Chapter 4
Functions and Mechanisms of the Human Ribosome-Translocon Complex

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Abstract The membrane of the endoplasmic reticulum (ER) in human cells harbors the protein translocon, which facilitates membrane insertion and translocation of almost every newly synthesized polypeptide targeted to organelles of the secretory pathway. The translocon comprises the polypeptide-conducting Sec61 channel and several additional proteins, which are associated with the heterotrimeric Sec61 complex. This ensemble of proteins facilitates ER targeting of precursor polypeptides, Sec61 channel opening and closing, and modification of precursor polypeptides in transit through the Sec61 complex. Recently, cryoelectron tomography of translocons in native ER membranes has given unprecedented insights into the architecture and dynamics of the native, ribosome-associated translocon and the Sec61 channel. These structural data are discussed in light of different Sec61 channel activities including ribosome receptor function, membrane

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insertion or translocation of newly synthesized polypeptides as well as the possible roles of the Sec61 channel as a passive ER calcium leak channel and regulator of ATP/ADP exchange between cytosol and ER.

Keywords Endoplasmic reticulum · Membrane protein biogenesis · Protein secretion · Protein targeting · Protein translocation · Sec61 channel

Introduction: Structure, Function, Dynamics and Connectivity of the Mammalian Endoplasmic Reticulum (ER)

A fascinating hallmark of nucleated human cells is their complex compartmentalization, separating the cellular interior into different organelles. While some organelles like mitochondria occur in a multicopy fashion others such as the endoplasmic reticulum (ER) are usually present in one copy under steady-state conditions. Like other organelles the ER fulfills a plethora of functions many of which are interwoven with its morphological heterogeneity. Despite the fact that the ER represents a continuous single-membrane network within nucleated cells, different structural variations are known (Baumann and Walz 2001; Schwarz and Blower 2016; Voeltz et al. 2002). From the perspective of localization, the ER radiates as the outer membrane of the nuclear envelope to the perinuclear space and peripheral regions all the way to the plasma membrane, where it is considered as cortical ER (Westrate et al. 2015). Not strictly correlated to this spatial distribution, the ER can morph between different shapes often referred to as sheets (or cisternae), tubules and tubular-matrices (Nixon-Abell et al. 2016). In addition, near the nucleus, where the height of a cell is usually much greater than in the periphery, another structural peculiarity of the ER can be formed, Terasaki ramps (Terasaki et al. 2013). This structure is based on helicoidal ramps connecting adjacent stacks of ER sheets in a "parking garage" like fashion (Guven et al. 2014). From the 6000 μm^3 (6 pl) total cell volume of a COS-7 cell the ER occupies 1500 μm^3 (1.5 pl), nine times the volume occupied by mitochondria (Valm et al. 2017). The dynamics of the mesh-like shaped ER are further underscored by its intracellular mobility allowing the ER to scan and explore the majority of the cytosolic volume within minutes. With such mobility rates it is not surprising that the ER is the organelle with the highest contact rate to other organelles (Valm et al. 2017; Shim 2017). This interconnectivity between different organelles and extended cellular structures such as the cytoskeleton usually relying on proteinaceous tethers were elegantly reviewed previously and are not further discussed here (Csordás et al. 2018; Phillips and Voeltz 2016; Zhang and Hu 2016; Gatta and Levine 2017). In their landmark papers sixty years ago, Palade and colleagues also distinguished different ER morphologies. From their electron microscopic images they concluded,

spot on, that the ER represents a "continuous, tridimensional reticulum" consisting of "cisternae [the term is used to designate a flat element of large size and irregular outline] which appear to communicate freely with the tubules". Furthermore, they wrote "although such cisternae may assume considerable breadth they seem to retain, in general, a depth of ~50 μm" and "the surface of the latter appears to be dotted with small, dense granules that cover them in part or in entirety" (Palade and Porter 1954). Nowadays, those original observations are coined by the key phrase rough sheets and smooth tubules, where rough and smooth refers to the presence or absence of the dense granules observed by Palade et al., i.e. ribosomes or polysomes attached to the cytosolic surface of the ER (Friedman and Voeltz 2011; Shibata et al. 2006; Pfeffer et al. 2012). Besides other factors, ribosome binding to the ER membrane is considered a major driving force for sheet formation (Shibata et al. 2010; Puhka et al. 2007). However, cells differ widely in the fraction of ER-bound ribosomes, from secretory cells in which almost all ribosomes are found at the ER to mature leukocytes in which the ER is barely detectable (Reid et al. 2014; Palade 1956). On average, half of all ribosomes and a third of all messenger RNAs are associated with the ER membrane of a typical mammalian cell (Reid et al. 2014). Important to note, the ratio of ER sheets to tubules is actively regulated by a cell and varies for example with cell type, cellular demands, and cell cycle stage (Puhka et al. 2007, 2012).

Similarly complex and versatile as the structural design of the mammalian ER is its function. With ribosomes bound to the membrane ER sheets are usually considered the primary domain for processes related to protein maturation including protein synthesis, membrane translocation or insertion, post-translational modification, folding, assembly as well as quality control and degradation. On the other hand, ER tubules with their higher surface-to-lumen ratio might be better suited for membrane-surface related ER functions such as lipid and steroid synthesis or inter-organelle signaling (Westrate et al. 2015). In addition, the ER represents the major intracellular calcium reservoir of mammalian cells (Brostrom and Brostrom 2003; Sammels et al. 2010). Under resting conditions the free calcium concentration of the ER lumen (>100 μM) exceeds the cytosolic counterpart (~50 nM) by several orders of magnitude, thus, generating a massive calcium gradient as prerequisite for efficient signaling purposes (Clapham 2007; Mogami et al. 1998; Suzuki et al. 2016). Hence, the ER is intimately linked to calcium signaling and related aspects such as muscle contraction, neuronal excitability, mitochondrial respiration or apoptosis (Berridge 2002; Berridge et al. 2003). Also, prominent, i.e. abundant, chaperones of the ER including BiP and calreticulin serve a dual function as calcium buffering protein on the one hand and folding chaperone on the other hand (Coe and Michalak 2009; Michalak et al. 2002). Common for ubiquitous calcium buffering chaperones is (i) a low affinity (k_d in mM range) paired with a high capacity (up to 50 calcium binding sites per molecule) for calcium binding and (ii) their folding activity relying on the ER calcium content (Lievremont et al. 1997; Meldolesi and Pozzan 1998; Ashby and Tepikin 2001). Given the continuous nature of both the membrane and the lumen between ER cisternae and tubules it is unclear to what extend different functions of the ER are spatially restricted or

domain specific. Upcoming high-resolution live cell imaging approaches will certainly find an answer to this question.

At the crossroad of ER structure and function appears the protein translocase where the tasks of ribosome binding, protein transport, and calcium signaling coalesce. This heteromultimeric protein complex of the ER membrane has gained much attention over the past decades starting from the biochemical identification to evolutionary conservation and functional characterization all the way to its structural organization. From a biochemist's point of view, the protein translocase could be considered as an enzyme catalyzing the membrane passage of otherwise impermeable substrates such as the roughly 3000 presecretory proteins (Rychkova and Warshel 2013), which are encoded by the human genome (https://www.proteinatlas.org/humanproteome/tissue/secretome#plasma). In order to allow membrane passage, the precursors of secretory proteins are characterized by a cleavable N-terminal signal peptide with its tripartite structure (a positively charged N-terminal region, termed N-region, a central region containing hydrophobic residues, termed H-region, and a slightly polar C-terminal region, C-region). In the case of membrane proteins without a cleavable signal peptide, the most N-terminal transmembrane helix typically serves as an ER targeting and membrane insertion signal. To handle this wide range of different soluble and membrane protein substrates (Fig. 4.1), the active center of the protein translocase is designed with a lack of substrate specificity. Notably, the translocon of higher eukaryotes is even more promiscuous than the translocons of lower eukaryotes, archaea, and bacteria (Gonsberg et al. 2017). Therefore, multiple accessory cofactors support the active center to solve the issue of substrate specificity (Table 4.1). Following the idea of Koshland's induced-fit theory of specificity, insufficient compatibility between the substrate and the active center of the translocase might also explain the imperfect sealing of the translocase observed for small molecules including calcium ions (Koshland 1958; Harsman et al. 2011c).

In the following sections we will summarize our current knowledge and concepts about the functions and mechanisms of the eukaryotic protein translocon starting with the active center, the Sec61 complex (Fig. 4.2), followed by different cofactors and how these components affect the enzyme's kinetic for membrane permeability of proteins or small molecules.

Structures and Functions of Isolated and Native Sec61 Complexes

Structural Esthetics of the Sec61 Complex

As stated earlier, the protein translocon of the ER represents a complex machinery with a variable structural architecture and dynamic stoichiometry. In the past, major emphasis was usually given to the Sec61 complex, which is considered the pivotal

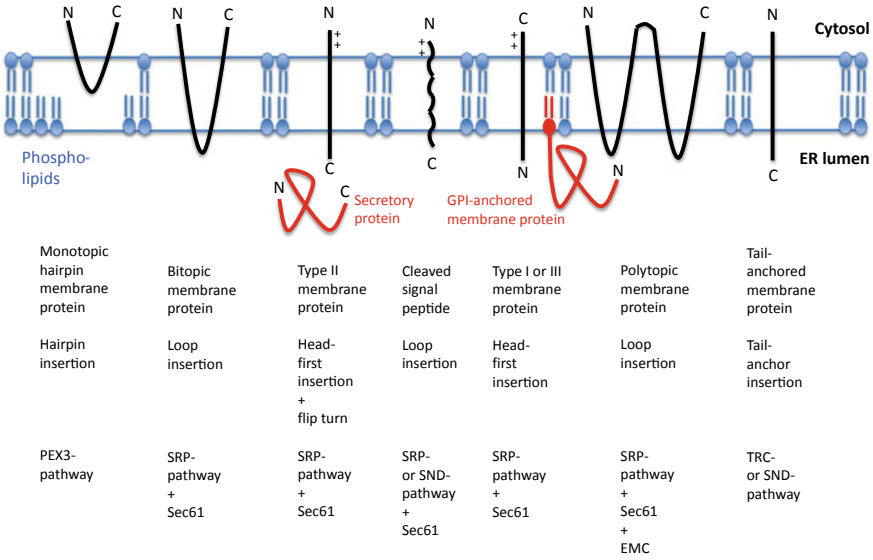


Fig. 4.1 Topologies of membrane proteins in the ER membrane. The cartoon depicts the membrane proteins of the ER membrane, together with their type, mechanism of membrane insertion, and targeting and insertion pathway. See text for details. We note that (i) bitopic and polytopic proteins can also have the opposite orientation, (ii) the shown bitopic protein is alternatively named double-spanning membrane protein, (iii) the shown polytopic protein is alternatively named tetra-spanning membrane protein, (iv) type I membrane proteins as well as bitopic and polytopic proteins with their N-terminus facing the ER lumen can be targeted to and inserted into the membrane via N-terminal signal peptides that are subsequently cleaved by ER luminal signal peptidase, (v) in case the shown type I membrane was not targeted by a cleavable signal peptide it is also defined as signal anchor protein, (vi) positively charged amino acid residues (+) play an important role in membrane protein orientation, i.e. typically, follow the positive inside rule. In the case of membrane proteins without N-terminal signal peptides, membrane insertion appears to involve the same components and mechanisms, which deliver secretory proteins and glycosylphosphatidylinositol (GPI)-anchored membrane proteins to the ER lumen. Subsequent to ER import, GPI-anchored membrane proteins become membrane anchored via their C-termini by GPI-attachment. N, N-terminus; C, C-terminus

subunit of the heteromultimeric translocon both structurally as its core subunit and enzymatically as its active center lowering the activation barrier for the membrane transport of polypeptides (Pfeffer et al. 2016; White and von Heijne 2008; Görlich and Rapoport 1993). Moreover, importance of the Sec61 complex as central component of a protein translocon is probably fortified best by its evolutionary conservation from bacteria and archaea to lower and higher eukaryotes (Calo and Eichler 2011; du Plessis et al. 2011; Park and Rapoport 2012; Dalal and Duong 2009). In all three domains of life the corresponding Sec61 complex is usually organized as a heterotrimeric protein ensemble consisting of a pore-forming α subunit accompanied by two smaller subunits, called β and γ (Fig. 4.3). Yet, nomenclature of the three Sec61 subunits is somewhat inconsistent. While we will

Table 4.1 Protein transport components/*complexes*/*networks* and associated proteins in HeLa cells

Component/subunit	Abundance	Location	Linked diseases
Calmodulin	9428	C	
Cytosolic chaperone network			
– Hsc70 (HSPA8)	3559		
– Hdj2 (DNAJA1)	660		
– Bag1 (HAP, RAP46)	46		
#NAC		C	
– NACα	1412		
– NACβ			
#SRP		C	
– SRP72	355		Aplasia, Myelodysplasia
– SRP68	197		
– SRP54	228		
– SRP19	33		
– SRP14	4295		
– SRP9	3436		
– 7SL RNA			
SRP receptor		ERM	
– SRα (docking protein)	249		
– SRβ	173		
hSnd1	?		
Snd receptor			
– hSnd2 (TMEM208)	81	ERM	
– hSnd3	?		
#Bag6 complex		C	
– TRC35 (Get4)	171		
– Ubl4A	177		
– Bag6 (Bat3)	133		
SGTA	549	C	
TRC40 (Asna1, Get3)	381	C	
TA receptor		ERM	
– CAML (CAMLG, Get2)	5		
– WRB (CHD5, Get1)	4		Congenital heart disease, down syndrome
ERM protein complex		ERM	
– EMC1	124		
– EMC2	300		
– EMC3	270		
– EMC4	70		
– EMC5 (MMGT1)	35		

(continued)

Table 4.1 (continued)

Component/subunit	Abundance	Location	Linked diseases
– EMC6 (TMEM93)	5		
– EMC7	247		
– EMC8	209		
– EMC9	1		
– EMC10	3		
#TMCO1	2013	ERM	Glaucoma, cerebrofaciothoracic dysplasia
PEX19	80	C	Zellweger syndrome
PEX3	103	ERM	Zellweger syndrome
#Sec62 (TLOC1)	26	ERM	Prostate cancer, lung cancer
#Sec61 complex		ERM	
– Sec61α1	139		Diabetes**, CVID, TKD
– Sec61β	456		Polycystic liver disease (PLD)
– Sec61γ	400		Glioblastoma
Alternative Sec61 complex			
– Sec61α2	?		
– Sec61β	456		
– Sec61γ	400		
ER chaperone network			
– Sec63 (ERj2)	168	ERM	Polycystic liver disease (PLD)
– #ERj1 (DNAJC1)	8	ERM	
– ERj3 (DNAJB11)	1001	ERL	Polycystic kidney disease (PKD)
– ERj4 (DNAJB9)	12	ERL	
– ERj5 (DNAJC10)	43	ERL	
– ERj6 (DNAJC3, $p58^{IPK}$)	237	ERL	Diabetes
– ERj7 (DNAJC25)	10	ERM	
– ERj8 (DNAJC16)	24	ERM	
– BiP (Grp78, HSPA5)	8253	ERL	Hemolytic uremic syndrome (HUS)
– Grp170 (HYOU1)	923	ERL	
– Sil1 (BAP)	149	ERL	Marinesco-Sjögren-syndrome (MSS)
#$Calnexin_{palmitoylated}$	7278	ERM	
#TRAM1	26	ERM	
TRAM2	40	ERM	
PAT-10	?		
#TRAP complex		ERM	
– TRAPα (SSR1)	568		
– TRAPβ (SSR2)			
– TRAPγ (SSR3)	1701		Congenital disorder of glycosylation (CDG), hepatocellular carcinoma

(continued)

Table 4.1 (continued)

Component/subunit	Abundance	Location	Linked diseases
– TRAPδ (SSR4)	3212		Congenital disorder of glycosylation (CDG)
#RAMP4 (SERP1)		ERM	
#Oligosaccharyltransferase		ERM	
– RibophorinI (Rpn1)	1956		
– RibophorinII (Rpn2)	527		
– OST48	273		Congenital disorder of glycosylation (CDG)
– OST4			
– TMEM258			
– DAD1	464		
– Stt3A*	430		Congenital disorder of glycosylation (CDG)
– Stt3B*	150		Congenital disorder of glycosylation (CDG)
– Kcp2			
– DC2			
– TUSC3			Congenital disorder of glycosylation (CDG)
– MagT1	33		
Signal peptidase (SPC)		ERM	
– SPC12	2733		
– SPC18*			
– SPC21*			
– SPC22/23	334		
– SPC25	94		
GPI transamidase (GPI-T)		ERM	
– GPAA1	9		
– PIG-K	38		
– PIG-S	86		
– PIG-T	20		
– PIG-U	42		
Signal peptide peptidase	424	ERM	
#p34 (LRC59, LRRC59)	2480	ERM	
#p180 (RRBP1)	135	ERM	
Kinectin 1 (KTN1)	263	ERM	

Alternative names of components/subunits are given in parentheses. We note that oligosaccharyltransferase exists as two paralogs, comprising Stt3a or Stt3b. Abundance is given in nM (Hein et al. 2015); 1 nM corresponds to roughly 1000 molecules/cell (Moran et al. 2010). C, cytosol; CVID, common variable immune deficiency; ERL, ER lumen; ERM, ER membrane; TKD, tubulo-interstitial kidney disease; *, catalytically active subunit; **, in mice; #, ribosome associated; ?, uncharacterized

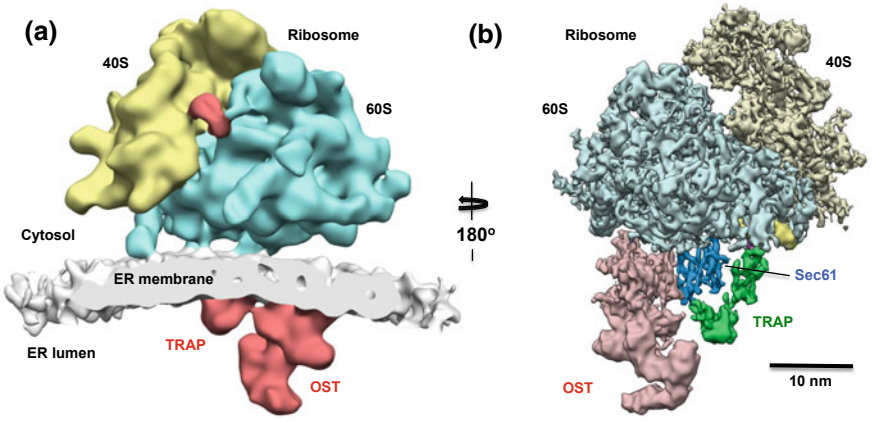


Fig. 4.2 Architecture of the ribosome associated protein translocon of the mammalian ER membrane. **a** In situ structure of the ER-associated mammalian ribosome after subtomogram-averaging at a resolution of about 20 Angström. The ribosome is present in rough microsomes and was imaged by cryo-electron tomography, using a FEI Titan Krios TEM and a FEI Falcon direct electron detector. The ER membrane was cut for better visibility of ER luminal electron densities (shown in red), the Sec61 complex is hidden by the phospholipid bilayer and the ribosome. An additional electron density (shown in red) was observed between 40S and 60S ribosomal subunits and indicates that a translating ribosome was imaged. **b** Further developments of cryo-EM instrumentation and computational algorithms allowed improvement of the ribosome-translocon structure to about 10 Angström resolution, which enables resolving separate transmembrane helices. Here, the membrane density was removed to highlight membrane integral parts of the translocon. Transmembrane helices for Sec61 complex, TRAP (translocon-associated protein), and OST (oligosaccharyl transferase) can be clearly distinguished under these conditions. Helix 51 of an rRNA expansion segment (shown in yellow) and ribosomal protein eL38 (shown in magenta) represent the contact site to the TRAP γ-subunit, but are partially hidden by other ribosomal densities in this view

adhere to the mammalian Sec61α-β-γ subunit terminology, these are referred to SecY-G-E in bacteria, SecY-β-E in archaea and Sec61p-Sbh1p-Sss1p in yeast, respectively (Auer et al. 1991; Hartmann et al. 1994; Kinch et al. 2002; Cao and Saier 2003; Görlich et al. 1992). Genetic studies in yeast and bacteria showed that the two subunits with highest sequence conservation across kingdoms, Sec61α and Sec61γ, are essential for protein translocation and cell viability, whereas the β subunit with lower sequence homology seems dispensable, hence some bacteria assemble only a dimeric SecYE complex (Nishiyama et al. 1994; Matlack et al. 1998; Tsukazaki et al. 2008). First structural insights for the arrangement of a trimeric Sec61 complex came from X-ray crystallography depicting the archaean SecYβE isolated from *Methanocaldococcus jannaschii* (Van den Berg et al. 2004). Subsequent crystal structures and cryo-electron microscopic (EM) studies using isolated pro- or eukaryotic Sec61 complexes confirmed the evolutionary conservation of its architecture (Egea and Stroud 2010; Becker et al. 2009; Gogala et al. 2014; Voorhees et al. 2014; Zimmer et al. 2008; Tanaka et al. 2015). Congruent for

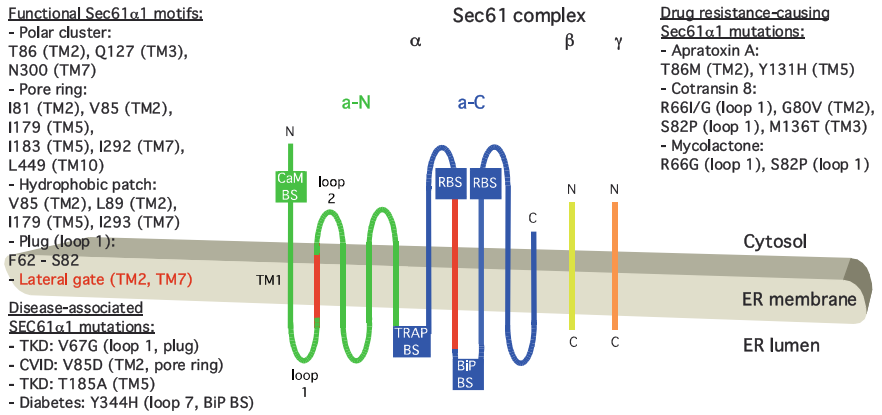


Fig. 4.3 Topology and functionally relevant domains of the heterotrimeric Sec61 complex. The membrane topology of the three subunits of the mammalian Sec61 complex is shown, highlighting binding sites (BS) of Ca^{2+}-calmodulin (CaM), the ribosome (R), the translocon-associated protein complex (TRAP), and immunoglobulin heavy-chain binding protein (BiP). Furthermore, functional motifs, disease associated mutations, and drug resistance causing mutations of the α-subunit are indicated, as are different targeting pathways (purple) to the Sec61 complex. The N- and C-terminal halves of the Sec61 α-subunit are shown in green and blue, respectively (a-N, a-C). Amino acid residues are given in single letter code; C, C-terminus; CVID, Common Variable Immune Deficiency; N, N-terminus; TKD, Tubulo-Interstitial Kidney Disease; TM, transmembrane helix. Notably, recent 3D reconstructions after single particle cryo-electron microscopic analysis of the yeast SEC complex, i.e. the Sec61 complex together with the Sec62/63/71/72 complex, suggested that in the post-translationally acting Sec61 complex, the Sec62/63 sub-complex interacts with the cytsoslic loops 6 and 8 on the cytosolic face of the Sec61 complex (i.e. clashes with ribosome binding), and that the ER luminal domain of Sec63 interacts with ER luminal loop 5 (i.e. clashes with TRAP binding)

all structures is the central, hourglass-like shaped Sec61α subunit forming the actual polypeptide-conducting channel. Its ten transmembrane helices are organized in a pseudo-symmetrical fashion generating an N-terminal half encompassing the cytosolic N-terminus and transmembrane helices 1–5 as well as a C-terminal half encompassing transmembrane helices 6–10 and the cytosolic C-terminus (Figs. 4.3 and 4.4). Loops connecting the ten transmembrane helices are numbered consecutively from 1 to 9 with the odd numbered ones located in the luminal (eukaryotic Sec61 complexes) or periplasmic (prokaryotic SecY complexes) space and even numbered ones in the cytosol. Interestingly, the cytosolic loops 6 and 8 share a functional conservation among all Sec61 homologs serving as universal docking port for different interaction partners including the polypeptide-delivering ribosome or SecA. Hence, loop 6 and 8 project in a lighthouse-like fashion into the cytosol directing incoming shipments. In its three-dimensional fold the Sec61α subunit forms in the plane of the membrane a central constriction called the pore ring. This structural element consists of six bulky, hydrophobic residues facing inwards to form a flexible gasket avoiding excessive membrane permeability of small

molecules during the transport of polypeptides (Figs. 4.4 and 4.5). The six pore ring residues are dispersed across the primary structure of Sec61α, yet, localized in critical transmembrane helices 2, 5, 7 and 10 as will be discussed below. Fitting to the hourglass analogy, the pore ring separates two opposing funnels. In the closed conformation of the Sec61 channel the cytosolic funnel is "empty" (water-filled), whereas the luminal/periplasmic facing funnel is occupied by a short helical domain of loop 1, aptly named the plug domain (Rapoport et al. 2017; Junne et al. 2006). However, it appears that plug domains of orthologous Sec61 complexes have different structures, consistent with the facts that this region is the least conserved in the amino acid sequence of different homologs and has a subordinate role for function and cell viability (Li et al. 2007; Junne et al. 2006; Li et al. 2016). In addition, to act as a molecular switching device allowing membrane passage or insertion of incoming precursor polypeptides the Sec61α subunit has a lateral gate, formed between the two sterically adjacent transmembrane helices 2 and 7. In the closed state the lateral gate is stabilized by a polar cluster consisting of three conserved polar residues residing in the helices 2, 3 and 7 (Voorhees and Hegde 2016a). Structural and molecular dynamics studies demonstrated that transition from a closed to an open state spreads the N- and C-terminal halves of Sec61α apart laterally between helices 2 and 7, termed rigid body movement, thereby opening up the lateral gate and permitting access to the lipid phase (Fig. 4.5) (Denks et al. 2014; Egea and Stroud 2010; Park et al. 2014; Hizlan et al. 2012). Vis-à-vis to the lateral gate the Sec61α subunit is associated with the tail-anchored Sec61γ protein (Fig. 4.4a). It wraps transversely around the N- and C-terminal half like a U-shaped clip, potentially restricting excessive mobility of the complex. The third subunit Sec61β, another tail-anchored protein in eukaryotes (the bacterial SecG ortholog has two transmembrane segments), contacts the N-terminal half of Sec61α in vicinity to transmembrane helices 1 and 4, whereas its cytosolic domain might serve a regulatory function for the transport process, as described below (Figs. 4.4 and 4.5).

Structural Dynamics of the Sec61 Complex

Based on the multitude of structural data of Sec61 complexes gathered with X-ray crystallography and cryo-EM three common themes emerge. First, opening of the Sec61 complex requires some kind of ligand binding, ligands being the substrates as well as allosteric effectors, which bind to other parts of the Sec61 complex as compared to the substrates. Visualized ligands contributing in the transition from the idle to an open Sec61 complex include the bacterial ATPase SecA, the translating ribosome, the ER membrane protein Sec63, or even pseudo-ligands like a heterologous anti-Sec61α F_{ab} fragment or an autologous copy of a second Sec61 molecule arising from crystal packing (Li et al. 2016; Zimmer et al. 2008; Gogala et al. 2014; Voorhees et al. 2014; Voorhees and Hegde 2016a; Itskanov and Park 2018; Wu et al. 2018; Egea and Stroud 2010; Braunger et al. 2018). Second, all of those ligands, native or pseudo, interact with the cytosolic docking port (loops 6 and 8) of the

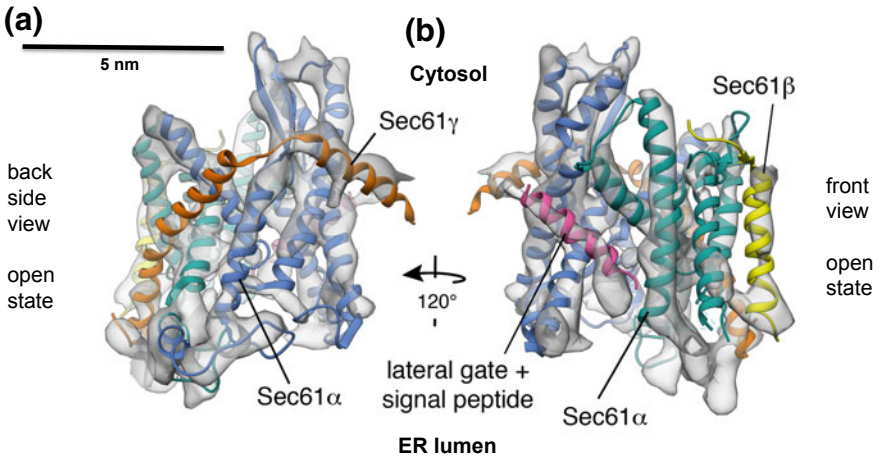
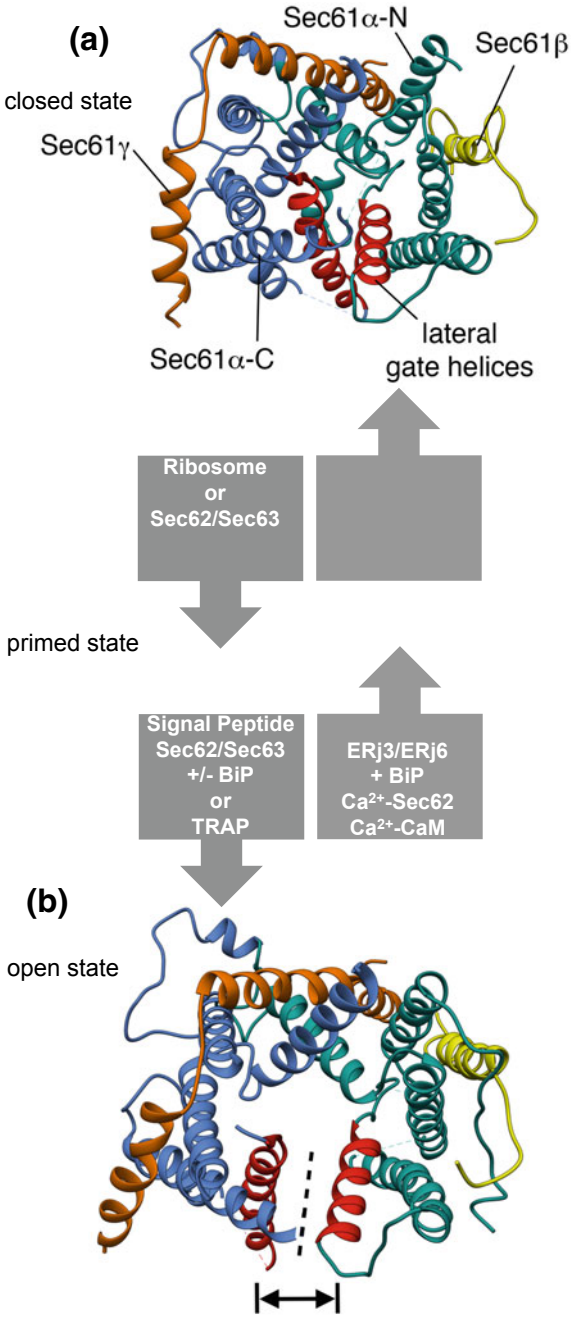


Fig. 4.4 Atomic model PDB 3jc2 for the heterotrimeric Sec61 complex, fitted into the ribosome-translocon structure, shown in Fig. 4.2b. **a**, **b** The laterally opened and translocating Sec61 channel is shown in two orientations, as seen form the plane of the membrane. b, In b the Sec61 complex is rotated counter clockwise in the plane of the membrane by 120° as compared to a. The N-terminal signal peptide of the translocating polypeptide is shown at the open lateral gate in magenta. The N- and C-terminal halves of the Sec61 α-subunit are shown in green and blue, respectively, the ß-subunit in yellow, and the γ-subunit in orange

Sec61 complex to initiate a conformational change (Figs. 4.2 and 4.5). Coming back to the enzymatic concept of the translocase such ligands probably act as catalysts that lower the activation energy (energy barrier) for the Sec61 channel gating reaction (Fig. 4.6). And third, the pivotal structural elements of the Sec61 complex mentioned before such as the universal docking port, the pore ring residues, the plug domain, the lateral gate helices, as well as the polar cluster stabilizing the latter seem to work hand in hand during the cycle of opening and closing the channel. Recent

Fig. 4.5 Atomic model for the structural dynamics of gating of the heterotrimeric Sec61 complex, as seen from the cytsosol. **a** Atomic model for the laterally closed Sec61 complex (PDB 3j7q). **b** Atomic model for the laterally opened Sec61 channel (PDB 3jc2). **a**, **b** N- and C-terminal halves of the Sec61 α-subunit, are shown in green and blue, respectively, lateral gate helices 2 and 7 are shown in red, and cytosolic loops are not shown for clarity. The ß-subunit is depicted in yellow and the γ-subunit in orange. At least three conformations of the Sec61 complex can be distinguished, (i) the closed state (closed even to calcium ions), (ii) a structurally ill-characterized primed state that is induced by interaction with either the ribosome or the Sec62/Sec63 complex, and (iii) the open state, which is induced by interaction with a strong signal peptide or N-terminal transmembrane helix of a precursor polypeptide or a weak signal peptide or N-terminal transmembrane helix plus allosteric effectors, such as TRAP or Sec62/Sec63 ± BiP (characterized by an open lateral gate and permeable to calcium ions). During protein translocation, the lateral gate is typically occupied by a signal peptide (Fig. 4.4b) and the central aqueous pore by the polypeptide chain in transit. We note that efficient closing of the Sec61 channel can also involve allosteric effectors, such as BiP with its ER luminal Hsp40-type co-chaperones ERj3 plus ERj6 or calcium-bound Sec62 plus calcium-bound calmodulin (CaM) ▶


(a)
closed state
Sec61α-N
Sec61β
Sec61γ
Sec61α-C
lateral
gate helices
Ribosome
or
Sec62/Sec63
primed state
Signal Peptide
Sec62/Sec63
+/- BiP
or
TRAP
ERj3/ERj6
+ BiP
Ca2+-Sec62
Ca2+-CaM
(b)
open state

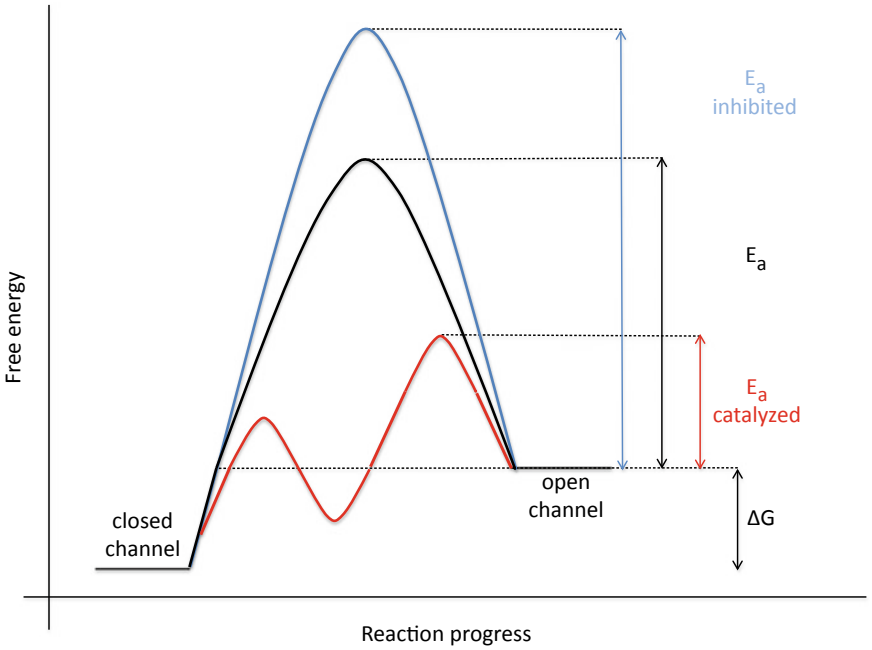


Fig. 4.6 Energetics and kinetics of Sec61 channel gating. The TRAP- or Sec63 ± BiP-mediated Sec61-channel gating is probably best considered in analogy to an enzyme-catalysed reaction. Accordingly, TRAP, Sec63 or BiP reduce the energetic barrier for full channel opening, which can apparently be reinforced by Sec61 channel inhibitors, such as cyclic heptadepsipeptides (e.g. CAM741) or certain eeyarestatins (e.g. ES1, ES24). At least in the case of ES1 and ES24, binding of the inhibitor within the channel pore arrests the channel in a partially open state (termed 'foot in the door'), which maybe identical with the primed state and is compatible with Ca^{2+}-efflux but not with full channel opening for protein translocation. TRAP and BiP contribute to full channel opening by direct interaction with ER luminal loops 5 or 7 of Sec61α (Fig. 4.6). SEC61A1 mutations can increase the energy barrier (E_a) for channel opening per se (V85D or V67G mutation) or indirectly, such as by interfering with BiP binding (Y344H mutation). Notably, all these effects are precursor specific because the N-terminal signal peptides are either efficient or inefficient in driving Sec61 channel opening. Typical for an enzyme-catalysed reaction, BiP can also support efficient gating of the Sec61 channel to the closed state, i.e. the reverse reaction

cryo-EM analyses of programmed and detergent extracted mammalian Sec61 complexes shed light on the orchestration of the opening process and its intermediary steps (Voorhees et al. 2014; Voorhees and Hegde 2016a, b). The closed, i.e. ligand-free, Sec61 complex is unable to conduct substrate transport due to a narrow pore ring and stabilized lateral gate topology (Fig. 4.5). Following the induced-fit model binding of a translating 80S ribosome to the cytosolic docking port of Sec61α induces a conformational change in the loops 6 and 8 reminiscent of a transitional state, also called the primed Sec61 complex. Specific interactions occur between the ribosomal components uL23, eL39, and 28S rRNA contacting conserved, basic residues in loop 6, loop 8, as well as the N-terminal helix of Sec61γ. As a result, the

ribosomal exit tunnel with the nascent polypeptide aligns right on top of the cytosolic funnel of the primed Sec61 complex. Conformational changes in the docking port upon ligand binding propagate through associated transmembrane helices and the rest of the Sec61 complex with two ramifications. Binding of a translating ribosome causes destabilization of the polar cluster generating (i) a crack in the cytosolic half of the lateral gate and (ii) exposing a hydrophobic patch in the cytosolic funnel of the Sec61 complex. This single hydrophobic patch is ideally positioned to attract the hydrophobic stretch encoded in the targeting signal (cleavable signal peptide or transmembrane helix) of incoming precursor polypeptides and intercalates the targeting signal in the lateral gate (Fig. 4.4b). This intercalation displaces lateral gate helix 2 in a way that the targeting signal takes over the space occupied by helix 2 in the closed state. The intercalation step supports a rigid body movement of the N- and C-terminal halves of Sec61α and the channel is fully open with the pore ring widened and the plug displaced (Fig. 4.5b). The open Sec61 complex than allows the substrate access axially across or laterally into the membrane. Still, the pore ring residues surround placidly the polypeptide in transit to preserve the permeability barrier for other small molecules and ions during the transport process (Li et al. 2016; Park and Rapoport 2011). Strikingly, this two-stage model of activation from a closed to a primed to an opened Sec61 complex was observed for both prokaryotic and eukaryotic Sec61 complexes activated by a substrate-engaged SecA or translating 80S ribosome using X-ray crystallography or cryo-EM, respectively. Therefore, regardless of the organism or mode of substrate delivery the fundamental principle of protein transport is conserved at both levels component-wise (the Sec61 complex) and mechanistically (temporary intercalation of a targeting signal at the lateral gate).

Functions of the Mammalian Sec61 Complex

The Sec61 complex is a great example how structural, biochemical, biophysical, and cell biological methodologies can complement and guide one another to unravel its structure-function relationship. Historically, the abbreviation “Sec” was introduced to the eukaryotic system by Randy Schekman and coworkers to define mutants in a yeast screen for hampered secretion of secretory enzymes (Spang 2015; Novick et al. 1980). A refined version of the screen searching for mutant yeast cells that fail to translocate a secretory precursor protein into the lumen of the ER identified Sec61p (Deshaies and Schekman 1987; Schekman 2002). Subsequently, Tom Rapoport’s research group cloned the mammalian ortholog of Sec61p and demonstrated in a couple of landmark papers its association with ribosomes and nascent chains, corroborating its central role in protein transport as polypeptide-conducting channel (Görlich et al. 1992; Görlich and Rapoport 1993; Hartmann et al. 1994; Kalies et al. 1994). Further crosslinking studies verified that the Sec61 complex also handles transmembrane helices and that targeting signals intercalate between the lateral gate helices 2 and 7, whose flexibility is a

prerequisite for efficient translocation (High et al. 1993; Plath et al. 1998; du Plessis et al. 2009; Spiess 2014). Also, the high affinity of the Sec61 complex for ribosome binding was demonstrated under more physiological conditions using a fluorescence resonance energy transfer (FRET) based assay or directly after siRNA-mediated depletion of the Sec61 complex in conjunction with electron microscopy (Benedix et al. 2010; Lang et al. 2012). These data are in agreement with structural biology showing ribosome binding and transport of incoming polypeptides by the Sec61 complex. Actually, the phrase "incoming polypeptides" covers two themes, the arrival of the polypeptide relative to its synthesis and the topology of the polypeptide. Polypeptides arrive at the Sec61 complex either co-translationally, i.e. as nascent chain emerging from the ribosomal exit tunnel during their synthesis, or post-translationally, i.e. after completion of synthesis and release from the ribosome. Both options are conserved across all organisms and the polypeptides, fully synthesized or nascent, are transported by the Sec61 complex before substantial folding occurs (Dudek et al. 2015). While the ribosome acts as activating ligand for the Sec61 complex under co-translational conditions, structural studies mentioned above as well as in vitro reconstitutions highlight the importance of the Sec62/63 protein complex or the SecA ATPase for the post-translational transport in eukaryotic and prokaryotic systems, respectively (Haßdenteufel et al. 2018; Lakkaraju et al. 2012b; Schlenstedt et al. 1990; Johnson et al. 2012; Panzner et al. 1995; Akimaru et al. 1991; Brundage et al. 1990; Driessen and Nouwen 2008). Differentiating features driving the co- or post-translational transport mode are manifold and encoded by the primary structure of the precursor polypeptide, encompassing both the actual targeting signal as well as downstream located stretches (Chatzi et al. 2017). For the second theme, topology, precursor polypeptides can be classified as follows. Besides the complete translocation across the membrane in case of secretory proteins and glycosylphosphatidylinositol (GPI)-anchored proteins including cleavage of their N-terminally located signal peptide multiple variations of membrane topology are reported and summarized together with the mode of membrane insertion in Fig. 4.1. To facilitate transport, polypeptides insert either in a horseshoe bend coordination, called loop insertion (N-terminus of the targeting signal faces the cytosol), or head-first (N-terminus of the targeting signal faces away from the cytosol) into the Sec61 complex. The loop insertion corresponds nicely with structural data and can be envisioned as a result of the targeting signal intercalation at the lateral gate with the downstream mature part creating the horseshoe shaped loop (Li et al. 2016; Park et al. 2014; Voorhees and Hegde 2016b). Loop insertion is considered the more productive mode for cleavable signal peptides and every other transmembrane helix of multi-spanning membrane proteins whose N-terminus faces the cytosol. On the other hand, the transmembrane helices of multi-spanning membrane proteins whose N-termini face away from the cytosol are inserted head-first (Gogala et al. 2014). Interestingly, head-first insertion seems to be the preferred mode of transport for the two groups of inversely oriented type I and type II single-spanning membrane proteins (Fig. 4.1). The transmembrane helix of type II membrane protein starts out with a head-first, or a type I, orientation (the N-terminus translocates across the

membrane) followed by an energetically unfavorable reaction of a 180° flip turn reversing orientation of the transmembrane helix to the final type II topology with the N-terminus now facing the cytosol (Devaraneni et al. 2011). This phenomenon of delayed topology determination allowing reorientation of transmembrane helices was also observed for an engineered poly-leucine model protein as well as a polytopic membrane protein (Seppälä et al. 2010; Goder and Spiess 2003). Two special cases of topology are represented by (i) monotopic hairpin proteins, whose membrane domains only dip into a membrane leaflet without traversing it and (ii) tail-anchored (also called type IV) membrane proteins, whose single transmembrane helix is located at the C-terminal end (Pataki et al. 2018; Borgese et al. 2009). Both classes of proteins seem to insert into the ER membrane Sec61-independently (Schrul and Kopito 2016; Yamamoto and Sakisaka 2012; Wang et al. 2014).

In addition to the snapshots of the transport process, live cell imaging and biophysical single-channel recordings from planar lipid bilayer experiments can address dynamic properties of the Sec61 complex over an extended period of time. As such, they can also shed light on the events during termination of polypeptide transport and provide insights into how quickly structural elements of the Sec61 channel "re-shape" to make the transition from the open back to the closed state (Figs. 4.5 and 4.6). The latter point is of particular interest given that the ER is considered the major intracellular calcium store in nucleated mammalian cells and the permeability of a powerful second messenger such as calcium across the ER membrane has to be precisely controlled (Clapham 2007). Indeed, studies with non-physiological molecules larger than a hydrated calcium ion show their permeation into the ER, likely via the Sec61 complex, and demonstrate the imperfect sealing of the mammalian channel for small molecules (Heritage and Wonderlin 2001; Roy and Wonderlin 2003; Le Gall et al. 2004). More recently in yeast, permeability of the physiological glutathione molecule was also shown to involve the Sec61 complex and two ER luminal proteins, Kar2 and Ero1 (Ponsero et al. 2017). Planar lipid bilayer experiments addressing the ion conductance of purified Sec61 complexes directly demonstrated its permeability for calcium with a main and sub-conductance state for calcium of 165 ± 10 pS and 733 ± 16 pS, which correlate to opening diameters of the pore from 5–7 to 12–14 Å, respectively (Lang et al. 2011a; Harsman et al. 2011a). Additional work with presecretory polypeptides and an inhibitor of protein synthesis showed the ion conductance of the Sec61 complex occurs at the end of protein translocation and the channel is fully closed only after washing off non-translating ribosomes (Wirth et al. 2003; Simon and Blobel 1991). The simultaneous use of ratiometric calcium sensitive dyes localized in the cytosol (such as Fura-2) and ratiometric biosensors for calcium in the ER lumen (such as D1ER) in combination with RNAi mediated gene silencing also demonstrated the calcium permeability of the vertebrate and invertebrate Sec61 complex in a cellular setting, under more physiological conditions (Lang et al. 2011a, b; Zhang et al. 2006; Gamayun et al. 2019).

Furthermore, work with dendritic cells showed that the Sec61 complex functions as polypeptide dislocase from endosomes during cross-presentation of extracellular

antigens via MHC-I molecules, extending the portfolio of possible Sec61 complex functions and locations (Zehner et al. 2015). However, to restrict mobility of the Sec61 complex (none of the subunits harbors a known ER retention signal) the cytosolic N-terminus of the β-subunit interacts with microtubules allowing stable ER-cytoskeleton interaction. The loss of Sec61β in *Caenorhabditis elegans* induced ER stress, enhanced Sec61 complex mobility and reduced the amount of membrane-attached ribosomes (Zhu et al. 2018). It becomes apparent that the Sec61 complex is a multi-functional player able to (i) bind ribosomes and other ligands, (ii) transport structurally very different substrates, and (iii) represents a calcium permeable channel of the mammalian ER membrane. How these functions are concerted in situ and the different allosteric regulators supporting the Sec61 complex will be discussed in the next sections.

Architecture of the Native Sec61 Complex, the Translocon

While the aforementioned structures of purified Sec61 complexes are informative the situation in vivo within the native membrane might be more challenging. For example, competing forces within a living cell influencing biochemical reactions and transport processes arise from macromolecular crowding taking account of specific and nonspecific interactions between macromolecules (Minton 2006; Ellis 2001; Zhou et al. 2008). The high protein and solute concentrations in both the cytosol and the ER lumen as well as proteins and lipids of the membrane in the immediate vicinity of the Sec61 complex might influence the channel and details of the transport mechanism. Similarly, the plethora of transported clients (Fig. 4.1) with varying amino acid sequences of their targeting signal or mature part requires substrate-specific adjustments of the transport reaction. Advancements in the field of cryo-electron tomography (CET) in conjunction with subtomogram analysis enable this technology to address the heterogeneity of local, native environments and address the structure of Sec61 complexes in situ (Koning et al. 2018; Lučić et al. 2013). So far, CET studies were conducted for eukaryotic Sec61 complexes from intact HeLa cells or ER membrane vesicles derived from canine pancreatic cells, human cell lines, primary fibroblasts, or green algae (Mahamid et al. 2016; Pfeffer et al. 2012, 2014, 2015, 2017; Braunger et al. 2018). While CET structures of the mammalian ribosome-associated Sec61 complex are in agreement with the main conclusions drawn from cryo-EM and crystal structures, tomography adds an important aspect. The native Sec61 complex is a team player and associates (at least) with two membrane protein complexes to form the native ER protein translocon (Fig. 4.2). The translocon-associated protein (TRAP) complex localizes in a stoichiometric manner next to the C-terminal half of the ribosome-engaged Sec61 complex and the oligosaccharyl-transferase (OST) complex approaches in a substoichiometric manner (present only in 40–70% of complexes) the N-terminal half (Pfeffer et al. 2016). Therefore, TRAP and OST are not occluding the lateral gate and targeting signal intercalation. These data are further supported by cryo-EM

studies of solubilized ribosome-bound translocon complexes (Ménétret et al. 2005, 2008; Braunger et al. 2018). Thus, the protein translocase seems dynamic by nature (Fig. 4.5). Not only the active center is subject to structural flexibility, also stoichiometry of subunits and partner components changes with different substrates or cellular cues. The functional implication of structurally visualized and other biochemically verified translocon components will be discussed next.

The Role of Allosteric Effectors of the Eukaryotic Sec61 Complex Previously Visualized by Structural Biology

In the living cell the Sec61 complex is continuously contacted and supported by an alternating repertoire of associated proteins. Up to now, the ribosome and three protein complexes were unequivocally identified by structural data and shown to affect gating and, therefore, functionality of the Sec61 complex (Fig. 4.5).

The Ribosome

As stated before, the ribosome contacts the Sec61 complex via charged residues in loop 6 and 8, the evolutionarily conserved docking port of the Sec61α subunit, inducing a conformational change from the closed to the primed state (Figs. 4.4 and 4.5). Mutational studies in yeast showed a phenotypic differentiation between mutants of Sec61α loop 6 and 8. While loop 8 mutants had a reduced binding affinity for 80S ribosomes, mutations in loop 6 inhibited co-translational transport without significantly affecting ribosome binding activity (Cheng et al. 2005). Similar studies in yeast and bacteria also highlighted the importance of the cytosolic C-terminus of Sec61α for both ribosome binding and viability. Positive charges in the C-terminus may interact with ribosomal rRNA to support positioning of the ribosome and protein translocation (Egea and Stroud 2010; Mandon et al. 2018). The ribosome also affects calcium permeability of the Sec61 channel. Protein synthesis inhibitors that arrest (e.g. emetine) or release (e.g. puromycin) the ribosome from the Sec61 complex can block or increase calcium efflux from the ER, respectively (Lang et al. 2011a; Klein et al. 2018; Gamayun et al. 2019). Thus, the ribosome acts as prominent modulator of the calcium leak from the ER opening an interesting connection between Sec61-mediated calcium efflux and protein synthesis by the ribosome. Furthermore, cryo-EM and CET studies have shown the interaction between the Sec61 complex and translating ribosome is not hermetically sealed. Instead, a considerable gap is visible between the N-terminal half of the Sec61 complex and the ribosome exit tunnel (Ménétret et al. 2007; Park et al. 2014; Voorhees et al. 2014; Pfeffer et al. 2015). This partially shielded micro-compartment could provide a space for the release and folding of cytosolic

domains of membrane proteins, or a location for quality control, repair and de-clogging factors probing the transport process (Malsburg et al. 2015; Kayatekin et al. 2018; Ast et al. 2016). A cryo-EM structure of the bacterial ribosome-translocon complex (RTC) during synthesis of a polytopic membrane protein showed electrostatic interactions between positively charged residues in the cytosolic loop connecting the two transmembrane helices of the model precursor and negative charges of the ribosomal rRNA helix 59 (H59). Thus, the ribosome could be another player in decoding the positive-inside rule acting in concert with the Sec61/SecY complex and the decoding being orchestrated in the gap volume (von Heijne 1989; Bischoff et al. 2014). Alternatively, the calcium sensor calmodulin can occupy this micro-compartment to monitor or minimize the calcium flux associated with the transport process (Erdmann et al. 2011). However, elusion of the nascent polypeptide into the gap demonstrates that the GTP-driven elongation process is not necessarily a driving force for translocation and opens up a space and time-window for the recruitment of regulatory factors (Conti et al. 2015).

Aside from docking to the Sec61 complex, the ribosomal surface is an enormous hub for the recruitment of other ligands. Ligands, cytosolic as well as membrane-bound, allow the fine-tuning of protein transport in response to various stimuli. A recent example addressing the plethora of ribosomal ligands, dubbed the mammalian "ribo-interactome", identified in addition to the 100 proteins constituting the canonical translation machinery 330 interactors with diverse functions. For example, the combination of high-throughput sequencing after UV crosslinking (iCLIP) and proximity-specific ribosome-profiling demonstrated that isoform 2 of the pyruvate kinase of the muscle, Pkm2, is enriched on ER-bound ribosomes near the A-site and acts as translational activator of ER destined mRNAs. A SILAC (stable isotope labeling by amino acids in cell culture) approach additionally verified that Pkm2-enriched ribosomes are contacting the Sec61 and OST complex, whereas the gamma subunit of the TRAP complex was found as general ribosomal interactor (Simsek et al. 2017). The cytosolic domain of the γ-subunit of the TRAP complex is in close proximity to the RNA expansion segments ES20L/ES26L and ribosomal protein eL38 of the 60S subunit (Pfeffer et al. 2016, 2017). Based on the structure of the bacterial RTC, the tip of a nascent targeting signal interacting with H59 and uL24 might come in close contact with the neighboring eL38 (Jomaa et al. 2016, 2017; Nguyen et al. 2018). Although not in conjunction with the translocon or the targeting signal, eL38 was shown to be a regulatory ribosomal protein that can support translation of subset of mRNAs carrying a specialized regulon motif in the 5' untranslated region (Xue et al. 2015). Analogous to a regulon motif, TRAP could trigger the regulatory function of eL38 to support translation in vicinity to the Sec61 complex.

Recent structural data of the OST complex also provided striking insights into its interaction with the ribosome (Bai et al. 2018; Wild et al. 2018; Braunger et al. 2018). The cytosolic C-terminus of Rpn1, one of the core subunits shared amongst the two paralogous OST complexes containing either Stt3A or Stt3B as catalytic subunit, interacts with the ribosome. Rpn1 forms a quadruple-helix bundle aligning in a cavity made from rRNA helices H19/H20, rRNA expansion segment ES7a and

ribosomal protein eL28 (Braunger et al. 2018). Importance of this interaction for efficient RTC formation was demonstrated by antibodies against the cytosolic Rpn1 segment which prevented ribosome targeting to and efficient protein translocation by the translocon (Yu et al. 1990). Though, the Rpn1 interaction with the ribosome is sterically hindered in case of the Stt3B-containging OST complex due to an additional sequence extension in a cytosolic loop and the presence of a paralog specific subunit (Braunger et al. 2018).

Besides the Sec61, TRAP, and OST complex the ribosome was shown to interact with additional membrane proteins of the ER, including palmitoylated Calnexin, ERj1, Sec62, or p180 (Table 4.1). These candidates support ribosome anchoring at the ER or serve as transient, regulatory proteins of the RTC. Calnexin, a type I membrane protein, is a lectin-like chaperone and assists maturation, folding and oligomerization of glycoproteins (Hebert et al. 1996). Upon modification by the ER palmitoyltransferase Dhhc6, palmitoylated calnexin associates with the RTC (next to the TRAP complex) to catch transported client proteins as they emerge from the Sec61 complex (Lakkaraju et al. 2012a; Wada et al. 1991; Chevet et al. 1999). Another single-pass type I membrane protein of the ER carrying a characteristic luminal J-domain, ERj1, can directly associate with ribosomes in the intact ER as was demonstrated by a FRET based assay employing antibody accessibility as a readout or from analysis of ribosome-associated ER membrane proteins in detergent extracts of canine pancreatic microsomes (Blau et al. 2005; Dudek et al. 2002; Benedix et al. 2010; Dudek et al. 2005). Using the same experimental strategies as well as surface plasmon resonance spectroscopy the double-spanning membrane protein Sec62 was shown to interact via two positively charged clusters encoded in the cytosolic N-terminus of Sec62 with the ribosome close to the exit tunnel (Müller et al. 2010). In case of p180, a single-pass type I membrane protein with a gigantic cytosolic coiled-coil domain, data are somewhat ambiguous with regard to what entity it actually attracts to the ER membrane. Besides a direct association of p180 to the ribosome as part of the RTC, some data highlight the possibility of p180 acting as direct mRNA anchor (Savitz and Meyer 1990, 1993; Cui et al. 2012, 2013; Dejgaard et al. 2010; Morrow and Brodsky 2001; Ueno et al. 2010, 2011). Regardless, both options would attract polysomes to the translocon to enhance protein translocation, substrate-specific or not. Further, predominantly cytosolic, ribosomal interactors supporting the substrate specific targeting of precursor polypeptides to the translocon will be discussed in the section “precursor protein targeting factors”.

The TRAP Complex

The human TRAP complex consists of four consecutively named subunits TRAPα to TRAPδ (Hartmann et al. 1993). With the exception of TRAPγ carrying four transmembrane helices and negligible luminal mass, the other TRAP subunits are single spanning type I membrane proteins with a cleavable targeting signal and a

luminal domain of over 100 amino acids (Bañó-Polo et al. 2017). A reasonable cytosolic mass comprised of roughly 60 amino acids is found only in TRAPα and TRAPγ. Despite the often discussed compositional heterogeneity of the native ribosome-associated translocon, TRAP seems to be a stoichiometric and permanent component of it (Braunger et al. 2018; Pfeffer et al. 2015). Functionally, TRAP supports protein translocation by the Sec61 complex in a substrate-specific manner. TRAP-mediated assistance was observed for precursor proteins with cleavable signal peptides or N-terminal transmembrane helices. Experiments based on biochemical reconstitution demonstrated for a small subset of substrates that only signal sequences with a strong translocon interaction and quick gating potential are able to be transported independently of TRAP (Fons et al. 2003). Similarly, in vitro studies testing mutations in the flanking charges of a type II signal anchor obscuring the positive-inside rule demonstrated the importance of TRAP for proper topogenesis of such a transmembrane helix handled by the Sec61 complex (Sommer et al. 2013; Baker et al. 2017; von Heijne 2006; von Heijne and Gavel 1988; Goder et al. 2004). Using quantitative proteomics to analyze changes of cellular protein abundance upon TRAP depletion revealed that signal peptides of TRAP-dependent clients exhibit a glycine-plus-proline content above and hydrophobicity below average (Nguyen et al. 2018). As "helix-breaking" residues the pronounced glycine-plus-proline content of TRAP-dependent signal peptides reduces their propensity of helix formation and likely their ability to (i) intercalate at the lateral gate of the Sec61 complex and (ii) displace the lateral gate helix 2 in order to advance the channel from the primed to the open state (Figs. 4.5 and 4.6). The reduced hydrophobicity of TRAP-dependent targeting signals probably reduces the likelihood for efficient binding/interaction with the single hydrophobic patch that opens up upon priming of the Sec61 complex by ribosome binding (Voorhees and Hegde 2016a). In the end, both parameters of TRAP clients reduce their ability to overcome the activation energy necessary to convert the primed state of the Sec61 complex into the open state in a reasonable dwell time (Figs. 4.5 and 4.6). CET provided an interesting view on how the TRAP complex could help to overcome this energetic deficit. To do so, a set of difference densities comparing the native canine TRAP complex with algal (lacking TRAPγ/δ) and TRAPδ-deficient human TRAP complexes demonstrated proximity of the luminal mass (likely the TRAPα/β subunits) to loop 5, the hinge region connecting the N- and C-terminal halves of Sec61α and permitting the rigid body movement (Pfeffer et al. 2017) (Figs. 4.5 and 4.7a). Different algorithms predicted for the luminal domains of both TRAPα and TRAPβ a beta-sandwich fold. This is a classical domain structure often found in immunoglobulins or lectins, protein classes specialized in binding to other polypeptide or carbohydrate moieties. Considering both proximity and domain fold, we assume that TRAP acts as allosteric effector of the Sec61 complex in a chaperone-equivalent fashion and catalyzes a reduction of the energy barrier enabling gating deficient signal peptides of TRAP-dependent substrates to open the Sec61 complex (Figs. 4.4, 4.5, 4.6 and 4.7). Alternatively, or additionally, TRAP could work as a ratchet on the nascent precursor polypeptides in transit into the ER lumen directly. Moreover, taking into consideration the vicinity between the cytosolic

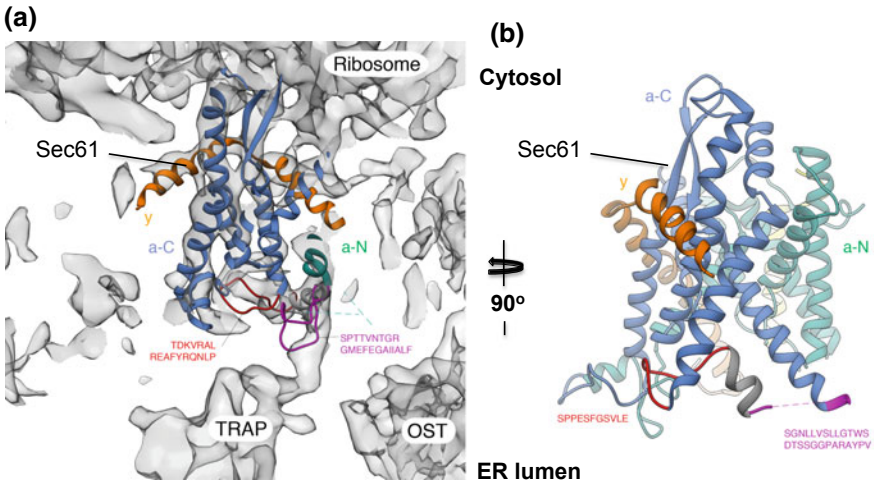


Fig. 4.7 Atomic model for the heterotrimeric Sec61 complex, fitted into the ribosome-translocon structure, shown in Fig. 4.2b and highlighting the ER luminal contact sites of TRAP and BiP, respectively. **a**, **b** The Sec61 channel is shown in two orientations, as seen from the plane of the membrane (PDB 3jc2, EMD 3069). The N- and C-terminal halves of the Sec61 α-subunit are shown in green and blue, respectively, and the γ-subunit in orange. **a** The same view of the Sec61 complex is shown as in Fig. 4.4b. However, most of the N-terminal half of the α-subunit and the complete ß-subunit were clipped for better visibility of the TRAP interaction site. The ER luminal domains of the TRAP α- and β-subunits interact with ER luminal loop 5 of the Sec61 α-subunit (connecting transmembrane helices 5 and 6), which is shown with the surrounding electron densities of the ribosome, TRAP, and OST. The primary structure of the TRAP binding site within loop 5 is N-terminal to the so-called hinge helix (connecting the N- and C-terminal halves of the Sec61 α-subunit; shown in grey) and is shown in magenta; the amino acid sequence C-terminal to the hinge region is shown in red. There is no atomic structure of TRAP, but secondary structure predictions for the ER luminal domains of the TRAP α- and β-subunits are consistent with a beta sandwich fold. **b** In (**b**) the Sec61 complex is rotated counter clockwise in the plane of the membrane by 90° as compared to (**a**). The substrate binding domain (SBD) of ER luminal Hsp70-type molecular chaperone BiP interacts with ER luminal loop 7 of the Sec61 α-subunit (connecting transmembrane helices 7 and 8), and is recruited to the Sec61 complex by ER membrane resident Hsp40-type co-chaperone Sec63 via the J domain of the latter. The BiP binding site within loop 7 includes the so-called minihelix (shown in grey) between the up-stream lying oligopeptide (shown in purple) and the down-stream lying oligopeptide (shown in red)

domain of TRAPγ and the ribosome (eL38, ES20L/ES26L) mentioned before, the TRAP complex acts as relay bridging incoming precursor information from the cytosol across the ER membrane to the luminal side to support the conformational switch of the Sec61complex necessary to accommodate TRAP-dependent substrates. This mechanism of action portraying TRAP as allosteric effector that supports opening of the Sec61 complex was further substantiated by two lines of evidence. One, live cell calcium imaging measurements showed that depletion of TRAP in human cells reduced the Sec61-mediated calcium efflux from the ER (Nguyen et al. 2018). Two, comparing the evolutionary conservation of the

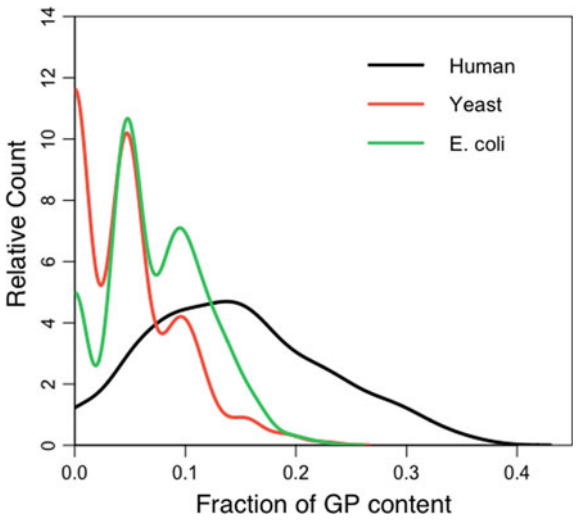


Fig. 4.8 Due to the presence of TRAP, the mammalian Sec61 complex can handle signal peptides with relatively high content of glycines and prolines, in contrast to the homologous complexes in yeast and bacteria. Client specificity of human TRAP was revealed by a combination of siRNA-mediated TRAP depletion in HeLa cells and quantitative proteomics plus differential protein abundance analysis. The combination of siRNA-mediated gene silencing, using two different siRNAs for each target and one non-targeting (control) siRNA, respectively with three replicates for each siRNA and label-free quantitative proteomic analysis plus differential protein abundance analysis was used to identify negatively affected proteins (i.e. TRAP clients). Subsequently, we used custom scripts to compute the glycine/proline (GP) content of signal peptide sequences of TRAP clients as the total fraction of glycine and proline in the respective signal peptide sequences (data not shown). We also used custom scripts to extract protein annotations for all human, *E. coli* and *S. cerevisiae* signal peptides from UniProtKB entries

glycine-plus-proline content of cleavable signal peptides encountered in TRAP-carrying humans and TRAP-deficient organisms such as yeast and *E. coli* showed a much higher glycine-plus-proline content in the former (Fig. 4.8). Thus, enabled by TRAP, the mammalian Sec61 complex can manage signal peptides with a higher content of glycines and prolines compared to its homologous ancestors in yeast and bacteria.

The OST Complex

In contrast to the monomeric oligosaccharyl-transferase in bacteria (PglB) and archaea (AglB) the human OST complex is represented by two multimeric paralogs, named after the catalytic core subunits Stt3A and Stt3B (Table 4.1). The latter two, which show highest sequence homology amongst the OST subunits to the prokaryotic monomers, catalyze the transfer of the lipid-linked glycan

($Glc_3Man_9GlcNAc_2$) onto the asparagine residue of a specific trimeric sequon motif (Asn-X-Ser/Thr, where X ≠ Pro) in the polypeptide chain (Larkin and Imperiali 2011). N-linked glycosylation in the mammalian ER can occur co-translationally, while the polypeptide is in transit, or post-translocationally. While the former is usually catalyzed by the Stt3A-containing OST complex skipped sequons are complemented by the Stt3B paralog which can also act post-translocationally to N-glycosylate C-terminally located sites within the polypeptide chain (Shrimal et al. 2015; Ruiz-Canada et al. 2009; Sato et al. 2012). Despite two paralogous OST complexes complementing each other one-third of Asn in glycosylation sequons are not modified (Petrescu et al. 2004; Kelleher and Gilmore 2006). Important for glycosylation is a conserved sequon binding motif (Trp-Trp-Asp) in the substrate binding groove of various Stt3 homologs, representing the active center of OST complexes (Bai et al. 2018). Structural data and early in vitro based assays demonstrated a distance of roughly 40–60 Å (equivalent to a distance spanned by 15–20 amino acids) from the translocon exit to the active center of Stt3 (Nilsson et al. 2003; Nilsson and von Heijne 1993; Wild et al. 2018; Bañó-Polo et al. 2011; Kowarik et al. 2002). The paralogous OST complexes in humans share a set of six subunits Rpn1, Rpn2, DAD1, OST4, OST48, and TMEM258. In the Stt3B-containing OST this set is supplemented by the catalytic Stt3B, and MagT1 or TUSC3 subunit. In case of the Stt3A paralog the complex-specific subunits are Stt3A, DC2 and Kcp2 (Cherepanova et al. 2016). The latter two, DC2 and Kcp2, were shown biochemically and by structural analysis to connect the Stt3A-containing OST to the Sec61 complex (Shrimal et al. 2017; Braunger et al. 2018). While the Stt3B-containing OST complex is a stand-alone unit, the Stt3A-containing OST can associate with the RTC, including TRAP, to form a co-translational super-complex as observed even in the native membrane (Wild et al. 2018; Pfeffer et al. 2014; Ruiz-Canada et al. 2009). Tethering of the Stt3A-containing OST to the RTC is ensured by two interfaces. The first interface refers to the specific association between DC2 and Stt3A. This interaction is mediated by cytosolic, membrane and luminal portions of both proteins including transmembrane helices 10–13 and the last cytosolic loop of Stt3A. Due to sequence variations between Stt3A and Stt3B in this contact area required for the DC2 association, DC2 binds selectively to the Stt3A paralog. Next, DC2 with the rest of the Stt3A-containing OST in tow binds to the Sec61 complex. The luminal loop of DC2 likely interacts with the N-terminal half of Sec61α as well as the C-termini of Sec61β and Sec61γ (Braunger et al. 2018). Put simply, the membrane-embedded part of the Stt3A-containing OST complex flanks the hinge region of Sec61α (Pfeffer et al. 2016). The second interface was mentioned earlier and refers to the quadruple-helix bundle of the cytosolic Rpn1 domain binding to the ribosome. Thus, the dissimilar subunit composition between the two alternative OST complexes explains both the paralog-specific association with the RTC and, by extrapolation, the different modes of action (co-translational Stt3A versus post-translocational Stt3B). The DC2 subunit is the bridging element that ties exclusively the Stt3A-containing OST to the N-terminal half of the Sec61 complex (Fig. 4.2). Further evidence showing the paralog-specific integration of the

Stt3A-containing OST- complex into the RTC comes from CET of Stt3 knockout cells. While the ratios of RTC populations carrying only TRAP or TRAP + OST densities were identical for wildtype and Stt3B knockout cells, the RTC population carrying TRAP + OST could not be observed at all in Stt3A knockout cells (Braunger et al. 2018). Yet, it is currently unclear if and how the OST complex might affect the Sec61-mediated calcium efflux. Although inhibitors of glycosylation such as tunicamycin trigger an elevated Sec61-mediated calcium efflux from the ER, this effect cannot be assigned to the inactivity of OST complexes rather than being a consequence of inaccurate protein maturation and subsequent BiP sequestration (Schäuble et al. 2012).

The Sec62/63 Complex

As discussed before, the Sec61 complex can handle incoming polypeptides either co-translationally or post-translationally. However, reasonable structural data depicting the organization of the post-translational eukaryotic Sec61 complex are scarce. So far, three studies highlighted the assembly of the detergent extracted, unoccupied, post-translational translocon complex from *S. cerevisiae* by cryo-EM (Itskanov and Park 2018; Wu et al. 2018; Harada et al. 2011). In yeast, the fully assembled post-translational translocon represents a heptameric protein ensemble referred to as the SEC complex (Deshaies et al. 1991; Panzner et al. 1995; Jermy et al. 2006). In the SEC assembly the trimeric Sec61 complex is accompanied by the tetrameric Sec62/63 complex. The latter consist of two essential, evolutionarily conserved membrane proteins, Sec62 and Sec63, and two dispensable subunits, Sec71 and Sec72. All seven subunits of the SEC complex are bundled in a 1:1 stoichiometry (Harada et al. 2011). Additionally, in vitro reconstitutions demonstrated that the functional SEC complex needs support from an ATP consuming Hsp70 chaperone of the ER lumen, Kar2p in yeast or BiP in mammals, for efficient post-translational transport (Panzner et al. 1995; Matlack et al. 1999; Brodsky et al. 1995; Brodsky and Scheckman 1993). Although the different cryo-EM structures did not include any BiP density, the data provided first insights into how the SEC complex is arranged to allow gating of the Sec61 complex and support transport of post-translational substrates. Most striking was the extensive interaction between Sec63 and the Sec61 complex including contacts in their cytosolic, membrane and luminal domains. Similar to the ribosome or the bacterial SecA ATPase, the cytosolic Brl domain of Sec63 interacts with loops 6 and 8 of Sec61α, the universal docking port, thereby "reserving" the docking port and blocking ribosome binding. Interestingly, as assumed for the interaction of the TRAPα/β subunits with the Sec61 complex, the Brl domain of Sec63 shows a canonical beta-sandwich fold for an antigen-antibody-like binding to loop 6. In the membrane, Sec63 (transmembrane helix 3) contacts all three subunits of the Sec61 complex in the hinge region opposite to the lateral gate including transmembrane helices 5 and 1 of Sec61α as well the membrane anchors of Sec61β and Sec61γ. In addition, the short luminal

N-terminus of Sec63 appears to intercalate on the luminal side of the channel between the hinge loop (Sec61α loop 5) and Sec61γ (Itskanov and Park 2018; Wu et al. 2018). Yeast viability assays with single point mutations introduced in the cytosolic or membrane contact area of Sec63 resulted in lethality, highlighting the importance of those contacts. On the other hand, mutations in the luminal interaction site did not affect cell viability (Wu et al. 2018). The two nonessential subunits, Sec71 and Sec72, sit on top of Sec63's Brl domain. Structurally, binding of the Sec62/63 complex to the Sec61 channel triggered opening of the lateral gate much wider than observed in any previous cryo-EM structure (Van den Berg et al. 2004; Voorhees and Hegde 2016a; Li et al. 2016; Zimmer et al. 2008; Egea and Stroud 2010). The functional implications for the translocon resulting from gating by the Sec62/63 complex are exquisite. First, targeting signals of many post-translational substrates are often less hydrophobic and therefore would have a lower chance to intercalate at the lateral gate in the primed Sec61 complex and drive further opening of the channel. However, in the post-translational SEC complex binding of the Sec62/63 complex seems to induce a fully opened channel that readily accommodates even "weak" or otherwise inefficient intercalating targeting signals (Trueman et al. 2011, 2012; Ng et al. 1996). Fitting to the concept of the Sec62/63 complex inducing wide opening of the lateral gate yeast Sec62 was found to mediate topology of moderately hydrophobic signal anchor proteins, in particular type II membrane proteins that undergo the energetically unfavorable 180° flip turn for reversing the initial type I orientation (Reithinger et al. 2013; Jung et al. 2014). Second, association of the Sec62/63 complex opposite to the lateral gate of the Sec61 complex might perturb binding of the co-translational acting Stt3A-containing OST complex. Thus, Sec63 might block both ribosomal binding and coordination of the Stt3A-containing OST complex found in most multicellular plants and metazoans (Cherepanova et al. 2016). This could imply that post-translational substrates are exclusively glycosylated post-translocationally and that the appearance of the Sec62/63 complex, which is absent in bacteria, occurred hand-in-hand with the gene duplications of specific OST complex subunits. Similar to metazoans, also yeast has two paralogous OST complexes containing either Ost3 or Ost6, the yeast homologs of TUSC3 and MagT1, respectively. Both variations of the OST complexes in yeast share the catalytic Stt3 subunit, which is more similar to the mammalian Stt3B subunit and explains why the OST complexes in yeast are considered stand-alone units (Wild et al. 2018). Interestingly, recent CET data from Stt3A depleted HEK cell microsomes observed a previously unidentified translocon population devoid of TRAP and OST (Braunger et al. 2018). The unknown density could represent the mammalian equivalent of the SEC complex with or without BiP. Third, binding of the Sec62/63 complex to the luminal end of the Sec61 channel might also interfere with functionality of the TRAP complex. Both accessory complexes Sec62/63 and TRAP appear on the luminal site of the channel in proximity to loop 5, which connects the N- and C-terminal halves of Sec61α. Interaction of the accessory complexes with loop 5 might support the rigid body movement during opening of the Sec61 complex, eventually in a substrate specific manner. Fourth, the extremely wide opening of the lateral gate triggered by binding

of the Sec62/63 complex might also impact the Sec61-mediated calcium efflux from the ER. While this issue can be tolerated in yeast, whose major intracellular calcium store is the vacuole rather than the ER, in mammalian cells the excessive leakage of calcium would need to be compensated by other factors. Work from our own group suggests that Sec62 and BiP are efficient regulators of the ER calcium leakage (Schäuble et al. 2012; Greiner et al. 2011; Linxweiler et al. 2013).

Similar to the situation in yeast, studies of protein transport in mammalian cells also show the substrate-specific involvement of Sec62 in ER import (Fig. 4.5). Mammalian Sec62 is required for the efficient transport of small precursor proteins (such as preproapelin), which are, due to their short length, transported post-translationally (Lang et al. 2012; Haßdenteufel et al. 2018; Johnson et al. 2013; Lakkaraju et al. 2012b). However, in contrast to yeast, the mammalian Sec62 protein experienced a gain of function as it is able to interact with the ribosome near the ribosomal exit tunnel and also supports the co-translational transport of certain substrates, such as the precursors of ERj3 and prion protein (Müller et al. 2010; Fumagalli et al. 2016; Ziska et al. 2019). Accordingly, cross-linking experiments using different stalled precursor polypeptides in transit through the mammalian translocon demonstrated the dynamic recruitment of allosteric regulators including Sec62. The model precursor preprolactin recruited accessory factors like TRAP, OST, and the translocating chain-associating membrane (TRAM) protein to the Sec61 complex. Yet, when ERj3 or prion protein were used as precursor the Sec62/63 complex instead of TRAP and TRAM was recruited to the channel to support translocation of those substrates with a rather slowly or inefficiently gating targeting signal (Conti et al. 2015; Fumagalli et al. 2016; Ziska et al. 2019). The gap between the Sec61 complex and the ribosome exit tunnel providing a space for the elusion of nascent polypeptides could be critical for the substrate-specific recruitment of regulatory factors. Another dynamic transition of the translocon during specific translocation events was observed for Sec62 and the SRP receptor, a protein targeting complex that, as will be discussed in more detail later, is required for co-translational targeting of precursor proteins to the Sec61 complex. To allow co-translational targeting the SRP receptor apparently displaces Sec62 from the SEC complex switching the Sec61 channel from Sec62- to SRP-dependent translocation (Jadhav et al. 2015). However, according to above mentioned experiments, SRP receptor and Sec62 can also act sequentially, i.e. after SRP-dependent targeting of precursors of ERj3 and prion protein, Sec62 can displace SRP receptor from the Sec61 channel and together with Sec63 support channel gating. Furthermore, in this scenario Sec63 has to be expected to take over loops 6 and 8 of Sec61α from the ribosome. Lastly, depletion of Sec63 from mammalian cells, which is neither accompanied by a loss of Sec62 nor compensated by increased levels of other major translocon components (Fig. 4.9), causes a substrate specific defect in protein translocation of membrane and secretory proteins, but is without effect on the Sec61-mediated calcium leakage (Lang et al. 2012; Mades et al. 2012; Haßdenteufel et al. 2018; Schorr et al. 2015; Fedeles et al. 2011). To a certain extent even the cryo-EM structures of the post-translational SEC complex reflect the idea of a dynamic transition and flexibility of the Sec62/63

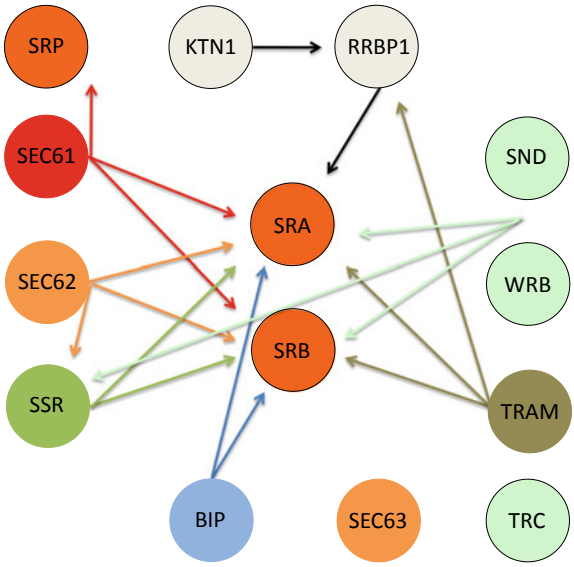


Fig. 4.9 Genetic interactions between ER targeting- or translocation-components, as revealed by a combination of siRNA-mediated component depletion in HeLa cells and quantitative proteomics plus differential protein abundance analysis. The combination of siRNA-mediated gene silencing, using two different siRNAs for each of the indicated targets and one non-targeting (control) siRNA, respectively with three replicates for each siRNA and label-free quantitative proteomic analysis plus differential protein abundance analysis was also used to identify positively affected proteins (i.e. compensatory mechanisms or genetic interactions, indicated as arrows)

complex. Both Sec62 and the characteristic luminal J-domain of Sec63 could not be sufficiently resolved in the particle analysis which might be due to their structural flexibility and dynamic integration into the translocon.

Additional Transport Components and Allosteric Effectors of the Sec61 Channel

In addition to the multimeric complexes from the previous section many more auxiliary transport components and allosteric effectors of the Sec61 channel, respectively, have been experimentally detected to transiently interact with the Sec61 complex. So far, these auxiliary components eluded structural visualization in combination with the polypeptide-conducting channel. Yet, a few examples will be named and summarized.

BiP, an Additional Allosteric Sec61 Channel Effector

One of the most abundant and versatile proteins within the ER lumen is the Hsp70-type molecular chaperone named BiP (Fig. 4.5). Originally identified as protein that binds non-covalently to free immunoglobulin heavy-chains its repertoire of functions was steadily extended as was reviewed before (Haas and Wabl 1983; Zimmermann 2016; Otero et al. 2010; Dudek et al. 2009; Ni and Lee 2007; Ma and Hendershot 2004). Consistent with its domain organization BiP reversibly binds to hydrophobic oligopeptides of loosely folded polypeptides in an ATP-regulated manner (Flynn et al. 1991). To do so, a flexible inter-domain linker region connects the N-terminal nucleotide binding domain (NBD) and C-terminal substrate-binding domain (SBD) of BiP (Kumar et al. 2011). Thus, binding and release of a substrate are coupled to an ATPase cycle that triggers conformational transition of BiP's different sub-domains and supports productive folding of a substrate (Smock et al. 2010; Marcinowski et al. 2011). For a complete and productive ATPase cycle BiP is part of a tripartite system. Aside from BiP, this system encompasses a J-domain carrying Hsp40-type co-chaperone and a nucleotide exchange factor (Table 4.1). ATP-bound BiP has a low affinity for substrates given that a sub-domain within the SBD called the lid is in the open conformation. Association with the characteristic J-domain of one of the multiple ER luminal Hsp40-type co-chaperones (e.g. Sec63) stimulates the ATPase activity of BiP and coincides with both a closure of the lid in the SBD and a drastic increase in substrate affinity. Reversal of this reaction is mediated by a nucleotide exchange factor that helps to replace ADP by ATP and convert BiP back into the low-affinity state with an open lid (Melnyk et al. 2015). The currently known repertoire of at least eight Hsp40 co-chaperones and two nucleotide exchange factors in human cells allows fine-tuning of the ATPase cycle of BiP and its function can be tailored for various substrates and/or occasions. The combinatorial assembly of the tripartite system with BiP at its center probably allows BiP to integrate its many known functions. Those functions relate to the import, folding/assembly, export, and degradation of polypeptides as well as regulation of folded proteins such as the UPR sensors IRE1, PERK, and ATF6 or the translocon component Sec61α (Dudek et al. 2009; Hennessy et al. 2000, 2005; Wang and Kaufman 2012; Walter and Ron 2011; Zhao and Ackerman 2006). For the latter, BiP was shown to support protein transport into the ER in two different modi operandi. One, as molecular ratchet BiP directly works on the incoming, unfolded precursor polypeptide in transit through the Sec61 complex (Tyedmers et al. 2003). The ratcheting supports both co- as well as post-translational translocating substrates (Liebermeister et al. 2001; Panzner et al. 1995; Brodsky and Scheckman 1993; Brodsky et al. 1995). Two, BiP acts as direct allosteric effector of the Sec61 complex for channel opening by binding to the luminal loop 7 of Sec61α (Fig. 4.7b). Prohibiting the binding of BiP to luminal loop 7 achieved either by BiP depletion or the introduction of a BiP repelling mutation in loop 7 caused a substrate-specific defect in transport for the precursors of ERj3 and prion protein as well as for the short, post-translationally transported

preproapelin (Schäuble et al. 2012; Lloyd et al. 2010; Haßdenteufel et al. 2018). Again, those are the precursors with a rather inefficiently gating targeting signal plus inhibitory features in the mature part that require support for opening the channel (Fig. 4.5) (Haßdenteufel et al. 2018). Given that both ERj3 and prion protein were demonstrated to recruit the Sec62/63 complex to the translocon, suggests that BiP together with the luminal J-domain of the Hsp40 co-chaperone Sec63 forms a functional unit acting as allosteric effector of the Sec61 complex for substrate-specific channel opening (Conti et al. 2015). Simultaneously, the activity of BiP at the Sec61 complex is required for proper sealing of the Sec61 channel to prevent excessive calcium leakage (Schäuble et al. 2012). Interestingly, studies of the calcium permeability across the ER membrane provide an explanation of how the different activities of BiP at the Sec61 complex (opening for protein substrates; closing for calcium) might be connected to different co-chaperones. On the one hand, depletion of the Sec63 co-chaperone, i.e. a membrane-bound J-domain, caused a substrate-specific defect in protein transport without detectable impact on the Sec61-mediated calcium leakage (Lang et al. 2012; Schorr et al. 2015). On the other hand, depletion of the ER luminal, i.e. soluble, J-domain containing protein ERj3 or ERj6 caused a calcium specific phenotype which was attributed to inefficient sealing of the Sec61 channel (Schorr et al. 2015). This idea of BiP closing the Sec61 channel by direct interaction was further substantiated by single channel recordings from planar lipid bilayer experiments. Addition of a loop 7 antibody, binding as pseudo-ligand to loop 7, prevented ion currents through the translocon by shifting the equilibrium of the Sec61 channel to the closed state (Schorr et al. 2015). Taken together, functionality of the abundant calcium buffering chaperone BiP is linked to two metabolites, ATP and calcium. Therefore, BiP might be able to supervise and orchestrate ER homeostasis by integrating proteostasis, calcium balance, and, as will be discussed later, energy homeostasis of the ER (Lang et al. 2017).

Auxiliary Transport Components of the ER Membrane

Other auxiliary translocon components have been detected that reside in the ER membrane and modify the translocating polypeptide or foster its transport. As such, cleavable signal peptides from precursor proteins that intercalate at the lateral gate can be cleaved off by an intramembrane protease called the signal peptidase complex (Chen et al. 2001; Evans et al. 1986; Dalbey and von Heijne 1992). The existence of two enzymatically active subunits in this complex may hint at the possibility, in analogy to oligosaccharyltransferase, that there may actually be two signal peptidase paralogs with either SPC18 or SPC21 (Table 4.1). Chemical crosslinking suggested that the signal peptidase complex is transiently recruited to the translocon via an interaction with the Sec61β subunit and recruitment depended on the presence of membrane-bound ribosomes (Kalies et al. 1998). Signal peptides can be further processed by the signal peptide peptidase and translocating nascent

chains can be modified by the GPI transamidase attaching a mixed lipid/sugar moiety called glycosylphosphatidylinositol (GPI) anchor to the C-terminus (Kapp et al. 2009; Lemberg and Martoglio 2002; Weihofen et al. 2002; Kamariah et al. 2011). Recently, attention was drawn to another ER membrane protein complex (EMC) that was first identified in yeast and later in human cells as heteromultimeric protein complex with 6 and 10 subunits, respectively (Jonikas et al. 2009; Christianson et al. 2011). Biochemical and cellular characterization of EMC characterized it as both stand-alone insertase for tail-anchored membrane proteins with a moderately hydrophobic transmembrane helix and as helper translocase likely in synergy with the Sec61 complex for the insertion of critical transmembrane helices of polytopic membrane proteins (Guna et al. 2017; Chitwood et al. 2018; Shurtleff et al. 2018). Besides those multimeric protein complexes other monomeric proteins have been shown to transiently contact or be in vicinity of the Sec61 complex (Table 4.1). Interestingly, it was deduced from sequence comparisons that TMCO1 is one of these proteins and, together with Get1 and EMC3, represents a remote Oxa1/Alg3/YidC homolog in the ER membrane (Anghel et al. 2017). In partial analogy with YidC, TMCO1 was found in association with both ribosomes and the Sec61 complex. It addition, it was found to be able to reversibly tetramerize and to restore calcium homeostasis upon calcium-overfilling of ER calcium stores (Wang et al. 2016). Using reconstituted proteoliposomes, TRAM was one of the first proteins found to provide substrate-specific assistance for the translocase (Görlich and Rapoport 1993). Follow-up studies demonstrated that precursor proteins with short charged N-terminal domains in their signal peptide require TRAM for efficient insertion into the lateral gate and that TRAM could regulate the cytosolic elusion of nascent chain domains into the gap between ribosome and translocon (Voigt et al. 1996; Hegde et al. 1998). Similar to Sec63 another J-domain containing membrane protein named ERj1 was demonstrated to associate with the Sec61 complex. ERj1 binds to ribosomes close to the exit tunnel and recruits BiP via the J-domain to both the Sec61 channel and incoming polypeptides (Dudek et al. 2005; Blau et al. 2005). However, the precise role of ERj1 in protein transport remains enigmatic. The dynamic recruitment of yet another auxiliary translocon component, RAMP4, can be triggered by transmembrane helices still buried within the ribosomal exit tunnel. Recruitment of RAMP4, also called stress-associated endoplasmic reticulum protein 1 (SERP1), is mediated by ribosomal protein uL22 which spans from the tunnel wall to the ribosomal surface. Thus, uL22 senses a transmembrane helix inside the ribosome and signals recruitment of RAMP4 to the translocon to prime it for subsequent transmembrane helix integration (Pool 2009; Yamaguchi et al. 1999). Also, bundles of transmembrane helices of certain polytopic membrane proteins were shown to reside in vicinity of the Sec61 complex for their collective release into the membrane during biogenesis (Cross and High 2009a, b; Sadlish et al. 2005; Ismail et al. 2008). In some cases, PAT-10 (protein associated with the ER translocon of 10 kDa) was found to interact in vicinity of the Sec61 complex and chaperone specific transmembrane helices of a polytopic membrane protein throughout its synthesis (Meacock et al. 2002; Ismail et al. 2008). To monitor arising problems with protein

folding or transport early and right at the translocon the UPR sensor Ire1 directly interacts with the Sec61 complex generating a rendezvous point for surveillance, signaling and processing endeavors (Plumb et al. 2015). Ire1 is a membrane-anchored endonuclease that can cleave ribosome-engaged mRNAs after it is activated by misfolded proteins in the ER. To initiate UPR signaling Ire1 cleaves its key substrate XBP1u mRNA, which was targeted as ribosome-nascent chain complex to the translocon. And, Ire1 cleaves other ER destined mRNAs in a process identified as regulated Ire1-dependent decay (RIDD) to reduce the folding and synthesis burden of the ER under stress conditions (Sundaram et al. 2017; Yanagitani et al. 2011; Hollien et al. 2009; Hollien and Weissman 2006). In addition to the signaling and processing mode of Ire1, its close interaction with the RTC allows it to act in a surveillance mode to cleave mRNAs whose translation products show signs of mis-folding in the ER (Acosta-Alvear et al. 2018). Connection between the Sec61 complex and the Ire1 branch of the UPR was also demonstrated on a genetic level using a multiplexed, genome scale CRISPR screening showing a reciprocal feedback loop between the two. During ER stress, subunits of the Sec61 complex were exclusively transcribed in response to activation of the Ire1 branch of the UPR. In turn, the loss of Sec61 complex subunits was compensated by selective activation of Ire1 signaling (Adamson et al. 2016).

Additional Allosteric Effectors in the Cytosol Interacting with the Sec61 Complex

Multiple cytosolically located proteins have been identified to interact with the Sec61 complex. Many of those are part of coexisting targeting machineries that are composed of cytosolic and membrane-bound components. These targeting pathways delivering precursor proteins to the translocon will be discussed later. Here, we will focus on calmodulin (Fig. 4.5), the ubiquitous calcium binding protein of the cytosol involved in different second messenger systems and the regulation of ion channels and other pivotal proteins (Chin and Means 2000). Some binding sites for calmodulin are called IQ-motifs to which calmodulin binds either in its apo- (calcium-free) or holo-form (Bähler and Rhoads 2002; Tidow and Nissen 2013). Such an IQ-motif was identified in the cytosolic N-terminus of mammalian Sec61α (Erdmann et al. 2011). A series of protein-protein interaction studies, planar lipid bilayer recordings, molecular modeling and live cell calcium imaging demonstrated the calcium-dependent binding of calmodulin to this IQ-motif to limit the Sec61-mediated calcium efflux during protein translocation. The introduction of charge deleting mutations masking the IQ-motif or the use of calmodulin inhibitors caused dysregulated calcium permeability of the Sec61 complex (Erdmann et al. 2011; Harsman et al. 2011b; Lang et al. 2011b). Interestingly, binding of calcium-calmodulin to the translocon seemed not to interfere with the protein transport activity of the Sec61 complex and goes in line with the observed targeting

function of calmodulin directing small precursors to the Sec61 complex (Shao and Hegde 2011; Erdmann et al. 2011). Thus, the N-terminus of Se61α serves as calmodulin docking site for the transport of certain precursors and regulation of calcium permeability without interference for the ribosome binding and formation of the RTC.

Small Molecules Directly Interfering with the Sec61 Complex

As discussed above, the efficient gating of the Sec61 channel is of crucial importance for its role in ER protein import and its potentially harmful role for calcium-homeostasis. In recent years a growing number of Sec61 channel inhibitors was identified, which to us are best discussed in light of the energetics and kinetics of Sec61 channel gating (Fig. 4.6). According to this point of view, inhibitor selectivity is based on the distinct efficiencies of different signal peptides in reducing the activation energy for Sec61 channel opening and the common principle that the bound inhibitors or ions may increase the energy barrier for opening of the Sec61 channel (Fig. 4.6). This view is supported by the observation that the ER import of the BiP- and Sec63-dependent preproapelin is sensitive to CAM741 (Haßdenteufel et al. 2018).

This common principle is e.g. demonstrated by the effect of Lanthanum ions on the channel. Binding of several Lanthanum ions to the Sec61 complex arrests the channel in the open state, restricts the Sec61 channel dynamics, and inhibits translocation of polypeptides. Molecular modeling indicated that Lanthanum binding sites cluster at the lateral gate (Erdmann et al. 2009). In addition, several structurally unrelated small molecules have non-identical binding sites in the Sec61 complex and also inhibit the Sec61 channel with respect to ER protein import (Fig. 4.3). The first described class of Sec61 inhibitors were the cyclic heptadepsipeptides, i.e. CAM741 and cotransins (such as CT8), which inhibit translocation of polypeptides in a precursor-specific manner (Garrison et al. 2005). Next, the natural compounds Apratoxin A and Mycolactone were characterized as Sec61 inhibitors and shown to have selective (Mycolactone) or non-selective (Apratoxin A) effects on ER protein import (Paatero et al. 2016; Baron et al. 2016). The binding sites of these Sec61 channel inhibitors were identified by clever strategies, which selected inhibitor resistant human cell lines. According to the analysis by MacKinnon et al., who obtained five mutations that showed resistance to CT8, four of which were in the plug and downstream region (R66I, R66G, G80V, and S82P), CT8 interacts with the short plug helix in loop 1 and transmembrane domains 2 and 3 of Sec61α (Fig. 4.3) (MacKinnon et al. 2014). In case of Mycolactone, the mutagenesis studies also identified the plug residues R66 and S82 in loop 1 as interaction site (Fig. 4.3) (Baron et al. 2016; McKenna et al. 2017). In contrast, the respective studies on Apratoxin A identified T86 and Y131 as binding site and

indicated a distinct binding mode (Fig. 4.3) (Paatero et al. 2016). This is consistent with the effects of yet another class of Sec61 channel inhibitors, i.e. the Eeyarestatins (ES1, ES24), where there are no mutagenesis studies available as of yet, on calcium permeability of the channel. Here, binding of the inhibitor within the channel pore arrests the channel in a partially open state (termed 'foot in the door'), which may be identical with the primed state and is compatible with calcium efflux but not with full channel opening for protein translocation (Gamayun et al. 2019). We note, however, that the Sec61 channel is also affected by a bacterial protein toxin, *Pseudomonas aeruginosa* Exotoxin A, which enters human cells by retrograde transport and inhibits ER export of immunogenic peptides. Exotoxin A binds near the calcium-calmodulin binding site to the N-terminal tail of Sec61α and arrests the channel in the closed state (Schäuble et al. 2014).

Modalities of Precursor Targeting Factors Delivering Substrates to the Translocon

As outlined above, membrane proteins of the secretory pathway are integrated into the ER membrane by either the Sec61-channel, the tail-anchored (TA) receptor, or by PEX3, and, possibly also by TMCO1 or the SND receptor (Table 4.1). Before a precursor polypeptide can be membrane integrated or fully translocated by the polypeptide conducting Sec61 channel, however, its respective mRNA or the precursor itself has to be specifically delivered, i.e. targeted to the Sec61 complex in the ER membrane (Fig. 4.3). Current knowledge about mRNA targeting to the ER membrane is scarce (see below). In contrast, the following detailed concept emerged for ER protein targeting: Apparently, a molecular triage operates for ER-destined precursor polypeptides during their synthesis on ribosomes in the cytosol, which determines the fates of nascent or fully synthesized but not yet folded polypeptides by the complex network of targeting signals in nascent chains or completed polypeptides and a whole variety of cytosolic factors (SND1, SRP, TRC40) (Table 4.1), which recognize these signals and have overlapping specificities. In addition, these factors chaperone the precursors for staying in solution and competent for ER targeting as well as subsequent membrane insertion into or translocation across the ER membrane. The common principle seems to be that the cytosolic factors in complex with their clients interact with heterodimeric receptors on the ER surface, which are associated with or in the neighborhood of Sec61 complexes. The respective receptors are termed Snd receptor (comprising Snd2 and Snd3), SRP receptor (comprising SRα and SRβ), or TA receptor (comprising WRB and CAML) (Table 4.1). In addition, there may be direct targeting of fully synthesized precursor polypeptides to the Sec62 protein in the ER membrane.

Targeting of Precursor Polypeptides to the Sec61 Complex in the ER Membrane

The original concept for targeting of precursor polypeptides to the ER was formulated in 1971 by G. Blobel and colleagues in the signal hypothesis (Blobel 1980). Accordingly, the N-terminal signal peptide of a nascent presecretory protein is recognized and bound by SRP at the ribosomal tunnel exit and mediates a translational attenuation. Next, the respective ribosome-nascent chain-SRP complex associates with the ER membrane via the heterodimeric SRP receptor (SR), which is membrane-anchored via the β-subunit (Blobel and Dobberstein 1975a, b; Gilmore et al. 1982a, b). Contact between SRP and SR drives the mutual hydrolysis of bound GTP and leads to hand-over of the ribosome-nascent chain complex to the Sec61 complex (Voorhees and Hegde 2015; Halic and Beckmann 2005; Egea et al. 2004; Halic et al. 2004; Jomaa et al. 2017). Thus, by definition, SRP also represents a, albeit ribosome-dependent, mRNA targeting device. This latter concept of SRP acting eventually as precursor-mRNA dependent particle was addressed in yeast by a combination of ribosome profiling and biochemical fractionation of membrane-attached and soluble ribosome populations. The genome-wide analysis of ribosome footprints (mRNA snippets protected by the ribosome during nuclease treatment) showed that non-coding mRNA elements of the 3′ UTR promote recruitment of SRP even before the encoded targeting signal is synthesized. However, such SRP loading motifs of the 3′ UTR alone were insufficient to direct translocation of a substrate into the ER (Chartron et al. 2016; Ingolia 2016). Two more studies employing comparative ribosome profiling strategies addressed functionality of the bacterial and yeast SRP in vivo. They highlighted the strong preference of SRP for transmembrane helices as "SRP recognon" regardless of their position relative to the N-terminus and, most surprisingly, the efficient targeting of precursors with just cleavable signal peptides in absence of SRP (Costa et al. 2018; Schibich et al. 2016). Rather than being at odds with the current SRP targeting dogma, in our eyes, those studies stretch the versatility of SRP and reconcile two important considerations. First, the lower abundance of the SRP compared to translating mono- or polysomes can be overcome by an mRNA dependent pre-recruitment step, probably stretching the time-window for the target recognition by SRP. Second, the crowded environment at the ribosomal tunnel exit sieged by many factors with competing function (reviewed in Pfeffer et al. 2016) can be eased by multiple iterations for SRP recognition not limited to recognition of the first transmembrane helix. In the late 1980s, identification of precursor proteins with the ability for SRP-independent ER targeting, such as small presecretory proteins in mammalian cells (many of which act as hormones in intercellular signaling or as antibacterial proteins in the immune sytem), TA-membrane proteins in mammalian and yeasts cells, and, GPI-anchored membrane proteins in yeast, suggested alternative ER targeting machineries (Yabal et al. 2003; Shao and Hegde 2011; Schlenstedt et al. 1990; Ast et al. 2013; Hann and Walter 1991). TA proteins are defined as single spanning membrane proteins with a characteristic C-terminally

located transmembrane helix (Fig. 4.1) (Kutay et al. 1993). Approximately 1% of the human genome code for TA proteins. However, not all of these have their functional locations in the secretory pathway (Kalbfleisch et al. 2007; Borgese and Righi 2010). TA proteins of the secretory pathway, such as the γ- and β-subunits of the Sec61 complex, the redox protein Cytochrome b_5, many apoptosis-associated proteins (such as the Bcl family members) and many vesicular transport components (such as Syntaxins and VAMPs), have to be targeted and inserted into the ER membrane (Borgese and Fasana 2011). Similar to SRP-mediated targeting, TA proteins are directed to the ER membrane via an ER membrane resident receptor complex. The minimal targeting machinery for TA proteins was termed transmembrane domain recognition complex (TRC) in mammalian cells (Table 4.1). The cytosolic ATPase TRC40 (also termed Asna1) with its hydrophobic binding pocket binds the TA protein and the heterodimeric receptor complex facilitates efficient ER targeting. The receptor may also facilitate the actual membrane insertion (Stefanovic and Hegde 2007; Schuldiner et al. 2008; Vilardi et al. 2011; Yamamoto and Sakisaka 2012). In addition, the TA targeting machinery involves a ribosome binding heterotrimeric complex (comprising Bag6, Ubl4A, and TRC35), which appears to act upstream of TRC40 (Mariappan et al. 2010).

Although about one dozen genes coding for yeast TA proteins were characterized as essential, knockout strains in the TA targeting pathway are viable, suggesting at least one additional route (Schuldiner et al. 2008). Indeed, in 2016 a high-throughput screening approach performed by the lab of Maya Schuldiner identified a novel targeting pathway in yeast, termed SRP-independent (SND) (Aviram et al. 2016). Three novel components were identified, characterized and termed Snd1, Snd2, and Snd3 (Table 4.1). Two hallmarks of the SND targeting pathway were described. First, similar to the SRP and TA targeting pathways, precursor polypeptides were targeted via the interplay of a cytosolic factor (termed Snd1) and a heterodimeric receptor located at the ER membrane (termed Snd2 and Snd3). Interestingly, Snd1 had previously been described as a ribosome-binding protein. Second, the SND pathway showed a preference for clients with a central transmembrane domain. In addition, the SND route was able to provide an alternative targeting pathway for substrates with a transmembrane helix at their extreme N- or C-terminus (Aviram et al. 2016). Sequence comparisons identified the previously described ER membrane protein TMEM208 as putative human Snd2 ortholog, which was termed hSnd2 (Zhao et al. 2013; Aviram et al. 2016). According to experiments, combining siRNA-mediated gene silencing and protein transport into the ER of semi-permeabilized human cells in cell-free transport assays, hSnd2 appears to have the same or at least a similar function as its yeast ortholog (Haßdenteufel et al. 2017; Casson et al. 2017). The TA membrane protein Cytochrome b_5 as well as some small presecretory proteins can be targeted to the ER or even the Sec61 complex in the mammalian cell-free assay. In brief, the human hormone precursor proteins preproapelin and prestatherin can use Sec62 as well as SR for ER targeting in the cell-free assay, which does not necessarily mean they actually do so in living cells (see below). Although smaller in overall size, prestatherin preferred SRα over Sec62-mediated targeting, whereas preproapelin

did the opposite, which may be related to the higher hydrophobicity of the prestatherin signal peptide (ΔG^{pred} −0.91 vs. −0.19). Taken together with our observation that C-terminal extension of preproapelin or prestatherin by the dihydrofolate reductase (i.e. by 187 amino acid residues) leads to Sec62 independence, our data support the hypothesis that small presecretory proteins use the SRP pathway for Sec61 targeting in human cells less efficiently, simply because the corresponding nascent chains are more likely released from ribosomes before SRP can efficiently interact (Haßdenteufel et al. 2018; Lakkaraju et al. 2012b; Schlenstedt et al. 1990). Therefore, these precursors have to use alternative targeting pathways. Notably, in yeast, low hydrophobicity of signal peptides and C-terminal signals for the attachment of GPI-anchors preclude effective use of SRP and, therefore, cause Sec62p- and TA-dependence (Aviram and Schuldiner 2014). In addition to SR and Sec62, co- and post-translational targeting of preproapelin and prestatherin can also involve both the TRC system and the recently identified SND pathway, albeit to different degrees for the two different precursors (Fig. 4.3) (Haßdenteufel et al. 2018). However, orthologs of Snd1 and Snd3 have not yet been characterized in the mammalian system and are subject of our current research.

Furthermore, some small model presecretory proteins were shown to be targeted to the mammalian ER membrane in an SRP-independent fashion by their interaction with the cytosolic protein calcium-calmodulin and its putative association with the calcium-calmodulin-binding site in the cytosolic N-terminus of the Sec61α protein (Fig. 4.3) (Shao and Hegde 2011). In terms of interconnections between pathways, it is interesting to note that calmodulin was found to inhibit rather than stimulate targeting of TA proteins to the ER membrane (Haßdenteufel et al. 2011).

Targeting of mRNAs to the ER Membrane

Apparently, the synthesis of many polypeptides can be initiated on ribosomes or large ribosomal subunits that are continuously attached to the ER-membrane (Potter et al. 2001). In these cases, the above discussed targeting pathways for precursor polypeptides may not be required for membrane insertion or translocation of the translation products by the Sec61 channel. Instead, mRNA targeting was suggested as an alternative ER targeting mechanism and the proteins RRBP1 (also termed p180) and kinectin 1 (KTN1) were suggested as possible mRNA receptors in the ER membrane (Table 4.1) (Savitz and Meyer 1990, 1993; Cui et al. 2012, 2013; Dejgaard et al. 2010; Morrow and Brodsky 2001; Ueno et al. 2010, 2011). So far, however, there is no clue about the possible specificity of this targeting reaction and to the best of our knowledge there is only a single example of a precursor polypeptide (Sec61ß), where mRNA targeting was found to be involved in subsequent Sec61-independent membrane insertion (Voigt et al. 2017). In contrast, polypeptides that lack a signal peptide for ER-targeting and whose synthesis was initiated on ER-bound ribosomes or large ribosomal subunits were shown to be recognized by the nascent chain associated complex (NAC). Apparently, this

interaction leads to release of the respective ribosomes from the ER membrane and completion of protein synthesis proceeds in the cytosol (Möller et al. 1998; Gamerdinger et al. 2015).

Additional Putative Functions of the Human Sec61 Channel

The mammalian Sec61 complex forms a dynamic and precursor gated channel, which can provide an aqueous path for polypeptides into the ER lumen and is regulated by various allosteric effectors (Fig. 4.5). When the aqueous path is open, it can apparently also provide a pore for efflux of calcium from the ER lumen into the cytosol. We suggest that this Sec61 feature is physiologically linked to the regulation of ATP import into the ER and the initiation of the intrinsic pathway to apoptosis, respectively. Furthermore, it is pathophysiologically linked to various human diseases, which we termed Sec61-channelopathies (Linxweiler et al. 2017; Haßdenteufel et al. 2014).

As outlined above, the ER of nucleated mammalian cells depends on an Hsp70-type molecular chaperone, termed BiP. BiP is present in the ER lumen in millimolar concentration and requires ATP for its action. Moreover, ATP hydrolysis by BiP results in ADP and, therefore, necessitates ADP removal from the ER. Until recently, mammalian proteins catalyzing the ATP uptake and the concomitant ADP release remained unknown on the molecular level. Screening databases for solute carriers (SLCs) that are located in the ER membrane, drew our attention to SLC35B1, which is predicted to have ten transmembrane domains (Fig. 4.10). Heterologously expressed SLC35B1 was found to be highly specific for ATP and ADP and to operate in antiport mode, to name just two of four characteristics it shares with the ATP transport activity, present in rough ER membranes. In addition, siRNA-mediated depletion of SLC35B1 from HeLa cells was found to reduce ER ATP levels and, therefore, BiP activity. Together these findings implied that SLC35B1 mediates ATP uptake into the ER plus ADP release from the ER in living cells. Therefore, SLC35B1 was named AXER, ATP/ADP exchanger of the ER membrane (Klein et al. 2018). According to a hypothetical structural model, human AXER can be expected to catalyze the equimolar exchange of adenosine di- and triphosphates by an alternating access mechanism, in which a single substrate binding site is made available either to the cytosolic ER surface or the ER lumen through conformational changes (Fig. 4.10). In human cells, AXER appears to be part of a regulatory circuit and a calcium-dependent signaling pathway, termed low energy response (lowER), acting in the vicinity of the ER and supplying sufficient ATP to the ER (Fig. 4.10). We suggest the following scenario for lowER: High ATP/ADP ratio in the ER allows BiP to limit calcium leakage from the ER via the Sec61 channel. Low ATP/ADP ratio due to increased protein import and folding or due to protein misfolding, leads to BiP dissociation from the Sec61 channel and, therefore, induces calcium leakage from the ER. In the cytosol, calcium binds to calmodulin (CaM) near the ER surface and activates AMP-activated protein kinase

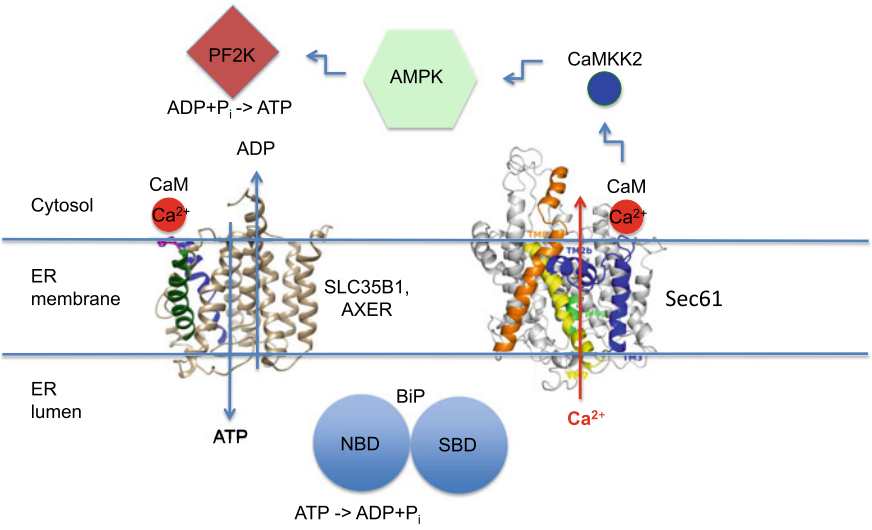


Fig. 4.10 ER low energy response (lowER) ensures a sufficient ATP supply of the mammalian ER. The given atomic structures of AXER (systematically termed SLC35B1) and Sec61 complex were derived from structure predictions. AXER, ATP/ADP exchanger in the ER membrane; AMPK, AMP-activated protein kinase; CaM, Calmodulin; CaMKK2, CaM kinase kinase 2; NBD, nucleotide binding domain of BiP; PF2K, 6-phospho-fructo-2-kinase; P_i, inorganic phosphate; SBD, substrate binding domain of BiP. See text for details

(AMPK), which in turn activates calcium-CaM kinase kinase 2 (CAMKK2) to eventually activate 6-phospho-fructo-2-kinase (PF2K). Activated PF2K causes increased ADP phosphorylation in glycolysis. The latter leads to ATP import into the ER via AXER, which is also activated by calcium efflux from the ER. Interestingly, mammalian AXER comprises an IQ motif in the cytosolic loop between transmembrane domains 2 and 3 (Fig. 4.10) and, thus, may also be activated by calcium-CaM. Normalization of the ER ATP/ADP ratio causes BiP to limit the calcium leakage and thus inactivates the signal transduction pathway. Sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA), which pumps calcium back into the ER lumen, balances the passive calcium efflux and protein phosphatase 2 (PP2) dephosphorylates AMPK. We note that activated AMPK was shown previously to lead to reduced cap-dependent translation and therefore ties the lowER to the unfolded protein response (UPR). While ADP is exported via AXER, phosphate may leave the ER via the Sec61 channel. Thus, under non-physiological or patho-physiological conditions, lowER can be expected to represent the first line of defense of a cell against ER stress. However, the details of this novel calcium-dependent signaling cascade from ER to cytosol remain to be worked out. Furthermore, future work will have to address the question if the mammalian ER membrane harbors additional ATP carriers. Additional open questions are discussed next.

Open Question

In all eukaryotic cells, nascent proteins, which are destined to membranes or the lumen of organelles of the endo- and exocytotic pathways or even the extracellular space, must be translocated across or integrated into the ER membrane. In mammals, most proteins are translocated concomitantly with their synthesis by cytosolic ribosomes (co-translationally), whereas many small presecretory proteins, which are essential for intercellular communication or pathogen defense, are post-translationally imported. A consensus on the major components of the ER-translocation machinery, almost 100 different proteins, is established, but their precise functions, as well as their spatial and temporal organization still remains largely elusive (Table 4.1).

Therefore the authors of this review, are trying to characterize the mammalian machinery for the co- and post-translational translocation of polypeptides into the ER and their accompanying covalent modifications in terms of composition, structure, as well as component functions and mechanisms. In addition, the obtained structural and mechanistic insights into the ER-translocation machinery are expected to provide a detailed understanding of the etiology of several human diseases and may even guide us to novel therapeutic strategies.

Systematic Knock Down of ER-Protein Translocation Machinery Components in Human Cells Combined with Characterization of Substrate Precursor Proteins and Compensatory Mechanisms by Quantitative Proteomic Analysis

Traditionally, the substrate specificities of mammalian protein transport components have been investigated in cell-free translation reactions in which a small set of model precursor proteins is synthesized one-by-one in the presence of reconstituted- or HeLa cell derived-ER membranes or in pulse/chase experiments in human cells that overproduce the model precursor of interest (Sharma et al. 2010; Dudek et al. 2015). These traditional approaches are suitable for addressing whether a certain component can stimulate ER import of a given precursor polypeptide. However, due to the bias of these experimental strategies, they fail to clearly define the characteristics of precursor polypeptides that lead to a certain dependence under physiological conditions. Therefore, we established a novel unbiased approach, which involves treatment of HeLa cells with either one of two different targeting siRNAs or a non-targeting siRNA, label-free quantitative proteomic analysis, and differential protein abundance analysis. As a proof of concept, HeLa cells were depleted of the Sec61-complex using two different *SEC61A1*-targeting siRNAs. We assessed the proteomic consequences of this knock-down via label-free quantitative

proteomics and differential protein abundance analysis relative to cells treated with non-targeting siRNA. Roughly, 50% of the HeLa cell proteome was quantified in these experiments. Our experimental strategy was successfully used to analyze the client spectrum of the Sec61-complex, an essential transport component. These results set the stage for subsequent analysis of precursor-specific auxiliary transport components, such as the TRAP-complex. As discussed above, signal peptide analysis of the TRAP clients revealed above-average glycine-plus-proline content as the distinguishing feature for TRAP dependence and, thus, suggested a hitherto undetected signal peptide feature and heterogeneity (Nguyen et al. 2018). Therefore, we proposed that this signal peptide heterogeneity may provide an opportunity for regulation of transport of a subset of precursor polypeptides and may be linked to both TRAP mechanism and the etiology of TRAP-linked congenital disorder of glycosylation in human patients. Since TRAPα was found to be subject to phosphorylation and calcium-binding, this phenomenon may also provide a potential regulatory mechanism for the TRAP-dependent subset of precursors. At present, we are analyzing the results from similar experiments on another auxiliary transport component, i.e. the Sec62/Sec63-complex. Notably, this complex is also affected by phosphorylation (the Sec63 subunit) and binding of calcium (the Sec62 subunit) and, therefore, may provide a similar opportunity for transport regulation for another set of precursor polypeptides. As noted above, our novel approach also identified several genetic interactions between targeting- and Sec61 channel gating-pathways (Fig. 4.9), which may eventually pave the way towards understanding yet another layer of regulatory phenomena.

Integrative Determination of the Molecular Architecture of the Native ER Translocon Core Complexes

Cryo-electron tomography (CET) in conjunction with subtomogram averaging provided a three-dimensional map firstly of the core of the native co-translational translocation machinery. Next, we want to explore Sec62/Sec63-dependent import structurally in situ using CET. Here, our approach is based on the recent publication by the Bill Skach lab (Conti et al. 2015). The respective publication had followed our work on the import requirements of the precursors of ERj3 and prion protein (Lang et al. 2012; Schäuble et al. 2012) and defined in a sophisticated combination of nascent precursor polypeptide chains, cross-linking, and 2D gel electrophoresis, when these precursor polypeptides recruit Sec62 and Sec63 to the Sec61-complex. Apparently, they do so at a comparatively late stage of chain growth, possibly when a polybasic motif within the mature part of the precursor enters the Sec61-channel. We will use these particular nascent polypeptide chains for CET under the established conditions.

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