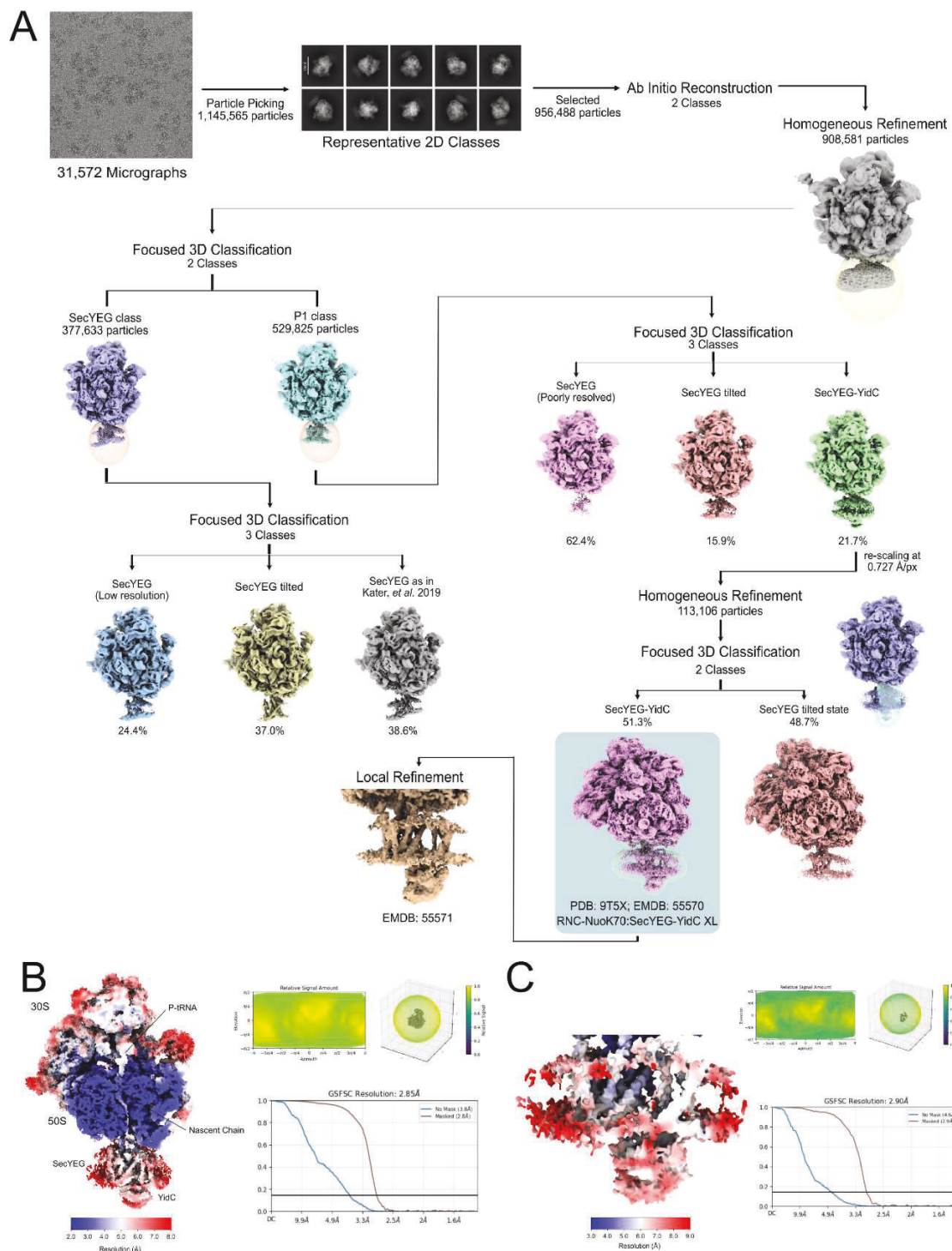


Appendix Figure S7. Docking geometry of SecYEG on RNC depends on the nascent chain.

- (A)** Orientation of SecY bound to Nuok⁸⁶-RNC within the SecYEG-YidC complex (ribbon representation) and that bound to FtsQ-RNC (PDB ID 6R7L, cylinder representation) (aligned on the 50S ribosomal subunit).
- (B)** Orientations of FtsQ-RNC and Nuok⁸⁶-RNC (aligned on the SecYEG complex). The 50S ribosomal subunit in the Nuok⁸⁶-RNC is tilted towards the N-terminal half of SecY (grey).



Appendix Figure S8. Mutations in the YidC paddle domain affect *E. coli* viability at low temperature. Complementation assay to test the effect of mutations within the paddle domain of YidC (substitution of Met-408/409 by indicated amino acids) under permissive (0.2% arabinose, "Ara") and the complementation (0.2 % glucose, "Glc") conditions. The assay was performed at 37 °C **(A)** and at 25 °C **(B, C)**. Panel B ("Glc") also appears in Figure 5B in the main text.



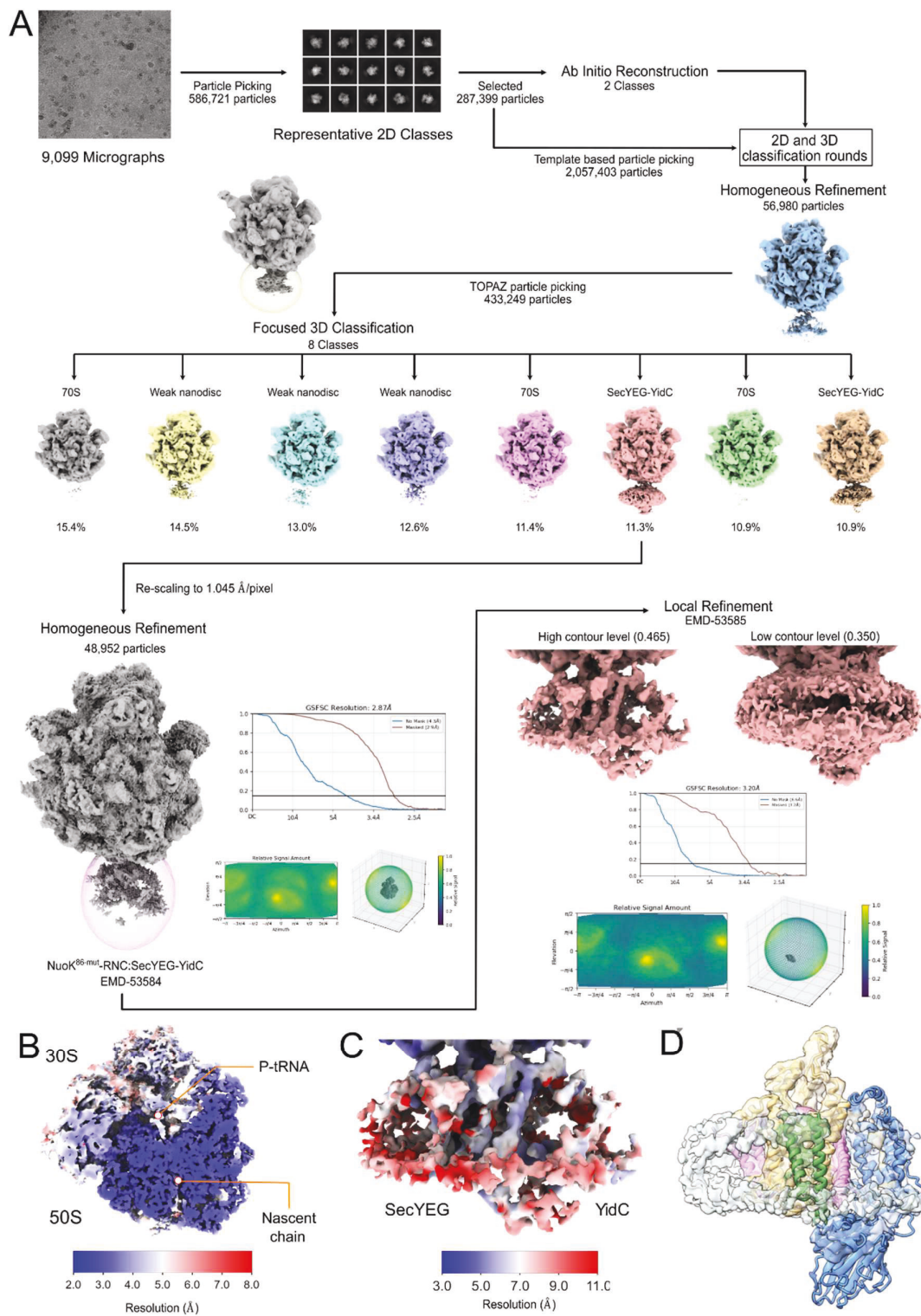
Appendix Figure S9. Cryo-EM data analysis, classification and resolution of the NuoK⁷⁰-RNC:SecYEG-YidC complex.

(A) From a total of 31,572 micrographs, 956,488 particles were selected after 2D classification using cryoSPARC, which yielded a total of 908,581 ribosomal particles (70S) with an extra density at the tunnel exit. Several rounds of 3D focused classification using masks around the tunnel exit/nanodisc region yielded one class with SecYEG and YidC present and two well-resolved

classes with only SecYEG present, one in the conformation as observed in Kater et al., one in the tilted conformation as observed in presence of YidC (see also Appendix Fig. 7). The SecY-YidC-containing map was refined to a final resolution of 2.85 Å average resolution. Local refinement on the SecYEG-YidC region resulted in map of 2.90 Å resolution.

(B, C) Globally and locally refined cryo-EM maps shown color coded according to local resolution.

Gold-standard Fourier Shell Correlation (GS-FSC) curves as well as angular distribution plots are shown for globally and locally refined maps.



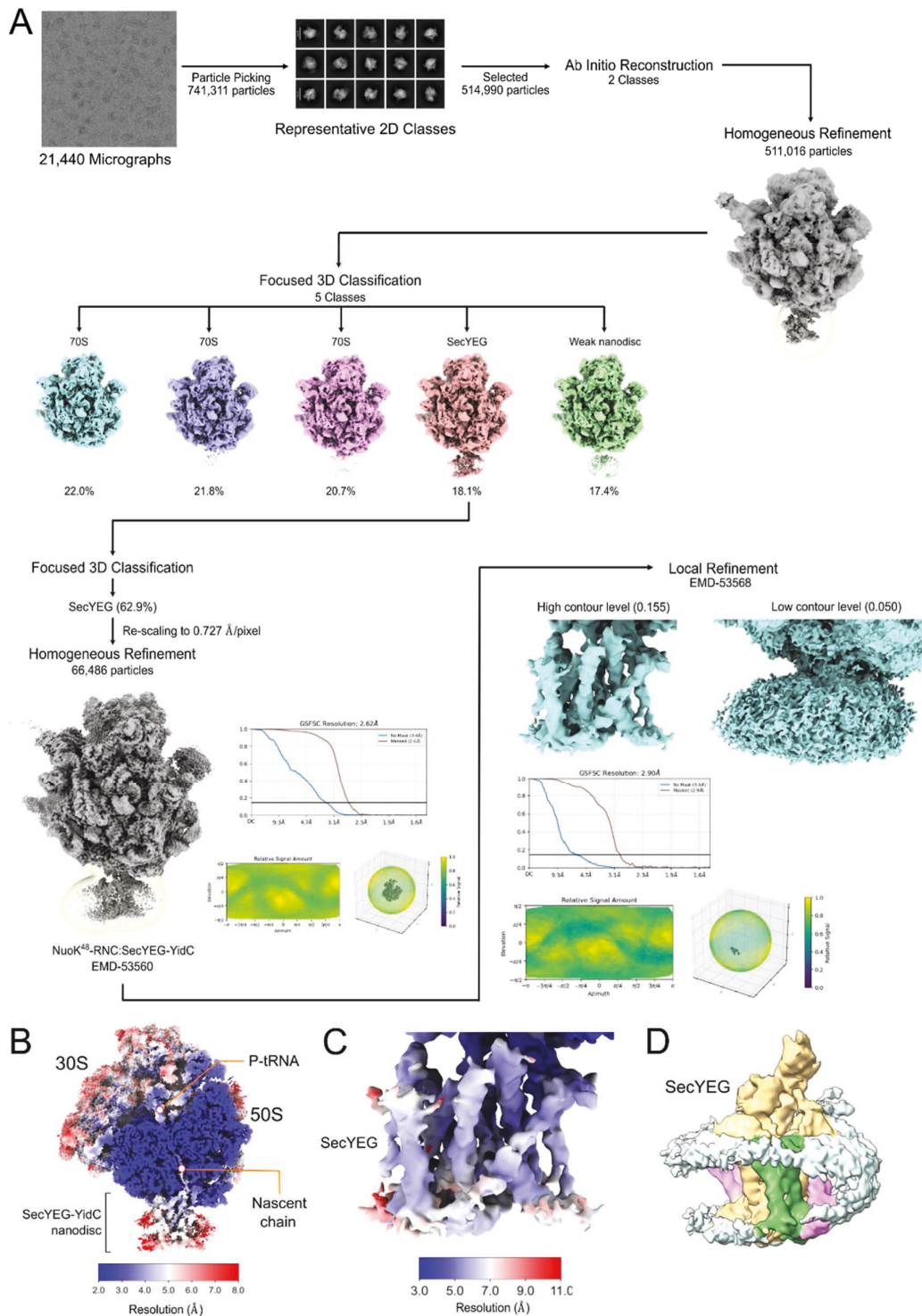
Appendix Figure S10. Cryo-EM data analysis, classification and resolution of the Nuok^{86mut}-RNC:SecYEG-YidC complex.

(A) From a total of 9,099 micrographs, 586,721 particles were picked and used for 2D classification using cryoSPARC, which yielded a total of 287,399 (70S) ribosomal particles with an extra density

at the tunnel exit. A similar procedure to optimize particle picking and 3D classification was performed as for the non-crosslinked NuoK⁸⁶-RNC:SecYEG-YidC complex (Appendix Fig. S6) yielding a class with 48,952 particles with well-defined density for SecYEG and YidC including the P1 domain below the tunnel exit. This class was refined to a final average resolution of 2.87 Å. Local refinement on the SecYEG/YidC region resulted in map of 3.20 Å resolution that showed density for YidC at low contour levels. Gold-standard Fourier Shell Correlation (GS-FSC) curves as well as angular distribution plots are shown for globally and locally refined maps.

(B, C) Globally and locally refined cryo-EM maps shown color coded according to local resolution.

(D) Cryo-EM reconstruction of the NuoK86mut-RNC:SecYEG-YidC complex. The focused refined map was colored based on the molecular model of the crosslinked SecYEG-YidC complex (Figure 2A). Note that also here extra density for YidC was present at the “back-of Sec” position suggesting that the architecture of SecYEG-YidC complex was not affected by the E36K, E72K double mutation in the NuoK nascent chain.



Appendix Figure S11. Cryo-EM data analysis, classification and resolution of the Nuok⁴⁸-RNC:SecYEG complex.

(A) From a total of 21,440 micrographs, 741,311 particles were picked and used for 2D classification using cryoSPARC, which yielded a total of 514,990 ribosomal particles (70S) with an extra density at the tunnel exit. This dataset was essentially process as outlined for the Nuok⁸⁶-RNC:SecYEG-

YidC dataset (Figure EV2). Here, focused 3D classification only yielded classes with no ligand or only SecYEG bound to the tunnel exit. The best-resolved class with 66,486 particles was refined to a final average resolution of 2.62 Å. Local refinement on the Nanodisc/SecYEG region resulted in map of 2.90 Å resolution that shown all α -helices of SecYEG clearly resolved. Gold-standard Fourier Shell Correlation (GS-FSC) curves as well as angular distribution plots are shown for globally and locally refined maps.

- (B, C) Globally and locally refined cryo-EM maps shown color coded according to local resolution.
- (D) Cryo-EM reconstruction of the NuoK⁴⁸-RNC:SecYEG complex. The focused refined map was colored based on the molecular model of the SecYEG complex (Figure 2A). No extra density for YidC was present at the “back-of-Sec” position suggesting that YidC is not stably recruited.

Appendix Table S1. AlphaFold 3 predictions of SecYEG-YidC complexes of different bacterial clades

No.	Bacterial clade	Species	Position relative to SecYEG	ipTM	pTM	Comments
1	Alphaproteobacterium	<i>Caulobacter vibrioides</i>	SecG	0,44	0,48	
2	Betaproteobacterium	<i>Neisseria meningitidis</i>	SecG	0,45	0,49	
3	Gammaproteobacterium	<i>Escherichia coli</i>	Back of Sec	0,69	0,71	
4	Gammaproteobacterium	<i>Pseudomonas aeruginosa</i>	Back of Sec	0,69	0,71	
5	Gammaproteobacterium	<i>Klebsiella pneumoniae</i>	Back of Sec	0,63	0,66	
6	Gammaproteobacterium	<i>Legionella pneumophila</i> Lens	Back of Sec	0,6	0,63	
7	Gammaproteobacterium	<i>Vibrio cholerae</i>	Back of Sec	0,55	0,58	
8	Gammaproteobacterium	<i>Xylella fastidiosa</i>	SecG	0,45	0,5	
9	Deltaproteobacterium	<i>Geobacter sulfurreducens</i>	SecG	0,45	0,49	
10	Epsilonproteobacterium/ Campylobacterota	<i>Campylobacter jejuni</i>	Back of Sec	0,63	0,66	
11	Acidithiobacillus class	<i>Acidithiobacillus ferrooxidans</i>	SecG	0,45	0,5	
12	Cyanobacteria	<i>Synechocystis</i> sp. PCC 6803	Back of Sec	0,5	0,53	
13	Cyanobacteria	<i>Synechococcus elongatus</i> PCC 7942	Back of Sec	0,51	0,55	
14	Firmicutes	<i>Bacillus subtilis</i> - YidC1	Back of Sec	0,59	0,63	YidC is rotated ~180 grad
15	Firmicutes	<i>Bacillus subtilis</i> - YidC2	SecG	0,53	0,59	
16	Firmicutes	<i>Lactococcus lactis</i> - YidC1	SecG	0,53	0,61	
17	Firmicutes	<i>Lactococcus lactis</i> - YidC2	SecG	0,51	0,58	
18	Firmicutes	<i>Staphylococcus aureus</i>	SecY TMH 6/TMH7	0,52	0,56	
19	Actinobacteria	<i>Streptomyces coelicolor</i>	Lateral gate	0,52	0,55	
20	Chloroflexi	<i>Chloroflexus aggregans</i>	Lateral gate	0,5	0,56	
21	Chlorobi	<i>Chlorobaculum tepidum</i>	Lateral gate	0,44	0,48	Model #1 (correct topology)
22	Fibrobacteres	<i>Fibrobacter succinogenes</i>	Lateral gate	0,43	0,47	
23	PVC — Chlamydiae	<i>Chlamydia trachomatis</i> serovar D	Back of Sec	0,42	0,47	YidC is rotated ~90 grad
24	Nitrospirae	<i>Nitrospira moscoviensis</i>	Inverted/non-physiological topology	0,52	0,54	
25	Thermodesulfobacteriota	<i>Nitratidesulfobacterium vulgare</i>	SecG	0,46	0,5	
26	Spirochaetes	<i>Borrelia burgdorferi</i>	Back of Sec	0,6	0,63	YidC is rotated ~180 grad
27	Tenericutes / Mollicutes	<i>Mycoplasma genitalium</i>	Lateral gate	0,5	0,54	
28	Deinococcus–Thermus	<i>Deinococcus radiodurans</i>	SecG	0,47	0,51	
29	Thermotogae	<i>Thermotoga maritima</i>	Lateral gate	0,4	0,46	
30	Aquificota	<i>Sulfurihydrogenibium azorense</i>	SecG	0,47	0,51	Model #2 (correct topology)

Appendix Table S2. Cryo-EM data collection, refinement and validation statistics

	NuoK ⁸⁶ -RNC: SecYEG-YidC (crosslinked) (EMD-53892) ^a (EMD-53893) ^b (EMD-53894) ^c (PDB 9RBF)	NuoK ⁷⁰ -RNC: SecYEG-YidC (crosslinked) (EMD-55570) ^a (EMD-55571) ^b (EMD-55598) ^c (PDB-9T5X)	NuoK ⁸⁶ -RNC: SecYEG-YidC (EMD-53587) ^a (EMD-53589) ^b	NuoK ^{86mut} - RNC: SecYEG- YidC (EMD-53584) ^a (EMD-53585) ^b	NuoK ⁴⁸ -RNC: SecYEG-YidC (EMD-53560) ^a (EMD-53568) ^b
Data collection and processing					
Magnification	165,000	165,000	130,000	130,000	165,000
Voltage (kV)	300	300	300	300	300
Electron exposure (e ⁻ /Å ²)	60	60	40	40	60
Defocus range (μm)	0.5-3.5	0.5-3.5	0.5-3.5	0.5-3.5	0.5-3.5
Pixel size (Å)	0.727	0.727	1.060	1.049	0.727
Symmetry imposed	C1	C1	C1	C1	C1
Initial particle images (no.)	1,420,623	1,145,565	2,300,222	2,057,403	741,311
Final particle images (no.)	113,368	58,063	70,670	48,952	66,486
Map resolution (Å)					
FSC threshold (0.143)	2.44 ^a , 3.76 ^b	2.85 ^a , 2.90 ^b	2.73 ^a , 3.19 ^b	2.87 ^a , 3.20 ^b	2.62 ^a , 2.90 ^b
Refinement					
Initial model used (PDB code)	7k00, 8qoa, AlphaFold	9RBF			
Model resolution (Å)	3.0	3.0			
FSC threshold (0.5)					
Model composition					
Non-hydrogen atoms	150,739	150,806			
Protein residues	6735	6723			
Nucleotides	4556	4556			
Ligands	ZN, MG, SPM	ZN, MG, SPM			
B factors (mean) (Å²)					
Protein	87.07	187.83			
Nucleotide	72.51	152.70			
Ligand	49.64	105.46			
R.m.s. deviations					
Bond lengths (Å)	0.006	0.005			
Bond angles (°)	0.728	0.597			
Validation					
MolProbity score	1.33	1.09			
Clashscore	3.37	1.02			
Poor rotamers (%)	0.42	0.16			
Ramachandran plot					
Favored (%)	96.79	95.79			
Allowed (%)	3.15	4.18			
Disallowed (%)	0.06	0.03			

^a Consensus refinement^b Local refinement^c Composite map

4.2 Probing for Lateral Gate Opening: LacY and the Translocon

Different structures of the Sec translocon in complex with the ribosome have already provided insights into the insertion process. However, many of these structures have important limitations: they were acquired in detergent micelles, which may influence protein-protein interactions; they capture only a single late-stage snapshot of the insertion process; or they were not assembled in a native state, but instead generated by mixing pre-formed RNCs with the insertase machinery *in vitro* [115, 120, 272-274]. A combination of cell-free protein expression, nanodisc reconstitution and cryo-EM presented in the previous chapter has delivered a new view on the organization and dynamics of the bacterial SecYEG-YidC insertase machinery in the lipid environment, in particular on YidC recruitment in response to the substrate emerging at the “back-of-Sec”, which was believed to be exclusive to (higher) eukaryotes [109, 112]. The translocon, as the primary docking point for the ribosome, should therefore be seen as a modular adaptable platform, rather than a static pore integrating IMPs exclusively through lateral gate opening. Here we further set out to employ our membrane protein insertion platform to probe for IMP insertion via the lateral gate.

Since neither FtsQ, nor the YidC-independent mutant NuoK^{E36K E72K} showed an opening of the lateral gate of SecYEG, we have considered introducing a different substrate. An interesting candidate is the lactose permease LacY, since it has an N-terminus facing the cytoplasm and relatively short loops connecting the helices. Another aspect making LacY even more interesting is the suggestion from previous studies that its folding is altered by interactions with YidC, which is able to control the topology of LacY during insertion [106, 275]. Furthermore, in cells lacking phosphatidylethanolamine (PE), LacY changes the topology of the first six TMHs with TMH 7 being exposed as an extramembrane domain, while the remaining five TMHs keep their orientation [276].

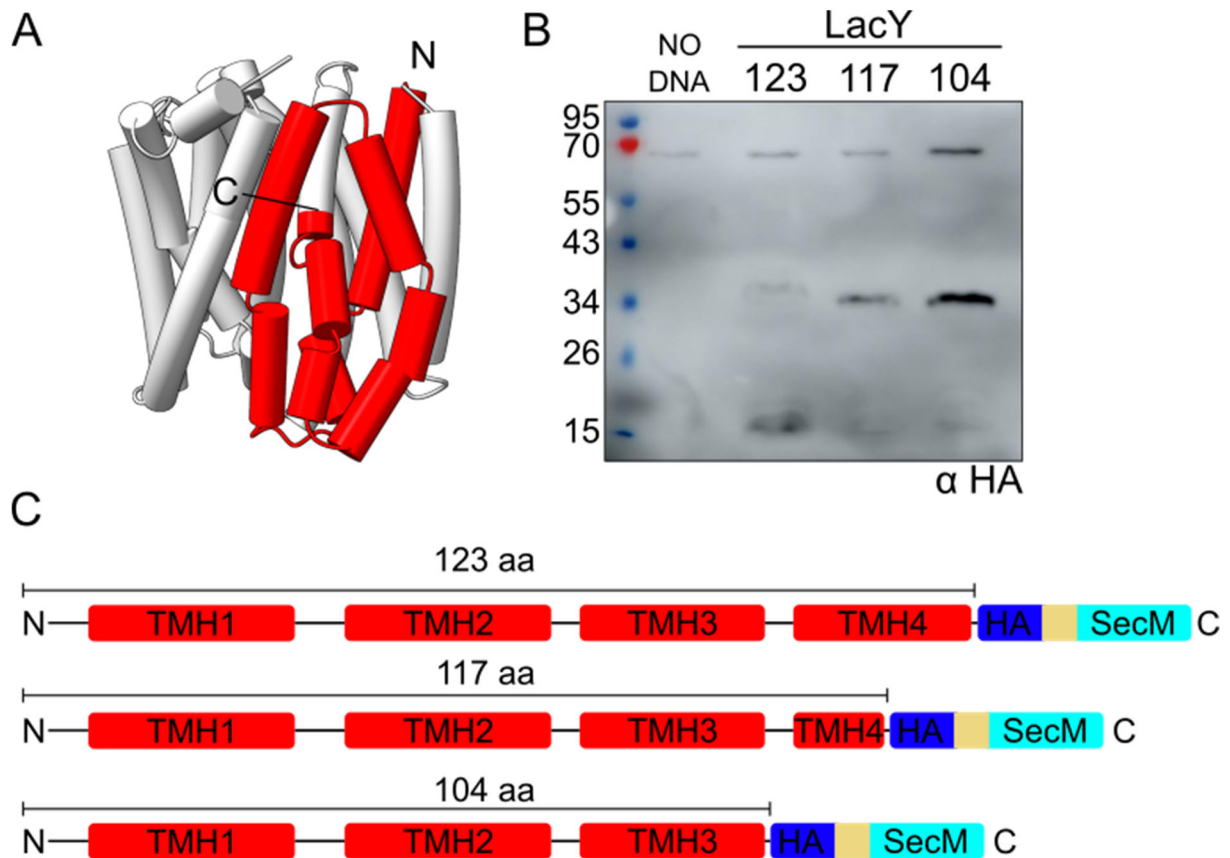


Figure 12: LacY constructs to investigate lateral gate opening (A) Crystal structure of LacY (PDB: 4ZYR) highlighted in red are the first 123 amino acids that were used for construct design. (B) Immunodetection of HA-tag for the used LacY constructs. The length corresponds to the construct itself and additional ~25 kDa for the attached tRNA. (C) Organization of the used LacY constructs with the length in amino acids.

Therefore, we designed constructs of nascent chains containing up to four TMHs of LacY connected to the HA-Tag for recognition, a GSG linker, and the optimized SecM stalling sequence, as described previously. The CFPS reactions showed prominent signal upon immunoblotting against the HA-tag, so all the nascent chains with lacY fragments were synthesized (Fig. 12 B). The longest construct, LacY¹²³, was selected for further experiments. MSP1E3D1 nanodiscs containing a single copy of SecYEG, as well as MSP2N2 nanodiscs containing both SecYEG and YidC, were employed to investigate potential cooperativity during the insertion process. The samples were processed as described by Busch *et al.* 2026 using the LacY¹²³-encoding plasmid. After purification and enrichment of the stalled RNC:SecYEG:YidC complexes, the samples were chemically crosslinked by the addition of glutaraldehyde, and flash-frozen until cryo-EM grid preparation.

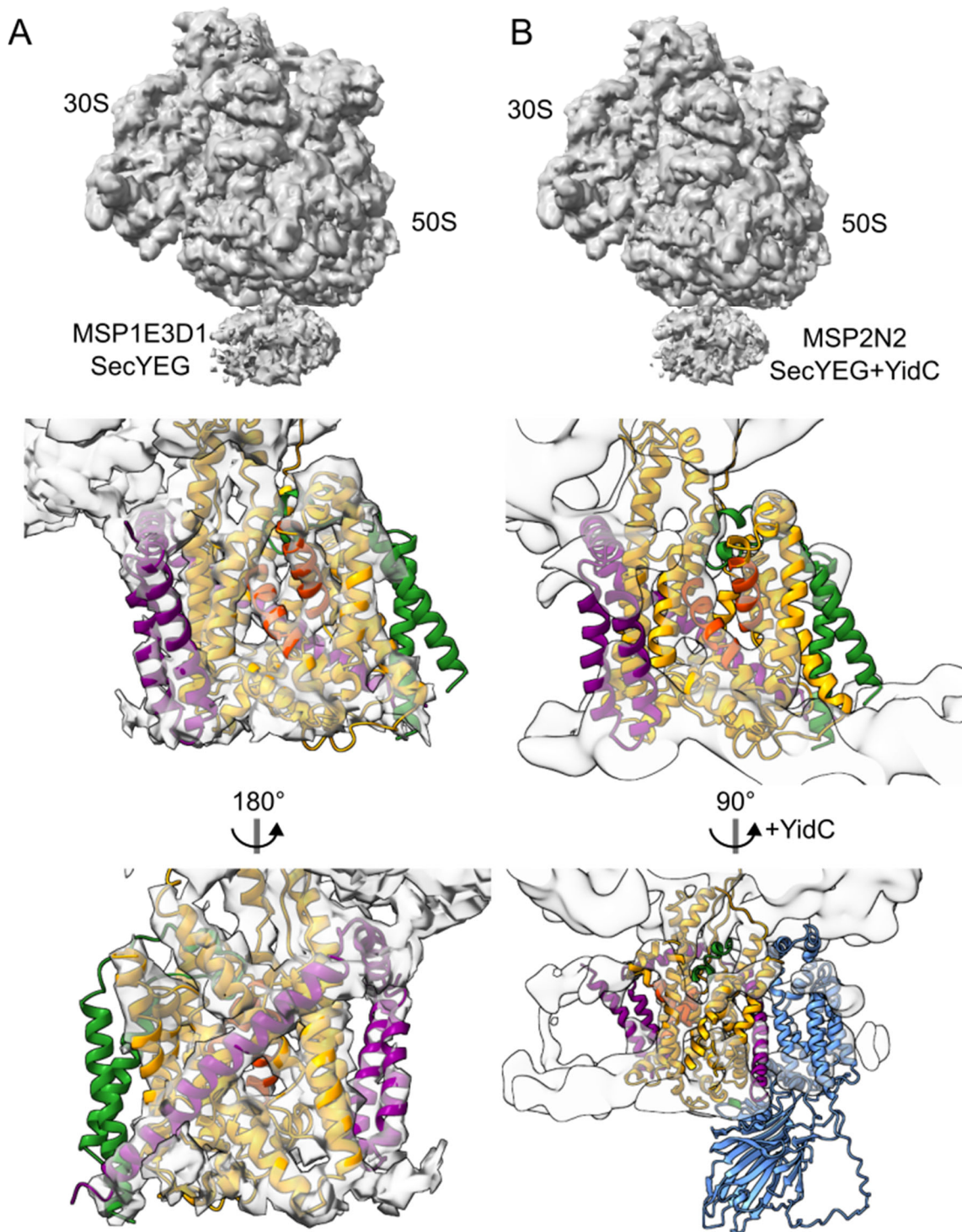


Figure 13: LacY¹²³ insertion does not trigger lateral gate opening. (A) 3D reconstruction confirms the presence of MSP1E3D1 filled with SecYEG at the ribosomal exit tunnel (Top). SecYEG model from Busch *et al.* 2026 with closed lateral gate (highlighted in orange) fitted in the cryo-EM volume, nearly all helices are resolved with no additional density attributable to LacY (Middle and bottom). (B) 3D reconstruction confirms the presence of MSP2N2 filled with SecYEG and YidC at the ribosomal exit tunnel (Top). SecYEG model from Busch *et al.* 2026 with closed lateral gate (highlighted in orange) fitted in the cryo-EM volume (Middle). Due to limited density resolution, the position of YidC behind SecYEG could not be verified (Bottom).

As observed previously for different FtsQ and NuoK constructs, the lateral gate of SecYEG in the MSP1E3D1 nanodisc remained in the closed conformation also for LacY¹²³ (Fig. 13 A) [277, 278]. Although the resolution was sufficient to accurately position the translocon within the 3D reconstruction, no additional density attributable to the substrate could be assigned. For the SecYEG-YidC complex in the MSP2N2 nanodisc, the overall resolution of SecYEG was lower than in the previous reconstruction, potentially due to increased flexibility associated with the larger nanodisc diameter (12 nm vs 17 nm; [279]). Consequently, no density corresponding to the LacY nascent chain could be identified in these reconstructions. In addition, YidC could not be reliably positioned in any of the classes obtained (Fig. 13B). At higher contour levels, weak density that might originate from the P1 domain of YidC may be recognizable; however, it appears shifted toward SecG relative to our previously published model and is not located in front of the lateral gate in contrast to before proclaimed studies [106, 107].

Discussion

When testing multiple N-terminal fragments of LacY prepared with our CFPS platform, cryo-EM analysis revealed that the lateral gate of SecYEG remained closed under all conditions examined. Furthermore, the nascent chain could not be visualized in any of the selected conditions, suggesting that no single conformational state could be sufficiently trapped for structural characterization by cryo-EM. As glutaraldehyde crosslinking also failed to stabilize a detectable nascent chain density, it is likely that the substrate adopts multiple conformations with a high degree of flexibility, thereby preventing its visualization by cryo-EM. We additionally observed that YidC was not trapped in a distinct position, which is consistent with at least two possible explanations. First, YidC may only be involved in folding later TMHs or specific stretches of the protein that were not captured by our construct [263]. Second, this observation could reflect the nanodisc system itself: either it provides enough lateral freedom for cargo movement to escape capture, or it lacks additional factors required for stable engagement. The latter possibility would stand in contrast to Dowhan and coworkers, who reported that no accessory proteins are strictly required for folding [276, 280].

To further investigate a potential cooperation between SecYEG and YidC during LacY or other substrate insertion, several complementary approaches could be pursued. As a short-term goal, larger insertase complexes could be reconstituted, for example by incorporating multiple YidC copies or additional subcomponents of the holo-translocon. A more technically demanding approach would be to couple the CFPS reaction to single-molecule FRET, which could enable faster screening of truncation constructs and help define the stretches during which YidC is recruited to facilitate folding or insertion. Finally, global ribosome profiling, similar to work from the Keenan lab, could help identify the factors recruited during membrane protein insertion in prokaryotes; this could be complemented by cryogenic electron tomography to visualize the process directly in living cells.

5. Lipid structure modulates the dynamics of the bacterial translocon SecYEG

Authors: Max Busch, Rocco Gentile, Holger Gohlke, Alexej Kedrov

Published: *In preparation*

Contribution: **Max Busch:** Conceptualization, Investigation, Formal Analysis, Data Curation, Visualization, Writing – Original Draft, Writing – Review & Editing

Rocco Gentile: Conceptualization, Software, Investigation, Formal Analysis, Visualization, Writing – Original Draft, Writing – Review & Editing

Holger Gohlke: Supervision, Formal Analysis, Resources, Project Administration, Funding Acquisition, Writing – Review & Editing

Alexej Kedrov: Conceptualization, Supervision, Formal Analysis, Resources, Project Administration, Funding Acquisition, Writing – Original Draft, Writing – Review & Editing

Lipid structure modulates the dynamics of the bacterial translocon SecYEG

Max Busch^{1,§}, Rocco Gentile^{2,§}, Holger Gohlke^{2,3,4,*}, Alexej Kedrov^{1,4,*}

¹ Synthetic Membrane Systems, Institute of Biochemistry, Heinrich Heine University Düsseldorf, 40225 Düsseldorf, Germany

² Institute for Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, 40225 Düsseldorf, Germany

³ Institute of Bio- and Geosciences (IBG-4: Bioinformatics), Forschungszentrum Jülich GmbH, 52425, Jülich, Germany

⁴ Interfaculty Center for Membrane Research, Heinrich Heine University Düsseldorf, 40225, Düsseldorf, Germany

§: These authors contributed equally.

*: Corresponding authors.

Prof. Dr. Holger Gohlke:

Email: gohlke@uni-duesseldorf.de

Prof. Dr. Alexej Kedrov:

Email: kedrov@hhu.de

Abstract

Bacterial cells transport hundreds of proteins across their cytoplasmic membrane via the evolutionarily conserved machinery built of the SecYEG protein translocon and the cytoplasmic ATPase SecA. The protein transport via SecA:SecYEG depends on the charge and fatty acids unsaturation of the surrounding lipids. The unsaturation degree determines the lipid packing and influences SecA binding at the interface, but its potential effects on the membrane-embedded translocon were not studied. By means of biochemical analysis and molecular dynamics simulation we show here that the fatty acid composition within the lipid bilayer affects the conformational dynamics of SecYEG. Elevated levels of unsaturated fatty acids shift the equilibrium of the lateral gate to its open state associated with the transport, while the equimolar ratio of saturated and unsaturated fatty acids stabilizes the idle closed conformation. Simulations in membranes with different cardiolipin species indicate the same dependence and so explain the stimulatory effect of tetra-oleoyl cardiolipin on translocation. Analysis of SecYEG:lipid interactions identified aromatic residues W293 and F294 within the lateral gate as potential sensors of the membrane environment. Replacing those with alanines greatly increased the translocation efficiency in the presence of saturated fatty acids, suggesting that the SecYEG dynamics and activity may be tuned by altering its interactions with lipids. Our findings establish a direct mechanistic link between membrane lipid packing and the translocon gating, revealing SecY as a lipid-responsive conformational switch.

Introduction

Protein transport across the bacterial membranes is an essential process for microbe viability and pathogenicity¹. The Sec pathway is one of the most conserved and minimalistic transport routes, which is built of the protein-conducting channel (SecYEG in *Escherichia coli*) embedded in the cytoplasmic membrane, and the motor ATPase SecA that powers translocation of unfolded polypeptide chains². The SecA:SecYEG complex facilitates translocation of unfolded polypeptide chains, which then either reside in the periplasm, are incorporated into the outer membrane, or are secreted into the extracellular environment. Extensive studies of SecA:SecYEG-mediated protein transport provided detailed structural and functional insights into how protein-protein interactions govern the targeting, engagement, and translocation of substrates³⁻⁵. In particular, they suggested that the dynamics of the ATPase SecA is coupled to the opening of the translocon, which includes displacement of the plug domain from the central pore, and repeated widening of the lateral gate⁶⁻⁸. In contrast, the understanding of the associated protein-lipid interactions remains comparatively limited, though the bacterial membrane is known to be a critical modulator of Sec-mediated transport⁹⁻¹¹. The electrostatic properties of the lipid membrane were recognized early as a decisive factor in translocation, and they have received much of attention over the decades^{12,13}. Anionic phospholipids, first of all phosphatidylglycerol (PG) and cardiolipin (CL), promote membrane association of the amphipathic N-terminal helix of SecA, even in the absence of SecYEG^{14,15}, and stimulate the translocation via SecA:SecYEG. More recently, lipid packing has been identified as another determinant of transport efficiency, as the loosely packed bilayer built of lipids with unsaturated fatty acids (UFAs) strongly augmented protein translocation¹⁶. Biophysical analysis and molecular dynamics simulations linked the abundance of UFAs to elevated SecA:membrane binding, followed by SecA:SecYEG association. Given the highly dynamic nature of membrane acyl-chain composition, these findings suggest that protein transport may be tuneable *in vivo* through lipid remodelling^{17,18}. Although the

efficiency of SecA binding to the lipid membrane qualitatively correlates with protein translocation, UFAs affect translocation 5- to 10-fold more strongly than they affect SecA-membrane interactions¹⁶. One possible explanation is that efficient protein translocation requires the cooperative action of multiple SecA molecules, amplifying the effect of reduced membrane binding on transport¹⁹⁻²¹. However, the specific properties of the lipid bilayer may also influence the dynamics of the embedded SecYEG, as lipid packing and the associated lateral pressure can modulate the activity of various protein complexes in bacteria and eukaryotes²²⁻²⁴. More specifically, lipid packing and lateral pressure could affect the translocon functional dynamics, e.g. by tuning the opening of the lateral gate, a conformational change required to insert the signal peptide, and possibly to let the polypeptide chain through²⁵ (Figure 1A). Here, we demonstrate that the fusion of SecA and SecYEG does not eliminate the dependence on the UFA content, indicating that the effect of the membrane composition goes beyond SecA recruitment (Figure 1B). All-atom molecular dynamics simulations and free energy analysis reveal how the lipid environment shifts the equilibrium of the SecY lateral gate toward either the open or closed state. The simulations point to the aromatic residues that build conformation-specific contacts with the surrounding lipids. Substitutions of those residues led to the creation of a “super-translocating” SecYEG mutant that further shows a reduced inhibitory effect of saturated fatty acids on SecA:SecYEG-mediated transport. It thus supports our claim that this mutant acts “lipid independent” and that the differences in translocation are only a result of SecA binding defects.

Results

Effect of SecA binding on translocation – SecYA-EG fusion construct

SecA:lipid interactions are recognized as a crucial factor for protein translocation, as SecA binding to the lipid membrane greatly enhances the translocation efficiency²⁶. It has been proposed that a conformational change upon membrane binding primes SecA for interactions with SecYEG and facilitates the assembly of the translocation machinery (Figure 1 B). Both the

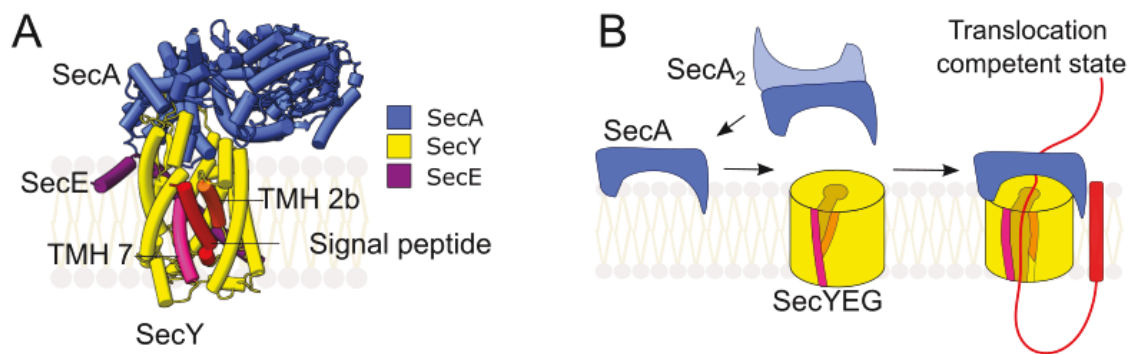


Figure 1. Structure of SecY with open lateral gate during signal peptide insertion and scheme describing SecA recruitment. (A) Structure of the substrate-engaged SecA:SecYE complex (PDB:6ITC). SecA is shown in blue, SecY in yellow, the lateral gate built by SecY TMHs 2b and 7 in orange and pink, and the inserted signal peptide in red. (B) SecA binding to the membrane and conversion from dimer to monomer is a step towards the assembly of the SecA:SecYEG translocation machinery.

lipid charge and packing modulate SecA:membrane interactions^{9,10,16,27}, so the translocation efficiency of SecA:SecYEG depends strongly on the physical properties of the membrane. To minimize SecA binding to the membrane and thereby investigate the intrinsic behaviour of SecYEG in different lipid environments, liposomes with 3 mol% DOPE-polyethylene glycol (PEG) were employed, as this molecular crowder should block recruitment to the membrane (Figure 2A). In liposome flotation assays, incorporation of PEG reduced SecA binding from 57% in DOPC:DOPG liposomes to 29% in DOPC:DOPG:PEG2000 liposomes. Despite this reduction, *in vitro* translocation assays still revealed an approximately ten-fold higher transport activity for SecYEG reconstituted in DOPC:DOPG liposomes compared with POPC:POPG liposomes (Figure 2B, C and Suppl. Figure 1). Thus, reducing membrane association of SecA did not abolish the lipid-dependent differences in translocation efficiency, consistent with the defects previously reported by Kamel et al.¹⁶. It remained elusive, however, whether the lipid composition has a specific functional effect on the membrane-embedded translocon SecYEG. In particular, variations in the lipid packing and the associated changes in the membrane lateral pressure could affect the translocation-associated dynamics of SecYEG. To probe this potential effect independently of the lipid-mediated recruitment of SecA, we aimed to assess the translocation activity of a fusion protein, where SecA was linked to the C-terminal end of SecY (further referred to as lipids (mono-UFA lipids), as those lipid compositions previously showed prominent differences in SecA:SecYEG-mediated transport¹⁶ (Suppl. Figure

SecYA-EG). That way, the ATPase remained in the immediate proximity to the translocon (Figure 2D and Suppl. Figure 2). Such a construct containing the proteins from *Thermus thermophilus* has been previously used in biochemical and single-molecule studies, which validated its functionality^{28,29}. In accordance with the previous design, we prepared a construct built of *E. coli* proteins, with a flexible glycine-serine linker between SecY and SecA to provide sufficient conformational flexibility for correct SecA:SecYEG assembly (Suppl. Figure 2 A). The corresponding AlphaFold model suggested that SecYA-EG acquired the same conformation like the translocon and the ATPase as individual entities (Suppl. Figure 2B). SecYA-EG was expressed in *E. coli* and isolated from the cell membranes via solubilization and affinity purification (Figure 2D). SDS-PAGE of the elution fraction manifested the expected band of SecYA fusion above 130 kDa, but also a prominent band at ~100 kDa. The latter band matched the molecular weight of SecA and showed the immunostaining signal against a poly-histidine sequence (Suppl. Figure 2E), thus it likely represents a fraction of the cleaved ATPase, which could be largely removed via size exclusion chromatography (SEC; Suppl. Figure 2C, D). The purified SecYA-EG was then reconstituted into liposomes containing either DOPC:DOPG (also referred to as bis-UFA lipids further in text) or POPC:POPG

relative to SecA:SecYEG assembled in the presence of free SecA, a similar lipid dependence was observed, as the transport in

2F, G). The activity of the reconstituted SecYA-EG was determined by measuring translocation of the urea-denatured precursor of the outer membrane protein A (proOmpA) into the lumen of the proteoliposomes. Although the overall transport efficiency of the fusion protein was reduced

POPC:POPG liposomes was approx. 20-fold lower than in DOPC:DOPG (Figure 2F). Those results reaffirm that the transport deficiencies observed before are most likely not exclusively caused by SecA binding deficiencies, but rather that the lipids also affect the membrane-embedded SecYEG translocon.

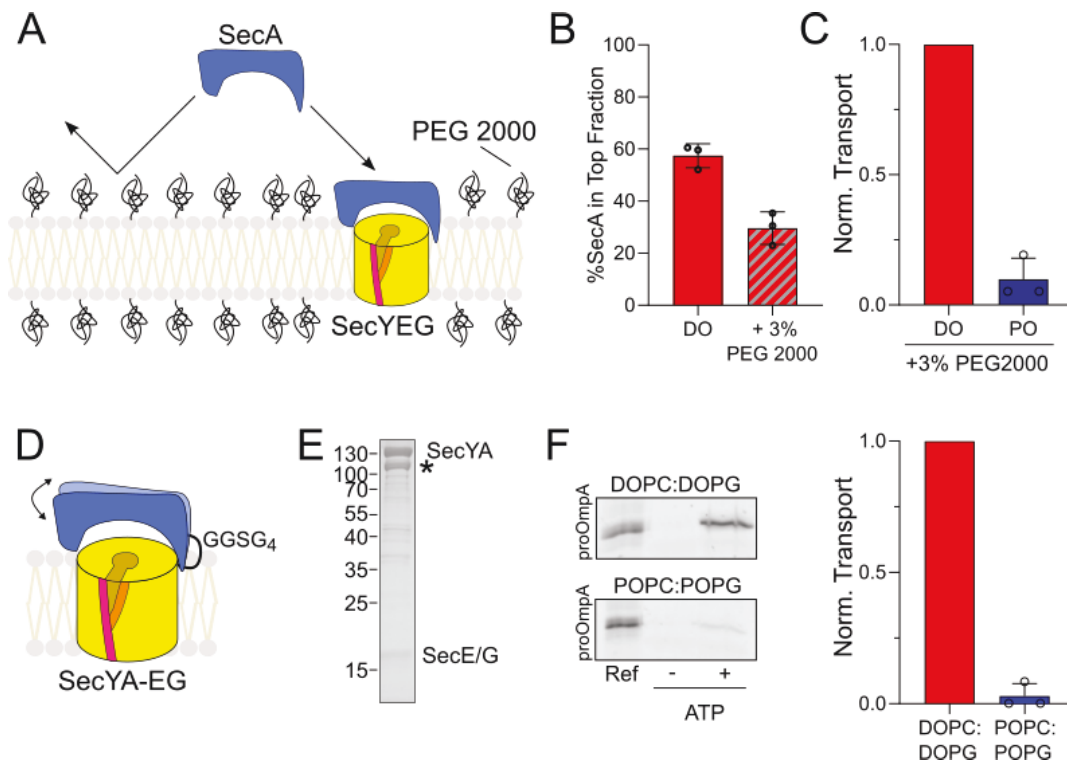


Figure 2. SecA binding prevention by a molecular crowder and characterization of the ATPase-translocon fusion construct SecYA-EG. (A) Schematic on SecA binding reduction by PEG 2000, while binding to the translocon is still possible (Compare Suppl. Figure 1). (B) Results of SecA floatation assay comparing the recovered SecA from Top fraction in presence or absence of 3% PEG2000. (C) Relative translocation efficiency of SecYEG in DOPC:DOPG:PEG2000 Liposomes vs POPC:POPG:PEG2000 Liposomes (molar ratio of 68.5:28.5:3 for both conditions). The translocation in DOPC:DOPG:PEG2000 liposomes was used for normalization. Bars indicate mean values based on technical triplicates (individual values are shown as circles), error bars indicate standard errors of the mean (SEM). (D) Schematics of the SecYA-EG construct, where SecA is genetically fused to the C-terminal end of SecY via a flexible linker (GGSG)₄. (E) Coomassie-stained SDS-PAGE of the SecYA-EG fusion construct isolated via IMAC. (F) Representative in-gel fluorescence of proOmpA used in the translocation assay with the SecYA-EG in indicated liposomes. In the presence of ATP (“+”), the fluorescently labelled preprotein proOmpA is translocated into the liposome lumen, where it is protected from the added protease. The reference sample (“Ref”; 2% of total proOmpA in the assay) is used to quantify the translocation efficiency. Right: Relative translocation efficiency of SecYA-EG. The translocation in DOPC:DOPG liposomes was used for normalization. Bars indicate mean values based on technical triplicates (individual values are shown as circles), error bars indicate standard errors of the mean (SEM).

Fatty acid composition modulates the core translocon

Within the heterotrimer SecYEG, only the central channel-forming subunit SecY and the C-terminal part of the subunit SecE are essential for translocation, while both the N-terminal TMHs 1 and 2 of SecE and the whole subunit SecG are poorly conserved, even among bacteria, and thought to play structural and regulatory roles³⁰⁻³². These ancillary elements are positioned at the periphery of the translocon, being largely exposed to the lipid environment (Suppl. Figure 3A). To probe whether they serve as sensors for the lipid composition, which can transmit the cues to the core translocation machinery, we designed translocon variants, where either the non-essential TMHs of SecE or the complete SecG subunit were deleted (further referred as SecYE^{ΔNG} and SecYE, respectively; Suppl. Figure 3A). The absence of the ancillary elements did not affect either solubilization or affinity purification of the designed translocon complexes (Figure 3A and Suppl. Figure 3B). To examine whether the isolated translocons were correctly folded, we assessed their thermal stability via differential scanning fluorimetry (nanoDSF). Upon heating, the full-length SecYEG and both SecYE^{ΔNG} and SecYE constructs manifested a change in the intrinsic fluorescence that occurred in a similar and narrow temperature range 42-45°C (Figure 3B, Suppl. Figure 3C, D, E). Thus, the deletion of the ancillary structural elements did not have a detrimental effect on the folding of the translocon, so the constructs were further used for functional analysis.

To assess the potential role of the ancillary domains in sensing the lipid environment, the full-length SecYEG, SecYE^{ΔNG}, and SecYE were reconstituted into liposomes containing either DOPC:DOPG or POPC:POPG lipids, and SecA-mediated translocation of proOmpA was analysed. The overall translocation was markedly reduced for both truncated variants, and the decline was most pronounced for SecYE that retained only approx. 30% of the transport activity in DOPC:DOPG (Figure 3C, D). The reduced activity was expected, as SecG is known to modulate SecA:SecYEG interactions and stimulate the transport^{33,34}. Conversely, the N-terminal helices of SecE approach the lateral gate of SecYEG at the periplasmic side thus, once

present, they may attenuate its translocation-related dynamics^{35,36}. Despite the differences in the magnitude of the translocation efficiency, all the examined variants exhibited similar decline in transport upon switching to POPC:POPG bilayers.

Thus, although the effect of the fatty acid architecture was not linked to the ancillary elements within the translocon, it must involve the channel-forming core subunit SecY.

A change in the membrane composition alters the opening of SecYEG lateral gate

Minor differences in the membrane composition can affect the dynamics of the embedded proteins, either via specific protein:lipid interactions or via bulk properties of the lipid bilayer, such as the lateral pressure³⁷⁻³⁹. While the effect of specific interactions can often be explained via structural analysis, elucidating the role of the bulk properties or transient interactions can be more challenging. To assess the influence of the lipid composition on the translocon dynamics, we set up all-atom unbiased MD simulations of the membrane-embedded SecYEG (Suppl. Table 1)⁴⁰ focusing on the conformation of the lateral gate, as its widening is a pre-requisite for insertion of a signal peptide at the early stage of the translocation, as well as transport of the polypeptide chain through the central pore^{41,42}. Hence, *E. coli* SecYEG in either closed or open conformations were simulated in DOPC:DOPG and POPC:POPG bilayers (Figure 4A), and the spontaneous dynamics was followed in five independent simulations (replicas), each 1 μs long. To track the state of the lateral gate, the distance between the centres of mass of the gate-forming TMH 2b (residues 82-96) and TMH 7 (residues 275-294) was used as a reaction coordinate (Figure 4B). The specific architecture of the lipid fatty acids appeared to have an effect on the SecYEG dynamics. When starting the simulations from the closed conformation (lateral gate width ~13 Å, Figure 4C), spontaneous transitions to the open state were observed in the presence of DOPC:DOPG in two out of five replicas ((Fischer's exact test, two-sided, $p = 0.444$) Figure 4D, Suppl. Figure 4). In contrast, the translocon remained in its closed conformation when modelled in POPC:POPG,

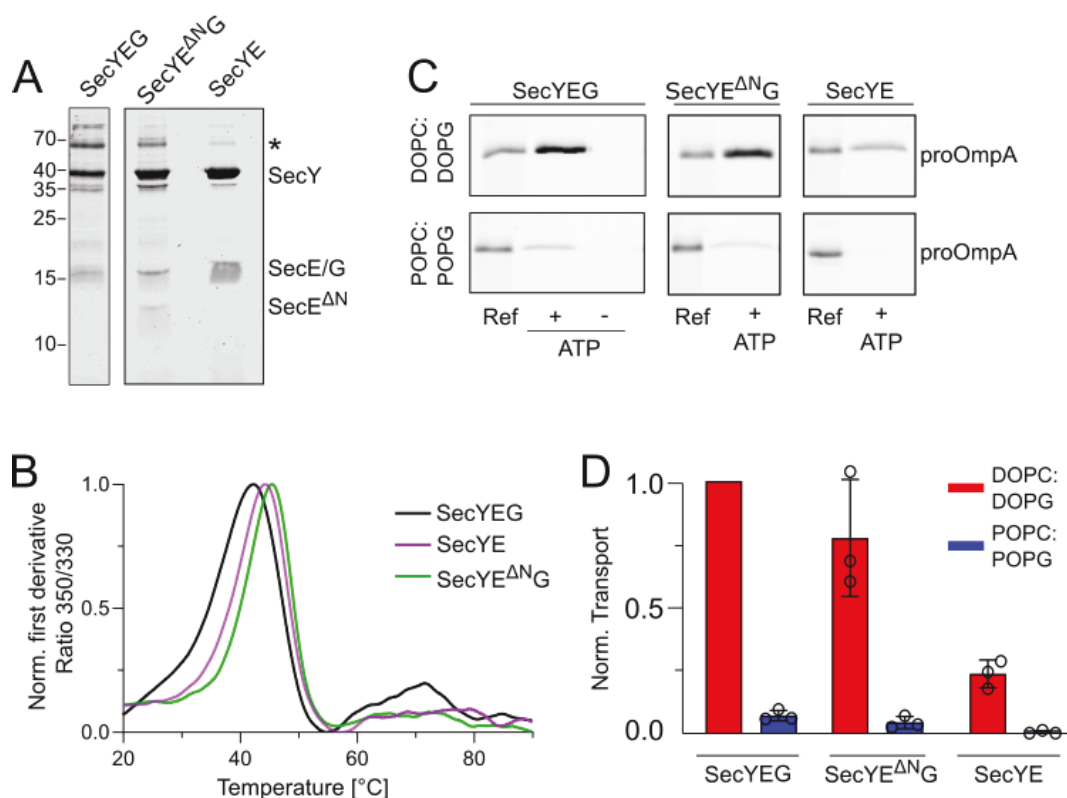


Figure 3. Truncated translocon variants SecYE Δ NG and SecYE retain the functional dependence on the lipid composition. (A) Coomassie-stained SDS-PAGE of the affinity-purified translocon variants. The translocon subunits are indicated. The band at ~60 kDa (indicated with an asterisk) likely corresponds to the co-purified membrane protein insertase YidC. (B) nanoDSF thermograms of the detergent-isolated translocons (shown as first derivative of the intrinsic fluorescence ratio at 350 nm and 330 nm) indicate cooperative unfolding events above 40 °C. (C) Representative in-gel fluorescence of proOmpA used in the translocation assay with the translocon variants in the indicated liposomes. In the presence of ATP (“+”), the fluorescently labelled preprotein proOmpA is translocated into the lumen, where it is protected from the added protease. The reference sample (“Ref”; 10% of total proOmpA in the assay) is used to quantify the translocation efficiency. (D) Translocation efficiency of the individual translocon variants in indicated lipid environments. The translocation via the full-length SecYEG in DOPC:DOPG was used as a reference for normalization. Bars indicate mean values based on technical triplicates. Individual values are shown as circles, and the standard errors of the mean (SEM) are shown as whiskers.

and the lateral gate motion was limited to minofluctuations (Figure 4E, Suppl. Figure 5). Contact analysis suggested that the hydrophobic residues M83, S87, I82 and I91 (TMH 2b) as well as I275, I278, F279 and L285 (TMH 7) remained in proximity, and could stabilize the closed conformation (Suppl. Figure 6). When the open state of SecYEG was taken as a starting point (lateral gate width ~17 Å, Figure 4F), no transition to the closed conformation was observed in DOPC:DOPG lipids (Figure 4G, Suppl. Figure 4),

while the POPC:POPG environment promoted closing of the lateral gate in two simulation replicas ((Fischer’s exact test, two sided $p = 0.444$, Figure 4H, Suppl. Figure 5). Thus, the bis-UFA lipids seem to favour the open state of SecYEG, which is associated with substrate translocation, so SecYEG:lipid interactions should be considered when interpreting the results of biochemical experiments. We also assessed whether the dynamics of the plug domain (TMH 2a) is affected by changes in

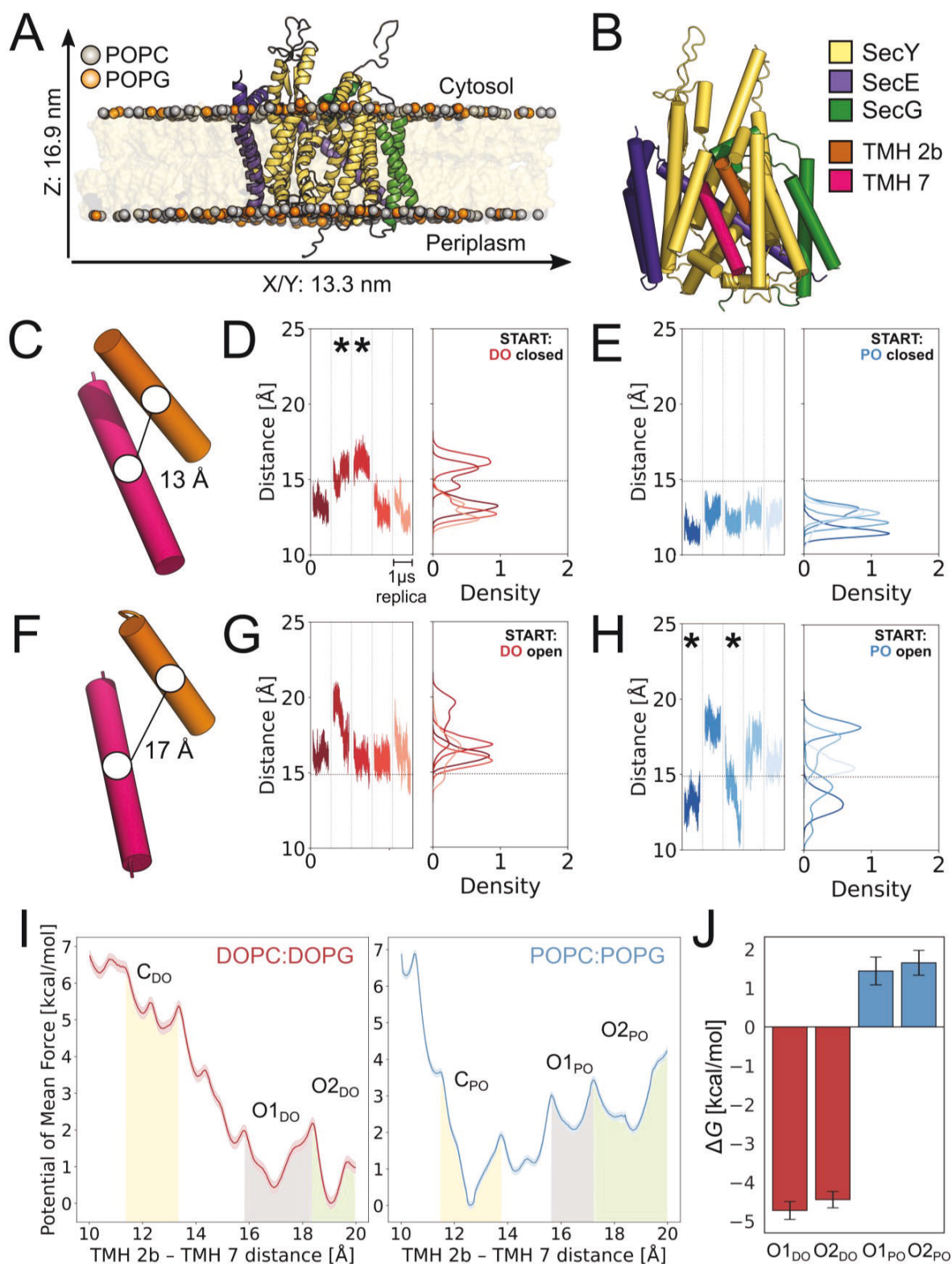


Figure 4. The lipid membrane composition influences the opening likelihood of the SecYEG lateral gate. (A) Visualization of SecYEG embedded in POPC:POPG bilayer. The dimensions of the simulation box are indicated. (B) SecYEG model in the closed conformation. (C) The lateral gate width in the closed conformation of SecYEG. The gate-forming TMH 2b and TMH 7 are shown as orange and pink tubes, respectively. The width is defined as the distance between the center of mass (COM) of the C_{α} of residues of TMH 2b and TMH 7. (D) Time evolution of the lateral gate width for independent

simulations replicas for the closed SecYEG in DOPC:DOPG, (*left*); The dotted horizontal line indicates the open/closed state threshold width (14.9 Å) in the AlphaFold3 model, identified as closed configuration representative. Vertical dotted lines separate individual replicas. Kernel density estimates (KDE) of the width distribution per replica (*right*). In all panels, distances above and below the threshold correspond to the open and closed conformations of the lateral gate. The simulations showing the switching between conformations are indicated with asterisks (*). **(E)** Time evolution of the gate width for independent simulations replicas (shaded blue) for the closed SecYEG in POPC:POPG, (*left*); KDE of the distance distribution per replica (*right*). **(F-H)** Same as (C-E), but calculated using the open conformation of SecYEG as a starting state for the simulations. **(I)** Potential of mean force (PMF) profiles describing the lateral gate opening in umbrella sampling MD simulations in both types of the lipid bilayer. The shaded areas along the PMF curves show the standard error of the mean (SEM). The shaded regions under the PMF profiles are the integration limits used to calculate K_{opening} (eq. 1). Three different states are identified: a single closed state (yellow, C_{DO} or C_{PO}), and two open states (grey, $O1_{\text{DO}}$ and $O2_{\text{PO}}$, and green, $O2_{\text{DO}}$ and $O2_{\text{PO}}$), respectively. **(J)** The free energy difference between the closed and open SecYEG conformations is reported as ΔG (kcal/mol $\pm$ SEM). Bars indicate the predicted ΔG . The SEM is also indicated, for DO (red) and PO (blue), considering two different open states (O1 and O2), respectively.

the membrane composition, given that its mobility is linked to translocon activation and transport⁴³. However, the membrane composition had no pronounced influence on the plug dynamics, and only small differences in root mean squared deviations (RMSD) were identified when comparing open and closed conformations. The plug in the open state was found to be slightly more mobile than in the closed state (Suppl. Figure 7), in agreement with the loss of stabilizing interactions in the translocation tunnel of SecYEG⁴³. To explain the lipid-specific dynamics of the lateral gate, we aimed to measure the energetics of its transition between open and closed states and analyse the effect of the lipid composition. For this purpose, we performed umbrella sampling simulations to compute a potential of mean force (PMF), as done previously by us to probe energetically unfavourable and, thus, rare transitions in other bacterial membrane proteins⁴⁴⁻⁴⁷. The umbrella sampling simulations started from either the closed or open SecYEG conformations (lateral gate widths of 12.8 Å and 19.3 Å, respectively), and according to the unbiased MD simulations where a spontaneous transition was observed, the width of the lateral gate was used as the reaction coordinate. For each transition, 20 windows were sampled in between the closed and open conformations with 1375 ns of sampling time each, of which the first 1250 ns were discarded as equilibration. In both cases, the likelihood densities showed a median overlap of

$28.8 \pm 5.0\%$ and $33.3 \pm 5.3\%$ between contiguous windows, respectively (Suppl. Figures 8-9), which are well-suited for PMF calculations^{46,48}. The standard error of the mean (SEM) was calculated from free energy profiles determined independently every 25 ns from the last 125 ns per window. The PMF of SecYEG opening in both lipid environments was converged and precise (Suppl. Figures 8-9).

The calculated PMF profiles are different between DOPC:DOPG and POPC:POPG bilayers (Figure 4I). In the mono-UFA environment, three distinct energy minima were observed, with positions ranging from 12.6 to 18.7 Å. The position of the global PMF minimum (12.6 Å; C_{PO}) matched the closed conformation of the lateral gate, while the other minima likely reflect sub-states of SecYEG upon the opening ($O1_{\text{PO}}$ and $O2_{\text{PO}}$). The presence of multiple energy minima correlates with varying dimensions of the gate known from available structures^{35,49,50}. Those variations are associated with the translocation cycle and depend on the presence of SecA or ribosome and the substrate protein. Furthermore, SecYEG can facilitate translocation of partially folded polypeptide chains and even bulky synthetic substrates up to 2 nm diameter, suggesting substantial plasticity, which must rely on a wide gate opening, such as $O2_{\text{PO}}$ (Suppl. Figure 8). Overall, the PMF in POPC:POPG reveals that the closed state is more favorable than the open ones, and the configurational free energy

difference between the minima of the closed and the most open state O2_{PO} is 2.1 ± 0.4 kcal/mol. In contrast, the global energy minimum of SecYEG in DOPC:DOPG was observed at 19.1 Å (O2_{DO}), corresponding to the widely open gate, although another minimum (O1_{DO}) was found in proximity, at 16.9 Å. The configurational free energy difference between the minima of the closed and the open state O2_{DO} is -4.7 ± 0.2 kcal/mol, which indicates that the open state is energetically favoured over the closed one (Figure 4I). Following previous work^{46,47,51} we have used PMFs of the closed-to-open transitions in different membrane compositions to compute free energies of SecYEG opening. In contrast to the configurational free energy differences, which are obtained from the relative depths of the PMF minima, these values were determined by integrating the probability distributions over the respective basins corresponding to the closed and open SecYEG configurations (Equations 1 and 2), yielding the opening free energies $\Delta G_{O2_PO} = 1.44 \pm 0.07$ kcal/mol, $\Delta G_{O2_DO} = -4.73 \pm 0.05$ kcal/mol. Using the first opening state (O1) instead of the second (O2) to define the local minimum of the open state did not significantly alter the free energy values, (Figure 4J, Equations 1-2). Thus, the open conformation of SecYEG is computed to be thermodynamically preferred over the closed one in DOPC:DOPG, but not in POPC:POPG, in line with the observations made during the unbiased MD simulations.

Cardiolipins affect the SecYEG conformation in the fatty acid-dependent manner

Next to phosphatidylglycerol lipids, cardiolipins (CL) contribute to the negative charge of the bacterial membrane, and they strongly augment SecA:SecYEG-mediated translocation^{52,53}. Notably, the stimulatory effect of cardiolipins could be reconstructed in liposomes supplemented with tetra-oleoyl CL (TOCL), but not with the CL variant containing both unsaturated (oleoyl-) and saturated (palmitoyl-) acyl chains, bis(PO)CL (Figure 5A)¹⁶. The translocation measured in POPC:TOCL bilayers was approx. 10-fold higher than in POPC:bis(PO)CL; however, less than a two-fold difference in SecA binding was observed, thus raising the question whether other factors influenced the functionality of the system¹⁶. To assess the influence of cardiolipin species on

SecYEG dynamics, we performed all-atom unbiased MD simulation of SecYEG in POPC enriched with either bis(PO)CL or TOCL, following the same computational approach as described above (Figure 5A). When starting from the closed conformation in POPC:bis(PO)CL bilayers, SecYEG remained closed throughout all simulations (Figure 5B, Suppl. Figure 10), but it repeatedly underwent spontaneous closure when the open state was modelled (three out of five replicas; Fischer's exact test, two sided $p = 0.444$, Figure 5C, Suppl. Figure 10). Thus, the conformational preference in the presence of bis(PO)CL greatly resembled that in the POPC:POPG environment and correlated with the low translocation efficiency reported earlier¹⁶. Differently, the initially closed SecYEG could spontaneously open in POPC:TOCL (two out of five replicas; Figure 5D, Suppl. Figure 11), and it remained constitutively open across all replicas, when the simulations started with the open conformation (Figure 5E, Suppl. Figure 11). Following the established umbrella sampling approach, we calculated PMFs in both membrane compositions (Suppl. Figures 12-13) and (Figure 5F). In POPC:bis(PO)CL, a broad global PMF minimum was identified for a lateral gate width of 12-14 Å, matching the closed state, while no marked local minima could be recognized beyond that. By contrast, SecYEG in POPC:TOCL manifested a single global minimum at 16.5 Å (Figure 5F), matching the O1 open state described before (Figure 5I). Thus, in the presence of bis(PO)CL, SecYEG alone could only exist in the closed state, while TOCL favoured the open conformation, again indicating that UFA-rich lipids contribute to stimulation of protein transport.

Dynamics of the lateral gate is associated with specific protein:lipid interactions

Both unbiased MD simulations and umbrella sampling simulations revealed a preference of SecYEG for the open conformation in UFA-rich lipids, thus corroborating the biochemical observations of the enhanced translocation activity. We questioned then whether differences in protein:lipid interactions can be deduced from the analysis *in silico*. To investigate how specific lipid species interact with the lateral gate in its open and closed conformations, we focused on

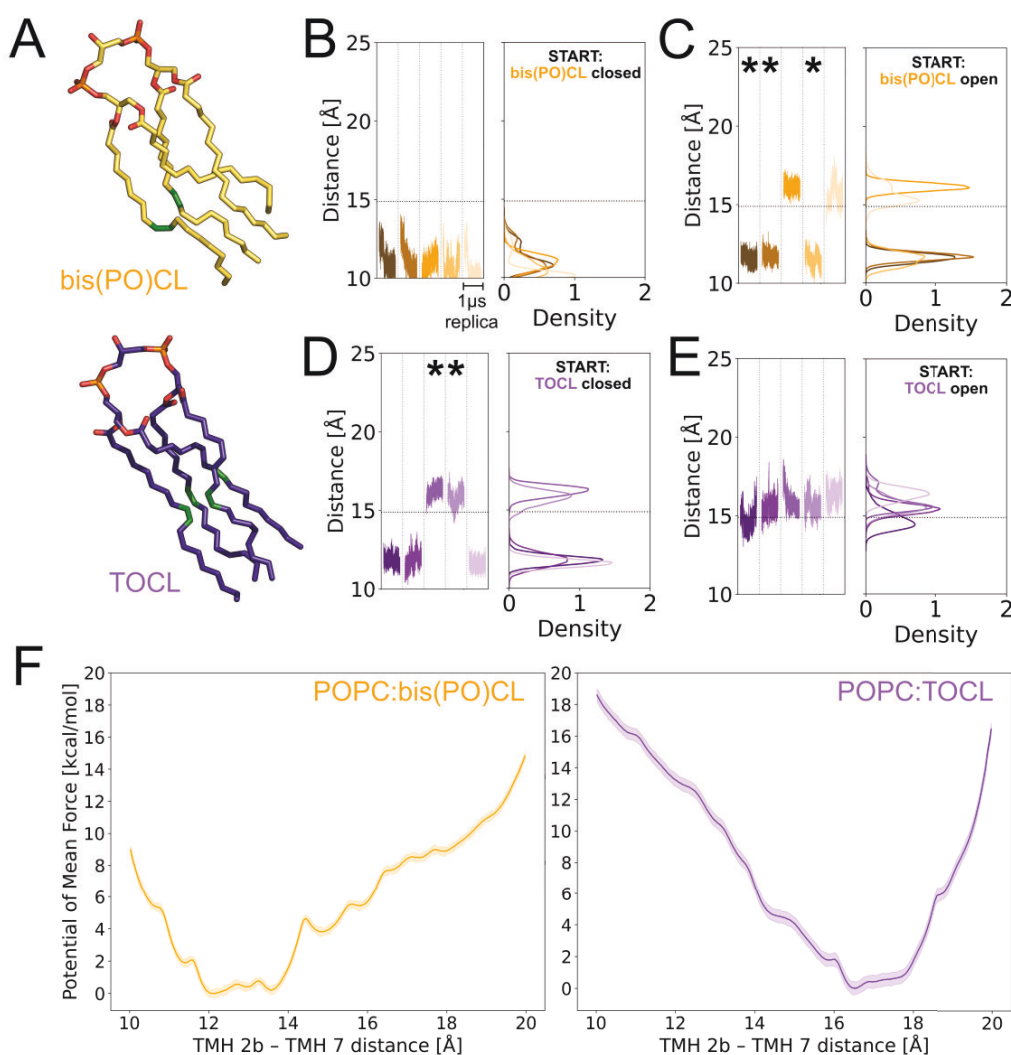


Figure 5. Effects of cardiolipin species on the conformational preference of the SecYEG lateral gate. (A) Cardiolipins used in this study: bis(PO)CL is shown as yellow sticks. TOCL is shown as purple sticks. The double bonds within the oleic fatty acids are shown in green. (B) Time evolution of the TMH 2b-7 inter-helical distance for independent simulation replicas (shaded orange) starting from the closed conformation of SecYEG in POPC:bis(PO)CL 70:15 systems, (left); kernel density estimates (KDE) of the distance distribution per replica (right). (C) Time evolution of the TMH 2b-7 inter-helical distance for independent simulation replicas (shaded orange) started from the open conformation of SecYEG in POPC:bis(PO)CL, (left); kernel density estimates (KDE) of the distance distribution per replica (right). (D and E) Same as B and C, calculated for SecYEG in POPC:TOCL. (F) Potentials of mean force (PMF) describing the lateral gate opening in POPC:bis(PO)CL (left, orange) and POPC:TOCL (right, purple) lipid bilayers. The shaded areas at the curves show the standard error of the mean (SEM).

the umbrella sampling simulations performed in POPC:POPG and DOPC:DOPG, as the resulting PMF profiles contained well-defined minima for both conformational states (Figure 4). In contrast, the cardiolipin-containing membranes strongly stabilized either the open (TOCL) or closed (bis(PO)CL) state (Figure 5), preventing an equivalent state-resolved comparison.

An initial analysis aimed to assess whether UFA-rich lipids influence headgroup distributions around SecYEG, potentially stabilizing open or closed conformations through interactions with specific SecY regions. Previous studies have shown that anionic lipids associate with lysine clusters in SecYEG and that PG can dock at the

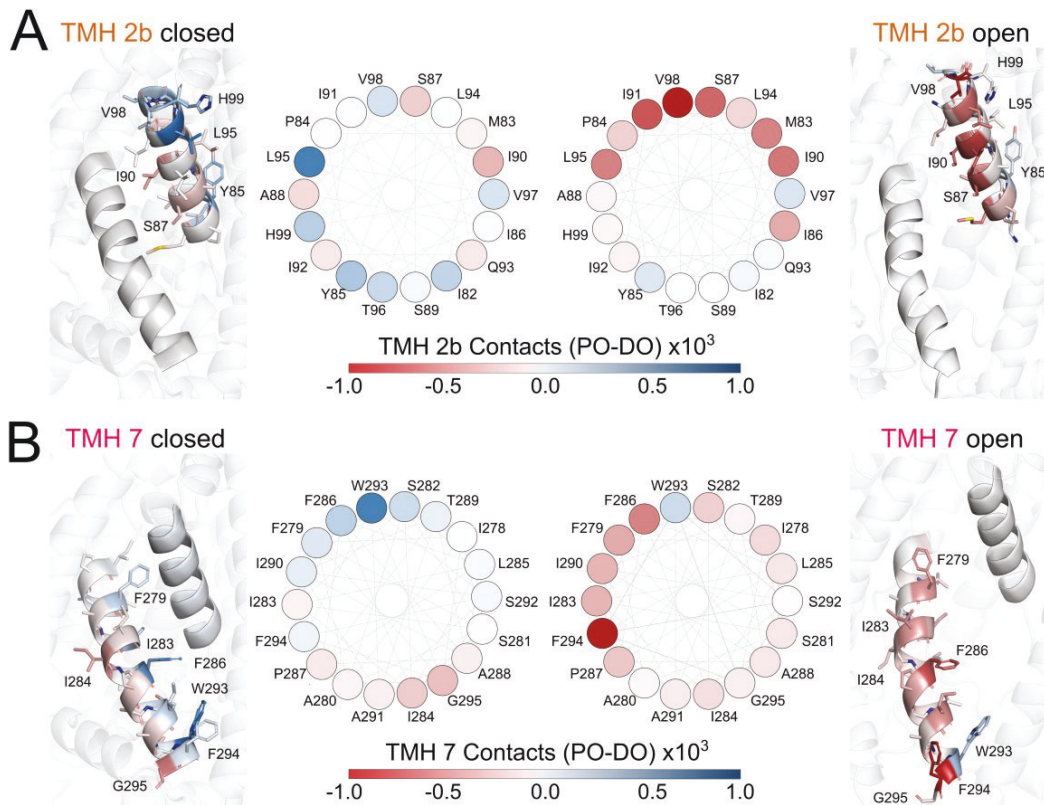


Figure 6. Analysis of SecY TMH 2b or TMH 7 contacts to lipids in the closed and open conformations. Helical wheel projections showing the per-residue difference in lipid contact frequency for either the closed (*left*) or open (*right*) conformations of the SecYEG lateral gate between umbrella sampling simulations in POPC:POPG (PO) or DOPC:DOPG (DO), expressed as the difference PO - DO for TMH 2b (**A**) and TMH 7 (**B**). The color intensity reflects the magnitude and direction of the contact difference, with red indicating more contacts in DO and blue indicating more contacts in PO. Structural representations of TMH 2b and TMH 7 are shown alongside the helical wheels, with residue side chains colored according to their contact difference. The closed (open) conformation of the lateral gate is the global energetic minimum in the PO (DO) PMF, respectively.

lateral gate in the open state¹⁵. However, 2D density maps of polar headgroups did not reveal consistent or interpretable patterns (Suppl. Figures 14-15), and subsequent analyses therefore focused on interactions involving the apolar fatty acid chains. For both analysed lipid compositions (DOPC:DOPG and POPC:POPG), protein-lipid contacts at the lateral gate were calculated on the local minima identified for the closed and open configuration (Figure 5A, B). TMH 2b manifested a pronounced asymmetry across the helix face: Aligned residues M83, S87, I91, L95, and V98 showed moderately increased contacts with bis-UFA lipids in the open configuration, whereas residues on the opposing side, such as I82, Y85, T96 and H99, displayed substantially elevated contacts in the presence of mono-UFA species in the closed configuration. A

particular case is represented by L95, which shows the highest contacts with the POPC:POPG membrane in the closed configuration and one of the largest contributions in the interaction with DOPC:DOPG in the open configuration (Suppl. Figures 16-18). A similar pattern is observed for SecY TMH 7, where many residues manifest conformation- and UFA-specific contacts with the surrounding lipids (Suppl. Figures 19-21). In particular, W293 (periplasmic end of the helix) builds extensive contacts with lipids in the POPC:POPG membrane in the closed state, while the neighbouring F294 interacts with DOPC:DOPG when the gate is in the open conformation. The positions of these residues may suggest a state-dependent rotation of TMH 7, in which the aromatic and hydrophobic surface accessible to

lipids shifts between the closed and open conformations of SecYEG. Taken together, the contact analyses of both TMH 2b and TMH 7 indicate that the UFA composition may modulate the lipid interactions at the lateral gate, and this may explain the difference in free energy deduced from the PMFs.

Taken together, the contact analyses of both TMH 2b and TMH 7 demonstrate that the UFA composition may modulate the lipid interactions at the lateral gate, and this may explain the large difference in free energy observed in the umbrella sampling.

Mutations at the lateral gate of SecY attenuate the lipid sensitivity of transport

The prominent difference in the protein:lipid interactions resolved at the level of the individual amino acids, first of all W293 and F294 (Figure 7A), suggests that those bulky residues may tune the lateral gate dynamics, also in response to the UFA composition⁵⁴. Interested in their possible functional effect, we mutated both W293 and F294 to alanine residues and set out to measure the resulting preprotein translocation. Although aromatic residues at the ends of TMHs may influence membrane protein folding, the mutant SecY^{AA}EG could be overexpressed, isolated and purified to homogeneity, with its SEC elution profile being comparable to that of the wild-type SecYEG (Suppl. Figure 22A, B)⁵⁵. Furthermore, nanoDSF analysis revealed a melting temperature of approximately 50°C (Suppl. Figure 22C), indicating that the overall structural integrity of the translocon remained intact. We reconstituted SecY^{AA}EG in POPC:POPG liposomes and compared its functionality to the wild-type SecYEG. Strikingly, SecY^{AA}EG manifested nearly 20-fold higher translocation of proOmpA, thus supporting the hypothesis that W293 and F294 may modulate the protein dynamics and the resulting functionality (Figure 7 B, C). We further set out to probe the sensitivity of SecY^{AA}EG to the UFA composition. While the activity of the wild-type translocon was up to 10-fold reduced when switching from DOPC:DOPG to POPC:POPG, the effect was reduced to approx. 2-fold for SecY^{AA}EG (Figure 7 D, E), while the ATPase activity of SecA did not differ for the different liposomes (Suppl. Figure 23). The overall transport in DOPC:DOPG liposomes to over 40%, exceeding known and well

characterized hyper- active SecYEG mutants, such as the PrlA4^{16,56}. A comparable trend was observed when cardiolipin species differing in UFA composition were compared. SecY^{AA}EG manifested prominent activity also in the presence of bis(PO)CL (Figure 7 F, G), in contrast to the wild-type translocon¹⁶. The remaining two-fold difference seen between the tested lipid compositions may be explained by altered SecA binding, as Kamel et al. reported lipid-dependent differences in SecA binding of a similar magnitude¹⁶.

Discussion

Protein transport through the Sec machinery is among the most conserved and essential processes in bacteria⁵⁷. Though several important aspects, such as mechanisms that couple transport and ATP hydrolysis, remain debated⁵⁸, the process is commonly described as a sequence of steps, including SecA binding to the membrane and SecYEG itself, membrane insertion of the signal peptide via the lateral gate, followed by ATP-dependent transport of the unfolded polypeptide chain through the opened translocon. Proteinaceous and physicochemical factors, such as chaperones and the proton gradient across the membrane, can modulate the translocation efficiency, but also the composition of the lipid bilayer has pronounced effects. Previously, we observed enhanced binding of SecA to the UFA-enriched lipid bilayers, which largely correlated with the improved translocation, suggesting an additional and underappreciated effect of the environment on the ATPase¹⁶. Here, we have questioned whether UFAs have an additional effect on dynamics of the membrane-embedded component, SecYEG. Consistently, biochemical analysis has indicated that the functionality of the translocon is indeed sensitive to the lipid composition, molecular simulations have shown that the fatty acids impact the conformational equilibrium of the SecY lateral gate, and the computed free energy of the transition between the closed and open states radically altered in response to changes in the UFA content. The UFA-rich lipids, including TOCL, promote the open state of SecYEG that is associated with the substrate translocation, in agreement with the *in vitro* experiments, thus also resolving

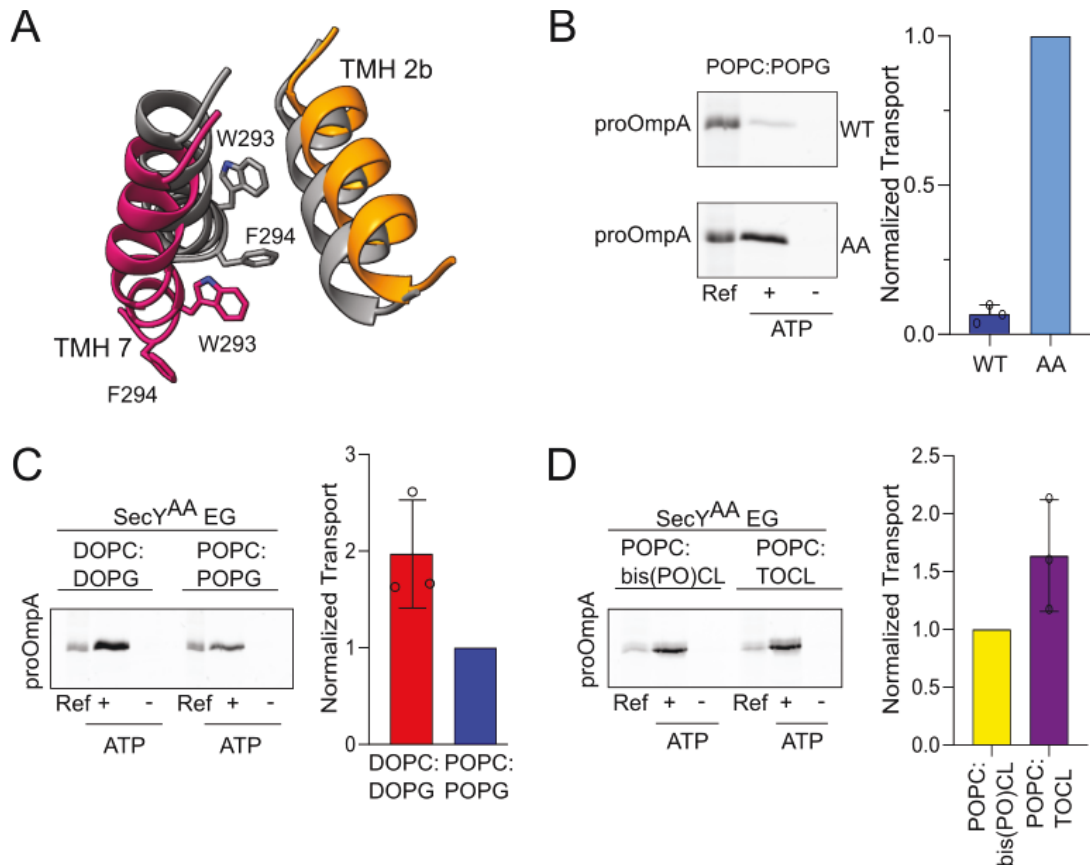


Figure 7. Characterization of the SecY^{AA}EG Mutant. (A) Comparison of the closed (in grey) and open (in pink/orange) conformations of the lateral gate. Positions of the lipid-binding residues W293 and F294 are indicated. (B) Representative in-gel fluorescence of proOmpA used in the translocation assay with the wild-type SecYEG (“WT”) and the mutant SecY^{AA}EG (“AA”) in POPC:POPG liposomes. Relative translocation efficiencies are shown as bar diagrams, with mean values $\pm$ SEM and individual measurements ($n=3$). (C) Representative in-gel fluorescence of proOmpA used in the translocation assay with SecY^{AA}EG in indicated liposomes. Relative translocation efficiencies are shown as bar diagrams, with mean values $\pm$ SEM and individual measurements ($n=3$). (D) Representative in-gel fluorescence of proOmpA used in the translocation assay with SecY^{AA}EG in indicated cardiolipin-containing liposomes. Relative translocation efficiencies are shown as bar diagrams, with mean values $\pm$ SEM and individual measurements ($n=3$).

inconsistencies observed in previous work¹⁶. On a molecular level, these effects can be explained by protein:lipid interactions at the lateral gate of SecY: The aromatic residues W293 and F294 at the periplasmic end of TMH 7 manifest extensive conformation-specific contacts with the surrounding lipids, so these interactions may influence the lateral gate dynamics. Strikingly, the translocon mutant lacking those bulky residues manifest enhanced activity in both mono-UFA and bis-UFA environments, while the remaining two-fold difference matches well the effect of UFAs on SecA binding. Thus, we propose that the fatty acid composition of the membrane may be a potent regulator of protein transport via the interactions

formed at the interface and within the core of the lipid bilayer.

Conformational dynamics of the lateral gate is a crucial factor for both protein transport and insertion of nascent membrane proteins via the translocon. Up to date, all small-molecule agents supposed to inhibit the translocon activity in eukaryotes and bacteria are aiming to suppress the lateral gate dynamics⁵⁹. Furthermore, the current model of SecA activity suggests that the ATP hydrolysis at the translocon is mainly required to facilitate widening of the gate or to maintain it in its open state, and not to physically move the substrate protein⁸. Thus, the effect of lipids on SecYEG dynamics and the functionality

could be expected, though it was not previously considered in experiments. Extensive investigations of SecYEG dynamics focused primarily on dynamics of the central plug domain. Using a similar methodology as presented here, MD simulations of SecYEG in DOPE:DOPG bilayers revealed how forced displacement of the plug destabilizes the hydrophobic interactions required to maintain the closed conformation, and also assessed the role of the physiologically relevant proton gradient⁶⁰. However, the opening of the gate via either signal peptide of SecA may likely precede the plug displacement, so our study contributes a valuable information on this early stage of the functional cycle. Notably, our unbiased simulations suggest that SecYEG may spontaneously switch between the closed and open conformations, which correlates with the recent single-molecule fluorescence analysis performed in *E. coli* lipid extract, and the functionally-enhanced mutant prlA4 manifested altered dynamics of the lateral gate, corroborating the view on the lateral gate as the major site to regulate the translocation.

While our comparative *in vitro* and computational analysis suggests a molecular mechanism of the lipid-based regulation, there are certain limitations, which should be kept in mind. The experiments have been performed in well-defined environments, which however do not represent the whole complexity of biological membranes, such as differences in ion concentrations across the membrane, the natural diversity of the lipid species, or interactions with the accessory proteinaceous factors. Those may certainly influence the functional activity of SecYEG, but do not question though the described UFA-dependent protein:lipid interactions. Our conclusions about the translocation efficiency are based on studying the model with the highly abundant substrate, proOmpA. As the lateral dynamics is associated with the emergence of the signal peptide, its unique sequence, and first of all the hydrophobicity, may contribute to the complexity of the protein:lipid interactions. The substrate-independent analysis of SecYEG dynamics in molecular simulations, on the other hand, could be applied more broadly, so it can be used for evaluation of new results, but also form the basis for further tests, where variations of lipid compositions can be tested. Then, more replica of the simulated systems should be used to go

beyond observing trends only. Remarkably, the computational analysis of protein:lipid interactions pointed to the aromatic residues at the lateral gate as potential sensors for the lipid environment. The behaviour of the double alanine mutants is in comparison with recently published data by Meier and coworkers rather unexpected as single alanine substitutions in position 293 and 294 did not lead to “super-translocators” but only a minor boost in translocation indicating a cooperativity of both residues, potentially indicating a cooperativity⁶¹. The elevated transport activity observed for SecY^{AA}EG may provide further insights into the evolutionary constraints shaping the Sec translocon. Although the SecY^{AA}EG mutant exhibits enhanced translocation efficiency, we cannot draw conclusions regarding its membrane-sealing properties or substrate proofreading capabilities comparable to the previously reported prlA4 mutant of SecY that is known to transport preproteins even in the absence of its signal peptide⁵⁶.

To summarize, our study provides a solid combination of *in vitro* and computational experiments on how membrane lipid composition, in particular unsaturated fatty acid content, regulates the conformational dynamics of the SecY lateral gate, and identifies the aromatic residues W293 and F294 as cooperative sensors that connect the lipid environment to the open-closed state of the translocon. Our results provide a solid foundation for such studies, including targeted mutagenesis of the lateral gate to dissect the relative contributions of individual residue:lipid contacts. Once resolved, an understanding of lipid-based regulation of Sec-dependent transport may be used for future strategies targeting this essential and conserved machinery in antimicrobial drug design.

Supporting information

This article contains supporting information.

Competing interest statement

The authors declare no competing financial interests.

Methods

Molecular cloning

SecYA-EG fusion construct was inspired by the work of the Tsukazaki group²⁸. Therefore, the *E. coli* SecA gene was amplified via PCR with an overhang of (GGSG)₂ on its N-terminal end and blunt-end ligated into the linearized vector of His-SecY, SecE and SecG (pEK20 previously described in van der Sluis et al. 2002 in which the stop codon of SecY was substituted with a sequence encoding the (GGSG)₂ linker⁶². The organization of the resulting construct is visualized in Suppl. Fig 2. The full-length SecYEG construct was expressed using pEK20 plasmid. The plasmid was further used to generate two truncated variants of the translocon, SecY^{ΔN}G and SecYE. To remove specific regions, either the base pairs encoding the two N-terminal helices of SecE (residues 20 to 64) or the entire secG gene, primers were designed to exclude those fragments by PCR followed by blunt-end ligation.

Expression and purification of the translocon variant

All translocon variations were overexpressed as earlier described in Kater et al., using the *E. coli* C41(DE3) ΔompFΔacrAB strain optimized for membrane protein expression^{35,63}. Cells were lysed (Microfluidizer, M-110P, Microfluidics Corp.) and subsequently debris was removed by centrifugation for 10 min at 20000 x g (Sorvall SS34 rotor, Thermo Scientific). Membranes were isolated by ultracentrifugation for 1 h at 200000 x g. Membranes were then solubilized for 1 h at 4 °C in solubilization buffer (50 mM Hepes/KOH, pH 7.4, 500 mM KOAc, 5% glycerol, 200 μM TCEP, 1% n-dodecyl β-maltoside (DDM; Glycon Biochemicals GmbH). The solubilized material was centrifuged at 21380 x g for 10 min at a tabletop centrifuge (Z 216 MK, Hermle Labortechnik GmbH) and the supernatant was incubated with Ni²⁺-NTA agarose resin (Macherey-Nagel GmbH & Co. KG) for 1 h at 4 °C. The beads were washed with 50 mM Hepes/KOH pH 7.4, 500 mM KOAc, 5% glycerol, 200 μM TCEP, 0.05% DDM, 10 mM imidazole. SecYEG was eluted with 50 mM Hepes/KOH pH 7.4, 150 mM KOAc, 5% glycerol, 200 μM TCEP, 0.05% DDM, 300 mM imidazole. Following a size exclusion chromatography (SEC) was performed in 50 mM Hepes/KOH pH 7.4, 150 mM KOAc, 5% glycerol using a Superdex 200 Increase GL 10/300 (Cytiva). Fractions containing SecYEG were concentrated using Amicon Ultra-4, Ultracel 30K centrifugal filters (Merck/Millipore).

SecA, SecB expression and purification

Overexpression of SecA and SecB in *E. coli* BL21(DE3) cells was induced with 0.5 mM IPTG and carried out for 2 h at 37 °C. Cells were then harvested by centrifugation and resuspended in buffer containing 20 mM Tris/HCl pH 7.5, 50 mM KOAc, 5 mM Mg(OAc)₂, 10% glycerol, 1 mM DTT, and a cOmplete™ protease inhibitor cocktail. Cells were lysed, and the lysate was clarified by centrifugation at 200000 × g for 1 h. The clarified lysate was incubated with Ni²⁺-NTA agarose resin for 1 h at 4 °C. The resin was then washed with buffer containing 20 mM Tris/HCl pH 7.5, 500 mM KOAc, 5 mM Mg(OAc)₂, 5% glycerol, 20 mM imidazole, and 1 mM DTT. The proteins were eluted using buffer containing 300 mM imidazole. Eluted fractions were concentrated using an Amicon Ultra-4 centrifugal filter device, molecular weight cut-off 50 kDa (SecA), and 10 kDa (SecB). The concentrated samples were then subjected to SEC using a Superose 6 Increase 10/300 GL (for SecA) and Superdex 200 Increase 10/300 GL (for SecB) columns equilibrated with 20 mM Tris/HCl pH 7.5, 50 mM KOAc, 5 mM Mg(OAc)₂, 5% glycerol.

proOmpA expression, purification and labeling

For overexpression of the single-cysteine mutant proOmpA_{C302S} in *E. coli* BL21(DE3) cells, cultures were grown at 37 °C with shaking at 180 rpm until OD₆₀₀ of 0.5 was reached. At this point, 4 mM sodium azide was added to inhibit ATPase-dependent protein secretion. After 10 min, overexpression was induced by the addition of 0.5 mM IPTG and carried out for 2 h. During this time, the overexpressed proOmpA accumulated in the cytoplasm in the form of inclusion bodies. Cells were harvested by centrifugation and lysed by *E. coli* Lysis Reagent (New England Biolab). The lysate was centrifuged for 15 min at 20000 × g to pellet inclusion bodies. The pellet was washed three times with 20 mM Tris/HCl pH 7.5 and 50 mM KOAc, followed by centrifugation after each wash. The washed pellet was solubilized in 8 M urea, 20 mM Tris/HCl pH 7.5, and flash frozen until further use. Labelling of proOmpA_{290Cys} with fluoresceine-5-maleimide was performed as described previously⁶⁴.

Protein reconstitution

Liposomes containing 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1-*rac*glycerol) (POPG) or 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-

glycero-3-phospho-(1'-rac-glycerol) (DOPG) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1',3'-bis(1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho)-glycerol (bis(PO)CL) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1',3'-bis(1,2-dioleoyl-*sn*-glycero-3-phospho)-glycerol (TOCL) (all Avanti Polar Lipids, Inc) were prepared as previously described¹⁶. For reconstitution, the liposomes (lipid concentration 5 mM) were incubated with 0.5% Triton X-100 for 10 min on 40 °C, subsequently, 10 min on ice, before 1 nmol of purified SecYEG/SecYA-EG (0.5 nmol for the comparison of SecYEG and SecY^{AA}EG in POPC:POPG liposomes was added to the 1000 nmol of lipids (1:2000 molar ratio). After 30 min on ice, the protein:lipid mixture was diluted to 400 µL with the protein's buffer, before the detergent was removed by rolling overnight at 4 °C using 100 mg Bio-Beads SM-2 sorbent (Bio-Rad Laboratories). Following, the proteoliposomes were pelleted by 30 min centrifugation at 100000 x g and afterwards resuspended in 200 µL buffer containing 50 mM Hepes/KOH pH 7.4, 50 mM KCl. The protein content of the pelleted fraction was analysed via SDS-PAGE.

In vitro translocation assay

For *in vitro* translocation assay of the fluorescently labelled proOmpA, the previously described protocols were followed with minor modifications^{16,65}. For proteoliposomes containing SecYEG WT, SecY^{ANG} and SecYE (10 µL) were mixed with 1 µM SecA and 0.5 µM SecB₄, 0.1 mg/ml BSA, 10 mM DTT, energy mix (10 mM phosphocreatine, 0.05 mg/ml phosphocreatine kinase), 10 mM Mg(OAc)₂ and 1 µM proOmpA. For proteoliposomes containing SecYA-EG, no free SecA was added. The reaction volume was adjusted to 50 µL by addition of reaction buffer (50 mM Hepes/KOH pH 7.4, 50 mM KCl) and incubated at 37 °C for 5 min before translocation was induced by addition of 6 mM ATP in respective tubes. After 15 min of incubation (30 min in case of SecYA-EG), a reference was taken before the reaction was stopped by degradation of non-translocated preprotein through 0.02 mg/mL proteinase K for 15 min. Following, 150 µL 30% TCA was added and incubated on ice for 15 min before aggregated material was pelleted via centrifugation for 10 min at 20,000 x g in a tabletop centrifuge (Z 216 MK, Hermle Labortechnik GmbH). The pellets were washed with 500 µL ice-cold acetone and sedimented via centrifugation for 5 min. Acetone was removed, followed by a 10 min drying step at

37 °C. The dried pellets were resuspended in 15 µL 2.5x SDS-PAGE sample buffer before being loaded on SDS-PAGE. The in-gel fluorescence of the protease-protected proOmpA was recorded with an Amersham Imager 680 and quantified via ImageQuant software (both GE Healthcare).

ATPase activity measurements

The ATPase activity was measured as described before by Kamel et al. with the Sigma Aldrich malachite green kit (MAK307)¹⁶. The reaction was started by addition of ATP and incubated for 15 min. before the reaction was stopped by transferring 25 µL of the reaction mix to 175 µL stopping solution (40 mM sulfuric acid). Finally, 80 µL of the stopped reaction mix was following mixed with 20 µL working reagent and the absorbance was measured at 595 nm.

SecA flotation assay

The flotation assay was performed as described before in Kamel et al. with minor variations¹⁶. SecA and the liposomes were incubated for 15 min at RT prior to centrifugation and for the fractionation following volumes were chosen: Top 125 µL, middle 250 µL and bottom 125 µL.

Construction of SecYEG models in the open and closed configuration

To our knowledge, there is no experimentally resolved 3D structure of the complete SecYEG from *E. coli* available. Fragmented experimental structures are available for the assembled SecA:SecYEG complex (RCSB_ID: 3DL8,⁶⁶), fragmented SecA:SecYE (RCSB_IDs: 6ITC,⁴⁹ 7XHA,⁵⁰) and fragmented SecA:YE including the substrate (RCSB_ID: 5EUL,²⁵). A complete structure model was thus generated using AlphaFold 3⁶⁷. The target sequences for SecY, SecE and SecG have Uniprot IDs P0AGA2, P0AG96, and P0AG99, respectively. A FASTA file with the three monomeric target sequences was prepared and uploaded to the server. The multiple sequence alignments were generated with the default AlphaFold3 workflow. AlphaFold3 provides confidence scores in the form of a weighted linear combination of the predicted TM-score (pTM) and the interface predicted TM-score (ipTM). The five resulting models all have a pTM score of 0.74 and an ipTM score of 0.70. The resulting structural model was then superimposed onto the already mentioned experimental structures (Suppl. Figure 24). The superposition revealed a higher RMSD in proximity to SecY, particularly at the lateral gate, as

the TMH 7 moves away from the TMH 2b, especially at the periplasmic side of the translocon. The dynamics of the lateral gate is similar upon the ribosome binding; the opening is occasionally more pronounced. This suggests that AlphaFold3 predicted a rather closed configuration of SecYEG. The starting structure for MD simulations of the open SecYEG state was thus modelled using TopModel⁶⁸ as already done for other bacterial transporters⁶⁹ using the model for the open state of SecA:SecYE (RCSB_ID: 6ITC) as a single template for the open conformation. The quality of the model was assessed with TopScoreSingle (TopScore = 0.25 (where values closer to 1 indicate a better model)⁷⁰).

Preparation of the starting structures for molecular dynamics simulations of SecYEG

The protonation states of protein residues were adjusted according to pH 7.4 using the Epik routine⁷¹⁻⁷³ in Maestro⁷⁴. The two conformations were embedded into a membrane composed of different mixtures of lipids (Table S1) using the PPM server⁷⁵ and solvated using PACKMOL-Memgen^{76,77}. A distance of at least 15 Å between the protein or membrane in the z-direction and the solvent box boundaries was kept. To obtain a neutral system, counter ions 0.15 M KCl were added that replaced solvent molecules.

Unbiased molecular dynamics simulations

The GPU particle mesh Ewald implementation from the AMBER23 suite of molecular simulations programs⁷⁸ with the ff14SB⁷⁹ and Lipid21⁸⁰ forcefields for the protein and membrane lipids, respectively, were used; water molecules and ions were parametrized using the TIP3P model⁸¹ and the Li and Merz 12-6 ions parameters^{82,83}. For each considered SecYEG system, five independent simulations (replica) of 1 μs in length were performed. Covalent bonds to hydrogens were constrained with the SHAKE algorithm⁸⁴ in all simulations, allowing the use of a time step of 2 fs. Details of the thermalization of the simulation systems are given below. All unbiased MD simulations showed structurally invariant protein structures and membrane phases, as evidenced by RMSD (Suppl. Figures 25 and 25) and electron density calculations (Suppl. Figures 27-30).

Relaxation, thermalization, and production runs of the obtained systems

An initial minimization step was performed with the CPU code pmemd⁸⁵. Each minimization was organized in four steps of 5000 cycles each, for a total of 20000 cycles of minimization. Afterward, each minimized system was thermalized in one stage from 0 to 300 K over 25 ps using the NVT ensemble and the Langevin thermostat⁸⁶, and the density was adapted to 1.0 g cm⁻³ over 975 ps using the NPT ensemble with a semi-isotropic Berendsen barostat⁸⁷ with the pressure set to 1 bar. The thermalization and equilibration were performed with the GPU code pmemd⁸⁵. There were three density equilibration steps with a total time of 4 ns. The sum of the thermalization, density adaptation, and equilibration took 5 ns. For each replica, 1 μs of production run using the GPU code of pmemd was performed in the NPT ensemble at a temperature of 300 K using the Langevin thermostat⁸⁶ and a collision frequency of 1 ps⁻¹. To avoid noticeable distortions in the simulation box size, semi-isotropic pressure scaling using the Berendsen barostat⁸⁷ and a pressure relaxation time of 1 ps was employed by coupling the box size changes along the membrane plane⁸⁸.

Analysis of MD trajectories of the prepared systems

The trajectories were analyzed with CPPTRAJ⁸⁹. The distance between the COM of backbone atoms of residues 82-96 and 275-294 was calculated to describe the lateral gate distance in SecYEG simulations. As in ref.⁹⁰, the software was also used for evaluating root mean square deviations (RMSD), non-native contact networks between TMH 2b and 7 and TMH 2b and TMH 7 with membrane components, electron density curves for membrane components, and lipid distributions around SecYEG. To evaluate interactions of the SecYEG lateral gate and the membrane, we computed the number of non-native contacts formed between the TMH 2b or 7 with the phospholipid lipophilic chains. A non-native contact was defined as a contact satisfying the distance cutoff of 5 Å without being already found in the initial reference frame. The same set of analyses was also carried out to analyze the configurations sampled during umbrella sampling. To unbiased the configurations t generated, we estimated a weight w^t according to Pagani & Gohlke 2018⁹¹. The reweighting is performed over the entire ensemble of each system and, then, normalized by dividing each w^t by the sum of all w^t for each system.

PMF and free energy calculation of the lateral gate opening

As in Gentile et al. 2024, the initial conformation used in every window for calculating a configuration free energy profile of the lateral gate opening were obtained from the first microsecond of the MD simulations of replica 3 of SecYEG in DOPC:DOPG and POPC:POPG simulations, replica 4 of SecYEG in POPC:TOCL simulations, and replica 5 of SecYEG in POPC:bis(PO)CL (Suppl. Table 1) ⁴⁶. These replicas were chosen according to the observed spontaneous transitions starting from either the closed or open SecYEG configurations to the latter state. Starting from the AlphaFold3 model conformation, SecY spontaneously opened. The distance d between the COM of C α atoms of residues 82-96 and 275-294 was used to select 21 intermediate opening configurations. The starting conformations were extracted from the representative trajectory, taking the respective snapshots where d showed the least absolute deviation to the average value obtained by binning d in windows that were 0.5 Å in width and with an evenly distributed separation of 0.5 Å ⁴⁴, and sampling was performed for 2 μs per window. d values were obtained every 2 ps and postprocessed with the Weighted Histogram Analysis Method implementation of WHAM 2.0.9 ⁹², removing the first 1250 ns as an equilibration phase of the system. The error was estimated by considering the last 125 ns only and by calculating the standard error for the mean from five independent free energy profiles that were determined every 25 ns during this time. The overlap between contiguous windows and the convergence of the PMF were validated (Suppl. Figure 8-9, 12-13).

Free energy difference for the closed-to-open transition of the lateral gate of SecYG from PMFs

For calculating the free energy difference for the closed-to-open transition of the lateral gate of SecYG from the PMF, the PMF $w(d)$ was integrated in the range of the basins B_1 and B_2 , which represent the closed and the open conformations of SecYEG, respectively, using eq. 1, which yielded the equilibrium constant K_{opening} . From K_{opening} , the difference in free energy was then obtained with eq. 2 ^{46,47}.

Equation 1

$$K_{\text{opening}} = \frac{\int_{B_1} e^{-\frac{w(d)}{k_B T}} dr}{\int_{B_2} e^{-\frac{w(d)}{k_B T}} dr}$$

Equation 2

$$\Delta G_{\text{opening}} = -RT \ln K_{\text{opening}}$$

d is the value of the reaction coordinate. The integration limits B_1 and B_2 included the flat regions delimited by the slope changes within the basins (lateral gate $d_1 = 11.5$ -13.7 Å and $d_2 = 17.2$ -20.0 for closed and open DO, and $d_1 = 11.3$ -13.4 Å and $d_2 = 18.3$ -20.0 for closed and open PO, respectively) that indicate the closed and open conformations of SecY, respectively (Figure 4I).

Acknowledgements

This study was funded by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG), project 267205415/CRC1208 (subproject A10 to A.K. and subproject A03 to H.G.) and, in part, by the DFG Research grant 1879/3 (A.K.) and a grant from the Ministry of Innovation, Science, and Research within the framework of the NRW Strategieprojekt BioSC (No. 313/323-400-00213) (H.G.). We thank Michael Kamel for inspiring discussions. We are grateful for the computational support by the “Zentrum für Informations- und Medientechnologie” at the Heinrich Heine Universität Düsseldorf and the computing time provided by the John von Neumann Institute for Computing (NIC) to H.G. on the supercomputer JUWELS at the Jülich Supercomputing Centre (JSC) (user IDs: metapro) ⁹³.

Author contributions

M.B.: Conceptualization, Investigation, Formal Analysis, Data Curation, Visualization, Writing – Original Draft, Writing – Review & Editing. **R.G.:** Conceptualization, Software, Investigation, Formal Analysis, Visualization, Writing – Original Draft, Writing – Review & Editing. **H.G.:** Supervision, Formal Analysis, Resources, Project Administration, Funding Acquisition, Writing – Review & Editing. **A.K.:** Conceptualization, Supervision, Formal Analysis, Resources, Project Administration, Funding Acquisition, Writing – Original Draft, Writing – Review & Editing.

References

- 1 Tsirigotaki, A., De Geyter, J., Sostaric, N., Economou, A. & Karamanou, S. Protein export through the bacterial Sec pathway. *Nat Rev Microbiol* **15**, 21-36 (2017). <https://doi.org/10.1038/nrmicro.2016.161>
- 2 Kusters, I. & Driessen, A. J. SecA, a remarkable nanomachine. *Cell Mol Life Sci* **68**, 2053-2066 (2011). <https://doi.org/10.1007/s00018-011-0681-y>
- 3 Castanie-Cornet, M. P., Bruel, N. & Genevau, P. Chaperone networking facilitates protein targeting to the bacterial cytoplasmic membrane. *Biochim Biophys Acta* **1843**, 1442-1456 (2014). <https://doi.org/10.1016/j.bbamcr.2013.11.007>
- 4 Komarudin, A. G. & Driessen, A. J. M. SecA-Mediated Protein Translocation through the SecYEG Channel. *Microbiol Spectr* **7** (2019). <https://doi.org/10.1128/microbiolspec.PSIB-0028-2019>
- 5 Bornemann, T., Holtkamp, W. & Wintermeyer, W. Interplay between trigger factor and other protein biogenesis factors on the ribosome. *Nat Commun* **5**, 4180 (2014). <https://doi.org/10.1038/ncomms5180>
- 6 Bauer, B. W., Shemesh, T., Chen, Y. & Rapoport, T. A. A "push and slide" mechanism allows sequence-insensitive translocation of secretory proteins by the SecA ATPase. *Cell* **157**, 1416-1429 (2014). <https://doi.org/10.1016/j.cell.2014.03.063>
- 7 Catipovic, M. A., Bauer, B. W., Loparo, J. J. & Rapoport, T. A. Protein translocation by the SecA ATPase occurs by a power-stroke mechanism. *EMBO J* **38** (2019). <https://doi.org/10.15252/embj.2018101140>
- 8 Allen, W. J. *et al.* Two-way communication between SecY and SecA suggests a Brownian ratchet mechanism for protein translocation. *Elife* **5** (2016). <https://doi.org/10.7554/eLife.15598>
- 9 Breukink, E., Demel, R. A., de Korte-Kool, G. & de Kruijff, B. SecA insertion into phospholipids is stimulated by negatively charged lipids and inhibited by ATP: a monolayer study. *Biochemistry* **31**, 1119-1124 (1992). <https://doi.org/10.1021/bi00119a021>
- 10 van der Does, C., Swaving, J., van Klompenburg, W. & Driessen, A. J. Non-bilayer lipids stimulate the activity of the reconstituted bacterial protein translocase. *J Biol Chem* **275**, 2472-2478 (2000). <https://doi.org/10.1074/jbc.275.4.2472>
- 11 Koch, S. *et al.* Lipids Activate SecA for High Affinity Binding to the SecYEG Complex. *J Biol Chem* **291**, 22534-22543 (2016). <https://doi.org/10.1074/jbc.M116.743831>
- 12 Lill, R., Dowhan, W. & Wickner, W. The ATPase activity of SecA is regulated by acidic phospholipids, SecY, and the leader and mature domains of precursor proteins. *Cell* **60**, 271-280 (1990). [https://doi.org/10.1016/0092-8674\(90\)90742-w](https://doi.org/10.1016/0092-8674(90)90742-w)
- 13 de Vrije, T., de Swart, R. L., Dowhan, W., Tommassen, J. & de Kruijff, B. Phosphatidylglycerol is involved in protein translocation across Escherichia coli inner membranes. *Nature* **334**, 173-175 (1988). <https://doi.org/10.1038/334173a0>
- 14 Breukink, E. *et al.* The C terminus of SecA is involved in both lipid binding and SecB binding. *J Biol Chem* **270**, 7902-7907 (1995). <https://doi.org/10.1074/jbc.270.14.7902>
- 15 Koch, S. *et al.* Two distinct anionic phospholipid-dependent events involved in SecA-mediated protein translocation. *Biochim Biophys Acta Biomembr* **1861**, 183035 (2019). <https://doi.org/10.1016/j.bbamem.2019.183035>
- 16 Kamel, M., Lowe, M., Schott-Verdugo, S., Gohlke, H. & Kedrov, A. Unsaturated fatty acids augment protein transport via the SecA:SecYEG translocon. *FEBS J* **289**, 140-162 (2022). <https://doi.org/10.1111/febs.16140>
- 17 Morein, S., Andersson, A., Rilfors, L. & Lindblom, G. Wild-type Escherichia coli cells regulate the membrane lipid composition in a "window" between gel and non-lamellar structures. *J Biol Chem* **271**, 6801-6809 (1996). <https://doi.org/10.1074/jbc.271.12.6801>
- 18 Siliakus, M. F., van der Oost, J. & Kengen, S. W. M. Adaptations of archaeal and bacterial membranes to variations in temperature, pH and pressure. *Extremophiles* **21**, 651-670 (2017). <https://doi.org/10.1007/s00792-017-0939-x>
- 19 Kusters, I. *et al.* Quaternary structure of SecA in solution and bound to SecYEG probed at the single molecule level. *Structure* **19**, 430-439 (2011). <https://doi.org/10.1016/j.str.2010.12.016>
- 20 Sardis, M. F. & Economou, A. SecA: a tale of two protomers. *Mol Microbiol* **76**, 1070-1081 (2010). <https://doi.org/10.1111/j.1365-2958.2010.07176.x>
- 21 de Keyzer, J. *et al.* Covalently dimerized SecA is functional in protein translocation. *J Biol Chem* **280**, 35255-35260 (2005). <https://doi.org/10.1074/jbc.M506157200>
- 22 Vanni, S., Hirose, H., Barelli, H., Antonny, B. & Gautier, R. A sub-nanometre view of how membrane curvature and composition modulate lipid packing and protein recruitment. *Nature Communications* **5** (2014). <https://doi.org/10.1038/ncomms5916>

- 23 Ballweg, S. *et al.* Regulation of lipid saturation without sensing membrane fluidity. *Nature Communications* **11** (2020). <https://doi.org/10.1038/s41467-020-14528-1>
- 24 Lee, A. G. How lipids affect the activities of integral membrane proteins. *Bba-Biomembranes* **1666**, 62-87 (2004). <https://doi.org/10.1016/j.bbamem.2004.05.012>
- 25 Li, L. *et al.* Crystal structure of a substrate-engaged SecY protein-translocation channel. *Nature* **531**, 395-399 (2016). <https://doi.org/10.1038/nature17163>
- 26 Koch, S., Driessen, A. J. M. & Kedrov, A. *Advances in Biomembranes and Lipid Self-Assembly* 41-85 (2018).
- 27 Wu, Z. C., de Keyser, J., Kedrov, A. & Driessen, A. J. Competitive binding of the SecA ATPase and ribosomes to the SecYEG translocon. *J Biol Chem* **287**, 7885-7895 (2012). <https://doi.org/10.1074/jbc.M111.297911>
- 28 Sugano, Y., Furukawa, A., Nureki, O., Tanaka, Y. & Tsukazaki, T. SecY-SecA fusion protein retains the ability to mediate protein transport. *PLoS One* **12**, e0183434 (2017). <https://doi.org/10.1371/journal.pone.0183434>
- 29 Kanaoka, Y. *et al.* AFM observation of protein translocation mediated by one unit of SecYEG-SecA complex. *Nat Commun* **16**, 225 (2025). <https://doi.org/10.1038/s41467-024-54875-x>
- 30 Nishiyama, K., Suzuki, H. & Tokuda, H. Role of the non-essential region encompassing the N-terminal two transmembrane stretches of Escherichia coli SecE. *Biosci Biotechnol Biochem* **64**, 2121-2127 (2000). <https://doi.org/10.1271/bbb.64.2121>
- 31 Nishiyama, K., Hanada, M. & Tokuda, H. Disruption of the gene encoding p12 (SecG) reveals the direct involvement and important function of SecG in the protein translocation of Escherichia coli at low temperature. *EMBO J* **13**, 3272-3277 (1994). <https://doi.org/10.1002/j.1460-2075.1994.tb06628.x>
- 32 Brundage, L., Hendrick, J. P., Schiebel, E., Driessen, A. J. & Wickner, W. The purified E. coli integral membrane protein SecY/E is sufficient for reconstitution of SecA-dependent precursor protein translocation. *Cell* **62**, 649-657 (1990). [https://doi.org/10.1016/0092-8674\(90\)90111-g](https://doi.org/10.1016/0092-8674(90)90111-g)
- 33 Das, S. & Oliver, D. B. Mapping of the SecA.SecY and SecA.SecG interfaces by site-directed in vivo photocross-linking. *J Biol Chem* **286**, 12371-12380 (2011). <https://doi.org/10.1074/jbc.M110.182931>
- 34 Matsumoto, G., Mori, H. & Ito, K. Roles of SecG in ATP- and SecA-dependent protein translocation. *Proc Natl Acad Sci U S A* **95**, 13567-13572 (1998). <https://doi.org/10.1073/pnas.95.23.13567>
- 35 Kater, L. *et al.* Partially inserted nascent chain unzips the lateral gate of the Sec translocon. *EMBO Rep* **20**, e48191 (2019). <https://doi.org/10.15252/embr.201948191>
- 36 Busch, M. *et al.* Substrate-induced assembly and functional mechanism of the membrane protein insertase SecYEG-YidC. *EMBO J* (2026). <https://doi.org/10.1038/s44318-026-00837-6>
- 37 Gullingsrud, J. & Schulten, K. Lipid bilayer pressure profiles and mechanosensitive channel gating. *Biophys J* **86**, 3496-3509 (2004). <https://doi.org/10.1529/biophysj.103.034322>
- 38 Kapsalis, C. *et al.* Allosteric activation of an ion channel triggered by modification of mechanosensitive nano-pockets. *Nat Commun* **10**, 4619 (2019). <https://doi.org/10.1038/s41467-019-12591-x>
- 39 Aguayo-Ortiz, R., Straub, J. E. & Dominguez, L. Influence of membrane lipid composition on the structure and activity of gamma-secretase. *Phys Chem Chem Phys* **20**, 27294-27304 (2018). <https://doi.org/10.1039/c8cp04138e>
- 40 Monje-Galvan, V., Warburton, L. & Klauda, J. B. Setting Up All-Atom Molecular Dynamics Simulations to Study the Interactions of Peripheral Membrane Proteins with Model Lipid Bilayers. *Methods Mol Biol* **1949**, 325-339 (2019). https://doi.org/10.1007/978-1-4939-9136-5_22
- 41 du Plessis, D. J., Berrelkamp, G., Nouwen, N. & Driessen, A. J. The lateral gate of SecYEG opens during protein translocation. *J Biol Chem* **284**, 15805-15814 (2009). <https://doi.org/10.1074/jbc.M901855200>
- 42 Lycklama, A. N. J. A., Wu, Z. C. & Driessen, A. J. M. Conformational dynamics of the plug domain of the SecYEG protein-conducting channel. *J Biol Chem* **286**, 43881-43890 (2011). <https://doi.org/10.1074/jbc.M111.297507>
- 43 Gumbart, J. & Schulten, K. The roles of pore ring and plug in the SecY protein-conducting channel. *J Gen Physiol* **132**, 709-719 (2008). <https://doi.org/10.1085/jgp.200810062>
- 44 Torrie, G. M. & Valleau, J. P. Non-Physical Sampling Distributions in Monte-Carlo Free-Energy Estimation - Umbrella Sampling. *J Comput Phys* **23**, 187-199 (1977). [https://doi.org/10.1016/0021-9991\(77\)90121-8](https://doi.org/10.1016/0021-9991(77)90121-8)

- 45 Doudou, S., Burton, N. A. & Henchman, R. H. Standard Free Energy of Binding from a One-Dimensional Potential of Mean Force. *J Chem Theory Comput* **5**, 909-918 (2009).
<https://doi.org/10.1021/ct8002354>
- 46 Gentile, R. *et al.* Molecular Mechanisms Underlying Medium-Chain Free Fatty Acid-Regulated Activity of the Phospholipase PlaF from *Pseudomonas aeruginosa*. *JACS Au* **4**, 958-973 (2024). <https://doi.org/10.1021/jacsau.3c00725>
- 47 Bleffert, F. *et al.* Structural, mechanistic, and physiological insights into phospholipase A-mediated membrane phospholipid degradation in *Pseudomonas aeruginosa*. *Elife* **11** (2022).
<https://doi.org/10.7554/eLife.72824>
- 48 Chen, P. C. & Kuyucak, S. Accurate determination of the binding free energy for KcsA-charybdotoxin complex from the potential of mean force calculations with restraints. *Biophys J* **100**, 2466-2474 (2011). <https://doi.org/10.1016/j.bpj.2011.03.052>
- 49 Ma, C. *et al.* Structure of the substrate-engaged SecA-SecY protein translocation machine. *Nat Commun* **10**, 2872 (2019). <https://doi.org/10.1038/s41467-019-10918-2>
- 50 Dong, L. *et al.* Structural basis of SecA-mediated protein translocation. *Proc Natl Acad Sci U S A* **120**, e2208070120 (2023). <https://doi.org/10.1073/pnas.2208070120>
- 51 Johnston, J. M., Wang, H., Provasi, D. & Filizola, M. Assessing the relative stability of dimer interfaces in g protein-coupled receptors. *PLoS Comput Biol* **8**, e1002649 (2012).
<https://doi.org/10.1371/journal.pcbi.1002649>
- 52 Gold, V. A. *et al.* The action of cardiolipin on the bacterial translocon. *Proc Natl Acad Sci U S A* **107**, 10044-10049 (2010). <https://doi.org/10.1073/pnas.0914680107>
- 53 Corey, R. A. *et al.* Specific cardiolipin-SecY interactions are required for proton-motive force stimulation of protein secretion. *Proc Natl Acad Sci U S A* **115**, 7967-7972 (2018).
<https://doi.org/10.1073/pnas.1721536115>
- 54 Covino, R. *et al.* A Eukaryotic Sensor for Membrane Lipid Saturation. *Mol Cell* **63**, 49-59 (2016). <https://doi.org/10.1016/j.molcel.2016.05.015>
- 55 Yau, W. M., Wimley, W. C., Gawrisch, K. & White, S. H. The preference of tryptophan for membrane interfaces. *Biochemistry* **37**, 14713-14718 (1998).
<https://doi.org/10.1021/bi980809c>
- 56 van der Wolk, J. P. *et al.* PrlA4 prevents the rejection of signal sequence defective preproteins by stabilizing the SecA-SecY interaction during the initiation of translocation. *EMBO J* **17**, 3631-3639 (1998). <https://doi.org/10.1093/emboj/17.13.3631>
- 57 Harris, A. J. & Goldman, A. D. The very early evolution of protein translocation across membranes. *PLoS Comput Biol* **17**, e1008623 (2021).
<https://doi.org/10.1371/journal.pcbi.1008623>
- 58 Allen, W. J. & Collinson, I. A unifying mechanism for protein transport through the core bacterial Sec machinery. *Open Biol* **13**, 230166 (2023). <https://doi.org/10.1098/rsob.230166>
- 59 Jin, J. *et al.* SecA inhibitors as potential antimicrobial agents: differential actions on SecA-only and SecA-SecYEG protein-conducting channels. *FEMS Microbiol Lett* **365** (2018).
<https://doi.org/10.1093/femsle/fny145>
- 60 Sobakinskaya, E. & Muh, F. Physical mechanisms of the Sec machinery operation. *Phys Chem Chem Phys* **26**, 27176-27188 (2024). <https://doi.org/10.1039/d4cp03201b>
- 61 Meier, M. *et al.* Functional Mapping and Engineering of the Sec Translocon Unlocked by a Cell-Free System. *bioRxiv*, 2025.2012.2009.688994 (2025).
<https://doi.org/10.64898/2025.12.09.688994>
- 62 van der Sluis, E. O., Nouwen, N. & Driessen, A. J. SecY-SecY and SecY-SecG contacts revealed by site-specific crosslinking. *FEBS Lett* **527**, 159-165 (2002).
[https://doi.org/10.1016/s0014-5793\(02\)03202-7](https://doi.org/10.1016/s0014-5793(02)03202-7)
- 63 Kanonenberg, K. *et al.* Shaping the lipid composition of bacterial membranes for membrane protein production. *Microb Cell Fact* **18**, 131 (2019). <https://doi.org/10.1186/s12934-019-1182-1>
- 64 Kusters, I. *et al.* Purification and functional reconstitution of the bacterial protein translocation pore, the SecYEG complex. *Methods Mol Biol* **619**, 131-143 (2010).
https://doi.org/10.1007/978-1-60327-412-8_8
- 65 Kedrov, A., Kusters, I., Krasnikov, V. V. & Driessen, A. J. A single copy of SecYEG is sufficient for preprotein translocation. *EMBO J* **30**, 4387-4397 (2011).
<https://doi.org/10.1038/emboj.2011.314>
- 66 Zimmer, J., Nam, Y. & Rapoport, T. A. Structure of a complex of the ATPase SecA and the protein-translocation channel. *Nature* **455**, 936-943 (2008).
<https://doi.org/10.1038/nature07335>

- 67 Abramson, J. *et al.* Accurate structure prediction of biomolecular interactions with AlphaFold 3. *Nature* **630**, 493-500 (2024). <https://doi.org/10.1038/s41586-024-07487-w>
- 68 Mulnaes, D. *et al.* TopModel: Template-Based Protein Structure Prediction at Low Sequence Identity Using Top-Down Consensus and Deep Neural Networks. *J Chem Theory Comput* **16**, 1953-1967 (2020). <https://doi.org/10.1021/acs.jctc.9b00825>
- 69 Lehnert, A. *et al.* Efficient microbial glucose-fructose conversion at ambient temperature through adaptive laboratory evolution and application for D-allulose production. *bioRxiv*, 2024.2012.2016.628640 (2024). <https://doi.org/10.1101/2024.12.16.628640>
- 70 Mulnaes, D. & Gohlke, H. TopScore: Using Deep Neural Networks and Large Diverse Data Sets for Accurate Protein Model Quality Assessment. *J Chem Theory Comput* **14**, 6117-6126 (2018). <https://doi.org/10.1021/acs.jctc.8b00690>
- 71 Greenwood, J. R., Calkins, D., Sullivan, A. P. & Shelley, J. C. Towards the comprehensive, rapid, and accurate prediction of the favorable tautomeric states of drug-like molecules in aqueous solution. *J Comput Aided Mol Des* **24**, 591-604 (2010). <https://doi.org/10.1007/s10822-010-9349-1>
- 72 Johnston, R. C. *et al.* Epik: pK(a) and Protonation State Prediction through Machine Learning. *J Chem Theory Comput* **19**, 2380-2388 (2023). <https://doi.org/10.1021/acs.jctc.3c00044>
- 73 Shelley, J. C. *et al.* Epik: a software program for pK(a) prediction and protonation state generation for drug-like molecules. *J Comput Aided Mol Des* **21**, 681-691 (2007). <https://doi.org/10.1007/s10822-007-9133-z>
- 74 Maestro (Schrödinger, LLC, New York, 2023).
- 75 Lomize, M. A., Pogozheva, I. D., Joo, H., Mosberg, H. I. & Lomize, A. L. OPM database and PPM web server: resources for positioning of proteins in membranes. *Nucleic Acids Res* **40**, D370-376 (2012). <https://doi.org/10.1093/nar/gkr703>
- 76 Martinez, L., Andrade, R., Birgin, E. G. & Martinez, J. M. PACKMOL: a package for building initial configurations for molecular dynamics simulations. *J Comput Chem* **30**, 2157-2164 (2009). <https://doi.org/10.1002/jcc.21224>
- 77 Schott-Verdugo, S., Muller, L., Classen, E., Gohlke, H. & Groth, G. Structural Model of the ETR1 Ethylene Receptor Transmembrane Sensor Domain. *Sci Rep* **9**, 8869 (2019). <https://doi.org/10.1038/s41598-019-45189-w>
- 78 Case, D. A. *et al.* AmberTools. *J Chem Inf Model* **63**, 6183-6191 (2023). <https://doi.org/10.1021/acs.jcim.3c01153>
- 79 Maier, J. A. *et al.* ff14SB: Improving the Accuracy of Protein Side Chain and Backbone Parameters from ff99SB. *J Chem Theory Comput* **11**, 3696-3713 (2015). <https://doi.org/10.1021/acs.jctc.5b00255>
- 80 Dickson, C. J., Walker, R. C. & Gould, I. R. Lipid21: Complex Lipid Membrane Simulations with AMBER. *J Chem Theory Comput* **18**, 1726-1736 (2022). <https://doi.org/10.1021/acs.jctc.1c01217>
- 81 Zhao, C. L. *et al.* Seven-Site Effective Pair Potential for Simulating Liquid Water. *J Phys Chem B* **123**, 4594-4603 (2019). <https://doi.org/10.1021/acs.jpcc.9b03149>
- 82 Li, P., Song, L. F. & Merz, K. M., Jr. Systematic Parameterization of Monovalent Ions Employing the Nonbonded Model. *J Chem Theory Comput* **11**, 1645-1657 (2015). <https://doi.org/10.1021/ct500918t>
- 83 Sengupta, A., Li, Z., Song, L. F., Li, P. & Merz, K. M., Jr. Correction to "Parameterization of Monovalent Ions for the OPC3, OPC, TIP3P-FB, and TIP4P-FB Water Models". *J Chem Inf Model* **61**, 3734-3735 (2021). <https://doi.org/10.1021/acs.jcim.1c00576>
- 84 Kräutler, V., Van Gunsteren, W. F. & Hünenberger, P. H. A fast SHAKE:: Algorithm to solve distance constraint equations for small molecules in molecular dynamics simulations. *Journal of Computational Chemistry* **22**, 501-508 (2001). [https://doi.org/10.1002/1096-987x\(20010415\)22:5<501::Aid-Jcc1021>3.0.Co;2-V](https://doi.org/10.1002/1096-987x(20010415)22:5<501::Aid-Jcc1021>3.0.Co;2-V)
- 85 Le Grand, S., Götz, A. W. & Walker, R. C. SPFP: Speed without compromise-A mixed precision model for GPU accelerated molecular dynamics simulations. *Comput Phys Commun* **184**, 374-380 (2013). <https://doi.org/10.1016/j.cpc.2012.09.022>
- 86 Quigley, D. & Probert, M. I. Langevin dynamics in constant pressure extended systems. *J Chem Phys* **120**, 11432-11441 (2004). <https://doi.org/10.1063/1.1755657>
- 87 Berendsen, H. J. C., Postma, J. P. M., Vangunsteren, W. F., Dinola, A. & Haak, J. R. Molecular-Dynamics with Coupling to an External Bath. *J Chem Phys* **81**, 3684-3690 (1984). <https://doi.org/10.1063/1.448118>
- 88 Lin, Y. Q., Pan, D. Y., Li, J. M., Zhang, L. X. & Shao, X. M. Application of Berendsen barostat in dissipative particle dynamics for nonequilibrium dynamic simulation. *J Chem Phys* **146** (2017). <https://doi.org/10.1063/1.4978807>

- 89 Roe, D. R. & Cheatham, T. E., 3rd. PTRAJ and CPPTRAJ: Software for Processing and Analysis of Molecular Dynamics Trajectory Data. *J Chem Theory Comput* **9**, 3084-3095 (2013). <https://doi.org/10.1021/ct400341p>
- 90 Gentile, R. *et al.* Molecular Insights into CLD Domain Dynamics and Toxin Recruitment of the HlyA E. coli T1SS. *J Mol Biol* **437**, 169485 (2025). <https://doi.org/10.1016/j.jmb.2025.169485>
- 91 Pagani, G. & Gohlke, H. On the contributing role of the transmembrane domain for subunit-specific sensitivity of integrin activation. *Sci Rep* **8**, 5733 (2018). <https://doi.org/10.1038/s41598-018-23778-5>
- 92 WHAM: the weighted histogram analysis method v. 2.0.9.
- 93 Alvarez, D. Jülich Supercomputing Centre. *Journal of large-scale research facilities* **7** (2021).

Supplemental information for

Lipid structure modulates the dynamics of the bacterial translocon SecYEG

Max Busch^{1,§}, Rocco Gentile^{2,§}, Holger Gohlke^{2,3,4,*}, Alexej Kedrov^{1,4,*}

Table of Contents

Suppl. Text 1. Standard Error of the Mean (SEM) propagation from PMF to $\Delta G_{\text{opening}}$	S5
Suppl. Table 1. Membrane systems generated with Packmol-Memgen and subjected to simulations and analyses.	S6
Suppl. Figure 1. Effects of PEG 2000 on SecA binding and protein translocation.	S7
Suppl. Figure 2. Design and characterization of the SecYA-EG fusion translocon.	S8
Suppl. Figure 3. Characterization of SecYEG and truncations.	S9
Suppl. Figure 4. Open-state occupancy of the TMH 2b–7 distance across independent replicas of the closed-DO (I, left) and open-DO (II, right) MD simulations.	S10
Suppl. Figure 5. Open-state occupancy of the TMH 2b–7 distance across independent replicas of the closed-PO (III, left) and open-PO (IV, right) simulations.	S10
Suppl. Figure 6. Differential TMH 2b-TMH 7 (top) and TMH 7-TMH 2b (bottom) contacts for distinct membrane compositions in relationship to the closed SecYEG simulations.	S11
Suppl. Figure 7. Plug (TMH 2a) structural invariance across simulations.	S12
Suppl. Figure 8. Evaluation of the PMF of SecYEG opening when embedded in a DOPC:DOPG membrane (system closed-DO (I)).	S13
Suppl. Figure 9. Evaluation of the PMF of SecYEG opening when embedded in a POPC:POPG membrane (system open-PO (IV)).	S13
Suppl. Figure 10. Open-state occupancy of the TMH 2b–7 distance across independent replicas of the closed-PO-bCL (VII, left) and open-PO-bCL (VIII, right) simulations.	S14
Suppl. Figure 11. Open-state occupancy of the TMH 2b–7 distance across independent replicas of the closed-PO-CL (V, left) and open-PO-CL (VI, right) simulations.	S14
Suppl. Figure 12. FIGURE RG_S6. Evaluation of the PMF of SecYEG opening when embedded in a POPC:TOCL membrane (system closed-PO-CL (V)).	S15
Suppl. Figure 13. Evaluation of the PMF of SecYEG opening when embedded in a POPC:bis(PO)CL membrane (system open-PO-bCL (VIII)).	S15
Suppl. Figure 14. 2D density maps of PC (left) and PGR (right) lipids around the closed SecYEG complex viewed from the cytoplasmic side of the membrane, in the presence of DOPC:DOPG (A) and POPC:POPG (B).	S16

Suppl. Figure 15. 2D density maps of PC (left) and PGR (right) lipids around the open SecYEG complex viewed from the cytoplasmic side of the membrane, in the presence of DOPC:DOPG (A) and POPC:POPG (B).	S17
Suppl. Figure 16. Differential lipid-TMH 2b contacts for distinct membrane compositions in relationship to the closed (top) and open (bottom) SecYEG simulations	S18
Suppl. Figure 17. Lipid-TMH 2b contacts for the DOPC:DOPG composition in relationship to the closed (pink) and open (red) SecYEG simulations.....	S19
Suppl. Figure 18. Lipid-TMH 2b contacts for the POPC:POPG composition in relationship to the closed (blue) and open (cyan) SecYEG simulations.	S19
Suppl. Figure 19. Differential lipid-TMH 7 contacts for distinct membrane compositions in relationship to the closed (top) and open (bottom) SecYEG simulations.	S20
Suppl. Figure 20. Lipid-TMH 7 contacts for the DOPC:DOPG composition in relationship to the closed (pink) and open (red) SecYEG simulations.....	S21
Suppl. Figure 21. Lipid-TMH 7 contacts for the POPC:POPG composition in relationship to the closed (blue) and open (cyan) SecYEG simulations.	S21
Suppl. Figure 22. SecY ^{AA} EG gel filtration and NanoDSF	S22
Suppl. Figure 23. ATPase activity is stimulated by proOmpA addition.	S23
Suppl. Figure 24. Structures of SecYEG homologs in open conformations.	S24
Suppl. Figure 25. Two-dimensional root-mean-square deviation (2D-RMSD) matrix comparing molecular dynamics simulation poses of SecYEG starting either from the closed or open configuration in DOPC:DOPG (DO) and POPC:POPC (PO) membranes, respectively.	S25
Suppl. Figure 26. Two-dimensional root-mean-square deviation (2D-RMSD) matrix comparing molecular dynamics simulation poses of SecYEG starting either from the closed or open configuration in POPC:TOCL (TOCL) and POPC:bis(PO)CL (bis(PO)CL) membranes, respectively	S25
Suppl. Figure 27. Atom density profiles of membrane components averaged over five independent unbiased MD simulations of SecYEG closed (left) and open (right) conformations in DOPC:DOPG membranes for the phospholipid tails (OL) and head groups (PC and PGR) molecules	S26
Suppl. Figure 28. Atom density profiles of membrane components averaged over five independent unbiased MD simulations of SecYEG closed (left) and open (right) conformations in POPC:POPG membranes for the phospholipid tails (OL, PA) and head groups (PC and PGR) molecules	S26
Suppl. Figure 29. Atom density profiles of membrane components averaged over five independent unbiased MD simulations of SecYEG closed (left) and open (right) conformations in POPC:TOCL membranes for the phospholipid tails (OL, PA) and head groups (PC and CL) molecules.	S27
Suppl. Figure 30. Atom density profiles of membrane components averaged over five independent unbiased MD simulations of SecYEG closed (left) and open (right) conformations in POPC:bis(PO)CL membranes for the phospholipid tails (OL, PA) and head groups (PC and CL) molecules.	S27
Supplemental References	S28

Supplemental Text

Suppl. Text 1. Standard Error of the Mean (SEM) propagation from PMF to $\Delta G_{\text{opening}}$.

The complete first-order standard error of the mean (SEM) of $\Delta G_{\text{opening}}$ from the SEM of the PMF $w(d)$ was calculated starting from defining the partition-function integrals coming from Equation 1:

$$A = \int_{B_1} e^{\frac{-w(d)}{k_B T}} dr$$

$$B = \int_{B_2} e^{\frac{-w(d)}{k_B T}} dr$$

Then:

$$K_{\text{opening}} = \frac{A}{B}$$

The uncertainty of the integral function was initially propagated similarly for A (reported) or B inside the used bins using:

$$SEM(A)^2 = \sum_{i \in B_1} \left(\frac{dr}{k_B T} e^{\frac{-w_i}{k_B T}} \right)^2 SEM(w_i)^2$$

The uncertainty was then propagated to K_{opening} . Since:

$$K_{\text{opening}} = \frac{A}{B}$$

Then,

$$\left(\frac{SEM(K_{\text{opening}})}{K_{\text{opening}}} \right)^2 = \left(\frac{SEM(A)}{A} \right)^2 + \left(\frac{SEM(B)}{B} \right)^2$$

Finally, the propagation was extended to $\Delta G_{\text{opening}}$ (Equation 2) using:

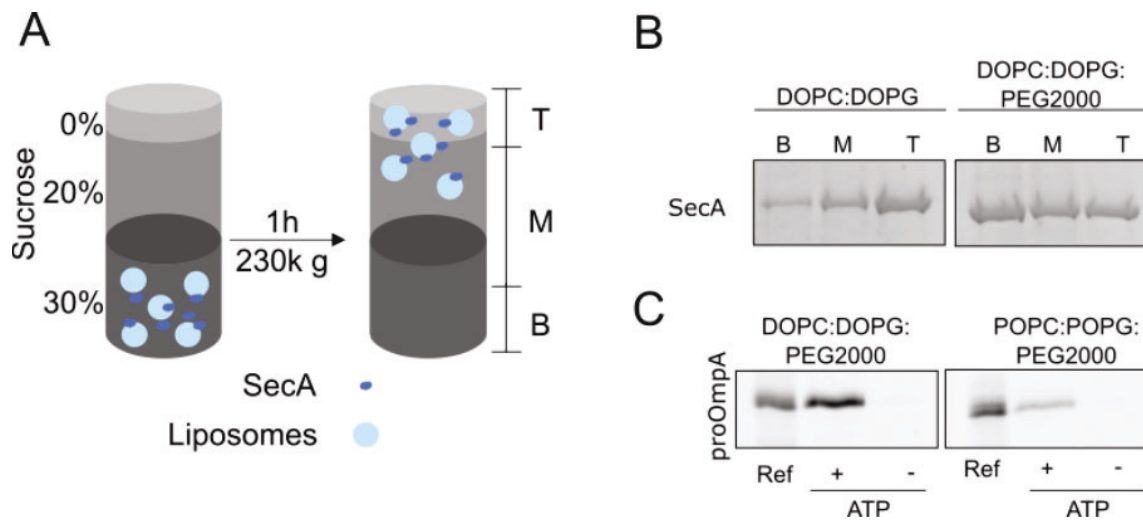
$$SEM(\Delta G) = RT \sqrt{\left(\frac{SEM(A)}{A} \right)^2 + \left(\frac{SEM(B)}{B} \right)^2}$$

Supplemental Table

Suppl. Table 1. Membrane systems generated with Packmol-Memgen and subjected to simulations and analyses.

System	SecYEG configuration	Membrane composition	Simulation replicas
closed-DO (I)	SecYEG closed	DOPC:DOPG 70:30	5
open-DO (II)	SecYEG open	DOPC:DOPG 70:30	5
closed-PO (III)	SecYEG closed	POPC:POPG 70:30	5
open-PO (IV)	SecYEG open	POPC:POPG 70:30	5
closed-PO-CL (V)	SecYEG closed	POPC:TOCL 70:15	5
open-PO-CL (VI)	SecYEG open	POPC:TOCL 70:15	5
closed-PO-bCL (VII)	SecYEG closed	POPC:POPOCL 70:15	5
open-PO-bCL (VIII)	SecYEG open	POPC:POPOCL 70:15	5

Supplemental Figures

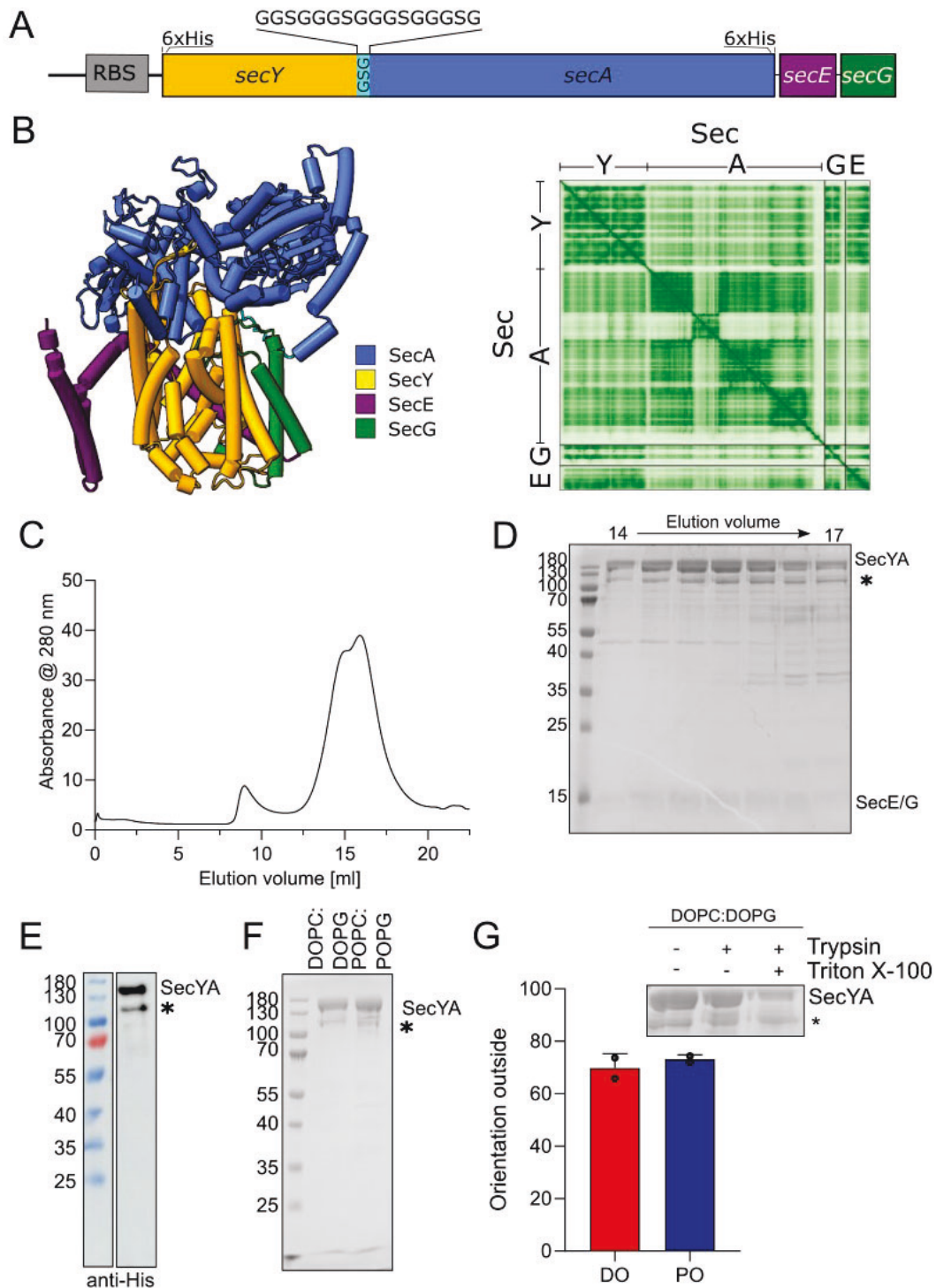


Suppl. Figure 1. Effects of PEG 2000 on SecA binding and protein translocation.

(A) Schematic of SecA flotation assay in a discontinuous sucrose gradient for studying SecA lipid interactions

(B) Comassie stained SDS-PAGE after flotation assay. Separated and TCA precipitated fractions from bottom (B), middle (M) and Top (T) are shown for the different liposomes.

(C) Representative in gel fluorescence of proOmpA used in translocation assay with SecYEG in indicated liposomes containing DOPC:DOPG:3%PEG2000 or just DOPC:DOPG lipids.



Suppl. Figure 2. Design and characterization of the SecYA-EG fusion translocon.

(A) Design of the cloned construct for expression of the SecYA-EG fusion translocon. The genes *secY* and *secA* are genetically fused via the spacer. *SecE* and *SecG* are expressed as individual proteins

(B) AlphaFold3-based model of the SecYA-EG fusion translocon and the corresponding PAE plot. The unstructured termini of *SecY* and *SecG* are not shown.

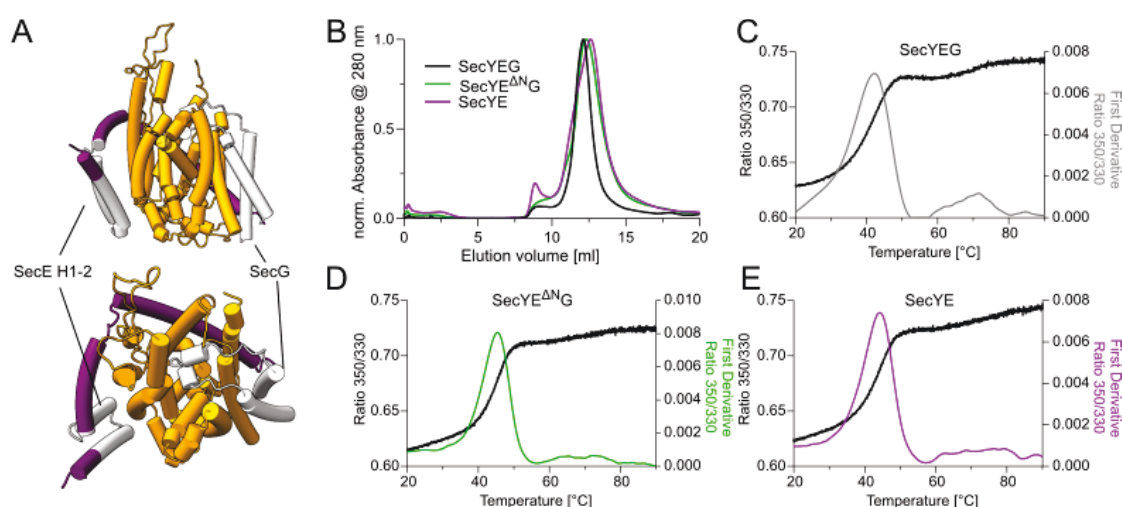
(C) Size exclusion chromatography (SEC) profile of the isolated SecYA-EG complex in DDM micelles.

(D) Coomassie-stained SDS-PAGE of the SEC fractions with SecYA-EG, elution volume 14 to 17. The isolated subunits are labelled. Free SecA is indicated with an asterisk.

(E) Anti-His Immunoblot of the SecYA-EG construct after gel filtration chromatography.

(F) SDS-PAGE of the SecYA-EG fusion construct reconstituted in DOPC:DOPG and POPC:POPG liposomes.

(G) SDS-PAGE of the trypsin-treated SecYA-EG reconstituted in DOPC:DOPG liposomes. The accessibility for the protease indicates exposure of SecYA to the exterior of the liposomes. SecYA orientation in different liposomes is estimated based on the reduction in the protein amount (bar plot below).

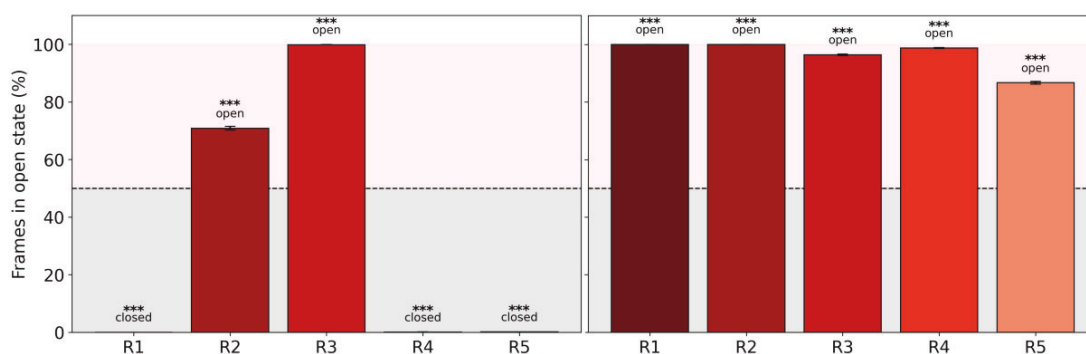


Suppl. Figure 3. Characterization of SecYEG and truncations.

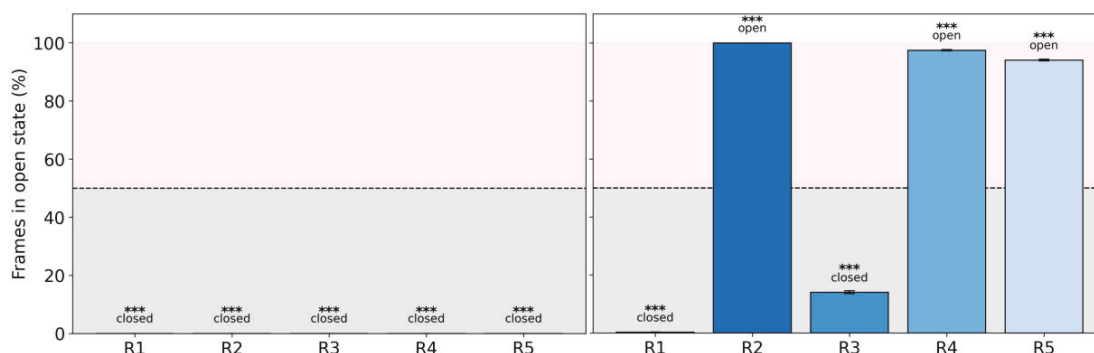
(A) Model of the full-length SecYEG, derived from Busch et al 2026. Residues lacking for the SecYE and SecYE^{ΔNG} variants are colored in white.

(B) Size-exclusion chromatogram of the isolated translocon variants

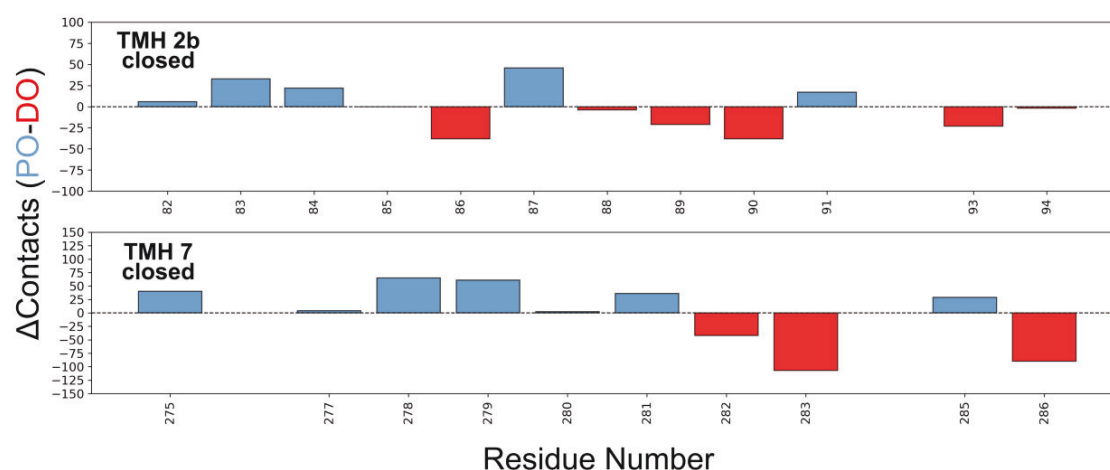
(C-E) NanoDSF measurements of SecYEG, SecYE^{ΔNG} and SecYE truncations. Ratio 350/330 as well as the first derivative is plotted against the temperature. Measurements indicate a stable complex unfolding at approx. 45°C.



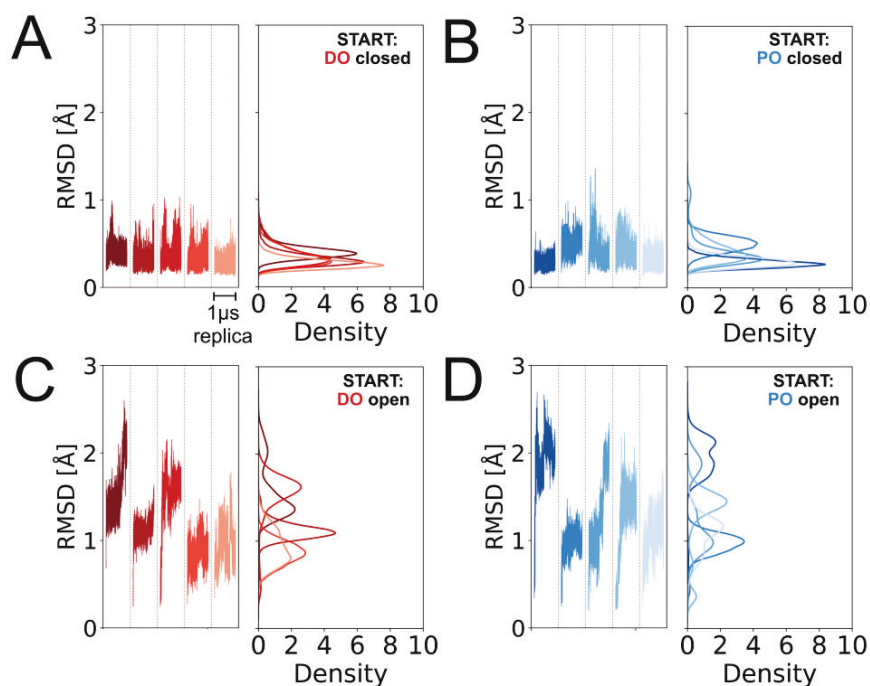
Suppl. Figure 4. Open-state occupancy of the TMH 2b–7 distance across independent replicas of the closed-DO (I, left) and open-DO (II, right) MD simulations. Bars represent the percentage of simulation frames in which the TMH 2b–7 distance exceeds the threshold, corresponding to the open state. Error bars indicate the 95% confidence interval of the mean open-state occupancy across independent replicas, estimated as mean $\pm$ SEM, treating each replica as a single independent observation. The dashed horizontal line marks equal occupancy (50%), separating closed-state preference (grey-shaded area) from open-state preference (pink-shaded area). Statistical significance was assessed for each replica using a two-sided binomial test under the null hypothesis of equal occupancy (percentage of frames in the open state = 50%). The number of frames classified as open was compared to the total number of frames in each trajectory. Significance levels are indicated by asterisks (n.s., $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). All replicas exhibit a statistically significant deviation from equal occupancy, indicating a preferential sampling of one conformational state.



Suppl. Figure 5. Open-state occupancy of the TMH 2b–7 distance across independent replicas of the closed-PO (III, left) and open-PO (IV, right) simulations. Bars represent the percentage of simulation frames in which the TMH 2b–7 distance exceeds the threshold, corresponding to the open state. Error bars indicate the 95% confidence interval of the mean open-state occupancy across independent replicas, estimated as mean $\pm$ SEM, treating each replica as a single independent observation. The dashed horizontal line marks equal occupancy (50%), separating closed-state preference (grey-shaded area) from open-state preference (pink-shaded area). Statistical significance was assessed for each replica using a two-sided binomial test under the null hypothesis of equal occupancy (percentage of frames in the open state = 50%). The number of frames classified as open was compared to the total number of frames in each trajectory. Significance levels are indicated by asterisks (n.s., $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). All replicas exhibit a statistically significant deviation from equal occupancy, indicating a preferential sampling of one conformational state.



Suppl. Figure 6. Differential TMH 2b-TMH 7 (top) and TMH 7-TMH 2b (bottom) contacts for distinct membrane compositions in relationship to the closed SecYEG simulations. The bar plot shows the change in contact frequency ($\Delta\text{Contacts}$) between either TMH 2b or TMH 7 protein residues and TMH 7 or TMH 2b protein residues, respectively, across two membrane compositions. Contacts were summed over molecular dynamics trajectory parts associated with the global minimum of the PMF considering only lipophilic chains of DO (dioleoyl) or PO (palmitoyl-oleoyl) lipids. The dashed horizontal line indicates no change ($\Delta = 0$). Bars above zero (blue) correspond to residues exhibiting increased contacts in PO relative to DO, while bars below zero (red) indicate increased contacts in DO relative to PO. The magnitude of each bar reflects the extent of the difference, identifying residues whose interactions in stabilizing the lateral gate are most sensitive to membrane composition change.



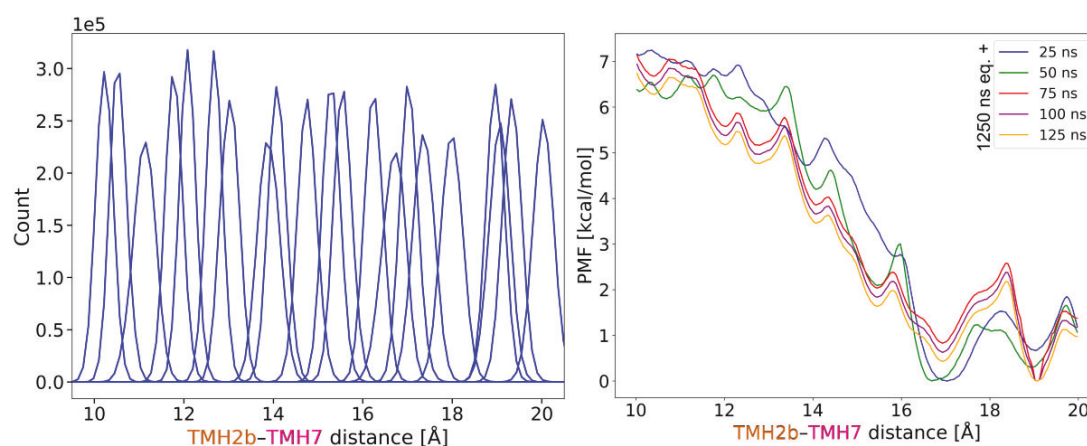
Suppl. Figure 7. Plug (TMH 2a) structural invariance across simulations.

(A) Time evolution of the TMH 2a RMSD for independent MD simulation replicas for the closed SecYEG in DOPC:DOPG 70:30 systems, (*left*); vertical dotted lines separate individual replicas. Kernel density estimates (KDE) of the RMSD distribution per replica (*right*).

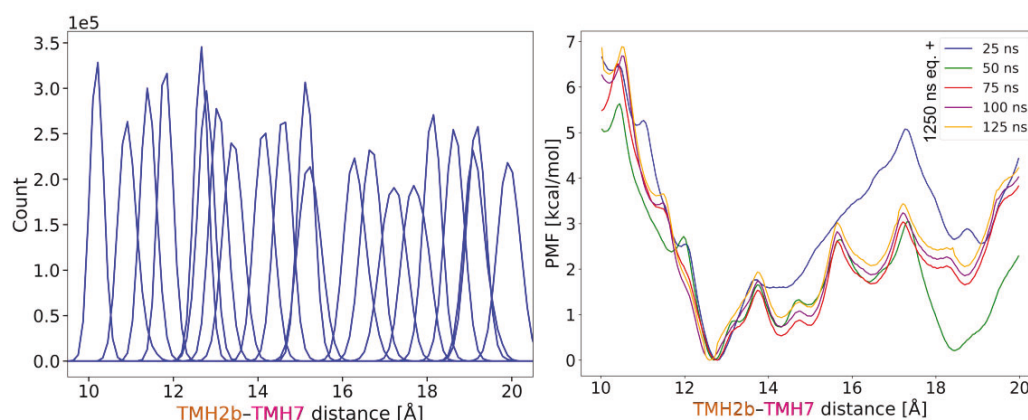
(B) Same as in A) but for POPC:POPG 70:30 systems.

(C) Time evolution of the TMH 2a RMSD for independent simulations replicas for the open SecYEG in DOPC:DOPG 70:30 systems, (*left*); kernel density estimates (KDE) of the RMSD distribution per replica (*right*).

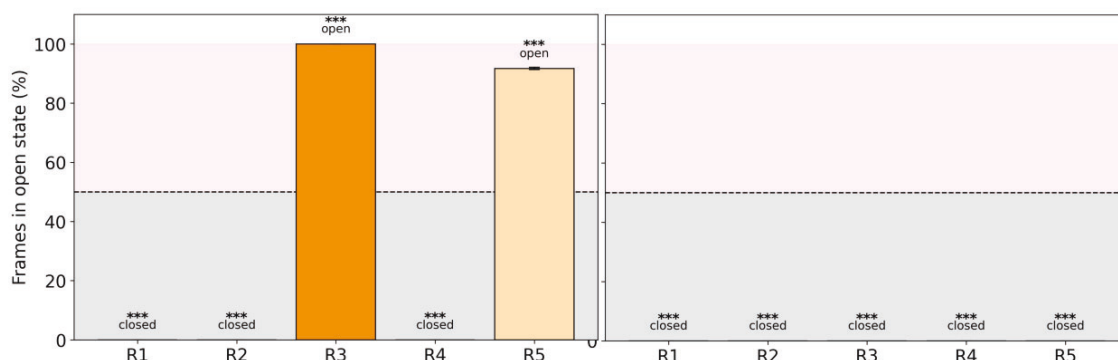
(D) Same as in (C) but for POPC:POPG 70:30 systems.



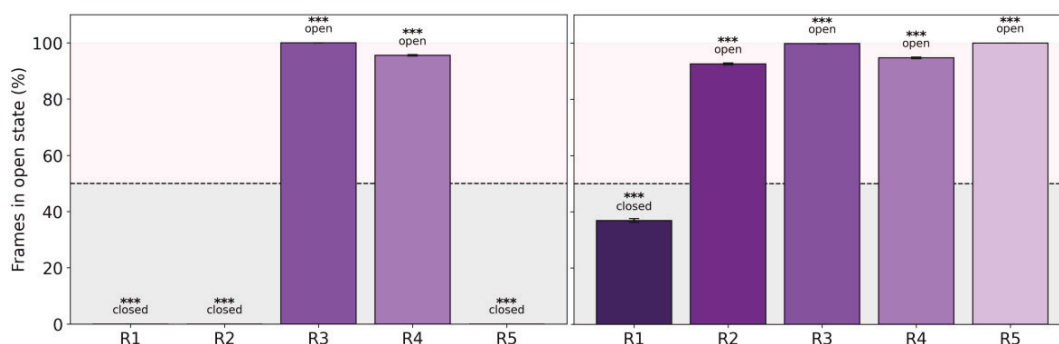
Suppl. Figure 8. Evaluation of the PMF of SecYEG opening when embedded in a DOPC:DOPG membrane (system closed-DO (I)). Histograms indicate sufficient overlap among the umbrella windows using a force constant of $4 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$: the minimum window overlap is 5.97% (mean $\pm$ SEM: $28.78 \pm 5.02\%$) between contiguous windows. Succession of PMFs with increased sampling times per window after 1250 ns of equilibration time. The plot indicates a converged PMF after 1375 ns of sampling.



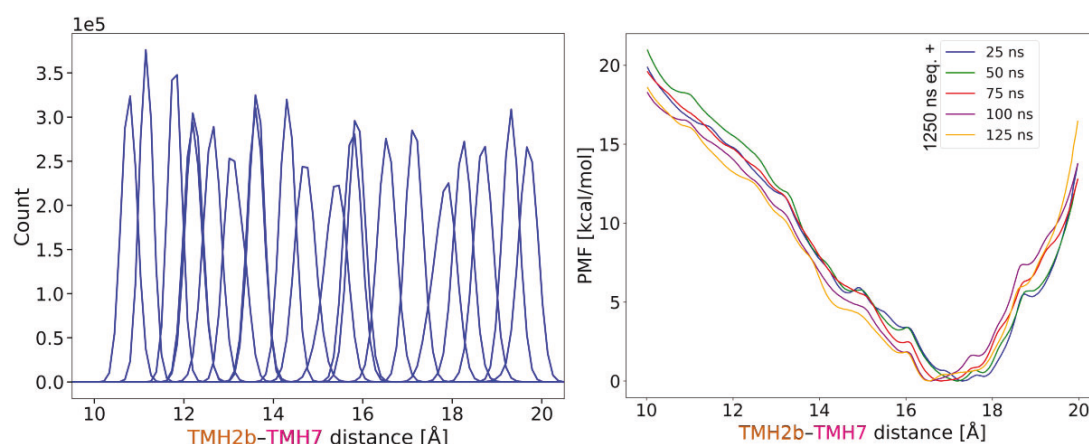
Suppl. Figure 9. Evaluation of the PMF of SecYEG opening when embedded in a POPC:POPG membrane (system open-PO (IV)). Histograms indicate sufficient overlap among the umbrella windows using a force constant of $4 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$: the minimum window overlap is 4.58% (mean $\pm$ SEM: $33.26 \pm 5.32\%$) between contiguous windows (*left*). Succession of PMFs with increased sampling times per window after 1250 ns of equilibration time. The plot indicates a converged PMF after 1375 ns of sampling (*right*).



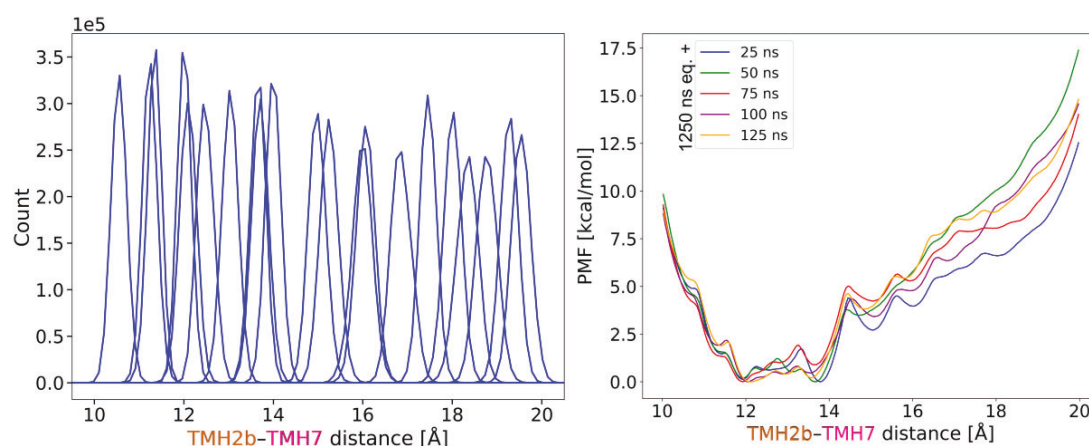
Suppl. Figure 10. Open-state occupancy of the TMH 2b–7 distance across independent replicas of the closed-PO-bCL (VII, left) and open-PO-bCL (VIII, right) simulations. Bars represent the percentage of simulation frames in which the TMH 2b–7 distance exceeds the threshold, corresponding to the open state. Error bars indicate the 95% confidence interval of the mean open-state occupancy across independent replicas, estimated as mean $\pm$ SEM, treating each replica as a single independent observation. The dashed horizontal line marks equal occupancy (50%), separating closed-state preference (grey-shaded area) from open-state preference (pink-shaded area). Statistical significance was assessed for each replica using a two-sided binomial test under the null hypothesis of equal occupancy (percentage of frames in the open state = 50%). The number of frames classified as open was compared to the total number of frames in each trajectory. Significance levels are indicated by asterisks (n.s., $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). All replicas exhibit a statistically significant deviation from equal occupancy, indicating a preferential sampling of one conformational state.



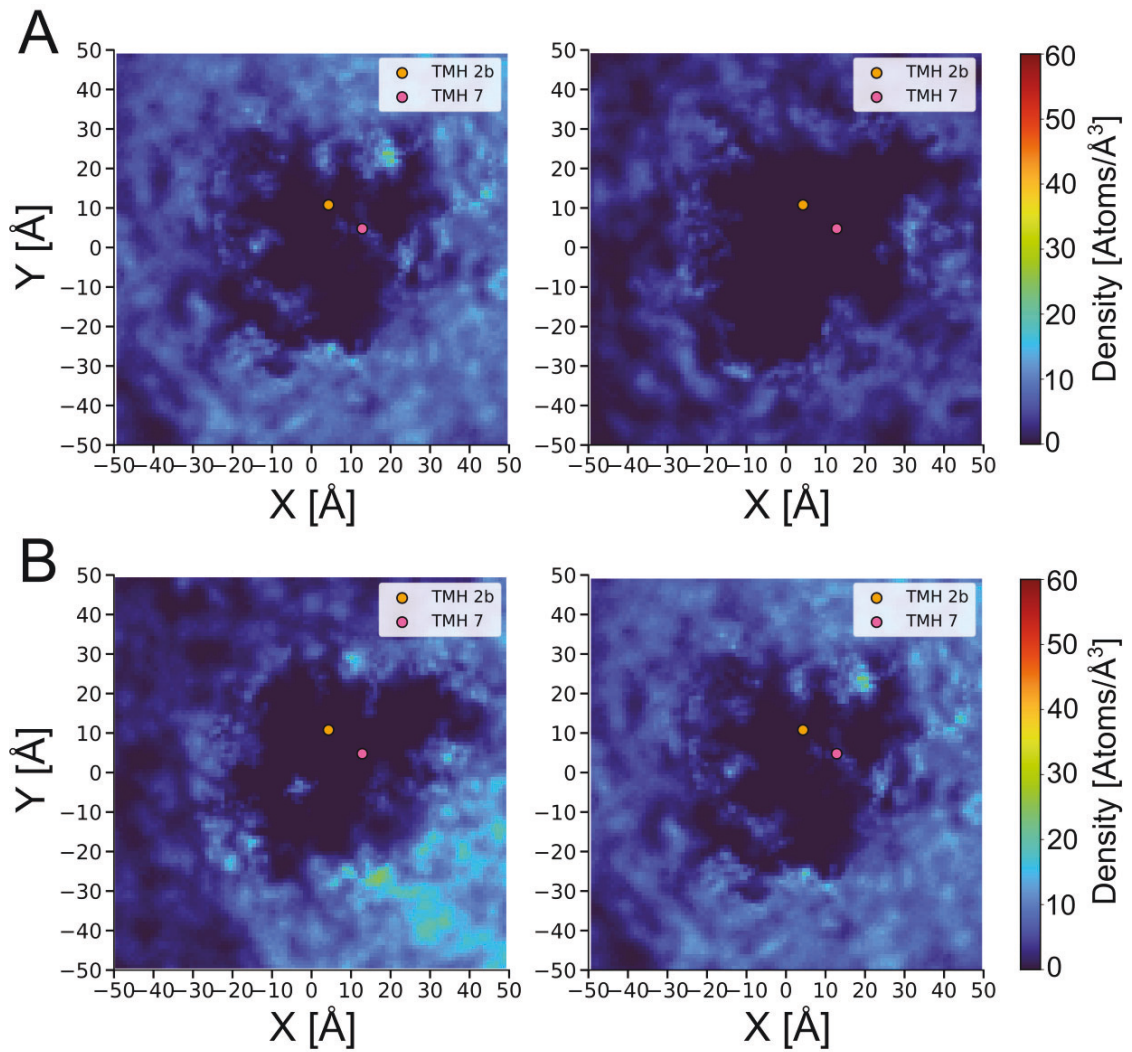
Suppl. Figure 11. Open-state occupancy of the TMH 2b–7 distance across independent replicas of the closed-PO-CL (V, left) and open-PO-CL (VI, right) simulations. Bars represent the percentage of simulation frames in which the TMH 2b–7 distance exceeds the threshold, corresponding to the open state. Error bars indicate the 95% confidence interval of the mean open-state occupancy across independent replicas, estimated as mean $\pm$ SEM, treating each replica as a single independent observation. The dashed horizontal line marks equal occupancy (50%), separating closed-state preference (grey-shaded area) from open-state preference (pink-shaded area). Statistical significance was assessed for each replica using a two-sided binomial test under the null hypothesis of equal occupancy (percentage of frames in the open state = 50%). The number of frames classified as open was compared to the total number of frames in each trajectory. Significance levels are indicated by asterisks (n.s., $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). All replicas exhibit a statistically significant deviation from equal occupancy, indicating a preferential sampling of one conformational state.



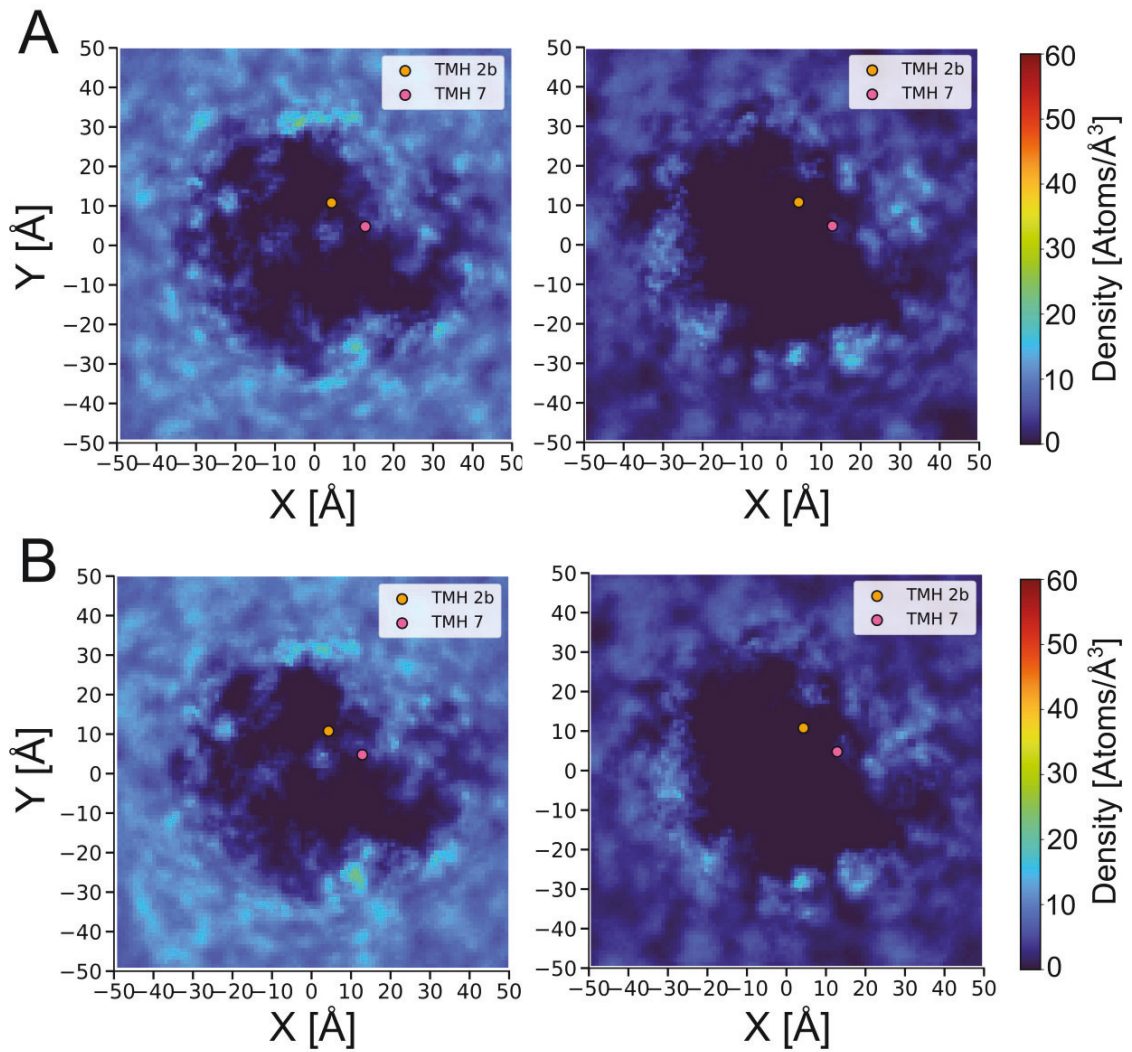
Suppl. Figure 12. FIGURE RG_S6. Evaluation of the PMF of SecYEG opening when embedded in a POPC:TOCL membrane (system closed-PO-CL (V)). Histograms indicate sufficient overlap among the umbrella windows using a force constant of $4 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$: the minimum window overlap is 6.83% (mean $\pm$ SEM: $31.90 \pm 6.04\%$) between contiguous windows (*left*). Succession of PMFs with increased sampling times per window after 1250 ns of equilibration time. The plot indicates a converged PMF after 1375 ns of sampling (*right*).



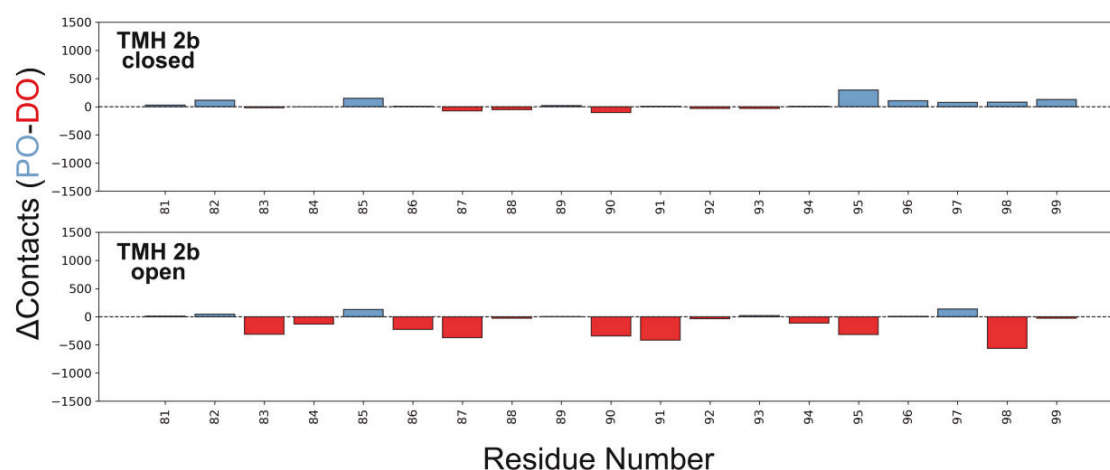
Suppl. Figure 13. Evaluation of the PMF of SecYEG opening when embedded in a POPC:bis(PO)CL membrane (system open-PO-bCL (VIII)). Histograms indicate sufficient overlap among the umbrella windows using a force constant of $4 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$: the minimum window overlap is 3.67% (mean $\pm$ SEM: $33.80 \pm 6.62\%$) between contiguous windows (*left*). Succession of PMFs with increased sampling times per window after 1250 ns of equilibration time. The plot indicates a converged PMF after 1375 ns of sampling (*right*).



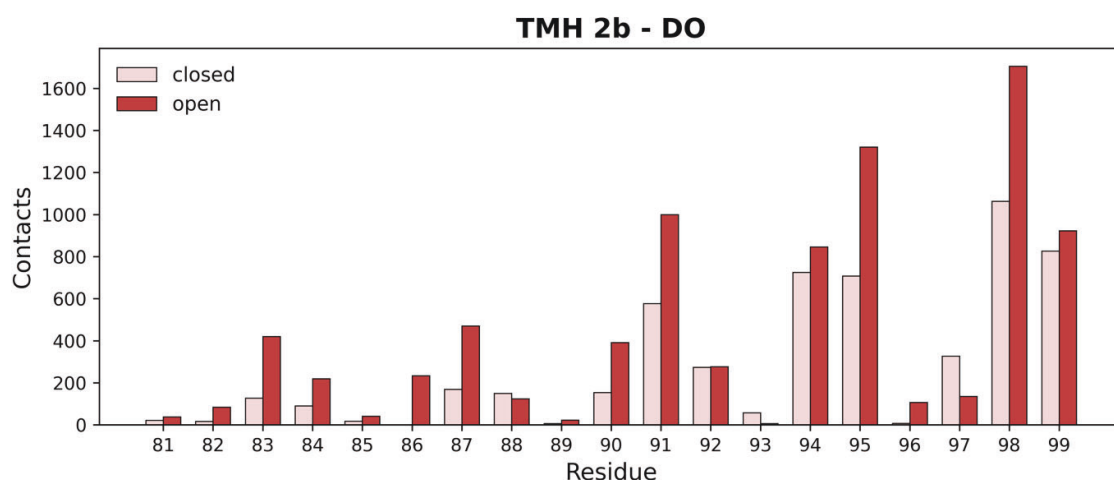
Suppl. Figure 14. 2D density maps of PC (left) and PGR (right) lipids around the closed SecYEG complex viewed from the cytoplasmic side of the membrane, in the presence of DOPC:DOPG (A) and POPC:POPG (B). The density map was obtained from the three-dimensional lipid density grid by splitting the system at the membrane midplane and integrating voxel occupancies separately over the cytoplasmic half of the bilayer. Orange and magenta markers indicate the COM of TMH 2b and TMH 7.



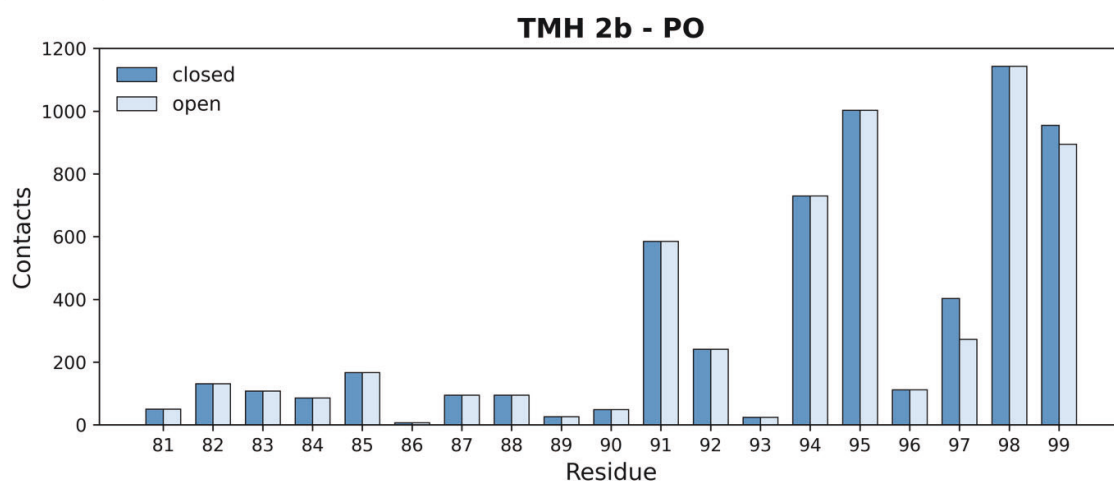
Suppl. Figure 15. 2D density maps of PC (left) and PGR (right) lipids around the open SecYEG complex viewed from the cytoplasmic side of the membrane, in the presence of DOPC:DOPG (A) and POPC:POPG (B). The density map was obtained from the three-dimensional lipid density grid by splitting the system at the membrane midplane and integrating voxel occupancies separately over the cytoplasmic half of the bilayer. Orange and magenta markers indicate the COM of TMH 2b and TMH 7.



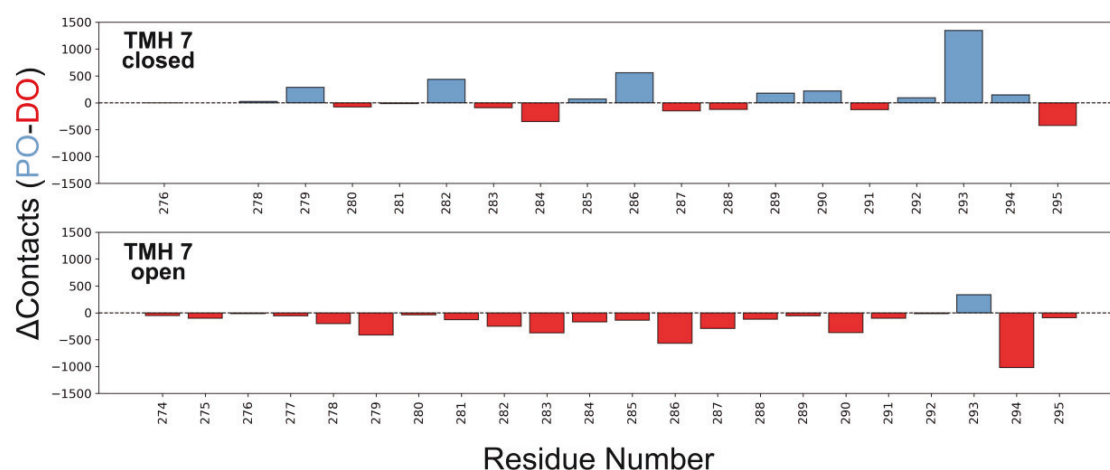
Suppl. Figure 16. Differential lipid-TMH 2b contacts for distinct membrane compositions in relationship to the closed (top) and open (bottom) SecYEG simulations. The bar plot shows the change in contact frequency ($\Delta\text{Contacts}$) between TMH 2b protein residues and lipid acyl chains across two membrane compositions. Contacts were summed over molecular dynamics trajectory parts associated with the global minimum of the PMF considering only lipophilic chains of DO (dioleoyl) or PO (palmitoyl-oleoyl) lipids. The dashed horizontal line indicates no change ($\Delta = 0$). Bars above zero (blue) correspond to residues exhibiting increased contacts in PO relative to the DO, while bars below zero (red) indicate increased contacts in DO relative to PO. The magnitude of each bar reflects the extent of the difference, identifying residues whose interactions with lipid tails are most sensitive to membrane composition change.



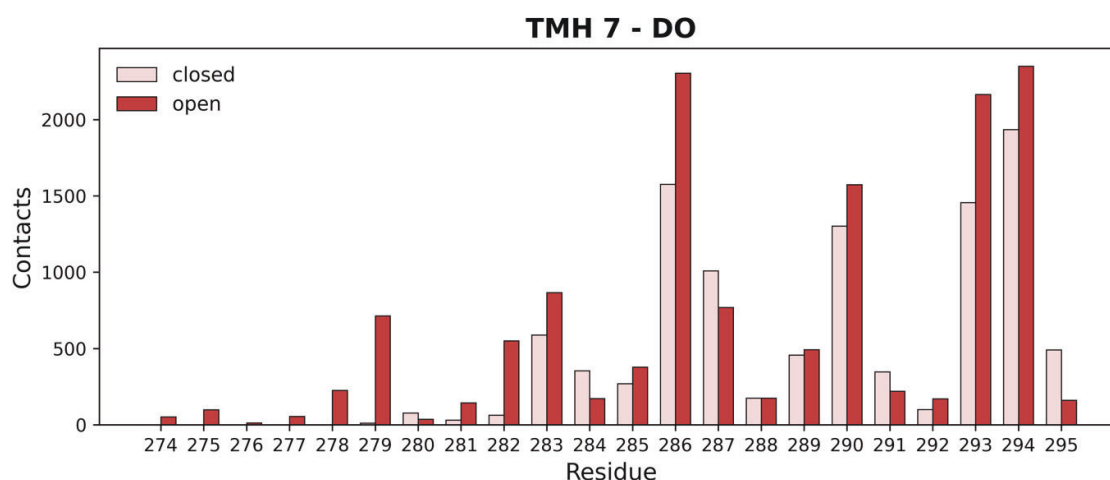
Suppl. Figure 17. Lipid-TMH 2b contacts for the DOPC:DOPG composition in relationship to the closed (pink) and open (red) SecYEG simulations. The bar plot shows the change in contact frequency between TMH 2b protein residues and lipid acyl chains across two protein conformations. Contacts were summed over molecular dynamics trajectory parts associated with the global minimum of the PMF considering only lipophilic chains of DO (dioleoyl) lipids.



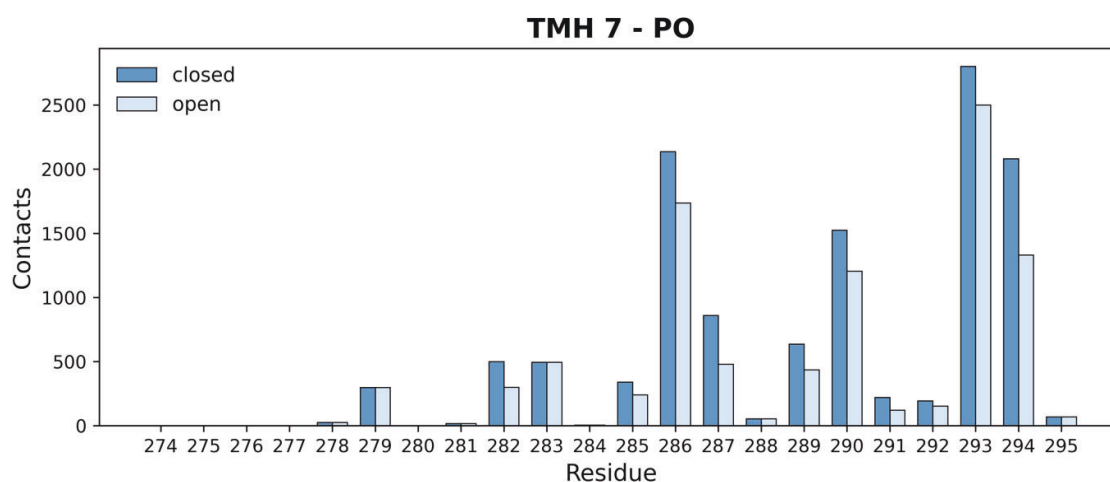
Suppl. Figure 18. Lipid-TMH 2b contacts for the POPC:POPG composition in relationship to the closed (blue) and open (cyan) SecYEG simulations. The bar plot shows the change in contact frequency between TMH 2b protein residues and lipid acyl chains across two protein configurations. Contacts were summed over molecular dynamics trajectory parts associated with the global minimum of the PMF global considering only lipophilic chains of PO lipids.



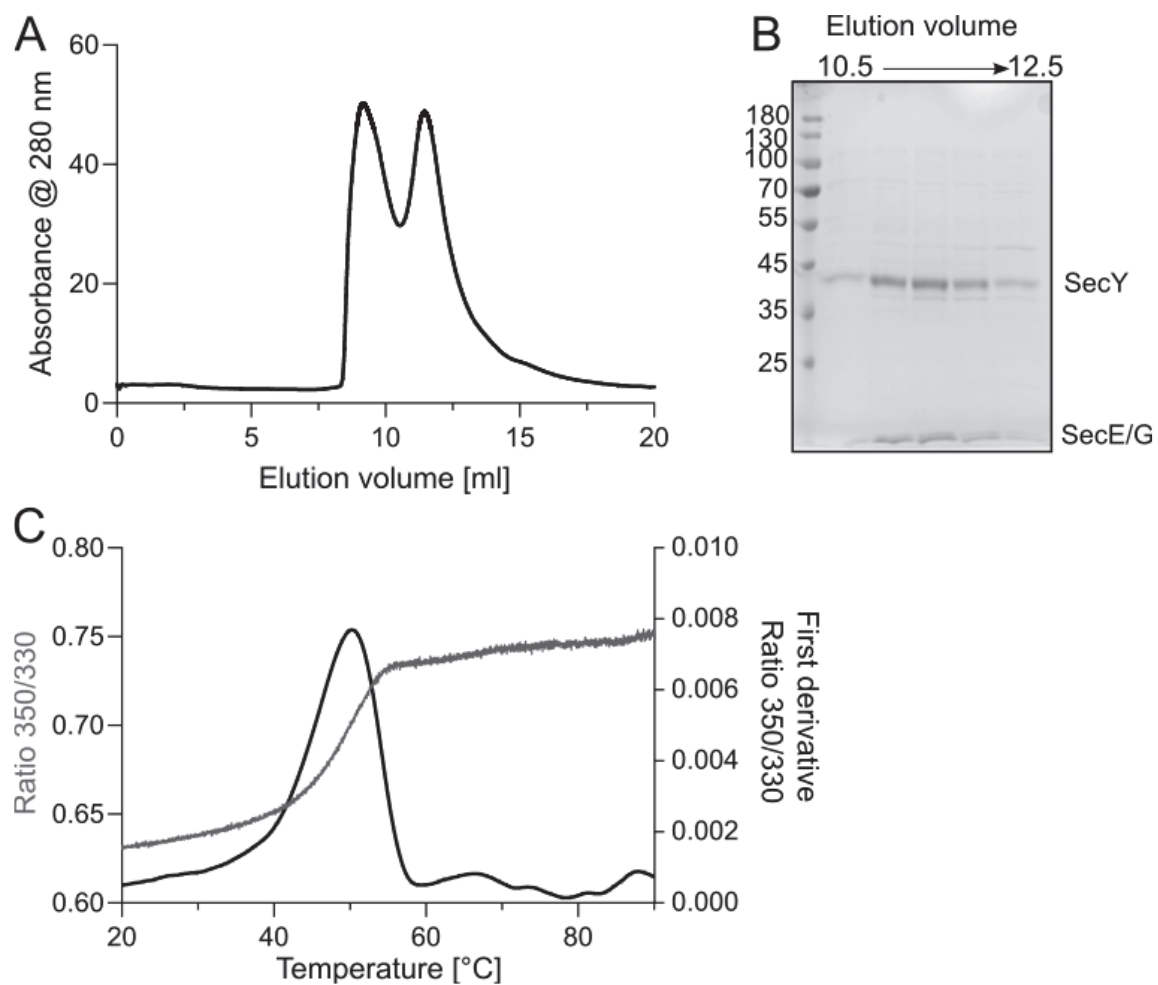
Suppl. Figure 19. Differential lipid-TMH 7 contacts for distinct membrane compositions in relationship to the closed (top) and open (bottom) SecYEG simulations. The bar plot shows the change in contact frequency (Δ Contacts) between TMH 7 protein residues and lipid acyl chains across two membrane compositions. Contacts were summed over molecular dynamics trajectory parts associated with the global minimum of the PMF considering only lipophilic chains of DO (dioleoyl) or PO (palmitoyl-oleoyl) lipids. The dashed horizontal line indicates no change ($\Delta = 0$). Bars above zero (blue) correspond to residues exhibiting increased contacts in PO relative to the DO, while bars below zero (red) indicate increased contacts in DO relative to PO. The magnitude of each bar reflects the extent of the difference, identifying residues whose interactions with lipid tails are most sensitive to membrane composition change.



Suppl. Figure 20. Lipid-TMH 7 contacts for the DOPC:DOPG composition in relationship to the closed (pink) and open (red) SecYEG simulations. The bar plot shows the change in contact frequency between TMH 7 protein residues and lipid acyl chains across two protein configurations. Contacts were summed over molecular dynamics trajectory parts associated with the global minimum of the PMF considering only lipophilic chains of DO (dioleoyl) lipids.



Suppl. Figure 21. Lipid-TMH 7 contacts for the POPC:POPG composition in relationship to the closed (blue) and open (cyan) SecYEG simulations. The bar plot shows the change in contact frequency between TMH 7 protein residues and lipid acyl chains across two protein configurations. Contacts were summed over molecular dynamics trajectory parts associated with the global minimum of the PMF considering only lipophilic chains of PO lipids.

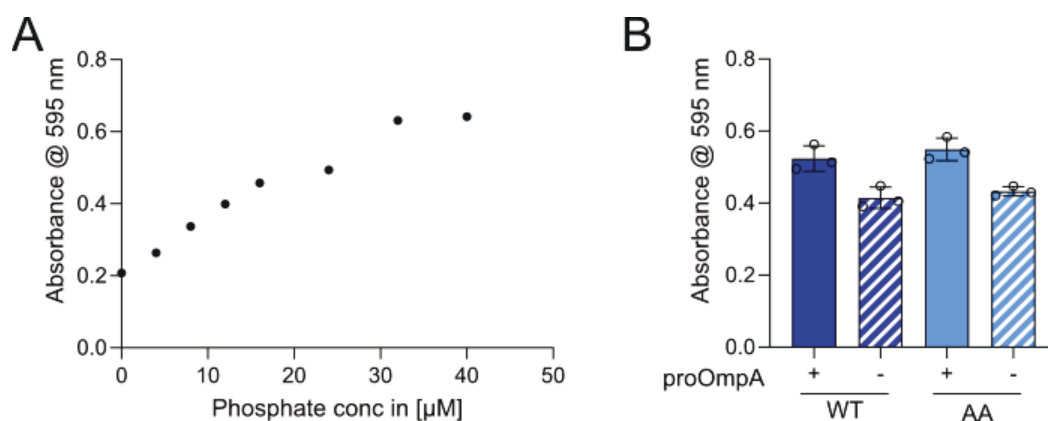


Suppl. Figure 22. SecY^{AA}EG gel filtration and NanoDSF.

(A) Size exclusion chromatography (SEC) profile of the isolated SecY^{AA}EG in DDM micelles.

(B) Corresponding Coomassie-stained SDS-PAGE from elution volume 10.5 to 12.5 ml.

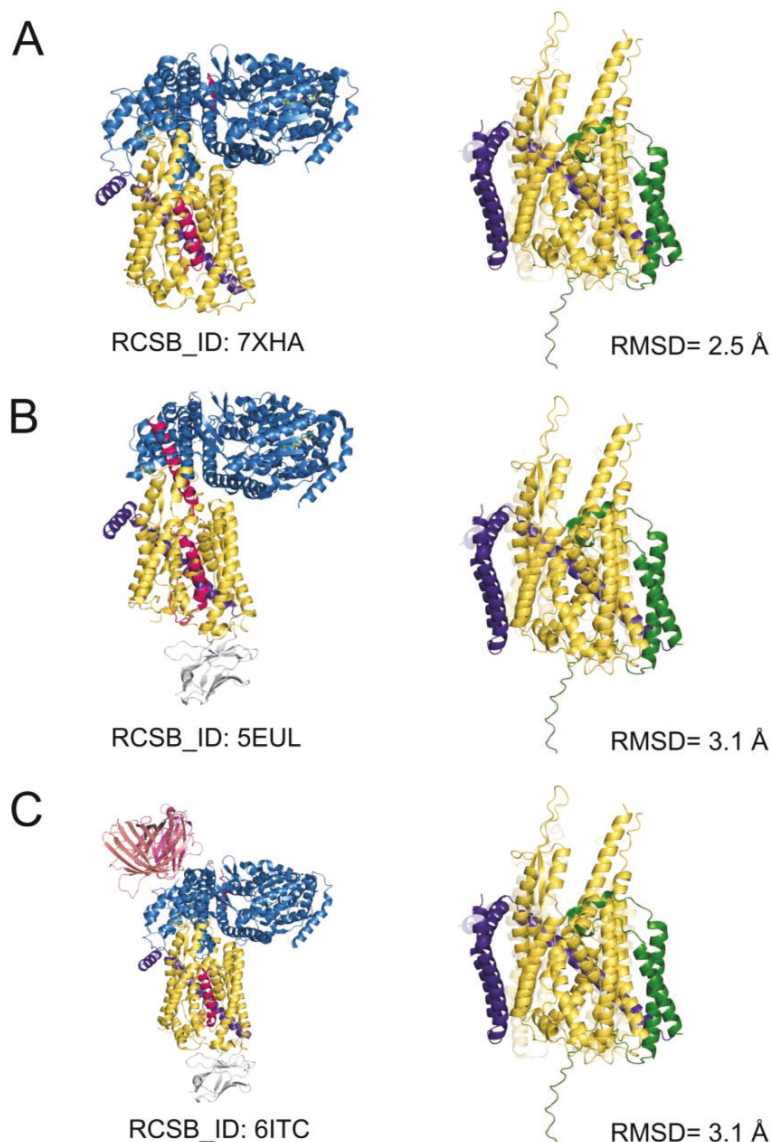
(C) NanoDSF measurements of SecY^{AA}EG mutant. Ratio 350/330 as well as the first derivative is plotted against the temperature. Measurements indicate a stable complex unfolding at approx. 50°C.



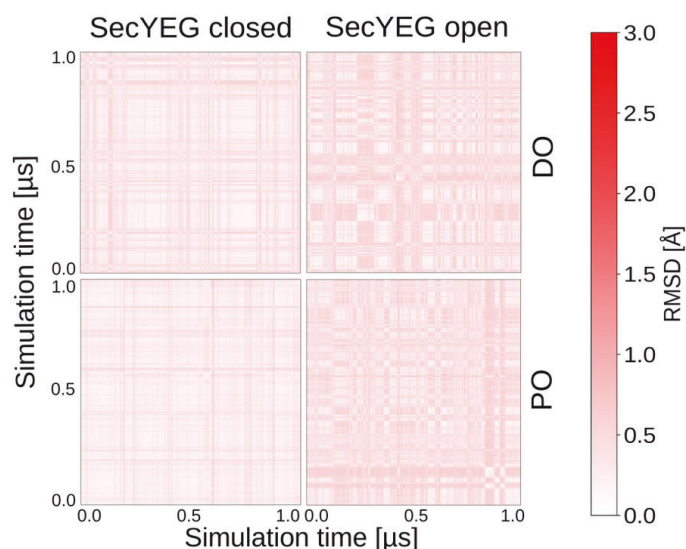
Suppl. Figure 23. ATPase activity is stimulated by proOmpA addition.

(A) Phosphorus standard as provided by Sigma-Aldrich malachite green ATPase kit (MAK307).

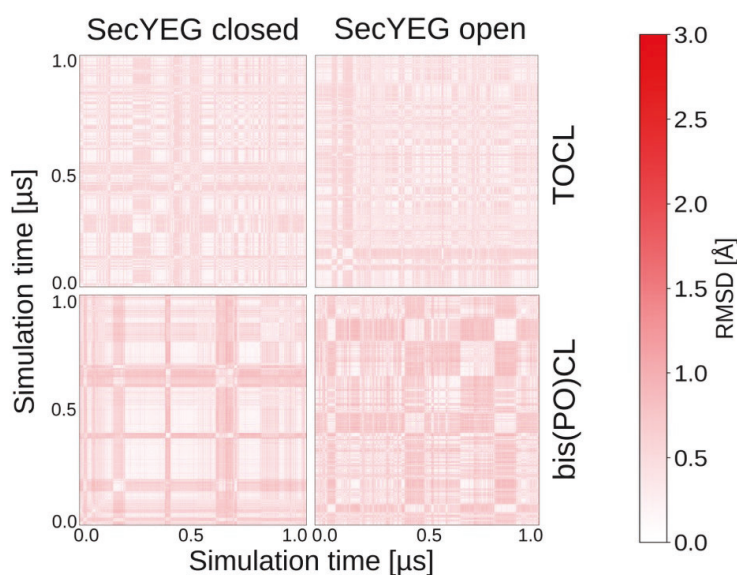
(B) Mean absorbance at 595 nm of conditions with proteoliposomes bearing SecYEG^{WT} or SecY^{AA}EG with and without proOmpA added. Individual measurements were conducted three times and each with three technical replicates.



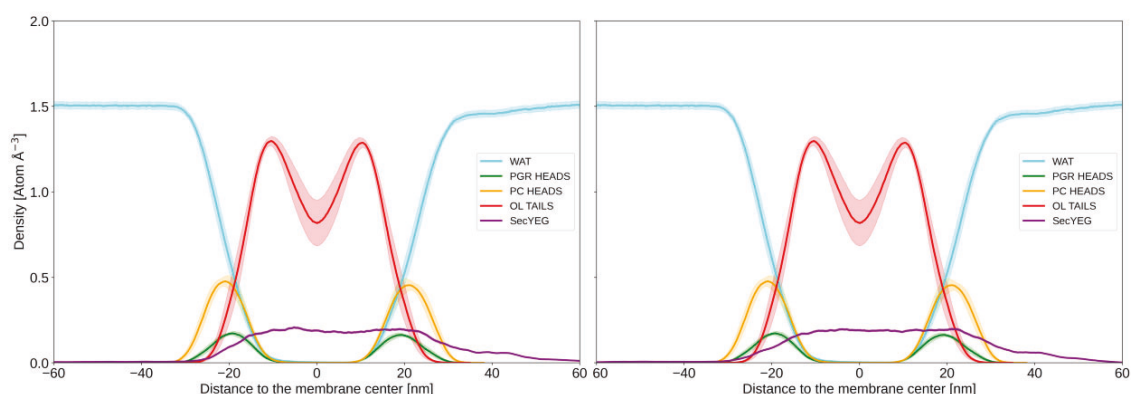
Suppl. Figure 24. Structures of SecYEG homologs in open conformations. **(A)** The translocon SecYE engaged with SecA and the substrate proOmpA(4Y)-sfGFP (RCSB_ID: 7XHA, ¹); **(B)** SecA-SecYE complex with a translocating polypeptide substrate (RCSB_ID: 5EUL, ²), and **(C)** substrate-engaged SecA-SecY protein translocation machine (RCSB_ID: 6ITC, ³) (left). SecY is shown as yellow ribbons, with fragmented SecE shown in purple. SecA is shown in blue, and the translocating polypeptide and the inserted signal sequence are shown in magenta. The nanobody at the periplasmic side is shown as grey cartoon (panels B and C); the eGFP is represented as pink cartoon (panel C). Right column: In all cases, the structural superposition of the structures with the obtained AlphaFold3 model shows high structural displacements in SecY (center), particularly in correspondence to the lateral gate area (TMHs 2b and 7) and neighbouring helices (TMH8). The dynamics of the lateral gate is similar upon the ribosome binding; the opening is occasionally more pronounced. This suggests that AlphaFold 3 predicted a rather closed configuration of SecYEG.



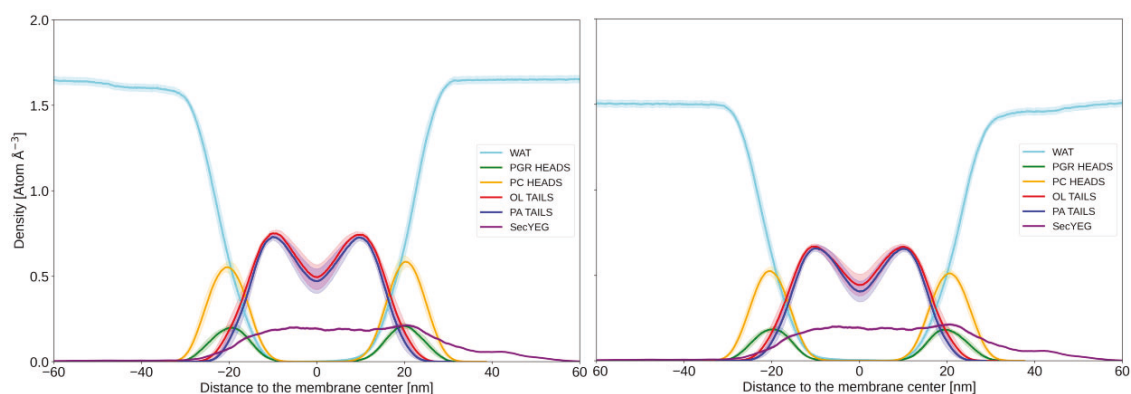
Suppl. Figure 25. Two-dimensional root-mean-square deviation (2D-RMSD) matrix comparing molecular dynamics simulation poses of SecYEG starting either from the closed or open configuration in DOPC:DOPG (DO) and POPC:POPC (PO) membranes, respectively. In all frames, the protein structures preserve their position during 1.0 μ s of simulation time with respect to the first frame (RMSD < 2 Å) across five different replicas. The 2D-RMSD of the SecYEG protein backbone was computed after superpositioning the C α atoms of SecYEG, with warmer colors denoting increased mobility.



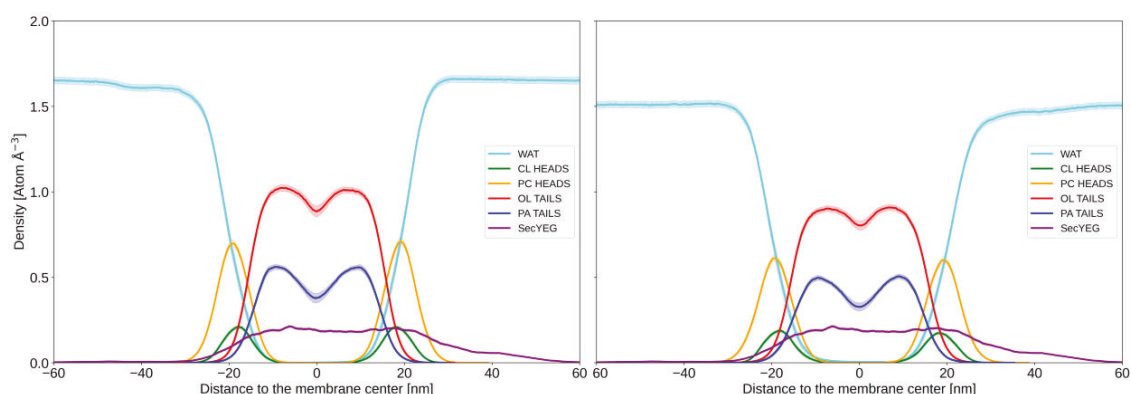
Suppl. Figure 26. Two-dimensional root-mean-square deviation (2D-RMSD) matrix comparing molecular dynamics simulation poses of SecYEG starting either from the closed or open configuration in POPC:TOCL (TOCL) and POPC:bis(PO)CL (bis(PO)CL) membranes, respectively. In all frames, the protein structures preserve their position during 1.0 μ s of simulation time with respect to the first frame (RMSD < 2 Å) across five different replicas. The 2D-RMSD of the SecYEG protein backbone was computed after superpositioning the C α atoms of SecYEG, with warmer colors denoting increased mobility.



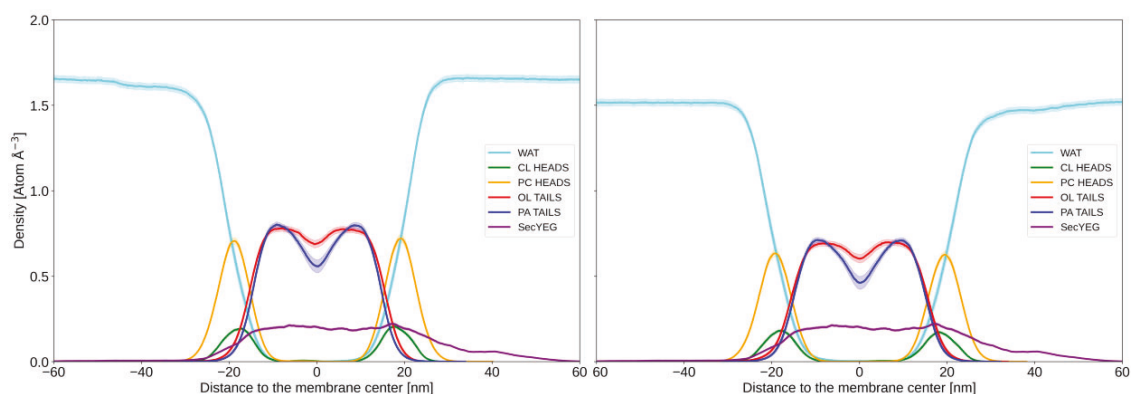
Suppl. Figure 27. Atom density profiles of membrane components averaged over five independent unbiased MD simulations of SecYEG closed (left) and open (right) conformations in DOPC:DOPG membranes for the phospholipid tails (OL) and head groups (PC and PGR) molecules. The obtained shapes correspond with those generally found by experiments and MD simulations for biomembranes and our previous work ⁴⁻⁶.



Suppl. Figure 28. Atom density profiles of membrane components averaged over five independent unbiased MD simulations of SecYEG closed (left) and open (right) conformations in POPC:POPG membranes for the phospholipid tails (OL, PA) and head groups (PC and PGR) molecules. The obtained shapes correspond with those generally found by experiments and MD simulations for biomembranes and our previous work ⁴⁻⁶.



Suppl. Figure 29. Atom density profiles of membrane components averaged over five independent unbiased MD simulations of SecYEG closed (left) and open (right) conformations in POPC:TOCL membranes for the phospholipid tails (OL, PA) and head groups (PC and CL) molecules. The obtained shapes correspond with those generally found by experiments and MD simulations for biomembranes and our previous work ⁴⁻⁶.



Suppl. Figure 30. Atom density profiles of membrane components averaged over five independent unbiased MD simulations of SecYEG closed (left) and open (right) conformations in POPC:bis(PO)CL membranes for the phospholipid tails (OL, PA) and head groups (PC and CL) molecules. The obtained shapes correspond with those generally found by experiments and MD simulations for biomembranes and our previous work ⁴⁻⁶.

Supplemental References

- 1 Dong, L. *et al.* Structural basis of SecA-mediated protein translocation. *Proc Natl Acad Sci U S A* **120**, e2208070120 (2023). <https://doi.org/10.1073/pnas.2208070120>
- 2 Li, L. *et al.* Crystal structure of a substrate-engaged SecY protein-translocation channel. *Nature* **531**, 395-399 (2016). <https://doi.org/10.1038/nature17163>
- 3 Ma, C. *et al.* Structure of the substrate-engaged SecA-SecY protein translocation machine. *Nat Commun* **10**, 2872 (2019). <https://doi.org/10.1038/s41467-019-10918-2>
- 4 Liu, Y. & Nagle, J. F. Diffuse scattering provides material parameters and electron density profiles of biomembranes. *Phys Rev E Stat Nonlin Soft Matter Phys* **69**, 040901 (2004). <https://doi.org/10.1103/PhysRevE.69.040901>
- 5 Gentile, R. *et al.* Molecular Mechanisms Underlying Medium-Chain Free Fatty Acid-Regulated Activity of the Phospholipase PlaF from *Pseudomonas aeruginosa*. *JACS Au* **4**, 958-973 (2024). <https://doi.org/10.1021/jacsau.3c00725>
- 6 Tristram-Nagle, S., Petrache, H. I. & Nagle, J. F. Structure and interactions of fully hydrated dioleoylphosphatidylcholine bilayers. *Biophys J* **75**, 917-925 (1998). [https://doi.org/10.1016/S0006-3495\(98\)77580-0](https://doi.org/10.1016/S0006-3495(98)77580-0)

6. Conclusion

Proteins need to acquire the correct fold at the right time and location to fulfill their intended function within the cell. Whereas soluble cytoplasmic proteins face the comparatively simpler challenge of attaining the correct fold, membrane proteins are subject to an additional layer of complexity during this process. In bacteria, insertion into the cytoplasmic membrane is mediated by SecYEG and/or YidC, potentially assisted by additional biogenesis factors. Proteins that are translocated across the cytoplasmic membrane in an unfolded state, either to mature within the periplasm by themselves or induced by their steric chaperone or to be subsequently inserted into the outer membrane, are exported via the post-translational translocation pathway of the general secretory (Sec) system. This work has combined biochemical, structural, and computational approaches to characterize this universally conserved system.

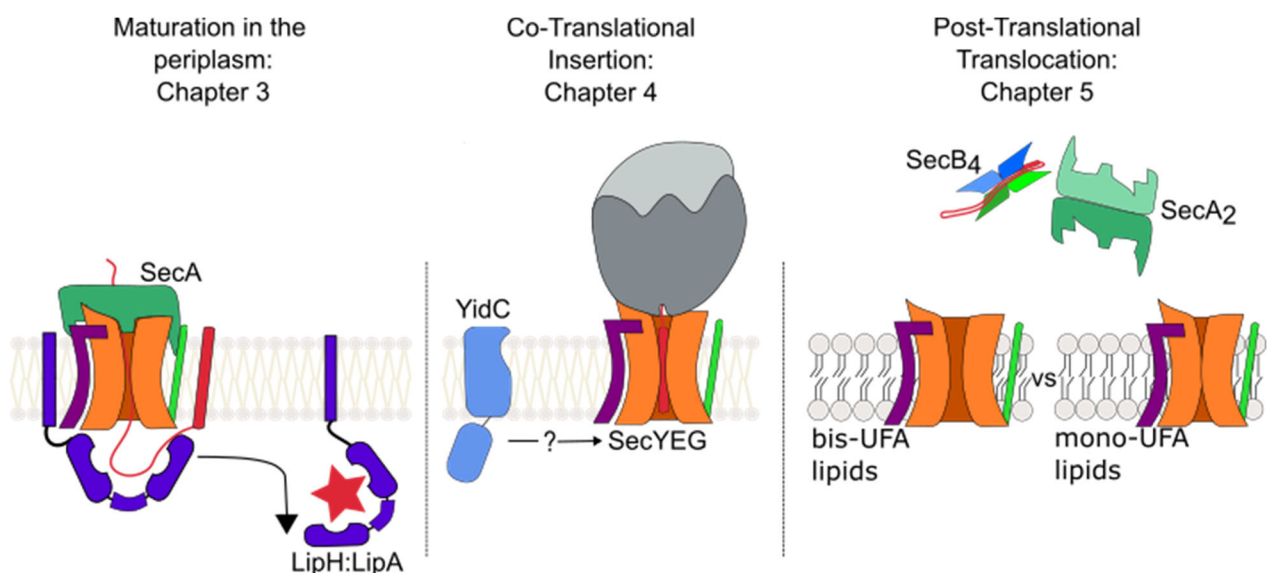


Figure 14: Graphical summary of the presented projects. (A) *P.aeruginosa* LipA requires its steric chaperone LipH for maturation before secretion. Within the highly dynamic chaperonic domain of the foldase, MD2 interacts tightly with the client whereas MD1 contacts only transiently. Further, promote negatively charged lipids the release of the lipase (B) The fatty acid composition within the lipid bilayer affects the conformational dynamics of SecYEG. Unsaturated fatty acids shift the equilibrium of the lateral gate to its open state, while saturated stabilizes the closed conformation. Especially two aromatic residues in TMH 7 manifest extensive conformation-specific contacts with the surrounding lipids influencing lateral gate dynamics. (C) The nascent chain does not enter the lateral gate of SecYEG but instead crosses the translocon towards its back side, where YidC is recruited in the nascent substrate-dependent manner. The SecY-YidC interface also promotes folding of the transmembrane helices before insertion. In the membrane YidC forms extensive contacts with the nascent chain, highlighting its key role

6.1 Conformational dynamics of the membrane-anchored foldase LipH from *Pseudomonas aeruginosa* governs recognition and release of its client lipase

The first chapter does not focus on the SecYEG translocon itself but instead addresses a subsequent step in the secretion pathway, namely the folding and maturation of a Sec substrate in the periplasm. Previous work from our group investigated the roles of general periplasmic chaperones and their interactions with the lipase, the studies presented here specifically examine its interplay with the dedicated steric chaperone LipH and its dynamics at the membrane interface [75].

A key insight emerging from these findings is that LipH does not operate as a rigid scaffold, but as a highly dynamic, membrane-tethered chaperone whose conformational dynamics enable sequential client capture, folding and release. A special focus was set on the thus far neglected variable domain of the foldase, accounting for one fifth of the full-length protein. In accordance with molecular dynamics simulations and SAXS analysis, the linker allows for extensive motions of the chaperoning domain leading to the hypothesis that the membrane-anchored LipH can sample a large area within the periplasm to bind the client lipase and potentially release it, after it achieved the proper fold.

On a more molecular level, our data, supported by biochemical assays and HDX-MS, suggests that the MD2-EHD interface of LipH first recognizes the N-terminal fragment of the lipase, and the folding and activation are completed after loading the remaining LipA into the chaperoning cavity. Hypothetically, such recognition could occur already during LipA translocation via the SecA:SecYEG machinery. It is further tempting to speculate that the LipH MD2 domain plays a leading role in recognition and binding of the client, while MD1 contributes to the chaperoning activity, such as correct positioning of LipA helices, and, possibly, release of the correctly folded lipase. Strikingly, the experiments also suggested a functional role of the membrane environment, more specifically the charged headgroups. Presence of the charged interface increased the dissociation constant of the client:chaperone complex from approximately 8 nM to 200 nM. A reason for this could be an increased dissociation, induced by the repulsion of the anionic charges clustering at MD1 and the EHD from the membrane mimetics.

These results provide a solid ground for *in vivo* studies going forward, which may test potential effects of specific point mutations at the LipH:LipA interface that will be essential to validate our model and to further elucidate the interplay. Such insights will deepen our understanding of the biogenesis of virulence factors in *P. aeruginosa* and related pathogens but may also facilitate production of the functional lipase in biotechnological applications.

6.2 SecYEG/YidC Mediated Membrane Protein Biogenesis

The findings presented in this chapter extend current models of membrane protein biogenesis by showing that the bacterial SecYEG:YidC machinery can operate through a substrate-dependent mechanism that goes beyond the classical lateral gate opening and release. While lateral gate opening has long been considered the primary route into the membrane, our data support an additional, evolutionarily conserved "back-of-Sec" insertion route that appears particularly suited to multipass membrane proteins. This observation aligns bacterial systems with observations made in eukaryotes and suggests that membrane protein insertion is more versatile than previously assumed [109, 112].

The central implication of this work is that the translocon should no longer be viewed exclusively as the static pore, but rather as a modular and adaptable platform whose architecture is shaped by the nascent chain that can lead to recruitment of other subunits, making the whole "Holo-translocon" idea more dynamic than the priorly claimed rather static super-complex [107]. The substrate-induced recruitment of YidC highlights this key principle: the insertion machinery self-organizes in response to the physicochemical properties and length of the emerging polypeptide, which matches with findings made in eukaryotes for multipass membrane proteins [111]. This dynamic assembly likely enables the cell to accommodate a diversity of membrane protein topologies without requiring fundamentally different machineries. Importantly, the observed pre-folding of transmembrane helices just after leaving the ribosomes at the hydrophobic pocket of the translocon provides insights into the energetic landscape of membrane protein insertion. By forming a secondary structure prior to membrane partitioning, nascent helices may minimize the energetic penalty associated with exposing polar backbone atoms to the hydrophobic lipid environment. This supports a model in which folding and insertion are tightly coupled processes, coordinated across the ribosome, SecYEG and YidC.

From an evolutionary perspective, the conservation of the "back-of-Sec" route across bacteria and eukaryotes indicate that this mechanism represents a fundamental solution to the challenge of inserting certain membrane proteins. While eukaryotic systems have evolved additional layers of complexity through multi-subunit assemblies, the bacterial SecYEG-YidC complex appears to be a minimal functional core capable of achieving similar outcomes. This raises the possibility that diversification of insertase architectures reflects adaptation to organism-specific demands rather than fundamentally different principles. This work further suggests that pathway selection is encoded within the substrate itself, rather than being dictated exclusively by dedicated targeting factors. A potential next step could be the *in vivo* extraction, with a following glutaraldehyde crosslinking to trap complexes.

Several open questions still arise. First, the dynamics of SecYEG-YidC assembly remain unclear: how rapidly does YidC associate and dissociate and are multiple YidC molecules involved during insertion of larger substrates? Second, the role of the native membrane environment, including lipid composition, membrane potential and

accessory factors such as SecDF, YajC, YibN or multiple copies of YidC, needs to be addressed to fully understand how the insertase operates *in vivo*. Third, the possibility of pathway switching during the insertion of a single protein, hinted to by the diversity of substrate features, represents an intriguing question for future investigation.

In our approach to investigating the membrane protein insertion through lateral gate opening, we unfortunately did not observe any structural rearrangements within the translocon despite its structure being fully resolved. The fact that it was not possible to account any volume for the nascent chain can have multiple reasons. One possibility could be that no distinct state becomes trapped within SecYEG, potentially together with the chaperone YidC, that could subsequently be fixated by glutaraldehyde crosslinking. Heterogeneity in the orientation of the four TMHs within the nanodisc could also result in insufficient signal averaging. Neither can we be sure that the disc has all “factors” required for insertion, though it has been shown for GPCRs that insertion can happen even in “empty” nanodiscs, not carrying any membrane protein insertase [281, 282].

In summary, this work advances a unifying framework in which membrane protein biogenesis is governed by dynamic, substrate-driven assembly of conserved machineries. By revealing the structural and mechanistic basis of the SecYEG-YidC interaction, it bridges gaps between bacterial and eukaryotic systems and opens new directions for exploring the complexity of membrane protein folding and insertion.

6.3 Lipid structure modulates the dynamics of the bacterial translocon SecYEG

The third chapter shifts the perspective on Sec-mediated protein translocation by positioning the lipid membrane not only as a passive environment, but as an active regulator of translocon function. While previous models have largely focused on SecA:lipid and protein:protein interactions that occur before translocation itself, the combined biochemical and computational results presented here demonstrate that the lipid composition directly reshapes the conformational landscape of SecYEG that results in changes in the translocation activity [163, 283, 284]. In particular, the finding that unsaturated lipid environments favor the open state of the lateral gate while saturated lipids stabilize the closed confirmation, mostly induced by Trp 293 and Phe 294 making protein:lipid contacts. Our work thereby provides a mechanistic framework to explain longstanding observations of lipid-dependent transport deficiencies.

Cells may regulate protein translocation not only through amounts of translocation competent assemblies, but also through adaptive remodelling of membrane composition, a concept that has been described before, for bacterial lipidomes under different environmental conditions [285]. More broadly, these findings support a model in which the SecYEG translocon operates as a lipid-sensitive molecular machine. In this context, lipid composition can be viewed as an additional regulatory layer that modulates protein function at the membrane level.

For future endeavours, an important challenge will be to validate these lipid-dependent conformational changes under more native conditions and to confirm the computational results by biochemical analysis such as EPR or the use of optical tweezers.

7. References

1. Anfinsen, C.B., et al., *The kinetics of formation of native ribonuclease during oxidation of the reduced polypeptide chain*. Proc Natl Acad Sci U S A, 1961. **47**(9): p. 1309-14.
2. Dill, K.A. and J.L. MacCallum, *The protein-folding problem, 50 years on*. Science, 2012. **338**(6110): p. 1042-6.
3. Levinthal, C., *How to fold graciously*. Mossbauer spectroscopy in biological systems, 1969. **67**: p. 22-24.
4. Dill, K.A., et al., *The protein folding problem: when will it be solved?* Curr Opin Struct Biol, 2007. **17**(3): p. 342-6.
5. Nolting, B. and D.A. Agard, *How general is the nucleation-condensation mechanism?* Proteins, 2008. **73**(3): p. 754-64.
6. Udgaonkar, J.B. and R.L. Baldwin, *NMR evidence for an early framework intermediate on the folding pathway of ribonuclease A*. Nature, 1988. **335**(6192): p. 694-9.
7. Wright, P.E., H.J. Dyson, and R.A. Lerner, *Conformation of peptide fragments of proteins in aqueous solution: implications for initiation of protein folding*. Biochemistry, 1988. **27**(19): p. 7167-75.
8. Hausrath, A.C., *A kinetic theory of tertiary contact formation coupled to the helix-coil transition in polypeptides*. J Chem Phys, 2006. **125**(8): p. 084909.
9. Arai, M., et al., *Microsecond hydrophobic collapse in the folding of Escherichia coli dihydrofolate reductase, an alpha/beta-type protein*. J Mol Biol, 2007. **368**(1): p. 219-29.
10. Ellis, R.J., *The general concept of molecular chaperones*. Philos Trans R Soc Lond B Biol Sci, 1993. **339**(1289): p. 257-61.
11. Hartl, F.U., A. Bracher, and M. Hayer-Hartl, *Molecular chaperones in protein folding and proteostasis*. Nature, 2011. **475**(7356): p. 324-32.
12. Houben, B., F. Rousseau, and J. Schymkowitz, *Protein structure and aggregation: a marriage of necessity ruled by aggregation gatekeepers*. Trends Biochem Sci, 2022. **47**(3): p. 194-205.
13. Castanie-Cornet, M.P., N. Bruel, and P. Genevaux, *Chaperone networking facilitates protein targeting to the bacterial cytoplasmic membrane*. Biochim Biophys Acta, 2014. **1843**(8): p. 1442-56.
14. Hartl, F.U., *Molecular chaperones in cellular protein folding*. Nature, 1996. **381**(6583): p. 571-9.
15. Hartl, F.U. and M. Hayer-Hartl, *Converging concepts of protein folding in vitro and in vivo*. Nat Struct Mol Biol, 2009. **16**(6): p. 574-81.
16. Veinger, L., et al., *The small heat-shock protein IbpB from Escherichia coli stabilizes stress-denatured proteins for subsequent refolding by a multichaperone network*. J Biol Chem, 1998. **273**(18): p. 11032-7.
17. Mayer, M.P., *Gymnastics of molecular chaperones*. Mol Cell, 2010. **39**(3): p. 321-31.
18. Xu, Z., A.L. Horwich, and P.B. Sigler, *The crystal structure of the asymmetric GroEL-GroES-(ADP)7 chaperonin complex*. Nature, 1997. **388**(6644): p. 741-50.
19. Hayer-Hartl, M., A. Bracher, and F.U. Hartl, *The GroEL-GroES Chaperonin Machine: A Nano-Cage for Protein Folding*. Trends Biochem Sci, 2016. **41**(1): p. 62-76.
20. Langer, T., et al., *Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding*. Nature, 1992. **356**(6371): p. 683-9.
21. Frydman, J., et al., *Folding of nascent polypeptide chains in a high molecular mass assembly with molecular chaperones*. Nature, 1994. **370**(6485): p. 111-7.

22. Lee, S., et al., *The structure of ClpB: a molecular chaperone that rescues proteins from an aggregated state*. Cell, 2003. **115**(2): p. 229-40.
23. Haslbeck, M., S. Weinkauf, and J. Buchner, *Small heat shock proteins: Simplicity meets complexity*. J Biol Chem, 2019. **294**(6): p. 2121-2132.
24. Ehrnsperger, M., et al., *Stabilization of proteins and peptides in diagnostic immunological assays by the molecular chaperone Hsp25*. Anal Biochem, 1998. **259**(2): p. 218-25.
25. Zwirowski, S., et al., *Hsp70 displaces small heat shock proteins from aggregates to initiate protein refolding*. EMBO J, 2017. **36**(6): p. 783-796.
26. de Marco, A., et al., *Chaperone-based procedure to increase yields of soluble recombinant proteins produced in E. coli*. BMC Biotechnol, 2007. **7**: p. 32.
27. Stull, F., J.M. Betton, and J.C.A. Bardwell, *Periplasmic Chaperones and Prolyl Isomerases*. EcoSal Plus, 2018. **8**(1).
28. De Geyter, J., et al., *Protein folding in the cell envelope of Escherichia coli*. Nat Microbiol, 2016. **1**(8): p. 16107.
29. Schiffrin, B., et al., *Skp is a multivalent chaperone of outer-membrane proteins*. Nat Struct Mol Biol, 2016. **23**(9): p. 786-793.
30. Schiffrin, B., et al., *Dynamic interplay between the periplasmic chaperone SurA and the BAM complex in outer membrane protein folding*. Commun Biol, 2022. **5**(1): p. 560.
31. Hiller, S., *Chaperone-Bound Clients: The Importance of Being Dynamic*. Trends Biochem Sci, 2019. **44**(6): p. 517-527.
32. Chum, A.P., et al., *Plasticity and transient binding are key ingredients of the periplasmic chaperone network*. Protein Sci, 2019. **28**(7): p. 1340-1349.
33. Jauss, B., et al., *Noncompetitive binding of PpiD and YidC to the SecYEG translocon expands the global view on the SecYEG interactome in Escherichia coli*. J Biol Chem, 2019. **294**(50): p. 19167-19183.
34. Gotzke, H., et al., *YfgM is an ancillary subunit of the SecYEG translocon in Escherichia coli*. J Biol Chem, 2014. **289**(27): p. 19089-97.
35. Kinsella, R.L., et al., *Defining the interaction of the protease CpaA with its type II secretion chaperone CpaB and its contribution to virulence in Acinetobacter species*. J Biol Chem, 2017. **292**(48): p. 19628-19638.
36. Rosenau, F., J. Tommassen, and K.E. Jaeger, *Lipase-specific foldases*. Chembiochem, 2004. **5**(2): p. 152-61.
37. Hobson, A.H., et al., *Activation of a bacterial lipase by its chaperone*. Proc Natl Acad Sci U S A, 1993. **90**(12): p. 5682-6.
38. Nishiyama, M., et al., *Structural basis of chaperone-subunit complex recognition by the type I pilus assembly platform FimD*. EMBO J, 2005. **24**(12): p. 2075-86.
39. Vetsch, M., P. Sebbel, and R. Glockshuber, *Chaperone-independent folding of type I pilus domains*. J Mol Biol, 2002. **322**(4): p. 827-40.
40. Pauwels, K., et al., *Chaperoning Anfinsen: the steric foldases*. Mol Microbiol, 2007. **64**(4): p. 917-22.
41. Diggle, S.P. and M. Whiteley, *Microbe Profile: Pseudomonas aeruginosa: opportunistic pathogen and lab rat*. Microbiology (Reading), 2020. **166**(1): p. 30-33.
42. Letizia, M., S.P. Diggle, and M. Whiteley, *Pseudomonas aeruginosa: ecology, evolution, pathogenesis and antimicrobial susceptibility*. Nat Rev Microbiol, 2025. **23**(11): p. 701-717.
43. de Bentzmann, S. and P. Plesiat, *The Pseudomonas aeruginosa opportunistic pathogen and human infections*. Environ Microbiol, 2011. **13**(7): p. 1655-65.
44. Strateva, T. and D. Yordanov, *Pseudomonas aeruginosa - a phenomenon of bacterial resistance*. J Med Microbiol, 2009. **58**(Pt 9): p. 1133-1148.

45. Sadikot, R.T., et al., *Pathogen-host interactions in Pseudomonas aeruginosa pneumonia*. Am J Respir Crit Care Med, 2005. **171**(11): p. 1209-23.
46. Bhagirath, A.Y., et al., *Cystic fibrosis lung environment and Pseudomonas aeruginosa infection*. BMC Pulm Med, 2016. **16**(1): p. 174.
47. Freschi, L., et al., *The Pseudomonas aeruginosa Pan-Genome Provides New Insights on Its Population Structure, Horizontal Gene Transfer, and Pathogenicity*. Genome Biol Evol, 2019. **11**(1): p. 109-120.
48. Chandler, C.E., et al., *Genomic and Phenotypic Diversity among Ten Laboratory Isolates of Pseudomonas aeruginosa PAOI*. J Bacteriol, 2019. **201**(5).
49. Friedman, L. and R. Kolter, *Genes involved in matrix formation in Pseudomonas aeruginosa PA14 biofilms*. Mol Microbiol, 2004. **51**(3): p. 675-90.
50. Mathee, K., *Forensic investigation into the origin of Pseudomonas aeruginosa PA14 - old but not lost*. J Med Microbiol, 2018. **67**(8): p. 1019-1021.
51. Mikkelsen, H., R. McMullan, and A. Filloux, *The Pseudomonas aeruginosa reference strain PA14 displays increased virulence due to a mutation in ladS*. PLoS One, 2011. **6**(12): p. e29113.
52. Stover, C.K., et al., *Complete genome sequence of Pseudomonas aeruginosa PAOI, an opportunistic pathogen*. Nature, 2000. **406**(6799): p. 959-64.
53. Bleves, S., et al., *Protein secretion systems in Pseudomonas aeruginosa: A wealth of pathogenic weapons*. Int J Med Microbiol, 2010. **300**(8): p. 534-43.
54. Filloux, A., *Protein Secretion Systems in Pseudomonas aeruginosa: An Essay on Diversity, Evolution, and Function*. Front Microbiol, 2011. **2**: p. 155.
55. Qin, S., et al., *Pseudomonas aeruginosa: pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics*. Signal Transduct Target Ther, 2022. **7**(1): p. 199.
56. Strateva, T. and I. Mitov, *Contribution of an arsenal of virulence factors to pathogenesis of infections*. Annals of Microbiology, 2011. **61**(4): p. 717-732.
57. Rice, L.B., *Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE*. J Infect Dis, 2008. **197**(8): p. 1079-81.
58. Driscoll, J.A., S.L. Brody, and M.H. Kollef, *The epidemiology, pathogenesis and treatment of Pseudomonas aeruginosa infections*. Drugs, 2007. **67**(3): p. 351-68.
59. Tacconelli, E., et al., *Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis*. Lancet Infect Dis, 2018. **18**(3): p. 318-327.
60. Mulani, M.S., et al., *Emerging Strategies to Combat ESKAPE Pathogens in the Era of Antimicrobial Resistance: A Review*. Front Microbiol, 2019. **10**: p. 539.
61. Jamali, H., et al., *Bacterial protein secretion systems: Mechanisms, functions, and roles in virulence*. Microb Pathog, 2025. **206**: p. 107790.
62. Costa, T.R., et al., *Secretion systems in Gram-negative bacteria: structural and mechanistic insights*. Nat Rev Microbiol, 2015. **13**(6): p. 343-59.
63. Overly Cottom, C., et al., *Surface protein machineries in Gram-negative bacteria*. J Cell Sci, 2025. **138**(18).
64. Burdette, L.A., et al., *Developing Gram-negative bacteria for the secretion of heterologous proteins*. Microb Cell Fact, 2018. **17**(1): p. 196.
65. Diard, M. and W.D. Hardt, *Evolution of bacterial virulence*. FEMS Microbiol Rev, 2017. **41**(5): p. 679-697.
66. Casadevall, A. and L.A. Pirofski, *Virulence factors and their mechanisms of action: the view from a damage-response framework*. J Water Health, 2009. **7 Suppl 1**: p. S2-S18.
67. Wu, H.J., A.H. Wang, and M.P. Jennings, *Discovery of virulence factors of pathogenic bacteria*. Curr Opin Chem Biol, 2008. **12**(1): p. 93-101.

68. Konig, B., et al., *Role of Pseudomonas aeruginosa lipase in inflammatory mediator release from human inflammatory effector cells (platelets, granulocytes, and monocytes)*. Infect Immun, 1996. **64**(8): p. 3252-8.
69. Tielen, P., et al., *Interaction between extracellular lipase LipA and the polysaccharide alginate of Pseudomonas aeruginosa*. BMC Microbiol, 2013. **13**: p. 159.
70. Stehr, F., et al., *Microbial lipases as virulence factors*. Journal of Molecular Catalysis B-Enzymatic, 2003. **22**(5-6): p. 347-355.
71. Chihara-Siomi, M., et al., *Purification, molecular cloning, and expression of lipase from Pseudomonas aeruginosa*. Arch Biochem Biophys, 1992. **296**(2): p. 505-13.
72. Rosenau, F. and K. Jaeger, *Bacterial lipases from Pseudomonas: regulation of gene expression and mechanisms of secretion*. Biochimie, 2000. **82**(11): p. 1023-32.
73. El Khattabi, M., et al., *Role of the lipase-specific foldase of Burkholderia glumae as a steric chaperone*. J Biol Chem, 2000. **275**(35): p. 26885-91.
74. Khan, F.I., et al., *The Lid Domain in Lipases: Structural and Functional Determinant of Enzymatic Properties*. Front Bioeng Biotechnol, 2017. **5**: p. 16.
75. Papadopoulos, A., et al., *The periplasmic chaperone Skp prevents misfolding of the secretory lipase A from Pseudomonas aeruginosa*. Front Mol Biosci, 2022. **9**: p. 1026724.
76. Wretling, B. and O.R. Pavlovskis, *Genetic mapping and characterization of Pseudomonas aeruginosa mutants defective in the formation of extracellular proteins*. J Bacteriol, 1984. **158**(3): p. 801-8.
77. Naskar, S., et al., *The structure and mechanism of the bacterial type II secretion system*. Mol Microbiol, 2021. **115**(3): p. 412-424.
78. El Khattabi, M., et al., *Specificity of the lipase-specific foldases of gram-negative bacteria and the role of the membrane anchor*. Mol Gen Genet, 1999. **261**(4-5): p. 770-6.
79. Douzi, B., et al., *Deciphering the Xcp Pseudomonas aeruginosa type II secretion machinery through multiple interactions with substrates*. J Biol Chem, 2011. **286**(47): p. 40792-801.
80. Nardini, M., et al., *Crystal structure of pseudomonas aeruginosa lipase in the open conformation. The prototype for family I.1 of bacterial lipases*. J Biol Chem, 2000. **275**(40): p. 31219-25.
81. Ollis, D.L., et al., *The alpha/beta hydrolase fold*. Protein Eng, 1992. **5**(3): p. 197-211.
82. Rashno, F., et al., *Insight into the aggregation of lipase from Pseudomonas sp. using mutagenesis: protection of aggregation prone region by adoption of alpha-helix structure*. Protein Eng Des Sel, 2018. **31**(11): p. 419-426.
83. Liebeton, K., A. Zacharias, and K.E. Jaeger, *Disulfide bond in Pseudomonas aeruginosa lipase stabilizes the structure but is not required for interaction with its foldase*. J Bacteriol, 2001. **183**(2): p. 597-603.
84. Verma, N., et al., *The Membrane-Integrated Steric Chaperone Lif Facilitates Active Site Opening of Pseudomonas aeruginosa Lipase A*. J Comput Chem, 2020. **41**(6): p. 500-512.
85. Frenken, L.G., et al., *An accessory gene, lipB, required for the production of active Pseudomonas glumae lipase*. Mol Microbiol, 1993. **9**(3): p. 579-89.
86. Ellis, R.J., *Steric chaperones*. Trends Biochem Sci, 1998. **23**(2): p. 43-5.
87. Pauwels, K., et al., *Crystallization and crystal manipulation of a steric chaperone in complex with its lipase substrate*. Acta Crystallogr Sect F Struct Biol Cryst Commun, 2005. **61**(Pt 8): p. 791-5.
88. Pauwels, K., et al., *Structure of a membrane-based steric chaperone in complex with its lipase substrate*. Nat Struct Mol Biol, 2006. **13**(4): p. 374-5.

89. de Oliveira Silva, Y.R., et al., *Architecture of an embracing lipase-foldase complex of the type II secretion system of Acinetobacter baumannii*. Structure, 2025. **33**(3): p. 601-612 e4.
90. Williamson, M.P., *The Structure and Function of Proline-Rich Regions in Proteins*. Biochemical Journal, 1994. **297**: p. 249-260.
91. Dittrich, J., et al., *Resolution of Maximum Entropy Method-Derived Posterior Conformational Ensembles of a Flexible System Probed by FRET and Molecular Dynamics Simulations*. J Chem Theory Comput, 2023. **19**(8): p. 2389-2409.
92. Viegas, A., et al., *Structural and dynamic insights revealing how lipase binding domain MD1 of Pseudomonas aeruginosa foldase affects lipase activation*. Sci Rep, 2020. **10**(1): p. 3578.
93. Jaeger, K.E., et al., *Bacterial lipases*. FEMS Microbiol Rev, 1994. **15**(1): p. 29-63.
94. Stephen White, G.v.H., Donald Engelman, *Cell Boundaries: How Membranes and Their Proteins Work (1st ed.)*. 2021: Garland Science.
95. von Heijne, G., *The membrane protein universe: what's out there and why bother?* J Intern Med, 2007. **261**(6): p. 543-57.
96. Mercier, E., et al., *Cotranslational Biogenesis of Membrane Proteins in Bacteria*. Front Mol Biosci, 2022. **9**: p. 871121.
97. Konovalova, A., D.E. Kahne, and T.J. Silhavy, *Outer Membrane Biogenesis*. Annu Rev Microbiol, 2017. **71**: p. 539-556.
98. Cymer, F., G. von Heijne, and S.H. White, *Mechanisms of integral membrane protein insertion and folding*. J Mol Biol, 2015. **427**(5): p. 999-1022.
99. Koebnik, R., K.P. Locher, and P. Van Gelder, *Structure and function of bacterial outer membrane proteins: barrels in a nutshell*. Mol Microbiol, 2000. **37**(2): p. 239-53.
100. Veenendaal, A.K., C. van der Does, and A.J. Driessen, *The protein-conducting channel SecYEG*. Biochim Biophys Acta, 2004. **1694**(1-3): p. 81-95.
101. Akimaru, J., et al., *Reconstitution of a protein translocation system containing purified SecY, SecE, and SecA from Escherichia coli*. Proc Natl Acad Sci U S A, 1991. **88**(15): p. 6545-9.
102. Asakura, Y., et al., *Maize mutants lacking chloroplast FtsY exhibit pleiotropic defects in the biogenesis of thylakoid membranes*. Plant Cell, 2004. **16**(1): p. 201-14.
103. Mori, H. and K. Cline, *Post-translational protein translocation into thylakoids by the Sec and DeltapH-dependent pathways*. Biochim Biophys Acta, 2001. **1541**(1-2): p. 80-90.
104. Helmers, J., et al., *The beta-subunit of the protein-conducting channel of the endoplasmic reticulum functions as the guanine nucleotide exchange factor for the beta-subunit of the signal recognition particle receptor*. J Biol Chem, 2003. **278**(26): p. 23686-90.
105. du Plessis, D.J., et al., *The lateral gate of SecYEG opens during protein translocation*. J Biol Chem, 2009. **284**(23): p. 15805-14.
106. Sachelaru, I., et al., *YidC occupies the lateral gate of the SecYEG translocon and is sequentially displaced by a nascent membrane protein*. J Biol Chem, 2013. **288**(23): p. 16295-16307.
107. Botte, M., et al., *A central cavity within the holo-translocon suggests a mechanism for membrane protein insertion*. Sci Rep, 2016. **6**: p. 38399.
108. Petriman, N.A., et al., *The interaction network of the YidC insertase with the SecYEG translocon, SRP and the SRP receptor FtsY*. Sci Rep, 2018. **8**(1): p. 578.
109. Smalinskaite, L., et al., *Mechanism of an intramembrane chaperone for multipass membrane proteins*. Nature, 2022. **611**(7934): p. 161-166.
110. Sundaram, A., et al., *Substrate-driven assembly of a translocon for multipass membrane proteins*. Nature, 2022. **611**(7934): p. 167-172.

111. Sundaram, A., et al., *Global analysis of translocon remodeling during protein synthesis at the ER*. Nat Struct Mol Biol, 2025. **32**(12): p. 2517-2525.
112. Yang, T.J., et al., *SND3 is the membrane insertase within a distinct SEC61 translocon complex*. Nat Commun, 2025. **16**(1): p. 9566.
113. du Plessis, D.J., N. Nouwen, and A.J. Driessen, *The Sec translocase*. Biochim Biophys Acta, 2011. **1808**(3): p. 851-65.
114. Lycklama, A.N.J.A. and A.J. Driessen, *The bacterial Sec-translocase: structure and mechanism*. Philos Trans R Soc Lond B Biol Sci, 2012. **367**(1592): p. 1016-28.
115. Kater, L., et al., *Partially inserted nascent chain unzips the lateral gate of the Sec translocon*. EMBO Rep, 2019. **20**(10): p. e48191.
116. Driessen, A.J., *How proteins cross the bacterial cytoplasmic membrane*. J Membr Biol, 1994. **142**(2): p. 145-59.
117. Van den Berg, B., et al., *X-ray structure of a protein-conducting channel*. Nature, 2004. **427**(6969): p. 36-44.
118. Tanaka, Y., et al., *Crystal Structures of SecYEG in Lipidic Cubic Phase Elucidate a Precise Resting and a Peptide-Bound State*. Cell Rep, 2015. **13**(8): p. 1561-8.
119. Zimmer, J., Y. Nam, and T.A. Rapoport, *Structure of a complex of the ATPase SecA and the protein-translocation channel*. Nature, 2008. **455**(7215): p. 936-43.
120. Frauenfeld, J., et al., *Cryo-EM structure of the ribosome-SecYE complex in the membrane environment*. Nat Struct Mol Biol, 2011. **18**(5): p. 614-21.
121. Ma, C., et al., *Structure of the substrate-engaged SecA-SecY protein translocation machine*. Nat Commun, 2019. **10**(1): p. 2872.
122. Mori, H. and K. Ito, *Different modes of SecY-SecA interactions revealed by site-directed in vivo photo-cross-linking*. Proc Natl Acad Sci U S A, 2006. **103**(44): p. 16159-64.
123. Das, S. and D.B. Oliver, *Mapping of the SecA.SecY and SecA.SecG interfaces by site-directed in vivo photocross-linking*. J Biol Chem, 2011. **286**(14): p. 12371-80.
124. Kuhn, P., et al., *The bacterial SRP receptor, SecA and the ribosome use overlapping binding sites on the SecY translocon*. Traffic, 2011. **12**(5): p. 563-78.
125. Oswald, J., et al., *The Dynamic SecYEG Translocon*. Front Mol Biosci, 2021. **8**: p. 664241.
126. Park, E. and T.A. Rapoport, *Mechanisms of Sec61/SecY-mediated protein translocation across membranes*. Annu Rev Biophys, 2012. **41**: p. 21-40.
127. Lycklama, A.N.J.A., Z.C. Wu, and A.J.M. Driessen, *Conformational dynamics of the plug domain of the SecYEG protein-conducting channel*. J Biol Chem, 2011. **286**(51): p. 43881-43890.
128. Dong, L., et al., *Structural basis of SecA-mediated protein translocation*. Proc Natl Acad Sci U S A, 2023. **120**(2): p. e2208070120.
129. Lycklama a Nijeholt, J.A., et al., *Characterization of the supporting role of SecE in protein translocation*. FEBS Lett, 2013. **587**(18): p. 3083-8.
130. Kihara, A., Y. Akiyama, and K. Ito, *FtsH is required for proteolytic elimination of uncomplexed forms of SecY, an essential protein translocase subunit*. Proc Natl Acad Sci U S A, 1995. **92**(10): p. 4532-6.
131. Brundage, L., et al., *The purified E. coli integral membrane protein SecY/E is sufficient for reconstitution of SecA-dependent precursor protein translocation*. Cell, 1990. **62**(4): p. 649-57.
132. Flower, A.M., L.L. Hines, and P.L. Pfennig, *SecG is an auxiliary component of the protein export apparatus of Escherichia coli*. Mol Gen Genet, 2000. **263**(1): p. 131-6.
133. Flower, A.M., *SecG function and phospholipid metabolism in Escherichia coli*. J Bacteriol, 2001. **183**(6): p. 2006-12.
134. Nishiyama, K., M. Hanada, and H. Tokuda, *Disruption of the gene encoding p12 (SecG) reveals the direct involvement and important function of SecG in the protein*

- translocation of Escherichia coli at low temperature*. EMBO J, 1994. **13**(14): p. 3272-7.
135. Kontinen, V.P., I.M. Helander, and H. Tokuda, *The secG deletion mutation of Escherichia coli is suppressed by expression of a novel regulatory gene of Bacillus subtilis*. FEBS Lett, 1996. **389**(3): p. 281-4.
 136. Suzuki, H., K. Nishiyama, and H. Tokuda, *Increases in acidic phospholipid contents specifically restore protein translocation in a cold-sensitive secA or secG null mutant*. J Biol Chem, 1999. **274**(43): p. 31020-4.
 137. Nishiyama, K., S. Mizushima, and H. Tokuda, *A novel membrane protein involved in protein translocation across the cytoplasmic membrane of Escherichia coli*. EMBO J, 1993. **12**(9): p. 3409-15.
 138. Hanada, M., K. Nishiyama, and H. Tokuda, *SecG plays a critical role in protein translocation in the absence of the proton motive force as well as at low temperature*. FEBS Lett, 1996. **381**(1-2): p. 25-8.
 139. Hennon, S.W., et al., *YidC/Alb3/Oxa1 Family of Insertases*. J Biol Chem, 2015. **290**(24): p. 14866-74.
 140. Wang, P. and R.E. Dalbey, *Inserting membrane proteins: the YidC/Oxa1/Alb3 machinery in bacteria, mitochondria, and chloroplasts*. Biochim Biophys Acta, 2011. **1808**(3): p. 866-75.
 141. Anghel, S.A., et al., *Identification of Oxa1 Homologs Operating in the Eukaryotic Endoplasmic Reticulum*. Cell Rep, 2017. **21**(13): p. 3708-3716.
 142. Dalbey, R.E., et al., *The membrane insertase YidC*. Biochim Biophys Acta, 2014. **1843**(8): p. 1489-96.
 143. Steinberg, R., et al., *Co-translational protein targeting in bacteria*. FEMS Microbiol Lett, 2018. **365**(11).
 144. McDowell, M.A., M. Heimes, and I. Sinning, *Structural and molecular mechanisms for membrane protein biogenesis by the Oxa1 superfamily*. Nat Struct Mol Biol, 2021. **28**(3): p. 234-239.
 145. Urbanus, M.L., et al., *Targeting, insertion, and localization of Escherichia coli YidC*. J Biol Chem, 2002. **277**(15): p. 12718-23.
 146. Samuelson, J.C., et al., *YidC mediates membrane protein insertion in bacteria*. Nature, 2000. **406**(6796): p. 637-41.
 147. Chen, Y., et al., *YidC Insertase of Escherichia coli: Water Accessibility and Membrane Shaping*. Structure, 2017. **25**(9): p. 1403-1414 e3.
 148. Kumazaki, K., et al., *Crystal structure of Escherichia coli YidC, a membrane protein chaperone and insertase*. Sci Rep, 2014. **4**(1): p. 7299.
 149. Klenner, C. and A. Kuhn, *Dynamic disulfide scanning of the membrane-inserting Pf3 coat protein reveals multiple YidC substrate contacts*. J Biol Chem, 2012. **287**(6): p. 3769-76.
 150. Jiang, F., et al., *Defining the regions of Escherichia coli YidC that contribute to activity*. J Biol Chem, 2003. **278**(49): p. 48965-72.
 151. Chen, Y., et al., *The role of the strictly conserved positively charged residue differs among the Gram-positive, Gram-negative, and chloroplast YidC homologs*. J Biol Chem, 2014. **289**(51): p. 35656-67.
 152. Wickles, S., et al., *A structural model of the active ribosome-bound membrane protein insertase YidC*. Elife, 2014. **3**: p. e03035.
 153. Geng, Y., et al., *Role of the Cytosolic Loop C2 and the C Terminus of YidC in Ribosome Binding and Insertion Activity*. J Biol Chem, 2015. **290**(28): p. 17250-61.
 154. Seitzl, I., et al., *The C-terminal regions of YidC from Rhodospirillum rubrum and Oceanicaulis alexandrii bind to ribosomes and partially substitute for SRP receptor function in Escherichia coli*. Mol Microbiol, 2014. **91**(2): p. 408-21.

155. Kedrov, A., et al., *Elucidating the native architecture of the YidC: ribosome complex*. J Mol Biol, 2013. **425**(22): p. 4112-24.
156. Denks, K., et al., *The Sec translocon mediated protein transport in prokaryotes and eukaryotes*. Mol Membr Biol, 2014. **31**(2-3): p. 58-84.
157. Rapoport, T.A., *Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes*. Nature, 2007. **450**(7170): p. 663-9.
158. Koch, H.G., M. Moser, and M. Muller, *Signal recognition particle-dependent protein targeting, universal to all kingdoms of life*. Rev Physiol Biochem Pharmacol, 2003. **146**: p. 55-94.
159. Driessen, A.J. and N. Nouwen, *Protein translocation across the bacterial cytoplasmic membrane*. Annu Rev Biochem, 2008. **77**: p. 643-67.
160. Smets, D., et al., *Protein Transport Across the Bacterial Plasma Membrane by the Sec Pathway*. Protein J, 2019. **38**(3): p. 262-273.
161. Owji, H., et al., *A comprehensive review of signal peptides: Structure, roles, and applications*. Eur J Cell Biol, 2018. **97**(6): p. 422-441.
162. Bornemann, T., W. Holtkamp, and W. Wintermeyer, *Interplay between trigger factor and other protein biogenesis factors on the ribosome*. Nat Commun, 2014. **5**: p. 4180.
163. Hartl, F.U., et al., *The binding cascade of SecB to SecA to SecY/E mediates preprotein targeting to the E. coli plasma membrane*. Cell, 1990. **63**(2): p. 269-79.
164. Komarudin, A.G. and A.J.M. Driessen, *SecA-Mediated Protein Translocation through the SecYEG Channel*. Microbiol Spectr, 2019. **7**(4).
165. Chatzi, K.E., et al., *SecA-mediated targeting and translocation of secretory proteins*. Biochim Biophys Acta, 2014. **1843**(8): p. 1466-74.
166. Chatzi, K.E., et al., *Preprotein mature domains contain translocase targeting signals that are essential for secretion*. J Cell Biol, 2017. **216**(5): p. 1357-1369.
167. De Geyter, J., et al., *Trigger factor is a bona fide secretory pathway chaperone that interacts with SecB and the translocase*. EMBO Rep, 2020. **21**(6): p. e49054.
168. Karamyshev, A.L. and A.E. Johnson, *Selective SecA association with signal sequences in ribosome-bound nascent chains: a potential role for SecA in ribosome targeting to the bacterial membrane*. J Biol Chem, 2005. **280**(45): p. 37930-40.
169. Kaushik, S., H. He, and R.E. Dalbey, *Bacterial Signal Peptides- Navigating the Journey of Proteins*. Front Physiol, 2022. **13**: p. 933153.
170. Heijne, G., *The distribution of positively charged residues in bacterial inner membrane proteins correlates with the trans-membrane topology*. EMBO J, 1986. **5**(11): p. 3021-7.
171. Perlman, D. and H.O. Halvorson, *A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides*. J Mol Biol, 1983. **167**(2): p. 391-409.
172. von Heijne, G. and L. Abrahmsen, *Species-specific variation in signal peptide design. Implications for protein secretion in foreign hosts*. FEBS Lett, 1989. **244**(2): p. 439-46.
173. Hegde, R.S. and H.D. Bernstein, *The surprising complexity of signal sequences*. Trends Biochem Sci, 2006. **31**(10): p. 563-71.
174. Rusch, S.L. and D.A. Kendall, *Interactions that drive Sec-dependent bacterial protein transport*. Biochemistry, 2007. **46**(34): p. 9665-73.
175. Valent, Q.A., et al., *Early events in preprotein recognition in E. coli: interaction of SRP and trigger factor with nascent polypeptides*. EMBO J, 1995. **14**(22): p. 5494-505.
176. Schlunzen, F., et al., *The binding mode of the trigger factor on the ribosome: implications for protein folding and SRP interaction*. Structure, 2005. **13**(11): p. 1685-94.

177. Liu, C.P., S. Perrett, and J.M. Zhou, *Dimeric trigger factor stably binds folding-competent intermediates and cooperates with the DnaK-DnaJ-GrpE chaperone system to allow refolding*. J Biol Chem, 2005. **280**(14): p. 13315-20.
178. Hoffmann, A., B. Bukau, and G. Kramer, *Structure and function of the molecular chaperone Trigger Factor*. Biochim Biophys Acta, 2010. **1803**(6): p. 650-61.
179. Saio, T., et al., *Structural basis for protein antiaggregation activity of the trigger factor chaperone*. Science, 2014. **344**(6184): p. 1250494.
180. Crooke, E. and W. Wickner, *Trigger factor: a soluble protein that folds pro-OmpA into a membrane-assembly-competent form*. Proc Natl Acad Sci U S A, 1987. **84**(15): p. 5216-20.
181. Deuerling, E., et al., *Trigger factor and DnaK cooperate in folding of newly synthesized proteins*. Nature, 1999. **400**(6745): p. 693-6.
182. Teter, S.A., et al., *Polypeptide flux through bacterial Hsp70: DnaK cooperates with trigger factor in chaperoning nascent chains*. Cell, 1999. **97**(6): p. 755-65.
183. Kandror, O., et al., *Trigger factor is involved in GroEL-dependent protein degradation in Escherichia coli and promotes binding of GroEL to unfolded proteins*. EMBO J, 1995. **14**(23): p. 6021-7.
184. Deuerling, E., et al., *Trigger Factor and DnaK possess overlapping substrate pools and binding specificities*. Mol Microbiol, 2003. **47**(5): p. 1317-28.
185. Ullers, R.S., et al., *Trigger Factor can antagonize both SecB and DnaK/DnaJ chaperone functions in Escherichia coli*. Proc Natl Acad Sci U S A, 2007. **104**(9): p. 3101-6.
186. Mayer, M.P., *The Hsp70-Chaperone Machines in Bacteria*. Front Mol Biosci, 2021. **8**: p. 694012.
187. Calloni, G., et al., *DnaK functions as a central hub in the E. coli chaperone network*. Cell Rep, 2012. **1**(3): p. 251-64.
188. Ferbitz, L., et al., *Trigger factor in complex with the ribosome forms a molecular cradle for nascent proteins*. Nature, 2004. **431**(7008): p. 590-6.
189. Schulze-Gahmen, U., et al., *Structure of the hypothetical Mycoplasma protein MPN555 suggests a chaperone function*. Acta Crystallogr D Biol Crystallogr, 2005. **61**(Pt 10): p. 1343-7.
190. Bechtluft, P., et al., *SecB--a chaperone dedicated to protein translocation*. Mol Biosyst, 2010. **6**(4): p. 620-7.
191. Fekkes, P., et al., *Preprotein transfer to the Escherichia coli translocase requires the co-operative binding of SecB and the signal sequence to SecA*. Mol Microbiol, 1998. **29**(5): p. 1179-90.
192. Bechtluft, P., et al., *Tight hydrophobic contacts with the SecB chaperone prevent folding of substrate proteins*. Biochemistry, 2010. **49**(11): p. 2380-8.
193. Dekker, C., B. de Kruijff, and P. Gros, *Crystal structure of SecB from Escherichia coli*. J Struct Biol, 2003. **144**(3): p. 313-9.
194. Huang, C., et al., *Structural basis for the antifolding activity of a molecular chaperone*. Nature, 2016. **537**(7619): p. 202-206.
195. Randall, L.L. and M.T. Henzl, *Direct identification of the site of binding on the chaperone SecB for the amino terminus of the translocon motor SecA*. Protein Sci, 2010. **19**(6): p. 1173-9.
196. Randall, L.L. and S.J. Hardy, *High selectivity with low specificity: how SecB has solved the paradox of chaperone binding*. Trends Biochem Sci, 1995. **20**(2): p. 65-9.
197. Kumamoto, C.A. and O. Francetic, *Highly selective binding of nascent polypeptides by an Escherichia coli chaperone protein in vivo*. J Bacteriol, 1993. **175**(8): p. 2184-8.

198. Randall, L.L., et al., *Binding of SecB to ribosome-bound polypeptides has the same characteristics as binding to full-length, denatured proteins*. Proc Natl Acad Sci U S A, 1997. **94**(3): p. 802-7.
199. Fekkes, P., T. den Blaauwen, and A.J. Driessen, *Diffusion-limited interaction between unfolded polypeptides and the Escherichia coli chaperone SecB*. Biochemistry, 1995. **34**(31): p. 10078-85.
200. Zahn, R., S. Perrett, and A.R. Fersht, *Conformational states bound by the molecular chaperones GroEL and secB: a hidden unfolding (annealing) activity*. J Mol Biol, 1996. **261**(1): p. 43-61.
201. Topping, T.B. and L.L. Randall, *Determination of the binding frame within a physiological ligand for the chaperone SecB*. Protein Sci, 1994. **3**(5): p. 730-6.
202. Baars, L., et al., *Defining the role of the Escherichia coli chaperone SecB using comparative proteomics*. J Biol Chem, 2006. **281**(15): p. 10024-34.
203. Eismann, L., et al., *Selective ribosome profiling reveals a role for SecB in the co-translational inner membrane protein biogenesis*. Cell Rep, 2022. **41**(10): p. 111776.
204. Bauer, B.W., et al., *A "push and slide" mechanism allows sequence-insensitive translocation of secretory proteins by the SecA ATPase*. Cell, 2014. **157**(6): p. 1416-1429.
205. Koonin, E.V. and A.E. Gorbalenya, *Autogenous translation regulation by Escherichia coli ATPase SecA may be mediated by an intrinsic RNA helicase activity of this protein*. FEBS Lett, 1992. **298**(1): p. 6-8.
206. Nithianantham, S. and B.H. Shilton, *Analysis of the isolated SecA DEAD motor suggests a mechanism for chemical-mechanical coupling*. J Mol Biol, 2008. **383**(2): p. 380-9.
207. Ding, H., I. Mukerji, and D. Oliver, *Nucleotide and phospholipid-dependent control of PPXD and C-domain association for SecA ATPase*. Biochemistry, 2003. **42**(46): p. 13468-75.
208. Bauer, B.W. and T.A. Rapoport, *Mapping polypeptide interactions of the SecA ATPase during translocation*. Proc Natl Acad Sci U S A, 2009. **106**(49): p. 20800-5.
209. Bhanu, M.K., P. Zhao, and D.A. Kendall, *Mapping of the SecA signal peptide binding site and dimeric interface by using the substituted cysteine accessibility method*. J Bacteriol, 2013. **195**(20): p. 4709-15.
210. Papanikolau, Y., et al., *Structure of dimeric SecA, the Escherichia coli preprotein translocase motor*. J Mol Biol, 2007. **366**(5): p. 1545-57.
211. Ernst, I., et al., *Large conformational changes of a highly dynamic pre-protein binding domain in SecA*. Commun Biol, 2018. **1**: p. 130.
212. Catipovic, M.A., et al., *Protein translocation by the SecA ATPase occurs by a power-stroke mechanism*. EMBO J, 2019. **38**(9).
213. Allen, W.J., et al., *Two-way communication between SecY and SecA suggests a Brownian ratchet mechanism for protein translocation*. Elife, 2016. **5**.
214. Corey, R.A., et al., *ATP-induced asymmetric pre-protein folding as a driver of protein translocation through the Sec machinery*. Elife, 2019. **8**.
215. Fessl, T., et al., *Dynamic action of the Sec machinery during initiation, protein translocation and termination*. Elife, 2018. **7**.
216. Krishnamurthy, S., et al., *Preproteins couple the intrinsic dynamics of SecA to its ATPase cycle to translocate via a catch and release mechanism*. Cell Rep, 2022. **38**(6): p. 110346.
217. Fekkes, P., C. van der Does, and A.J. Driessen, *The molecular chaperone SecB is released from the carboxy-terminus of SecA during initiation of precursor protein translocation*. EMBO J, 1997. **16**(20): p. 6105-13.
218. Breukink, E., et al., *The C terminus of SecA is involved in both lipid binding and SecB binding*. J Biol Chem, 1995. **270**(14): p. 7902-7.

219. Itskanov, S. and E. Park, *Mechanism of Protein Translocation by the Sec61 Translocon Complex*. Cold Spring Harb Perspect Biol, 2023. **15**(1).
220. Peterson, J.H., C.A. Woolhead, and H.D. Bernstein, *The conformation of a nascent polypeptide inside the ribosome tunnel affects protein targeting and protein folding*. Mol Microbiol, 2010. **78**(1): p. 203-17.
221. Adams, H., et al., *The presence of a helix breaker in the hydrophobic core of signal sequences of secretory proteins prevents recognition by the signal-recognition particle in Escherichia coli*. Eur J Biochem, 2002. **269**(22): p. 5564-71.
222. Kuhn, A., H.G. Koch, and R.E. Dalbey, *Targeting and Insertion of Membrane Proteins*. EcoSal Plus, 2017. **7**(2).
223. Nagai, K., et al., *Structure, function and evolution of the signal recognition particle*. EMBO J, 2003. **22**(14): p. 3479-85.
224. Ataide, S.F., et al., *The crystal structure of the signal recognition particle in complex with its receptor*. Science, 2011. **331**(6019): p. 881-6.
225. Miller, J.D., H.D. Bernstein, and P. Walter, *Interaction of E. coli Ffh/4.5S ribonucleoprotein and FtsY mimics that of mammalian signal recognition particle and its receptor*. Nature, 1994. **367**(6464): p. 657-9.
226. Focia, P.J., et al., *Heterodimeric GTPase core of the SRP targeting complex*. Science, 2004. **303**(5656): p. 373-7.
227. Egea, P.F., et al., *Substrate twinning activates the signal recognition particle and its receptor*. Nature, 2004. **427**(6971): p. 215-21.
228. Jomaa, A., et al., *Structures of the E. coli translating ribosome with SRP and its receptor and with the translocon*. Nat Commun, 2016. **7**: p. 10471.
229. Holtkamp, W., et al., *Dynamic switch of the signal recognition particle from scanning to targeting*. Nat Struct Mol Biol, 2012. **19**(12): p. 1332-7.
230. Akopian, D., et al., *Signal recognition particle: an essential protein-targeting machine*. Annu Rev Biochem, 2013. **82**: p. 693-721.
231. Parlitz, R., et al., *Escherichia coli signal recognition particle receptor FtsY contains an essential and autonomous membrane-binding amphipathic helix*. J Biol Chem, 2007. **282**(44): p. 32176-84.
232. Stjepanovic, G., et al., *Lipids trigger a conformational switch that regulates signal recognition particle (SRP)-mediated protein targeting*. J Biol Chem, 2011. **286**(26): p. 23489-97.
233. Eitan, A. and E. Bibi, *The core Escherichia coli signal recognition particle receptor contains only the N and G domains of FtsY*. J Bacteriol, 2004. **186**(8): p. 2492-4.
234. Bacher, G., et al., *Regulation by the ribosome of the GTPase of the signal-recognition particle during protein targeting*. Nature, 1996. **381**(6579): p. 248-51.
235. Rawat, S., et al., *SecA drives transmembrane insertion of RodZ, an unusual single-span membrane protein*. J Mol Biol, 2015. **427**(5): p. 1023-37.
236. Wang, S., C.I. Yang, and S.O. Shan, *SecA mediates cotranslational targeting and translocation of an inner membrane protein*. J Cell Biol, 2017. **216**(11): p. 3639-3653.
237. Huber, D., et al., *SecA Cotranslationally Interacts with Nascent Substrate Proteins In Vivo*. J Bacteriol, 2017. **199**(2).
238. Steinberg, R., et al., *Posttranslational insertion of small membrane proteins by the bacterial signal recognition particle*. PLoS Biol, 2020. **18**(9): p. e3000874.
239. Pross, E., et al., *Membrane Targeting and Insertion of the C-Tail Protein SciP*. J Mol Biol, 2016. **428**(20): p. 4218-4227.
240. Duong, F. and W. Wickner, *The SecDFyajC domain of preprotein translocase controls preprotein movement by regulating SecA membrane cycling*. EMBO J, 1997. **16**(16): p. 4871-9.

241. Becker, T., et al., *Structure of monomeric yeast and mammalian Sec61 complexes interacting with the translating ribosome*. Science, 2009. **326**(5958): p. 1369-73.
242. Cheng, Z., et al., *Identification of cytoplasmic residues of Sec61p involved in ribosome binding and cotranslational translocation*. J Cell Biol, 2005. **168**(1): p. 67-77.
243. Mercier, E., et al., *Lateral gate dynamics of the bacterial translocon during cotranslational membrane protein insertion*. Proc Natl Acad Sci U S A, 2021. **118**(26).
244. Zhu, Z., S. Wang, and S.O. Shan, *Ribosome profiling reveals multiple roles of SecA in cotranslational protein export*. Nat Commun, 2022. **13**(1): p. 3393.
245. Catipovic, M.A. and T.A. Rapoport, *Protease protection assays show polypeptide movement into the SecY channel by power strokes of the SecA ATPase*. EMBO Rep, 2020. **21**(11): p. e50905.
246. Urbanus, M.L., et al., *Sec-dependent membrane protein insertion: sequential interaction of nascent FtsQ with SecY and YidC*. EMBO Rep, 2001. **2**(6): p. 524-9.
247. Wu, Z.C., et al., *Competitive binding of the SecA ATPase and ribosomes to the SecYEG translocon*. J Biol Chem, 2012. **287**(11): p. 7885-95.
248. Facey, S.J., et al., *The mechanosensitive channel protein MscL is targeted by the SRP to the novel YidC membrane insertion pathway of Escherichia coli*. J Mol Biol, 2007. **365**(4): p. 995-1004.
249. Kauffman, P., et al., *Consecutive Steps of Membrane Insertion of the Two-spanning MscL Protein by Insertase YidC*. J Mol Biol, 2025. **437**(10): p. 169074.
250. Samuelson, J.C., et al., *Function of YidC for the insertion of M13 procoat protein in Escherichia coli: translocation of mutants that show differences in their membrane potential dependence and Sec requirement*. J Biol Chem, 2001. **276**(37): p. 34847-52.
251. Chen, M., et al., *Direct interaction of YidC with the Sec-independent Pf3 coat protein during its membrane protein insertion*. J Biol Chem, 2002. **277**(10): p. 7670-5.
252. van Bloois, E., et al., *F(1)F(0) ATP synthase subunit c is targeted by the SRP to YidC in the E. coli inner membrane*. FEBS Lett, 2004. **576**(1-2): p. 97-100.
253. Aschtgen, M.S., et al., *The C-tail anchored TssL subunit, an essential protein of the enteroaggregative Escherichia coli Sci-I Type VI secretion system, is inserted by YidC*. Microbiologyopen, 2012. **1**(1): p. 71-82.
254. Peschke, M., et al., *SRP, FtsY, DnaK and YidC Are Required for the Biogenesis of the E. coli Tail-Anchored Membrane Proteins DjlC and Flk*. J Mol Biol, 2018. **430**(3): p. 389-403.
255. Peschke, M., et al., *Distinct Requirements for Tail-Anchored Membrane Protein Biogenesis in Escherichia coli*. mBio, 2019. **10**(5).
256. Guna, A., et al., *A Tale of Two Pathways: Tail-Anchored Protein Insertion at the Endoplasmic Reticulum*. Cold Spring Harb Perspect Biol, 2023. **15**(3).
257. Hegde, R.S. and R.J. Keenan, *Tail-anchored membrane protein insertion into the endoplasmic reticulum*. Nat Rev Mol Cell Biol, 2011. **12**(12): p. 787-98.
258. Schuldiner, M., et al., *The GET complex mediates insertion of tail-anchored proteins into the ER membrane*. Cell, 2008. **134**(4): p. 634-45.
259. McGilvray, P.T., et al., *An ER translocon for multi-pass membrane protein biogenesis*. Elife, 2020. **9**.
260. Kalinin, I.A., et al., *Features of membrane protein sequence direct post-translational insertion*. Nat Commun, 2024. **15**(1): p. 10198.
261. Nicolaus, F., et al., *Residue-by-residue analysis of cotranslational membrane protein integration in vivo*. Elife, 2021. **10**.
262. Wu, H., L. Smalinskaite, and R.S. Hegde, *EMC rectifies the topology of multipass membrane proteins*. Nat Struct Mol Biol, 2024. **31**(1): p. 32-41.

263. Zhu, L., H.R. Kaback, and R.E. Dalbey, *YidC protein, a molecular chaperone for LacY protein folding via the SecYEG protein machinery*. J Biol Chem, 2013. **288**(39): p. 28180-94.
264. Houben, E.N., et al., *Nascent Lep inserts into the Escherichia coli inner membrane in the vicinity of YidC, SecY and SecA*. FEBS Lett, 2000. **476**(3): p. 229-33.
265. Wagner, S., et al., *Biogenesis of MalF and the MalFGK(2) maltose transport complex in Escherichia coli requires YidC*. J Biol Chem, 2008. **283**(26): p. 17881-90.
266. van Bloois, E., et al., *Distinct requirements for translocation of the N-tail and C-tail of the Escherichia coli inner membrane protein CyoA*. J Biol Chem, 2006. **281**(15): p. 10002-9.
267. Celebi, N., et al., *Membrane biogenesis of subunit II of cytochrome bo oxidase: contrasting requirements for insertion of N-terminal and C-terminal domains*. J Mol Biol, 2006. **357**(5): p. 1428-36.
268. Nagamori, S., I.N. Smirnova, and H.R. Kaback, *Role of YidC in folding of polytopic membrane proteins*. J Cell Biol, 2004. **165**(1): p. 53-62.
269. Smalinskaite, L. and R.S. Hegde, *The Biogenesis of Multipass Membrane Proteins*. Cold Spring Harb Perspect Biol, 2023. **15**(4).
270. Schulze, R.J., et al., *Membrane protein insertion and proton-motive-force-dependent secretion through the bacterial holo-translocon SecYEG-SecDF-YajC-YidC*. Proc Natl Acad Sci U S A, 2014. **111**(13): p. 4844-9.
271. Xie, K., et al., *Different regions of the nonconserved large periplasmic domain of Escherichia coli YidC are involved in the SecF interaction and membrane insertase activity*. Biochemistry, 2006. **45**(44): p. 13401-8.
272. Mitra, K., et al., *Structure of the E. coli protein-conducting channel bound to a translating ribosome*. Nature, 2005. **438**(7066): p. 318-24.
273. Bischoff, L., et al., *Visualization of a polytopic membrane protein during SecY-mediated membrane insertion*. Nat Commun, 2014. **5**: p. 4103.
274. Menetret, J.F., et al., *Ribosome binding of a single copy of the SecY complex: implications for protein translocation*. Mol Cell, 2007. **28**(6): p. 1083-92.
275. Serdiuk, T., et al., *YidC assists the stepwise and stochastic folding of membrane proteins*. Nat Chem Biol, 2016. **12**(11): p. 911-917.
276. Vitrac, H., et al., *The lipid-dependent structure and function of LacY can be recapitulated and analyzed in phospholipid-containing detergent micelles*. Sci Rep, 2019. **9**(1): p. 11338.
277. Kamel, M., *SecYEG/YidC-Mediated Membrane Protein Biogenesis*, in *Biochemistry*. 2023, Heinrich Heine Universität Düsseldorf: Düsseldorf.
278. Busch, M., et al., *Substrate-induced assembly and functional mechanism of the membrane protein insertase SecYEG-YidC*. EMBO J, 2026.
279. Denisov, I.G., et al., *Directed self-assembly of monodisperse phospholipid bilayer Nanodiscs with controlled size*. J Am Chem Soc, 2004. **126**(11): p. 3477-87.
280. Dowhan, W. and M. Bogdanov, *Lipid-dependent membrane protein topogenesis*. Annu Rev Biochem, 2009. **78**: p. 515-40.
281. Gao, T., et al., *Characterization of de novo synthesized GPCRs supported in nanolipoprotein discs*. PLoS One, 2012. **7**(9): p. e44911.
282. Yang, J.P., et al., *Cell-free synthesis of a functional G protein-coupled receptor complexed with nanometer scale bilayer discs*. BMC Biotechnol, 2011. **11**: p. 57.
283. Kamel, M., et al., *Unsaturated fatty acids augment protein transport via the SecA:SecYEG translocon*. FEBS J, 2022. **289**(1): p. 140-162.
284. van der Does, C., et al., *Non-bilayer lipids stimulate the activity of the reconstituted bacterial protein translocase*. J Biol Chem, 2000. **275**(4): p. 2472-8.

285. Siliakus, M.F., J. van der Oost, and S.W.M. Kengen, *Adaptations of archaeal and bacterial membranes to variations in temperature, pH and pressure*. Extremophiles, 2017. **21**(4): p. 651-670.

8. List of Publications

Busch, M., Rosales-Hernandez, C., Kamel, M., Schaumkessel, Y., van der Sluis, E. O., Berninghausen, O., ... & Kedrov, A. (2026). Substrate-induced assembly and functional mechanism of the membrane protein insertase SecYEG-YidC. *The EMBO Journal*, 1-25.

Busch, M., Loschwitz, J., Papadopoulos, A., Reiners, J., Steinchen, W., Calvagna, V., ... & Kedrov, A. (2026). Conformational dynamics of the membrane-anchored foldase LipH from *Pseudomonas aeruginosa* governs recognition and release of its client lipase. *Journal of Biological Chemistry*, 302(8).

Dali, S., Schultz, M., Köster, M., Kamel, M., **Busch, M.**, Steinchen, W., ... & Schipper, K. (2025). Specific phosphoinositide interaction of Jps1 is a key feature during unconventional secretion in *Ustilago maydis*. *Journal of Biological Chemistry*, 301(6).

Papadopoulos, A., **Busch, M.**, Reiners, J., Hachani, E., Baeumers, M., Berger, J., ... & Kedrov, A. (2022). The periplasmic chaperone Skp prevents misfolding of the secretory lipase A from *Pseudomonas aeruginosa*. *Frontiers in Molecular Biosciences*, 9, 1026724.

9. Acknowledgement

First, I would like to thank Professor **Alexej Kedrov**. For all the support I received during the years in his team. Your guidance from my first months, when I was still a bachelor student to the scientific discussions during my Ph.D., the opportunity to exchange ideas as equals, I truly appreciated. But throughout our shared time, I did not only learn on a scientific level, but how you lead a team, always being approachable, and how you think and analyse so many different projects at the same time is just remarkable and you have been a great role model for me.

I also want to thank my second supervisor Professor **Lutz Schmitt**. Thank you for the support and input throughout many of my progress reports, your critical questions that made me rethink and optimize my projects. Also, granting full access to your laboratories and devices was a major help over the time, in your institute, I never felt like someone from the other building but a member of the team.

I further want to thank Professor **Karl Erich Jaeger**. Your insights in the *Pseudomonas aeruginosa* field, the discussions and support were of high value for our work.

I would like to thank Professor **Sander Smits** for the input in my progress reports. A big thank you also to Dr. **Jan Stindt** for his input and for the organization of, and support during, the teaching obligations.

I want to thank Dr. **Cordula Kruse**, **Martina Wesemann**, **Silke Mavaro** and **Corinna Franzkowiak** for managing countless things in the background making the day-to-day work for all of us possible.

I further want to thank all the collaborators who contributed to this work over all these years in any way: first and foremost, **Cristian Rosales Hernandez** for the amazing and relentless job you did for the SecYEG-YidC project and all the other involved people from LMU Munich: Professor **Roland Beckmann**, Dr. **Thomas Becker** and Dr. **Otto Beringhausen**. And the people who contributed with their work, without knowing in which way their results would be used in the future: **Yulia Schaumkessel** and Dr. **Eli van der Sluis**. Also, the people involved in our other projects: **Jennifer Loschwitz**, Dr. **Jens Reiners** and Dr. **Wieland Steinchen** for the support on the LipH project and **Rocco Gentile**, as well as Professor **Holger Gohlke** for the work in the translocon dynamics project.

I also want to thank the “Season 1 PhD students” of the Synthetic Membrane Systems team. The environment you created made it incredibly easy to decide to pursue a Ph.D. in Alexej’s team. **Atha**, the time we spent together taught me a lot about science. Thank you for being there throughout my first few years. Thank you, **Maryna** for all the fun in the lab and being (involuntarily) the responsible person so the lab would not sink into chaos. A huge Thanks to you, **Michael**, for being more than just a colleague! Your love for science is contagious and has helped me throughout my Ph.D. But also, for the time outside of the lab, showing me your hometown, Cairo, the countless evenings spent having a beer or two, playing Skat, and much more. I am glad I met you!

Further, I would like to thank the current Synthetic Membrane Systems team! First, **Nhi**, the greatest office mate someone could ask for! Thank you for proof-reading nearly anything I wrote in whatever context. Further, **Marius**, **Debbie** and **Pauline** for a good time in and outside the lab, for actively participating in many incredible stupid discussions and tolerating the chaos I sporadically made in the lab.

I would like to thank all my previous students: **Mariam**, **Nadia**, **Antonia**, and **Lisa** for all the nice work you have done and making it so easy to work with you! A special thanks goes to **Vini** for his fantastic contribution to our paper.

I also want to thank **Marten**. Your scientific guidance was a big help but I enjoyed our shared time outside of university even more. The many trips to old town and football games near and far were a great pleasure and I am more than happy to call you a friend!

I thank **Miriam** for her sheer endless optimism. I think I have never seen someone so enthusiastic and passionate about nearly everything we spoke about. It was a great help during the tougher times, especially the writing.

I want to thank all the members of the Institute of Biochemistry for all the helpful discussions about science and the great atmosphere, but maybe even more for the 'non-scientific' conversations about Bundesliga and Champions League and the games we played together.

I would also like to thank **Julian** and **Sergiy** – being able to exchange experiences with people in a similar situation was incredibly helpful.

At this point, I would also like to thank **Alexander Lübeck**. During my school years, you took me to my first lectures at the Heinrich Heine University and you were certainly a major reason why I became so enthusiastic about life sciences.

A huge thank you goes to **Hakan**, **Raphael**, and **David**. The countless hours spent with you in the fencing hall in Solingen or somewhere else around the world were among the most formative years of my life. I learned a lot about hard work, dedication, success, and defeat.

I am also deeply grateful to my friends who have been by my side since early childhood for all the time we have shared together. **Maximilian**, **Lennart**, **Julian**, and **Taso**, I am very grateful to have you in my life. Likewise, I want to thank **Lucas**, **Hauke**, and **Felix** for the years we spent together at school and beyond.

I want to thank the friends I made during my PhD, which made this time in Düsseldorf and elsewhere special. Thank you **Paweł**, **Anna Rita**, **Farhad**, **Julian**, **Julia**, **Cassy** and **Charly**. Without you all the last few years would not have been such a blast!

Another person I would like to thank is my brother **Maik**, who is always there for me. Growing up with me as your big brother, might not have been easy, I know, I was not always the easiest. I am grateful to have you in my life!

I want to express my deepest gratitude to my parents, **Oliver** and **Susanne**! Your support and encouragement throughout my whole life brought me here. Your nurturing made me the person I am to this day. This accomplishment is as much yours as it is mine.

Finally, I want to thank the person without whom none of this would have been possible: **Meryem**. Your support throughout this emotional rollercoaster, always having my back and knowing you would cheer me up no matter how my day went, was the greatest source of strength during this journey. I am incredibly proud and grateful to have you by my side.

10. Declaration

Ich versichere an Eides statt, dass die vorliegende Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Diese Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen oder erfolgreichen Promotionsversuche unternommen.

(Ort, Datum)

Max Busch