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STATE-OF-THE-ART REVIEW

Membrane protein biogenesis at the ER: the highways and byways

Sarah O'Keefe, Martin R. Pool and Stephen High

School of Biological Sciences, Faculty of Biology, Medicine and Health, University of Manchester, UK

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Correspondence
S. O'Keefe, M. R. Pool and S. High, School of Biological Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester M13 9PT, UK
Tel: +44 161 275 1526 (SO'K); +44 161 275 5392 (MRP); +44 161 275 5070 (SH)
E-mails: sarah.okeefe@manchester.ac.uk (SO'K); martin.r.pool@manchester.ac.uk (MRP); stephen.high@manchester.ac.uk (SH)

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The Sec61 complex is the major protein translocation channel of the endoplasmic reticulum (ER), where it plays a central role in the biogenesis of membrane and secretory proteins. Whilst Sec61-mediated protein translocation is typically coupled to polypeptide synthesis, suggestive of significant complexity, an obvious characteristic of this core translocation machinery is its surprising simplicity. Over thirty years after its initial discovery, we now understand that the Sec61 complex is in fact the central piece of an elaborate jigsaw puzzle, which can be partly solved using new research findings. We propose that the Sec61 complex acts as a dynamic hub for co-translational protein translocation at the ER, proactively recruiting a range of accessory complexes that enhance and regulate its function in response to different protein clients. It is now clear that the Sec61 complex does not have a monopoly on co-translational insertion, with some transmembrane proteins preferentially utilising the ER membrane complex instead. We also have a better understanding of post-insertion events, where at least one membrane-embedded chaperone complex can capture the newly inserted transmembrane domains of multi-span proteins and co-ordinate their assembly into a native structure. Having discovered this array of Sec61-associated components and competitors, our next challenge is to understand how they act together in order to expand the range and complexity of the membrane proteins that can be synthesised at the ER. Furthermore, this diversity of components and pathways may open up new opportunities for targeted therapeutic interventions designed to selectively modulate protein biogenesis at the ER.

Introduction

Integral membrane proteins are often anchored into their host membrane via one or more hydrophobic polypeptide segments, or transmembrane domains (TMDs), that span the entire width of the phospholipid bilayer. These so-called 'transmembrane' proteins (TMPs) represent ~ 25% of human genes, are diverse

Abbreviations
BiP, immunoglobulin binding protein; CCDC47, coiled-coil domain containing 47 protein, also known as calumin; EMC, ER membrane complex; ER, endoplasmic reticulum; GET, guided entry of tail-anchored proteins; hSnd2, human SRP-independent protein 2, also known as TMEM208; NAC, nascent polypeptide-associated complex; NOMO, nodal modulating protein; PAT, protein associated with the ER translocon; RNC, ribosome-nascent chain; SGTA, small glutamine-rich tetratricopeptide repeat-containing protein alpha; SND, SRP-independent proteins or pathway; SPC, signal peptidase complex; SRP, signal recognition particle; TA, tail-anchored; TMCO1, transmembrane and coiled-coil domains 1 protein; TMD, transmembrane domain; TMEM147, transmembrane protein 147; TMEM208, transmembrane protein 208; TMP, transmembrane protein; TRAM, translocating chain-associated membrane protein; TRAP, translocon-associated protein; TRC40, transmembrane recognition complex of 40 kDa, also known as Asna1.

in structure and perform a plethora of essential cellular functions [1]. The endoplasmic reticulum (ER) is a major site for the biogenesis of such integral membrane proteins, acting as their entry point into the secretory pathway, an elaborate network tasked with the synthesis, folding and transport of both membrane and secretory proteins (Fig. 1A) [2,3]. Given the molecular crowding of the cytosol and the biophysical constraints of the lipid bilayer, most TMPs enter a dedicated ER targeting pathway(s) as soon as an appropriate subcellular targeting signal has emerged from the ribosome. Upon arrival at the ER, these nascent polypeptides are threaded into and across its membrane via specialised protein translocation channels that typically act concomitantly with translation [4].

Amongst these ER translocation channels, the heterotrimeric Sec61 complex (α, β, γ subunits), or Sec61 'translocon' [5], is the principal protein-conducting channel through which secretory proteins are fully translocated across the ER membrane. TMPs also access the Sec61 complex, however, in contrast to secretory proteins, they are only partially translocated, with their TMD(s) exiting the Sec61 complex via a lateral gate. This enables stable membrane integration and thereby constrains their membrane topology as they navigate the secretory pathway towards the plasma membrane. Single-span TMPs can be grouped into different types based on their structural features and the location of their N and C termini relative to the ER membrane (Fig. 1B). Here, we will classify them as type I, type II or type III TMPs and tail-anchored (TA) proteins (Fig. 1B; [6]). Based on the features of their first TMD, this characterisation can, in principle, also be extrapolated to multi-span TMPs (type I-like, type II-like, type III-like). However, given that the membrane insertion of multiple TMDs is not necessarily sequential and may also be co-operative [7,8], such an approach may be of limited use when trying to understand the biogenesis of multi-span TMPs.

It is increasingly clear that TMP biogenesis at the ER is a substrate-selective and mechanistically diverse process that involves a range of molecular machines well beyond the canonical Sec61 translocon [9]. Herein, we review the rapidly expanding field of co-translational membrane protein biogenesis at the mammalian ER; that is, when membrane insertion is concomitant with ribosomal polypeptide synthesis. Focussing on the mechanisms of ER targeting, together with protein translocation across, and TMD insertion into, the ER membrane, where relevant, we draw upon molecular details obtained in bacterial and yeast systems so as to gain prospective insight into mammalian mechanisms of co-translational TMP biogenesis that are yet to be fully elucidated.

ER membrane targeting: the SRP-delivery system

Within the arsenal of 'accessory components' employed by the Sec61 complex [9], the signal recognition particle-(SRP) and its ER membrane-localised cognate binding partner, the SRP receptor, constitute the first key players that are encountered by the majority of proteins destined for the secretory pathway. Together, these complexes mediate protein targeting to the ER [4], typically by virtue of an N-terminal hydrophobic stretch of amino acids [13], or signal sequence, that acts as a 'molecular postcode' and, in many cases, is cleaved [11] from the newly synthesised polypeptide once it is committed to membrane translocation and/or insertion.

Not all polypeptides that are destined for the ER are equipped with a so-called cleavable N-terminal signal sequence (Fig. 1B). Hence, in the case of type II and III TMPs their hydrophobic TMD(s) act as 'signal-anchor' sequences, emulating the functions of N-terminal ER signal sequences and targeting nascent TMPs to the ER prior to their integration into the membrane bilayer [7,8]. Thus, whether cleavable or not, these hydrophobic regions within TMPs act as 'signal flares', efficiently recruiting and interacting with the SRP at an early stage during the synthesis of the nascent polypeptide. Hence, the SRP-delivery system predominantly operates co-translationally, targeting a range of structurally diverse single- and multi-span TMP clients to the ER for co-translational membrane insertion. Notable exceptions include TMPs whose ER targeting and integration occurs after protein synthesis is completed (post-translationally), as best exemplified by the TA proteins (cf. Fig. 1B) [14–16]. In yeast, the proteome-wide effects of rapid SRP depletion suggest it is essential for the efficient ER targeting of TMPs utilising their TMDs as signal-anchor sequences [17]. In contrast, SRP is only required for the ER delivery of ~ 14% of yeast proteins with cleavable N-terminal signal sequences [17]. Likewise in bacteria, SRP is essential for membrane targeting of inner-membrane proteins utilising signal-anchor sequences. However, it is dispensable for the targeting of many secreted precursor proteins with N-terminal signal sequences [18,19]. Although it is generally assumed that SRP plays a wider role in the ER targeting of proteins that bear N-terminal signal sequences in mammalian cells, proteome-wide analyses that directly test this hypothesis are presently lacking.

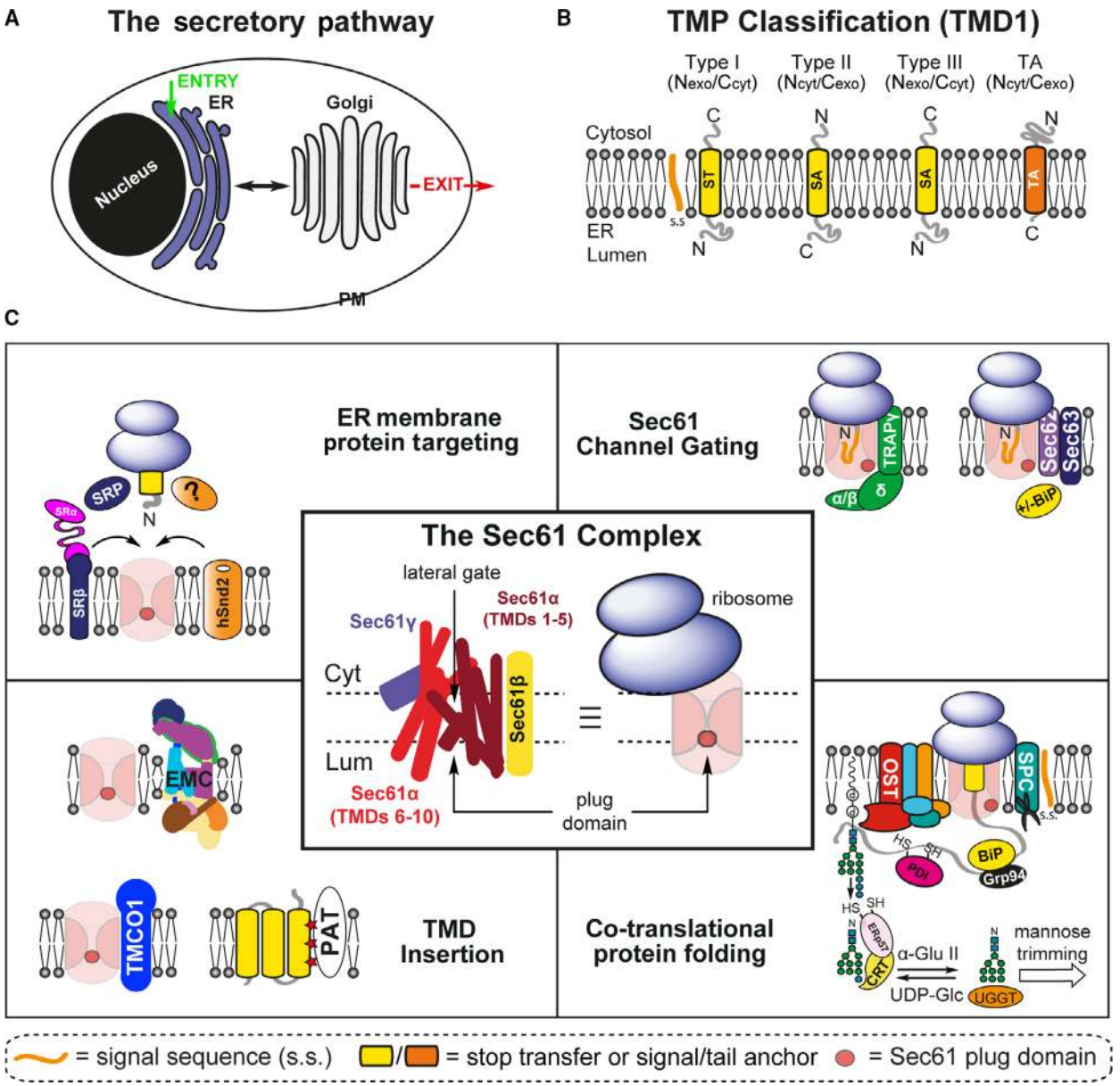
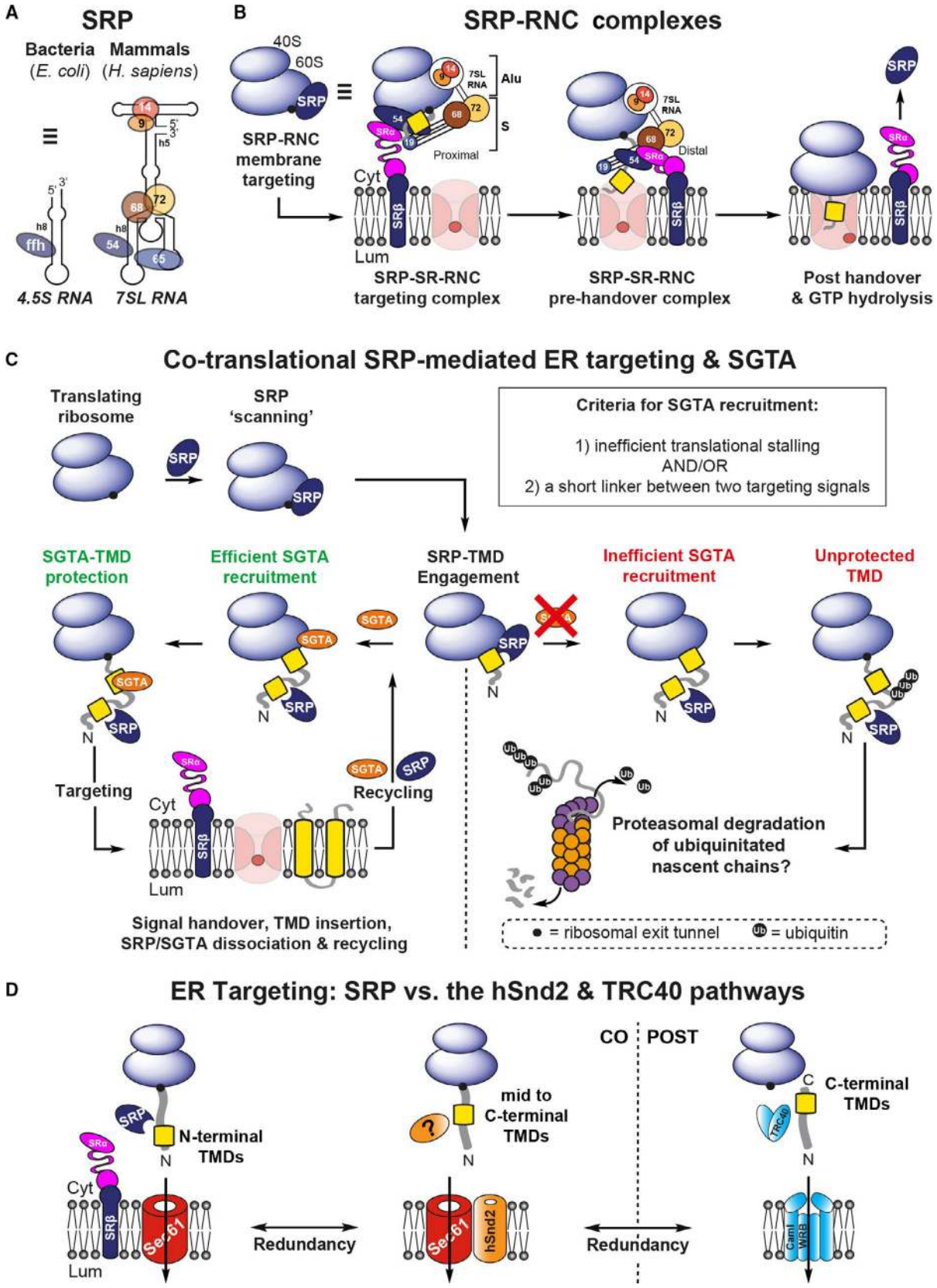


Fig. 1. Accessing the secretory pathway via Sec61: 'many hands make light work' (A) Newly synthesised secretory proteins and TMPs are targeted to and translocated into the ER lumen in order to enter the secretory pathway (green arrow). Mature proteins that have progressed through the Golgi apparatus are then delivered to the plasma membrane (PM) where they may be either incorporated or secreted (red arrow). (B) Representative structures of four classes of single-span TMPs: type I TMPs are equipped with an N-terminal signal sequence (s.s.), a lumenally translocated N terminus and a stop-transfer sequence (ST) which acts as the TMD; type II and type III TMPs do not possess an N-terminal signal sequence and have a signal-anchor sequence (SA) and, respectively, translocate their C and N termini into the ER lumen; tail-anchored (TA) proteins are topologically and structurally similar to type II TMPs, but their extremely short C-terminal region necessitates that their insertion into the ER occurs post-translationally. (C) The Sec61 complex can call on a diverse repertoire of additional cellular machineries to facilitate various aspects of its role in co-translational TMP biogenesis including: ER targeting (top left inset), Sec61 channel gating (top right inset), TMD insertion and TMD folding/assembly (bottom left inset). Additional events, such as N-linked glycosylation (via OST, oligosaccharyltransferase complex), signal sequence cleavage (via SPC, signal peptidase complex) and ER chaperone-mediated lumenal folding (see BiP, binding immunoglobulin protein; Grp94; PDI, protein disulphide isomerase; ERp57; CRT, calreticulin), are also coupled to the actions of the Sec61 translocon (bottom right inset), and we direct the reader to recent articles that review these processes [9–12]. Schematics are illustrative only and are not drawn to scale.



In mammals, the cytosolic targeting factor SRP is a multimeric complex of six protein subunits assembled onto a core 7S RNA (Fig. 2A) [20–22], and is first recruited by signal sequences/anchors from within the ribosomal exit tunnel [23,24]. As the exit tunnel typically shields the first ~ 40 amino acid residues of nascent polypeptide chains from the cytosol [25], such early SRP recruitment is presumed to occur via ribosome-nascent chain (RNC)-induced structural rearrangements within the actively translating ribosome [26]. Once recruited, SRP binds to the ribosome at a location that is partly occupied by the nascent polypeptide-associated complex (NAC); a co-translational chaperone that enhances SRP-dependent targeting by increasing the fidelity of signal sequence recognition by SRP [27] and preventing the promiscuous interaction of ribosomes with Sec61 [28]. Thus, co-translationally bound and NAC-regulated SRP [27] is suitably poised to 'scan' and engage ER signal sequences/anchors as soon as they emerge from the ribosome [22,24,29,30].

Cellular levels of SRP are significantly lower than the near stoichiometric concentrations of ribosomes and NAC [27]. Thus, if an ER targeting signal is not encountered quickly, SRP rapidly dissociates from the RNC complex, effectively cycling on and off ribosomes in search of a substrate signal sequence [31]. In the case of timely SRP engagement by an ER signal sequence/anchor, a process that may be enhanced by certain nonoptimal, 'translation slowing' mRNA codons located downstream of the signal sequence coding region [32], ribosomal translation is transiently stalled by the Alu domain of SRP [33,34; see Fig. 2B, left]. This translational stalling effectively maintains the nascent chain in a 'translocation competent' state during the time window available for successful ER delivery, as dictated by the limiting number of SRP receptor targeting sites [33].

Once at the membrane, and co-ordinated by the concerted actions of two GTPases, the signal sequence binding subunit of SRP (SRP54) and the membrane-tethered alpha subunit of the SRP receptor (SRα), the now quiescent SRP-RNC complex engages the SRP receptor [35]. Complex formation between SRP and its receptor leads to repositioning of both SRP54 and SRα relative to the SRP RNA from the so-called 'proximal' site to an alternative 'distal' site (Fig. 1B). This generates a 'prehandover complex', where the Sec61 binding site of the ribosome that was previously occluded by SRP54 now becomes accessible, whilst also blocking GTP hydrolysis by SRP54 and SRα. Subsequent arrival of this complex at the Sec61 translocon triggers handover of the ribosome and nascent chain from the SRP/SRP receptor complex concomitantly with GTP hydrolysis by SRP54 and SRα. Posthandover, and following the opening of the Sec61 translocon (see Gating of the Sec61 complex), translation is resumed as membrane translocation and/or membrane insertion of the nascent polypeptides takes place (Fig. 2B, right) and SRP is recycled for additional rounds of ER targeting [22].

Since the 'signal hypothesis' was postulated [36–39], the mammalian SRP-delivery system outlined above

Fig. 2. Getting there in one piece: an SRP- and SGTA-assisted route to the ER membrane (A) A representation of bacterial and mammalian SRP complexes indicating conserved RNA helices [54]. Assembled on the highly base-paired 7SL SRP RNA, the six mammalian SRP proteins are organised into a functionally independent Alu domain, responsible for translational pausing, and an S domain which mediates SRP binding to signal sequences/anchors and the SRP receptor. Subunits of the S domain are essential for SRP function, whereas those of the Alu domain are dispensable [22]. (B) SRP-dependent ER membrane targeting involves distinct SRP-RNC-mediated events. The RNC with a signal sequence/anchor is first bound by SRP. SRP next associates with the SRα subunit of the SRP receptor via its SRP54 subunit, located at a 'proximal site' of the SRP RNA that is close to the ribosomal exit tunnel. Subsequent interaction of the SRP receptor with the Sec61 translocon permits a structural rearrangement, in which SRα and SRP54 are relocated to a conserved 'distal site' in the SRP RNA that primes for the handover of signal sequences to the Sec61 translocon. Signal handover, most likely coupled with the hydrolysis of GTP, drives the dissociation of SRP from the SRP receptor [22]. Hydrophobic targeting signals are depicted as signal anchors only (and not signal sequences) for simplicity. (C) A model of SRP-dependent targeting to the ER membrane: SRP 'scans' the emerging nascent chain of a translating ribosome for hydrophobic targeting signals, binds the ribosome-nascent chain complex (RNC) and delivers it to the ER membrane via its interaction with the SRP receptor (RNC-ER membrane docking), prior to membrane insertion [22]. If translational pausing is inefficient, or there is only a short linker between two transmembrane domains, SGTA may be recruited to protect the second hydrophobic region until it is membrane inserted (left). If such regions of hydrophobicity emerge from the RNC and remain un-chaperoned (right), these nascent chains may become ubiquitinated and hence targeted for proteasomal degradation [44]. For clarity, only a membrane protein with a short linker between two signal-anchor sequences is depicted as an example. (D) Three ways to the ER. If a TMD is located at the N terminus or towards the middle of the protein, TMPs are targeted to the ER via SRP as described in parts A-C. However, if a TMD is located at the C terminus, TMPs are targeted post-translationally to the ER via the mammalian equivalent (TRC40) of the GET pathway in yeast [14]. Alternatively, hSnd2, the human orthologue of a component of the SND pathway in yeast [47], is involved in the biogenesis of TMPs whose TMD is located in the mid to C-terminal region of the protein. hSnd2 additionally demonstrates redundancy with the SRP and TRC40 pathways.

has been extensively characterised [22,40]. Nevertheless, novel aspects of SRP-mediated co-translational delivery to the ER continue to emerge. For example, it has been suggested that SGTA [41], a cytosolic quality control component involved in the post-translational targeting of TA proteins to the ER [14,41–43] also contributes to co-translational ER targeting. Hence, SGTA may facilitate the biogenesis of TMPs containing two or more closely spaced hydrophobic signals by binding prematurely exposed TMDs that do not recruit the substoichiometric SRP [44] (Fig. 2C). In this way, the actions of SGTA would complement the ER targeting role of SRP by shielding potentially vulnerable TMDs in the nascent polypeptide from inappropriate, and possibly damaging, interactions [44]. Such recruitment of SGTA thereby protects nascent membrane proteins from potential proteasomal degradation until they engage the Sec61 complex and initiate membrane insertion (Fig. 2C) [44]. Exactly how SGTA is recruited to and associates with the ribosome and/or other ribosome-associated chaperones such as NAC [45], why it does not compete with SRP despite its comparative cytosolic abundance (~ 1 μM versus ~ 5–10 nM) [43,46] and how it is released upon delivery to the ER membrane are all questions that remain to be answered.

Despite the range of precursor proteins that are catered for by the SRP-delivery system, at least two other ER targeting pathways are operational in the cytosol: a mammalian version [16] of the co-translational, SRP-independent or, 'SND' pathway first identified in yeast [47]; and a post-translational route for TA protein biogenesis known as the TRC40 pathway in mammals [14]. Whilst these pathways function in parallel, they are also most likely overlapping and/or partially redundant in terms of their substrate specificity. In principle, SRP typically caters for signal sequences/anchors that are located at or near the N terminus of nascent polypeptides, SND favours signal anchors that are more central and TRC40 deals with C-terminal tail anchor sequences (Fig. 2D) [17,47,48]. Only one mammalian orthologue of the three components which make up the SND pathway in yeast has been identified to date [16,47]. However, this component, known as TMEM208 or hSnd2 [49], has been implicated in the Sec61-mediated biogenesis of short secretory proteins [50] and single- and multi-span TMPs [16,51,52], with the wider hSnd2/SND targeting pathway able to compensate for an absence of the SRP or TRC40/GET pathways in yeast and mammals [16,47,51]. The partial redundancy of these three ER targeting pathways (see [17,47]), which most likely allows cells to efficiently target membrane proteins under a wide range of physiological conditions and/or external stresses, probably explains why the hSnd2/SND pathway remained undiscovered for so long [48].

Whilst bacteria typically contain only one location, the inner membrane, to which newly synthesised proteins are delivered, mammalian SRP must correctly discriminate between the multiple membrane systems that are accessible from the eukaryotic cytosol. When compared to its bacterial equivalent, mammalian SRP displays not only greater structural complexity (Fig. 2A) but also increased functional complexity, as evidenced by its early recruitment to the nascent chain, regulation by the NAC complex and capacity to induce a translational arrest. When combined with the 'fail-safe' option provided by SGTA recruitment, we speculate that this additional complexity of eukaryotic SRPs has most likely evolved to enhance the specificity of, and lengthen the window for, nascent chain targeting to the ER membrane; a feat of increasing importance for TMPs containing multiple TMDs located after the initial ER signal sequence that may even require additional rounds of SRP-mediated targeting to the ER [17,19,53].

We further suggest that in the event that SRP fails to engage the TMD of a protein client, whether as a consequence of a nonfunctional SRP-delivery pathway, a more C-terminal location of its ER targeting signal or for some other reason, the hSnd2/SND pathway provides an alternative and/or additional system to ensure that co-translational protein clients continue to be targeted to the ER membrane. We anticipate that uncovering the mechanistic details of the hSnd2/SND pathway will help to delineate the extent of the substrates that it caters for, why SRP 'loses' its ability to engage TMDs as a polypeptide chain extends and whether the hSnd2/SND pathway integrates with the SRP-dependent delivery pathway at the ER membrane.

Gating of the Sec61 complex

Following signal sequence/anchor-mediated SRP-dependent delivery and transfer of RNCs to the Sec61 complex (cf. Fig. 2), these hydrophobic targeting signals perform a second key action to fulfil their role as an 'ER-entry tag'; they must open the Sec61 channel. Organised into two distinct halves, with TMDs 1–5 and 6–10 surrounding a central pore (Fig. 3A), the hourglass-shaped conduit of Sec61α appears empty on the cytosolic side of the ER membrane whereas a ring of hydrophobic residues, known as the 'plug domain', seals the ER lumenal side of the pore [5,55,56], effectively preventing the free movement of small molecules across the Sec61 translocon when inactive (see [57]).

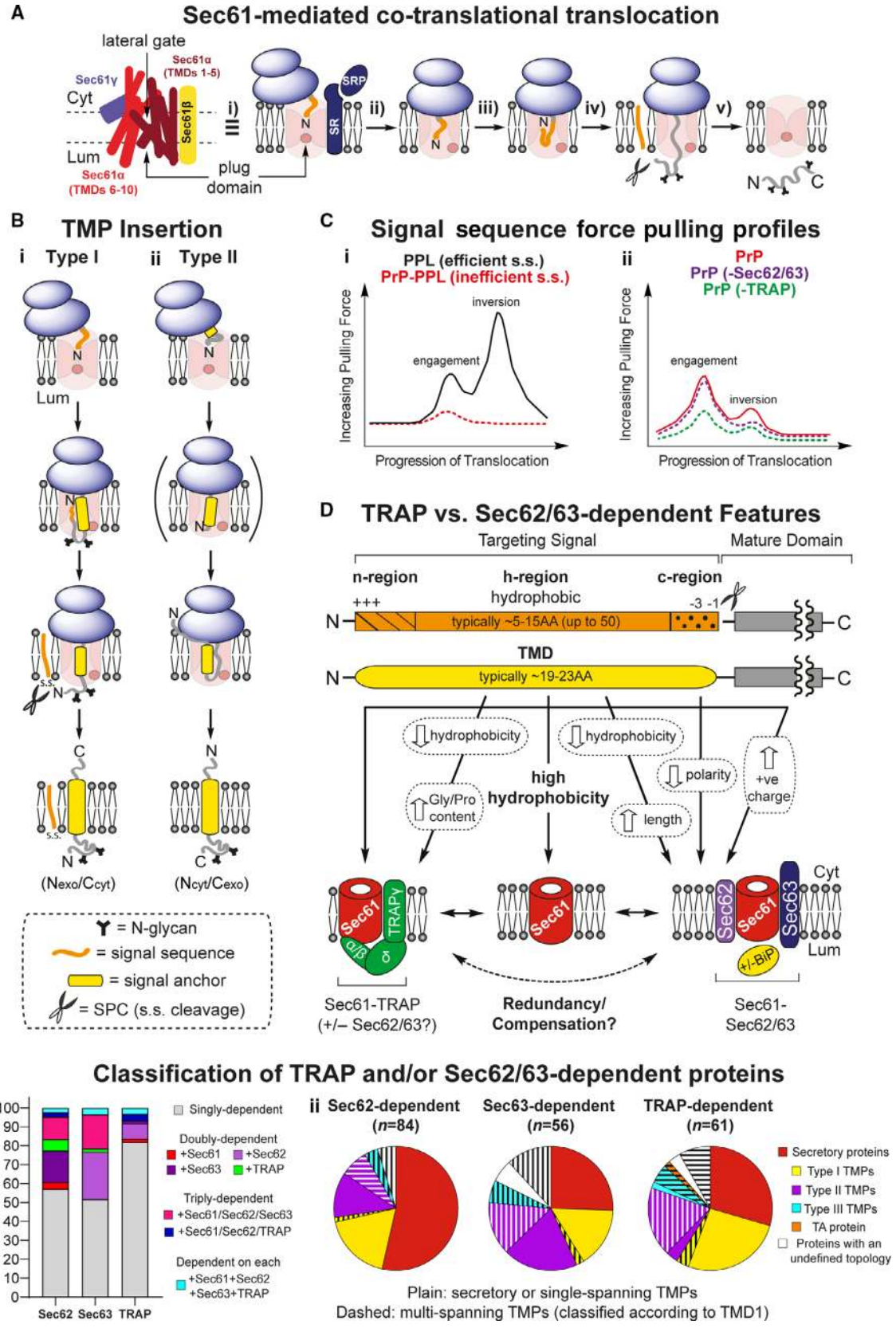
Opening of the Sec61 channel involves three major steps. First, the channel is primed to open via the docking of RNCs to the Sec61 complex (Fig. 3Ai). Secondly, docking-induced conformational changes 'crack' the cytosolic end of the lateral gate (comprised by TMDs 2, 3, 7 and 8) which facilitates the engagement and intercalation of the signal sequence/anchor between TMDs 2 and 7. Thirdly, the signal sequence/anchor-mediated displacement of TMD2 finally results in movement of the plug domain and opening of the lateral gate (Fig. 3Aii) [5,55,56]. *In situ* studies of the native Sec61 translocon suggest that the lateral gate can be opened by ribosome binding alone, even in the absence of a nascent chain [58], although in the case of bacterial SecYEG [59] the presence of a signal sequence on a nascent chain enhances opening of the equivalent lateral gate [60]. These conformational changes enable co-translational movement of the growing polypeptide chain across the membrane and into the ER lumen (Fig. 3Aiii,iv) [5,55,56]. In contrast to these actively translocated hydrophilic regions of polypeptide, hydrophobic targeting signals are laterally inserted into the lipid bilayer via the lateral gate. In the case of N-terminal signal sequences, they are cleaved from the nascent chain by the signal peptidase complex (SPC) [11] (Fig. 3Aiv) and, ultimately, subject to further processing and/or degradation by signal peptide peptidase [61]. In contrast, signal-anchor sequences form a stable membrane tether for the newly synthesised TMPs. Following translation termination and the exit of newly synthesised polypeptides from the channel, the ribosome dissociates from the Sec61 complex and the Sec61α plug domain returns to its original position (Fig. 3Av).

The central role of the Sec61 translocon during the biogenesis of secretory proteins, type I and type II TMPs has been well established for many years (Fig. 3A,B; see Membrane insertion via the EMC for type III TMPs). However, there is now a growing body of evidence that signal sequences can provide an additional, as yet poorly defined, level of control during membrane translocation [62]. Highly diverse in terms of their hydrophobicity, length, charge and specific amino acid composition [8,13,63], ER targeting signals appear to regulate the opening, or 'gating' of the Sec61 translocon [62], particularly since SRP effectively caters for targeting signals seemingly irrespective of their intrinsic ability to gate the translocon which, in some cases, is 'inefficient' and 'slow' (Fig. 3C) [64,65].

The defining structural feature of an archetypically efficient and 'strongly gating' signal sequence appears to be that its core h-region (Fig. 3D) is of sufficient hydrophobicity to successfully engage with, and insert 'head-on' into, the Sec61 translocon (Fig. 3A, stage ii) and then subsequently re-orientate to form a hairpin conformation within the channel (Fig. 3A, stage iii). During both of these apparently discrete stages, the RNC is subject to a distinct force that pulls the nascent chain away from the ribosome (Fig. 3Ci) [64]. In contrast, if an 'inefficient' signal sequence is appended to the same polypeptide, the nascent chain experiences a single, weaker pulling force that reflects its inability to successfully engage the translocon and undergo in-channel re-orientation (Fig. 3Ci) [64]. We speculate that hydrophobic signal-anchor sequences [66,67], some of which can also reorient inside the Sec61 translocon [68], will be subject to pulling forces comparable to those experienced by signal sequences during their membrane insertion at the ER [65,69]. Likewise, it seems plausible that the profiles and strength of the pulling forces experienced by signal sequences and/or signal anchors may be influenced by the drivers and determinants of signal orientation/TMD topology, such as the 'positive-inside' rule, the degree of N-terminal folding and, perhaps, even the lipid composition of the bilayer [7,8].

In cases where inefficient signal sequences (see [70]) and/or signal anchors require extra help to gate the translocon, the Sec61 complex may employ 'gating assistants'. Hence, the tetrameric TRAP (translocon-associated protein) complex, composed of α, β, γ and ∂ subunits [71–73], and/or the Sec62/Sec63 proteins [74] (Fig. 3D), contribute to the second pulling event that occurs during secretory protein translocation (Fig. 3Cii) [75]. Despite subtle differences in the features that facilitate their recruitment (Fig. 3D), these gating assistants exhibit a common propensity to assist signal sequences/anchors that are of lower hydrophobicity [72,74] and/or contain clusters of positive charge in regions of polypeptide that must be translocated [74–76]. Furthermore, given that different gating assistants can contribute to the efficient translocation of the same protein substrate, whether a secretory protein or TMP (Fig. 3Ei, Tables 1–4) [74], and cellular levels of TRAP-β are upregulated following depletion of Sec62 [74], it seems likely that the TRAP complex and Sec62/Sec63 perform overlapping, but nonidentical, functions during Sec61-mediated protein translocation [62].

Such a notion is supported by the behaviour of the mammalian prion protein (PrP) during its ER translocation [75] where TRAP is required for both signal sequence engagement with Sec61 and its in-channel inversion, whereas Sec62/63 only influence the latter event, and to a lesser extent [75] (see Fig. 3cii). Thus, it appears that Sec62/63 supports the translocation of



polypeptides with suboptimal ER targeting features that may first be recognised by the cytosolic portion of TRAP [76], prior to TRAP-mediated opening of the Sec61 complex [72,75–78]. Previous studies established that Sec62/63 contribute strongly to Sec61 gating during the post-translational translocation of certain substrates, including short secretory proteins [50]. However, structural studies strongly suggest that in order to stabilise RNCs that co-translationally engage the Sec61 translocon, either alone [5], or in its TRAP-assisted mode [78], the Sec62/63 complex must adopt a different conformation to its posttranslational one [9,79–81].

By subjecting a broader range of TRAP- and Sec62/63-dependent clients of the Sec61 translocon [72,74] to detailed force-pulling studies [64,65,69,75,76], it should be possible to discover mechanistic detail about these gating assistants that is currently lacking. Currently unanswered questions include: is the constitutively Sec61-bound TRAP complex [78] the only 'gating assistant' capable of exerting a pulling force on RNCs (see [75]); can the Sec62/63 complex compensate for the loss of TRAP-mediated assistance; is TRAP- and/or Sec62/63-assisted translocation used to regulate the flux of protein substrates through the Sec61 translocon [72,74]? Likewise, although the translocating chain-associated membrane (TRAM1) protein is closely associated with the active Sec61 translocation complex [7,8], a recent 'global' analysis suggests that it is not a gating assistant [82]. Rather it seems that TRAM1 may facilitate the egress of hydrophobic regions from the Sec61 lateral gate into the phospholipid bilayer [82], a possibility that clearly merits further exploration. Answering these questions may also help us to finally reconcile the long-standing enigma surrounding the broad sequence diversity that is seen across ER targeting signals [13].

Membrane insertion via the EMC

Analogous to the recruitment of 'gating assistants' by ER targeting signals that the Sec61 complex finds challenging, the multisubunit ER membrane complex (EMC) (Fig. 4A) provides a membrane insertase for TMDs that also appear to be more 'demanding' of the ER translocon [83,84]. The EMC was first implicated in membrane protein biogenesis when gene disruption of its subunits was found to have pleiotropic effects on the expression of multi-span TMPs in several species [85–88]. Multi-span TMPs were subsequently found to be significantly enriched amongst putative EMC-dependent protein clients (Fig. 4A, Table 5) [89,90], and the EMC was also identified as an ER membrane insertase that can facilitate the post-translational insertion of certain TA proteins [15,91]. In light of several recent structural studies, we now have a better

Fig. 3. 'With a little help from my friends': TRAP and/or Sec62/63-assisted gating of Sec61 (A) Schematics of the heterotrimeric Sec61 complex (α, β, γ) and the regulation of Sec61α via its plug domain and lateral gate during the co-translational translocation of secretory proteins [5,9]. Schematics are not drawn to scale. (B) Models for the Sec61-mediated insertion of type I and type II single-pass TMPs. (Bi) For type I TMPs, a cleavable N-terminal signal sequence enters the Sec61 translocon 'headfirst' and is then inverted allowing the subsequent stop-transfer sequence to become laterally inserted as a TMD with an N_{exo}/C_{cyt} topology [8]. (Bii) The N_{cyt}/C_{exo} topology of type II TMPs necessitates that they are membrane inserted in the opposite orientation; this may be achieved either by the 'headfirst' insertion of a signal-anchor sequence followed by its inversion within the Sec61 translocon (shown in brackets) or via a 'hairpin integration' mechanism whereby the signal-anchor engages the translocon in a looped conformation [8]. (C) Schematic overview of the pulling forces experienced by; (Ci) the protein substrate preprolactin equipped with its normal, archetypically strong, signal sequence (PPL, black solid line) versus its replacement with an inefficient signal sequence (*PPL, red dashed line; see [64]); (Cii) the Prion protein (PrP) in control cells (red solid line) and cells depleted of the Sec62/63 (purple dashed line) or the TRAP complex (green dashed line) [75]. (D) TRAP- and/or Sec62/63-dependent cleavable signal (orange) or signal-anchor (yellow) sequences typically include reduced hydrophobicity in the signal and/or clusters of positive charge in the early mature domain [72,74,75]. Cleavable signal sequences are N-terminal and typically composed of three regions: a polar n-region that facilitates signal sequence insertion/inversion at the ER translocon (hatched orange section), a central hydrophobic h-region that is recognised by SRP (plain orange) and a polar C-terminal region that contains the site for signal sequence cleavage (dotted orange). Other features of ER signal sequences that necessitate a Sec61-'gating assistant' include a high glycine/proline content (TRAP complex) [72], a longer core h-region (Sec62/63 [74]) and regions of decreased polarity (Sec62/63; [74]). Whilst favouring different groups of clients, the roles of the TRAP complex and Sec62/63 may be partially redundant. (E) Classification of membrane and secretory protein clients of Sec62, Sec63 and TRAP based on previous global studies [72,74]. (Ei) Proteins that were negatively affected by the absence/depletion of one or more of Sec62, Sec63 and TRAP were classified (see Eii) as secretory proteins, single-span TMPs (type I, type II, type III, TA protein, undefined) or multi-span TMPs (type I-like, type II-like, type III-like, undefined) based on their features and the topology of their first TMD. The proportion of the putative clients that belong to each of these groups is shown as a percentage of the total number of proteins (*n*) that were a negatively affected in each case: Sec62 ($n = 84$), Sec63 ($n = 56$) and TRAP ($n = 61$) (Tables 1–4). Proteins that do contain an ER targeting sequence and any subunits of Sec61 'gating assistant' were discounted from the analysis. Figure 3D has been reproduced from Ref. [62].

Table 1. Negatively affected secretory proteins and TMPs following Sec61 and accessory factor depletion; analysis of data presented in Ref. [74]. Putative Sec63 clients that were identified in cells transiently depleted of Sec63 but not in Sec63 knockout cells are denoted in brackets (X).

Uniprot ID	Protein name	Protein class	Single/Multi	Sec61A1	Sec62	Sec63	TRAP	TMD number
Q10589	BST2	Type II TMP	Single	X	X	X		1
Q9H6E4	CCDC134	Secretory	N/A	X	X	X		0
O75503	CLN5	Type II TMP	Single	X	X	X		1
Q96HD1	CRELD1	Type I-like	Multi	X	X	X		2
Q9UBS4	DNAJB11	Secretory	N/A	X	X	(X)		0
Q9UM22	EPDR1	Secretory	N/A	X	X		X	0
Q96AY3	FKBP10	Secretory	N/A	X	X			0
P06280	GLA	Secretory	N/A	X	X	X		0
Q14554	PDIA5	Secretory	N/A	X	X	(X)		0
Q15262-2	PTPRK	Secretory	N/A	X	X	X		0
Q9NXG6	P4HTM	Type II TMP	Single	X	X			1
O00584	RNASET2	Secretory	N/A	X	X	(X)		0
Q99470	SDF2	Secretory	N/A	X	X	(X)		0
Q9H173	SIL1	Secretory	N/A	X	X	(X)	X	0
Q08357	SLC20A2	Type III-like	Multi	X	X	X		12
Q9ULF5	SLC39A10	Type III-like	Multi	X	X		X	7
O15533	TAPBP	Type I	Single	X	X			1
Q15582	TGFBI	Secretory	N/A	X			X	0
Q9Y3A6	TMED5	Type I	Single	X	X	(X)	X	1

understanding of how the evolutionarily conserved EMC acts in concert with the Sec61 complex to perform two important, yet apparently discrete, roles during co-translational TMP biogenesis [92]. Firstly, the EMC acts as a membrane insertase that enables the stable integration of certain types of TMD into the lipid bilayer [92–95]. Hence, following SRP-dependent delivery to the ER (cf. Fig. 2), membrane proteins destined to assume a type III orientation do not employ the canonical Sec61 translocon [97]. Rather, following engagement of the SRP receptor, these nascent type III TMPs can uniquely access the membrane insertase activity of the EMC [96,97, our unpublished data]; an action that may potentially be assisted by the Sec61 complex acting as a ribosomal docking site and/or via Sec61-stimulated release of ribosome-nascent chains from the SRP receptor (see [98]). Secondly, the EMC acts as a chaperone/holdase for multi-span TMPs with TMDs that contain suboptimal features [99–101].

Whilst some multi-span TMPs have cleavable ER targeting signals (cf. Fig. 1B), many employ signal-anchor sequences to enable their SRP-dependent delivery to the ER. These signal-anchor sequences form the first TMD and, as for single-span TMPs (cf. Fig. 1B), this sequence can be inserted into the ER membrane either with its N terminus remaining in the cytosol (type II-like, see Fig. 4) or with its N terminus translocated into the lumen (type III-like, see Fig. 4). When a type III-like multi-span TMP is truncated to enable the membrane insertion of its first TMD to be studied in isolation, its integration can be mediated by the EMC alone [96]. Hence, TMDs that assume a type III orientation, either in the context of a single-span TMP or as the first TMD of a multi-span TMP, employ the EMC for the membrane insertion. The ability of type III and type III-like TMPs to access the membrane insertase capacity of the EMC also provides a molecular level explanation of their unusual capacity to bypass the otherwise extremely potent blockade of the Sec61 translocon that can be achieved using small molecule inhibitors such as ipomoeassin-F and mycolactone [97,102–105]. We also note that type III and type III-like TMPs appear to show a reduced dependence on Sec61 translocon gating assistants when compared to obligate Sec61 clients (Fig. 3E, Table 5, cf. secretory proteins, type I and type II TMPs versus type III TMPs), further supporting their use of an alternative site for translocation into and across the ER membrane.

Whilst the precise molecular mechanisms that enable the integration of type III and type III-like TMDs via the EMC remain to be determined, a conserved hydrophilic vestibule formed by the EMC3, EMC4 and EMC6 subunits within the cytosolic side of the bilayer is most likely its *de facto* insertase site [92–95]. Like other members of the Oxa1 'superfamily' of membrane protein biogenesis factors [106,107], EMC3 is a structural homologue of YidC [95], a bacterial insertase which acts downstream of SRP and whose flexible cytosolic domains transiently contact SecY (a Sec61α

Table 2. Negatively affected secretory proteins and TMPs in Sec62 knockout HeLa cells; analysis of data presented in Ref. [74]. Protein substrates with an undefined topology of the 1st TMD (Uniprot) are in red.

Uniprot ID	Protein name	Protein class	Single/Multi	Sec61A1	Sec62	Sec63	TRAP	TMD number
P11117	ACP2	Type I TMP	Single		X		X	1
O14672	ADAM10	Type I TMP	Single		X		X	1
P78536	ADAM17	Type I TMP	Single		X			1
P20933	AGA	Secretory	N/A		X			0
Q9NRZ7	AGPAT3	Type II-like	Multi		X			2
Q9NRZ5	AGPAT4	Undefined	Multi		X	X		4
Q8N6S5	ARL6IP6	Undefined	Multi		X	X		3
Q86WA6	BPHL	Secretory	N/A		X			0
Q10589	BST2[a]	Type II TMP	Single	X	X	X		1
Q6Y288	B3GALTL	Type II TMP	Single		X			1
P54289	CACNA2D1	Type I TMP	Single		X			1
Q8NFZ8	CADM4	Type I TMP	Single		X	X		1
O43852-1	CALU	Secretory	Single		X			0
O43852-4	CALU	Secretory	Single		X			0
Q9H6E4	CCDC134[a]	Secretory	N/A	X	X	X		0
Q9BWS9	CHID1	Secretory	N/A		X			0
O75503	CLN5	Type II TMP	Single	X	X	X		1
Q9H8M5	CNNM2	Type III-like	Multi		X	X		4
P12109	COL6A1	Secretory	N/A		X	X		0
P16870	CPE	Secretory	N/A		X	X		0
Q9H3G5	CPVL	Secretory	N/A		X			0
Q96HD1	CRELD1[a]	Type I-like	Multi	X	X	X		2
P07858	CTSB	Secretory	N/A		X			0
P07339	CTSD	Secretory	N/A		X			0
Q9UBR2	CTSZ	Secretory	N/A		X			0
Q9UBS4	DNAJB11[a]	Secretory	N/A	X	X	(X)		0
Q13217	DNAJC3	Secretory	N/A		X			0
Q9UM22	EPDR1[a]	Secretory	N/A	X	X		X	0
Q9NZ08	ERAP1	Type II TMP	Single		X			1
Q96DZ1	ERLEC1	Secretory	N/A		X			0
Q9BS26	ERP44	Secretory	N/A		X			1
O75063	FAM20B	Type II TMP	Single		X	X		1
P98173	FAM3A	Secretory	N/A		X			0
P26885	FKBP2	Secretory	N/A		X			0
Q9Y680	FKBP7	Secretory	N/A		X			0
Q96AY3	FKBP10[a]	Secretory	N/A	X	X			0
Q9NWM8	FKBP14	Secretory	N/A		X			0
P10253	GAA	Secretory	N/A		X			0
Q14697-2	GANAB	Secretory	N/A		X			0
Q92820	GGH	Secretory	N/A		X			0
P06280	GLA[a]	Secretory	N/A	X	X	X		0
P08236	GUSB	Secretory	N/A		X		X	1
P23229	ITGA6	Type I TMP	Single		X			1
Q14573	ITPR3	Type II-like	Multi		X			6
Q7Z4H8	KDELC2	Secretory	N/A		X			0
P49257	LMAN1	Type I TMP	Single		X			1
Q9UIQ6	LNPEP	Type II TMP	Single		X		X	1
Q8TDW0	LRRC8C	Type II-like	Multi		X	X		4
Q17RY6	LY6K	Secretory	N/A		X	X		0
Q14165	MLEC	Type I TMP	Single		X			1
Q13724	MOGS	Type II TMP	Single		X			1
P17050	NAGA	Secretory	N/A		X			0
P13591	NCAM1	Type I TMP	N/A		X	X		1
Q8TEM1	NUP210	Type I TMP	Single		X			1

Table 2. (Continued).

Uniprot ID	Protein name	Protein class	Single/Multi	Sec61A1	Sec62	Sec63	TRAP	TMD number
Q9UHG3	PCYOX1	Secretory	N/A		X			0
Q14554	PDIA5[a]	Secretory	N/A	X	X	(X)		0
Q92508	PIEZO1	Undefined	Multi		X			36
O95427	PIGN	Type II-like	Multi		X			15
Q8TEQ8	PIGO	Undefined	Multi		X			14
Q8NBL1	POGLUT1	Secretory	N/A		X			0
P42785	PRCP	Secretory	N/A		X			0
Q13162	PRDX4	Secretory	N/A		X			0
P14314	PRKCSH	Secretory	N/A		X			0
Q13308-6	PTK7	Type I TMP	Single		X			1
P10586	PTPRF	Type I TMP	Single		X			1
P23470	PTPRG	Type I TMP	Single		X	X		1
Q15262-2	PTPRK[a]	Secretory	N/A	X	X	X		0
Q9NXG6	P4HTM[a]	Type II TMP	Single	X	X			1
Q15293	RCN1	Secretory	N/A		X			0
O00584	RNASET2[a]	Secretory	N/A	X	X	(X)		0
Q9HB40	SCPEP1	Secretory	N/A		X			0
Q99470	SDF2[a]	Secretory	N/A	X	X	(X)		0
P07093	SERPINE2	Secretory	N/A		X			0
P51688	SGSH	Secretory	N/A		X			0
Q9H173	SIL1[a]	Secretory	N/A	X	X	(X)	X	0
Q08357	SLC20A2[a]	Type III-like	Multi	X	X	X		12
Q9ULF5	SLC39A10[a]	Type I-like	Multi	X	X		X	7
O15533	TAPBP[a]	Type I TMP	Single	X	X			1
Q9Y3A6	TMED5[a]	Type I TMP	Single	X	X	(X)	X	1
O14656	TOR1A	Secretory	N/A		X			0
Q8NFQ8	TOR1AIP2	Type II TMP	Single		X			1
Q8NBZ7	UXS1	Type II TMP	Single		X	X		1
Q9ULK5	VANGL2	Type II-like	Multi		X	X		4
Q9BWQ6	YIPF2	Type II-like	Multi		X	X		5

[a]Protein substrates that were also negatively affected following Sec61 depletion.

orthologue) during membrane protein insertion [108,109]. By analogy with YidC, it may be envisaged that an equivalent cytosolic region of EMC3, for example the methionine-rich C1 loop and/or C terminus [95], might somehow selectively capture type III and/or type III-like TMDs and direct them towards the EMC insertase site. We additionally speculate that, as for YidC [110,111], positively charged regions within one or more cytosolic domains may promote RNC binding and thereby enable co-translational insertion via the EMC. Like YidC and SecYEG in bacteria [110], the EMC may support co-translational membrane insertion both alone and when acting in concert with the Sec61 complex. In the latter case, this would provide a flexible site for the membrane insertion of multi-span proteins containing closely spaced TMDs with distinct requirements for Sec61 and EMC-mediated integration (Fig. 4B; see also [83]).

The role of the EMC during the co-translational biogenesis of multi-span TMPs is not strictly limited to protein clients whose first TMD is type III-like (Fig. 4A), additionally extending to the stabilisation and/or insertion of downstream TMDs [99–101,112,113] irrespective of the orientation of the first TMD [89,90]. The decisive feature for the EMC-dependence of these multi-span TMPs is reduced hydrophobicity and/or increased polarity or charge, as further evidenced by the ability to create artificial EMC dependency by introducing polar/charged residues into a TMD [90,99,100]. Likewise, the observation that a multi-span TMP containing such a suboptimal TMD is diverted into to a pre-emptive ribosome quality control pathway in the absence of a functional EMC [114] implicates the EMC in a chaperone-like protective role akin to, but distinct from, that of the Sec61 translocon (Fig. 4C) [115].

Informed by structural and functional studies [92–97], we propose that, together, the Sec61 complex and the EMC provide a flexible hub for co-translational membrane insertion which can effectively mitigate the potentially error-prone biogenesis of a diverse range of client TMPs. We anticipate that future studies will

Table 3. Negatively affected secretory proteins and TMPs in Sec63 knockout HeLa cells; analysis of data presented in Ref. [74]. Protein substrates with an undefined topology of the 1st TMD (Uniprot) are in red.

Uniprot ID	Protein name	Protein class	Single/Multi	Sec61A1	Sec62	Sec63	TRAP	TMD number
Q9NRZ5	AGPAT4	Undefined	Multi		X	X		4
Q9H6U8	ALG9	Type III-like	Multi			X		8
Q9HDC9	APMAP	Type II TMP	Single			X		1
Q8N6S5	ARL6IP6	Undefined	Multi		X	X		3
P15848	ARSB	Secretory	N/A			X		0
P98194-7	ATP2C1	Type II-like	Multi			X		10
Q10589	BST2[a]	Type II TMP	Single	X	X	X		1
Q7KYR7	BTN2A1	Type I TMP	Single			X		1
Q8NFZ8	CADM4	Type I TMP	Single		X	X		1
Q9H6E4	CCDC134[a]	Secretory	N/A	X	X	X		0
Q4G0I0	CCSMST1	Type I TMP	Single			X		1
P13987	CD59	Secretory	N/A			X		0
Q8TCZ2	CD99L2	Type I TMP	Single			X		1
O75503	CLN5[a]	Type II TMP	Single	X	X	X		1
P10909	CLU	Secretory	N/A			X		0
Q9H8M5	CNNM2	Type III-like	Multi		X	X		4
Q9BT09	CNPY3	Secretory	N/A			X		0
Q8NBJ5	COLGALT1	Secretory	N/A			X		0
P12109	COL6A1	Secretory	N/A		X	X		0
P16870	CPE	Secretory	N/A		X	X		0
Q96HD1	CRELD1[a]	Type I-like	Multi	X	X	X		2
P81605	DCD	Secretory	N/A			X		0
P52429	DGKE	Undefined	Single			X		1
Q9BW60	ELOVL1	Undefined	Multi			X		7
O75063	FAM20B	Type II TMP	Single		X	X		1
P06280	GLA[a]	Secretory	N/A	X	X	X		0
Q68CQ7	GLT8D1	Type II TMP	Single			X	X	1
Q70UQ0	IKBIP	Type II TMP	Single			X		1
A1L0T0	ILVBL	Undefined	Single			X		1
P53708	ITGA8	Type I TMP	Single			X		1
Q8TDW0	LRRC8C	Type II-like	Multi		X	X		4
Q6NSJ5	LRRC8E	Type II-like	Multi			X		4
Q17RY6	LY6K	Secretory	N/A		X	X		0
Q9UKM7	MAN1B1	Type II	Single			X		1
Q8N4S9	MARVELD2	Type II-like	Multi			X		6
Q10469	MGAT2	Type II TMP	Single			X		1
P13591	NCAM1	Type I TMP	Single		X	X		1
Q8N5Y8	PARP16	Type II TMP	Single			X		1
P23470	PTPRG	Type I TMP	Single		X	X		1
Q15262-1	PTPRK	Type I TMP	Single			X		1
Q15262-2	PTPRK[a]	Secretory	N/A	X	X	X		0
P02753	RBP4	Secretory	N/A			X		0
Q08357	SLC20A2[a]	Type III-like	Multi	X	X	X		12
P46977	STT3A	Type II-like	Multi			X		13
Q66K14	TBC1D9B	Undefined	Single			X		1
Q9P2C4	TMEM181	Undefined	Multi			X		9
Q6ZXV5	TMTC3	Undefined	Multi			X		9
Q8N2C7	UNC80	Undefined	Multi			X		4
Q8NBZ7	UXS1	Type II TMP	Single		X	X		1
Q9ULK5	VANGL2	Type II-like	Multi		X	X		4
Q9BWQ6	YIPF2	Type II-like	Multi		X	X		5

[a]Protein substrates that were also negatively affected following Sec61 depletion.

Table 4. Negatively affected secretory proteins and TMPs in siRNA-mediated TRAP depleted HeLa cells; analysis of data presented in Ref. [72]. Protein substrates with an undefined topology of the 1st TMD (Uniprot) are in red.

Uniprot ID	Protein name	Protein class	Single/Multi	Sec61A1	Sec62	Sec63	TRAP	TMD number
P11117	ACP2	Type I TMP	Single		X		X	1
O14672	ADAM10	Type I TMP	Single		X		X	1
Q9BRR6	ADPGK	Secretory	N/A				X	0
Q9NW15-2	ANO10	Type II-like	Multi				X	8
Q9H6X2	ANTXR1	Type I TMP	Single				X	1
Q9BXK5	BCL2L13	Undefined	Single				X	1
P08962	CD63	Type II-like	Multi				X	4
Q8N129	CNPY4	Secretory	N/A				X	0
P08572	COL4A2	Secretory	N/A				X	0
O75629	CREG1	Secretory	N/A				X	0
O00622	CYR61	Secretory	N/A				X	0
P61803	DAD1	Type II-like	Multi				X	3
P39656	DDOST	Type I TMP	Single				X	1
O15121	DEGS1	Undefined	Multi				X	6
P00533	EGFR	Type I TMP	Single				X	1
Q9UM22	EPDR1[a]	Secretory	N/A	X	X		X	0
P02751	FN1	Secretory	N/A				X	0
Q68CQ7	GLT8D1	Type II TMP	Single			X	X	1
Q5VW38-2	GPR107	Type I-like	Multi				X	7
P08236	GUSB	Secretory	N/A		X		X	0
Q8TCT9	HM13	Type III-like	Multi				X	9
P56937	HSD17B7	Type III TMP	Single				X	1
P08069	IGF1R	Type I TMP	Single				X	1
P06756	ITGAV	Type I TMP	Single				X	0
Q8IWB1	ITPRIP	Type I TMP	Single				X	1
Q08380	LGALS3BP	Secretory	N/A				X	0
Q12907	LMAN2	Type I TMP	Single				X	1
Q9UIQ6	LNPEP	Type II TMP	Single		X		X	1
Q643R3	LPCAT4	Undefined	Multi				X	2
Q9H0U3	MAGT1	Type I TMP	Single			(X)	X	1
Q8NHP6	MOSPD2	TA protein	Single				X	1
P15941	MUC1	Type I TMP	Sinle				X	1
P54802	NAGLU	Secretory	N/A				X	0
Q969V3	NCLN	Type I TMP	Single				X	1
Q9UMX5	NENF	Secretory	N/A				X	0
Q99519	NEU1	Secretory	N/A				X	0
Q5JPE7	NOMO2	Type I TMP	Single				X	1
H0Y858	N/A	Type I TMP	Single				X	1
Q96E52	OMA1	Undefined	Single				X	1
Q9UBV2	SEL1L	Type I TMP	Single				X	1
Q13214	SEMA3B	Secretory	N/A				X	0
Q9H173	SIL1[a]	Secretory	N/A	X	X	(X)	X	0
P11166	SLC2A1	Type II-like	Multi				X	12
Q8TB61	SLC35B2	Undefined	Multi				X	9
Q9ULF5	SLC39A10[a]	Type III-like	Multi	X	X		X	7
P04920	SLC4A2	Type II-like	Multi				X	11
P35610	SOAT1	Type II-like	Multi				X	9
Q15005	SPCS2	Type II-like	Multi				X	2
Q8TCJ2	STT3B	Type II-like	Multi				X	13
Q15582	TGFBI[a]	Secretory	N/A	X			X	0
P55061	TMBIM6	Type II-like	Multi				X	7
Q9Y3A6	TMED5[a]	Type I TMP	Single	X	X	(X)	X	1
Q6UW68	TMEM205	Undefined	Multi				X	4
A0PJW6	TMEM223	Undefined	Multi				X	2

Table 4. (Continued).

Uniprot ID	Protein name	Protein class	Single/Multi	Sec61A1	Sec62	Sec63	TRAP	TMD number
Q8N2U0	TMEM256	Type III-like	Multi				X	2
O14773	TPP1	Secretory	N/A				X	0
Q15629	TRAM1	Type II-like	Multi				X	8
Q13454	TUSC3	Type I-like	Multi				X	4
Q9GZX9	TWSG1	Secretory	N/A				X	0
Q5T9L3	WLS	Type II-like	Multi				X	8
P41221	WNT5A	Secretory	N/A				X	0

[a]Protein substrates that were also negatively affected following Sec61 depletion.

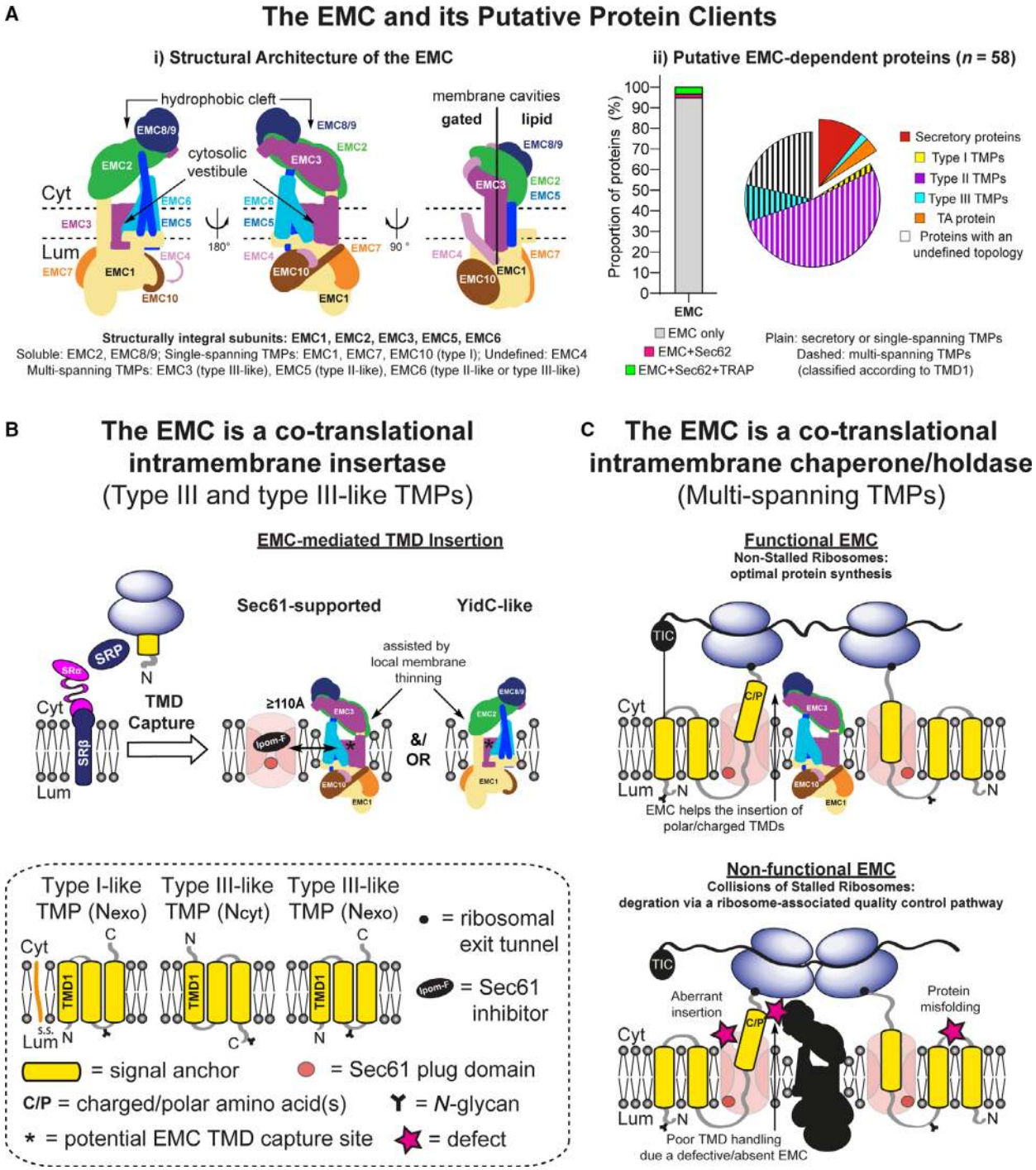
establish how individual TMDs are directed to either the EMC or Sec61 complex as appropriate, how the EMC governs TMD release into the membrane and further explore the regulation and potential interplay between the insertase activity of the EMC and its role as a chaperone/holdase. Given that EMC disruption negatively affects the levels of several secretory proteins [89], some of which also require Sec62 and/or TRAP (Fig. 4A) [72,74], we speculate that Sec61 dgating assistants and/or the EMC may also exert some, as yet undefined, regulatory role during Sec61-mediated co-translational translocation. Likewise, how the actions of this Sec61/EMC membrane insertion hub are co-ordinated with other recently identified TMD insertases/assemblases such as TMCO1 and the PAT complex (see New routes for insertion and folding: TMCO1 and the PAT complex) and various membrane protein complexes responsible for co-translational modifications including N-glycosylation (for a review see [9]) is a fascinating question.

New routes for insertion and folding: TMCO1 and the PAT complex

TMCO1 belongs to the same family of membrane protein insertases as EMC3/YidC (see [95,106,107]), and it can transiently associate with the ribosome-bound Sec61 complex [117]. In addition to the Sec61 complex, TMCO1 associates with CCDC47 [118] and the Nicalin-TMEM147-NOMO complex [119] to form a higher order collective referred to as the 'TMCO1 translocon' (Fig. 4A), which is implicated in the biogenesis of multispan TMPs [117]. Like the EMC, the TMCO1 translocon appears widely conserved and its disruption leads to reduced cellular fitness [120] and various organismal phenotypes [117,121]. Thus, although the precise biochemical function of the TMCO1 translocon remains unclear, structural and functional analogy with the EMC suggest that it may integrate insufficiently hydrophobic TMDs alone. Alternatively, given its association with the active Sec61 complex, it might assist the 'core' Sec61 translocon with the membrane insertion of suboptimal TMDs and/or help to shield newly integrated TMDs during the biogenesis and assembly of multi-span TMPs [117]. As with the EMC, we anticipate that a fuller understanding of TMCO1 protein clients, together with high-resolution structures of Sec61-TMCO1 bound RNCs, will be required to provide a unifying model for the concerted actions of the Sec61 and TMCO1 complexes. Despite clear parallels between the EMC and TMCO1 complex, including their potential dual activities as both a TMD insertase and chaperone/holdase [92,117], one feature firmly sets them apart; subunits of the TMCO1 translocon do not stably associate in the absence of ribosomes [117]. Thus, unlike the EMC, the TMCO1 translocon appears to exist as a short-lived entity that transiently assembles and disassembles according the needs of the Sec61 complex and the RNCs that it is presented with. The availability of TMCO1 subunits for transient assembly into the TMCO1 translocon may also be regulated by ER calcium levels. Hence, TMCO1 subunits homotetramerise to form calcium release channels in response to critically high levels of ER lumenal calcium, but rapidly disassemble once calcium levels are restored [121].

Structural evidence that the EMC has distinct TMD chaperone and TMD insertase activities is only beginning to emerge [92]. However, a protein complex that acts as a *bona fide* chaperone for TMDs that have exited the Sec61 translocon has recently been identified, firmly establishing the physiological necessity of such components [122]. The existence of the PAT complex was first apparent from *in vitro* studies of multispan TMP biogenesis, which characterised a component named PAT10 [123]. Following its lateral exit from the Sec61 translocon, the first TMD of a model multi-span TMP was shown to next encounter PAT10 and remain associated with this component as subsequent TMDs from the same nascent multi-span TMP were integrated via the Sec61 complex [123–125]. Only now do we know that PAT10 is in fact a protein

called Asterix, which forms an obligate heterodimer with CCDC47 that has been termed the PAT complex [122] (Fig. 5B). Most significantly, the PAT complex chaperones the assembly of multi-span TMPs, acting after their TMDs are inserted into the membrane but before protein folding is complete (Fig. 5C) [122]. Furthermore, whilst the substrate-binding Asterix subunit co-translationally engages membrane inserted TMDs with charged/polar residues that are exposed to the lipid bilayer, the PAT complex may remain associated with client TMDs even after translation termination, effectively shielding suboptimal TMDs until they are correctly packed into a natively structured multi-span TMP [122–124]. Interestingly an earlier genome-wide



screen had implicated both Asterix (also known as WDR83OS) and hSnd2 (see ER membrane targeting: the SRP-delivery system) in the biogenesis of multi-span TMPs [52].

Significantly, both the apparent preference of the PAT complex for TMDs of a more hydrophilic nature and its ability to engage TMDs irrespective of their transmembrane orientation [122,123] mirror the chaperone/holdase activity of the EMC (cf. Fig. 4). Furthermore, at least some multi-span TMPs that are dependent on the insertase activity of the EMC also require the PAT complex in order to assume a native conformation [122]. However, the EMC does not compensate for loss of the PAT complex, whilst the dependence of a TMP client on the PAT complex is unaffected if the EMC is bypassed during membrane insertion [122], suggesting that any functional redundancy between the two complexes is limited. Rather, it seems likely that the molecular basis for TMD recognition by each complex is sufficiently distinct that specific protein clients are able to access the chaperone activity of one complex whilst being precluded from engaging with the other (Fig. 5C). In the case of TMDs that are inserted via the Sec61-containing TMCO1 translocon, one possibility is that hydrophilic TMDs may be sequentially handed over to the PAT complex until the assembly of a multi-span TMP is complete (Fig. 5C) [126]. In this scenario, the interaction of CCDC47 with the Sec61-bound ribosome near its exit tunnel could provide an important physical link that enables client TMDs to access the substrate-binding Asterix subunit of the PAT complex. However, it should be noted that at present there is no direct evidence that TMCO1-associated CCDC47 is also in complex with Asterix [117,126].

Whether or not Asterisk is an as yet unidentified component of the TMCO1 translocon, or specific to a distinct PAT complex (Fig. 5A,B), is an urgent question that needs to be addressed. Likewise, determining how specific TMDs are directed to the PAT complex, how it helps these proteins assemble into a native fold and how correctly folded TMPs are eventually released, are all key steps towards fully understanding exactly how these recently identified Sec61 assistants contribute to the biogenesis of multi-span TMPs. Given that CCDC47 was named calumin on the basis of its calcium binding properties [127], and that calcium levels affect the homomerisation state of the TMCO1 subunit [121], the possibility that calcium levels might influence the biogenesis of multi-span TMPs via CCDC47 and/or the TMCO1 subunit should also be considered (for a review see [128]).

Concluding remarks: where do we go from here?

Over the past few years, our understanding of the molecular machineries that can be recruited by, and in at least one case completely bypass, the core Sec61 complex has skyrocketed. Various studies have redefined the roles of cytosolic components, including NAC and SGTA, during co-translational TMP biogenesis at the ER, discovered new routes for TMD insertion via the EMC and TMCO1 translocon, and identified the

Fig. 4. The role of the EMC in co-translational integration: two sides of the same coin (A) Schematics of the human EMC depicting its tripartite organisation in the ER membrane: a basket-shaped cytosolic region comprised of EMC2 and either of the functional paralogues EMC8 or EMC9; a membrane spanning core containing both gated and lipid-filled membrane cavities; and an L-shaped ER lumenal domain comprised of EMC1, EMC7 and EMC10 [92,94,95]. The insertase site formed by EMC3/EMC4/EMC6 is near the cytosolic vestibule, whilst the hydrophobic cleft may have a role in client TMD capture [92,94,95]. EMC subunits identified as structurally integral are based on depletion studies of individual subunits which destabilise the wider EMC complex [116] and the classification of EMC subunits relies on structural studies and/or topology prediction software, with the topology of EMC4 remaining ambiguous and that of EMC6 dependent on its assembly with EMC5 [92,94,95]. (Aii) The types of potential client proteins that were negatively affected using a mass-spectrometry-based proteomic approach in EMC2, EMC4 and EMC6 knockout HeLa cells are indicated [89,90]. The percentage of each substrate class is shown relative to the total number of putative EMC clients (n = 58) in the combined datasets [89,90], clearly illustrating that the majority of EMC clients are multi-span proteins. In a small number of cases, EMC clients are also dependent on Sec62 and/or TRAP as indicated [72,74]; see also Fig. 3E, Table 5). Proteins that do contain an ER targeting signal were discounted for the purposes of this analysis. (B) A unifying model for the co-translational insertion of type III TMPs whereby, following presumed SRP-dependent targeting to the ER membrane, the noncanonical Sec61 complex supports the insertase activity of the EMC via its gated cavity in some way [92, 97, our unpublished data]. As suggested for TMD1 of type III-like multi-span TMPs [96], and analogous to the bacterial insertase YidC, co-translational TMD insertion may also be facilitated by the EMC acting alone. These depictions are not representative of the relative orientations of Sec61, the EMC and the RNC which are currently unknown, although it has been proposed that the minimum distance from the Sec61 lateral gate to the membrane spanning core of the EMC is ~ 110 Å [94]. (C) The chaperone/holdase activity of the EMC, which stabilises and/or inserts suboptimal TMDs of multi-span TMPs, manifests via the lipid cavity [92]. Absence of a functional EMC leads to defective TMD handling resulting in the degradation of multi-span TMPs via a pre-emptive ribosome-associated quality control pathway [114]. TIC, translation initiation complex.

Table 5. Negatively affected secretory proteins and TMPs in EMC2, EMC4 or EMC6 knockout HeLa cells; analysis of data presented in Refs [89,90]. Protein substrates with an undefined topology of the 1st TMD (Uniprot) are in red, whilst those that are negatively affected by Sec62, Sec63 or TRAP depletion are in blue. Hydrophobicity values (ΔGapp) were calculated using https://dgpred.cbr.su.se/index.php?p=home [67]. Polar/charged amino acid residues: N, Q, Y, T, S, D, E, R, K, H.

Uniprot ID	Protein	Localisation	Protein Class	Single/ Multi	TMD Number	Signal/TMD Sequence	ΔGapp (ss/TMD1)	Polar/charged AAs (ss/TMD1)	Sec62/63/TRAP-dependent?
Q9UBR2	CATZ[a]	Lysosome	Secretory	N/A	N/A	ss: MA**RR**GPGW**R**PLLLLVLLAGAA**Q**G	2.23	4	N/A
Q9UM22	EPDR1[a]	Lysosome	Secretory	N/A	N/A	ss: MPG**R**APL**RT**VPGALGAWLLGGLWAW**T**LCGLC**S**LGAVG	1.67	5	Sec62, TRAP
Q92820	GGH[a]	Lysosome	Secretory	N/A	N/A	ss: MA**S**PGCLLCVLGLLLCGAA**S**LE**L**S	-0.28	4	Sec62
P13284	GILT[a]	Lysosome	Secretory	N/A	N/A	ss: M**T**L**S**PLLLFLPPLLLLL**D**VP**T**AAV**Q**A	1.19	5	N/A
P10619	PPGB[a]	Lysosome	Secretory	N/A	N/A	ss: MI**R**AAPPPLFLLLLLLLLLV**S**WA**SR**G**E**A	-1.84	5	N/A
Q9BRK5	SDF4[a]	Golgi	Secretory	N/A	N/A	ss: MVWPWVAMA**SR**WGPLIGLAPCCLWLLGAVLLM**D**A**S**A	0.28	4	N/A
O95470	SGPL1	ER	Type III	Single	1	V**D**ACLGGFLIVFM**EK**AG**Y**PL	2.59	4	N/A
P37268	FDFT1[b]	ER	TA protein	Single	1	**YS**PI**Y**L**S**FVMLLAAL**S**W**QY**L**TT**L	-0.24	9	N/A
O95159	ZPL1	Golgi	TA protein	Single	1	**R**AGLLLLLGLLGFLALLALM**SR**L	-4.19	3	N/A
Q9NZC3	GDE1[b]	PM	Type II-like	Multi	2	LLGPF**S**FLLLVLLLV**TRS**PV	-0.85	4	N/A
Q9UJG1	MSPD1[a]	ER, Golgi	Undefined	Multi	2	LL**T**VFLGVVCIAALMLP**T**L	-1.5	2	N/A
Q8N511	TMEM199	ER	Undefined	Multi	2	ALVI**T**IF**N**FIV**T**VVAAFVC**TY**	-1.13	5	N/A
Q96S66	CLCC1[b]	ER, Golgi, Nucleus	Type I-like	Multi	3	ss: MLC**S**LLLC**E**CLLLVAG**Y**A**H**	-0.15	4	N/A
Q9UHQ4	BAP29	ER	Type III-like	Multi	3	WAAVA**T**FL**Y**A**E**IGLILIFCLPFI	-1.22	3	N/A
P51572	BAP31[b]	ER	Type III-like	Multi	3	W**T**AVA**T**FL**Y**A**E**VFVVLLLCIPFI	-1.36	4	N/A
P21926	CD9	PM	Type II-like	Multi	4	FGF**N**FIFWLAGIAVLAIGLWL**R**F	-2.72	2	N/A
Q6NSJ5	LRC8E	ER	Type II-like	Multi	4	VLA**EY**L**T**VAMLMIGVFGC**T**L**Q**V**T**	0.09	6	N/A
Q5BJF2	SGMR2[a]	ER, Nucleus	Type II-like	Multi	4	WLLGL**Y**FL**SH**IPI**T**LFM**D**L**Q**AVL	0.87	6	N/A
P30408	T4S1[a]	Undefined	Type II-like	Multi	4	**R**CIG**HS**LVGLALLCIAA**N**ILL**Y**F	-1.17	5	N/A
P27449	VATL	Undefined	Type III-like	Multi	4	**YA**SFFAVMGA**S**AAMVF**S**ALGAA**Y**	0.96	5	N/A
Q15125	EBP[a]	ER, Nucleus	Undefined	Multi	4	**T**W**H**ILAGLF**S**V**T**GVLVV**TT**WLL**S**	-0.65	7	N/A
Q9H6R6	ZDHHC6	ER	Undefined	Multi	4	IIALGVIAIC**ST**MAMI**DS**VLW**Y**W	-0.233	5	N/A
P35610	SOAT1[a]	ER	Type II-like	Multi	5	**T**I**YH**MFIALLILFIL**ST**LVV**DY**I	-2.91	7	TRAP

Table 5. (Continued).

Uniprot ID	Protein	Localisation	Protein Class	Single/ Multi	TMD Number	Signal/TMD Sequence	ΔGapp (ss/TMD1)	Polar/charged AAs (ss/TMD1)	Sec62/63/TRAP-dependent?
Q9GZM5	YIPF3[b]	Golgi	Type II-like	Multi	5	L**Y**GPLMLVF**T**LVAILL**H**GM**KT**	0.03	5	N/A
Q9UNQ0	ABCG2[a]	Mitochondria	Type II-like	Multi	6	IA**Q**IIV**T**VVLGLVIGAI**Y**FGL	-1.28	3	N/A
P55061	BI1	ER	Type II-like	Multi	6	**K**V**Y**A**S**FALCMFVAAAGA**Y**V	0.43	4	N/A
Q14643	ITPR1	ER	Type II-like	Multi	6	LL**R**L**TK**ILLAIL**D**CV**H**V**TT**IFPI	1.1	7	N/A
O43688	PLPP2[a]	PM	Undefined	Multi	6	WVFVLL**D**VLCLLVA**S**LPFAIL**T**L	-1.8	3	N/A
Q96HH6	TMM19	Undefined	Undefined	Multi	6	MI**TN**IVIL**S**LIIC**S**LAFWII**S**M	-2.58	5	N/A
P61073	CXCR4[a]	Endosome, Lysosome, PM	Type III-like	Multi	7	**T**I**YS**IIFL**T**GIVG**N**GLVILVMG**Y**	-0.18	6	N/A
O60353	FZD6	PM	Type III-like	Multi	7	MF**T**FLL**T**CIFLPLL**R**G**HS**LF	0.03	5	N/A
Q4KMQ2	ANO6[a]	PM	Type II-like	Multi	8	G**YYTQ**MLLLAAVVGVACFL**Y**G**Y**L	-2.08	6	N/A
P43003	EAA1[a]	PM	Type II-like	Multi	8	AFVLL**T**V**T**AVIVG**T**ILGF**T**L**R**	-1.35	5	N/A
Q93050	VP11	Undefined	Type II-like	Multi	8	AP**YT**II**T**FPFLFAVMFG**D**F	0.97	4	N/A
Q13488	VP33	Undefined	Type II-like	Multi	8	VALV**Q**LFLP**T**AAA**YT**CV**SR**L	2.31	6	N/A
Q96S97	MYADM[a]	Undefined	Undefined	Multi	8	LL**R**LL**Q**LV**ST**CVAF**S**LVA**S**VGAW	0.06	6	N/A
Q8WWI5	CTL1[a]	Mitochondria, PM	Type II-like	Multi	9	IPWLLLFILFCIGMGFICGF**S**IA	-3.41	1	N/A
P23634	ATB4[a]	PM	Type II-like	Multi	10	V**T**LIIL**E**IAAII**S**LVL**S**F**YR**	-0.82	6	N/A
P98196	AT11A	Endosome, ER, PM	Type II-like	Multi	10	F**RR**VA**N**F**Y**FLIIFLV**Q**LII**DT**	-0.16	7	N/A
Q9HD20	AT131[b]	ER	Type II-like	Multi	10	**R**LALL**RR**L**T**VLPFAGLL**Y**PAWL	-4.24	5	N/A
P04920	B3A2[a]	Undefined	Type II-like	Multi	10	L**D**AVL**E**VPVPV**R**FLFLLLG	2.65	3	N/A
P51790	CLCN3	Endosome, Golgi	Type II-like	Multi	10	AW**S**GWLVV**T**L**T**GLA**S**GALAGLI	0.74	4	N/A
Q8IWA5	CTL2	Undefined	Type II-like	Multi	10	IICCVFLLLAIVG**Y**VAVGIIAW**T**	-2.41	2	N/A
P20020	AT2B1[a]	PM	Type II-like	Multi	11	V**T**LIIL**E**IAAIV**S**LGL**S**F**Y**	0.05	5	N/A

Table 5. (Continued).

Uniprot ID	Protein	Localisation	Protein Class	Single/ Multi	TMD Number	Signal/TMD Sequence	ΔGapp (ss/TMD1)	Polar/charged AAs (ss/TMD1)	Sec62/63/TRAP-dependent?
Q9H2H9	S38A1[a]	PM	Type II-like	Multi	11	LAFALA**NT**GILLFLVLL**TS**V**T**LL	-2.57	5	N/A
Q8WUX1	S38A5[a]	PM	Type II-like	Multi	11	LA**Y**AMA**HT**GVIFFLALLLCIALL	-3.24	3	N/A
Q9H7F0	AT133 [a]	Undefined	Undefined	Multi	11	LAIV**S**LGVIC**S**GGFLLLLL**Y**WM	-2.39	3	N/A
O60503	ADCY9	PM	Type II-like	Multi	12	**RR**F**RY**ALF**Y**IGFACLLW**S**I**Y**FAV	-2.47	7	N/A
Q5BKT4	AG10A	ER	Type II-like	Multi	12	F**S**AAL**S**C**T**FLV**S**CLLF**S**AF**SR**AL	-0.67	7	N/A
P30825	CTR1	PM	Type II-like	Multi	12	**T**F**D**LVALGVG**ST**LGAGV**Y**VLA	1.53	5	N/A
Q96SL1	DIRC2	Lysosome	Type II-like	Multi	12	V**Y**G**RR**WLVLLLF**S**LLAFV**Q**GLVW	-1.94	5	N/A
P31641	SC6A6[b]	PM	Type II-like	Multi	12	FVL**S**VAGGFVGLG**N**VW**R**FP**Y**LC**Y**	1.91	5	N/A
O15439	MRP4	Undefined	Undefined	Multi	12	C**Y**W**KSY**LVLGIF**T**LI**EES**A	2.39	8	N/A
Q92508	PIEZ01	Endosome, PM	Undefined	Multi	12	LLAACLL**R**F**S**GL**S**LV**Y**LLFLLLL	-3.17	4	Sec62
Q96T83	SL9A7	Endosome, Golgi	Undefined	Multi	12	**R**LLLLPLLLGWGL**R**VAAAA	0.17	2	N/A
Q8NBI5	S43A3[b]	Undefined	Undefined	Multi	12	LL**T**GLL**E**CLGFAGVLFGWP**S**LVF	-0.23	3	N/A

[a]Proteins identified via a mass-spectrometry proteomics-based approach using EMC2 and EMC4 knockout cells [89].; [b]Those that were also identified in EMC6 knockout cells [90].

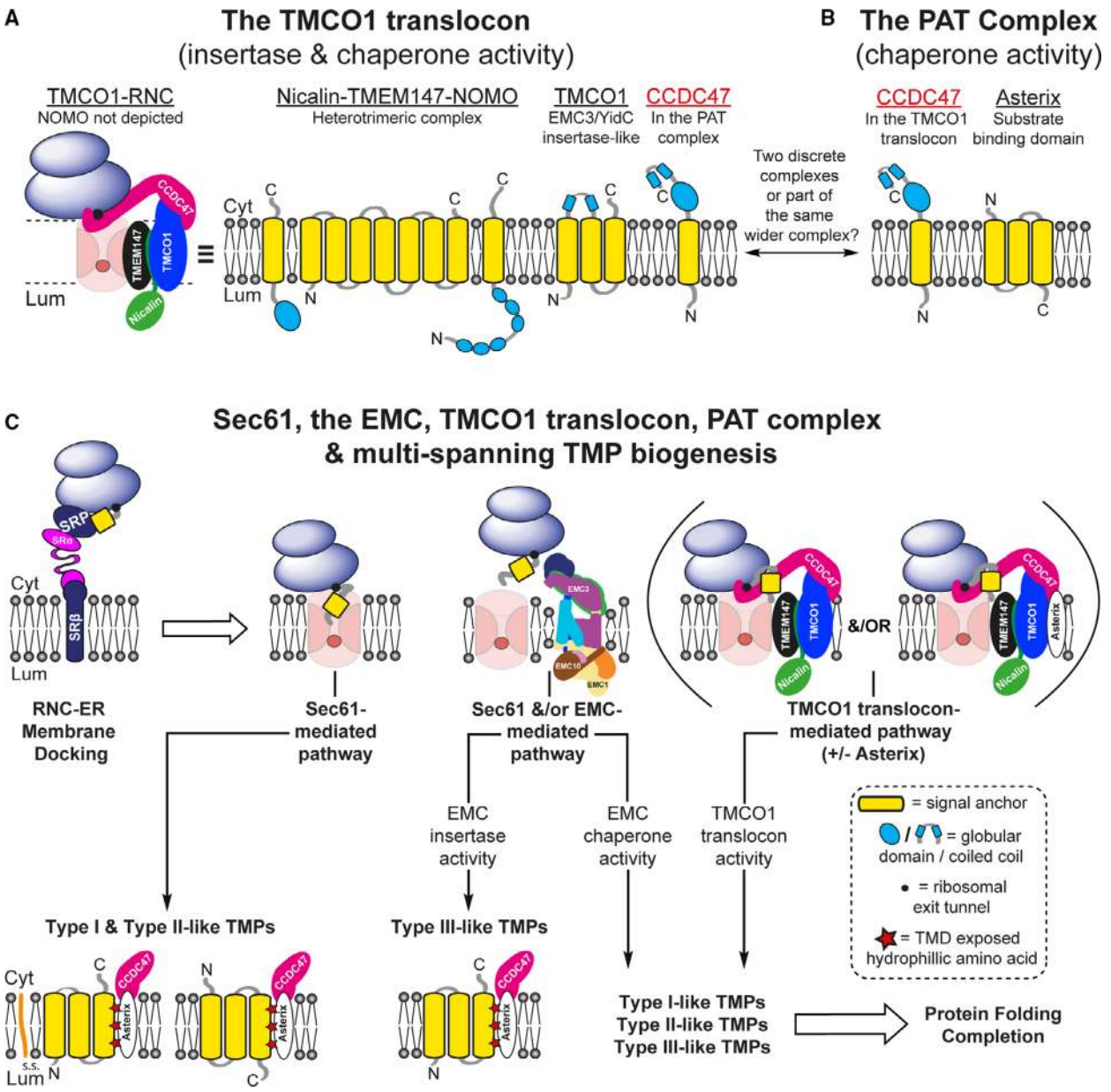


Fig. 5. TMCO1 translocon and PAT complex: one or two more Sec61-assistants for multi-span TMPs? (A) Schematics of the TMCO1 translocon and the topologies and domain structures of each subunit [117]: TMEM147 is the core subunit of the Nicalin-TMEM147-NOMO complex [119] which, when assembled into the Sec61-TMCO1-RNC complex (NOMO not depicted), lines a lipid cavity at the centre of this transient complex. (B) The CCDC47 and Asterix subunits of the PAT complex form a stable heterodimer [122], but its precise relationship to the TMCO1 translocon, if any, is unclear [126]. Based on its proximity to the ribosomal exit tunnel [117], CCDC47 may be able to sense hydrophilic TMD residues and recruit Asterix into the wider TMCO1 complex. Alternatively, the PAT complex may function as an independent entity, closely associated with the Sec61 complex [123], that shields and assembles TMDs following their initial membrane insertion [122]. (C) A snapshot of multi-span TMP biogenesis at the ER. Following SRP-dependent targeting, TMPs may access the ER membrane via a Sec61-mediated pathway, a Sec61/EMC-mediated pathway or a TMCO1 translocon-mediated pathway. Irrespective of the initial mechanism of TMD integration in the ER membrane, the PAT complex can associate with membrane inserted TMDs that would otherwise expose polar residues to the lipid bilayer, and chaperone them until they can be assembled with other TMDs to form a stable multi-span TMP.

PAT complex as a TMD chaperone and assemblase. Likewise, we now understand that Sec62, Sec63 and the TRAP complex modulate and enhance the capabilities of the core Sec61 translocon in order to expand its client base. When all of these elements are taken together, they provide an amazingly flexible platform that is capable of synthesising an incredibly diverse and challenging range of secretory and TMP clients. On the basis of our current understanding, we propose that the Sec61 complex provides the central component of this flexible platform, acting as a dynamic hub for membrane translocation at the ER. This begs the question as to how alternative membrane insertion pathways or particular Sec61-assistants are engaged by different client TMPs and how their actions can be co-ordinated; a feat that becomes increasingly complex when one considers the recent finding that both homomeric and heteromeric TMP complexes can begin their assembly co-translationally [129,130].

Another important contribution to our increased knowledge and understanding of TMP biogenesis has been the discovery and characterisation of small molecules and toxins, which selectively inhibit the Sec61 translocon [131–133]. Hence, via the study of individual proteins [6,97,104] together with a global proteomics-based approach [103], it was the resistance of type III and type III-like TMPs to such compounds which revealed a previously unanticipated level of complexity that was incompatible with the prevailing models of TMP biogenesis [7,8,83]. Given that many of the TMP clients of the Sec61 complex are drug targets [133], Sec61 inhibitors are promising candidates for therapeutic development; particularly since they appear well tolerated *in vivo* [134–137] and have demonstrated promising analgesic [134], antibacterial [138], anti-inflammatory [135], antitumour [136] and antiviral [139–141] activity.

As evidenced by studies of proteins from influenza and SARS-CoV-2 viruses, the antiviral activity of these compounds typically relies on their host-targeted inhibition of the canonical Sec61 translocon, effectively blocking the biogenesis of important viral proteins at the host cell ER [97,103]. Dengue and Zika viruses likewise co-opt the TMP biosynthetic machinery of the host cell ER [141], whilst cell-based studies of influenza, HIV and dengue have firmly established proof of concept for the inhibition of viral growth and propagation through the selective perturbation of Sec61-mediated protein translocation [139]. Thus, Sec61 inhibitors may provide one route for developing much needed broad-spectrum agents that can be mobilised against many different viruses [142]. Likewise, the discovery of the EMC, TMCO1 translocon and PAT complex make them valid candidates for developing complementary small molecule inhibitors which target the biogenesis of specific classes and/or groups of TMPs at the ER. In short, as we gain more insight into the components, pathways and molecular mechanisms utilised by our cells to create functional membrane proteins, this knowledge will in turn present us with new and exciting opportunities to modulate these processes for the benefit of human health [143,144].

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

SO'K, MRP and SH wrote and edited the manuscript.

The work mentioned as "our unpublished data" in the section entitled "Membrane insertion via the EMC" and the legend to Figure 4 has now been accepted for publication in Communications Biology.

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