Evidence for the relocalization of the ribosome-translocon complex as a key event for the emergence of endoplasmic reticulum during eukaryogenesis

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CARILO ISAAC

ABSTRACT

The origin of the eukaryotic cell is regarded as a major evolutionary innovation driving the emergence of complexities and diversities of life forms. However, the origin and evolutionary developments governing eukaryotic cellular complexities are unresolved questions that are obscured by controversies. Phylogenomic analysis of environmentally sampled DNA reveals the existence of eukaryotic features in the genomes of organisms belonging to an archaeal clade, the Asgard superphylum. This positions members of the Asgard superphylum as the closest archaeal relatives of eukaryotes. Although eukaryotes possess an internal membrane system which makes them unique from prokaryotes, no internal membrane has been observed in cultured organisms belonging to Asgard superphylum. The 2 billion years of separation between prokaryotes and eukaryotes makes it difficult to reconstruct specific events leading to the appearance of internal membrane in eukaryotes. Moreover, since eukaryotic internal membranes were derived from the endoplasmic reticulum (ER), the origin of the ER is crucial for understanding the emergence of internal membranes. Strikingly, the genomes of Asgard archaea encode homologues of the eukaryotic ER-resident translocon components, Sec61-OST-TRAP. The evolutionarily conserved ribosome-translocon complex mediates translocation of secretory proteins and co-translational insertion of membrane proteins into the eukaryotic endoplasmic reticulum (ER) membrane and the prokaryotic plasma membrane. However, the prokaryotic translocon components sit in the plasma membrane whilst the eukaryotic translocon resides in the ER. Possibly, the best tools available in understanding the emergence of eukaryotic internal membranes are the transmembrane proteins associated with these membrane systems. This distinction in their localization provides an opportunity to explore how the Sec61 translocon may have undergone relocalization from the plasma membrane of eukaryotic ancestor to an internal membrane (proto-ER) during eukaryogenesis. Understanding how the Sec61-OST-TRAP translocon may have re-located from the plasma membrane to an internal membrane (proto-ER) during eukaryogenesis would reveal the underlying principles governing the emergence of eukaryotic intracellular cellular complexity. In this study, fluorescently-tagged protein components of Sec61-OST-TRAP, including an S-layer protein, from *Candidatus Prometheoarchaeum syntrophicum* strain, MK-D1, are targeted to the ER when heterologously expressed in HeLa cells. Fluorescently tagged-signal peptides of the signal peptide-bearing proteins belonging to the Sec61-OST-TRAP are ER-localized and not plasma membrane-localized, demonstrating that the signal peptides are responsible for targeting signal peptide-containing proteins to the ER. Also, an N-glycosylatable EGFP fused to these signal peptides are N-glycosylated further revealing that MK-D1 signal peptides

targets EGFP to the ER. Therefore, MK-D1 signal peptides are inter-compatible with the eukaryotic Sec-61 translocon. AlphaFold-2 based modelling of MK-D1 signal peptides with human Sec61α reveals that the MK-D1 signal peptides mechanistically intercalate the Sec61α lateral gate helices as observed with the interaction between eukaryotic signal peptides and the translocon.

AlphaFold-2 modelling of the Sec61-TRAP-OST complex reveals significant structural similarities with human Sec61-TRAP-OST complex. The Sec61 translocon inhibitor, Eeyarestatin-I, inhibited the co-translational insertion of new Sec61α subunit into the ER. Taken together, this data demonstrates that the location of existing ribosome-translocon apparatus, at the protein level, determines the future placement of yet to be translated translocon subunits. The solved X-ray crystal structure of the cytoplasmic domain of OST1 (subunit of OST complex) reveals a 4-helix bundle with the same topology as the human domain, although the helices have slightly different angles relative to each other compared with the human OST1. This cytoplasmic domain of MK-D1 OST1 was demonstrated to interact with purified human 80S ribosomes, as reported in other studies for the interaction between cytoplasmic domain of human OST1 and human ribosomes. Thus, MK-D1 OST1 participates in the recruitment of ribosomes to the Sec61/OST/TRAP translocon at the cell membrane for translation and translocation of membrane proteins and proproteins. Helix-by-helix overlay between the MK-D1 OST1 and human OST1 reveals surface patches basic amino acid residues clustering in the regions that may interact with ribosome. Together, these data demonstrate that the Asgard and eukaryotic Sec61/OST/TRAP translocons are structurally and functionally similar, and mutually compatible. The eukaryotic ribosome-translocon complex is able to translate and direct Asgard translocon proteins to the ER, and the Asgard translocon complex can interact with eukaryotic ribosomes via OST1 subunit.

Taken together, the inter-compatibility of Asgard and eukaryotic ribosome-translocon complexes has implications for ER biogenesis. This principle predicts that during eukaryogenesis, under positive selection pressure, the relocation of a few translocon complexes to an emerging internal membrane (proto-ER) will have favorably propagated the new translocon location, eventually leading to their loss from the cell membrane.

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CARILO Isaac

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LIST OF ABBREVIATIONS AND SYMBOLS

Å	Angstrom
α	Alpha
AF-2	AlphaFold-2
β	Beta
γ	Gamma
BLAST	Basic Local Alignment Search Tool
CHX	Cycloheximide
C-terminal	Carboxy terminal
$CaCl_2$	Calcium chloride
DNA	Deoxyribonucleic acid
E. coli	Eschericia coli
EM	Electron microscopy
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
ES1	Eeyarestatin I
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
His	Histidine
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KCl	Potassium Chloride
kDa	Kilodaltons
M	Molar
MK-D1	*Candidatus* Prometheoarchaeum syntrophicum
$MgCl_2$	Magnesium chloride
Na	Sodium
NaCl	Sodium Chloride
Ni-NTA	Nickel-nitrilotriacetic acetic
mM	Millimolar
nm	nanometer
N-terminal	Amino terminal
°C	Degrees celcius
OST	Oligosaccharyltransferase

OSTI	Oligosaccharyltransferase subunit 1
PDB	Protein Data Bank
PEG	Polyethylene glycol
SDS PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SP	Signal peptide
TM	Transmembrane domain
TRAP	Translocon associated protein
TRIS	Trisaminomethane
X-ray	X-radiation
µL	microlitre
µM	micromolar
µm	micrometer

1. INTRODUCTION

Generally, signal peptides (SPs) and transmembrane domains (TMDs) either alone or in combination, function as targeting determinants that guarantee initial plasma membrane or ER targeting as well as their subsequent membrane integration or translocation (S. Lang et al., 2022; Sakaguchi et al., 1992; Wu & Hegde, 2023). The signal recognition particle (SRP) play a key role in co-translational targeting of secretory and transmembrane proteins to the plasma membrane of prokaryotes or ER of eukaryotes after their emergence from the ribosome(Cross et al., 2009; Grudnik et al., 2009; Keenan et al., 2001; K. Wild et al., 2004). Protein targeting mediated by signal recognition particle (SRP) is a universally conserved process in all domains of life (Akopian et al., 2013; Grudnik et al., 2009; Saraogi & Shan, 2011). At the mouth of the cytosolic ribosome exit tunnel, emerging nascent polypeptides containing either an N-terminal signal peptide (SP) or transmembrane domain (TMD) are recognized by signal recognition particle (SRP) (Egea et al., 2005; Halic et al., 2006; Luirink, 2004; Nyathi et al., 2013; K. Wild et al., 2004). This initial association involving the ribosome, emerging chain and SRP slows down or arrests translation (Walter & Blobel, 1980, 1982). The SRP-ribosome-nascent chain complex is targeted to the ER and docks at the Sec61 translocon via an interaction involving the SRP and its plasma membrane or ER-bound cognate receptor (SRP receptor or SR) (Görlich et al., 1992; K. U. Kalies et al., 1994; Keenan et al., 2001) and an interaction between ribosome and Sec61 translocon (Gilmore et al., 1982; D. I. Meyer et al., 1982). A crucial step during targeting of proteins to the prokaryotic cytoplasmic membrane or ER membrane is the transfer of the ribosome nascent chain from the SRP to the Sec61/SecYEG translocon via the signal receptor (SR) (Aviram & Schuldiner, 2017). The binding of SRP with its receptor triggers transfer of the nascent chain to the Sec61 translocon complex in a hand-off mechanism (Jomaa et al., 2017; Kobayashi et al., 2018; Park & Rapoport, 2012). Afterwards, the SRP dissociates from the ER-bound ribosome-nascent-chain complex. Ribosome binding to Sec61 initiates conformational changes that partially destabilizes the lateral gate of the translocon, priming it for engagement with the signal peptide or transmembrane domain (Berg et al., 2004; Voorhees et al., 2014; Voorhees & Hegde, 2016). The initial insertion can be spontaneous or may require substrate specific auxiliary components (Migliaccio et al., 1992; Wiedmann et al., 1987). Finally, the signal peptides are proteolytically cleaved at the luminal side of the ER by membrane-bound signal peptidase complex (SPC) (Liaci et al., 2021).

In eukaryotes, the core Sec61 channel forms a larger translocon associating with the multimeric translocon-associated protein (TRAP) and oligosaccharyltransferase (OST) complexes (Pfeffer et al., 2014). In the Sec61-OST-TRAP translocon, the TRAP complex aids ribosome docking and nascent polypeptide insertion into the Sec61 translocon, and participates in protein folding (Jaskolowski et al., 2023; Karki et al., 2023; Sommer et al., 2013) while the OST complex mediates N-linked glycosylation (Pfeffer et al., 2014; Ramírez et al., 2019; R. Wild et al., 2018). The TRAP complex is not found in bacteria (Russo, 2020; Sommer et al., 2013), and the bacterial oligosaccharyltransferase is a single polypeptide chain (Gerber et al., 2013; B. H. Meyer & Albers, 2014; Napiórkowska et al., 2018). Eukaryotic homologs of OST and TRAP protein subunits occur in the genomes of organisms belonging to the Asgard superphylum (Eme et al., 2023; Xie et al., 2022; Zaremba-Niedzwiedzka et al., 2017). Understanding these differences in the emergence of the eukaryotic Sec61-OST-TRAP translocon and their contribution to the development of eukaryotic cellular complexity are key questions for the eukaryogenesis field. Additionally, ER homologs of the widely conserved Oxa-1/YidC/Alb3 have been identified as the integral components of the multipass translocon and guided entry for tail-anchored (GET) proteins pathway (Anghel et al., 2017), and they utilize common mechanisms in their membrane insertion (McDowell et al., 2021).

Eukaryotes are distinguished from prokaryotes by the presence of an endomembrane system (Vellai & Vida, 1999) and eukaryote-specific protein components (Hartman & Fedorov, 2002). However, the origin and evolutionary developments that govern eukaryotic cellular complexities is an unresolved question that is obscured by controversies. Evolutionary models that have been postulated to explain the evolution of intracellular structures and endomembrane system in eukaryotes generally revolve around autogenous (non-symbiotic)(Baum, 2015) and endosymbiotic theories (Martin et al., 2001; Martin et al., 2015). Endosymbiogenic scenarios representing eukaryotes as the merger between an archaeal host and an alphaproteobacterial endosymbiont (mitochondrial ancestor) are the currently preferred models for eukaryogenesis (Imachi et al., 2020; Martijn & Ettema, 2013). While much evidence exists for the mitochondrial origin from alphaproteobacteria (Fitzpatrick et al., 2006; Gray et al., 2001; Thiergart et al., 2012, 2012). The origin of the eukaryotic internal membrane system and other eukaryotic-specific traits remains unclear. Possibly, the best tools available in understanding the emergence of eukaryotic internal membranes are the transmembrane proteins associated with each membrane. The endoplasmic reticulum (ER) is regarded as the precursor of the endomembrane system (Ozansoy & Denizhan, 2009) and harbors prominent transmembrane protein complexes. In particular, the eukaryotic ribosome-translocon complex (Sec61-OST-

TRAP) resides in the endoplasmic reticulum (ER) membrane(Gemmer & Förster, 2020) and facilitates the translocation of nascent polypeptides into the ER lumen and integration of transmembrane proteins into the ER membrane (Braunger et al., 2018; O'Keefe et al., 2022). However, in prokaryotes, the homologous SecYEG translocon is resident in the cytoplasmic membrane (Driessen & Nouwen, 2008). While both translocons transverse membranes, it is clear that they differ in their specific membrane localization. This distinction provides an opportunity to explore how the Sec61 translocon may have undergone relocalization during eukaryogenesis.

Phylogenetic analyses of the preprotein translocase channel SecY/Sec61 and the OST complex catalytic subunit (STT3) indicate that the eukaryotic versions of these translocon subunits are more closely related to their Asgard archaea counterparts than to other prokaryotic homologs (Liu et al., 2021; Zaremba-Niedzwiedzka et al., 2017). Asgard archaea are predicted to have a complete Sec61αβγ complex and many of the components of the OST and translocon-associated proteins (TRAP) complexes (Eme et al., 2023). In particular, re-localization of the ribosome-translocon apparatus to the emerging internal membrane system has been proposed as a key event during biogenesis of a proto-ER in the last universal common eukaryotic ancestor (Baum & Baum, 2014; Gould et al., 2016; Ozansoy & Denizhan, 2009). The presence of eukaryotic homologues of Sec61-OST-TRAP in these distant prokaryotic ancestors (Eme et al., 2023; Muñoz-Gómez et al., 2017; Petrů et al., 2021) identifies Asgard archaea as unique system for tracing the relocalization of translocon components during eukaryogenesis. Additionally, the core membrane insertion components of other ER-resident insertases (GET pathway, ER membrane complex, TMCO1 translocon) that cooperate with the Sec61-OST-TRAP during membrane protein biogenesis are structural homologs and functional homologs (possess an oxa1-like architecture) (Anghel et al., 2017) proposed to evolve from an archaeal origin (Petrů et al., 2021).

A growing body of evidence reveal that a number of protein machines in Asgard are inter-compatible with their eukaryotic partners (Akıl et al., 2020, 2022; Akıl & Robinson, 2018; Survery et al., 2021). Mechanistically, it is probable that the re-localization of core Asgard membrane translocases and insertases to the proto-ER is a milestone event for the recruitment of diverse proteins to the proto-ER during eukaryogenesis. However, no experimental evidence exists to support this hypothesis. The study sought to investigate how the translocon location was inherited, and the extent of inter-compatibility between components of the Asgard ribosome-translocon-complex and their eukaryotic counterparts. Although a wealth of

knowledge about the morphology and physiology of the eukaryotic ER exists, understanding the key factors driving the formation of the ER is essential to revealing the key concepts in cell biological events in eukaryotes.

2. LITERATURE REVIEW

2.1 The Trees of life

Living organisms were broadly divided into prokaryotes (bacteria) and eukaryotes based on their cellular organisation (Stanier & Niel, 1962). Shortly after this, Archaea were first regarded as a unique domain of life when 16S ribosomal (r) RNA analysis distinguished them from Eukarya and Bacteria domain (Fox et al., 1977; Woese et al., 1990; Woese & Fox, 1977). This led to the construction of the three domains of life comprising of bacteria, archaea and eukarya (Woese & Fox, 1977), replacing the original bacteria-eukaryote division. All organisms originally assigned to the archaeal domain were all extremophiles until an expanded application of 16S rRNA analysis revealed broad distribution of uncultivated archaea in normal biological niches such as soil, ocean water, sea water, marine and fresh water sediments, and human intestinal flora (Chaban et al., 2006; DeLong, 1998). Archaea are unicellular organisms possessing an S-layer cell envelope covering single cytoplasmic membranes with no internal organelles.

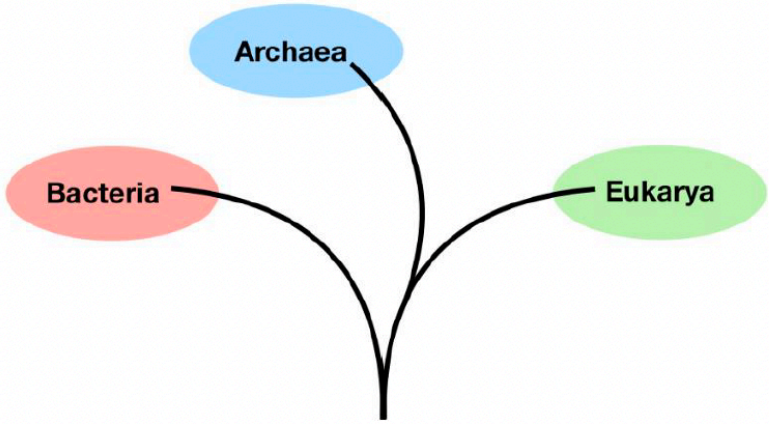


Fig. 2.1: The three-domain tree of life (Eme et al. 2017) modified from (Woese et al. 1977)

2.1.1 The two-domain tree of life

The three-domain theory of life was later challenged when analysis of ribosomal subunits identified eukaryotes to emerge from within Archaea domain (Koonin, 2010; Lake, 1988; Lake et al., 1984). This modified the three-domain tree of life to suit a two-domain tree (Eocyte hypothesis), with bacteria and archaea as the two primary domains whereas eukaryotes cluster with archaea domain (Koonin, 2010; Lake, 1988; Lake et al., 1984).

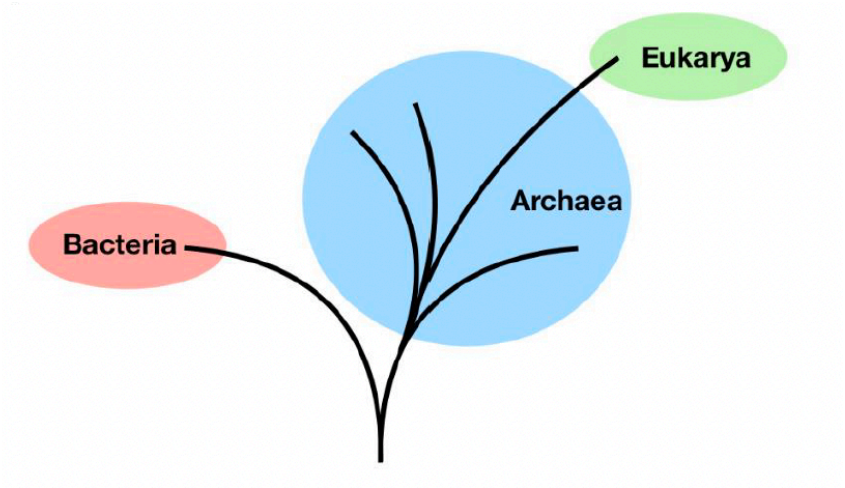


Fig. 2.1.1: The two-domain tree of life (Eme et al. 2017) modified from (Lake et al. 1988).

2.1.2 Archaeal origin of eukaryotes

Phylogenetic studies provided evidence for the emergence of eukaryotes from within or as a relative to an archaeal clade "TACK" superphylum, originally comprising of the phyla Thaumarchaeota, Aigarchaeota, Crenarchaeota and Korarchaeota (Ettema et al., 2011; Guy & Ettema, 2011; Martijn & Ettema, 2013; Williams et al., 2012; Yutin & Koonin, 2012). Increasing number of eukaryotic features were identified in this archaeal clade, suggesting that the archaeal ancestor was more eukaryotic in nature than previously thought (Bernander et al., 2011; Koumandou et al., 2013; Lindås et al., 2008; Martijn & Ettema, 2013; Yutin & Koonin, 2012).

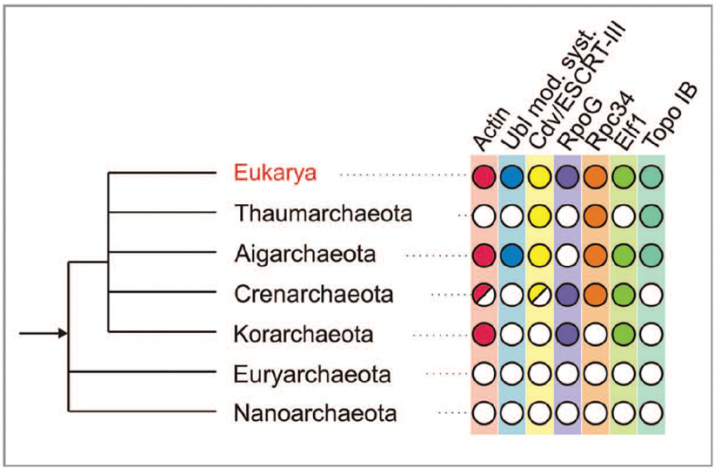


Fig. 2.1.2: Eukaryotic signature genes in TACK group (Bernander et al. 2011)

2.2 Asgard superphylum

Phylogenomic analysis of deep-sea sediments sampled off-shore in Norway revealed the existence of significant eukaryotic features in the genomes of archaeal organisms belonging to Asgard superphylum. The name 'Asgard' was derived from the Gods of Norse Mythology, was proposed for this archaeal clade (Zaremba-Niedzwiedzka et al., 2017), originally composed of Lokiarchaeota, Thorarchaeota, Odinarchaeota, Heimdallarchaeota. The 'Lokiarchaeota' phyla, and was proposed to represent the nearest archaeal relative to eukaryotes (Spang et al., 2015, 2018). Further metagenomic analysis of deep-sea sediments shed more light on the existence Asgard archaeal members that were monophyletic with already discovered Lokiarchaeota (Seitz et al., 2016; Spang et al., 2015, 2018; Zaremba-Niedzwiedzka et al., 2017). Additional studies have identified other members of the Asgard superphylum: Hermodarchaeota, Helarchaeota, Sifarchaeota, Gerdarchaeota, Tyrarchaeota, Sigynarchaeota, Freyrarchaeota, Hoderarchaeota, Balderarchaeota, Njordarchaeota, Wukongarchaeota and Hodarchaeales (Cai et al., 2020; Eme et al., 2023; Farag et al., 2021; Liu et al., 2021; Seitz et al., 2019; Xie et al., 2022). Prominent eukaryotic signature proteins such as components of oligosaccharyl transferase complex (OST complex)/ N-glycosylation complex), ESCRT complex proteins, membrane trafficking proteins, several components of the ubiquitin system, cytoskeletal components and informational proteins have been identified in the genome of Asgard superphylum (Cai et al., 2020; Imachi et al., 2020; Neveu et al., 2020; Seitz et al., 2019; Zaremba-Niedzwiedzka et al., 2017). The discovery of these genomic eukaryotic features in Asgard superphylum positioned them as the closest relatives of eukaryotes (Heinz & Domman, 2017; Stairs & Ettema, 2020).

2.2.1 Asgard superphylum: A novel Archaeal link to eukaryotes

Controversies surround the origin of eukaryotic cells. Phylogenetic studies have provided supportive evidence that eukaryotes evolved as a relative to or branching from within the archaeal 'TACK' superphylum (Cox et al., 2008; Foster et al., 2009; Guy et al., 2014; Lasek-Nesselquist & Gogarten, 2013; Spang et al., 2015; Williams et al., 2012). The 'TACK' is an acronym representing archaeal clade superphylum of the phyla Thaumarchaeota, Aigarchaeota, Crenarchaeota and Korarchaeota (Guy & Ettema, 2011). Contrary to the proposed three-domain tree of life (Fox et al., 1977; Woese & Fox, 1977), the Eocyte hypothesis (two-domain theory) which describes eukaryotes to emerge from within Archaeal domain was postulated afterwards (Koonin, 2010; Lake, 1988; Lake et al., 1984). Although the idea of two-domain

theory has been challenged (Da Cunha et al., 2017, 2018), phylogenomic evidence has substantially supported the branching of eukaryotes from within Archaeal domain (Neveu et al., 2020; Spang et al., 2015, 2018; Williams et al., 2012). These evidences have apparently overthrown the three-domain tree of life (Heinz & Domman, 2017; Stairs & Ettema, 2020; Williams et al., 2012), with the new tree of life having Bacteria and Archaea as the two main distinct primordial life forms (Heinz & Domman, 2017; Williams et al., 2012). A number of eukaryotic signature proteins (Hartman & Fedorov, 2002) have been identified in some archaeal organisms belonging to the 'TACK' lineages, providing substantial evidence to the archaeal origin of eukaryotes (Ettema et al., 2011; Lindås et al., 2008; Yutin & Koonin, 2012). Deep sea metagenomic studies revealed novel discovery of the genome of Deep-Sea Archaeal Groups (DSAG) 'Lokiarchaeum', and two low abundant DSAG-related lineages; designated as Loki2 (with high GC content) and Loki3 (with low GC content) lineages. Lokiarchaeota and Lokiarchaeum were named after the sampling location, Loki's Castle (Jorgensen et al., 2012; Pedersen et al., 2010). Loki's Castle is deep vent field at Arctic Mid-Ocean Ridge (AMOR). The Loki's Castle was named after a God from Norse Mythology, identified with unresolved scholarly disagreements (Von Schnurbein, 2000)

Phylogenetic analysis affiliated these archaeal groups to a special class of 'TACK' superphylum. Further phylogenetic analysis identified the Lokiarchaeum and, Loki2 and Loki3 lineages to be monophyletic and were later affiliated to 'Lokiarchaeota' (Spang et al., 2015). According to Spang et al. (2015), Loki3 was identified to represent the deepest branch of Lokiarchaeota whilst Lokiarchaeum and Loki were deduced to be sister lineages (Fig. 2.2.1). Interestingly, inclusion of eukaryotes in the phylogenetic analysis positioned them within the Lokiarchaeota, supporting a strong evolutionary association between Lokiarchaeota and eukaryotes (Spang et al. 2015).

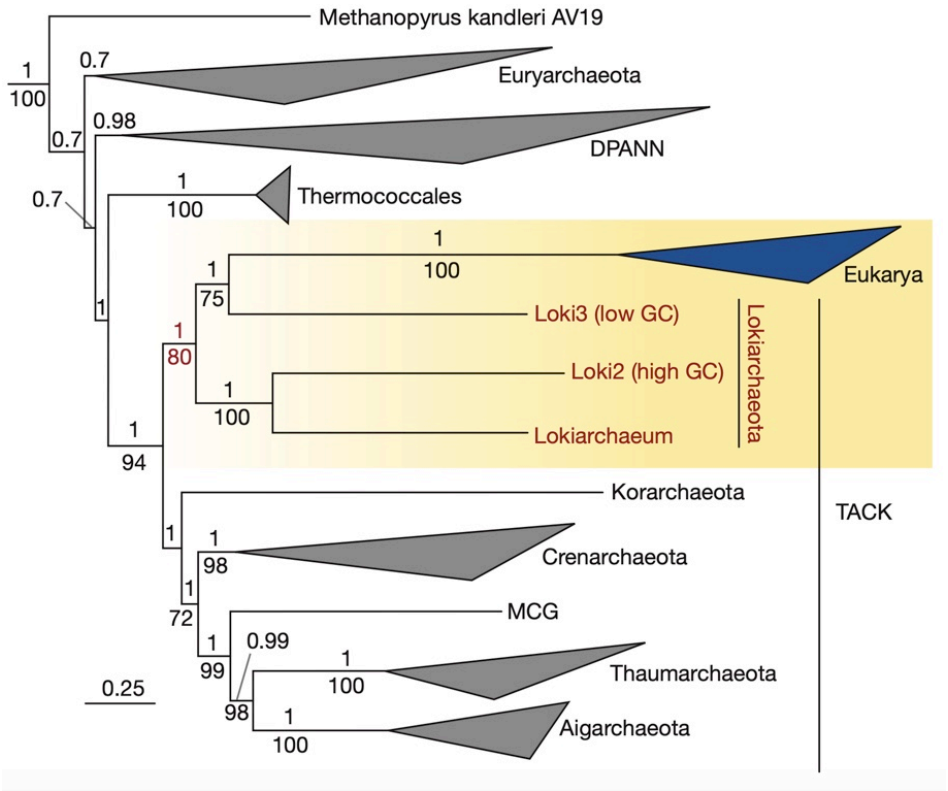


Fig. 2.2.1: Phylogenomic placement of eukaryotes in Lokiarchaeota (Spang et al. 2015).

Subsequent metagenomic studies identified several archaeal contigs in the White Oak River (WOR) estuary located at North Carolina. Genome reconstruction of these contigs sampled from sulfate–methane transition zone (SMTZ) unveiled another distinct but closely related archaea to previously discovered Lokiarchaeota (Fig. 2.5). This group of archaea was named as Thorarchaeota (Seitz et al. 2016) as a sister clade to Lokiarchaeota.

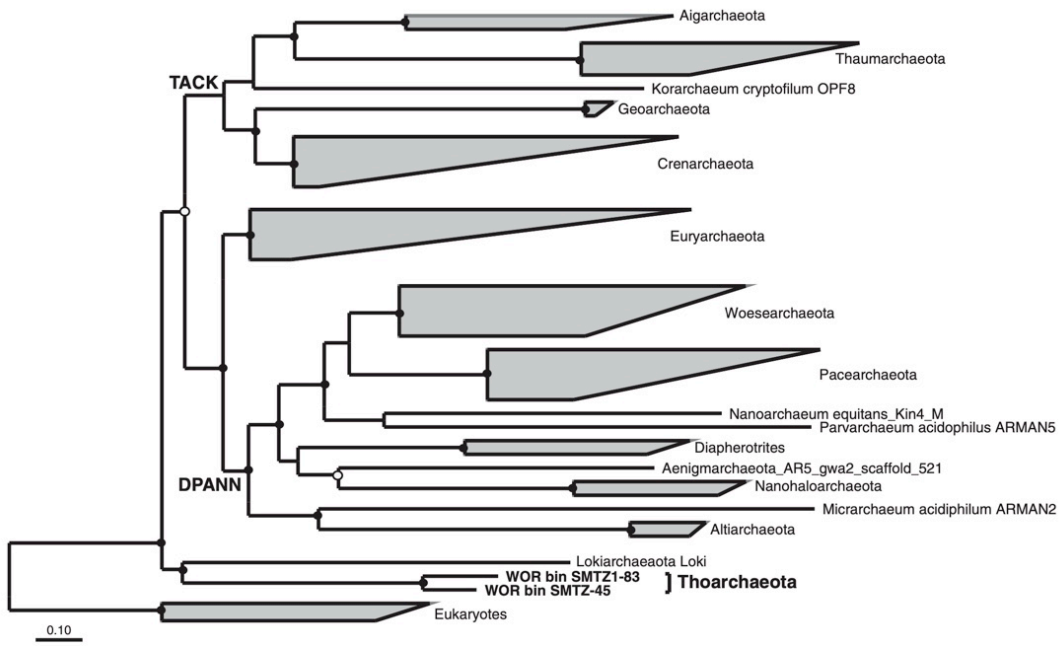


Fig. 2.2.2: Affiliation of White Oak River (WOR) bin reconstructed genomes (WOR bin SMTZ1-83 and SMTZ-45) to Thorarchaeota (Seitz et al. 2016).

In further metagenomic studies, aquatic sediments sampled from seven geographically distinct sites (Loki's Castle and White Oak River inclusive) revealed sequences belonging to two additional candidate phyla that were related to already described Lokiarchaeota and Thorarchaeota in the same archaeal clade. These additional Lokiarchaeota- and Thorarchaeota-related lineages were named Odinarchaeota and Heimdallarchaeota. This archaeal clade was named 'Asgard' superphylum (Zaremba-Niedzwiedzka et al., 2017) after realm of the gods in Norse mythology (Von Schnurbein, 2000). The Asgard superphylum form monophyletic group with eukaryotes. Some prominent eukaryotic signature proteins were identified in these genomes of Asgard superphylum include homologues of N-glycosylation pathway components, eukaryotic ESCRT (I, II and III), a wide set GTPases, actin homologues, gelsolins, ubiquitin modifier system and components of eukaryotic protein translocation (Fig. 2.6) (Zaremba-Niedzwiedzka et al., 2017)

Metagenomic-assembled genome data obtained from deep-sea sediments in the hydrocarbon-rich Guaymas Basin identified another Asgard phylum, Helarchaeota (Figure 12). This archaeal lineage was similarly found to contain ESPs as described in other candidate Asgard phyla (Seitz et al. 2019).

Expanded metagenomic analysis of sampled DNA from 11 locations around the world strongly positioned eukaryotes as a well-nested clade within the Asgard archaea. Notably, a new group of Asgard archaea called Hodarchaeales (proposed order falling within Heimdallarchaeia), was

identified to as the closest prokaryotic relatives of eukaryotes. Hodarchaeales were found to reveal key features characteristic of the last Asgard
archaea and eukaryotes common ancestor (LAECA), thus shedding more light on the identity and nature of the last common ancestor of Asgard archaea and eukaryotes (Eme et al. 2023).

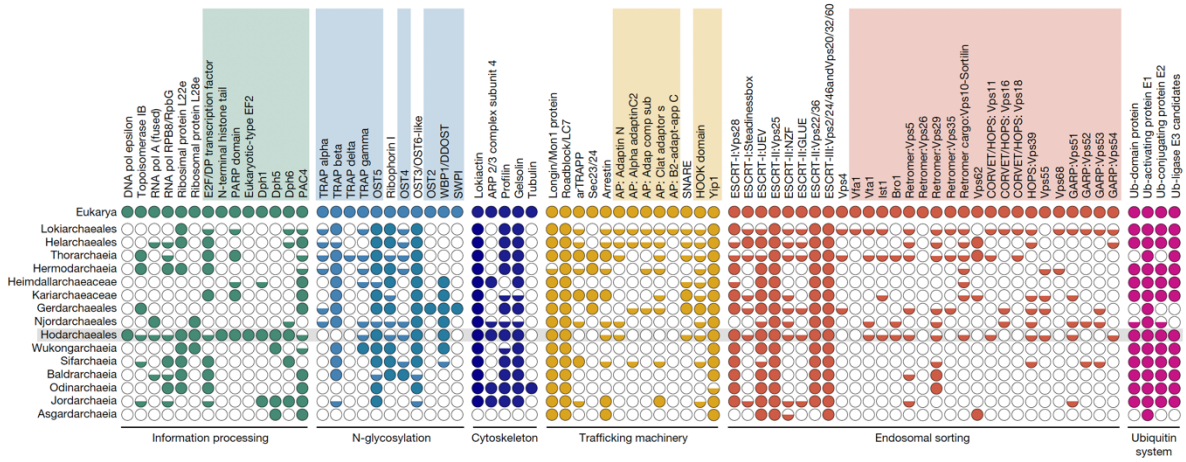


Fig. 2.2.3: Distribution of Asgard homologs of eukaryotic proteins (Eme et al. 2023).

2.2.2 The controversy of Asgard affiliation with eukaryotes

Earlier conclusions arising from these environmental sampling, phylogenomic assessment and eukaryotic link to Archaea (Spang et al. 2015) were initially challenged (Da Cunha et al. 2017). Although strong affiliation of eukaryotes to Asgard superphylum were postulated from metagenomic-reconstructed genomes of deep-sea archaea, no candidate Asgard organism had been successfully isolated and cultured back then (Cai et al., 2020; Neveu et al., 2020; Seitz et al., 2016, 2019; Spang et al., 2015, 2018; Zaremba-Niedzwiedzka et al., 2017). The possibility of contamination from eukaryotic sources, incomplete genome reconstructions, bioinformatic errors and homologous recombination of identified sequences with some eukaryotic DNA sequences were some of the contrary speculations that were proposed against Asgard archaeal affiliation with eukaryotes (Da Cunha et al., 2017, 2018; Spang et al., 2018). Additionally, because no representative Archaea from the Asgard superphylum has been cultivated, it raised doubtful considerations about their physiology, cell biology and ultimate acceptance of the two-domain tree of life. However, accumulation of experimental evidence from different environmental sources backed by different methodologies posited the proposal that eukaryotes emerged from ‘Asgard’ superphylum (Cai et al., 2020; Liu et al., 2018; Neveu et al., 2020; Seitz et al., 2016, 2019; Zaremba-Niedzwiedzka et al., 2017).

2.2.3 Eukaryotic insights and imaging of Asgard archaeal isolates

The decade long isolation, culturing and genomic characterization of Lokiarchaeota strain (*MK-D1*) led to the proposal of a new model for eukaryogenesis, termed as the entangle–engulf–endogenize (E^3) model (Imachi et al. 2020). Imaging of MK-D1 reveals no eukaryotic organelle-like structure *(Fig 2.3)*. However, eighty eukaryotic signature proteins were identified in *MK-D1* sequences. Among these ESPs include a hypothetical eukaryotic-like oligosaccharyltransferase STT3 subunit (Imachi et al. 2020). This study represented the first isolation and culturing of the *MK-D1* strain which features the nearest archaeal relative to eukaryotes with unique metabolism and physiology (Imachi et al. 2020). In another study, the scanning electron microscopy of the Asgard strain, Loki-b35, reveals no internal membrane but possess Lokiactin, one of the prominent and highly conserved eukaryotic signature proteins (Rodrigues-Oliveira et al., 2023).

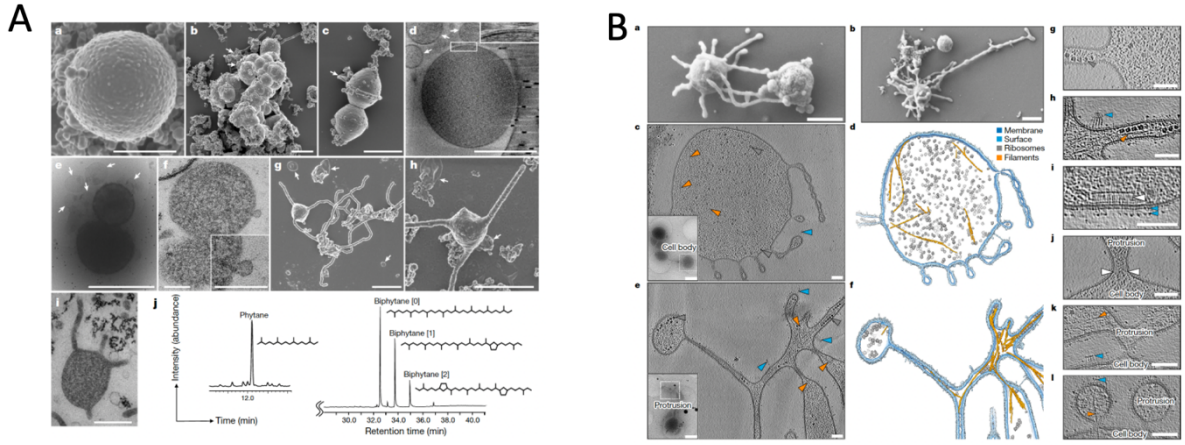


Fig. 2.3: (A) Microscopy characterization and lipid composition of MK-D1 (Imachi et al. 2020) and (B) CryoET images of enrichment cultures of Loki-B35 (Rodrigues-Oliveira et al. 2023)

2.3 Theories of eukaryogenesis

The origin of the eukaryotic cell is regarded as a major evolutionary innovation driving the emergence of complexities and diversities of life forms (Szathmáry & Smith, 1995; West et al., 2015). However, the origin and evolutionary developments governing eukaryotic cellular complexities is an unresolved question that is obscured by controversies (Zachar & Szathmáry, 2010, 2017). Two hypothesis, autogenous and endosymbiotic theories, have been widely proposed to explain the evolution of eukaryotes (Baum, 2015; W. F. Martin et al., 2015). Autogenous theories posit that eukaryotes emerged by the incremental modification of cellular

structures of a single ancestral protoeukaryotic lineage (Baum, 2015; Cavalier-Smith, 1975) whereas according to endosymbiotic theories, eukaryotes emerged as a cellular fusion between a host and an endosymbiont (Archibald, 2015; W. Martin et al., 2001; W. F. Martin et al., 2015). Earlier autogenous models postulate the development of eukaryotic organization from single cyanophyte-like ancestor, uralga (Taylor, 1976). Although endosymbiotic theories are widely accepted, a number of competing endosymbiotic models have been proposed to identify the nature of the engulfing host and the endosymbiont (Martin et al. 2015). Earlier endosymbiotic models proposed fusion between two ancestral partners; mycoplasma endosymbiont and an amoeboid host cell (Kowallik & Martin, 2021), an archaeal endosymbiont and a bacterial host (Lake & Rivera, 1994; Poole & Neumann, 2011) alphaproteobacterial endosymbiont and an archaeal host (Moreira & López-García, 1998). Advances in phylogenomic and metagenomic studies support eukaryogenesis as an endosymbiotic merging of alphaproteobacterial endosymbiont and a host archaeon (Eme et al., 2017; Imachi et al., 2020; Koonin, 2015; Lane & Martin, 2010; López-García & Moreira, 2015). Consistent with endosymbiotic models, eukaryotes possess two sets of ribosomes; archaeal ribosomes in the cytosol and bacterial ribosomes in the mitochondrion (Maier et al., 2013).

Overwhelming evidence exists for the alphaproteobacterial origin of the eukaryotic mitochondria (Chang et al., 2010; Gray et al., 1999, 2001; Kurland & Andersson, 2000; B. F. Lang et al., 1999). Genomic analysis further assigned the closest relatives of the mitochondria to alphaproteobacterium belonging the Rikettsiales order (Emelyanov, 2003; Fitzpatrick et al., 2006) or specifically *Rickettsia prowazekii* (Andersson, 1998; Andersson et al., 1998). The presence of an internalized mitochondrial endosymbiont bioenergetically favored the development of eukaryote-specific traits and innovations (Lane, 2014; Lane & Martin, 2010; Raval et al., 2022).

Identification of eukaryotic features in the genomes of archaeal organisms belonging to the Asgard clade positions them as the closest relatives of eukaryotes (Eme et al., 2023; Zaremba-Niedzwiedzka et al., 2017).The unique morphology and metabolic features of cultured representatives of Asgard superphylum highlights them as the potential endosymbiotic partner of aphaproteobacteria during eukaryogenesis (Imachi et al. 2020; Rodrigues-Oliveira et al. 2023).

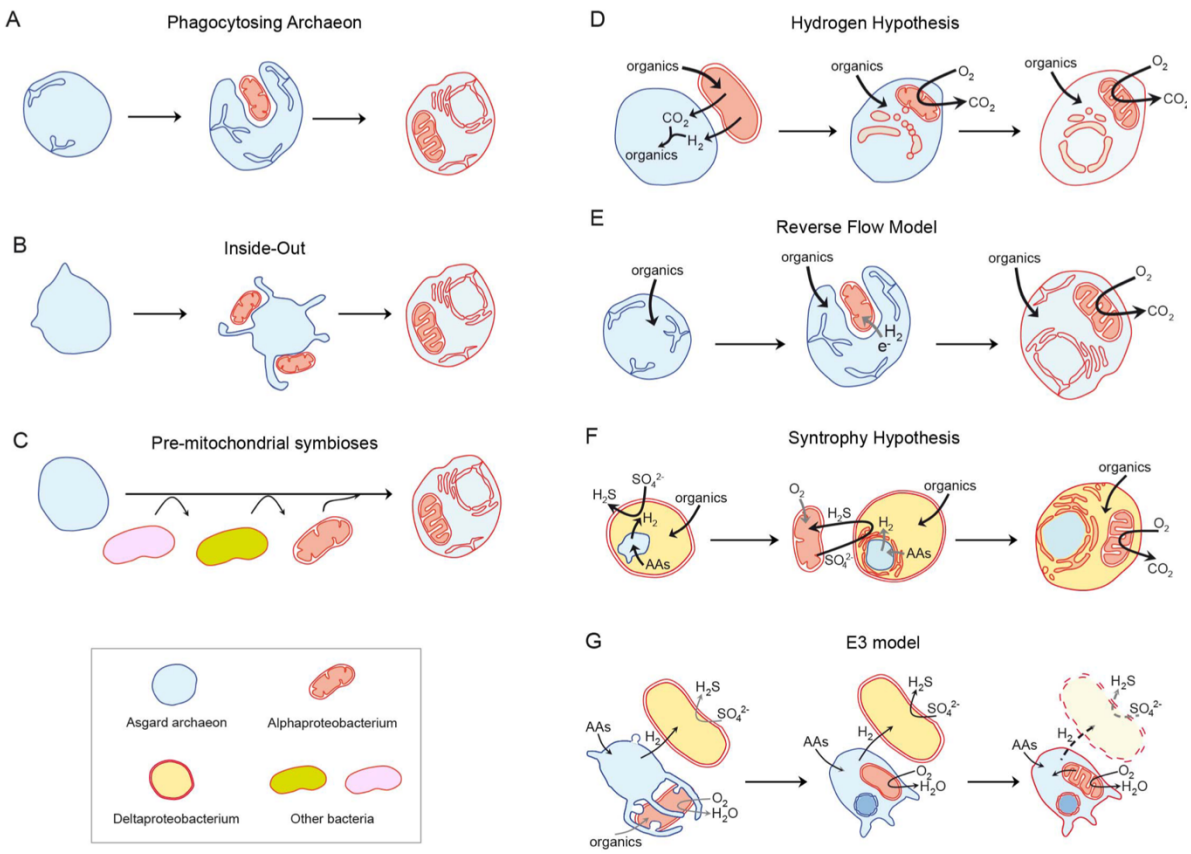


Fig. 2.3 Current models of eukaryogenesis based on the symbiotic merging of archaeal and bacterial partners. (A–C) Depict a selection of scenarios lacking a specified basis for the symbiosis. (D–E) Correspond to more detailed models postulating specific syntrophic interactions (López-García & Moreira, 2023).

2.3.1 Evolution of the eukaryotic endomembrane system

An elaborate endomembrane system and other intracellular complexities are prominent eukaryote-specific traits that completely distinguishes eukaryotes from prokaryotes. However, the evolutionary process governing the emergence of endomembrane system in eukaryotes remains unclear. The endoplasmic reticulum is regarded as the precursor of the endomembrane system (Gould et al., 2016). The outside-in (invagination) and inside-out (expansion) models are two widely known models that have been postulated to account for the emergence of a primordial ER, giving rise to the components of the eukaryotic endomembrane system. Whilst the outside-in hypothesis postulates that specialized invagination of the plasma membrane gave rise to differentiated internal compartments (Blobel, 1980; Cavalier-Smith, 2002; Jékely, 2003), the inside-out model proposes that extracellular protrusions on plasma membrane fused to give rise to a cytoplasmic network and endomembrane system (Baum & Baum, 2014). According to the outside-in model, the evolution of the internal membrane system is associated with origin

of food uptake, either by phagocytosis or endocytosis (Cavalier-Smith, 1987). However, further deliberations led to the proposal that elaboration of the membranous secretory system was an early event in the evolution of the endomembrane system (Jékely, 2003, 2007) . The emergence of the endomembrane system is associated with an initiated engulfment process with failed digestion. Inheritance of residual internal structures led to the evolution of a proto-ER (Ozansoy & Denizhan, 2009). This model was consistent with previous hypothesis that invagination of the plasma membrane allowed the formation of internalized extracellular pockets with high surface area for trapping captured food and secreted enzymes (De Duve, 1969; De Duve & Wattiaux, 1966). The outside-in models remained conventional for decades (Blobel, 1980; Cavalier-Smith, 1987; W. Martin, 2005; Poole & Neumann, 2011; Wilson & Dawson, 2011) until the inside-out hypothesis was proposed (Baum and Baum, 2014; Baum 2015).

On the basis of the widely accepted endosymbiotic theory (López-García and Moreira, 2023), the inside-out hypothesis proposes that extracellular protrusions/blebs of an ancestral prokaryote expanded and fused around an ectosymbiotic mitochondrial ancestor. The bleb-fusion event gave rise to the cytoplasm with the spaces between blebs forming the endoplasmic reticulum, a precursor of the eukaryotic secretory system. Additional bleb-fusion events led to the formation of a continuous plasma membrane around the symbiont (Baum and Baum, 2014). Observation of protrusions on the surface of two cultured Asgard archaea, '*Candidatus* Prometheoarchaeum syntrophicum (MK-D1) and *Candidatus* Lokiarchaeum ossiferum strain (Loki-B35) supports protrusion-mediated mitochondrial acquisition as the path to eukaryogenesis (Imachi et al. 2020; Baum and Baum, 2020; Rodrigues-Oliveira et al. 2023).

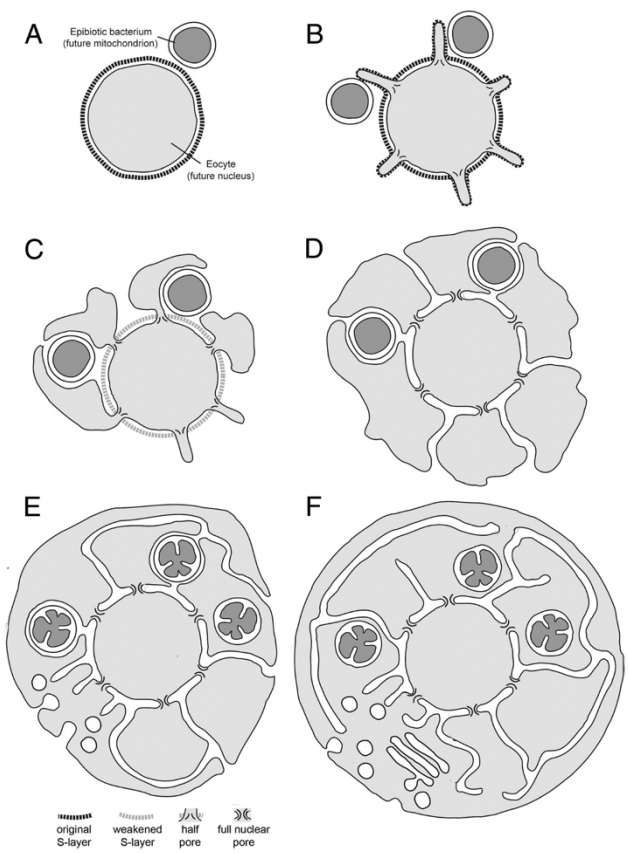


Fig. 2.3.1: Inside-out model for the evolution of eukaryotic cell organization (Baum and Baum, 2014). Membrane-derived extracellular protrusions expanded and fused around the mitochondrial ancestor. Protrusion-fusion events led to the formation of the cytoplasm with the spaces in between the cytoplasm giving rise to the ancient endoplasmic reticulum, which later evolved into the eukaryotic secretory and endomembrane system (Baum and Baum, 2014).

A more appealing model for the evolution of the ER-derived endomembrane system is hinged on the observation that bacteria and archaea secrete outer membrane vesicles (OMVs) into the environment (Grimm et al., 1998; E. Lee et al., 2009; Schwechheimer & Kuehn, 2015). This model postulates that after endosymbiosis between a host archaeon and a proto-mitochondrial endosymbiont, the endosymbiont continued to secrete OMVs into the cytosol of the host. The archaeal ribosome-translocon-OST machinery alternatively integrated into the flux of OMVs secreted by the endosymbiont. Incorporation of the host ribosome-translocon-OST into this vesicular compartment led to the formation of an ancient ER as the precursor of the endomembrane system. Additionally, the outward-directed vesicular flux from endosymbiont to the plasma membrane also led to the transformation of the host archaeal membrane lipids into bacterial lipids (Gould et al. 2016).

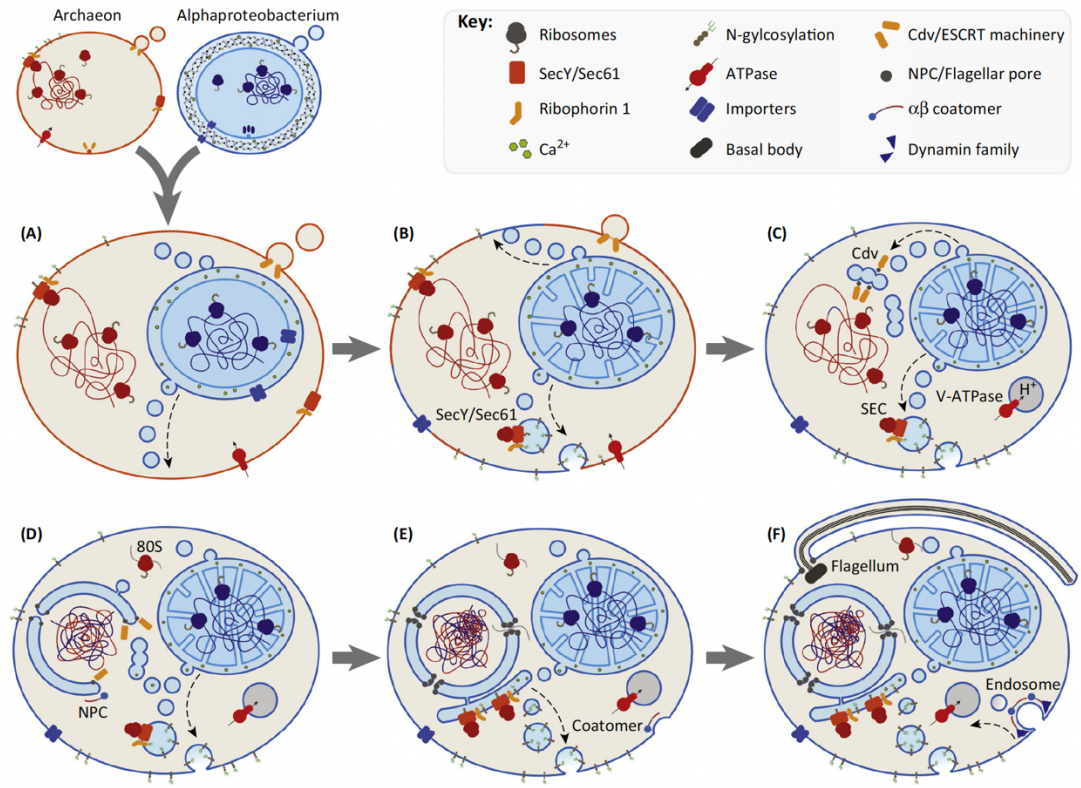


Fig. 2.3.2: A Model for the Evolutionary Origin of the Eukaryotic Endomembrane System after endosymbiosis between a host archaeon and alphaproteobacterial endosymbiont, the endosymbiont continued to release OMVs into the cytosol of the host. The flux of OMVs became an alternative target for the core translocon (ribosome-OST complex) of the host, giving rise to an ancient ER. Continuous flux OMV to the plasma membrane led to the transformation of the host plasma membrane components from from ether-linked isoprenes to ester-linked fatty acids (Gould et al. 2016).

2.3.2 The Endoplasmic reticulum

In eukaryotes, the ER primarily provides an isolated environment for protein folding, modification and maturation. It serves as the main entry point into the secretory pathway(Johnson & van Waes, 1999), and insertion of ER-associated membrane proteins into the membrane (Borgese & Fasana, 2011; Nyathi et al., 2013; Shao & Hegde, 2011). The ER coordinates the activities of the endomembrane system (Almeida & Amaral, 2020) and responsible for the processing of one-third of the human proteome(Chen et al., 2005). In eukaryotes, several combinations of ER membrane-bound translocon complexes have been identified, with the Sec61-OST-TRAP translocon being the most abundant (Gemmer, Chaillet, van Loenhout, et al., 2023)

2.4 Eukaryotic and prokaryotic Signal Recognition Particle (SRP)

Protein targeting to the protein translocation apparatus/membrane insertases of prokaryotic plasma membrane/ eukaryotic ER is mediated by the universally conserved signal recognition particle (SRP) (Keenan et al., 2001; Lutcke, 1995). The mammalian SRP is composed of one RNA molecule, 7SL RNA, and six SRP proteins: SRP9, 14, 19, 54, 68 and 72 (Massenet, 2019). The archaeal SRP is composed of SRP RNA, SRP19 and SRP54 (Calo & Eichler, 2011; Zwieb & Bhuiyan, 2010) whereas the bacterial SRP is composed of 4.5 SRP RNA and SRP 54 (Ffh) (Batey et al., 2000; Poritz et al., 1990). Across all domains of life, the SRP54 is involved in binding to and targeting the signal peptide to the prokaryotic plasma membrane/eukaryotic ER membrane (Miller et al., 1993; Zopf et al., 1990).

Although the archaeal SRP represents an intermediate between mammalian and bacterial versions (Rosendal et al., 2003), it shares the similarities of mammalian SRP54, SRP19, and 7S RNA (Egea et al., 2005; Eichler & Moll, 2001; Gupta et al., 2017; Zwieb & Bhuiyan, 2010). Although no homologs of other eukaryotic SRP proteins, SRP9/14 and SRP68/72, have been identified in archaeal SRP system, the archaeal 7SL RNA contains potential binding sites for these proteins (Eichler and Moll, 2001). This suggests that eukaryotic SRP system was inherited from an archaeal ancestor (Baum & Baum, 2014; Calo & Eichler, 2011; Luirink, 2004). It also reveals the possibility for human SRP proteins to interact with archaeal SRP RNA or that the appearance of these proteins occurred after divergence of eukaryotes from within archaeal clade (Bhuiyan, 2000). This implies that the functional homologs of the SRP9/14 and SRP68/72 not identifiable by current genome analysis might be present in other archaeal systems (Calo & Eichler, 2011).

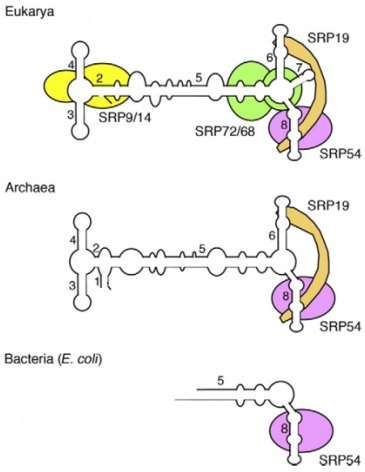


Fig. 2.4: Eukaryotic, archaeal and bacterial SRP (Calo & Eichler, 2011)

2.4.1 Signal recognition particle receptor (SR receptor)

In eukaryotes the signal recognition particle receptor (SR receptor or SR) exists as a membrane-bound heterodimer SRα - SRβ (Tajima et al., 1986). The SRβ is an integral membrane protein which provides a binding site for the SRα, thereby anchoring the peripheral SRα to the ER membrane (Miller et al., 1995). The prokaryotic SR exists as monomeric FtsY, the prokaryotic homolog of SRα that is peripherally anchored to the plasma membrane (Angelini et al., 2006). The eukaryotic SRβ subunit controls transfer of the RNC (Fulga, 2001) whereas the prokaryotic FtsY (prokaryotic homologue of SRα) functions as both the SRα and SRβ to mediate RNC transfer (P. Kuhn et al., 2011).

The SRβ belongs to Ras-like GTPases associated with the development of secretory endomembrane system (Jékely, 2003). Within the Ras-like GTPases family, only SRβ is ER membrane resident using its N-terminal transmembrane helix (Ogg et al., 1998). It has been demonstrated that the GTP binding domain of eukaryotic SRβ can sufficiently dimerize with SRα and recruit SRP-RNCs to the translocon (Legate, 2000; Legate & Andrews, 2003; Ogg et al., 1998). Sequence search reveals that homologs of this SRβ-GTP binding domain occurs in members of the Asgard superphylum (Tran et al., 2024), supporting the idea that early diversification events in ancient Ras GTPases led to the appearance of eukaryotic SRβ.

2.4.2 The SecYEG/ Sec61 translocon

The universally conserved SecYEG (in prokaryotes) or Sec61 complex (in eukaryotes) is a protein conducting channel (PCC) involved in the translocation of secretory proteins across and integration of transmembrane proteins into the prokaryotic cytoplasmic membrane and eukaryotic endoplasmic reticulum (Berg et al., 2004; Park & Rapoport, 2012). The central and essential component of the PCC is Sec61α (in mammals) or Sec61p (in *Saccharomyces cerevisiae*), or SecY (in prokaryotes) (Akimaru et al., 1991; Brundage et al., 1990; Gorlich, 1993), and is composed of ten transmembrane domains surrounding a central pore (Voorhees et al., 2014; Voorhees & Hegde, 2016). Transmembrane domains, TMD 1-5 and TMD 6-10, of SecY form two linked halves which dimerizes to form the hydrophilic pore at their dimerization interface (Lewis & Hegde, 2021). This hydrophilic pore forms the channel for translocating hydrophilic polypeptides across the membrane (Lewis & Hegde, 2021). The hydrophilic pore or channel is plugged by a short helix (Berg et al., 2004; Lewis & Hegde, 2021) which must be displaced to allow protein translocation through the channel (Berg et al., 2004; Voorhees & Hegde, 2016) . It has been proposed that SecY originated as a YidC homolog which dimerized

to form a channel by juxtaposition of their individual hydrophilic grooves (Lewis & Hegde, 2021). The Sec61/SecY also possesses a lateral gate which opens sideways to release transmembrane domains into the lipid bilayer (Voorhees et al., 2014; Voorhees & Hegde, 2016). Opening of the Sec lateral gate also destabilizes the plug helix to allow translocating polypeptides to pass through the central pore (Gogala et al., 2014; Park et al., 2014; Voorhees & Hegde, 2016). The central component of the channel, Sec61α/Sec61p/SecY, associates with Sec61β and γ (in mammals and archaea), or Sbh1p and Sss1p (in *S. cerevisiae*), or Sec G and E (in bacteria) to form the Sec complex (Berg et al., 2004; Park et al., 2014). The Sec61γ/SecE clamps and keeps together the two linked halves of the Sec61α subunit whereas the function of the Sec61β/SecG is not clearly known (Berg et al., 2004). The structure of SecYEG is shown in Fig. 2.4 below.

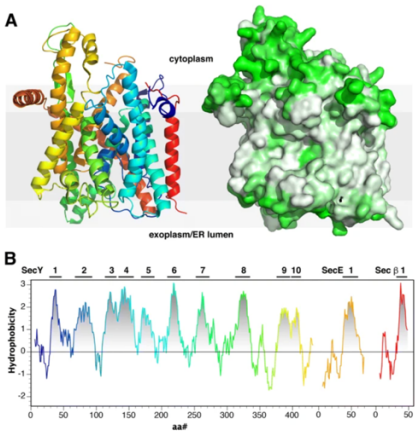


Fig. 2.4.2: (A) Ribbon structure of the archaeal SecYEβ translocon from *Methanococcus janaschii* (1RHZ) (left) and a surface representation showing hydrophilic residues in green (right). (B) Hydrophobicity plot showing the 10 TMDS of SecY, SecE and Sec61β (Spiess et al., 2019).

2.4.3 Inhibitors of SecYEG/Sec61-mediated protein translocation

A number of natural and synthetic compounds inhibit Sec61-mediated translocation (Itskanov et al., 2023; K. Kalies & Römisch, 2015; Luesch & Paavilainen, 2020; Pauwels et al., 2021; Van Puyenbroeck & Vermeire, 2018). Most of the Sec61 inhibitors such as eeyarestatin I (ESI), coibamide A, mycolactone, cotransin, decatransin, apratoxin F, ipomoeassin F, cyclotriazadisulfonamide (CADA) tested so far bind to the lipid-exposed pocket formed by the partially open lateral gate and stabilize the plug domain of Sec61 (Itskanov et al. 2023). In effect, Sec61 translocon stays in in a closed state, thereby preventing the protein-translocation channel from opening (Itskanov et al. 2023). Cotransin and CADA have been shown to inhibit

translocation in a client-specific manner (Garrison et al., 2005; Pauwels et al., 2021; Rehan et al., 2023), whereas other inhibitors block Sec61-mediated translocation in a client non-selective manner (Kalies and Römisch, 2015; Luesch and Paavilainen, 2020; Itskanov et al. 2023).

2.4.4 Mechanism of protein targeting to the prokaryotic plasma membrane or eukaryotic endoplasmic reticulum

Targeting of proteins to the prokaryotic plasma membrane or eukaryotic endoplasmic reticulum is mediated by N-terminal signal peptides (SPs) and/or transmembrane domains (TMDs or signal anchors) of proteins (Hatsuzawa et al., 1997; S. Lang et al., 2022; Wu & Hegde, 2023; Zheng & Nicchitta, 1999). As translation begins in the cytosol, emerging polypeptides harboring N-terminal SP or TMD are recognized by signal recognition particle (SRP) to form SRP-ribosome-nascent chain complex (SRP-RNC) (Egea et al., 2005; Halic et al., 2006; Jomaa et al., 2022; Luirink, 2004; Nunnari & Walter, 1992; Nyathi et al., 2013; K. Wild et al., 2004). The formation of the SRP-RNC in the cytosol either temporarily arrests or slows down translation (Lakkaraju et al., 2008; Walter & Blobel, 1980, 1981, 1982). The SRP-RNC is targeted to the prokaryotic plasma membrane/ER where the SRP interacts with its cognate membrane bound signal recognition particle receptor (SRP receptor or SR) (Bacher et al., 1996; Gilmore et al., 1982; Keenan et al., 2001) and the ribosome docks at the SecY/Sec61 translocon (Gorlich, 1993; K. U. Kalies et al., 1994). The interaction between the SRP and SR triggers the transfer of the nascent chain to the Sec61/SecY translocon complex (Fulga, 2001; Jomaa et al., 2017; Kobayashi et al., 2018; Park & Rapoport, 2012) whereas ribosome binding to Sec61 initiates conformational changes that partially destabilizes the lateral gate of the translocon, priming it for engagement with the signal peptide (Berg et al., 2004; Voorhees et al., 2014; Voorhees & Hegde, 2016). Afterwards, the SRP dissociates from the ER-bound ribosome-nascent-chain complex in a GTP hydrolysis-dependent manner (J. H. Lee et al., 2021; Shan et al., 2004, 2009). Translation resumes at this point (Gilmore et al., 1982; Walter & Blobel, 1981). The signal peptide explores exposed hydrophobic groove of Sec61 and engages the lateral gate in a manner which destabilizes the Sec61 central helix plug domain (Gogala et al., 2014; Voorhees et al., 2014; Voorhees & Hegde, 2016). Displacement of the helix plug is concomitant with the widening of the lateral gate towards the membrane and axial opening of the channel across the membrane (Egea & Stroud, 2010; Gogala et al., 2014; Li et al., 2016; Pfeffer et al., 2015; Voorhees & Hegde, 2016). The signal peptide undergoes a flip-turn to reorient its N-terminus to the cytosol (Goder, 2003; Lumangtad & Bell, 2020). However, if the

first hydrophobic element is a TMD, this sequence intercalates at the lateral gate of the translocon, before moving into the lipid bilayer (Heinrich et al., 2000). Once the first TMD gets embedded into the membrane, successive TMDs are serially inserted into the ER membrane by complex mechanisms involving the Sec61 and other translocation factors (Rapoport et al., 2004; Smalinskaitė et al., 2022; Sundaram et al., 2022). As translation resumes, the growing chain is pushed deeper into the translocon leading to the ultimate insertion and translocation of the elongating chain through the central pore into the ER lumen (Gogala et al., 2014; Hessa et al., 2005; Park & Rapoport, 2012; Voorhees et al., 2014; Voorhees & Hegde, 2016). The initial insertion of the elongating chain can be spontaneous or may require substrate specific auxiliary components (Migliaccio et al., 1992; Wiedmann et al., 1987). Finally, the signal peptides are proteolytically cleaved at the luminal side of the ER by membrane-bound signal peptidase complex (SPC) (Liaci et al., 2021) as shown in Fig 2.5.

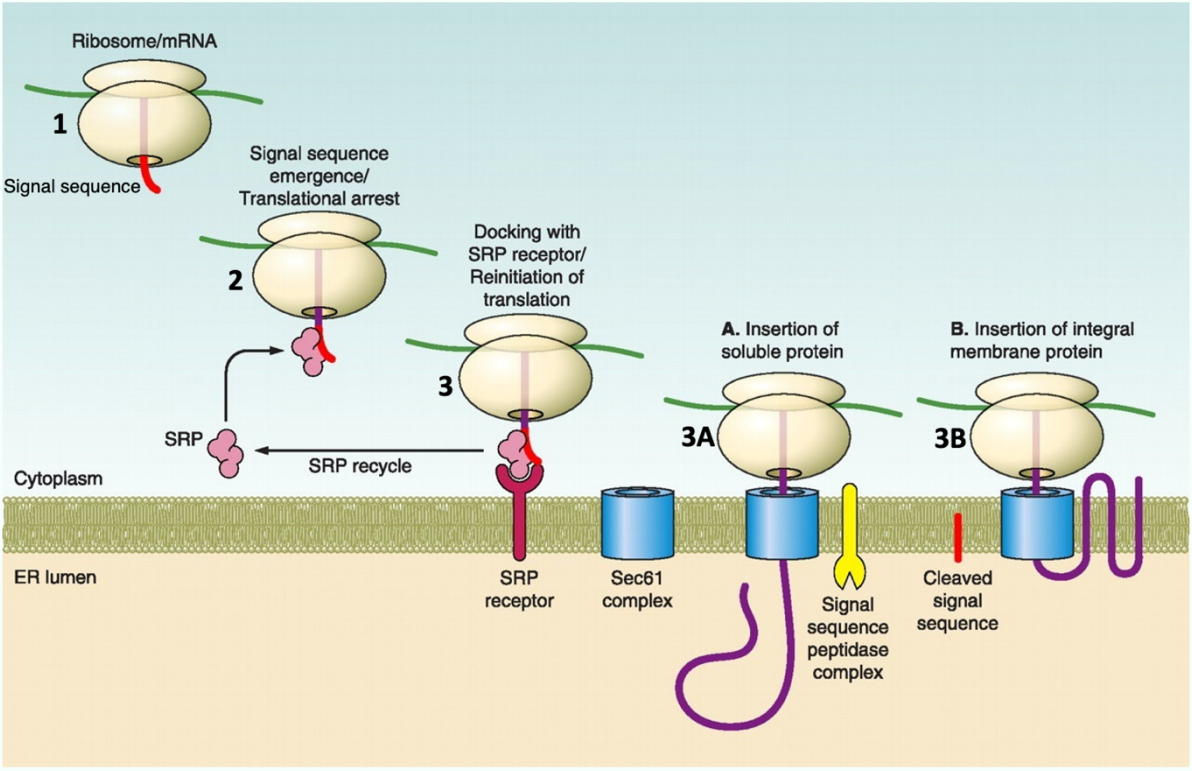


Fig. 2.4.4: Mechanism of ER protein targeting with signal recognition particle (Guerriero & Brodsky, 2012)

2.4.5 Mechanism of signal recognition particle (SRP)-mediated protein targeting

At the initiation of translation, N-terminal signal peptides of nascent polypeptides emerging from the ribosome are recognized by the signal recognition particle to form the ribosome nascent chain – signal recognition particle RNC-SRP complex (Nyathi et al., 2013). The RNC-

SRP complex is then targeted to the ER membrane where it interacts with ER resident signal recognition (SR) receptor in a GTP-dependent manner (Keenan et al., 2001). Prior to the formation of the complex between SRP and SR at the ER membrane, the GTP binding sites of SRP54 (of RNC-SRP complex) and SRα (of SR) are unoccupied (Rapiejko & Gilmore, 1994, 1997) Cooperative binding of GTP to SRP54 (of RNC-SRP complex) and SRα (of SR) is essential for high-affinity interaction between SRP and SR in the ER membrane (Rapiejko & Gilmore, 1994; Song et al., 2000). This interaction induces conformational changes in the RNC-SRP-SR complex leading to the dissociation and transfer of signal peptide to the Sec61 translocon (Bacher et al., 1996). Finally, the bound GTPs are hydrolyzed, after which the SRP recycles back to the cytoplasm to initiate a new round of targeting (Rapiejko & Gilmore, 1994; Song et al., 2000).

2.4.6 Signal peptides

Signal peptides are short peptide sequences (about 20-30 amino acid residues) located at the N-terminal of proteins and responsible for prokaryotic plasma membrane or eukaryotic ER targeting (Owji et al., 2018; Von Heijne, 1985, 1990). Signal peptides lack significant sequence homology but are generally characterized by a tripartite (3-domain) structure composed of 1-5 positively charged residues at the amino terminal (N-region), a stretch of hydrophobic 7-15 residues at the middle region (H-region) and a slightly polar 3-7 residues at carboxy region (C-region) having a signal peptide cleavage site (Garnier et al., 1980; Hegde & Bernstein, 2006; Liaci & Förster, 2021; Von Heijne, 1985, 1990). Although signal peptides are exceptionally diverse in their sequence information, their recognition by the translocon is under stringent requirements, suggesting that they interact with translocon in diverse ways (Rehan et al., 2023). Since the hydrophobic core mediates targeting and membrane insertion(Haeuptle et al., 1989), the ability of signal peptides to interact with SRP and Sec61 translocon is typically dependent on their total length and hydrophobicity of the H-region (Freudl, 2018; Goldstein et al., 1991; Hatsuzawa et al., 1997; Hikita & Mizushima, 1992).

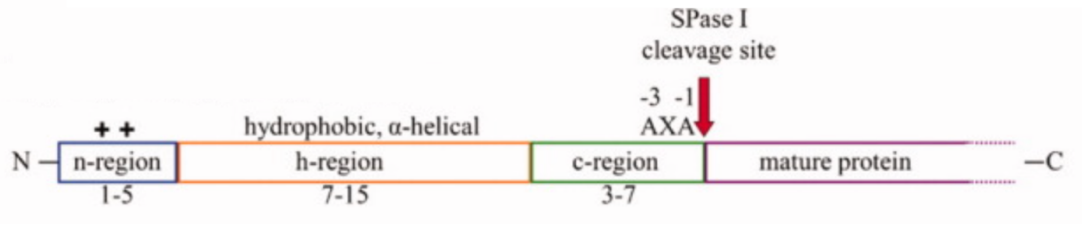


Fig. 2.4.6: Tripartite structure of an N – terminal signal peptide (Auclair et al., 2012).

2.5 Accessory components of the SecYEG/Sec61 translocon

In prokaryotes, the core machinery for insertion of transmembrane proteins is the universally conserved SecYEG/β translocon in prokaryotes (Berg et al., 2004; Egea & Stroud, 2010; Ito, 1984; Manting & Driessen, 2000; Osborne et al., 2005; Park et al., 2014) which is homologous to Sec61 translocon in eukaryotes (Deshaies & Schekman, 1987; Johnson & Van Waes, 1999; Mothes et al., 1994; Park & Rapoport, 2012; Pfeffer et al., 2015). Additional components associate with the prokaryotic SecYEG to facilitate protein translocation and transmembrane insertion (Komar et al., 2016). In bacteria, protein translocation through the SecYEG translocon is aided by the cytoplasmic ATPase, SecA (Akimaru et al., 1991; Dalal et al., 2012; Deville et al., 2011; Economou & Wickner, 1994; Karamanou et al., 1999; Lill et al., 1989; Neumann-Haefelin, 2000; Osborne & Rapoport, 2007; S. Wang et al., 2017; Zhu et al., 2022). However, no archaeal version of SecA has been identified (Eichler, 2000; Pohlschröder et al., 2004). In bacteria and archaea, the evolutionarily conserved YidC (Luirink et al., 2001) either alone (Kumazaki et al., 2014) or in coorporation with SecYEG mediates insertion of membrane proteins into the plasma membrane (Borowska et al., 2015; Houben et al., 2002; Kiefer & Kuhn, 2018; A. Kuhn & Kiefer, 2017; Kumazaki et al., 2014; Mercier et al., 2022; Petriman et al., 2018; Sachelaru et al., 2017; Scotti et al., 2000; Steudle et al., 2021). In bacteria, additional membrane insertion factors, SecDFYajC, have been shown to associate with YidC and SecYEG to facilitate insertion of bacterial membrane proteins (Duong, 1997; Nouwen & Driessen, 2002; Pogliano & Beckwith, 1994; Schulze et al., 2014; Tsukazaki et al., 2011). In archaea, only SecDF (No archaeal YajC homolog) has been identified as auxiliary factors associating with SecYEG and YidC to accelerate translocation into and across the membrane (Eichler, 2003; Hand et al., 2006; Pohlschröder et al., 2004). However, the eukaryotic Sec61 associates with the TRAP complex, OST complex, and the multipass translocon (PAT-BOS-GEL complexes) to facilitate the insertion co-translational of membrane proteins at the ER (Gemmer, Chaillet, van Loenhout, et al., 2023; Smalinskaitė et al., 2022; Sundaram et al., 2022).

2.5.1 Transmembrane insertion proteins

Majority of integral membrane proteins are α-helical transmembrane domain (TMD) proteins (Popot, 1993). Beta barrel membrane proteins occur exclusively in the outer membrane of bacteria, mitochondria and chloroplast (Gabriel et al., 2001; Ruiz et al., 2006; Wimley, 2003). In eukaryotes, most of the transmembrane proteins are initially targeted, inserted and assembled in the endoplasmic reticulum (ER) membrane (Martínez-Gil et al., 2011; Shao &

Hegde, 2011) before they are sorted into vesicles and transported to their final subcellular destinations (Pryer et al., 1992; Rothman, 1994).

Biogenesis of TMD proteins proceeds by the initial targeting and co-translational insertion into the bacterial plasma membrane or eukaryotic endoplasmic reticulum membrane (Blobel & Dobberstein, 1975; Johnson & Van Waes, 1999; Katz et al., 1977; Mercier et al., 2022; Park et al., 2014; Rapoport et al., 1996, 2017; Schnell & Hebert, 2003; Shao & Hegde, 2011; Walter & Lingappa, 1986). Transmembrane domains containing targeting determinants for targeting and insertion of transmembrane proteins are referred to as signal anchors (SA). As with signal peptide proteins, initial SA targeting is mediated by the engagement of exposed TMDs with the SRP (Chartron et al., 2016; Letourneur & Cosson, 1998; Schibich et al., 2016).

In prokaryotes, the core machinery for insertion of transmembrane proteins is the universally conserved SecYEG/β translocon in prokaryotes (Berg et al., 2004; Egea & Stroud, 2010; Ito, 1984; Manting & Driessen, 2000; Osborne et al., 2005; Park et al., 2014) which is homologous to Sec61 translocon in eukaryotes ((Deshaies & Schekman, 1987; Gorlich, 1993; Johnson & Van Waes, 1999; Mothes et al., 1994; Pfeffer et al., 2015; Rapoport et al., 2017; Schekman, 2002). The SecYEG/ Sec61 is gated in two ways; lateral opening of the gate mediates insertion of transmembrane helices into the prokaryotic plasma membrane or eukaryotic ER membrane, and the axial opening of the gate across the membrane translocate elongating segments into the lumen of the ER (Cymer et al., 2015; Martoglio et al., 1995; Rapoport et al., 1996). During membrane protein biogenesis, transmembrane regions of elongating polypeptides move from the central pore of the SecY/61 translocon into the lipid bilayer via the lateral gate (Osborne & Rapoport, 2007; Singer et al., 1987).

Sec61 is principally involved in ribosome docking (Braunger et al., 2018; K. U. Kalies et al., 1994; Patterson et al., 2015; Voorhees et al., 2014), and widely associated with mediating translocation of secretory proteins and co-translational insertion of type I and II transmembrane proteins into the ER membrane (Devaraneni et al., 2011; Gogala et al., 2014; Gorlich, 1993; Heinrich et al., 2000; High et al., 1991; Higy et al., 2004; Martoglio et al., 1995; Oliver et al., 1995; Patterson et al., 2015; Walter & Lingappa, 1986). Additionally, Sec61 translocon recruits an arsenal of other membrane insertion factors (McGilvray et al., 2020; Smalinskaitė et al., 2022; Sundaram et al., 2022).

2.5.2 Tail-anchored proteins

Another group of membrane proteins utilizing unique pathway for their membrane insertion are the Tail-anchored proteins (TA). Tail-anchored proteins are characterized by a C-terminal domain containing hydrophobic transmembrane segment, located about 30 residues from the last amino acid at the C-terminus end (Borgese et al., 2003; Kutay et al., 1993; Yabal et al., 2003). Therefore, membrane insertion of TA proteins occurs post-translationally because the hydrophobic C-terminal tail emerges from the ribosome exit tunnel after translation is terminated, with about the last 30 amino acid residues still within the ribosome. The C-terminal TMD anchors the protein to the membrane (Borgese et al., 2003). Examples of TA proteins include the ER resident proteins Sec61 β , Sec61 γ and cytochrome b5 (Borgese et al. 2003).

The eukaryotic tail-anchored protein targeting factor, TRC40/GET3, originated from an archaeal ancestor (Borgese & Righi, 2010). The TRC40/GET3 is also called Asna-1 because of its prior phylogenetic affiliation with bacterial homolog, ArsA ATPase, known to confer resistance to arsenites and antimonials (Rosen, 2002). As with the eukaryotic TRC40, the archaeal version (ArsA) of Asna1 possess similar transmembrane domain binding features and can functionally replace TRC40 in eukaryotic ER membranes (Favaloro et al., 2010; Sherrill et al., 2011). The absence of this feature in bacterial ArsA suggests a possibility for the inheritance of eukaryotic GET3 targeting factor from an archaeal ancestor whilst the bacterial version remained solely dedicated to arsenite extrusion or heavy metal resistance (Borgese & Righi, 2010). Although no homolog of eukaryotic GET1-GET2 tail-anchored insertase has been identified in Asgard yet, eukaryotic GET2 is structurally similar to EMC6-like proteins of archaeal origin, except that the TMD3 of GET2 has a cytoplasmic extension functionally adapted for interaction with the TA targeting factor TRC40. The structural similarity between GET2 and archaeal EMC6-like proteins reveals an archaeal origin of GET2 which was functionally diversified for TA insertion (Lewis & Hegde, 2021).

2.5.3 The ER membrane complex (EMC)

Additional ER associated machineries have been identified to chaperone insertion of a variety of multipass membrane proteins. The ER membrane complex (Jonikas et al., 2009) has been identified to mediate insertion of the first TMD of most multipass membrane proteins followed by the insertion of successive TMDs by the Sec61translocon (Chitwood et al., 2018). Therefore, the EMC cooperates with Sec61 to insert a particular class of signal anchor proteins.

Additionally, the EMC facilitate insertion of transmembrane proteins with low hydrophobicity (Chitwood et al., 2018; Coelho et al., 2019). Whilst Sec61 mediates translocation of signal peptide-containing proteins and membrane proteins with N-terminus facing the cytosol (N_{cyto} orientation) (Voorhees & Hegde, 2016), EMC is important for the insertion of signal anchors with N-terminus facing the exoplasmic side (N_{exo} orientation) (Chitwood et al. 2018). Conversely, signal anchors destined to be inserted in N_{cyto} typology would be skipped by EMC and inserted by Sec61 translocon in the N_{cyto} typology (Wu and Hegde, 2023). In summary, the EMC mediates insertion of TMDs whose features disqualify their membrane insertion by the classical Sec61 translocon and GET/TRC40 pathway (Chitwood et al., 2018; Guna & Hegde, 2018; O'Keefe et al., 2022).

2.5.4 The Multipass translocon (MPT)

A 'multipass translocon (MPT)' composed of PAT (protein associated with the translocon), BOS (Back of Sec61) and GEL (GET- and EMC-like), specialized for the biogenesis of multipass membrane proteins has been observed in mammalian cells (Sundaram et al. 2022). This is consistent with the identification of major translocon types in the ER microsomes; Sec61–TRAP, Sec61–OSTA–TRAP, Sec61-multipass, Sec61-multipass–TRAP (Gemmer, Chaillet, van Loenhout, et al., 2023). Although Sec61 is not directly involved in insertion of a particular class of membrane proteins (Wu and Hegde, 2023; Smalinskaitė et al. 2022), it functions primarily as the ribosome docking site (Kalies et al. 1994) and also involved in the recruitment of ER insertion chaperones and PAT-BOS-GEL complexes (Smalinskaitė et al. 2022). Homologs of the GEL sub-complex have been identified in archaea (Lewis and Hegde, 2021).

2.5.5 Evolutionary origin of transmembrane insertases

SecY/Sec61 is an evolutionary conserved membrane protein channel widely known to mediate the co-translational translocation/insertion of nascent polypeptide chains into the periplasmic membrane of prokaryotes or eukaryotic ER membrane (Osborne et al. 2005; Park and Rapoport, 2012). SecY, the central component of SecYEG/Sec61 has been shown to originate from the Oxa-1/Alb3/YidC superfamily of insertases (Lewis and Hegde, 2021). Homologues of EMC3 (core component of EMC complex) and TMCO1 (a component of the multipass transcolon) exists in Asgard superphylum (Lewis and Hegde, 2021). YidC possess a conserved U-shaped hydrophilic groove that mediate insertion of certain membrane proteins at an amphiphilic protein-lipid interface (Kumazaki et al., 2014).

Identification of oxa1-like features in the ER-resident proteins (WRB/Get1, EMC3 and TMCO1) unveil the existence of oxa1 family insertases in the endoplasmic reticulum (Anghel et al. 2017). The ER-resident oxa1 superfamily insertases shares structural similarity with YidC and utilize common mechanisms in their membrane insertion (McDowell et al. 2021). Asgard homologs of eukaryotic EMC3 and EMC6 has been identified (Lewis and Hegde, 2021), suggesting that the EMC3-EMC6 subcomplex is an evolutionarily conserved feature proposed to have originated from archaea before the emergence of eukaryotes (Hegde, 2022). The presence of a hydrophilic cytosolic funnel is an evolutionarily conserved feature of all membrane insertion and protein conducting channels (Kumazaki et al., 2014; Voorhees et al., 2014). Additionally, key residues in the hydrophilic and capping cavity of YidC have been shown to be homologous to the SecY's hydrophilic funnel and pore ring residues respectively (Lewis and Hedge, 2021). Therefore, it has been proposed that SecY evolved as a YidC homolog, which formed a translocation channel by connecting two protein-conducting hydrophilic grooves in an antiparallel homodimerization manner after gene duplication and fusion events (Lewis and Hegde 2021). Taken together, these suggest a common evolutionary origin of the core components of membrane protein translocation and insertion machineries (Hartman & Fedorov, 2002).

2.5.6 N-linked glycosylation in eukaryotes

In animals, fungi and plants N-glycosylation is catalysed by the assemblage of hetero-octameric oligosaccharyltransferase (OST) complex. Subunits comprising the mammalian OST complex are DAD1 (Ost2p in yeast), N33/Tusc3 and IAP3 (Ost3p and Ost6p in yeast), OST48 (Wbp1p in yeast), ribophorin I (Ost1p in yeast), ribophorin II (Swp1p in yeast), and STT3A and STT3B (Stt3p in yeast) (Mohorko et al., 2011). The mammalian OST exists in two isoforms depending on the presence of either of the paralogues STT3A or STT3B in the complex (Kelleher et al., 2003). Whilst protein N-glycosylation in prokaryotes is very simple and occurs post-translationally at the inner periplasmic face, the process is complex in yeast/higher eukaryotes and occurs co-translationally in the lumen of endoplasmic reticulum (Kelleher & Gilmore, 2006). This occurrence reflects the requirement of relatively complex OST structure for most of the co-translational N-glycosylation in yeast and higher eukaryotes (Bai et al., 2018).

2.5.7 Comparison of the protein glycosylation machinery in archaea, bacteria and eukaryotes

Among the three domains of life, eukarya, bacteria and archaea (Woese and Fox 1977), STT3 is the only conserved subunit (Zufferey et al., 1995) consisting of an N-terminal multi-spanning transmembrane region and a C-terminal globular domain (Feldman et al., 2005; Igura et al., 2008a; Jaffee & Imperiali, 2011; Kim et al., 2005; Li et al., 2010). As opposed to the multi-subunit OST in eukaryotes (Kelleher and Gilmore, 2006), the prokaryotic OST exists as a single polypeptide enzyme (only STT3 homologue); Archaeal glycosylation B (AglB) in archaea and protein glycosylation B (PglB) in bacteria (Feldman et al., 2005; Igura et al., 2008b). Also, in lower eukaryotes such as *Trypanosoma spp* and *Leishmania spp*, the OST exists as single subunit enzyme composed entirely of STT3 (Izquierdo et al., 2009; Jones et al., 2005; Parsaie Nasab et al., 2008).

2.6 Discovery of eukaryotic OST complex subunits in Asgard superphylum

Findings from deep sea metagenomic studies revealed the presence of eukaryotic signature proteins (ESPs) (Hartman & Fedorov, 2002) in the genomes of archaea belonging to the Asgard superphylum (Zaremba-Niedzwiedzka et al., 2017). Notable among these ESPs is the N-glycosylation machinery identified in Asgard superphylum (Spang et al., 2015, 2018; Zaremba-Niedzwiedzka et al., 2017). Apart from the catalytic subunit, STT3 which universally occurs in all domains of life (i.e AglB in archaea and PglB in bacteria), two additional eukaryotic-like OST subunits (OST3/OST6-like and Ribophorin I) were identified in Asgard superphylum (Imachi et al., 2020; Zaremba-Niedzwiedzka et al., 2017). Further to the discovery of Helarchaeota, samples obtained from coastal sediments (mangrove, mudflat and seagrass bed) were and subjected to metagenome analysis uncovered an additional Archaeal phylum, 'Gerdarchaeota' (Cai et al. 2020). In addition to ESPs that have been similarly described in related archaeal phyla, Cai et al. (2020), identified eukaryotic homologues of DAD1/OST2, one of the components eukaryotic N-glycosylation complex, within the Gerdarchaeotal metagenomic-assembled genomes. In addition to these three previously identified eukaryotic-like OST subunits, Asgard archaeal homologs of all five additional OST subunits (OST2/DAD1, OST4, OST5/TMEM258, SWP1/Ribophorin II and WBP1/OST48), belonging to all three OST subcomplexes, were identified in the genomes of newly identified Asgard archaea, Hodarchaeales, a proposed order within Heimdallarchaeia (Eme et al., 2023).

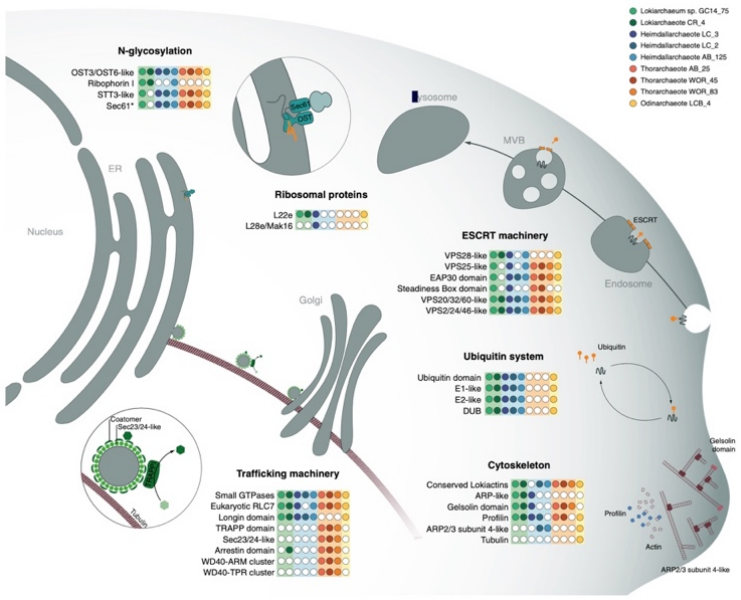


Fig 2.6: Eukaryotic signatures in Asgard archaea (Zaremba-Niedzwiedzka et al. 2017). Schematic representation of a eukaryotic cell in which ESPs that have been identified in Asgard archaea are highlighted, including their phylogenetic distribution pattern. The overall picture indicates that the archaeal ancestor of eukaryotes already contained many key components underlying the emergence of cellular complexity that is characteristic of eukaryotes. DUB, deubiquitinating enzyme; MVB, multi-vesicular body; ER, endoplasmatic reticulum (Zaremba-Niedzwiedzka et al., 2017).

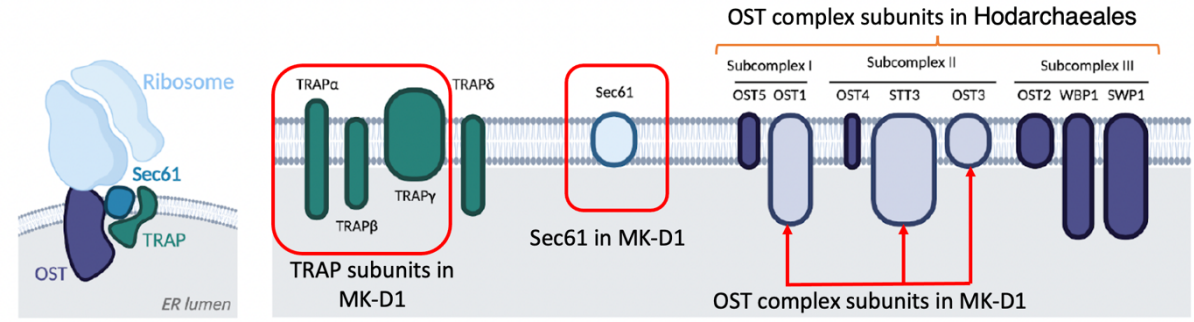
2.6.1 TRAP complex

The translocon-associated protein (TRAP) complex is an ER-resident auxiliary complex interacting with the Sec61 translocon and ribosome (Dejgaard et al., 2010; Pfeffer et al., 2017; Shibatani et al., 2005). Yeast has no homologs of the TRAP complex (Čiplys et al., 2011). The TRAP complex is a heterotetrameric complex composed of four membrane proteins (TRAPα/SSR1, TRAPβ/SSR2, TRAPγ/SSR3 and TRAPδ/SSR4) (Hartmann et al., 1993). The TRAPα/SSR1, TRAPβ/SSR2 and TRAPδ/SSR3 form a hydrophobic cradle-like domain at the lumenal side (below the exit of the Sec61 pore) that interacts with emerging nascent polypeptides, whereas the TRAPγ/SSR3 sits in the membrane (Jaskolowski et al., 2023). The lumenal domain of TRAPα interacts with translocated proteins (Jaskolowski et al., 2023). A loop in the cytosolic domain of TRAPγ (referred to as TRAPγ finger), and a C-terminal domain of the TRAP alpha (referred to as TRAPα anchor) together interacts with the ribosome (Jaskolowski et al., 2023; Pauwels et al., 2023). The TRAP-ribosome interactions additionally induce local ER membrane thinning to accommodate the V-shaped conformation formed by

the Sec61-TRAP complexes (Karki et al., 2023). TRAP also mediates the translocation of substrates whose signal peptide features (such as low hydrophobicity) limits their interaction with the Sec61 translocon (Fons et al., 2003; Nguyen et al., 2018). Taken together, the TRAP complex interacts with the Sec61 translocon and ribosome to facilitate translocation of nascent chains, biogenesis of membrane and secretory proteins, and essential for ER quality control (Jaskolowski et al., 2023; Nagasawa et al., 2007; Russo, 2020; Sommer et al., 2013).

2.6.2 Asgard translocon components

Homologues of eukaryotic Secαβγ, and some components of eukaryotic OST and TRAP were first identified in the genomes of Lokiarchaeota (Zaremba-Niedzwiedzka et al., 2017). Later findings revealed that the genomes of Asgard archaea, Hodarchaeales, contain homologues of a full set of eukaryotic Sec-OST-TRAP complex (Fig. 2.7.1), (Eme et al., 2023). These findings reveal that the genome of Asgard superphylum encode homologs of N-linked glycosylation machineries, its closely associated Sec61 translocon and accessory components of the translocon associated protein complex (TRAP) (Eme et al., 2023).



MK-D1: Candidatus Prometheoarchaeum syntrophicum

Fig. 2.6.2. The Sec-OST-TRAP complex in Hodarchaeales (Eme et al. 2023)

2.6.3 Inter-compatibility of Asgard and eukaryotic protein machineries

A growing body of evidence reveal that protein machines in Asgard are inter-compatible with their eukaryotic partners providing supporting evidence for evolutionary affiliation of eukaryotes to Asgard archaea. Asgard genomes encode actin-regulating proteins that regulate mammalian actin polymerization and depolymerization at the protein level, one of the prominent features of eukaryotes (Akıl et al., 2022; Akıl & Robinson, 2018). Asgard SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins interact with their eukaryotic partners during membrane fusion events (Neveu et al., 2020). It has been demonstrated that eukaryotic ESCRT (Endosomal Sorting Complex Required for Transport)

originated from Asgard archaea (Lu et al., 2020), and that ESCRT machinery functionally share ubiquitin-mediated membrane scission properties with their eukaryotic counterparts (Hatano et al., 2022).

2.6.4 AlphaFold-2 in protein structure prediction and modelling

Protein structural determination is central to understanding the functional and mechanistic roles of proteins in living systems. Although more than 150,000 protein structures have been experimentally determined (Berman, 2000; wwPDB consortium et al., 2019), this forms a small percentage of myriads of protein sequences (Mitchell et al., 2019; Steinegger et al., 2019) whose three dimensional structures have not yet been determined. Experimental resolution of protein structures is limited by several technical difficulties such as inability of some proteins to form crystals for X-ray diffraction (Kendrew et al., 1958; Perutz et al., 1960), relatively small proteins sizes required for NMR analysis ((Markwick et al., 2008; Wüthrich, 2001), and large protein sizes required for CryoEM analysis (Jonic & Vénien-Bryan, 2009).

To bridge the gap between the relatively small number of experimentally-derived protein structures and billions of protein sequences, a number computational techniques that can predict and model protein structures using protein sequence have been developed (Dill et al., 2008). Although these computational techniques have been useful, in some instances, the ability of a computational technique to accurately predict a protein structure close to experimental accuracy remains unresolved (Dill et al., 2008). Over the recent years, deep learning techniques have been introduced into computational predictive tools, which have brought revolutionary impacts in protein modelling (LeCun et al., 2015; Pakhrin et al., 2021; Torrisi et al., 2020; J. Wang et al., 2018). However, the deep learning-based computational methods that have been in use so far are constrained by low atomic accuracy, especially in the absence of homologous protein structures (Abriata et al., 2019; Pearce & Zhang, 2021; Senior et al., 2020). Latest advancement in protein structure prediction and modelling is the AlphaFold2 and AlphaFold3 and which combines novel deep learning reinforcement techniques and learning processes based on the evolutionary, physical and geometric constraints of a protein structure (Abramson et al., 2024; Jumper et al., 2021). As opposed to the other computational techniques, AlphaFold2 can accurately predict protein structures near to experimental accuracy even in the absence of homologous structures, outperforming other computational techniques (Jumper et al., 2021). Even further, AlphaFold3 can predict protein-ligand interactions at a far greater accuracy (Abramson et al., 2024).

3. METHODOLOGY

3.1 NCBI BLAST search of MK-D1 protein sequences

The proteins sequences of MK-D1 translocon were BLAST searched in NCBI PDB against the MK-D1 genome. The NCBI accession codes of the protein sequences are found in Table 2.1.

Table 3.1: NCBI accession codes for sequences used or discussed in the study

	Human	MK-D1
OST1	NP_002941.1	WP_147663064.1
OST3/6	NP_067050.1	QEE15371.1
STT3	NP_001265432.1	WP_147662255.1
TRAP-α	NP_003135.2	WP_147662165.1
TRAP-β	NP_003136.1	WP_147662978.1
TRAP-γ	NP_001295126.1	WP_147664586.1
Sec61α	NP_037468.1	WP_162306565.1
Sec61β	NP_003136.1	WP_147663065.1
Sec61γ	NP_001012474.1	WP_147664346.1
S-layer	-	QEE17131.1
Human OST1	KAI2531360.1	-

3.1.1 Protein expression and purification

The cytosolic (C-terminal) domains of MK-D1 OST1 (residues 449 to 607) and human OST1 (residues 465-607) and the extracellular (N-terminal) domains of MK-D1 OST1 (residues 33 to 425) were codon optimized for expression in *Escherichia coli*, synthesized (GeneScript) and placed in the pSY5 vector (Nag et al., 2009). The plasmids were transformed into BL-21 *Escherichia coli* and grown in Luria Bertani (LB) broth supplemented with 100 µg/mL ampicillin. The cells were grown to an optical density (OD_{600nm} =0.6) at 37 °C in an orbital shaker at 200 rpm and induced with 200 mM isopropyl-D-1-thiogalactopyranodside (IPTG) and allowed to shake at 18 °C overnight. The cells were harvested and resuspended in binding buffer (50mM Tris pH 8, 20 mM imidazole, 500 mM NaCl) supplemented with 1% Triton X-100. The cells were sonicated on ice using an ultrasonic cell disrupter Vibra-Cell (Sonics). The cell lysate was clarified by centrifugation at 15, 000 rpm for 30 min at 4 °C. The supernatant was mixed with 2 ml of Ni-NTA agarose (FUJIFILM Wako Chemicals) allowed to rotate gently for 30 min on a TAITEC Rotator RT-5 at 4 °C. The supernatant was allowed to drain through the column and washed with the 10 mL binding buffer. The resin was then washed with 6 mL

washing buffer (50mM Tris pH 8, 50 mM imidazole, 500 mM NaCl). The column trapped His-tagged protein was treated with PreScission protease (HRV protease) in 2 ml desalting buffer (20 mM Tris-HCl, pH=7.5, 150 mM NaCl) and His-tag cleavage reaction was left overnight at 4°C. Flow-through fraction was collected, column was washed with 10 ml desalting buffer, and 2 ml fractions were collected in batches. Protein fractions were pooled together and concentrated to 500 µl by centrifugation at 5000 x g for 20 min in a 10 kDa molecular mass cut-off Amicon concentrator. The protein was separated on Sephadex 200 (Sec 70) gel filtration column. purified by gel chromatography (Bio-Rad) by standard protocols (Akil & Robinson, 2018). The pure protein fractions were identified by SDS–PAGE, pooled and concentrated with 10k MWCO centrifuge filters (Merck). The proteins were exchanged to the crystallization buffer (10 mM Tris-HCl, pH 7.5, 30 mM NaCl) and concentrated to 10 mg/ml protein. The pure protein was flash-frozen in liquid nitrogen in small aliquots, or used freshly.

3.1.2 Site-directed mutagenesis and selenomethionine incorporation

A plate colony of BL-21 was transformed with psy5 plasmid containing MK-D1 OST1 mutant plasmid (Ile84Met), and inoculated into 5ml LB broth supplemented with 100 µg/ml ampicillin. The culture was incubated at 37°C for 12 hours at 200rpm in an incubator shaker. To 100 ml of M9 medium, 5 ml of starter culture was added and incubated at 37°C overnight at 200 rpm. The 100 ml overnight culture was added to 1 L freshly prepared M9 medium (1X M9 salt solution, 1 mM $MgSO_4$, $CaCl_2$, 0.4% Glucose, 1X BME Vitamin) and incubated at 37°C for 30 min at 200 rpm until OD_{600nm} reached 0.6. To the culture, 100 mg each of the following amino acids Lysine, Threonine, Isoleucine, Leucine, Valine, and Phenylalanine were directly added to the culture, and incubated at 37°C for 30 min at 200 rpm. The culture was then placed on ice for 30 min, induced with 200 mM IPTG (final concentration) and incubated at 18°C overnight at 200 rpm. The cells were harvested for downstream protein purification process as described already above.

PCR-based site directed mutagenesis was used to introduce mutations at position 532 (Ile532Met) of the cytosolic domain of OST1 using QuikChange® site-directed mutagenesis kit following manufacturer's protocol. A plate colony of BL-21 transformed with the plasmid and inoculated into 5ml LB broth supplemented with 100 µg/ml ampicillin. The culture was incubated at 37°C for 12 hours at 200rpm in an incubator shaker. To 100 ml of M9 medium, 5 ml of starter culture was added and incubated at 37°C overnight at 200 rpm. The 100 ml overnight culture was added to 1 L freshly prepared M9 medium (1X M9 salt solution, 1 mM

$MgSO_4$, $CaCl_2$, 0.4% Glucose, 1X BME Vitamin) and incubated at 37°C for 30 min at 200 rpm until OD_{600nm} reached 0.6. To the culture, 100 mg each of the following amino acids Lysine, Threonine, Isoleucine, Leucine, Valine, and Phenylalanine were directly added to the culture, and incubated at 37°C for 30 min at 200 rpm. The culture was then placed on ice for 30 min, induced with 0.2 mM IPTG, and incubated at 18°C overnight at 200 rpm. The cells were harvested for downstream protein purification process as described already above.

3.1.3 Crystallization, structure determination, model building and refinement

Crystallization screening of purified MK-D1 C-terminal OST1 was performed by sitting-drop vapour diffusion method. (protein: reservoir ratio = 0.2 µl: 0.2 µl), using the Gryphon 1.453 robot. Two screening kits, PACT PREMIER and JCGS (Molecular dimensions), were used for initial crystal screening and screened plates were kept at 20°C. After one day, protein crystals were observed in three screening conditions. Optimized crystals were grown from 0.1 M Bis-Tris propane, pH 6.5, 18% PEG 3350, 0.3 M NaF. Protein crystals were harvested, and flash frozen in liquid nitrogen for X-ray diffraction. X-ray data were collected on BL41XU ($\lambda = 1.0$ Å) SPring-8 on a Pilatus 6M detector. The selenomethionine-grown protein crystals diffracted to superior resolution, 1.95 Å. No appreciable selenium signal was detected indicating that these proteins did not have a significant amount of incorporated selenomethionine. Data were indexed, scaled, and merged following standard protocols (Nag et al., 2009). Molecular replacement and refinement were carried out using an AlphaFold2 generated model

Table 3.1: X-ray data collection and refinement statistics for cytoplasmic domain MK-D1 OST1.

	MK-D1 OST1 Cytoplasmic domain WP_147663064.1 (PDB code 8WHN)
Crystals	
Crystallization conditions	0.1M Bis-Tris propane, pH 6.5 18% PEG 3350 0.3 M NaF
Lattice	$P22_12_1$
a, *b*, *c* (Å)	41.4 58.7 67.6
α, β, γ (°)	90.0, 90.0, 90.0
Data collection	
Beamline	BL41XU, SPring-8
Wavelength (Å)	1.0
Resolution (Å)	20.0-1.95 (2.00-1.95)
R_{merge}	10.6 (158.5)
R_{meas}	11.6 (171.9)
R_{pim}	4.7 (66.0)
$I/\sigma(I)$	8.0 (1.3)
$CC_{1/2}$	(0.783)
Completeness (%)	99.7 (99.9)
Redundancy	6.3 (6.6)
Refinement	
Resolution (Å)	20.0-1.95 (2.02-1.95)
No. reflections	12432 (1230)
R_{work} / R_{free}	21.8/24.6 (40.6/41.1)
No. atoms	
Protein	1258
Water	39
B factors	
Protein	62.1
Water	54.3
r.m.s deviations	
Bond lengths (Å)	0.006
Bond angles (°)	0.76
Ramachandran Plot	
Favoured (%)	98.6
Outliers (%)	0.0

3.2 AlphaFold-2 modelling

The Sec61α-signal peptide co-predictions and MK-D1 Sec61-OST-TRAP complex modelling was done with AlphaFold-2 (Jumper et al., 2021). Predicted aligned errors (PAE) and pLDDT plots were used to assess the quality of the predictions.

3.2.1 Structural visualization

The cytosolic domain of MK-D1 OST1, models generated by AlphaFold-2 and PDB accession codes used in the study were visualized using PyMOL (Rigsby & Parker, 2016).

3.3 Human ribosome purification

80S ribosomes were purified from HeLa cells cultured in MEM media. Briefly, HeLa cells in ten 15 cm diameter petri dishes at 70-80% confluency were washed 3 times with 5 ml cold PBS (pH 7.4) and scraped off in 1 ml cold Buffer A (20mM HEPES, pH 7.4, 100 mM KOAc, 7.5 mM $Mg(OAc)_2$). The cells were ruptured using a needle with repeated suction and release for about 30 times. The cell lysate was centrifuged at 20,000 x g for 10 min at 4 °C. The supernatant was gently layered on top of a 10-40% sucrose density gradient in buffer A and centrifuged at 28,000 rpm for 4.5 h at 4 °C using the SW28 rotor (Beckman Coulter, Brea, CA, USA). The gradients were fractionated from the top to the bottom using a Gradient Master (BioComp, Fredericton, NB, Canada). The fractions corresponding to the 80S peak (Fig. S11*A*) were collected, concentrated using a 100 kDa Amicon concentrator and concentration was measured at A_{260nm}. As shown in Fig. 2.1, the pure 80S human ribosomes were confirmed by imaging of negative stained samples by electron microscopy (Tomono et al., 2024) (Fig. S11*B*).

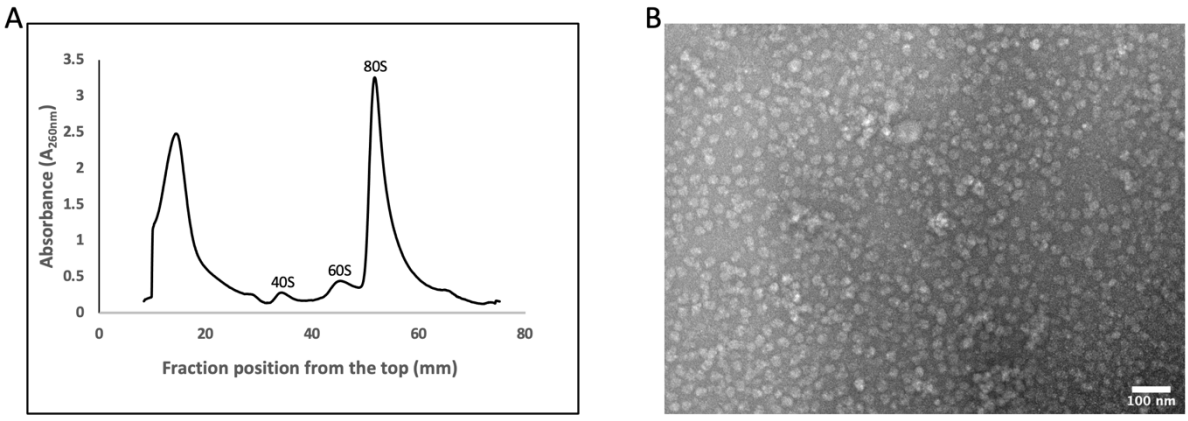


Fig. 3.1 (A) Purification profile and (B) negative staining electron microscopy of pure 80S human ribosomes. Pure 80S human ribosomes were purified from HeLa cells by sucrose density gradient centrifugation and confirmed by negative staining electron microscopy.

Semi-purified human ribosomes were prepared by an alternate protocol (Belin et al., 2010). Briefly, cells were harvested by scraping in 10 mL cold DPBS pH 7.4, and centrifuged for 5 min at 500 x g, 4 °C. Cell pellets were sequentially resuspended in three 100 µL-additions of Buffer B (250 mM sucrose, 250 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4). The cell

suspension was then treated with NP-40 detergent to a final concentration of 0.7%. Detergent-treated cells in Buffer B were incubated on ice for 10-15 min with gentle pipetting at 5 min intervals and then centrifuged at 750 x g for 10 min at 4 °C. The supernatant fraction was clarified by centrifugation at 12,500 x g for 10 min at 4 °C. 4 M KCl solution was added to give a final concentration of 0.5 M KCl. The KCl-adjusted supernatant was layered over a 1 ml sucrose cushion (1 M sucrose, 0.5 M KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4) in a 3 ml polycarbonate tube. This was balanced with buffer C (250 mM sucrose, 0.5 M KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4) and centrifuged at 250,000 x g for 2 h at 4 °C in an ultracentrifuge. The translucent pellet was rinsed twice with 200 µL cold water and resuspended with three 100 µl additions of buffer D (25 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4).

Cells extracts for pulldowns were prepared from HeLa cells cultured to 70-80% confluency in 15 cm petri dish and washed 3 times with cold PBS (pH 7.4). The cells were scraped with 100 µL lysis buffer (20mM HEPES, pH 7.4, 100 mM KOAc, 7.5 mM $Mg(OAc)_2$, 1% NP-40) into 1.5 ml tubes and allowed to rotate gently for 30 min on a TAITEC Rotator RT-5 at 4 °C. The cell lysate was centrifuged at 14,000 rpm for 10 min, and 100 µL of the supernatant was used for pull-down assay.

3.3.1 Western blotting

His-tagged versions of the C-terminal (cytosolic) domains of MK-D1 OST1 and human OST1 and the extracellular N-terminal domains of MK-D1 OST1 were affinity purified on a Ni-NTA agarose columns (FUJIFILM Wako Chemicals). Tag-based pull-down assay on Ni-sepharose His SpinTrap™ columns (Cytiva) was used to probe for interactions between the purified ribosomes and His-tagged versions of the cytosolic domain of MK-D1 OST1, cytosolic domain of human OST1, and N-terminal domain of MK-D1 OST1. Briefly, the Ni-sepharose column was first equilibrated with Buffer A (20mM HEPES, pH 7.4, 100 mM KOAc, 7.5 mM $Mg(OAc)_2$. The Ni-sepharose column was mixed with 50uL of 8 mg/ml of each affinity-purified His-tagged proteins in buffer A, and allowed to stand for 5 minutes. A column not bound with His-tagged protein was also used as a negative control (beads). The Ni-sepharose resin was washed 3 times with 800 µL of Buffer A. Next, 50 µL of 80 nM pure Human 80S ribosomes, 50 uL of semi purified (semi) ribosomes (A_{260nm} 8 mg/ml), and 100 µL of HeLa lysate were added to their respective columns and washed 3 times with 800 µL of Buffer A. Elution was performed with 400uL of elution buffer (20mM HEPES, pH 7.4, 100 mM KOAc,

7.5 mM $Mg(OAc)_2$, 250mM Imidazole). After the pull-down assay, eluted fractions representing bait-prey complexes were concentrated to 100 uL, and 15 µL aliquots from each sample were subjected to Western blot detection using anti-ribosomal protein S3 (RPS3, Cell Signaling Technology, Inc.). A 15 µL of 30 nM pure 80S Human ribosomes was included in the western blots as a positive control. Western blot transfer was visualized using the Amersham Imager 680 (Cytiva).

3.4 Cell culture and imaging

HeLa cells were grown in Minimum Essential Media (MEM, Sigma-Aldrich) supplemented with L-glutamine and 10% fetal bovine serum (FBS) (Nichirei), and incubated at 37 °C, 5% CO_2. Mycoplasma contamination in cell cultures was routinely tested using the PCR mycoplasma detection set (Takara Bio). At approximately 70% confluence, HeLa cells were co-transfected to express each of EGFP-fused MK-D1 proteins with an endoplasmic reticulum (ER) localizing Sec61β mCherry construct (Zurek et al., 2011), or a plasma membrane marker (mCherry-tagged FERM domain of Ezrin), using the Xfect transfection reagent (Takara Bio). At 24 h post-transfection, cells were washed with PBS (pH 7.4), fixed with 4% paraformaldehyde (Nacalai Tesque, Inc.) in PBS for 15 min at room temperature, mounted with Fluoro-KEEPER antifade reagent with DAPI (Nacalai Tesque, Inc.), and imaged using FluoView FV1200 confocal microscope (Olympus).

3.4.1 Quantification of co-localization

A 10.3 x 10.3 µm section from each set of images (EGFP and mCherry channels) was cropped, merged and used for colocalization analysis in Image J. The plugin, colocalization finder, was used to generate the Pearson correlation coefficient, R, of the colocalization after merging the cropped sections.

3.4.2 Prediction softwares

The signal peptide prediction software, signal P 6.0 (Teufel et al., 2022), was used to predict the strength of MK-D1 S-layer protein the MK-D1 translocon signal peptide proteins OST1 and TRAPα. The subcellular localization of MK-D1 translocon components and S-layer protein in eukaryotic cells was predicted with DeepLoc 2.0 (Thumuluri et al., 2022). AlphaFold2 (Jumper et al., 2021) was used for modelling complexes of the MK-D1 Sec-OST-TRAP.

3.4.3 Structural visualization and representation

Protein structures, both X-ray resolved and AlphaFold-2 based models were visualized and represented using the *PyMOL* molecular *visualization* program (Rigsby & Parker, 2016).

3.5 Sec61 inhibition assay

HeLa cells grown to a confluency of approximately 70% were co-transfected with mCherry human Sec61 and EGFP using the Xfect transfection reagent (Takara Bio). At 20 h post transfection, cycloheximide (CHX) was added to a final concentration of 100 μg/mL for 3 h at 37 °C to halt protein synthesis. The cycloheximide (CHX)-treated media was washed thrice with 1 mL PBS (pH 7.4) within 1 min. The media was exchanged to CHX-free media to allow expression either in the absence or presence of 8 μM Eeyarestatin 1 (ES1)(McKibbin et al., 2012) at 37 °C. Cells were washed, fixed and imaged as described above after 3 h.

3.6 Co-translational N-Glycosylation assay

An N-glycosylation reporter was created by introducing an N-glycosylation acceptor site (N147T) into EGFP (Losfeld et al., 2012). MK-D1 signal peptides were fused to the N-glycosylation reporter. HeLa cells at 70% confluence were transfected with DNA encoding the N-glycosylation reporter and treated with or without 1 mg/ml tunicamycin at 9 h post transfection. Similarly, HeLa cells were transfected with the N-glycosylation reporter without signal peptide, treated with and without tunicamycin were used as a control. At 24 h post-transfection, the cells were placed on ice for 10 min and washed three times with cold PBS (pH 7.4). The cells were scraped off with 100 μL of 1X Laemmli sample buffer and boiled at 95 °C for 5 min. The samples were cooled and 15 μL of each sample was used for SDS-PAGE. Western blot analysis was performed with anti-EGFP primary antibody (Cell Signaling Technology) and visualized with Amersham imager 680 (Cytiva).

3.7 Statistical information

The protein localization experiments were repeated twice. Pull-down and Western blots were performed twice.

3.8 Data availability

The atomic co-ordinates and structural factor data have been deposited in the PDB database under the accession code 8WHN.

4. RESULTS

4.1 Signal peptide prediction and ER localization scores for MK-D1 translocon proteins and S-layer protein

The ER localization server SignalP 6.0 (Teufel et al., 2022) was used to predict the presence and strength of the of MK-D1 translocon signal peptide proteins in addition to an S-layer protein. Many MK-D1 transmembrane proteins, including OST1, TRAPα and TRAPβ, were predicted to have eukaryotic-like signal sequences with probabilities of 0.77, 1.00 and 0.99, respectively; These results are comparable with the probabilities for the human proteins of 1.00, 0.81 and 1.00, respectively (Table 1). The subcellular localization prediction server, DeepLoc. 2.0 (Thumuluri et al., 2022), was used to predict the subcellular localization of the M-D1 proteins in a eukaryotic cell (Table 4.1) of the MK-D1 translocon proteins and an S-layer protein assuming they were expressed in human cells. However, the eukaryotic subcellular localization software predicted the localization of these MK-D1 membrane proteins to a variety of possible eukaryotic membranes in comparison to the strong ER localization predicted for the human translocon subunits (Table 4.1).

Table 4.1: Signal peptide and subcellular localization prediction scores for MK-D1 Sec-OST-TRAP and their human equivalents

	Signal peptide prediction (SignalP 6.0)		ER localization score (DeepLoc 2.0)	
Protein	**MK-D1**	**Human**	**MK-D1**	**Human**
OST1	0.77	1.00	0.45	0.85
OST3/OST6	-	-	0.68	0.82
STT3	-	-	0.55	0.92
TRAP-α	1.00	1.00	0.47	0.93
TRAP-β	1.00	1.00	0.23	0.89
TRAP-γ	-	-	0.51	0.90
Sec61α	-	-	0.57	0.56
Sec61β	-	-	0.62	0.82
Sec61γ	-	-	0.35	0.85
S-layer	0.69	-	0.37	-

SP, signal peptide probability. CM, ER, L/V, and Golgi are the predicted probabilities to be localized to the cell membrane, endoplasmic reticulum, lysosome/vacuole or Golgi apparatus, respectively. S-layer protein predictions are also included for MK-D1 as a reference.

4.2 Localization of MK-D1translocon signal peptide proteins in HeLa cells

To experimentally test whether the MK-D1 preproteins exhibit a preferred location in eukaryotic cells, MK-D1 OST1, TRAPα and the cell surface S-layer protein as EGFP fusion proteins were ectopically expressed in HeLa cells (4.20 A-C). In all three cases, these MK-D1 cell surface proteins co-localized with an mCherry ER marker and did not localize to the cell membrane (Fig. 4.21). By contrast, EGFP alone showed no co-localization with the ER marker (Fig. 4.1D). Quantification of ER co-localization shows a co-occurrence of EGFP and mCherry fluorescence in the ER network (S Fig. 4.1).

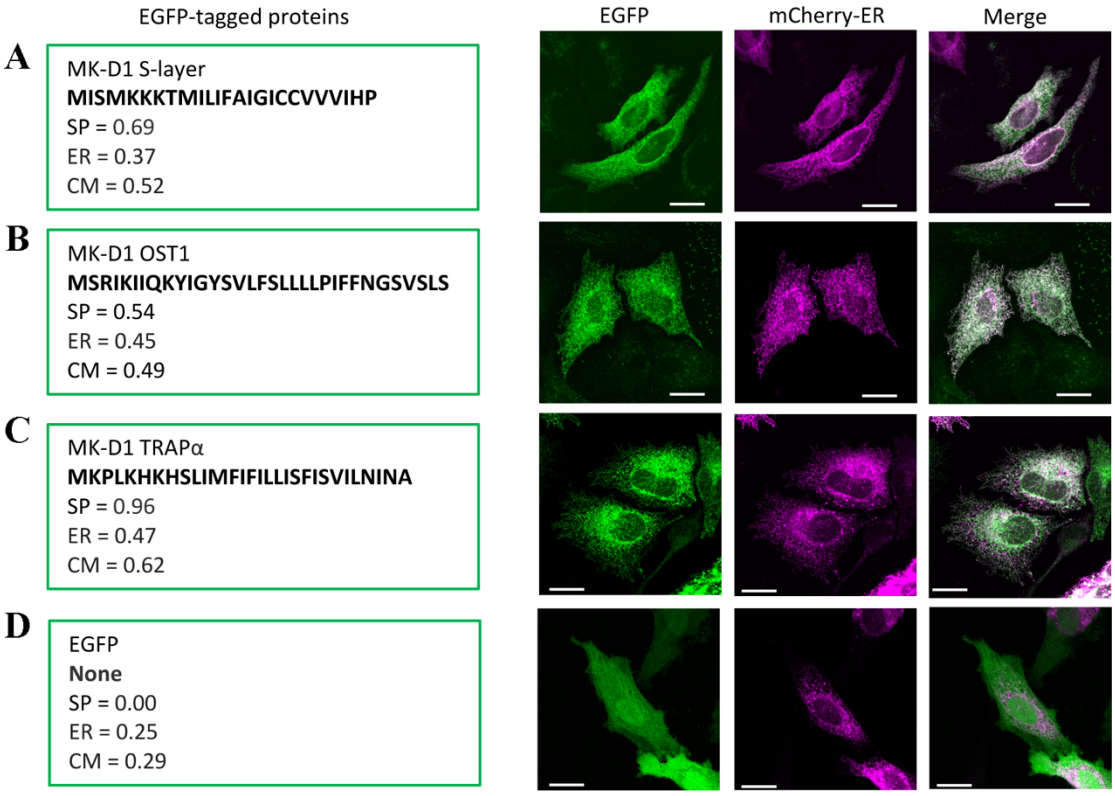


Fig. 4.20 Representative images of the localization of MK-D1 full length signal peptide-containing proteins on transfection in HeLa cells. Cells were co-transfected to express EGFP-fused signal peptide-containing proteins and an ER-localizing mCherry construct. At 24 h post-transfection, cells were fixed and imaged using the confocal microscope. EGFP (green),

mCherry (purple) and merged images are shown. *A*, MK-D1 S-layer protein. *B*, MK-D1 OST1. *C*, MK-D1 TRAPα. *D*, EGFP alone. The signal peptide sequences for each MK-D1 protein (bold), SignalP 6.0 signal peptide prediction scores (SP), and DeepLoc 2.0 localization probabilities for the endoplasmic reticulum (ER) and cell membrane (CM) are given for each EGFP construct.

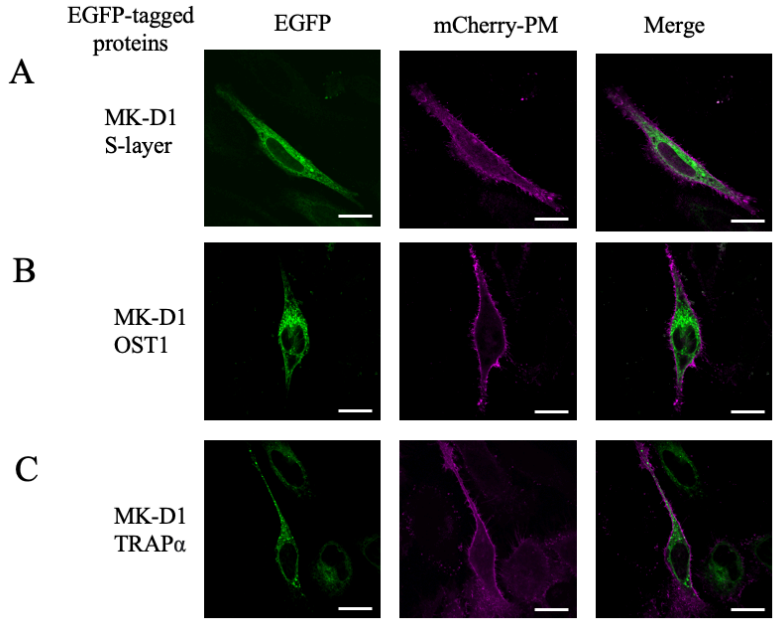


Fig. 4.21: ER-Localizaton of MK-D1 full length signal peptide proteins in the presence of plsama membrane marker in HeLa cells. HeLa cells were co-transfected to express EGFP-fused MK-D1 proteins with a plasma membrane localizing mCherry construct. At 24 hr post-transfection, cells were fixed and imaged using confocal microscope. (Scale bar = 20 μm)

To test whether signal peptides were responsible for targeting the full-length MK-D1 signal peptide proteins to the ER (Fig 4.20), each of their signal peptides were fused to EGFP. HeLa cells were co-transfected to express MK-D1 S-layer protein, TRAPα, TRAPβ and OST1 fused to EGFP with an ER marker. Signal peptide of Human OST1 fused to EGFP was co-transfected with the ER marker as a positive control. All MK-D1 signal peptide-EGFP fusions localized to the ER (Figure 4.22 A-B), as did the positive control human OST1 (Fig. 4.22C). Quantification of co-localization using Pearson's correlation coefficient reveals a correlation between EGFP and mCherry localization in the ER network (S Fig 4.2). Additionally, the signal peptide-EGFP chimeras did not localize to the plasma membrane of HeLa cells (Fig. 4.23).

AlphaFold-2 was used to co-predict the interaction of the signal peptides with the human Sec61α. AlphaFold-2 co-predictions reveal that signal peptides of MK-D1 S-layer, OST1, TRAPα and TRAPβ intercalate at the lateral gate helices of human Sec61α (Fig. 4.22), as similarly observed by (Voorhees & Hegde, 2016) in the interaction between pre-prolactin signal peptide and Sec61α

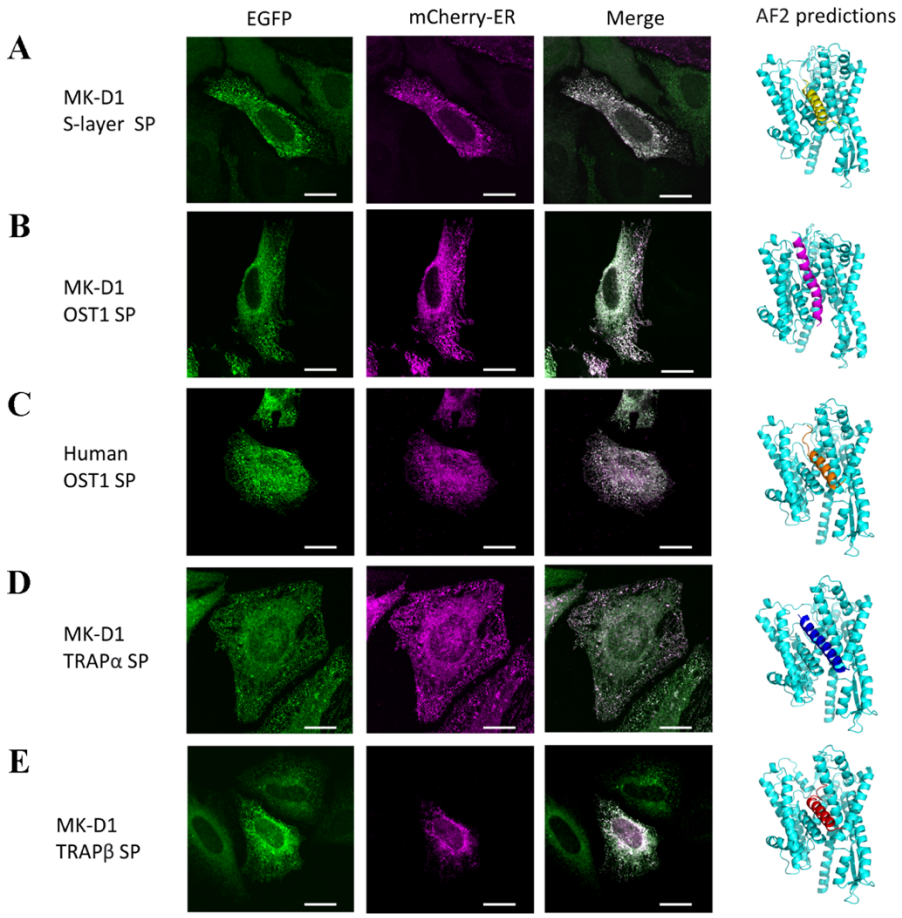


Fig. 4.22: Representative images of the localization of MK-D1 signal peptide-EGFP chimeras on transfection in HeLa cells. Signal peptides (SP) alone, from MK-D1 proteins, were fused to EGFP and co-transfected with the ER marker into HeLa cells as in Fig. 4.2. *A*, MK-D1 S-layer protein SP. *B*, MK-D1 OST1 SP. *C*, Human OST1 SP control. *D*, MK-D1 TRAPα SP. *E*, MK-D1 TRAPβ SP. AF2 co-predictions are shown as cartoons for the human Sec61α and each of the signal peptides. Sec61α, cyan; S-layer SP, yellow; MK-D1 OST1 SP, magenta; Human OST1 SP, orange; MK-D1 TRAPα SP, blue; MK-D1 TRAPβ SP, red. Scale bar = 20 μm. AF2 statistics are given in S Fig 4.2 .

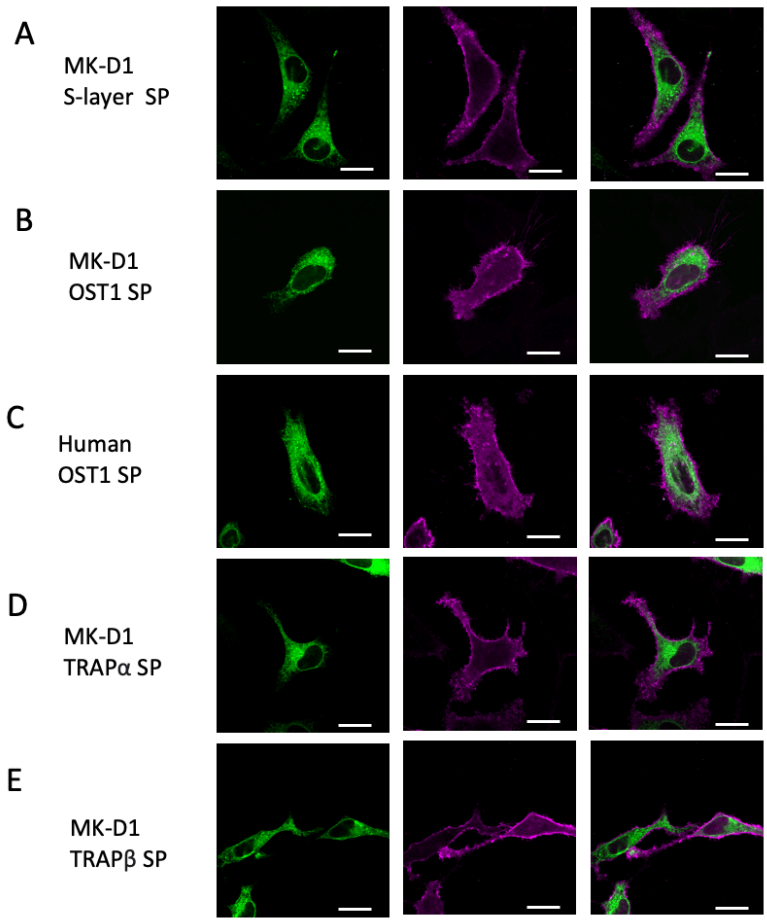


Fig. 4.23: Localizaton of MK-D1 signal peptide-EGFP chimeras in HeLa cells relative to mCherry plasma membrane marker.

Signal peptides of MK-D1 were fused to the EGFP. Each of the signal peptide-EGFP chimeras were co-transfected with the mCherry plasma membrane-targeting construct in HeLa cells. The signal peptide of Human ribophorin I fused with EGFP was used as a control. The cells were fixed at 24hr post transfection and imaged with confocal microscope.

4.2.1 Processing and glycosylation at membrane bound-translocons

To investigate whether the MK-D1 signal peptides undergo processing and can potentially target proteins to the ER for co-translational glycosylation by the human translocon, we expressed these signal peptides fused to a variant of EGFP containing an N-glycosylation acceptor site (gEGFP, N147T) (Gallo et al., 2022). HeLa cells transfected with these constructs were cultured with or without the N-glycosylation inhibitor tunicamycin (Yoo et al., 2018) and subsequently analyzed by Western blot using an EGFP primary antibody. In control experiments, gEGFP alone showed similar migration patterns with or without the N-

glycosylation inhibitor (Fig. 4.24), suggesting that gEGFP lacking a signal peptide is not glycosylated. Fusion of the human OST1 signal peptide to gEGFP displayed comparable migration in the presence of the N-glycosylation inhibitor, indicative of signal peptide cleavage by Sec61. In the absence of inhibitor, the band migrated at higher molecular weight, consistent with glycosylation. Similarly, fusion of the MK-D1 OST1 and TRAPβ signal peptides to gEGFP exhibited similar patterns to human OST, indicating both signal peptide cleavage and glycosylation. The TRAPα signal peptide fused to gEGFP showed two bands in the presence of tunicamycin, as it did in the absence of inhibitor, indicating partial signal peptide cleavage and glycosylation. Conversely, the S-layer fusion protein migrated as a higher molecular weight band than gEGFP in the presence of tunicamycin, indicating no cleavage of the predicted signal peptide. In the absence of tunicamycin, a shift to higher molecular weight indicated glycosylation (Fig. 4.24).

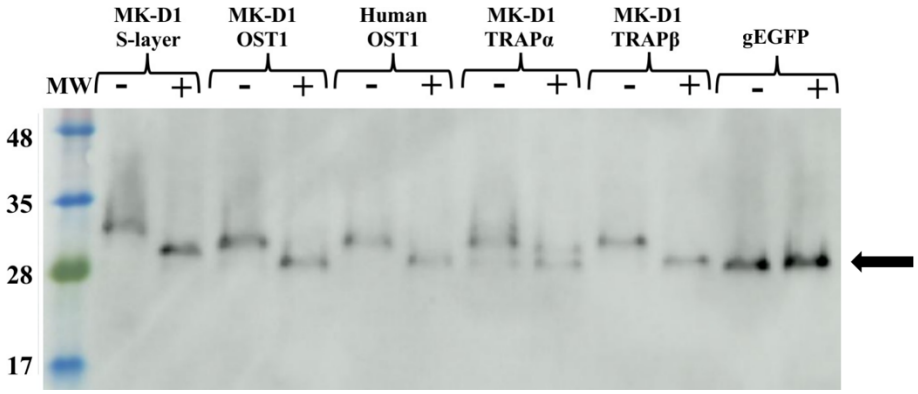


Fig. 4.24: Glycosylation and peptide processing of the signal peptide-EGFP chimeras with an N-glycosylation acceptor site upon transfection into HeLa cells. The Western blot was produced from total cell samples probed with an anti-EGFP primary antibody. + and – indicate the cells were grown in the presence or absence of tunicamycin an N-linked glycosylation inhibitor, respectively. gEGFP refers to EGFP with an N-glycosylation acceptor site but without a signal peptide. This construct is not targeted to the ER, and its migration position (size, black arrow) is the same as the processed, non-glycosylated signal peptide-EGFP chimeras. Migration at higher molecular weight positions in the + tunicamycin lanes indicate lack of cleavage of the signal peptides. Migration at higher molecular weight positions of each chimera in the - tunicamycin lane relative to the + tunicamycin lane, indicates glycosylation. The full Western blot is shown in Fig. S 4.4.

4.2.2 Localization of non-signal peptide MK-D1 transmembrane proteins in HeLa cells.

Each of the EGFP-fused MK-D1 transmembrane proteins TRAPγ, OST3/OST6 and STT3 were co-transfected with mCherry ER marker. Each of these MK-D1 transmembrane proteins co-localizes to the ER with the ER marker (Fig. 4.25 A, 4B, 4C) and did not localize to the plasma membrane (Fig. 4.26). Association between the ER-localization of the EGFP-fused MK-D1 transmembrane protein and mCherry ER marker using Pearson's correlation reveals co-occurrence of ER localization after dual transfection and expression (Fig S 4.5 A-C).

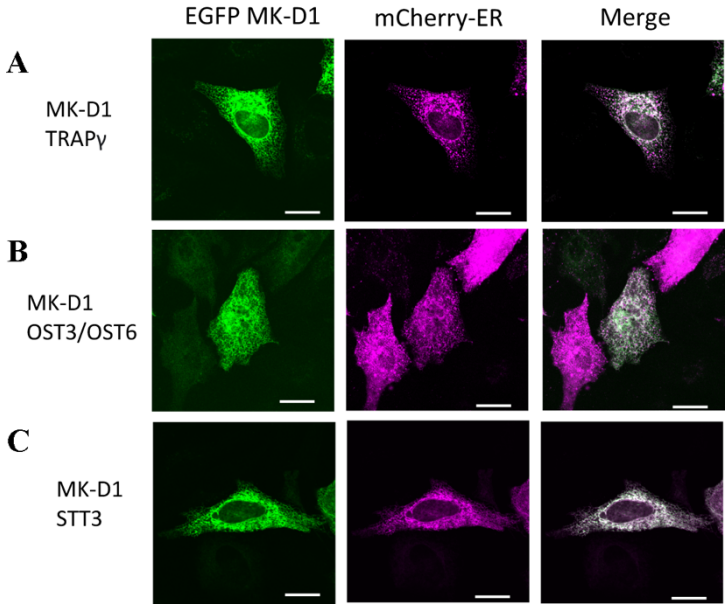


Fig. 4.25: Representative images of the localization of non-SP MK-D1 transmembrane proteins on transfection in HeLa cells. HeLa cells were co-transfected to express each of EGFP-fused MK-D1 *A*, TRAPγ, *B*, OST3/OST6 and *C*, STT3 with the ER localizing mCherry construct. At 24 h post-transfection, cells were fixed and imaged with the confocal microscope. Quantification of the colocalization is found in Fig. S 4.5 A-C. Scale bar = 20 µm.

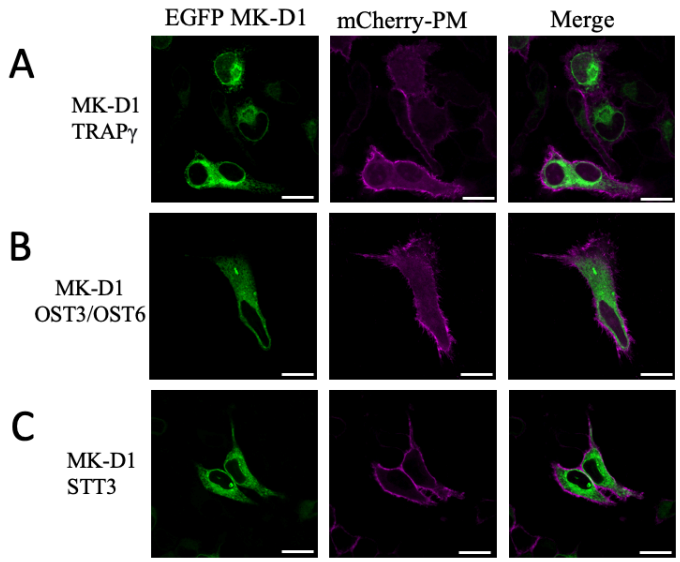


Fig. 4.26: Localizaton of non-signal peptide MK-D1 transmembrane proteins relative to plasma membrane marker in HeLa cells.

HeLa cells were co-transfected to express EGFP-fused MK-D1 proteins with a plasma membrane localizing mCherry construct. At 24 hr post-transfection, cells were fixed and imaged using confocal microscope. Scale bar = 20 µm.

Additionally, each of the EGFP fusion proteins of the three components of the MK-D1 Sec61αβγ preprotein translocase complex were co-expressed pairwise with their corresponding human Sec61αβγ subunits mCherry fusion proteins (Fig. 4.27). In each case, the EGFP-MK-D1 fusion proteins and human mCherry fluorescence co-localized (Fig. 4.27), and quantification of the co-localization reveals a co-occurrence of the ER-distribution for each pair (Fig S 4.5 A-C). Moreover, the distribution of the MK-D1 Sec61αβγ preprotein translocase complex proteins relative to an mCherry plasma membrane marker shows that MK-D1 Sec61αβγ did not localize to the plasma membrane (Fig. 4.28)

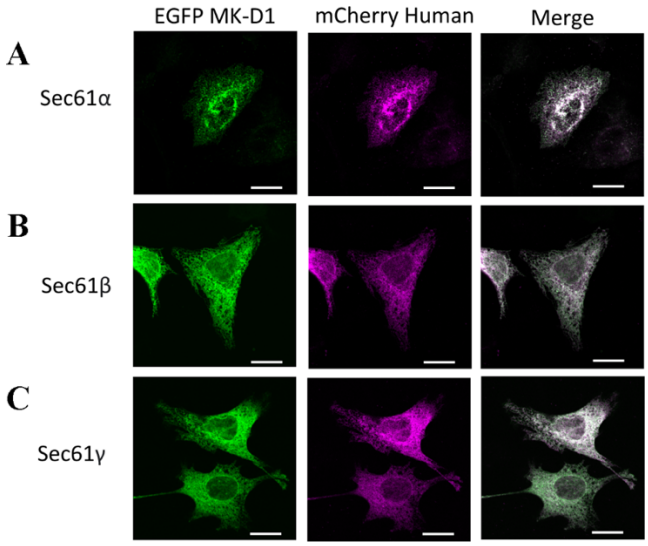


Fig. 4.27: Representative images of the localization of MK-D1 Sec61 proteins on transfection in HeLa cells. Each of EGFP-tagged MK-D1 *A*, Sec61α, *B*, Sec61β and *C*, Sec61γ was co-transfected with its corresponding mCherry fused-human counterpart into HeLa cells. Quantification of the colocalization is found in Fig. S 4.5 D-F. Scale bar = 20 µm. Scale bar = 20 µm.

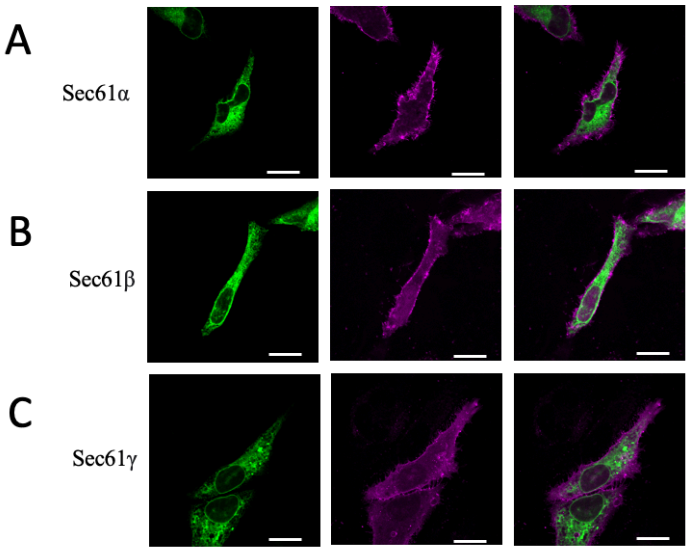


Fig. 4.28: Localizaton of MK-D1 MK-D1 Sec61αβγ preprotein components relative to plasma membrane marker in HeLa cells. Each of the three components of the MK-D1 Sec61αβγ preprotein translocase complex as EGFP fusion proteins were co-transfected with a plasma membrane localizing mCherry construct. At 24 hr post-transfection, cells were fixed and imaged using confocal microscope. Scale bar = 20 µm.

4.2.3 Eeyarestatin I-mediated Sec61 inhibition in HeLa cells

The central component of the human Sec61 translocon complex, Sec61α, was blocked with Eeyarestatin I (ES1), after transient halting of protein synthesis with cycloheximide (CHX). After 3 h, cells not treated with the Sec61α inhibitor exhibited robust Sec61α expression (Fig. 4.29A), while cells incubated with the Sec61α inhibitor showed little Sec61α expression (Fig. 4.29B).

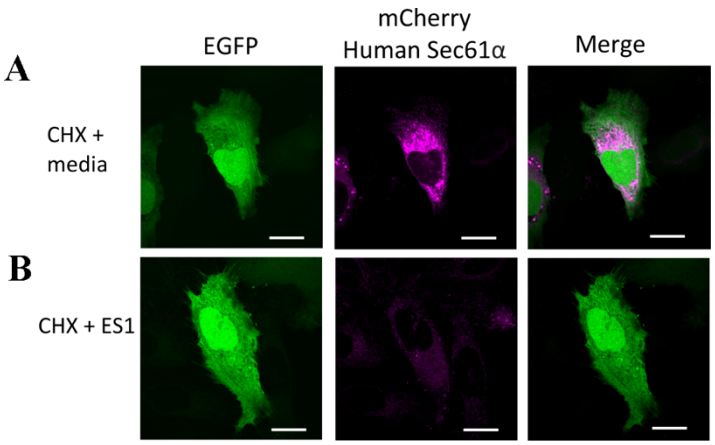


Fig. 4.29: Representative images of the effect of Sec61 inhibition on the localization of MK-D1 newly synthesized Sec61 proteins in HeLa cells. *A-C*, HeLa cells were co-transfected with EGFP and mCherry fused human Sec61α. At 20 h post-transfection, cycloheximide (CHX) was added to a final concentration of 100 µg/ml for 3 h, followed by *A*, the addition of media or *B*, addition of Eeyarestatin 1 (ES1) for 3h before imaging. Scale bar = 20 µm.

4.3 Inter-compatibility of MK-D1 and human translocons

To probe the global structural similarities between the MK-D1 and eukaryotic Sec61/OST/TRAP translocons, AF2 predicted models (Jumper et al., 2021)of MK-D1 Sec61, OST and TRAP complexes and superimposed them onto the eukaryotic Sec61/OST/TRAP translocon cryo-electron microscopy (cryoEM) structure. AlphaFold-2 (Jumper et al., 2021) was used to model larger complexes for MK-D1 Sec61-OST-TRAP complex. The modelled MK-D1 translocon complex was superimposed unto already published human D1 Sec61-OST-TRAP translocon complex (PDB code: 8B6L). Superimposition reveals similar domain organization and high structural similarity between MK-D1 and human translocon components

(Fig. 4.30A). The PAE and pLDDT plots for the complex modelling are shown in Fig. S 4.6 - 4.6.3.

The X-ray crystal structure of the cytoplasmic domain of MK-D1 OST1 was determined at 1.95 Å. The atomic co-ordinates and structural factor data for the cytosolic domain of MK-D1 OST1 have been deposited in the PDB database under the accession code 8WHN. The cytosolic domain of MK-D1 OST1 forms a 4-helix bundle architecture with the same topology as the human version (Fig. 4.29 B, C), although the helices have slightly different angles relative to each other. The X-ray resolved crystal structure of the cytosolic domain of MK-D1 OST1 shares high structural similarity with its corresponding AlphaFold-2 predicted structure (Fig. S 4.6.1). The cytosolic domain of the human OST1 was obtained after loading the PDB accession code, 8B6L, into the *PyMOL* molecular *visualization* program (Fig 4.29 C).

His-tag based pull-down assay reveals that the cytosolic domain of the MK-D1 OST1 can interact with pure 80S human ribosomes (Fig. 4.30 D). It was also demonstrated to recruit ribosomes from semi-purified and crude (lysate) states (Fig. 4.30 D). However, His-tagged N-terminal extracellular domain of MK-D1 OST1 did not interact with pure 80S human ribosomes. Similarly, His-tagged N-terminal extracellular domain of MK-D1 OST1 did not interact with ribosomes in semi-purified and crude (lysate) states (Fig. 4.30 D). Comparison of the surface residues of the human and MK-D1 cytosolic domains of OST1 reveals surface patches of basic amino acid residues that cluster in regions that may be able to interact with the RNA components of the ribosome (Fig 4.31).

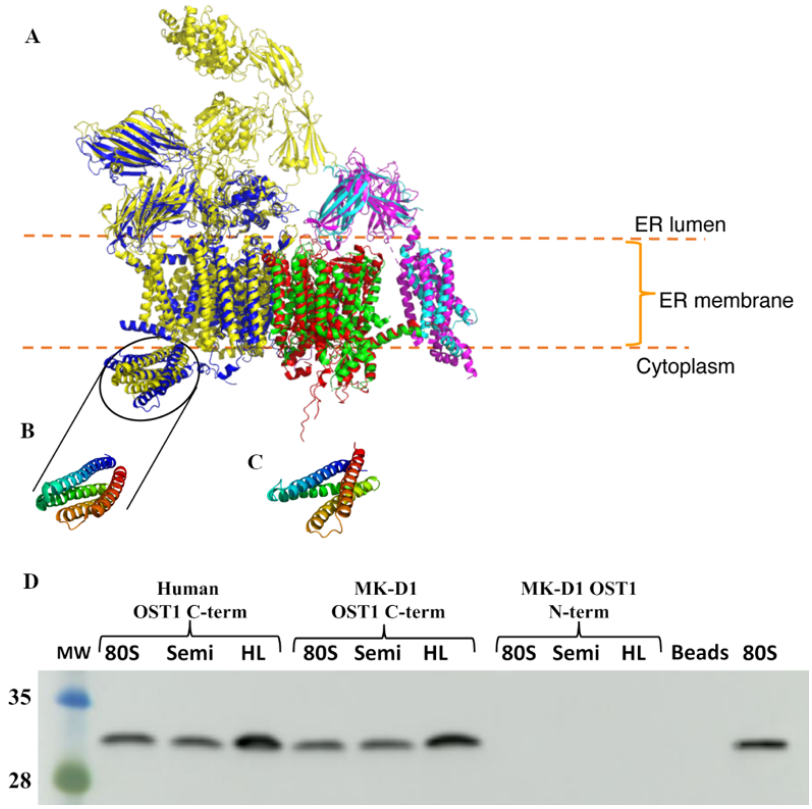
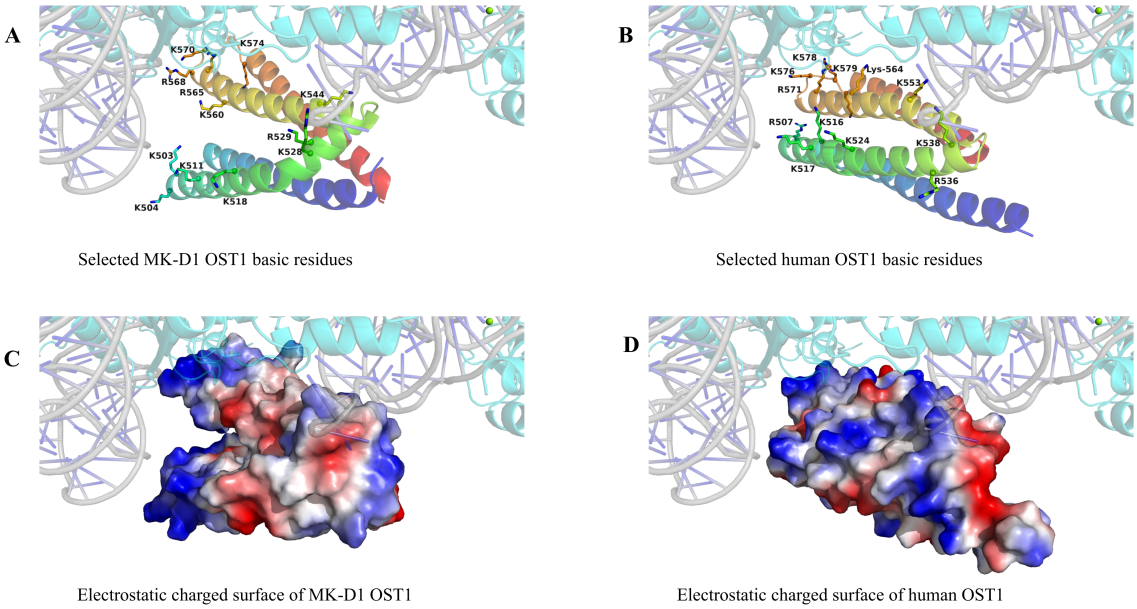


Fig. 4.30: The structural and functional relationship between the MK-D1 and human translocons. *A*, AF2 predicted models for MK-D1 Sec61 (red), OST (blue), and TRAP (cyan) complexes superimposed onto the human Sec61 (green), OST (yellow), and TRAP (pink) translocon structure (Gemmer, Chaillet, Van Loenhout, et al., 2023) (PDB 8B6L). AF2 statistics are given in Fig. S 4.6 – 4.6.3, Structure of the human OST1 cytosolic domain (ribosome interacting domain) obtained from the pymol representation of the PDB accession code 8B6L.. *C*, A 1.85 Å X-ray crystal structure of the cytosolic domain of MK-D1 OST1. *D*, Pull-down assay showing interactions of His-tagged versions of the cytosolic domain of MK-D1 OST1(C-term), cytosolic domain of human OST1 (C-term) and the extracellular domain of MK-D1 OST1 (N-term) with various preparations of ribosomes. 80S, highly purified ribosomes; Semi, semi-purified ribosomes; HL, HeLa cell clarified lysate. Beads, control experiment without His-tagged protein, and the final lane the 80S standard. The Western blot is probed with an antibody against ribosomal protein S3 (RPS3). The full Western blot is shown in Fig. S 4.7. The quality of the 80S ribosomes is shown in Fig. 3.1.

Comparison of the surface residues of the human and MK-D1 cytoplasmic C-terminal domains of OST1 reveals surface patches of basic residues that cluster in regions that may be able to interact with the RNA components of the ribosome (Fig. 4.31).



E

MAFFT sequence alignment for the cytosolic domains of MK-D1 OST1 and Human OST1

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CLUSTAL format alignment by MAFFT (v7.511)

MK-D1     449 RKQRKDYEEGEESFIEQSVPI--------RELTRFVTLIEEKNAILLDIEKADSDLRRKR 500
Human     465 KDPAAEARMKVACITEQVLTLVNKRIGLYRHFDETVNRYKQSR----DISTLNSGKKSLE 520
                :.   : .    .: ** :.:         *.: . *.  ::..    **.. :*. :  .

MK-D1     501 IQKKVYTKTVKNYQNKLKELNEESIPFKRILIETGGQIQSIIQKLDFLEAEKISVKDSVK 560
Human     521 TEHKALTSEIALLQSRLKTEGSDLCDRVSEMQKLDAQVKELVLK-SAVEAERLVAG---K 576
                ::*. *. :   *.:**  ..:       : : ..*::.:: * . :***:: .     *

MK-D1     561 LLKDRYKRGKLPSKAAYERLSSDMIKQLASSQNKIDRYINELRAYII 607
Human     577 LKKDTYIEN--------EKLISGKRQELVT---KIDHILDAL----- 607
              * ** * ..         *:* *.  ::*.:   ***: :: *
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Fig. 4.31: Potential ribosome binding residues on the OST1 cytosolic domain. *A*, Rainbow-colored cartoons of the MK-D1 OST1 cytosolic domain superimposed onto the *B*, human OST1 cytosolic domain structure (PDB 8B6L). Selected basic residues are shown and labeled. The surface charge representations of *A* and *B*, have been shown in C and D respectively. A portion of the ribosome is shown with RNA in gray and proteins in cyan. *E*, A structure-based sequence alignment of MK-D1 and human OST1 cytosolic domains. The selected basic residues in *A*, *B* are highlighted in boxes. The residues were selected based on their proximity to the ribosome in the model (*A*) or structure (*B*) as predicted by .

4.4 Model for translocon migration and the appearance of ER-like internal membrane during eukaryogenesis

Taken together, experimental data obtained in this study was used to infer a model for the evolution of ribosome-translocon complexes and the development an ER-like internal membrane during eukaryogenesis. In the top route of Fig. 4.32 below, invagination (Cavalier-Smith, 2010) or extrusion of the plasma membrane ((Baum & Baum, 2014) produced an isolated compartment with the co-migration ribosome translocon component to this new structure. Finally, a vesicle trafficking system evolved to return some membrane proteins to the plasma membrane. In the bottom route of Fig. 4.8, models proposing that the ER evolved from an endosymbiont in an Asgard host will have inverted translocons, making it difficult to insert new proteins into or translocate proteins appropriately across the ancient ER. Therefore, a vesicle trafficking system is required to properly transfer translocon components from the cell membrane of the Asgard and incorporate them into the internal membrane in the right orientation (Fig. 4.32).

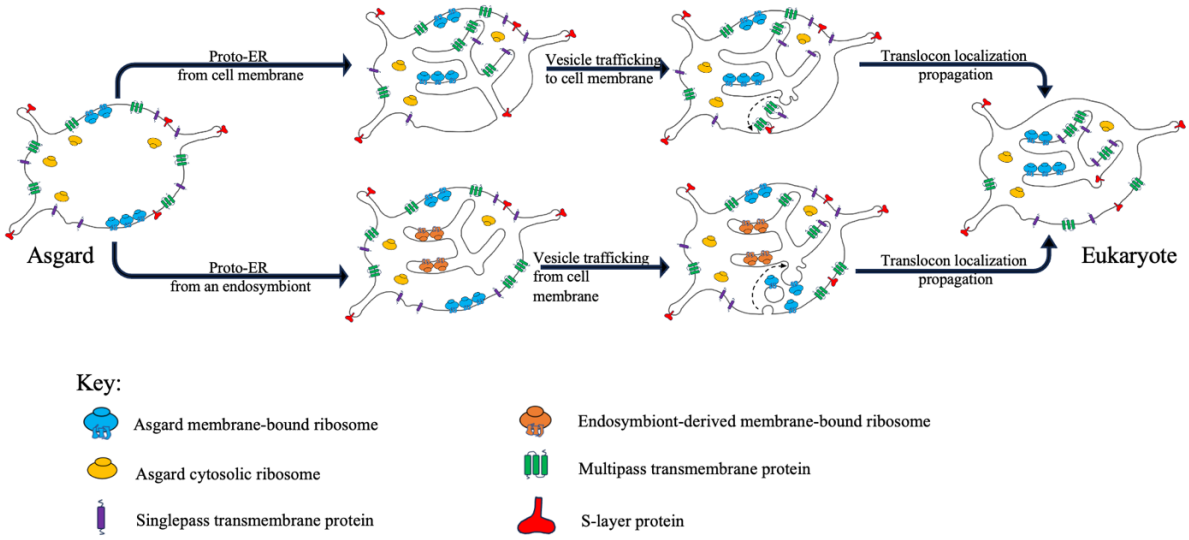

Fig. 4.32: Hypothetical models of ER biogenesis mediated by translocon-location propagation. The top route represents models where the proto-ER arises from Asgard cell membrane invagination or expansion. Bottom route from models where the proto-ER arises from an endosymbiont residing within an Asgard cell.

5. DISCUSSIONS

Controversies surround the origin of eukaryotes. In particular, the emergence of eukaryotic endomembrane (internal) system is one of the key events for the development of intracellular complexities in eukaryotes. Metagenomic and phylogenetic studies affiliate members of the Asgard superphylum closely to eukaryotes. The presence of eukaryotic features in the genomes of Asgard superphylum not only positions them as the eukaryotic ancestors (Spang et al. 2015; Zaremba-Niedzwiedzka et al. 2017; Spang et al. 2018; Eme et al. 2023) but also as endosymbiotic partner of the mitochondrial ancestor in endosymbiotic models (Imachi et al. 2020; López-García and Moreira, 2023; Rodrigues-Oliveira et al. 2023). Although the origin of eukaryotic internal membrane is not clearly understood, membrane proteins associated with internal membranes are useful indicators for tracing the emergence of eukaryotic internal membranes. Specifically, eukaryotic translocon components in the genomes of Asgard superphylum highlight them as potential systems for the development of eukaryotic internal membranes because internal membranes are considered to be ER-derived. However, prokaryotic translocon components sits in the plasma membrane whereas eukaryotic counterparts reside in the endoplasmic reticulum. The mechanism governing the migration of the translocon components from the plasma membrane to an internal membrane (proto-ER) during eukaryogenesis remains unclear.

In this study, NCBI BLAST search revealed homologs of the components of the eukaryotic Sec-OST-TRAP in Asgard archaeon (Zaremba-Niedzwiedzka et al. 2017). AlphaFold-2 predictions (Jumper et al. 2021; Mirdita et al. 2022) of the MK-D1 Sec-OST-TRAP complex subunits reveal high structural similarity with their eukaryotic counterparts (Figure 4.30 A).

Co-localization experiments showed that signal peptide-containing MK-D1 transmembrane proteins fused to EGFP are ER-localized when heterologously expressed in HeLa cells. Additionally, each of the N-terminal signal peptides of MK-D1 S-layer protein, TRAP α and TRAP β, fused to EGFP are localized to the ER as did the positive control, Human OST1. Taken together, these observations indicate that MK-D1 signal peptides can interchangeably interact with the Sec61 translocon. AlphaFold-2 co-predictions of Sec61α and each of the MK-D1 signal peptides showed that the MK-D1 signal peptides intercalate the lateral gate helices of human Sec61α in a similar orientation as observed by (Voorhees and Hegde, 2016) in a signal peptide-engaged Sec61 complex, and similar to the human OST1 signal peptide-Sec61α co-prediction (Fig. 4.22). While the question of Asgard translocon functional activity is worth considering, it is clear from the fluorescence images of the hybrid transmembrane GFP

constructs that GFP is functional and that the MK-D1 Sec61/OST/TRAP translocon subunits are directed to the ER (Fig. 4.24). Taken together, these data indicate that Asgard signal peptides are compatible with the eukaryotic Sec61α translocase. Homologs of the GTP binding domain of SRβ have been identified in members of the Asgard superphylum as Ras GTPases supporting the idea that early diversification events in ancient Ras GTPases led to the appearance of eukaryotic SRβ (Jékely, 2003).

The structural similarity between AF2-predicted MK-D1 Sec61 complex (Figure 4.30) and human Sec61 complex (PDB: 8B6L) suggests similar mechanisms in engagement of signal peptides of MK-D1 OST I, TRAP α and TRAP β as seen with human OST1 signal peptide.

The eukaryotic OSTC/DC2 integrate the OST complex into Sec61 translocon (Shrimal et al. 2017) to form the core ribosome-translocon OST complex (Braunger et al. 2018; Ramirez et al. 2019). Human OSTC/DC2 has three transmembrane domains (TM1-TM3) whilst yeast OST3/OST5 has four transmembrane domains (TMD1-TMD4). Sequence-based alignment reveals that the three TMDs of OSTC/DC2 shares distinct sequence similarity with TMD2-TMD4 of OST3/OST6. Therefore, eukaryotic OSTC/DC2 can be considered as an N-terminally truncated OST3/OST6 (Shrimal and Gilmore, 2019). Eukaryotic homologs of OST3/OST6 have been found in Asgard archaea, and a docking model reveals that yeast OST3 can functionally replace mammalian OSTC/DC2 in OST complex due to marked sequence similarities between their transmembrane domains (Wild et al. 2018). In this study, localization of MK-D1 OST3/OST6 to the ER membrane further supports this hypothesis. Human Sec61β and Sec61γ which are tail-anchored proteins (Hartmann et al. 1994), co-localizes with their distant archaeal homologs, MK-D1 Sec61β and Sec61γ (Fig. 4.27), respectively in the ER membrane. The core components of the GET pathway, TRC40 and GET 1, for membrane insertion of tail-anchored proteins in eukaryotes have been discovered in Asgard (Anghel et al. 2017). Moreover, it has been shown that archaeal TRC 40 interacts with the transmembrane helices of TA proteins and mediates their integration into the ER membrane raising the possibility that the post-translational tail-anchored insertion pathway originated from Asgard archaea (Favaloro et al. 2010; Sherrill et al. 2011). In the present study, homologous components of the eukaryotic core translocon, Sec-TRAP-OST complex (Braunger et al. 2018; Ramirez et al. 2019), exists in Asgard archaeon MK-D1.

Eukaryotic Sec61 translocon is the core machinery for co-translational insertion of transmembrane proteins at the ER membrane (Park and Rapoport, 2012) and also cooperates with other transmembrane intertases for the biogenesis of a subset of membrane proteins (Bai

and Li, 2023). Multipass transmembrane proteins whose signal anchors are in the N_{cyto} orientation utilize Sec61 translocon for their insertion into the ER membrane (Li et al., 2016; Voorhees and Hegde, 2016). Signal anchors of ER-localized Asgard multipass transmembrane proteins used in this study exhibit N_{cyto} orientation, hence might probably engage Sec61 translocon to mediate their membrane insertion into the ER. Additionally, Sec61 complex is involved in the recruitment of PAT-BOS-GEL complex to chaperone and facilitate the insertion of a particular class of membrane proteins (Smalinskaitė et al. 2022). Considering the central role played by Sec61 in membrane protein biogenesis, it was proposed that Sec61 is the nexus of membrane protein integration and might probably be involved in recruiting itself to the proto-ER under positive selection during eukaryogenesis. Eeyarestatin I-mediated Sec61 inhibition (Cross et al. 2009; Itskanov et al. 2023) revealed little expression of mCherry-fused Sec61α compared with EGFP (Fig. 4.29 A,B). This data supports the idea that Sec61 is involved in self-recruiting itself to the proto-ER during translocon re-localization. Notably, the relocalization of Asgard Sec61 to the proto-ER under positive selection was primarily essential to the co-translational membrane insertion of membrane proteins to the proto-ER.

Moreover, other ER insertion machinery such as ER membrane complex (Jonikas et al. 2009), TMCO1 insertase-complex (McGilvray et al. 2020) and the PAT-BOS-GEL complex (Smalinskaitė et al. 2022; Sundaram et al. 2022) have been shown to be involved in the insertion of multipass membrane proteins whose transmembrane helices cannot efficiently engage Sec61 (Chitwood et al. 2018; McGilvray et al. 2020; Chitwood and Hegde, 2020; Culver and Mariappan, 2020). The GET-1 and EMC3 which are core insertases of the GET pathway and ER membrane complex respectively, are evolutionary conserved eukaryotic homologs of the oxa1/YidC/Alb3 insertases. The TMCO1-OPTI (PAT complex) dimer is similar to the GET1-GET2 (tail-anchored insertase complex) and EMC3-EMC6 (catalytic insertase of EMC complex) (Lewis and Hegde, 2021). Identification of core components associated with the GET-pathway (TRC-40, GET-1 in Asgard), EMC complex (EMC3 and EMC6) and TMCO1 translocon (TMCO1) in Asgard reveals the evolutionary conservation of other transmembrane chaperones and insertases in Asgard archaea. Localization of MK-D1 Sec61αβγ to the ER membrane (Fig. 4.27) implies that the core components of the transmembrane insertion machinery in eukaryotes are functionally replaceable with their Asgard archaea counterparts. Although, components of the PAT and BOS are lacking in prokaryotes, homologs of the GEL complex (TMCO1-OPTI) occur in Asgard (Lewis and

Hegde, 2021) and may cooperate with the ubiquitous SecY to facilitate the biogenesis of multipass membrane proteins.

The MK-D1 OST1 domain forms a 4-helix bundle with the same topology as the human domain, although the helices have slightly different angles relative to each other. In eukaryotes, this OST1 cytosolic domain interact with ribosome (Braunger et al. 2018). Western blot analysis using a ribosome-specific antibody shows that the C-terminal domains of OST1 were able to recruit ribosomes from samples with different levels of ribosome purity, indicating a direct and specific interaction. This finding suggests that MK-D1 OST1 participates in the recruitment of MK-D1 ribosomes to the Sec61/OST/TRAP translocon at the cell membrane for translation and translocation of membrane proteins and preproteins. Comparison of the surface residues of the human and MK-D1 cytoplasmic C-terminal domains of OST1 reveals surface patches of basic residues that cluster in regions that may be able to interact with the RNA components of the ribosome. Taken together, these data demonstrate that the Asgard and eukaryotic Sec61/OST/TRAP translocons are structurally and functionally similar and show a level of mutual compatibility. Namely, the eukaryotic ribosome-translocon complex can translate, process and direct Asgard translocon proteins to the ER, and the Asgard translocon complex can interact with eukaryotic ribosomes.

Eukaryotes are distinguished from prokaryotes by the presence of eukaryotic signature proteins (Doolittle 1998; Hartman and Fedorov, 2002), an endomembrane system, cytoskeleton and other intracellular complexities (Vellai and Vida, 1999). A number of theories have been proposed to explain the evolution of eukaryotes (Baum 2015; Martin et al. 2015; Gould et al. 2016; Donoghue et al. 2023; López-García and Moreira, 2023). Identification of eukaryotic signature proteins in members of the Asgard superphylum positions them as the closest phylogenomic affiliates to eukaryotes (Spang et al. 2015; Spang et al. 2018; Eme et al. 2023). The widely accepted endosymbiotic models favoring eukaryogenesis posits that mitochondrial acquisition from α-proteobacteria endosymbiont bioenergetically favored the co-evolution of internal structures and protein re-localization events in the proto-eukaryote (Gould et al. 2016; Imachi et al. 2020). Additionally, the endoplasmic reticulum is regarded as the founding stock of the eukaryotic endomembrane system (Ozansoy and Denizhan, 2009; Gould et al. 2016). In this study, the localization of Asgard homologs of eukaryotic Sec-TRAP-OST translocon to the ER membrane provides experimental insights into the re-localization of Asgard translocon components as an early event for eukaryogenesis. In view of the experimental data from this study, two models were postulated; plasma membrane derived proto-ER and proto-ER derived

from an endosymbiont (Fig. 4.32). According to the model for proto-ER derived from the plasma membrane, infoldings of the plasma membrane bearing ribosome translocon components as the docking apparatus is regarded as an early landmark for creating special compartment responsible for protein modification. The eventual segregation of plasma membrane infoldings from the plasma membrane gave rise to a special secretory compartment as the seed for the endomembrane system. Next, is the development of a membrane trafficking system to redirect proteins to the plasma membrane and extracellular sites. The second model in the lower route represents an internal proto-ER generated by the mitochondrial endosymbiont. In this scenario, the ribosome docking apparatus derived from the endosymbiont assumes an inverted orientation. However, vesicular transport from the plasma membrane bearing Asgard ribosome translocon apparatus replaces inverted ribosome translocon of alphaproteobacterium. This establishes the mounting of the ribosome docking apparatus in the proper orientation (Fig 4.32). The possibility that Sec61 is involved in the biogenesis of future ER-targeted Sec61α was explored. In this study, eeyarestatin-mediated inhibition of the biogenesis of Sec61α subunit reveals the key role played by Sec61 translocon in coordinating the recruitment of nascent Sec61 Sec61α to the ER membrane. Core subunits of the SecYEG/Sec61, GET/TRC40, EMC and PAT-BOS-GEL complexes are evolutionarily associated (Lewis and Hegde, 2021) and structurally homologous to the oxa1/YidC/Alb3 family of insertases (Anghel et al. 2017). Since these core machineries involved in the insertion of membrane proteins at the ER membrane are also present in the eukaryotic ancestor, Asgard, it implies that key mechanistic features would be shared and maintained over evolutionary period.

Taken together, the study provides experimental evidence for the inter-compatibility of human and Asgard translocon apparatus. Therefore, re-localization of the Asgard ribosome translocon apparatus from the plasma membrane to an internally arising proto-ER is a key event for the emergence of the endomembrane system during eukaryogenesis. Additionally, members of the Asgard superphylum has become potential candidates for functional characterization of proto-eukaryotic protein machineries during eukaryogenesis.

6. CONCLUSIONS

Asgard translocon components exhibit preferred localization to the ER when in eukaryotic cells. The relocalization of the Asgard translocon components has implications for the development of the ER. The study unveils the inter-compatibility of Asgard and human translocons. Asgard and eukaryotic Sec61/OST/TRAP compatibility is consistent with models of eukaryogenesis in which the eukaryotic cell membrane and cytoplasm are derived from an Asgard archaeon. The study provides evidence for key events during the emergence of a proto-ER.

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8. SUPPLEMENTARY TABLES AND FIGURES

8.1 Supplementary Table

Table S 3.1: Signal peptide and eukaryotic cell localizations predicted for human and MK-D1 translocon components.

	Human					MK-D1				
Protein	**SP**	**CM**	**ER**	**L/V**	**Golgi**	**SP**	**CM**	**ER**	**L/V**	**Golgi**
OST1	1.00	0.2974	**0.8539**	0.3249	0.5281	0.77	**0.4928**	0.4491	0.5406	0.3896
OST3/6	-	0.4104	**0.8222**	0.4776	0.7766	-	0.2012	**0.6849**	0.3375	0.4546
STT3	-	0.2676	**0.9103**	0.2198	0.5628	-	0.3056	**0.5482**	0.2918	0.2968
TRAP-α	1.00	0.2115	**0.9329**	0.6124	0.7756	1.00	0.6247	0.4706	**0.6728**	0.3788
TRAP-β	1.00	0.1857	**0.8883**	0.2093	0.3706	1.00	**0.5493**	0.2311	0.5200	0.2683
TRAP-γ	-	0.1407	**0.9088**	0.1638	0.6022	-	0.1468	0.5100	**0.6175**	0.2737
Sec61α	-	0.4400	**0.5555**	0.3185	0.2664	-	0.2875	**0.5734**	0.3097	0.2100
Sec61β	0.16	0.4051	**0.8263**	0.2655	0.6076	-	0.2468	**0.6042**	0.4534	0.5575
Sec61γ	-	0.3844	**0.8462**	0.5236	0.5854	-	0.0690	0.3514	0.3797	**0.5174**
S-layer	-	-	-	-	-	0.69	**0.5185**	0.3739	0.3732	0.1124

SP, signal peptide probability. CM, ER, L/V, and Golgi are the predicted probabilities to be localized to the cell membrane, endoplasmic reticulum, lysosome/vacuole or Golgi apparatus, respectively. S-layer protein predictions are also included for MK-D1 as a reference.

8.2 Supplementary Figures

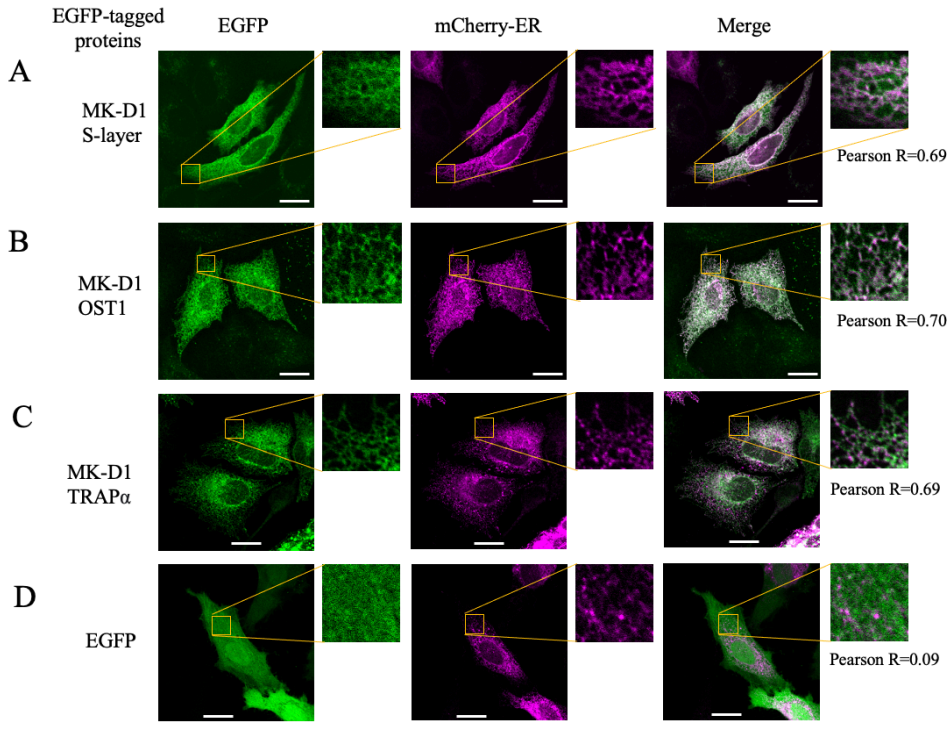

Fig S 4.1: Quantification of co-localization for signal peptide MK-D1 transmembrane proteins in HeLa cells

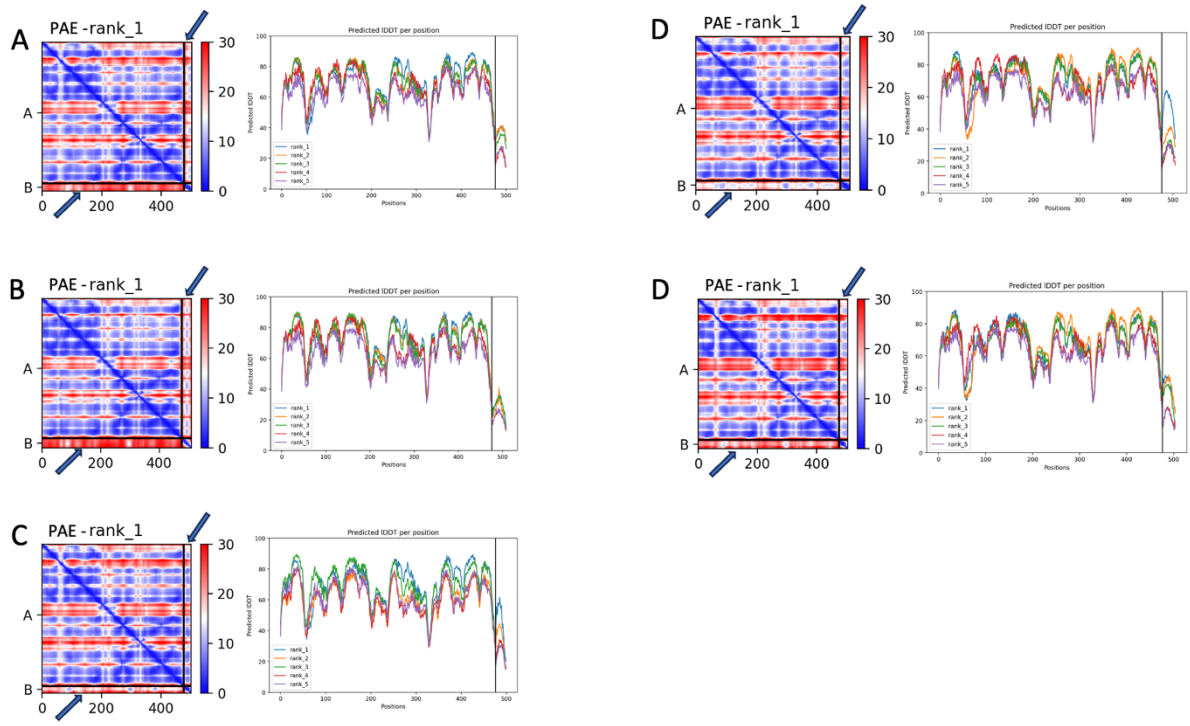

Fig S 4.2: AlphaFold2 co-predictions of human Sec61α with the signal peptides of A) MK-D1 S-layer B) MK-D1 OST1 C) Human OST1 D) MK-D1 TRAPα E) MK-D1 TRAPβ. Blue arrows represent PAE for the corresponding signal peptide.

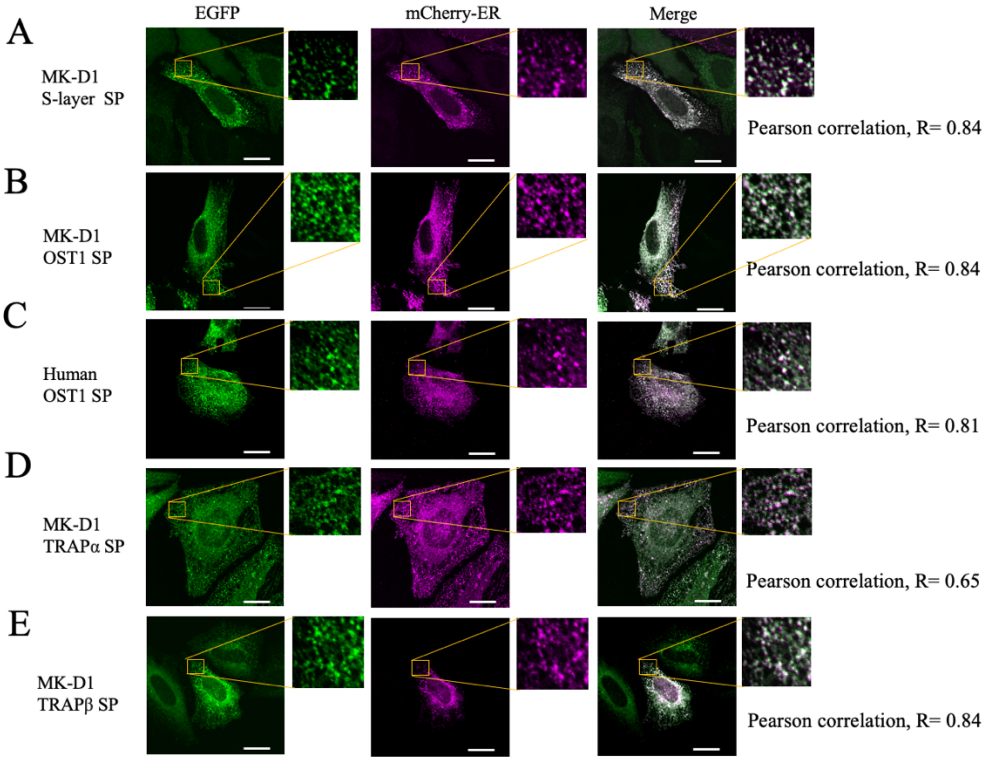

Fig. S 4.3: Quantification of ER localization of MK-D1 signal peptide-EGFP chimeras in HeLa cells. Signal peptides of MK-D1 were fused to the EGFP. Each of the signal peptide-EGFP chimeras were co-transfected with the mCherry ER-targeting construct in HeLa cells. The signal peptide of Human ribophorin I fused with EGFP was used as a control. The cells were fixed at 24 hr post transfection and imaged with confocal microscope.
Sections of the confocal images were cropped and used for colocalization analysis. The Pearson correlation coefficient, R, was used to measure the the co-occurrence of the co-localization.

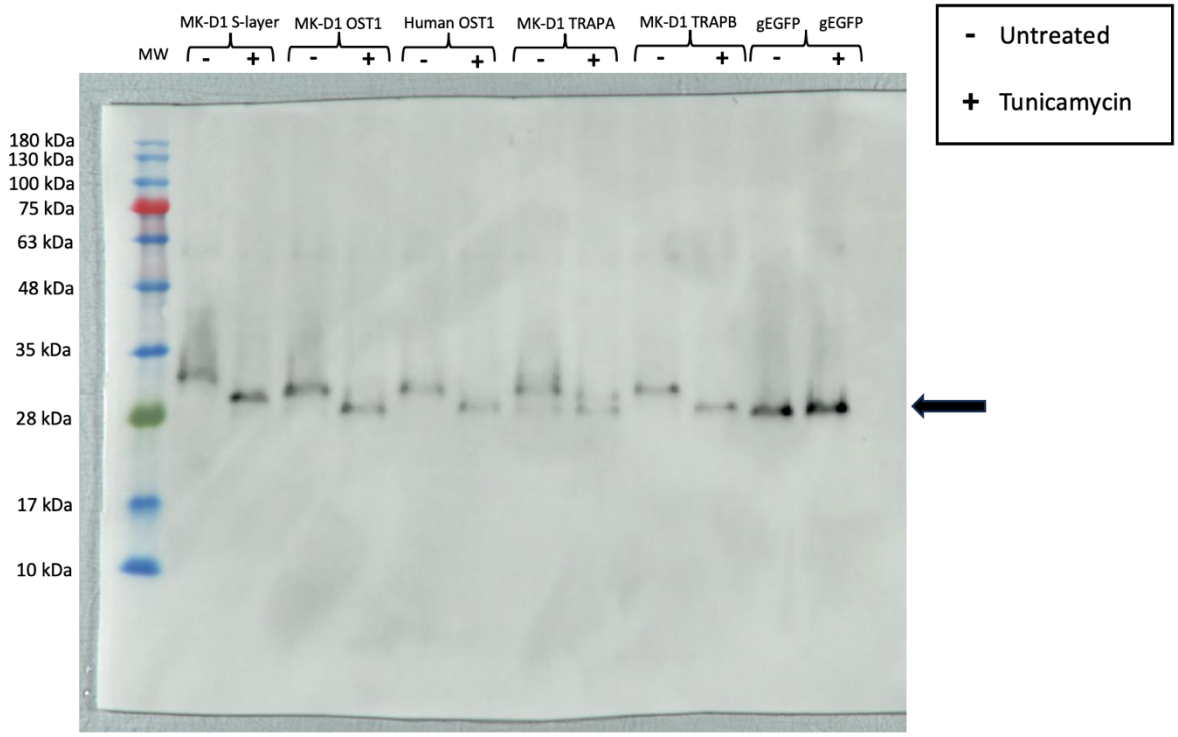

Fig. S 4.4: Glycosylation and peptide processing of the signal peptide-EGFP chimeras bearing an N-glycosylation acceptor site upon transfection into in HeLa cells. The Western blot was produced from total cell samples probed with an anti-EGFP primary antibody. + and – indicate the cells were grown in the presence or absence of tunicamycin an N-linked glycosylation inhibitor, respectively. gEGFP refers to EGFP with an N-glycosylation acceptor site but without a signal peptide. This construct is not targeted to the ER, and its migration position (size, black arrow) is the same as the processed, non-glycosylated signal peptide-EGFP chimeras. Migration at higher molecular weight positions in the + tunicamycin lanes indicate lack of cleavage of the signal peptides. Migration at higher molecular weight positions of each chimera in the - tunicamycin lane relative to the + tunicamycin lane, indicates glycosylation. The full Western blot is shown in Fig. S6.

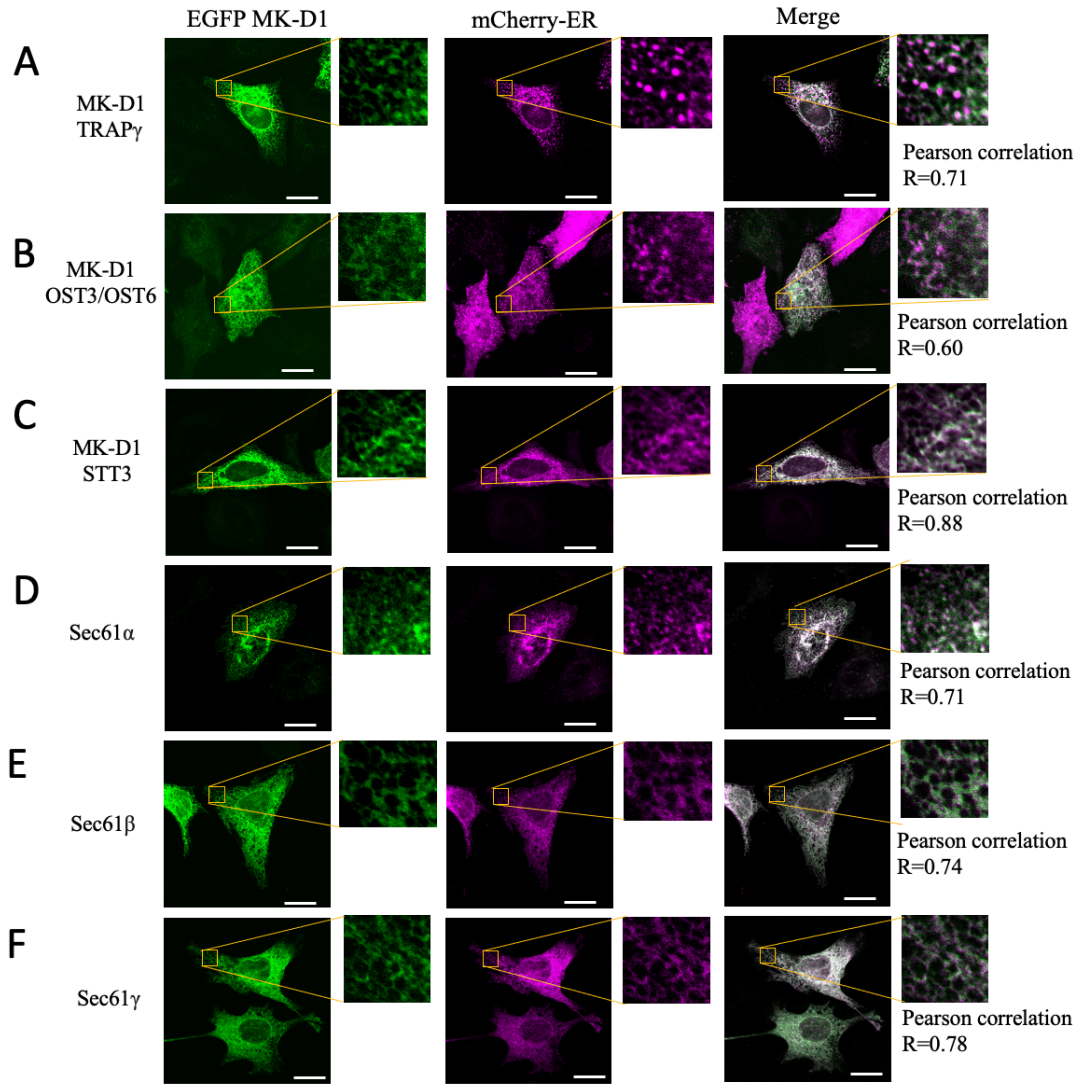

Fig S 4.5: Quantification of ER-localization of non-signal peptide MK-D1 transmemebrane proteins in HeLa cells. Each of EGFP-tagged TRAPPγ (a), OST3/OST6 (b), STT3 (c) was co-transfected with mCherry endoplasmic reticulum localizing construct. Each of the MK-D1 Sec61α (d), Sec61β and Sec61γ (c) was co-transfected with its corresponding mCherry fused-human counterpart, Sec61α, Sec61γ and Sec61β respectively, into HeLa cells. Sections of the images were cropped, merged and used statistical analysis of co-localization using Image J plugin, co-localization finder.

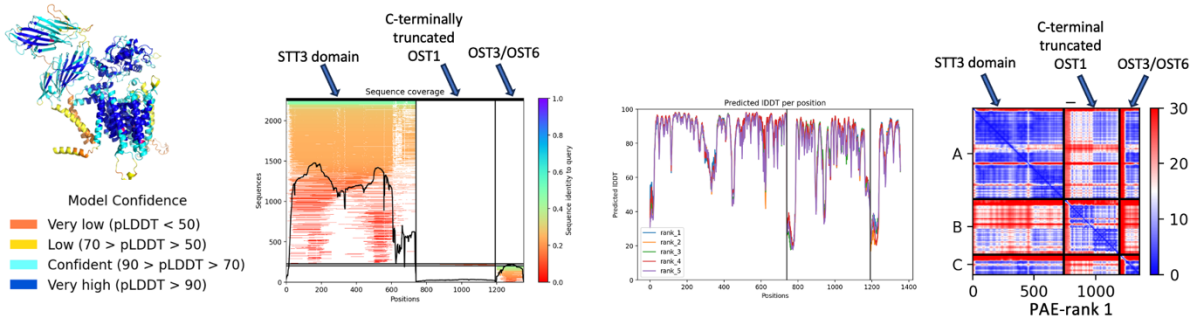

Fig S 4.6: **pLDDT and PAE plots for AlphaFold2 prediction of MK-D1 OST complex without the cytosolic domain of OST1**. Amino acid sequences for transmembrane and catalytic domains (1-740 residues) of MK-D1 STT3 subunit were used for the AF2 prediction. For the OST1 subunit, sequences for the C-terminal domain (residues 449 to 607) were deleted and the remaining sequences representing N-terminal and transmembrane domains (residues 1-448) were used for the AF2 multimer prediction. With OST3/OST6, full amino acid sequence was used to generate AF2 prediction for MK-D1 OST complex.

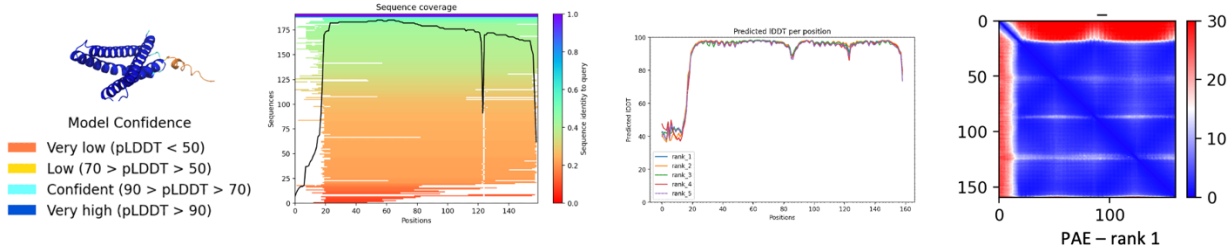

Fig. S 4.6.1: **pLDDT and PAE plots for AlphaFold2 prediction of the C-terminal domain (residues 449 to 607) of MK-D1 OST1.**

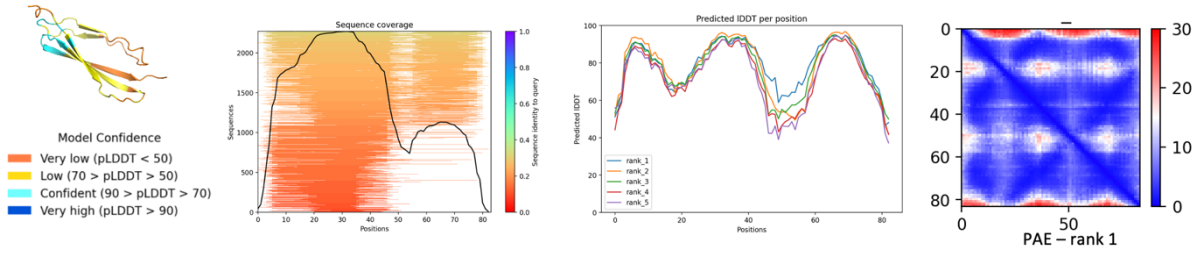

Fig. S 4.6.2: **pLDDT and PAE plots for AlphaFold2 prediction of MK-D1 TRAPβ domain**. The amino acid sequences, 334-416, representing the TRAPβ domain were used to generate AF2 model for MK-D1 TRAPβ domain.

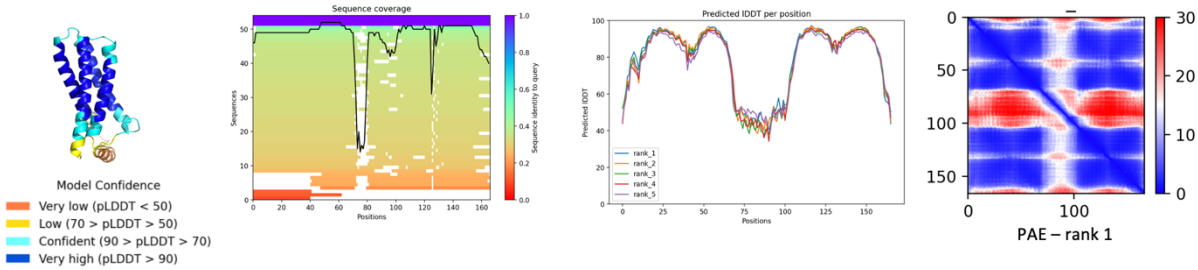

Fig S 4.6.3: pLDDT and PAE plots for AlphaFold2 prediction of MK-D1 TRAPγ.

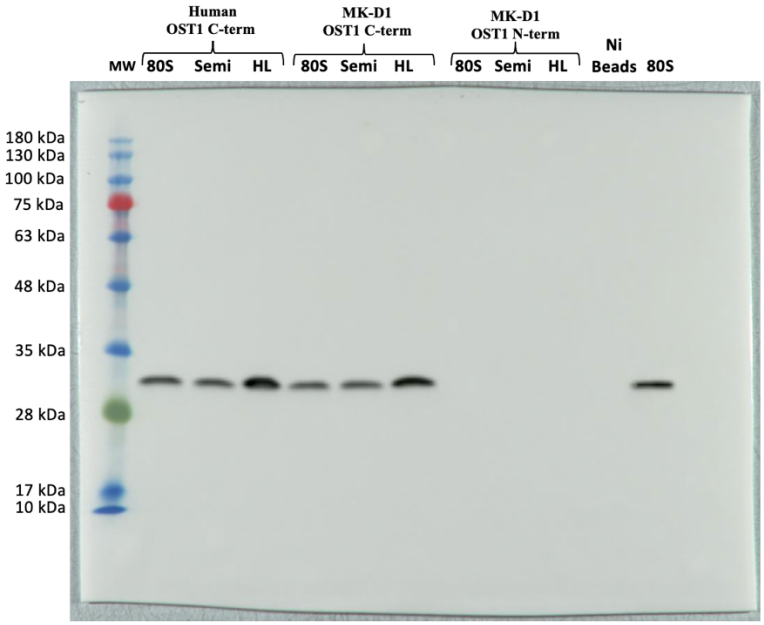

Fig. S 4.7: Full western blot for pull-down based interactions between His-tagged versions of the cytosolic domain of MK-D1 OST1(C-term), cytosolic domain of human OST1 (C-term) and the extracellular domain of MK-D1 OST1 (N-term) with various preparations of ribosomes. 80S, highly purified ribosomes; Semi, semi-purified ribosomes; HL, HeLa cell clarified lysate. Beads, control experiment without His-tagged protein, and the final lane the 80S standard. The Western blot is probed with an antibody against ribosomal protein S3 (RPS3). The quality of the 80S ribosomes is shown in Fig. 3.1.