The FEBS Journal

FEBS PRESS science publishing by scientists

STATE-OF-THE-ART REVIEW

The largely unexplored biology of small proteins in pro- and eukaryotes

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Keywords
antimicrobial peptides; cytochrome oxidase; mRNA targeting; protein targeting; Sec61 complex; SecYEG translocon; Small membrane proteins; stress response; YidC

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(Received 27 January 2021, revised 11 March 2021, accepted 26 March 2021)

doi:10.1111/febs.15845

The large abundance of small open reading frames (smORFs) in prokaryotic and eukaryotic genomes and the plethora of smORF-encoded small proteins became only apparent with the constant advancements in bioinformatic, genomic, proteomic, and biochemical tools. Small proteins are typically defined as proteins of < 50 amino acids in prokaryotes and of less than 100 amino acids in eukaryotes, and their importance for cell physiology and cellular adaptation is only beginning to emerge. In contrast to antimicrobial peptides, which are secreted by prokaryotic and eukaryotic cells for combatting pathogens and competitors, small proteins act within the producing cell mainly by stabilizing protein assemblies and by modifying the activity of larger proteins. Production of small proteins is frequently linked to stress conditions or environmental changes, and therefore, cells seem to use small proteins as intracellular modifiers for adjusting cell metabolism to different intra- and extracellular cues. However, the size of small proteins imposes a major challenge for the cellular machinery required for protein folding and intracellular trafficking and recent data indicate that small proteins can engage distinct trafficking pathways. In the current review, we describe the diversity of small proteins in prokaryotes and eukaryotes, highlight distinct and common features, and illustrate how they are handled by the protein trafficking machineries in prokaryotic and eukaryotic cells. Finally, we also discuss future topics of research on this fascinating but largely unexplored group of proteins.

Introduction

Defining the total number of different proteins in a given cell is a major analytical challenge, and several predictions were originally implemented in genome annotation studies for reducing the complexity of these analyses. The initial algorithms used by genomic studies to identify ORFs worked with a cutoff of 150 nucleotides in prokaryotes and 300 nucleotides in eukaryotes, disregarding translation products of < 50 or 100 amino acids [1–6]. This not only reduced the number of theoretical translation products to an editable level, but also assumed no relevant function for proteins smaller than 50/100 amino acids. Nevertheless, there were exceptions and the importance of some small proteins, such as the antimicrobial peptides (AMPs), was recognized early on. AMPs constitute a large group of small secreted proteins that target

Abbreviations
AMPs, antimicrobial peptides; CaM, calmodulin; CLD, cathelin-like domain; GET, guided entry of tail-anchored proteins; PrAMP, proline-rich AMPs; RNCs, ribosome-associated nascent chain; smORFs, small open reading frames; SND, SRP-independent targeting; SRP, signal recognition particle.

preferentially the bacterial membrane [7], but also intracellular targets, such as the ribosome [8]. One of the best-studied AMPs is the 26-amino-acid-long cationic peptide melittin, found in the venom of the European honeybee *Apis mellifera*. Melittin was first described in 1952 [9] and shown to have numerous antibacterial effects and to synergistically enhance the activity of conventional antibiotics [9]. Melittin was also used as model substrate for studying protein secretion pathways in eukaryotes and bacteria [10,11]. Another group of small proteins that gained attention from the beginning were small membrane proteins that are genetically linked to larger membrane protein complexes [12]. They were initially identified as accessory subunits of respiratory complexes [13,14] and shown to be required for complex stability [15–17] or co-factor insertion [18,19]. Nevertheless, the large abundance of small proteins encoded in the genomes of eukaryotic and prokaryotic cells became only visible with the constant improvements in genome, proteome, and bioinformatic approaches [20–26]. This is exemplified by the prediction of about 4000 different small protein families in the human microbiome by a comparative genomic study of about 1700 metagenomes [27]. Approx. 30% of these putative small proteins are predicted to be secreted or membrane-bound, and ~ 50% are not represented in reference genomes [27]. Computational studies predict more than 140 000 small ORFs (smORFs) in the yeast genome, but only a small fraction is confirmed at the protein level [28–30]. Bioinformatic studies also revealed ~ 600 000 putative smORFs in the noncoding *Drosophila* genome [28,31,32]. However, subsequent filtering indicated that only ~ 4500 of these smORFs are conserved between different *Drosophila* species and for only ~ 400 smORFs evidence of transcription was found in transcriptome studies during embryonic development [31,33]. For humans, the UniProt data bank lists > 5000 proteins of < 50 amino acids, most of them uncharacterized. A recent analysis using ribosome profiling, computational analyses, mass spectrometry-based proteomics, and CRISPR-*Cas9* knockout studies identified several hundred small proteins in humans, which are important for cell growth by largely unknown mechanisms [34].

Despite their obvious abundance, there is so far no uniform nomenclature for small proteins and many different terms have been used, for example, smORF-encoded polypeptides (SEPs) [35], small single transmembrane domain proteins (STMD proteins) [12], micropeptides [36,37], miniproteins [38], or microproteins [39]. Although these terms highlight their small size as common denominator, they fall short in describing the enormous functional diversity of small proteins in prokaryotes and eukaryotes. Furthermore, for differentiating between proteolytic cleavage products and ribosome-generated products, the term micropeptide could be misleading and the term 'small protein' is probably a better choice. A classification of small proteins based on function [1] would be more appropriate, but such a classification is still limited by the small number of detailed functional studies. This highlights the importance of functional studies on small proteins in different species. Equally important are studies on how cells handle folding, targeting, and transport of small proteins, because our current understanding about these processes is almost exclusively based on studies with larger proteins [40–42]. The current review focuses on the large variety of small proteins in prokaryotes and eukaryotes and their functional diversity. Furthermore, it highlights some recent developments on protein trafficking of small proteins.

Antimicrobial peptides

Antimicrobial peptides are oligopeptides of varying length that target different organisms ranging from viruses to parasites [43]. They are secreted by prokaryotic and eukaryotic cells as a defense mechanism against pathogens and for controlling proliferation of competitors [44] (Fig. 1). The *Antimicrobial Peptide Database* lists about 3000 peptides, of which more than 2700 are ribosomally synthesized. In addition, about 13 000 synthetic peptides have been shown to target Gram-negative or Gram-positive bacteria (DBAASP v3.0; https://dbaasp.org/home). About 90% of all naturally occurring AMPs are produced by animals and plants and have a length of < 50 amino acids. AMPs produced by bacteria account for about 8% of all known AMPs, and the remaining 2% are produced by fungi. Bacterial AMPs are also referred to as bacteriocins, a heterogeneous group of peptides, which differ largely in size, structure, and mode of action [44]. They can be classified by several criteria, including the producing strain, the mechanism of action, or the secretion mechanism [45]. Bacteriocins of Gram-negative bacteria are often divided into microcins, the larger colicin-like bacteriocins, such as the 626-amino-acid-long colicin 1a [46], and the very large tailocins, which consist of several subunits and are related to bacteriophage tail proteins [47]. Although they have their antimicrobial activity in common, only microcins of Gram-negative bacteria and the class I and class II bacteriocins of Gram-positive bacteria can be referred to as small proteins/

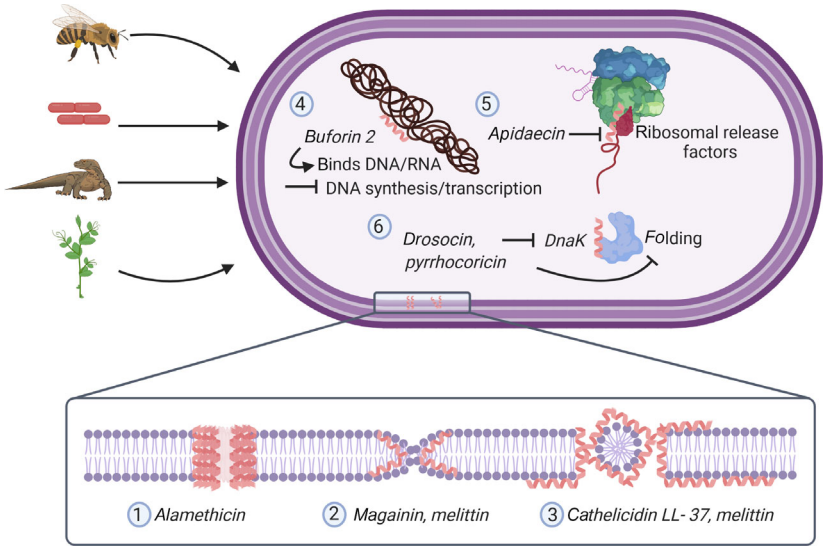

Fig. 1. Membrane-destabilizing and intracellular effects of antimicrobial peptides. Different organisms ranging from mammals to bacteria secrete antimicrobial peptides. These short peptides destabilize the bacterial cytoplasmic membrane by different modes of action: (1) barrel stave model of pore formation. 8-10 molecules of the fungal AMP *alamethicin* from barrels in the bacterial membrane, which induces cellular leakage. (2) AMPs such as *magainin 1/2* can bend the phospholipid bilayer and form toroidal pores together with phospholipids. (3) Carpet model. After accumulation on the phospholipid bilayer, for example, *cathelicidin LL-37* can disrupt the membrane in a detergent-like mechanism. Extensively studied melittin from the honeybee venom was shown to disintegrate the bacterial membrane by a combination of toroidal pore formation and carpet mechanism. Other AMPs act on intracellular targets: (4) DNA synthesis and transcription are inhibited by binding to DNA and RNA, for example, by histone-like *buforin 2*. (5) PrAMP *apidaecin* traps ribosomal release factors and thus inhibits translational termination. (6) Protein folding is inhibited by the PrAMPs *drosocin* or *pyrrhocoricin* via binding to the chaperone DnaK.

peptides. Microcins are small peptides with (class I) or without (class II) post-translational modifications, which are synthesized as precursor peptides [48]. They enter their bacterial target by hijacking nutrient-uptake systems, most frequently iron-uptake systems [49]. Inside the target cell, they compromise a variety of essential processes, such as translation [50] or ATP production [51].

Vertebrate AMPs are amphipathic peptides that bind via electrostatic interactions to the lipopolysaccharide surface of Gram-negative bacteria, the teichoic acids of the Gram-positive peptidoglycan, and anionic phospholipids of the bacterial cell membrane [52]. They belong to two main classes, the defensins and the cathelicidins, which are both produced as prepropeptides and cleaved to generate the active peptide. Defensins are characterized by six conserved cysteine residues, which stabilize a β-sheet core via disulfide bridges [53]. In addition to their antimicrobial activity, they also exhibit immune-modulatory activity [54]. Cathelicidins are small and mainly amphipathic α-helical peptides that are primarily found in vertebrates [7]. They are secreted as prepropeptides with an N-terminal signal peptide, a cathelin-like domain (CLD), and the carboxy-terminal mature peptide. CLDs show high sequence homology to cathelin, a cysteine protease inhibitor isolated from pigs' leukocytes [55]. The pro-CLD is cleaved after secretion, and the cationic mature domain is released. One example is the cathelicidin-like AMP secreted by the Komodo dragon [56–58]. The saliva of the Komodo dragon contains a cocktail of highly pathogenic bacteria, and a single bite is usually sufficient to inflict lethal sepsis in its prey. The Komodo dragon itself is highly resistant against these bacteria because it secretes a large variety of potential AMPs, which include not only cathelicidin-like AMPs but also histone-derived AMPs [56]. Additional AMPs have been characterized, like histatins found in the oral cavity of mammals [59] or magainin 1/2, which are secreted by amphibian skin granular glands [60]. Histatins are cationic peptides with a high histidine content [61], and they target both the membrane and intracellular targets [62]. Magainin 1 and magainin 2 are cationic peptides of almost identical amino acid sequences, which form α-helices when in contact with membranes [63].

The amphipathic and cationic nature of most AMPs is essential for their membrane-destabilizing effect on

the cytoplasmic membrane [64,65] (Fig. 1). The effect of AMPs on the outer membrane and the peptidoglycan layer is generally less explored, but AMPs are assumed to cross the outer membrane and the cell wall spontaneously. Disruption of the permeability barrier of the outer membrane probably involves displacement of divalent cations [66,67] and inhibition of cell wall synthesis [68,69]. For the destabilizing effect on the cytoplasmic membrane, several modes of action have been proposed (Fig. 1):

1 The barrel stave model of pore formation predicts that AMPs vertically insert into the membrane, forming membrane barrels that dissipate the electrochemical gradient. This was shown for the fungal AMP alamethicin [70], which forms a pore of ~ 18–26 Å that consists of 8–10 alamethicin monomers [71].
2 AMP-induced bending of the phospholipid bilayer and connecting the inner and outer leaflets of the membrane is suggested to form toroidal pores, consisting of both peptides and phospholipids. This was first shown for magainin, which forms large pores (30–50 Å) consisting of 4–7 peptides and 90 phospholipids [72]. The toroidal pore model is in particular attractive for very short AMPs. The thickness of the bilayer is typically 32–38 Å, and ~ 20 amino acids are required to span the phospholipid bilayer in an α-helical conformation [73]. However, the destabilizing effect of short AMPs might also be caused by membrane thinning [74], an effect that is also induced by short transmembrane domains of larger membrane proteins [75–77].
3 The carpet mechanism proposes a detergent-like disruption of the membrane by AMPs accumulating first on the bilayer surface and then disintegrating the membrane above a threshold concentration of the AMP monomer [78]. This mechanism was shown for the human AMP cathelicidin LL-37.

However, it is important to emphasize that these models are largely based on *in vitro* studies using model membranes and that both lipid composition and lipid/protein ratios influence the consequences of AMP–phospholipid interactions [74,79,80]. Furthermore, some AMPs, such as melittin, appear to act via a mechanism that combines the carpet mechanism with the formation of a toroidal pore [65,79].

AMPs that target the membrane or the cell wall have been analyzed in great detail [7,44,81], but several AMPs act on intracellular targets (Fig. 1). Proline-rich AMPs (PrAMPs) constitute a class of short, nonlytic peptides that are produced by plants and animals [82–84] (http://dramp.cpu-bioinfor.org/). Apidaecin, a PrAMP isolated from *Apis mellifera* [85], was shown to inhibit bacterial translation by trapping release factors on the ribosome [8]. Other PrAMPs, such as drosocin or pyrrhocoricin, bind to the Hsp70 chaperone DnaK and inhibit protein folding [86,87]. AMPs with similarity to histones, such as buforin 2, bind to DNA and RNA and inhibit DNA synthesis and transcription [88,89]. Uptake of AMPs acting on intracellular targets is facilitated by peptide transporters, such as SbmA or YgdD in Gram-negative bacteria [90,91]. In light of the drastic increase in drug-resistant pathogens, AMPs are intensively explored as alternative strategy against infections [81,92].

The expanding universe of small proteins in prokaryotes and eukaryotes

In contrast to AMPs, the abundance and functional relevance of small proteins in prokaryotes and eukaryotes have been largely overlooked in the past and received considerable attention only in recent years [1,21,26,35,93–95]. They are encoded by smORFs [21,96–98], alternative ORFs (altORFs) [21,99,100], upstream ORFs (uORFs) [21], or RNAs that were initially considered to be noncoding, such as circular RNAs [101,102]. Not included in the growing number of small proteins are peptides that derive from proteolytic cleavage of larger proteins. Despite their small size and the large absence of detectable motifs, small proteins have been implicated in multiple cellular processes.

Small proteins involved in stress response and metabolic regulation

Maintaining cellular homeostasis relies on a coordinated response to changing environmental conditions. In eukaryotes, the accumulation of unfolded proteins in the endoplasmic reticulum triggers the unfolded protein response (UPR) [103], which involves the downregulation of protein synthesis and the simultaneous upregulation of chaperones and proteases. It was recently shown that the absence of the small protein PIGBOS leads to enhanced UPR and apoptosis [39] (Fig. 2A). PIGBOS is located to the outer mitochondrial membrane and interacts with ER protein CLCC1, suggesting that ER–mitochondria contact sites are critical for the UPR. Another small protein regulating UPR is the recently identified FORCP, which is encoded by what was thought to be a long noncoding RNA (lncRNA) [104]. FORCP is an ER-localized protein with two predicted transmembrane domains. Upon ER stress, FORCP inhibits basal

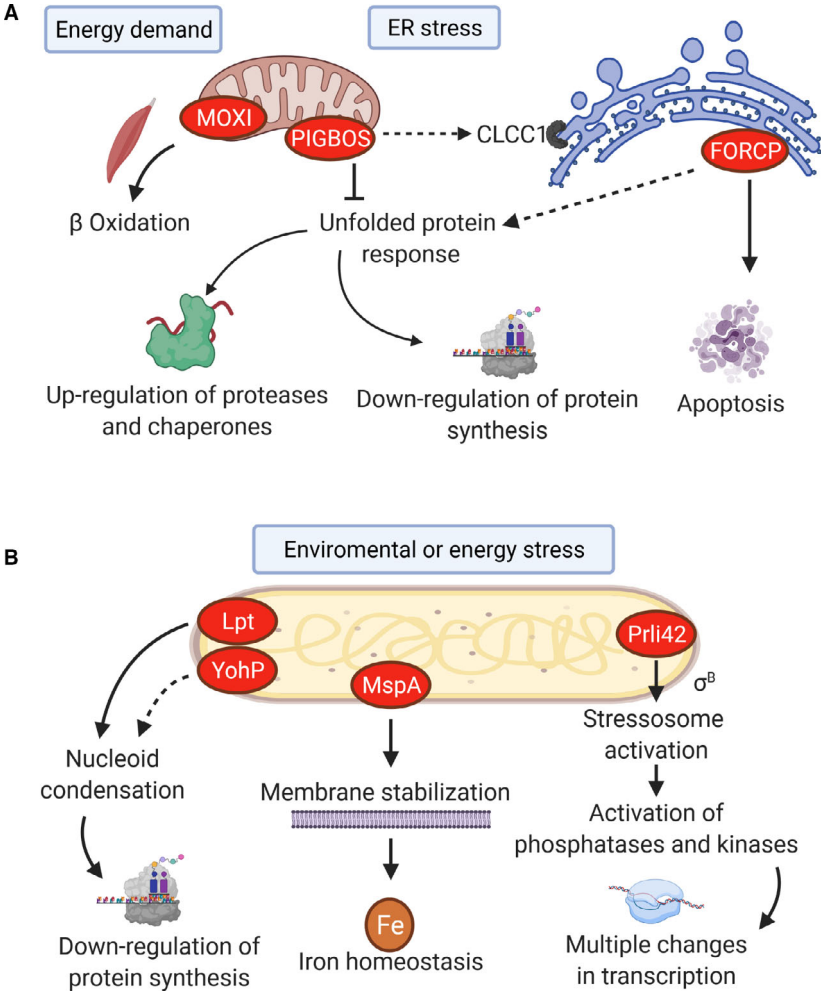

Fig. 2. Small proteins are involved in different stress–response mechanisms. Small proteins involved in different stress–response mechanisms are displayed in red. (A) In eukaryotes, the small proteins PIGBOS and FORCP influence the UPR, which leads to upregulation of proteases and chaperones and downregulation of protein synthesis when cells encounter ER stress. PIGBOS inhibits the UPR and interacts with the ER protein CLCC1. FORCP induces apoptosis in colorectal cancer cells. The small mitochondrial protein MOXI regulates β-oxidation and exercise performance when muscle cells show a high energy demand. (B) In prokaryotes, small membrane proteins YohP and Lpt can lead to nucleoid condensation, followed by downregulation of protein synthesis under nonfavorable conditions. Membrane stabilizing effects and iron homeostasis were shown to depend on the small membrane protein MspA in *S. aureus*. Stressosome activation is mediated by small protein Prli42, which was shown to play a major role in activation of SigB in *Listeria*. This activation leads to changes in the transcription of ~ 150 different genes.

proliferation and induces apoptosis [104]. However, the molecular mechanisms by which PIGBOS or FORCP regulates apoptosis in response to stress are unknown. The mitochondrial apoptotic pathway is further regulated by the cytoprotective mitochondrial-derived small protein humanin. Humanin was first discovered in neurons of patients suffering from Alzheimer's disease [105]. Humanin is encoded by an alternative ORF within the mitochondrial 16S rRNA gene and translated in both the mitochondrial matrix and the cytosol, resulting in products of 21 or 24 amino acids, respectively [106]. Humanin suppresses apoptosis by preventing Bax activation [107,108] and as such acts as a pro-survival factor for cells exposed to stress conditions. Mitochondrial-derived small proteins are also involved in regulating metabolic activities during stress conditions. This has been shown for MOXI, a 56-amino-acid-long protein that regulates β-oxidation in muscle cells [109]. MOXI is a nuclear-encoded protein that is tethered to the inner mitochondrial membrane. MOXI interacts with the mitochondrial trifunctional protein MTP, which catalyzes the final steps of long-chain fatty acid oxidation. The absence of MOXI leads to reduced exercise performance, indicating that it is required for coordinating β-oxidation with energy demand. In *Arabidopsis thaliana*, flowering is controlled by the transcriptional regulator CONSTANS in response to day length. However, the production of two small proteins, mP1a and mP1b, delayed flowering by tethering CONSTANS to an inhibitory protein [110].

An increasing number of small proteins are also identified in prokaryotes, but a biological function has been assigned for only a few of them [1,26,111,112]. The *E. coli* proteome database lists currently about 150 small proteins, but most small proteins are probably not yet identified [26,95]. Intriguingly, the production of many small proteins increases when *E. coli* encounter stress conditions, such as temperature stress or oxidative stress, or when cells are switched from

rich medium to minimal medium or enter stationary phase [93]. Many of these stress-induced small proteins are located to the inner membrane and basically exist of a single transmembrane domain with only a few residues exposed to the cytoplasm or periplasm [113]. Besides hydrophobicity, a common denominator of many small membrane proteins in *E. coli* is their positive net charge resulting in high pI values (e.g., YohP: 9.3; AzuC: 10.3; YkgR: 8.14) although exceptions exist (e.g., YshB: 4.35). These pI values are overall slightly lower than the pI values of AMPs, and the lower lysine and arginine content probably prevents membrane destabilization by these native membrane proteins. One exception is the amphiphilic AzuC for which DBAASP predicts an antimicrobial activity. The hydrophobic core of AzuC is likely too short to span the membrane completely, and AzuC is probably only peripherally attached to the membrane [113,114]. While the over-representation of basic residues is also observed in larger membrane proteins [115], the position of positively charged residues at the N or C termini in small membrane proteins allows for specific interactions with the polar head groups of phospholipids and determines their topology in the membrane [113,114]. The positive charges can also favor interactions with other proteins, in particular when oligomerization [114] generates highly charged clusters in the membrane. The positive net charge might additionally facilitate the interaction with nucleic acids, and this assumption is supported by data showing that the expression of the *L. rhamnosus* small membrane protein Lpt in *E. coli* results in nucleoid condensation [116], which is also observed when the endogenous small membrane protein YohP is overproduced [114] (Fig. 2B). However, it is unknown whether this is also observed at physiological YohP levels. A nucleoid-condensing activity of some small proteins in prokaryotes is also in line with data demonstrating that some eukaryotic AMPs, such as Buforin, show a histone-like activity that is inactivated by replacing positive residues [7]. Nucleoid condensation is associated with reduced transcription and protein synthesis [117], which is a conserved strategy for preventing stress-induced cellular damage [118,119]. Thus, some small membrane proteins might protect cells against stress by reducing transcription through nucleoid condensation.

Small membrane proteins can also shape membrane thickness and fluidity [74], an important determinant for stress tolerance [120]. Membrane fluidizers have been shown to induce chaperone networks, and the fluidity of the membrane or of membrane domains may serve as a molecular switch for stress signaling processes [121]. Thus, one mode of action of small membrane proteins might be that they re-enforce the cytoplasmic membrane as ultimate barrier against external stressors, as discussed for the small membrane protein MspA from *S. aureus* [122] (Fig. 2B). On the contrary, a membrane-destabilizing effect is observed for some small proteins belonging to the toxin–antitoxin systems [123]. These systems consist of a stable toxin and an unstable RNA or protein antitoxin, which counteracts toxicity [124]. One example is the *tisB*/*istR1* toxin–antitoxin system. TisB is a 29-amino-acid-long small membrane protein, which is under SOS control and produced in response to DNA damage [125,126]. Accumulation of TisB in the membrane causes membrane thickening [74] and membrane depolarization, which subsequently reduces the cellular ATP level [127]. Production of TisB is also linked to the formation of persister cells [125,126], a metabolically largely inactive subpopulation of highly stress-resistant cells that are a major cause of antibiotic treatment failure and chronic infections [128].

In Gram-positive bacteria, the general stress response is initiated by the stressosome, a more than 1 MDa large cytosolic protein complex [129]. The stressosome senses changes in the extracellular environment and activates the transcription factor σ^B (SigB), which in turn regulates more than 150 genes in *Listeria monocytogenes* or *B. subtilis* [130]. Activation of σ^B depends on the small membrane protein Prli42, which is suggested to tether the stressosome to the membrane and to provide the link between stress sensing and stressosome activation [131].

In *Klebsiella pneumoniae*, a pathogenic bacterium that causes a wide range of human diseases, hypervirulence is associated with a hypermucoviscosity phenotype (HMV). The 58-amino acid membrane protein RmpD was recently shown to be required for the HMV phenotype and to be conserved among hypervirulent *Klebsiella* strains [132]. Based on structure and sequence predictions, it is suggested that RmpD has an α-helical structure with an N_{out}-C_{in} topology in the membrane. The positively charged C terminus of RmpD is located in the cytosol and conserved between different *Klebsiella* strains. It likely provides a docking site for protein–protein interactions that mediate virulence, but this is not experimentally verified yet [132].

In addition to their importance in regulating stress response and pathogenicity, small proteins are also important players in adjusting metabolic activity to nutrient availability. In the phytopathogen *Agrobacterium tumefaciens*, three arginine-rich small proteins, containing a domain of unknown function (DUF1127), were demonstrated to control the

expression of several genes in late exponential and stationary growth phase. Deletion of the corresponding genes leads to increased cell aggregation and biofilm formation. Detailed analyses indicated the largest effects on genes relevant for phosphate uptake, glycine/serine homeostasis, and nitrate respiration [133]. In cyanobacteria, several small proteins were found to be involved in metabolic regulation [112]. In *Synechocystis* sp., the small soluble protein AcnSP was shown to modulate aconitase activity and thereby regulating carbon flow into the TCA cycle [134]. Moreover, the two small proteins IF7 and IF17 (inactivating factor) regulate nitrogen uptake by inactivating glutamine synthetase (GS) in *Synechocystis* under nitrogen-rich conditions [135]. In contrast to many other bacteria, which regulate GS activity by reversible adenylation, GS activity in cyanobacteria is exclusively regulated by the abundance of IF7 and IF17 [112]. A positive regulation of GS activity by small proteins was observed in the archaeal model organism *Methanosarcina mazei*; here, the 23 amino acids containing small protein sp26 were shown to stimulate the GS activity [136].

More than 40% of all characterized proteins depend on metals for activity or stability [137,138], for example, > 1000 human proteins contain zinc-binding domains (UniProt-Human reference proteome; https://www.uniprot.org/proteomes/), and small proteins play crucial roles in maintaining metal homeostasis. In *Escherichia coli*, they regulate metal uptake, as shown for MgtS, which regulates Mg^{2+} uptake by the P-loop ATPase MgtA [139] or MntS, which regulates the intracellular Mn^{2+} concentration [140]. In *Salmonella typhimurium*, the small protein MgtR promotes the degradation of the Mg^{2+} transporters MgtA and MgtB by the membrane-bound protease FtsH, whereas the small protein MgtU seems to protect MgtB from degradation [141]. Small proteins, like the conserved CopZ-like proteins [142,143], also serve as metallochaperones and deliver copper for metalloprotein biogenesis while simultaneously preventing copper-induced toxicity [144]. CopZ production in some species is induced by ribosomal frameshifting during translation of the *copA* mRNA [99,100], which encodes for a Cu-exporting ATPase [144].

A common theme that emerges from multiple studies on small proteins in prokaryotes and eukaryotes is that they appear to execute their biological activity mainly by modulating the activity of other proteins or protein complexes. Although small proteins are generally too small to execute enzymatic activity, their small size helps them to dock onto bigger proteins/protein complexes. Furthermore, considering that translation is a rather slow process [145], their small size allows for a much faster production and a rapid fine-tuning of metabolism to different growth conditions. As such, small proteins may act as intracellular signaling molecules analogous to extracellular signaling molecules, such as cytokines, and they may regulate enzymatic activity by noncovalent interactions. This could explain why many small proteins are upregulated upon stress conditions or environmental changes [146]. In *Salmonella*, for example, it was shown that the small membrane protein PmrR inhibits the enzymatic activity of the inner membrane protein LpxT, which is responsible for increasing the negative charge of the bacterial lipopolysaccharide and thus allows bacteria to sense and modify the cell surface in response to the environment [147].

Small proteins as accessory subunits and assembly factors of membrane protein complexes

The importance of small proteins was initially recognized by the observation that many protein complexes of the respiratory chains contained small proteins as accessory subunits [12]. One example is bovine complex I, which consists of 45 different subunits, 14 of which are small, single-spanning membrane proteins [148]. Other examples are small membrane protein subunits of terminal oxidases (complex IV) [149].

The available data suggest at least two distinct functions of small membrane proteins for membrane protein complexes: (a) providing a hydrophobic surface that favors helix–helix interactions and subsequently stabilizes subunit interactions in larger protein complexes and (b) co-factor insertion into respiratory complexes. A stabilizing effect of KdpF on the potassium transporter KdpFABC has been documented and is explained by the localization of KdpF between the two transporter subunits KdpA and KdpB [150,151] (Fig. 3). A similar stabilizing effect was observed for the small CcoQ subunit of cbb_3-type cytochrome oxidase [15,17]. cbb_3-type cytochrome oxidase shows reduced assembly and activity in the absence of CcoQ, which appears to primarily prevent stable binding of the *c*-type cytochrome subunit CcoP to the catalytic core of the enzyme [17]. Small membrane proteins also stabilize respiratory supercomplexes [152,153]; this was shown for CcoH, a small membrane subunit of cbb_3-type cytochrome oxidase [16], which is required for stabilizing a supercomplex consisting of the cytochrome bc_1 complex, the membrane-bound *c*-type cytochrome c_y, and cbb_3-type cytochrome oxidase [154] (Fig. 3). In mitochondria, the lncRNA-encoded small protein mitoregulin binds cardiolipin and promotes

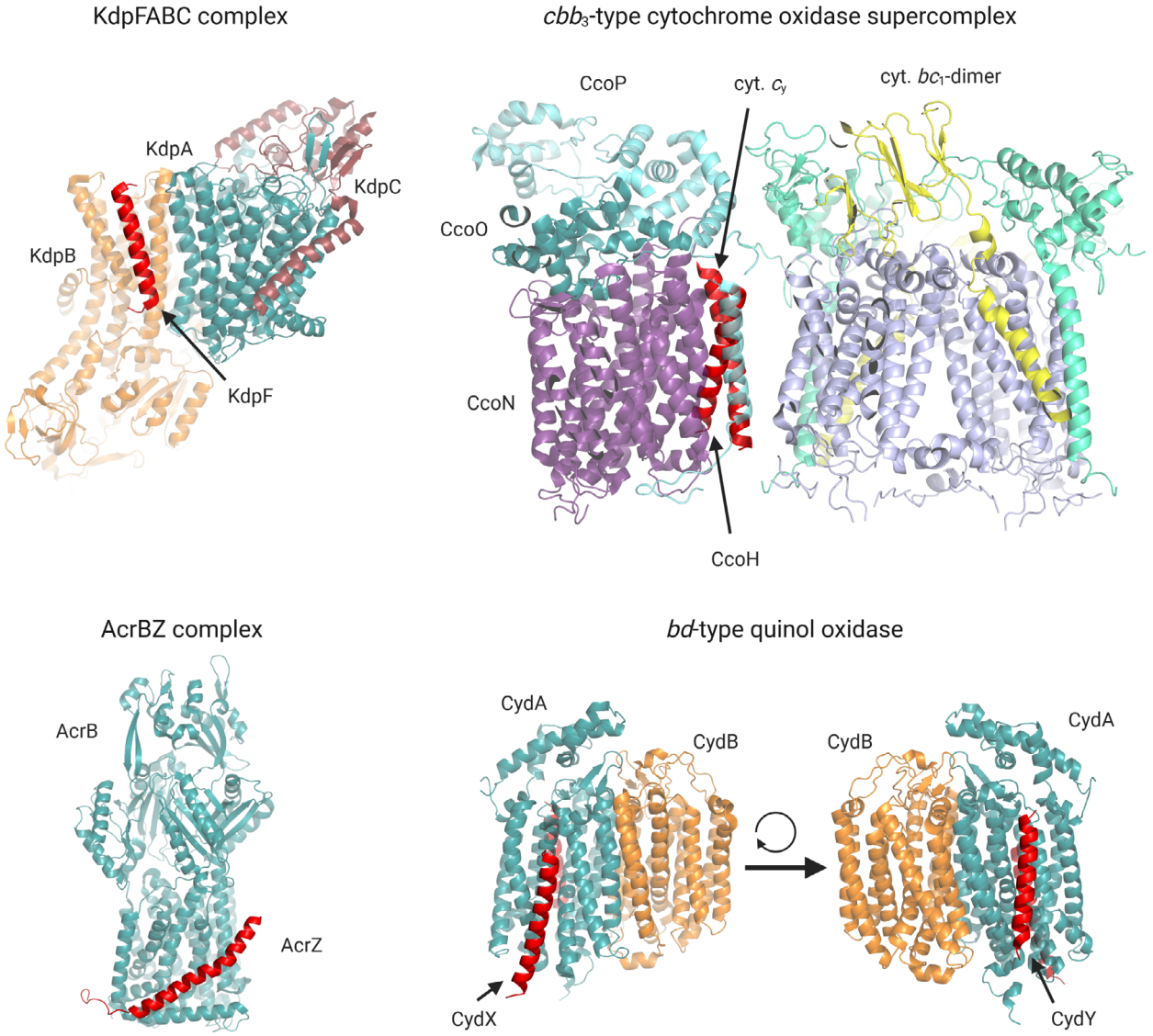

Fig. 3. Small membrane proteins as accessory subunits of membrane protein complexes. Crystal structures of membrane protein complexes with associated small membrane protein subunits were retrieved from the RCSB protein data bank using the following accession codes: AcrBZ complex (PDB 4CDI), cbb_3-type cytochrome oxidase supercomplex (PDB 6XKW), KdpFABC complex (PDB 5MRW), and *bd*-type quinol oxidase (PDB 6RX4). The small membrane protein subunit CcoQ of cbb_3-type cytochrome oxidase and the periplasmic domain of cytochrome c_y are not resolved in the current structure. The associated small proteins are displayed in red color.

supercomplex formation by providing a molecular tether [155]. It is important to emphasize that small membrane proteins not only lead to the structural stabilization of protein complexes, but also influence their activity directly. This has been shown for AcrZ, which stabilizes and allosterically regulates the AcrAB multidrug efflux pump [156,157] (Fig. 3). Another example is CydY, a small single-membrane subunit of *bd*-type quinol oxidases, which is proposed to regulate enzyme activity by blocking access of dioxygen to the catalytic site [158] (Fig. 3).

The role of small membrane proteins in co-factor insertion has been demonstrated for CydX, another small membrane subunit of the *E. coli bd*-type quinol oxidase [159], and CcoS, an assembly factor for cbb_3-type cytochrome oxidase [19,160]. CydX was shown to be required for the assembly of the di-heme catalytic site in *bd*-oxidase [158,161], while the absence of CcoS results in the formation of a cbb_3-type cytochrome oxidase that lacks the heme *b*-Cu_B catalytic site [18,19]. Despite their apparent similar role, CydX and CcoS do not show significant sequence similarity and they

might work by increasing the stability of assembly intermediates and thus by expanding the time window for co-factor insertion. Alternatively, by binding to co-factor-containing proteins, they might prevent the dissociation of noncovalently linked co-factors, such as heme *b* [18]. Small proteins also enable ferredoxin-dependent electron transfer to photosynthetic complex I in cyanobacteria. Here, the positively charged C terminus of NdhS likely traps ferredoxin, which in turn promotes electron transfer to the NdhI catalytic subunit [162].

The cellular machinery handling small proteins

Folding of small proteins

While the abundance and functional diversity of small proteins are slowly emerging, it is still largely unknown how folding and transport of these proteins occur within cells. Proteins usually fold into their unique native structures because the presence of multiple weak interactions overcomes the energy barrier of folding [163]. Due to the small number of possible intramolecular interactions, stable folding of small proteins *in vitro* is more difficult to obtain and usually requires the stabilization by disulfide bridges, metal ion chelation, or oligomerization [164–166]. NMR spectroscopy of 27 small proteins from 9 different bacterial and archaeal strains indeed demonstrated that most of them are unstructured or only partially folded [22]. However, bioinformatic predictions suggest an increased trend for acquiring a folded conformation upon intermolecular interactions [22]. For several AMPs, it is suggested that they obtain their α-helical structure upon the interaction with the anionic membrane surface or the hydrophobic lipid environment [43,167,168]. A recent study on Uperin 3.5, an AMP from the Australian toadlet *Uperoleia mjobergii*, demonstrated that a secondary structure switch in the presence of membranes is important for its antimicrobial activity [169]. The contribution of the lipid environment to folding is also observed for the small *E. coli* membrane protein YohP, which exists as monomer in solution but dimerizes in the membrane [114]. Dimerization is dependent on a single glycine residue, and the correct orientation of this residue for stable helix–helix interaction is obviously only obtained within the membrane [114].

Transport of small proteins

For proteins that have to be secreted across membranes or to be inserted into the membrane, the small size and the lack of secondary structures also pose a significant challenge for the cellular protein transport machinery. Proteins to be transported usually contain an N-terminal targeting motif, the so-called signal sequence or signal anchor sequence that directs proteins to protein transport channels located in the endoplasmic reticulum membrane of eukaryotes or in the cytoplasmic membrane of bacteria [40,77,170]. Signal sequences usually have a length of 25–30 amino acids and a tripartite structure. They consist of a positively charged N-region, a central hydrophobic H-region, and a polar C-terminal region that contains the signal peptidase cleavage site [171] (Fig. 4A). The signal sequence cleavage site is missing in signal anchor sequences of membrane proteins, and here, the signal anchor sequence serves also as transmembrane domain [172]. These signal sequences serve a dual function: They act as identification tags for protein targeting factors, and they are required for opening/gating the protein transport channels [173–178].

Two conceptionally different targeting systems operate in prokaryotic and eukaryotic cells, the co- and post-translational targeting pathways [42,179,180] (Fig. 4B). Co-translational targeting is mediated by the universally conserved signal recognition particle (SRP) and its membrane-bound receptor (SRαβ in eukaryotes or FtsY in prokaryotes) [179,181–183]. In eukaryotes, the SRP targeting system is responsible for the majority of soluble and membrane proteins destined to the ER, while in bacteria, the SRP pathway is preferentially used for inner membrane proteins [184–186]. The ribosome-bound SRP binds to its substrates as they emerge from the ribosomal peptide tunnel and targets the translating ribosome (ribosome-associated nascent chain, RNCs) to the trimeric Sec61 protein transport channel in the ER membrane or to the SecYEG protein transport channel in the bacterial membrane. In bacteria, the SRP pathway can target RNCs also to the YidC insertase, which serves as additional integration site for single-spanning membrane proteins and membrane proteins lacking large periplasmic domains [187–189]. Co-translational substrate recognition by SRP is, however, limited to substrates with a minimal length of 45–50 amino acids [190–193]. This is rationalized by the length of the ribosomal peptide tunnel, which shields ~ 35 amino acids, and the need for 10–15 hydrophobic amino acids outside of the ribosomal tunnel for stable SRP binding [190,192,194]. Thus, most small proteins in bacteria and eukaryotes are just too small to be co-translationally recognized by the SRP targeting system. The post-translational protein targeting pathways in prokaryotes and eukaryotes act on substrates after their release from the

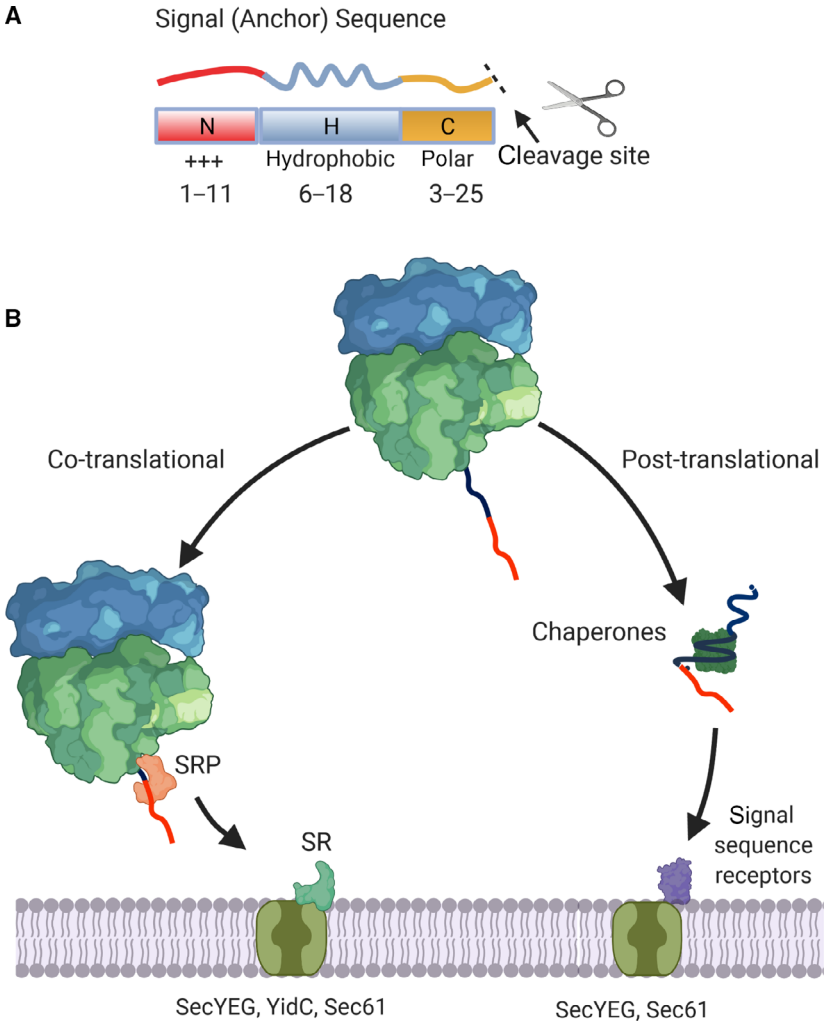

Fig. 4. Co-translational and post-translational targeting of membrane/secretory proteins. (A) The signal (anchor) sequence consists of three domains: the positively charged N-domain, the hydrophobic H-domain, and the polar C-terminal domain, which contains the signal peptidase cleavage site. The signal sequence of integral membrane proteins is called signal anchor sequence because the signal sequence serves as transmembrane domain and is not cleaved. (B) Co-translational targeting of proteins is mediated by the SRP in prokaryotes and eukaryotes. SRP targets the RNC to a free translocon in the inner membrane/ the ER membrane, where SRP binds to its receptor (SR). In prokaryotes, SRP targets primarily nascent membrane proteins, which are integrated into the cytoplasmic membrane via the SecYEG translocon or the YidC insertase. In eukaryotes, SRP targets both membrane proteins and secretory proteins to the Sec61 complex in the ER membrane. Post-translational targeting involves different chaperones, which keep the translated protein in a transport-competent state. The protein is then guided to a signal sequence receptor associated with the SecYEG translocon in prokaryotes or the Sec61 complex in eukaryotes for translocation.

ribosome (Fig. 4B). Substrates are kept in a translocation-competent state by cytosolic chaperones, before they are trapped by signal sequence receptors that are usually associated with the Sec61 complex in eukaryotes or the SecYEG translocon in bacteria.

Transport of small proteins in eukaryotes

Early studies on small secretory proteins in eukaryotes, such as prepromelittin, prepropeptide GLa, or prepro-cecropin A, had indeed shown that their targeting to the ER was independent of the SRP system and occurred post-translationally [11,195–197]. This was validated by the identification of several post-translational targeting systems for small secretory proteins. The post-translational translocation of the pre-CC2 chemokine and other small proteins of less than 100 amino acids was strictly dependent on Sec62 [198] (Fig. 5). Sec62 is an integral ER protein that serves as alternative signal sequence receptor and is also involved in Sec61 channel gating. In yeast, a permanently assembled complex for post-translational translocation exists. It is composed of the trimeric Sec61 complex and the heterotetrameric Sec62/Sec63/Sec71/Sec72 complex. This is different in mammals, where the assembly of Sec62/Sec63 with the trimeric Sec61 complex is substrate-dependent [42]. In addition to the Sec62-dependent transport, the transport of other small proteins in eukaryotes involves the post-translational GET pathway (guided entry of tail-anchored proteins) [199]. This pathway was initially identified as being responsible for targeting and insertion of tail-anchored proteins. These proteins cannot be co-translationally recognized by SRP, because their C-terminal transmembrane domain only becomes accessible after translation is terminated. In the absence of the GET pathway, targeting of at least one small protein, prestatherin, was shown to be dependent on the SND pathway (SRP-independent targeting), which operates in parallel to the SRP and GET

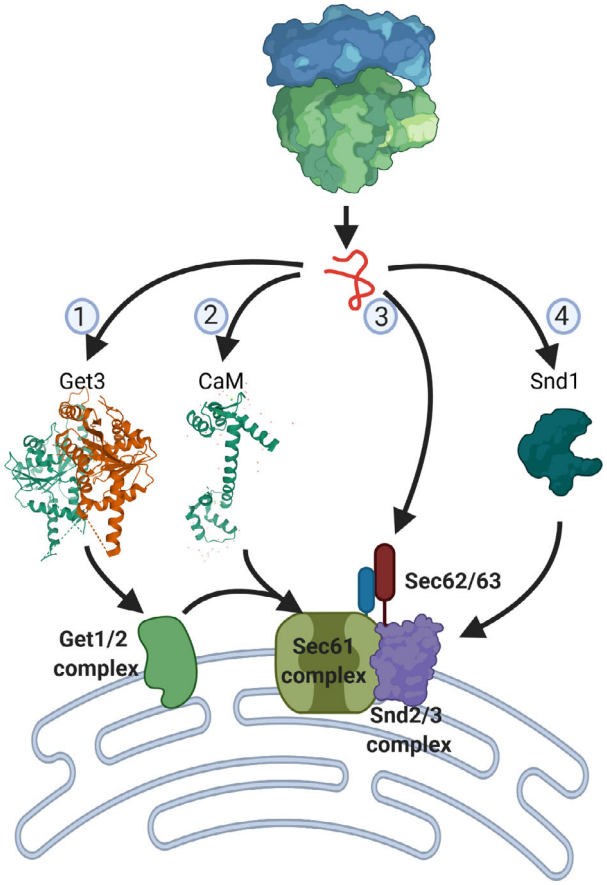

Fig. 5. Targeting and transport of small secretory proteins in eukaryotes. Small secretory proteins can engage multiple post-translational targeting pathways on their way to the endoplasmic reticulum membrane. (1) After their release from the ribosome, they are bound by the dimeric Get3–chaperone complex in the cytosol and delivered to the membrane-bound GET1/2 complex. They are then handed over to the Sec61 complex, which is the major protein transport channel in the ER membrane. The GET pathway is best characterized for its importance for the insertion of tail-anchored proteins. (2) Some small secretory proteins are bound by the cytosolic Ca^{2+}-binding protein calmodulin (CaM), which can deliver them directly to the Sec61 complex. (3) The Sec62/63 complex associates with the Sec61 complex and provides a membrane-bound signal sequence receptor for small secretory proteins. Furthermore, it is involved in channel gating of the Sec61 complex. (4) The SND pathway operates in parallel to the SRP and GET pathways. Proteins are recognized by the cytosolic targeting factor Snd1, which can also bind to the ribosome. Snd1 delivers its substrates to the membrane-bound SND2/3 complex, which can then associate with the Sec61 complex.

pathways in yeast and in mammalian cells [200–202]. Calmodulin (CaM)-dependent targeting has also been demonstrated for some small proteins, such as prepro-cecropin A or β-defensin 2 [203]. Here, CaM serves as a chaperone that binds in a sequence-dependent manner to the signal sequence and keeps the small protein in a translocation-competent conformation. Due to its ability to bind to the Sec61 complex [204], CaM is able to target small proteins to the Sec61 complex for transport.

The available data demonstrate that there is a significant heterogeneity in the targeting systems engaged by small secretory proteins in eukaryotes (Fig. 5), which probably reflects the diversity of signal sequences [205] and the large differences in hydrophobicity and charge within the mature region of small proteins [170]. Importantly, both Sec62 and CaM are important elements of eukaryotic stress–response pathways [206,207], which is probably critical for adapting small protein secretion to different metabolic conditions.

Transport of small proteins in prokaryotes

Bacterial AMPs also engage variable transport systems, including specific ABC transporter [208,209], or the SecYEG translocon [210]. AMPs are mainly translated as inactive precursor proteins, which become active upon protease cleavage [44]. In addition, some AMPs, such as lantibiotics, are post-translationally modified by incorporating thioether-based ring structures before they are transported via the ABC transporter LanT [211]. In combination with specific immunity proteins, these dedicated transport systems are important for protecting the producing cell against AMP-induced damage [212].

In contrast to eukaryotes and AMP secretion in prokaryotes, the mechanisms that target and transport small non-AMP proteins in bacteria are largely unknown. Considering that some small membrane proteins, such as YshB, are highly abundant during stress conditions or in stationary phase [113], specific targeting and transport systems likely exist. Early studies were focused on phage-derived small membrane proteins, and their targeting and insertion were initially considered to occur spontaneously [213,214]. However, later studies identified YidC as insertion site for these membrane proteins [189,215]. Targeting of the best-studied phage proteins, M13 procoat and PF3 coat, did not reveal any SRP dependency, unless their size was extended by fusing them to additional domains [216,217]. This also highlights a particular problem when studying targeting and transport of small proteins: Their detection is quite often based on fused protein tags that facilitate their immune detection [95,113]. However, adding those tags, which are sometimes larger than the protein itself, does not only hold the risk of interfering with function or intermolecular interactions, these tags will also increase the length of

a small protein. Considering a threshold level of 45 amino acids for SRP binding [190], adding a protein tag might well route a small membrane protein into the co-translational SRP pathway although it is not a native SRP substrate. One way to overcome this problem, while simultaneously utilizing a sensitive detection method, is radioactive labeling. This approach has been employed for small proteins both *in vitro*, using *in vitro* transcription/translation systems [114,185,218], and *in vivo*, using pulse labeling [114].

As discussed above, most small proteins in bacteria are too short to be co-translationally recognized by SRP, pointing to alternative targeting systems. One possibility is the involvement of the ATPase SecA. SecA is the main targeting factor for secretory proteins in bacteria [219,220] and delivers them to the SecYEG translocon for transport [174]. SecA mainly acts post-translationally [221,222], although it can also interact with RNCs [223–225]. So far, there are only few studies addressing the involvement of SecA in small protein targeting. For a 56-amino-acid-long truncated variant of the multispanning membrane protein YidC, a SecA-dependent transport was observed [218], while no SecA involvement was observed for native small proteins [114]. However, considering the diverse targeting requirements of eukaryotic small proteins [170], further small protein substrates need to be analyzed.

Unexpectedly, a site-directed cross-linking approach both *in vivo* and *in vitro* identified SRP as contact partner for two small membrane proteins in *E. coli*, YohP and YkgR [114]. Different to the canonical SRP interaction, these contacts were ribosome-independent, demonstrating that the bacterial SRP can recognize its substrates also post-translationally. Further biochemical analyses validated that SRP and its receptor FtsY were essential for post-translational insertion of YohP into the membrane. Liposome studies furthermore revealed that YohP insertion required either the SecYEG translocon or YidC in addition to SRP/FtsY [114]. This is in line with data showing that SRP can target simple membrane proteins to either the SecYEG translocon or the YidC insertase for insertion [187]. A possible post-translational role of SRP has also been shown in eukaryotic cells for tail-anchored proteins [226] and in chloroplasts during the integration of nuclear-encoded proteins into the thylakoid membrane [227,228]. This demonstrates that ribosome binding is not a prerequisite for SRP function.

The SRP/FtsY dependence of small membrane proteins in *E. coli* raises the question of their exact function during insertion. The requirement for the SRP receptor FtsY suggests that SRP does not execute a mere chaperone function for keeping small proteins in a transport-competent conformation. This assumption is supported by the observation that chaperones such as DnaK are unable to support YohP insertion [114]. The most straightforward role of SRP would be that it recognizes small membrane proteins in the cytosol after they have been released from the ribosome and then targets them to the SecYEG- or YidC-bound FtsY for insertion [187,229–231]. However, this scenario would depend on the presence of a significant pool of cytosolic SRP that is not bound to ribosomes. Considering the low abundance of SRP in prokaryotes [173,232] and its high affinity for ribosomes [233,234], the pool of non-ribosome-bound SRP is probably rather low. If SRP is primarily required for insertion, rather than for targeting small substrates from the cytosol to the membrane, then how do small proteins find the membrane?

One possible answer to this question comes from the observation that *in vivo* the majority of the YohP mRNA was found to be localized to the membrane [114] (Fig. 6). Membrane localization of the YohP mRNA was independent of the ribosome-binding site and also independent of any 5′- or 3′-UTRs, suggesting that the targeting information is retained with the coding sequence of *yohP*. Control mRNAs encoding cytosolic proteins did not show membrane enrichment [114]. The concept of mRNA targeting was first described for mRNAs encoding secretory proteins destined to the ER [235], but there is further evidence for specific mRNA targeting to different subcellular localizations in eukaryotes [236–238]. In prokaryotes, translation-independent targeting of mRNAs encoding for membrane proteins has also been described [239–242] and is probably linked to the higher uracil content of membrane protein-encoding mRNAs [243].

Although the molecular details of mRNA targeting in bacteria are still largely unknown, the current data suggest that bacteria use a rather unique targeting and insertion pathway for small membrane proteins. This pathway is initiated by an mRNA targeting step that delivers the mRNA to the membrane for subsequent translation (Fig. 6). Whether mRNA binding to the membrane requires a specific membrane-bound mRNA receptor is currently unknown. Both SecY and YidC specifically interact with ribosomal RNAs during co-translational insertion [244–247], demonstrating their intrinsic affinity for RNA. Furthermore, it was recently shown that RNA can adsorb unassisted onto phospholipid bilayers [248]. Thus, the presence of SecYEG and YidC and the lipid surface might in principle be sufficient for mRNA binding to the membrane. However, then the questions arise how specificity of mRNA targeting is achieved [242]. Uracil-rich mRNAs encoding for

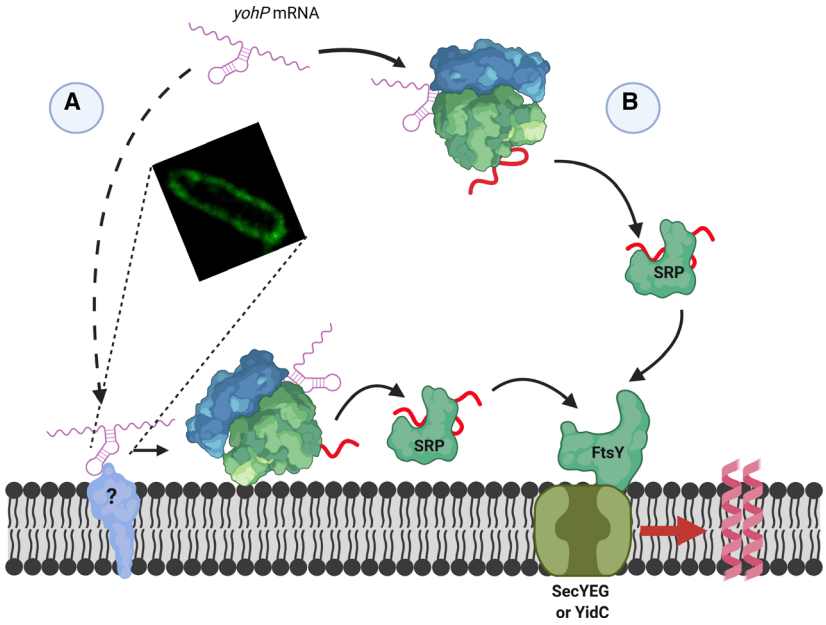

Fig. 6. Transport of small membrane proteins in bacteria. Targeting and transport of small membrane proteins have been mainly studied for YohP, an *E. coli* membrane protein of 27 amino acids. (A) The *yohP* mRNA is targeted translation-independently to the inner membrane of *E. coli* (inset). mRNA localization was determined by using the fluorescently labeled phage protein MS2, which binds to the MS2 recognition sequence that was fused to the 3′-end of the *yohP* mRNA [114]. The identity of the putative membrane-bound mRNA receptor is unknown. After the mRNA targeting step, the mRNA is translated by membrane-bound ribosomes and released from the ribosome. SRP binds to the translated YohP post-translationally and targets it to the SRP receptor FtsY, which can associate with either the SecYEG translocon or the YidC insertase. YohP is then inserted by either SecYEG or YidC into the membrane, where it dimerizes. (B) Some *yohP* mRNA is likely also bound by cytosolic ribosomes, which then synthesize YohP in the cytosol before it is also post-translationally targeted to the SecYEG- or YidC-bound FtsY for insertion.

membrane proteins have been shown to specifically interact with cold-shock proteins [249], which could confer a certain specificity on mRNA targeting. It is generally assumed that in bacteria, transcription and translation are coupled [250–252]. However, the small size of mRNAs encoding small proteins probably prevents such a coupled transcription/translation [252]. Thus, small mRNAs might escape ribosome binding in the cytosol and reach the membrane first, where they are then translated by membrane-bound ribosomes, which account for ~ 10–20% of all ribosomes in bacteria [253]. Translation at the membrane also offers the advantage that phospholipid surfaces promote secondary structure formation of small proteins, which would facilitate the subsequent recognition by SRP [254,255]. SRP would then primarily be required to deliver the small membrane protein to the next available and FtsY-primed protein transport channel, either SecYEG or YidC.

Outlook

Recent work on small proteins in prokaryotes and eukaryotes has provided a first glimpse into the largely unexplored biology of small proteins. The recognition that we are probably just looking at the tip of the small protein iceberg has ignited several initiatives for investigating the diversity and functional heterogeneity of small proteins. This included the first virtual conference on ‘Small proteins, big Questions’, organized by Gigi Storz, Joe Wade, Todd Gray, and Kai Papenfort in January 2021 and establishing a research priority program on ‘Small proteins in prokaryotes, an unexplored world’ by Ruth Schmitz-Streit and funded by the Deutsche Forschungsgemeinschaft (German Science Foundation, DFG). These initiatives will help to further establish the important function of small proteins in cell physiology. Several key areas of research on small proteins have been recently defined [21]. An additional key aspect is to understand why cells produce a large number of different small membrane proteins and how these proteins help to protect cells under unfavorable conditions. Considering the similarity between small membrane proteins and AMPs, why is the large production of small membrane proteins in most cases not causing membrane disruption? The information on the folding state of small

proteins and how folding is eventually implemented by protein–protein contacts requires detailed studies on the interactome of small proteins. This will also help to explore the diversity of protein trafficking systems that appear to be involved in handling small secretory protein in eukaryotes [170]. Small protein trafficking in prokaryotes has barely been touched on and is another focus for future research. Equally important is a better understanding about the evolution of small proteins. Did small membrane proteins evolve from duplicating coding sequences for transmembrane domains from multispanning membrane proteins? Alternatively, do they represent ancient building blocks for modern-day membrane proteins [256,257]? Many small proteins seem to act by modulating the activity or stability of other proteins/protein complexes, and the advantage over modulation by classical post-translational modifications is not entirely clear. In summary, studying small protein biology brings us back to fundamental questions of cell physiology and likely holds the premise for many future surprises.

Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG) to HGK (DFG grants KO2184/8; KO2184/9 (SPP2002); SFB1381, Project ID 403222702; and RTG 2202, Project-ID 278002225). Funding sources were not involved in the design of the article, writing the article, or decision to submit the article for publication. Figures were created with BioRender.com.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

RS and HGK designed the concept of the paper and wrote the paper.

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