

Review

Elucidating Protein Translocon Dynamics with Single-Molecule Precision

Madeline M. Davis,¹ Rajan Lamichhane,¹ and Barry D. Bruce^{1,2,3,4,*}

Translocons are protein assemblies that facilitate the targeting and transport of proteins into and across biological membranes. Our understanding of these systems has been advanced using genetics, biochemistry, and structural biology. Despite these classic advances, until recently we have still largely lacked a detailed understanding of how translocons recognize and facilitate protein translocation. With the advent and improvements of cryogenic electron microscopy (cryo-EM) single-particle analysis and single-molecule fluorescence microscopy, the details of how translocons function are finally emerging. Here, we introduce these methods and evaluate their importance in understanding translocon structure, function, and dynamics.

Single-Molecule Studies Are Achieving Key Insights into Translocon Structural Dynamics

Most studies of protein translocons have identified or characterized translocon components via genetic or biochemical approaches, including isotopic labeling and *in vitro* import assays [1,2], chemical cross-linking and circular dichroism [3], mutagenesis [4–6], and X-ray crystallography [7,8]. These are powerful tools for identifying and confirming the role of particular components, yet they are limited by measuring the bulk activity of a large ensemble and yield an average value, often providing poor temporal control, masking the nuances of transient complex interactions, and ultimately obscuring the mechanistic details of translocation. For several translocons, a lack of mechanistic resolution has led to the debate of multiple mechanistic models for both substrate recognition and motor domains that drive translocation [9,10]. However, the past two decades have brought significant advancements in single-molecule observations of protein structure and protein–protein interactions, allowing for the differentiation of multiple discrete states and the refining of previous mechanistic models [11–13].

Many of these advancements have been made using single-molecule fluorescence techniques, such as fluorescence resonance energy transfer (FRET) in conjunction with the high resolution and enhanced signal-to-noise ratio of total internal reflection fluorescence (TIRF) microscopy [14,15] (Table 1). Recent advances have also been made in single-particle cryogenic electron microscopy (cryo-EM), including improvements in direct electron detectors and computational analysis, which have allowed detailed structural characterizations of large protein structures at 2–5 Å resolutions [16,17]. In combination with native extraction and purification of membrane proteins, cryo-EM now stands among the most powerful tools for determining discrete structural states of individual proteins and protein complexes [16,17]. Additional methods, such as atomic force microscopy (AFM), enable force measurements of single-molecule events to provide detailed analysis of protein structure and folding dynamics [18,19]. Finally, single-channel ion conductivity studies can determine the substrate conditions of membrane protein channel opening and closure [20–22].

The general secretory (Sec) system of Gram-negative bacteria, such as *Escherichia coli* (SecYEG), stands among the best-characterized protein translocation pathways [23]. In

Highlights

Single-molecule Förster resonance energy transfer experiments have elucidated key details of the SecA-SecY translocation mechanism.

Structural details of both post- and co-translational mechanisms have been resolved by cryogenic electron microscopy (cryo-EM), including the conformation of the co-translational quaternary complex and a post-translational translocation intermediate.

Human disease mutants of Sec61 have been structurally resolved by cryo-EM, providing a foundation for understanding the role of the translocon-associated complex in some disorders.

Detailed structural analysis of high-resolution cryo-EM structures of Tom40, a mitochondrial translocation channel, has led to an updated mechanistic model for preprotein entry and exit.

Single-particle tracking has revealed the dynamics of plastid translocon components *in vivo*.

¹Department of Biochemistry and Cellular and Molecular Biology, University of Tennessee at Knoxville, Knoxville, TN 37996, USA

²Department of Microbiology, University of Tennessee at Knoxville, Knoxville, TN 37996, USA

³Graduate Program in Genome Science and Technology, University of Tennessee at Knoxville, Knoxville, TN 37996, USA

⁴Chemical and Biomolecular Engineering, University of Tennessee at Knoxville, Knoxville, TN 37996, USA

*Correspondence: bbruce@utk.edu (B.D. Bruce).

Table 1. Applications of Common Single-Molecule Methods

Method	Type(s) of resolution	Specific applications	Temporal resolution	Spatial, force, and/or electro-physiological resolution	Molecular context	Sample preparation
Single-molecule fluorescence microscopy	Spatial and temporal	FRET	≤ 100 ms [108,109]	≤ 1 nm [108]	Interdyer distances: intra- or intermolecular conformational dynamics	Usually purified and immobilized via tethering to slide
		Photo-activated localization microscopy (PALM)	< 10 s [110]	20–40 nm [111,112]	Localization or trajectory-based: diffusion and mobility states for single proteins or protein complexes in native context	Live- or fixed-cell imaging [112,113]
Single-particle transmission electron microscopy	Spatial	Cryo-EM	N/A	2–10 Å [17]	Global structure, <i>in vitro</i> : entire protein or protein complex structure outside of native environment	Purified and fixed via vitrification
		Cryogenic electron tomography (cryo-ET)		≤ 1 nm [114]	Global structure, <i>in situ</i> : entire protein or protein complex structure within cellular or organellar context	Fixed via vitrification
Single-molecule force spectroscopy	Spatial, force, and temporal	Optical tweezers (OT)	≥ 0.1 ms [115]	≥ 2 Å; ≥ 0.2 pN [115]	Force-induced responses: Intra- or intermolecular changes in conformation or processivity <i>in vitro</i>	Usually purified
		Atomic force microscopy (AFM)	< 2 μ s [116,117]	≥ 1 nm; ≤ 1 pN [117,118]	Force-induced responses: mechanical properties of proteins, typically protein folding and unfolding	Usually purified; can be reconstituted into artificial membranes
Single-channel conductivity	Electro-physiological and temporal	Patch-clamp	≤ 1 ms [119]	≤ 1 pA [120]	Single-channel conductance: kinetics of ion currents across membrane protein channels	Live whole-cell or purified and reconstituted into planar lipid bilayers

eukaryotic cells, the study of protein import has largely focused on Sec61 of the endoplasmic reticulum (ER), the major subunits of which are homologous to those of SecYEG (Box 1) [24–26]. Other eukaryotic translocons include TOM/TIM and TOC/TIC of the mitochondrial and chloroplast outer and inner membranes, respectively (Box 2) [9,27]. Here, we outline recent applications of single-molecule techniques to the study of these systems, with a particular emphasis on the bacterial SecYEG pathway.

Single-Molecule Studies of the Bacterial Sec System

Fluorescence Techniques Predominate Single-Molecule Studies of the Bacterial Translocon

While both soluble and membrane-targeted preproteins are translocated through SecYEG, these two classes are generally targeted to the translocon via separate pathways (Box 1). Soluble preproteins follow a post-translational route, in which a cytosolic chaperone, SecB, binds the nascent polypeptide chain and maintains its translocation-competent state. After translation is complete, SecB targets the preprotein to SecA, the ATPase motor of translocation that binds SecYEG to form the post-translational holotranslocon.

The localization and lateral mobility of SecA and SecYEG within their native membranes were observed by dual-color super-resolution photoactivated localization microscopy (PALM) [21]. PALM overcomes the resolution limit of light diffraction by stochastically activating individual fluorophores, thereby providing temporal separation of otherwise spatially indistinguishable fluorescent events [28,29]. This method revealed high levels of SecYEG-SecA colocalization and three distinct mobility states for SecA: (i) a rapid diffusion along the membrane, likely

corresponding to a scanning period of SecA in search of SecYEG; (ii) a short-lived, transiently mobile state corresponding to typical integral membrane protein lateral diffusion; and (iii) a long-lived immobile state that possibly represents SecA associating with the holotranslocon [26]. This approach also indicated a generally homogenous spatial distribution of the translocon and SecA along the plasma membrane, with the exception of possible active sites displaying higher concentrations. Notably, under these native expression conditions, SecYEG was not concentrated along cellular and membrane structures, and SecA was primarily membrane bound. Both of these observations are in contrast with previously spatial distributions observed under less native conditions, emphasizing the importance of *in vivo* studies using native expression systems [30,31].

Single-molecule FRET (smFRET) has been commonly utilized to interrogate the precise mechanistic role of SecA in post-translational translocation [32–37]. One hypothetical SecA mechanism has been called the ‘power-stroke’ model, in which a two-helix extension or ‘finger’ of ATP-bound SecA transiently and noncovalently interacts with amino acids on the preprotein to push it through the channel [34]. While bulk FRET assays previously informed this model [35], the high temporal and spatial resolution of smFRET was necessary for correlating the conformational changes of the power-stroke cycle with the corresponding stages of the SecA ATPase cycle [34]. These findings led to a current power-stroke hypothesis, which postulates that, upon ATP binding, the SecA two-helix finger (THF) pushes the preprotein into the SecY channel. ATP hydrolysis then prompts a retraction of the finger, while the preprotein is held in place by the SecA clamp domain. Phosphate release then loosens the clamp domain, and the preprotein passively slides through the complex until SecA binds another ATP molecule, and the cycle repeats.

Box 1. Conservation of Bacterial and Eukaryotic Sec Systems

While many bacterial translocons have been described [121], the Sec pathway is often characterized as the primary protein translocator of bacterial plasma membranes and is conserved across all life domains [24]. This universal conservation emphasizes the importance of protein transport systems in providing selective passage for proteins through cellular membranes. SecYEG of *E. coli* shares significant structural and mechanistic conservation with eukaryotic Sec61 of the ER membrane (Figure 1) [25,26].

The mechanistic similarities are perhaps strongest in the co-translational pathway, whereby transmembrane proteins are inserted into the bilayer (Figure 1) [122]. In both eukaryotes and bacteria, this process begins in the cytosol with the binding of the signal recognition particle (SRP) to the translating ribosome and to the nascent polypeptide, halting translation [123]. This complex is then received at the membrane by the SRP receptor (eukaryotes: ‘SR’; *E. coli*: ‘FtsY’), which guides the complex to the translocon. The ribosome is positioned above the translocon, and translation resumes. Transmembrane domains of the nascent polypeptide are inserted into the bilayer through a lateral gate within the translocon. Meanwhile, the SRP and its receptor are recycled back to their respective compartments for another round of targeting [123]. In eukaryotes, oligosaccharyltransferase (OST) associates with the co-translational translocon to glycosylate nascent polypeptides [64] and facilitates post-translocational glycosylation of soluble proteins within the lumen [124]. The role of a final component of the eukaryotic co-translational system, the translocon-associated protein (TRAP) complex, is not well understood [125].

Both translocons also facilitate post-translational translocation of soluble proteins (Figure 1) [25,26,126]. In bacteria, proteins destined for the periplasm are held in a translocation-competent state by chaperone SecB, which guides them to SecA, the ATPase motor of translocation. The nature of the role of SecA in protein insertion through the SecYEG channel has long been debated, but current models often include a combination of Brownian ratcheting and power-stroke processivity [35,37,127]. For eukaryotes, the post-translational pathway involves several cytosolic factors, including Sec62/63, Sec71, Sec72, and Hsp70 [62]. These factors maintain the protein substrate in a translocation-competent state and activate Sec61 for translocation [62].

For either route, substrate selectivity is provided by a signal peptide, which is cleaved by a signal peptidase (SPase) upon transport through the bilayer. However, a third pathway, represented by the membrane protein insertase YidC, can sometimes circumvent the signal peptide targeting system by directly facilitating membrane protein insertion (Figure 1A) [128]. YidC also associates with SecYEG during co-translational translocation [128].

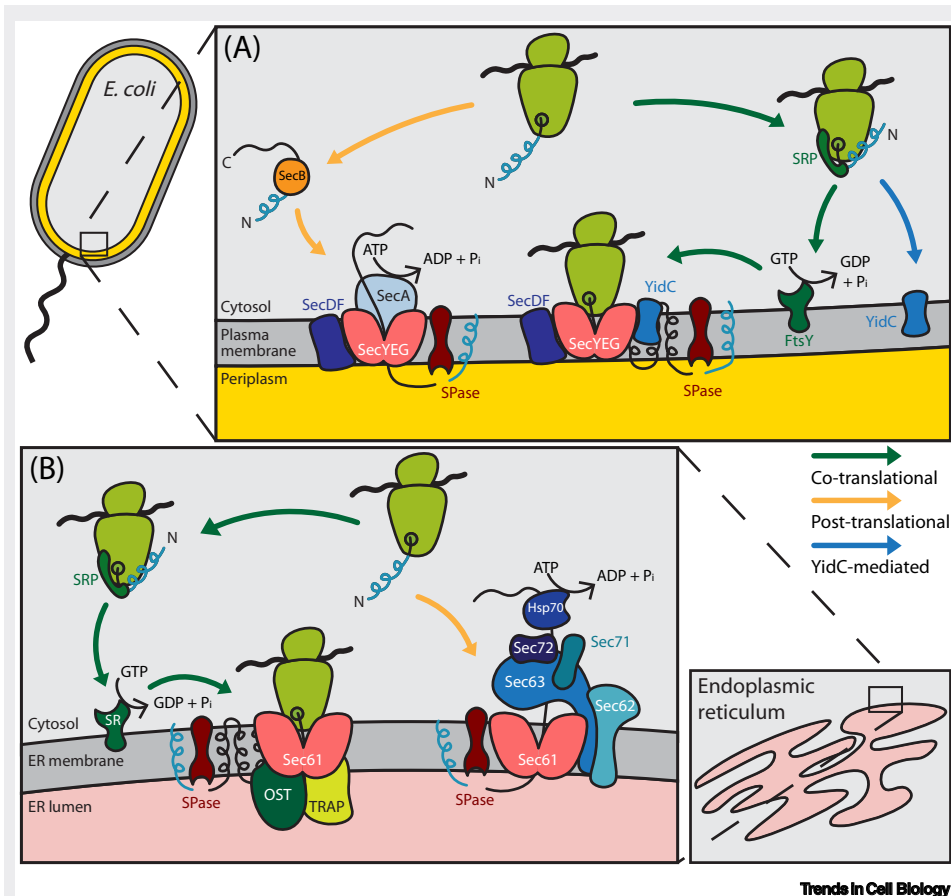


Figure 1. Mechanistic Models for Sec-Mediated Protein Translocation. (A) Model for translocation through bacterial SecYEG. (B) Model for translocation through eukaryotic Sec61. Signal peptide represented by blue segment of nascent polypeptide chain. Green arrows indicate co-translational pathways. Yellow arrows indicate post-translational pathways. Blue arrow indicates the YidC-mediated pathway. Abbreviations: *E.coli*, *Escherichia coli*; ER, endoplasmic reticulum; OST, oligosaccharyltransferase; SPase, signal peptidase; SRP, signal recognition particle; TRAP, translocon-associated protein complex.

smFRET has also informed a ‘Brownian-ratchet’ model for the SecA mechanism, in which the passive diffusion of the translocated protein through the translocon channel in the forward and reverse directions is transiently limited by blockages in the channel. These blockages are thought to bias the movement of the protein toward the forward direction [36]. Further smFRET experiments monitored movements of the cytoplasmic plug domain of the translocon throughout the SecA ATP cycle, providing an updated Brownian-ratchet model: (i) signal sequence-dependent but ATP-independent, ‘unlocking’ or opening of the translocon; (ii) ATP-dependent plug opening; (iii) a pre-processive translocon stage; (iv) ATP-dependent, forward processive translocation; and (v) ATP-independent, fast channel closing [37] (Figure 1). Although these models vary in their emphasis on the processivity of translocation, both have been refined and strengthened by the ability of smFRET to synchronize individual ATP hydrolysis events into a structurally and kinetically coordinated mechanism.

Preproteins destined for the inner plasma membrane generally follow an alternative, co-translational route to SecYEG (Box 1). In this pathway, transmembrane domains (TMDs) in the nascent chain act

as signal sequences for the GTPase signal recognition particle (SRP), which binds these domains, halts translation, and targets the ribosomal nascent chain complex (RNC) to the GTPase SRP receptor FtsY in the inner membrane [26]. After intercepting the RNC-SRP complex, FtsY coordinates the arrangement of the co-translational holotranslocon, positioning the RNC so that the ribosome exit tunnel is aligned directly above the cytosolic face of SecY. smFRET results suggest that the 4.5S RNA moiety of the SRP serves as a molecular scaffold for the conformational shift of the SRP-FtsY GTPase complex [38,39]. This coordination primes FtsY for GTP hydrolysis and for handing off the RNC to SecYEG. The discovery of this dramatic conformational shift, ~ 100 Å, also revealed that large RNA molecules can serve as molecular scaffolds for the transfer of proteins within complex cellular mechanisms, contributing to the number of known modes of nucleic acid-mediated protein movement.

Following the insertion of the nascent chain into the translocation channel, translation resumes and the TMDs of the nascent chain are inserted laterally into the bilayer through a gate in the SecY pore. In addition to the co-translational quaternary complex (formed by the RNC, SRP, SR, and SecYEG), a membrane protein insertase (YidC) can associate with SecYEG to aid and facilitate protein translocation (Box 1). YidC has also been shown to independently insert small, hydrophobic membrane proteins into the bilayer [40]. Natively expressed YidC was imaged *in vivo* by super-resolution fluorescence microscopy, and single-particle tracking of the resulting images resolved three discrete mobility states: one fast-moving and two slow-moving subpopulations [26]. These distinct diffusion rates likely correspond to one 'free' YidC state and two associated states, each of which probably represents a different holotranslocon composition. These differing associated states support the idea that the holotranslocon varies its subunit composition situationally [26].

Cryogenic Electron Microscopy and Tomography

Cryo-EM has also arisen as a powerful technique for single-particle analysis, following remarkable innovations in direct electron detection and advances in computational analysis [13,16,41,42]. This high-resolution method has been used extensively to study the bacterial Sec system [43–47],

Box 2. Organellar Protein Import Pathways

The general import pathway of plastid protein import involves two major translocase complexes: TOC and TIC of the outer and inner membranes, respectively (Figure 1A) [9,129]. Although these complexes may vary in composition depending on plastid maturity or subtype [130], most photosynthesis-related preproteins are initially perceived by Toc34 and Toc159, preprotein receptors of the outer chloroplast membrane [131,132]. These receptors coordinate their GTPase cycles to bind transit peptides, the N-terminal targeting sequences of plastid-targeted preproteins, and subsequently transfer them to the translocation pore of beta-barrel Toc75 [133]. In the intermembrane space, Toc75 and Tic22 chaperone the passage of the transit peptide to the inner membrane, where the preprotein is intercepted by the TIC complex [134,135]. The inner-membrane channel is at least partially formed by Tic20, which is putatively scaffolded by Tic110 and Tic40. Tic110 also scaffolds the heat shock protein (Hsp) ATPase motors (i.e., cpHsp70, Hsp90C, and Hsp93) that drive import into the stroma to completion [136,137]. Upon reaching the stroma, the transit peptide is cleaved by the stromal processing peptidase (SPP), and the mature domain of the imported protein carries out its plastidial function [138].

Five pathways for mitochondrial protein import have been described, each recognizing a different kind of targeting sequence [27]. Most mitochondrial proteins are imported via the classic import pathway (Figure 1B) [139], in which N-terminal, amphipathic-helix targeting signals, called presequences, are intercepted preferentially by Tom20, one of three receptors at the TOM complex [140], although Tom22 or Tom70 can also act as the initial receptor [141]. Electrostatic interactions are hypothesized to stabilize the passage of presequences through the beta-barrel channel of Tom40 [90]. Upon arrival at the inner membrane, Tim23 and Tim50 of the TIM23 complex bind the presequence. The preprotein is then imported into the matrix through the Tim23 channel, which is closely associated with another integral membrane translocase, Tim17 [142]. Peripheral membrane protein Tim44 links the TIM23 complex with the presequence translocase-associated motor (PAM) complex, the primary motor subunit of which, mtHsp70, hydrolyzes ATP to complete import. Upon entry into the matrix, the presequence is cleaved by the mitochondrial processing peptidase (MPP) [138].

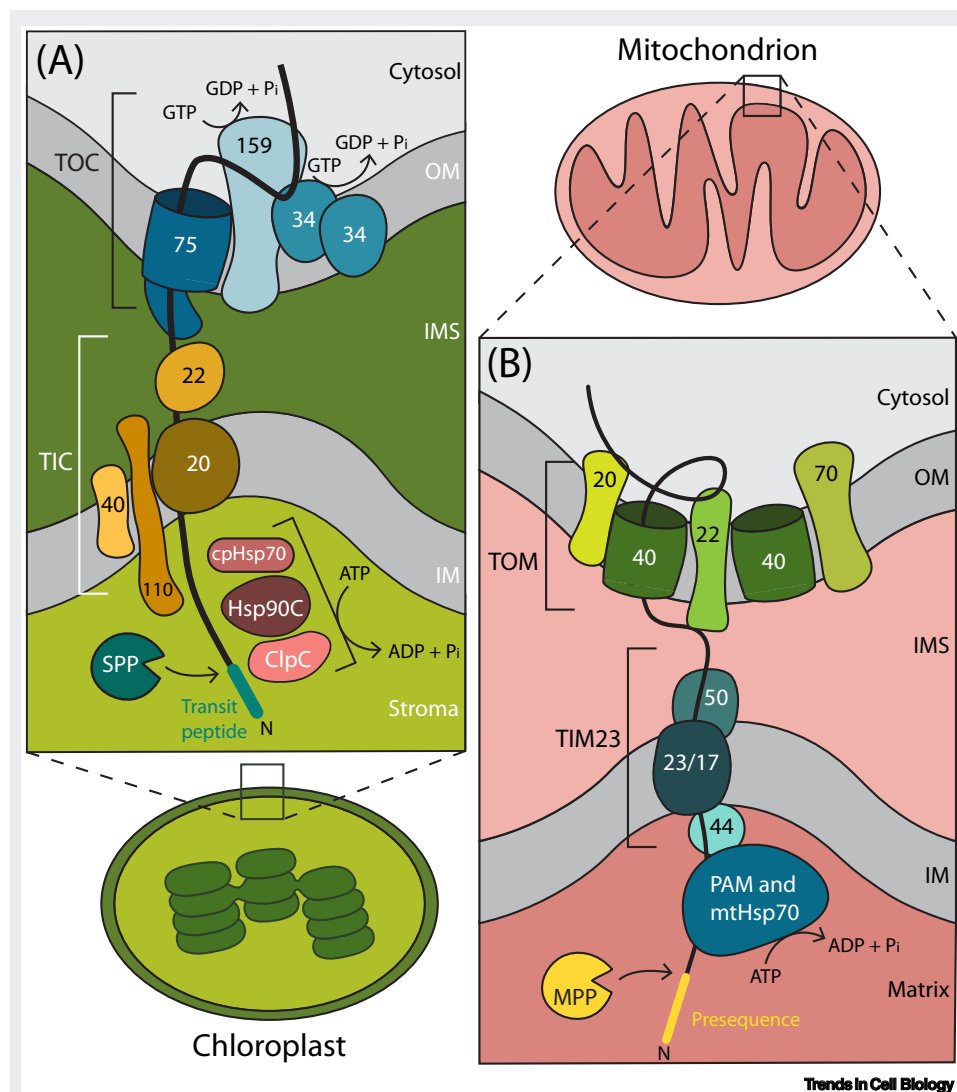
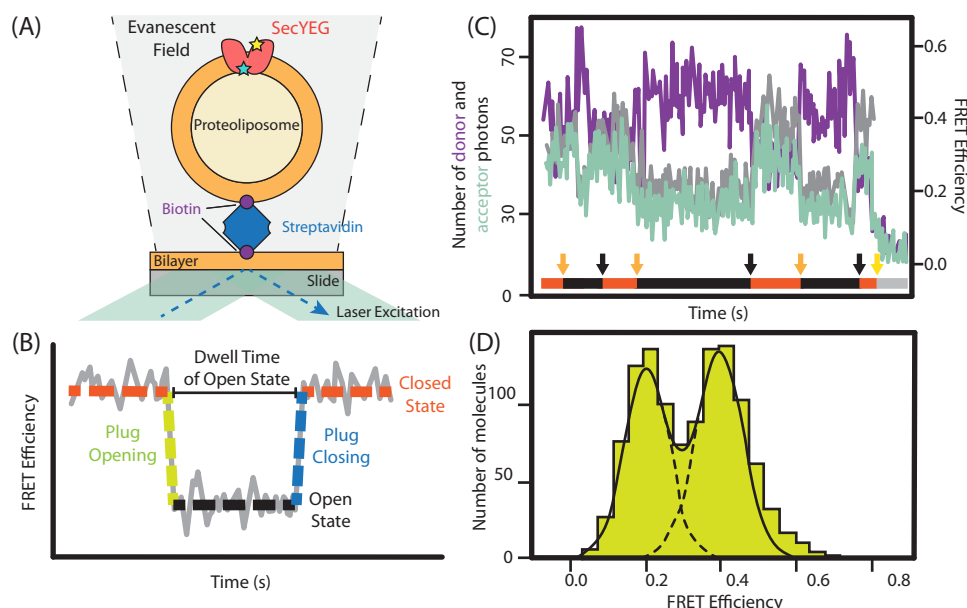


Figure 1. Models for Protein Import into Chloroplasts and Mitochondria. (A) Model for protein import into chloroplasts via the general import pathway. (B) Model for mitochondrial protein import through the classic import pathway. Abbreviations: IM, inner membrane; IMS, intermembrane space; OM, outer membrane; MPP, mitochondrial processing peptidase; SPP, stromal processing peptidase.

including an interrogation of the structure of the co-translational quaternary complex [48]. Resolved to a final average of 4.8 Å, this structure reveals the conformation of the SRP-FtsY-RNC complex in its activated state, as well as the localization of the translocon and the signal sequence of the nascent chain within the quaternary complex [48]. These results led to the proposal of a cargo handover mechanism of co-translational targeting that aligns with the smFRET results mentioned earlier, in which the transfer of the nascent chain to the translocon requires movement of the FtsY/SRP complex from the tetraloop end to the distal end of the SRP RNA moiety [38,39]. Cryogenic electron tomography (cryo-ET), which varies the angle of electron interrogation to create 3D reconstructions, has also been used to study membrane protein insertion via YidC [49]. In combination with high-resolution solid-state NMR, cryo-ET was used to compare RNC binding to YidC in its native membrane to RNC binding with purified YidC reconstituted into synthetic



Trends in Cell Biology

Figure 1. Single-Molecule Fluorescence Resonance Energy Transfer (smFRET) Analysis of SecY Channel Opening. (A) Immobilization scheme of a SecYEG proteoliposome to a glass slide for total internal reflection fluorescence (TIRF) microscopy. The translocon is fluorescently labeled with donor and acceptor dyes at the mobile plug domain (blue star) and at a stationary cytosolic loop (yellow star). The laser beam is reflected back through the slide, creating an evanescent field directly adjacent to the slide surface, which excites the donor dye. (B) Predicted FRET efficiency states corresponding to plug movements throughout translocation. A starting closed-channel state (red) corresponds to high FRET efficiency. The plug domain moves farther away from the cytosolic loop during translocation initiation (green), leading to a low-efficiency, open-channel state (black). After translocation, the channel rapidly closes (blue) and the FRET efficiency returns to closed-state levels. (C) Fluorescence intensity traces for translocation of preprotein through a single SecYEG translocon. Purple and green traces correspond to donor and acceptor emissions, respectively, while the gray trace represents the FRET efficiency. Beginning in a closed state (red line), the translocon initiates transport (orange arrows) and remains in an open state throughout translocation (black line). Post translocation, the channel closes (black arrow) again. This cycle repeats with varying duration of open and closed states until one of the fluorescent dyes is bleached (yellow arrow, gray line). (D) A histogram reporting the FRET efficiencies of 300 individual translocation events. Adapted from [37].

membranes. The prevalence of RNC binding was higher for YidC in its native membrane than for reconstituted YidC, supporting the conclusion that native membranes provide a better setting for examining membrane proteins [49].

Cryo-EM has also been used to study the structure of post-translational translocon complexes [50] (Figure 2). SecA-SecY complexes were reconstituted into membrane scaffold protein (MSP)-supported lipid nanodiscs for cryo-EM analysis, resulting in a 3.5-Å density map that revealed the position of the translocated polypeptide throughout the entire translocation channel (i.e., from the entry point at SecA in the cytosol to its exit site on the extracellular side of the SecY channel) [50]. The positioning of the preprotein in relation to the SecA THF and clamp domains in this structure is consistent with previous smFRET data [34]. Both studies support a SecA transition state in which the clamp on the polypeptide and the THF is not closely interacting with the polypeptide during retraction [34,50].

Single-Molecule Force Measurements of Bacterial Translocation Components

Single-molecule force spectroscopy, including optical tweezers and AFM, has also had an important role in the investigation of bacterial protein transport. A novel ‘pull-and-paste’ atomic force method was introduced to study the translocon-independent insertase role of YidC [51]. In this

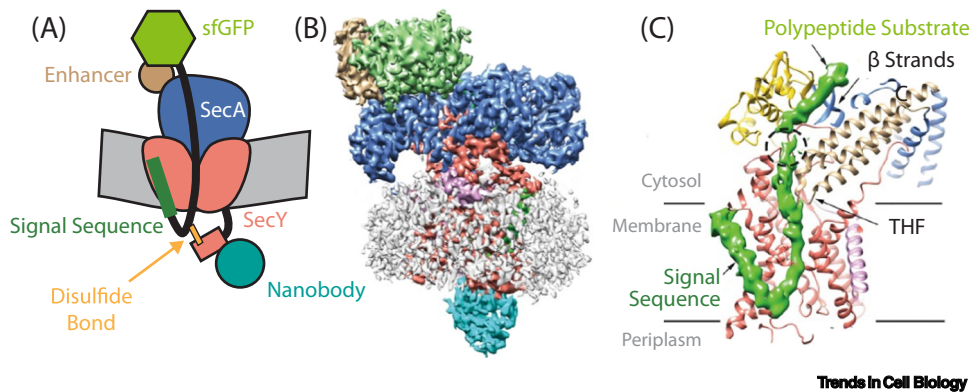


Figure 2. Cryo-Electron Microscopy (Cryo-EM) Structure of SecA-SecY Engaged with Imported Polypeptide. (A) Model of the translocation complex. Super-folder GFP (sfGFP, green) is partially imported by the SecA-SecY complex and cross-linked (disulfide bond, orange) to the SecY plug domain (pink rectangle) just past its signal sequence (dark green). (B) Density map of the translocon and SecA engaged with the sfGFP substrate (lipid nanodisc in gray). (C) Ribbon model emphasizing the position of the imported preprotein within the translocation channel, the two-helix finger of SecA, and the beta strands of the clamp domain. Adapted from [50]. Abbreviation: THF, two-helix finger.

method, a known YidC substrate, lactose permease (LacY), could be transiently tethered to the AFM cantilever tip by a C-terminal polyglycine tail. Thus, a single LacY molecule from one membrane-mimetic layer was transferred to another layer containing YidC, where the now unfolded LacY bound and folded into the membrane with the same characteristics as native LacY. By contrast, transfer to ‘empty’ membrane mimetic layers failed to result in correct LacY binding and folding. These single-molecule reconstitution results support the independent function of YidC as a membrane protein insertase and present a new, high-resolution method for investigating the dynamics of membrane protein folding [51].

Force measurements have also given insight into the structural dynamics of the secretion mechanism, particularly regarding SecA and SecYEG. Single-molecule AFM was used to compare wild-type SecA with a mutant lacking its protein-binding domain (PBD), allowing for the synchronization of the ATPase cycle with PBD conformational shifts [52]. The results suggest that, in a nearly native environment, ATP-driven dynamics of SecA are largely attributable to PBD motion, although other segments of SecA contribute to these dynamics during the ATP hydrolysis transition state. AFM was also used to analyze the oligomeric status of SecYEG while translocating various precursors [53]. These data suggest that SecYEG stoichiometry varies depending on the imported precursor. While these data are discordant with other studies of the stoichiometry of the active translocon [26,54], they raise an interesting possibility that could be tested in live cells for specific precursors. The AFM data further suggested that SecYEG is more likely to be active if its subunits (SecY, SecE, and SecG) assemble in the presence of SecA [53]. The results suggest that subunit assembly in the presence of known binding partners leads to higher activity of other protein complexes. These findings all point to the ability of AFM to provide near-native experimental conditions for the observation of individual translocation, protein-folding, and conformation events.

Single-Channel Ion Conductivity Measurements of SecYEG Channel Opening

Single-molecule electrophysiological assays have also been used to study the bacterial translocon. Single-channel ion conductivity data suggest that the RNC alone can fully open the SecYEG channel and that binding to an ‘empty’ ribosome lacking a nascent polypeptide results in partial opening [55]. Additionally, isolated signal peptides were unable to open the channel, suggesting that the ribosome and/or the act of translation are the primary modulators of pore opening. A later approach used complementary biophysical techniques, including single-

channel ion conductivity and high-speed AFM assays, to independently confirm the presence of two discrete SecA THF insertion states, further refining and strengthening the power-stroke model of post-translational translocation [56].

Single-Molecule Studies of Sec61

In eukaryotes, proteins are translocated across the ER membrane through Sec61, the three major subunits of which are homologous to the constituents of the SecYEG heterotrimer [25,26]. The function of the SecY and SecE subunits of SecYEG, corresponding to Sec61 α and Sec61 γ , respectively, are universally conserved, such that Sec61 also facilitates the co-translational insertion of ER-targeted membrane proteins or the post-translational translocation of soluble proteins into the ER lumen [57] (Box 1).

The structure of the eukaryotic translocon has been thoroughly investigated by cryo-EM and cryo-ET in various complexed states [58–67], including the native structure of the co-translational ribosome-Sec61 complex found in isolated canine ER vesicles [68]. Resolved to ~9 Å, two ‘noninserting’ states, either empty ribosomes or those occupied by tRNAs and translocating soluble proteins, were revealed by subtomogram averaging [68]. Regardless of the noninserting state, the only observed conformation of the Sec61 lateral gate was the open conformation. These findings are consistent with a previously proposed, low-energy ‘sliding’ model [69], which suggests that a constitutively open lateral gate in the ribosome-Sec61 complex allows hydrophobic TMDs to pass directly into the bilayer, while soluble domains preferentially move into the ER lumen through the main channel. The sub-nanometer resolution of these structures strengthens the sliding model by providing highly detailed structural information in a native, ER-vesicle setting [68].

Cryo-ET was also used to resolve structural differences between disease-linked mutants of the translocon-associated protein (TRAP) complex (Box 1), as well as TRAP complexes from evolutionarily divergent organisms [70]. These comparative structures allowed for the placement of the four TRAP subunits within the assembled mammalian complex structure, providing a basis for understanding the role of TRAP in glycosylation disorders [70].

The membrane protein insertion role of Sec61 has also been interrogated by cryo-EM [71]. The structure of a purified complex representing a translocation intermediate (in which the signal peptide has been engaged by the Sec61 lateral gate but has not yet been cleaved) was resolved to 3.6 Å and compared with other, previously defined Sec61 states to examine the conformational changes that accompany Sec61 channel opening [71]. These findings informed a molecular model for the gating of the Sec61 channel by hydrophobic signal sequences, which begins with the binding of the ribosome to Sec61, a priming event for further transmembrane signal sequence engagement. This binding weakens key contact sites of the lateral gate closure. Subsequently, a nearby hydrophobic region attracts the hydrophobic signal sequence, which displaces a TM helix to access the lipid bilayer. Together, these single-particle analyses by cryo-EM have provided detailed structural snapshots of multiple translocation states, allowing for updated models of co-translational eukaryotic translocation.

Single-channel electrophysiological data have also provided detailed information on the effect of Sec61-binding partners on channel opening and closure. For example, single-channel Ca²⁺ conductance recordings in planar lipid bilayers suggested a role for Sec61 in maintaining Ca²⁺ homeostasis [72,73]. Initially, two major conductance populations were shown for Sec61 channels, demonstrating that Sec61 is permeable to Ca²⁺ ions [72]. The calcium-binding protein calmodulin was also shown to associate with the Sec61 channel, and further single-channel

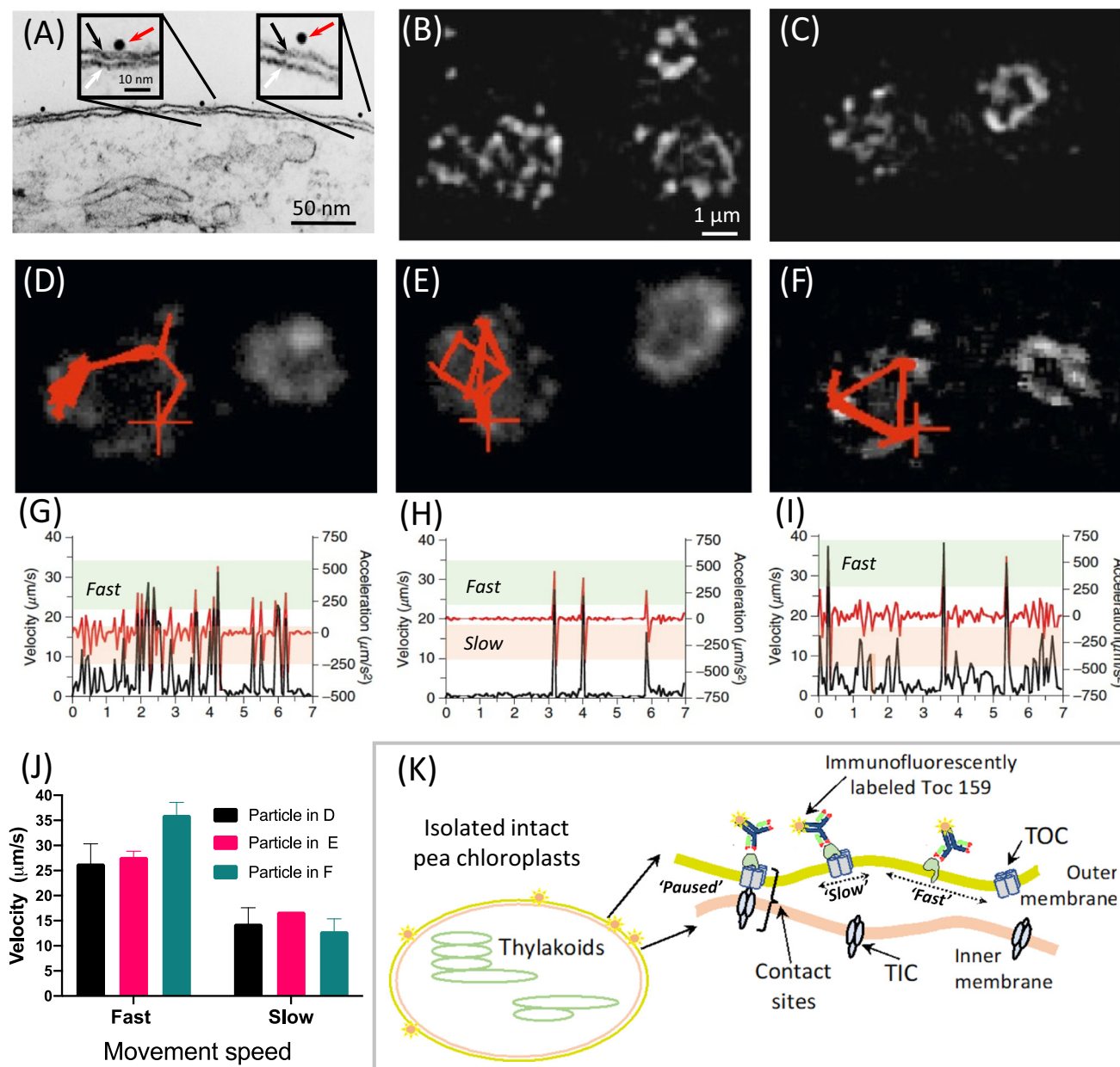
recordings suggested that calmodulin stymies Ca^{2+} leakage from the ER by mediating Sec61 channel closure [73]. A similar electrophysiological approach revealed that the Sec61 channel closes either spontaneously or can be induced to close by associating with binding immunoglobulin protein (BiP) [73–75], and the involvement of BiP in closing the Sec61 channel has been observed *in vivo* [74]. These results helped generate a model to describe the gating of the Sec61 channel, where the binding of a precursor signal peptide to the closed Sec61 complex triggers channel opening, either directly by altering the channel diameter or indirectly by binding to BiP on the luminal surface [76–79].

Single-Molecule Studies of Organellar Protein Import Systems

While the prokaryotic and eukaryotic secretion systems allow passage across a single bilayer, proteins entering organelles of endosymbiotic origin must traverse two bilayers to reach the organelle interior. This requirement led to the development of a dual system in which translocons of the outer membrane (TOM or TOC) coordinate with those in the inner membrane (TIM or TIC) of mitochondria and chloroplasts, respectively, at closely associated regions known as ‘contact sites’ (Box 2) [80,81]. For either organelle, the initial step of translocation is the recognition of signal sequences by outer membrane receptors [9,27].

In the TOC complex of plastids, a small GTPase, Toc34, acts as a signal sequence receptor. smFRET analyses revealed the conformational states of Toc34 in the presence and absence of signal peptides and various guanine nucleotides [82]. In the absence of nucleotide and signal sequence, Toc34 rested as a GDP-bound homodimer. However, the addition of signal peptide prompted a slight opening of the dimeric interface, and addition of GTP or its analog resulted in further opening to an active, monomeric state. These findings suggest that signal peptide binding is a prerequisite to nucleotide exchange and have informed a revised model of the TOC import mechanism. TIRF microscopy and single-particle tracking were used to analyze the mobility of another chloroplast signal peptide receptor, Toc159, in isolated chloroplast membranes [83] (Figure 3). Bound preproteins were visualized using immunogold labeling of the precursor and 5-nm gold particles coupled to Protein A. To complement the static nature of immunogold labeling, whole-chloroplast TIRF imaging was used to observe single Toc159 molecules by immunofluorescent labeling. Single-particle tracking revealed three distinct mobility states: (i) rapid movement of $\sim 30 \mu\text{m/s}$; (ii) a slower movement of $\sim 14 \mu\text{m/s}$; and (iii) no movement. A hypothetical correlation of these mobility states with Toc159 signal recognition states is depicted in Figure 3K. Single-channel conductance recordings have also provided insight into TOC complex function by revealing cation selectivity, transit-peptide specificity, a hydrophilic core, and two pore diameters for Toc75, a beta-barrel that forms the translocation pore [84,85]. Cryo-EM structures of TOC complexes isolated from native membranes will be needed to better understand the dynamic associations of these proteins, and further single-molecule fluorescence experiments could help elucidate the native mobility of more translocon components by using a dual- or multicolor approach.

Mitochondrial protein import has also been investigated by single-molecule methods, including cryo-EM and single-channel electrophysiology. Among the earliest structural dynamics studies, single- and multiple-channel recordings showed the cation selectivity and voltage-gated opening and closure of the beta-barrel Tom40, which serves as the translocation channel of the outer membrane [86,87]. These early electrophysiological data approximated a pore diameter of $\sim 16 \text{ \AA}$, an estimate that is well in line with current TOM complex structures resolved by cryo-EM [88]. Comparative analysis of a TOM structure resolved to $6.8 \text{ \AA}$ [88] with previous structural characterizations [89] determined that the TOM complex structure is conserved between *Neurospora crassa* and *Saccharomyces cerevisiae*, suggesting conservation of mitochondrial import mechanisms



Trends in Cell Biology

Figure 3. Single-Particle Tracking of Translocon Subunits Using Transmission Electron Microscopy (TEM) and Total Internal Reflection Fluorescence (TIRF) Microscopy. (A) TEM micrograph of immunogold-labeled, bound preprotein (small subunit of Rubisco) trapped on the outer envelope of isolated pea chloroplasts with low ATP levels. Black arrows indicate outer envelope; white arrows indicate inner envelope; and red arrows indicate a 5-nm gold particle. (B,C) Immunofluorescent detection using TIRF imaging of an Alexa 488-immunolabeled Toc159 subunit on the outer surface of isolated pea chloroplasts. Note the lack of chlorophyll autofluorescence. (D–F) Single-particle tracking (in red) of individual, Toc159-labeled particles for 7 s. (G–I) The velocity and acceleration of the single particle in the trace immediately above [for (D–F), respectively]. The particle shows three behaviors: 'fast' movement, with velocities observed within the upper green box; a 'slow' moving particle, with velocities observed within the pink box; and periods of no movement or 'paused' mobility. (J) The mean and standard deviation of the 'fast' and 'slow' velocities for the particles observed in (D–F). (K) One possible model to reflect the location and interactions resulting in the varying behavior the single Toc159 particles observed in (D–E). Derived from [83] (B–I).

across fungi and yeast. The location of each subunit within its resolved structure was also identified, and an updated molecular model of import across the outer mitochondrial membrane was devised. More recent cryo-EM analysis provided even greater resolution for dimeric (3.1 Å) and tetrameric (4.1 Å) TOM core complexes from yeast [90]. These structures showed that negatively charged residues in the Tom40 pore may have an important mechanistic role by attracting positively charged residues in the presequences of imported preproteins [90]. The path of imported proteins through Tom40 was also resolved by cryo-EM, which found distinct exit sites for presequence-containing and presequence-lacking preproteins [91]. Future single-molecule assays, such as smFRET, could clarify the structural dynamics of these exit sites throughout translocation.

The protein import machineries of other eukaryotic organelles have also been characterized, including the Pex1/Pex6 complex of peroxisomes. Single-particle cryo-EM analysis resolved the structure of this AAA-ATPase complex to 7.3 Å, revealing a unique, alternating arrangement of the two subunits in a double ring [92].

To the best of our knowledge, this handful of studies comprise the majority of single-molecule studies of protein import into eukaryotic organelles (apart from the Sec61 pathway into the ER). Although organellar protein import remains under-represented in the realm of single-molecule study, we remain optimistic that future studies will use the high resolution of such techniques to investigate further details of their mechanisms and functions, such as has been revealed for the bacterial Sec system.

Concluding Remarks

Taken together, these findings clearly demonstrate the ability of single-molecule methods to reveal new details of translocon functional and structural dynamics. Yet, these translocation systems all hold questions of interest in common (see Outstanding Questions). From an energetics perspective, the true cost of protein transport (in terms of ATP molecules per translocated aminoacyl residue) remains unclear, considering the wide-ranging estimates that have been reported [93,94]. A second line of inquiry demanding further study is the basis of specificity for the recognition and binding of targeting sequences (e.g., signal peptides, presequences, and transit peptides) to their respective translocons. Many data have been reported on the intrinsic motifs within these sequences that act as cellular zip codes for protein delivery systems, yet the molecular mechanisms of targeting sequence recognition are less well understood [95,96]. A final area of study that will further benefit from single-molecule work is the role of translocons in the folding and assembly of membrane proteins, especially multispanning integral membrane proteins.

Looking forward, future single-molecule methodologies will likely be applied to specific, translocation-related problems in the medical, agricultural, and biotechnological industries. For example, given that several human disease mutations are deficient in Sec61 translocation [97], and that the Sec61 complex is a target for various pathogen-derived toxins [98], single-molecule work will almost certainly be used in therapeutic drug design or the creation of pathogen-resistant structural variants [60,99,100]. Similarly, disruption of mitochondrial protein import has also been linked to different human diseases, including neurodegenerative disorders [101,102]. While cryo-EM and cryo-ET have resolved the structure of the core TOM complex, further structural determination of disease-linked mutants could offer aid in therapeutical research efforts.

Further study of plastid protein translocation is also relevant to a range of areas, from the potential control of *Plasmodium*, the non-green-plastid-containing organism that causes malaria [103], to the possibility of engineering plastid protein import for novel crop improvement strategies [104]. One such strategy would increase resistance to plant pathogens in which the type-III virulence

Outstanding Questions

What is the basis of specificity for the recognition of targeting sequences (signal peptides, presequences, and transit peptides) by the translocons?

How do translocons function in the folding and assembly of proteins, especially multispanning transmembrane proteins?

What is the true energetic cost of preprotein translocation, and what is the mechanism for force generation?

How does a cell utilize and regulate the dual targeting of proteins to mitochondria and plastids?

effectors reside in chloroplasts [105]. Finally, further single-molecule work in the field of bacterial protein secretion may lead to advances in recombinant protein expression, which is foundational to many areas of biotechnology and pharmaceutical drug design. One such advancement might be engineering the ability to control the synthesis rate of a recombinant secretory system to increase the capacity of the cell for the production of hormones and other small molecules [106]. Similarly, research has been undertaken to increase the ability of protein synthesis and secretion in Gram-positive bacteria, such as *Bacillus subtilis*, an industrial strain important for the production of many commercially relevant proteins [107]. We look forward to the application of single-molecule techniques in these areas, which emphasize the important roles of protein translocation in medical research, agricultural and plant sciences, and applied innovations in biotechnology.

Acknowledgments

Support provided from the Gibson Family Foundation, the Dr Donald L. Akers Faculty Enrichment Fellowship, and the National Science Foundation (EPS-1004083) support to B.D.B. In addition, M.M.D. and B.D.B. have been supported via a JDRD Award from UTK/ORNL Science Alliance. M.M.D. has been supported as a GATE Fellow from the Science Alliance at University of Tennessee, Knoxville. Imaging on the Nikon TIRF and JEOL 1400-Flash TEM was provided by the AMIC at University of Tennessee, Knoxville with support from ORE SARIF funds and the National Science Foundation (MRI-1828300), respectively. R.L. was supported from New Lab Start-Up funds from the College of Arts and Sciences, University of Tennessee, Knoxville.

Declaration of Interests

The authors declare no competing interests.

References

1. Blobel, G. and Dobberstein, B. (1975) Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. *J. Cell Biol.* 67, 835–851
2. Hessa, T. *et al.* (2007) Molecular code for transmembrane-helix recognition by the Sec61 translocon. *Nature* 450, 1026–1030
3. Driessen, A.J. (1993) SecA, the peripheral subunit of the *Escherichia coli* precursor protein translocase, is functional as a dimer. *Biochemistry* 32, 13190–13197
4. Saparov, S.M. *et al.* (2007) Determining the conductance of the SecY protein translocation channel for small molecules. *Mol. Cell* 26, 501–509
5. Baba, T. *et al.* (1990) Characterization of cold-sensitive secY mutants of *Escherichia coli*. *J. Bacteriol.* 172, 7005–7010
6. Baba, T. *et al.* (1994) A cytoplasmic domain is important for the formation of a SecY-SecE translocator complex. *Proc. Natl. Acad. Sci. U. S. A.* 91, 4539–4543
7. Dekker, C. *et al.* (2003) Crystal structure of SecB from *Escherichia coli*. *J. Struct. Biol.* 144, 313–319
8. Zimmer, J. *et al.* (2008) Structure of a complex of the ATPase SecA and the protein-translocation channel. *Nature* 455, 936–943
9. Schnell, D.J. (2019) The TOC GTPase receptors: regulators of the fidelity, specificity and substrate profiles of the general protein import machinery of chloroplasts. *Protein J.* 38, 343–350
10. Denks, K. *et al.* (2014) The Sec translocon mediated protein transport in prokaryotes and eukaryotes. *Mol. Membr. Biol.* 31, 58–84
11. Hashemi Shabestari, M. *et al.* (2017) Recent advances in biological single-molecule applications of optical tweezers and fluorescence microscopy. *Methods Enzymol.* 582, 85–119
12. Ott, W. *et al.* (2017) Single-molecule force spectroscopy on polypeptides and receptor-ligand complexes: The current toolbox. *J. Struct. Biol.* 197, 3–12
13. Koning, R.J. *et al.* (2018) Advances in cryo-electron tomography for biology and medicine. *Ann. Anat.* 217, 82–96
14. Algar, W.R. *et al.* (2019) FRET as a biomolecular research tool - understanding its potential while avoiding pitfalls. *Nat. Methods* 16, 815–829
15. Krainer, G. *et al.* (2019) Structural dynamics of membrane-protein folding from single-molecule FRET. *Curr. Opin. Struct. Biol.* 58, 124–137
16. Bai, X.C. *et al.* (2015) How cryo-EM is revolutionizing structural biology. *Trends Biochem. Sci.* 40, 49–57
17. Cheng, Y. (2018) Single-particle cryo-EM-How did it get here and where will it go. *Science* 361, 876–880
18. Thoma, J. *et al.* (2018) Single-molecule force spectroscopy of transmembrane beta-barrel proteins. *Annu. Rev. Anal. Chem. (Palo Alto, Calif)* 11, 375–395
19. Ando, T. (2018) High-speed atomic force microscopy and its future prospects. *Biophys. Rev.* 10, 285–292
20. Weatherill, E.E. and Wallace, M.I. (2015) Combining single-molecule imaging and single-channel electrophysiology. *J. Mol. Biol.* 427, 146–157
21. Knyazev, D.G. *et al.* (2020) Voltage sensing in bacterial protein translocation. *Biomolecules* 10, 78
22. Dabney-Smith, C. *et al.* (1999) The C terminus of a chloroplast precursor modulates its interaction with the translocation apparatus and PIRAC. *J. Biol. Chem.* 274, 32351–32359
23. Tsirigotaki, A. *et al.* (2017) Protein export through the bacterial Sec pathway. *Nat. Rev. Microbiol.* 15, 21–36
24. Stephenson, K. (2005) Sec-dependent protein translocation across biological membranes: evolutionary conservation of an essential protein transport pathway (review). *Mol. Membr. Biol.* 22, 17–28
25. Park, E. and Rapoport, T.A. (2012) Mechanisms of Sec61/SecY-mediated protein translocation across membranes. *Annu. Rev. Biophys.* 41, 21–40
26. Seinen, A.B. and Driessen, A.J.M. (2019) Single-molecule studies on the protein translocon. *Annu. Rev. Biophys.* 48, 185–207
27. Wiedemann, N. and Pfanner, N. (2017) Mitochondrial machineries for protein import and assembly. *Annu. Rev. Biochem.* 86, 685–714
28. Coelho, S. *et al.* (2020) Ultraprecise single-molecule localization microscopy enables in situ distance measurements in intact cells. *Sci. Adv.* 6, eaay8271
29. Bayle, V. *et al.* (2021) Single-particle tracking photoactivated localization microscopy of membrane proteins in living plant tissues. *Nat. Protoc.* 16, 1600–1628

30. Adachi, S. *et al.* (2015) Dynamic nature of SecA and its associated proteins in *Escherichia coli*. *Front. Microbiol.* 6, 75
31. Cabelli, R.J. *et al.* (1991) Characterization of membrane-associated and soluble states of SecA protein from wild-type and SecA51(TS) mutant strains of *Escherichia coli*. *J. Biol. Chem.* 266, 24420–24427
32. Ernst, I. *et al.* (2018) Large conformational changes of a highly dynamic pre-protein binding domain in SecA. *Commun. Biol.* 1, 130
33. Vandenberg, N. *et al.* (2019) The preprotein binding domain of SecA displays intrinsic rotational dynamics. *Structure* 27, 90–101
34. Catipovic, M.A. *et al.* (2019) Protein translocation by the SecA ATPase occurs by a power-stroke mechanism. *EMBO J.* 38, e101140
35. Bauer, B.W. *et al.* (2014) A ‘push and slide’ mechanism allows sequence-insensitive translocation of secretory proteins by the SecA ATPase. *Cell* 157, 1416–1429
36. Allen, W.J. *et al.* (2016) Two-way communication between SecY and SecA suggests a Brownian ratchet mechanism for protein translocation. *Elife* 5, e15598
37. Fessl, T. *et al.* (2018) Dynamic action of the Sec machinery during initiation, protein translocation and termination. *Elife* 7, e35112
38. Shen, K. *et al.* (2012) Activated GTPase movement on an RNA scaffold drives co-translational protein targeting. *Nature* 492, 271–275
39. Shen, K. *et al.* (2013) Molecular mechanism of GTPase activation at the signal recognition particle (SRP) RNA distal end. *J. Biol. Chem.* 288, 36385–36397
40. Kedrov, A. *et al.* (2013) Elucidating the native architecture of the YidC: ribosome complex. *J. Mol. Biol.* 425, 4112–4124
41. Lyumkis, D. (2019) Challenges and opportunities in cryo-EM single-particle analysis. *J. Biol. Chem.* 294, 5181–5197
42. McMullan, G. *et al.* (2016) Direct electron detectors. *Methods Enzymol.* 579, 1–17
43. Kater, L. *et al.* (2019) Partially inserted nascent chain unzips the lateral gate of the Sec translocon. *EMBO Rep.* 20, e48191
44. Botte, M. *et al.* (2016) A central cavity within the holo-translocon suggests a mechanism for membrane protein insertion. *Sci. Rep.* 6, 38399
45. Park, E. *et al.* (2014) Structure of the SecY channel during initiation of protein translocation. *Nature* 506, 102–106
46. Jomaa, A. *et al.* (2016) Structures of the *E. coli* translating ribosome with SRP and its receptor and with the translocon. *Nat. Commun.* 7, 10471
47. Frauenfeld, J. *et al.* (2011) Cryo-EM structure of the ribosome-SecYE complex in the membrane environment. *Nat. Struct. Mol. Biol.* 18, 614–621
48. Jomaa, A. *et al.* (2017) Structure of the quaternary complex between SRP, SR, and translocon bound to the translating ribosome. *Nat. Commun.* 8, 15470
49. Baker, L.A. *et al.* (2018) Combined (1)H-detected solid-state NMR spectroscopy and electron cryotomography to study membrane proteins across resolutions in native environments. *Structure* 26, 161–170
50. Ma, C. *et al.* (2019) Structure of the substrate-engaged SecA-SecY protein translocation machine. *Nat. Commun.* 10, 2872
51. Serdiuk, T. *et al.* (2017) Pull-and-paste of single transmembrane proteins. *Nano Lett.* 17, 4478–4488
52. Chada, N. *et al.* (2018) Single-molecule observation of nucleotide induced conformational changes in basal SecA-ATP hydrolysis. *Sci. Adv.* 4, eaat8797
53. Mao, C. *et al.* (2013) Stoichiometry of SecYEG in the active translocase of *Escherichia coli* varies with precursor species. *Proc. Natl. Acad. Sci. U. S. A.* 110, 11815–11820
54. Taufik, I. *et al.* (2013) Monitoring the activity of single translocons. *J. Mol. Biol.* 425, 4145–4153
55. Knyazev, D.G. *et al.* (2013) The bacterial translocon SecYEG opens upon ribosome binding. *J. Biol. Chem.* 288, 17941–17946
56. Winkler, K. *et al.* (2020) Interaction of the motor protein SecA and the bacterial protein translocation channel SecYEG in the absence of ATP. *Nanoscale Adv.* 2, 3431–3443
57. Pohlschroder, M. *et al.* (1997) Protein translocation in the three domains of life: variations on a theme. *Cell* 91, 563–566
58. Bai, L. *et al.* (2018) The atomic structure of a eukaryotic oligosaccharyltransferase complex. *Nature* 555, 328–333
59. Wild, R. *et al.* (2018) Structure of the yeast oligosaccharyltransferase complex gives insight into eukaryotic N-glycosylation. *Science* 359, 545–550
60. Gerard, S.F. *et al.* (2020) Structure of the inhibited state of the Sec translocon. *Mol. Cell* 79, 406–415
61. Gogala, M. *et al.* (2014) Structures of the Sec61 complex engaged in nascent peptide translocation or membrane insertion. *Nature* 506, 107–110
62. Wu, X. *et al.* (2019) Structure of the post-translational protein translocation machinery of the ER membrane. *Nature* 566, 136–139
63. Itskanov, S. and Park, E. (2019) Structure of the posttranslational Sec protein-translocation channel complex from yeast. *Science* 363, 84–87
64. Braunger, K. *et al.* (2018) Structural basis for coupling protein transport and N-glycosylation at the mammalian endoplasmic reticulum. *Science* 360, 215–219
65. Shanmuganathan, V. *et al.* (2019) Structural and mutational analysis of the ribosome-arresting human XBP1u. *Elife* 8, e46267
66. O'Donnell, J.P. *et al.* (2020) The architecture of EMC reveals a path for membrane protein insertion. *Elife* 9, e57887
67. McGilvray, P.T. *et al.* (2020) An ER translocon for multi-pass membrane protein biogenesis. *Elife* 9, e56889
68. Pfeffer, S. *et al.* (2015) Structure of the native Sec61 protein-conducting channel. *Nat. Commun.* 6, 8403
69. Cymer, F. *et al.* (2015) Mechanisms of integral membrane protein insertion and folding. *J. Mol. Biol.* 427, 999–1022
70. Pfeffer, S. *et al.* (2017) Dissecting the molecular organization of the translocon-associated protein complex. *Nat. Commun.* 8, 14516
71. Voorhees, R.M. and Hegde, R.S. (2016) Structure of the Sec61 channel opened by a signal sequence. *Science* 351, 88–91
72. Lang, S. *et al.* (2011) Sec61 complexes form ubiquitous ER Ca²⁺ leak channels. *Channels (Austin)* 5, 228–235
73. Erdmann, F. *et al.* (2011) Interaction of calmodulin with Sec61alpha limits Ca²⁺ leakage from the endoplasmic reticulum. *EMBO J.* 30, 17–31
74. Schauble, N. *et al.* (2012) BiP-mediated closing of the Sec61 channel limits Ca²⁺ leakage from the ER. *EMBO J.* 31, 3282–3296
75. Hassdenteufel, S. *et al.* (2018) Chaperone-mediated Sec61 channel gating during ER import of small precursor proteins overcomes Sec61 inhibitor-reinforced energy barrier. *Cell Rep.* 23, 1373–1386
76. Dudek, J. *et al.* (2015) Protein transport into the human endoplasmic reticulum. *J. Mol. Biol.* 427, 1159–1175
77. Pfeffer, S. *et al.* (2016) Organization of the native ribosome-translocon complex at the mammalian endoplasmic reticulum membrane. *Biochim. Biophys. Acta* 1860, 2122–2129
78. Zimmermann, R. (2016) Components and Mechanisms of import, modification, folding, and assembly of immunoglobulins in the endoplasmic reticulum. *J. Clin. Immunol.* 36, 5–11
79. Lang, S. *et al.* (2012) Different effects of Sec61alpha, Sec62 and Sec63 depletion on transport of polypeptides into the endoplasmic reticulum of mammalian cells. *J. Cell Sci.* 125, 1958–1969
80. Inoue, K. (2011) Emerging roles of the chloroplast outer envelope membrane. *Trends Plant Sci.* 16, 550–557
81. Horvath, S.E. *et al.* (2015) Role of membrane contact sites in protein import into mitochondria. *Protein Sci.* 24, 277–297
82. Lumme, C. *et al.* (2014) Nucleotides and substrates trigger the dynamics of the Toc34 GTPase homodimer involved in chloroplast preprotein translocation. *Structure* 22, 526–538
83. Reddick, L.E. *et al.* (2008) Nano-scale characterization of the dynamics of the chloroplast Toc translocon. *Methods Cell Biol.* 90, 365–398
84. Hinnah, S.C. *et al.* (2002) The chloroplast protein import channel Toc75: pore properties and interaction with transit peptides. *Biophys. J.* 83, 899–911
85. Hinnah, S.C. *et al.* (1997) Reconstitution of a chloroplast protein import channel. *EMBO J.* 16, 7351–7360
86. Kunkele, K.P. *et al.* (1998) The preprotein translocation channel of the outer membrane of mitochondria. *Cell* 93, 1009–1019
87. Kunkele, K.P. *et al.* (1998) The isolated complex of the translocase of the outer membrane of mitochondria. Characterization of the

- cation-selective and voltage-gated preprotein-conducting pore. *J. Biol. Chem.* 273, 31032–31039
88. Bausewein, T. *et al.* (2017) Cryo-EM structure of the TOM core complex from *Neurospora crassa*. *Cell* 170, 693–700 e7
 89. Model, K. *et al.* (2008) Cryo-electron microscopy structure of a yeast mitochondrial preprotein translocase. *J. Mol. Biol.* 383, 1049–1057
 90. Tucker, K. and Park, E. (2019) Cryo-EM structure of the mitochondrial protein-import channel TOM complex at near-atomic resolution. *Nat. Struct. Mol. Biol.* 26, 1158–1166
 91. Araisio, Y. *et al.* (2019) Structure of the mitochondrial import gate reveals distinct preprotein paths. *Nature* 575, 395–401
 92. Blok, N.B. *et al.* (2015) Unique double-ring structure of the peroxisomal Pex1/Pex6 ATPase complex revealed by cryo-electron microscopy. *Proc. Natl. Acad. Sci. U. S. A.* 112, E4017–E4025
 93. Shi, L.X. and Theg, S.M. (2013) Energetic cost of protein import across the envelope membranes of chloroplasts. *Proc. Natl. Acad. Sci. U. S. A.* 110, 930–935
 94. Alder, N.N. and Theg, S.M. (2003) Energy use by biological protein transport pathways. *Trends Biochem. Sci.* 28, 442–451
 95. Holbrook, K. *et al.* (2016) Functional analysis of semi-conserved transit peptide motifs and mechanistic implications in precursor targeting and recognition. *Mol. Plant* 9, 1286–1301
 96. Owji, H. *et al.* (2018) A comprehensive review of signal peptides: structure, roles, and applications. *Eur. J. Cell Biol.* 97, 422–441
 97. Lang, S. *et al.* (2017) An update on Sec61 channel functions, mechanisms, and related diseases. *Front. Physiol.* 8, 887
 98. Paatero, A.O. *et al.* (2016) Apratoxin kills cells by direct blockade of the Sec61 protein translocation channel. *Cell Chem. Biol.* 23, 561–566
 99. Yotsu, R.R. *et al.* (2018) Buruli ulcer: a review of the current knowledge. *Curr. Trop. Med. Rep.* 5, 247–256
 100. Demangel, C. and High, S. (2018) Sec61 blockade by mycolactone: a central mechanism in Buruli ulcer disease. *Biol. Cell* 110, 237–248
 101. Gunewald, A. *et al.* (2019) New insights into the complex role of mitochondria in Parkinson's disease. *Prog. Neurobiol.* 177, 73–93
 102. Harbauer, A.B. *et al.* (2014) The protein import machinery of mitochondria—a regulatory hub in metabolism, stress, and disease. *Cell Metab.* 19, 357–372
 103. Boucher, M.J. and Yeh, E. (2019) Disruption of apicoplast biogenesis by chemical stabilization of an imported protein evades the delayed-death phenotype in malaria parasites. *mSphere* 4, e00710-18
 104. Sadali, N.M. *et al.* (2019) Differentiation of chromoplasts and other plastids in plants. *Plant Cell Rep.* 38, 803–818
 105. Li, G. *et al.* (2014) Distinct *Pseudomonas* type-III effectors use a cleavable transit peptide to target chloroplasts. *Plant J.* 77, 310–321
 106. Karyolaimos, A. *et al.* (2019) *Escherichia coli* can adapt its protein translocation machinery for enhanced periplasmic recombinant protein production. *Front. Bioeng. Biotechnol.* 7, 465
 107. Neef, J. *et al.* (2020) Relative contributions of non-essential Sec pathway components and cell envelope-associated proteases to high-level enzyme secretion by *Bacillus subtilis*. *Microb. Cell Factories* 19, 52
 108. Holden, S.J. *et al.* (2010) Defining the limits of single-molecule FRET resolution in TIRF microscopy. *Biophys. J.* 99, 3102–3111
 109. Lerner, E. *et al.* (2018) Toward dynamic structural biology: two decades of single-molecule Förster resonance energy transfer. *Science* 359, eaan1133
 110. Deschout, H. *et al.* (2016) Complementarity of PALM and SOFI for super-resolution live-cell imaging of focal adhesions. *Nat. Commun.* 7, 13693
 111. Galbraith, C.G. and Galbraith, J.A. (2011) Super-resolution microscopy at a glance. *J. Cell Sci.* 124, 1607–1611
 112. Shen, H. *et al.* (2017) Single particle tracking: from theory to biophysical applications. *Chem. Rev.* 117, 7331–7376
 113. Henriques, R. *et al.* (2011) PALM and STORM: unlocking live-cell super-resolution. *Biopolymers* 95, 322–331
 114. Sanchez, R.M. *et al.* (2020) Subnanometer-resolution structure determination in situ by hybrid subtomogram averaging – single particle cryo-EM. *Nat. Commun.* 11, 3709
 115. Zhang, X. *et al.* (2013) High-resolution optical tweezers for single-molecule manipulation. *Yale J. Biol. Med.* 86, 367–383
 116. Rico, F. *et al.* (2013) High-speed force spectroscopy unfolds titin at the velocity of molecular dynamics simulations. *Science* 342, 741–743
 117. Edwards, D.T. and Perkins, T.T. (2017) Optimizing force spectroscopy by modifying commercial cantilevers: Improved stability, precision, and temporal resolution. *J. Struct. Biol.* 197, 13–25
 118. Heinisch, J.J. *et al.* (2012) Atomic force microscopy – looking at mechanosensors on the cell surface. *J. Cell Sci.* 125, 4189–4195
 119. Gezelle, J. *et al.* (2021) Promises and pitfalls of parasite patch-clamp. *Trends Parasitol.* Published online February 24, 2021. <https://doi.org/10.1016/j.pt.2021.02.002>
 120. Shlyonsky, V. and Gall, D. (2019) The OpenPicoAmp-100k: an open-source high-performance amplifier for single channel recording in planar lipid bilayers. *PLoS Arch.* 471, 1467–1480
 121. Green, E.R. and Mecsas, J. (2016) Bacterial secretion systems: an overview. *Microbiol. Spectr.* 4, VMBF-0012-2015
 122. Nyathi, Y. *et al.* (2013) Co-translational targeting and translocation of proteins to the endoplasmic reticulum. *Biochim. Biophys. Acta* 1833, 2392–23402
 123. Akopian, D. *et al.* (2013) Signal recognition particle: an essential protein-targeting machine. *Annu. Rev. Biochem.* 82, 693–721
 124. Ruiz-Canada, C. *et al.* (2009) Cotranslational and posttranslational N-glycosylation of polypeptides by distinct mammalian OST isoforms. *Cell* 136, 272–283
 125. Russo, A. (2020) Understanding the mammalian TRAP complex function(s). *Open Biol.* 10, 190244
 126. Cranford-Smith, T. and Huber, D. (2018) The way is the goal: how SecA transports proteins across the cytoplasmic membrane in bacteria. *FEMS Microbiol. Lett.* 365, fry093
 127. Gouridis, G. *et al.* (2013) Quaternary dynamics of the SecA motor drive translocase catalysis. *Mol. Cell* 52, 655–666
 128. Kiefer, D. and Kuhn, A. (2018) YidC-mediated membrane insertion. *FEMS Microbiol. Lett.* 365, fry106
 129. Richardson, L.G.L. and Schnell, D.J. (2020) Origins, function, and regulation of the TOC-TIC general protein import machinery of plastids. *J. Exp. Bot.* 71, 1226–1238
 130. Dutta, S. *et al.* (2014) A split-ubiquitin yeast two-hybrid screen to examine the substrate specificity of atToc159 and atToc132, two Arabidopsis chloroplast preprotein import receptors. *PLoS ONE* 9, e95026
 131. Schlieff, E. *et al.* (2002) Structural and guanosine triphosphate/diphosphate requirements for transit peptide recognition by the cytosolic domain of the chloroplast outer envelope receptor, Toc34. *Biochemistry* 41, 1934–1946
 132. Smith, M.D. *et al.* (2004) atToc159 is a selective transit peptide receptor for the import of nucleus-encoded chloroplast proteins. *J. Cell Biol.* 165, 323–334
 133. Chotewutmontri, P. *et al.* (2017) Plastid protein targeting: preprotein recognition and translocation. *Int. Rev. Cell Mol. Biol.* 330, 227–294
 134. Rudolf, M. *et al.* (2013) In vivo function of Tic22, a protein import component of the intermembrane space of chloroplasts. *Mol. Plant* 6, 817–829
 135. O'Neil, P.K. *et al.* (2017) The POTRA domains of Toc75 exhibit chaperone-like function to facilitate import into chloroplasts. *Proc. Natl. Acad. Sci. U. S. A.* 114, E4868–E4876
 136. Kovacheva, S. *et al.* (2007) Further in vivo studies on the role of the molecular chaperone, Hsp93, in plastid protein import. *Plant J.* 50, 364–379
 137. Kovacheva, S. *et al.* (2005) In vivo studies on the roles of Tic110, Tic40 and Hsp93 during chloroplast protein import. *Plant J.* 41, 412–428
 138. Teixeira, P.F. and Glaser, E. (2013) Processing peptidases in mitochondria and chloroplasts. *Biochim. Biophys. Acta* 1833, 360–370
 139. Vogtle, F.N. *et al.* (2009) Global analysis of the mitochondrial N-proteome identifies a processing peptidase critical for protein stability. *Cell* 139, 428–439
 140. Abe, Y. *et al.* (2000) Structural basis of presequence recognition by the mitochondrial protein import receptor Tom20. *Cell* 100, 551–560
 141. Yamano, K. *et al.* (2008) Tom20 and Tom22 share the common signal recognition pathway in mitochondrial protein import. *J. Biol. Chem.* 283, 3799–3807
 142. Demishtein-Zohary, K. *et al.* (2015) GxxxG motifs hold the TIM23 complex together. *FEBS J.* 282, 2178–2186