

Review

# Cotranslational sorting and processing of newly synthesized proteins in eukaryotes

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**Ribosomes interact with a variety of different protein biogenesis factors that guide newly synthesized proteins to their native 3D shapes and cellular localization. Depending on the type of translated substrate, a distinct set of cotranslational factors must interact with the ribosome in a timely and coordinated manner to ensure proper protein biogenesis. While cytonuclear proteins require cotranslational maturation and folding factors, secretory proteins must be maintained in an unfolded state and processed cotranslationally by transport and membrane translocation factors. Here we explore the specific cotranslational processing steps for cytonuclear, secretory, and membrane proteins in eukaryotes and then discuss how the nascent polypeptide-associated complex (NAC) cotranslationally sorts these proteins into the correct protein biogenesis pathway.**

## The various functions of ribosome-associated protein biogenesis factors

Newly synthesized proteins are cotranslationally processed by various protein biogenesis factors that interact with the ribosome at the nascent peptide exit tunnel. These factors exert a variety of different substrate-specific activities on nascent polypeptides, including: (i) enzymatic modification of the N terminus [1–3]; (ii) folding [4–8]; (iii) transport to the endoplasmic reticulum (ER) [9–11]; (iv) membrane translocation [12–14]; and (v) membrane insertion [15–17]. The biogenesis path of a newly synthesized protein is dictated by the factor that binds to the ribosome exit site. The accurate selection of nascent chains by appropriate protein biogenesis factors is thus fundamental for proper protein function in cells, and unspecific binding of nascent chain processing factors to translating ribosomes would cause protein misfolding and mislocalization, with detrimental effects on cell function and viability [18]. How the access of protein biogenesis factors to ribosomes is controlled has long been a mystery, although recent studies reveal the presence of a master regulator that orchestrates cotranslational protein biogenesis steps at the ribosome tunnel exit, the **NAC** (see [Glossary](#)) [19–21].

Here, we provide an overview of ribosome-associated protein maturation and transport factors in eukaryotes: the N-terminal-modifying enzymes and chaperones assisting in the nascent chain folding of **cytonuclear proteins** are first described before we discuss how nascent chains are targeted to the ER for translocation or membrane insertion. We then review recent mechanistic insights into how nascent polypeptides are cotranslationally sorted into the correct biogenesis pathways by the ubiquitous ribosome-associated factor NAC, noting throughout what questions remain to be addressed.

## Cotranslational processing of cytonuclear proteins

### Modification of nascent chain N terminus

Most cytonuclear proteins (80–90%) are initially processed by ribosome-associated enzymes that modify the N terminus of the nascent chain [3,22–26]. In eukaryotes, nine different cotranslational N-terminal-modifying enzymes have been described (Figure 1). These can be divided into three

## Highlights

Precise and efficient processing of nascent polypeptides by appropriate ribosome-associated factors is essential for proper protein function and localization.

Recent studies demonstrate that nascent chain binding by cotranslational factors is precisely orchestrated and not only triggered by random collisions of the factors with translating ribosomes.

Newly synthesized proteins are sorted at the ribosomal tunnel exit and directed into specific cotranslational protein biogenesis pathways.

The nascent polypeptide-associated complex (NAC) is important for the regulation of cotranslational processing.

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classes: the **methionine aminopeptidases (METAPs)**, the **N-acetyltransferases (NATs)**, and the **N-myristoyltransferases (NMTs)** [1,3,22]. METAPs remove the N-terminal methionine that is encoded by the universal translation start codon AUG [27]. NATs and NMTs transfer an acetyl group and a myristoyl group, respectively, to the  $\alpha$ -amino group of the nascent chain N terminus [1,28]. A large fraction of cytonuclear substrates (~40%) require two subsequent enzymatic modifications: first, methionine excision by METAPs, followed by acetylation or myristoylation by NATs or NMTs, respectively (Figure 1, left). The remaining fraction requires only one enzymatic processing step through a NAT family member that directly acetylates the initiator methionine encoded by the translation start codon (Figure 1, right) [1,22–24].

METAPs are universally conserved zinc-dependent metalloproteases that bind near the ribosomal tunnel exit and process nascent chains starting at a length of ~50 amino acids [19,29]. In eukaryotes, two structurally related METAPs are expressed in the cytosol, METAP1 and METAP2 [30,31]. They have similar substrate specificities but different ribosome interaction mechanisms [19,32]. Both enzymes have a tight methionine-binding pocket located deep in the core of the protease, which is accessible only to substrates that have a small and uncharged amino acid at the second position following the N-terminal methionine (A, C, G, P, S, T, or V) [30,31]. Cytonuclear proteins with other amino acids at the second position cannot be processed by METAPs and thus retain their N-terminal methionine (Figure 1).

NATs are a large family of enzymes that catalyze the transfer of an acetyl group from the donor acetyl-coenzyme A to a free  $\alpha$ -amino group of another protein or peptide [1,33]. In eukaryotes, five different ribosome-associated NATs have been described that cotranslationally acetylate the N termini of proteins (NatA, NatB, NatC, NatD, and NatE). These NATs differ in the composition of their subunits and display different substrate specificities (Figure 1). NatA and NatD acetylate the neo-N terminus of a substrate generated by methionine excision via METAPs. NatA has the largest substrate pool (~50%) in the NAT family and acetylates all substrates cleaved by METAPs except proteins with an N-terminal proline. NatD has the smallest substrate pool with only two proteins identified to date, histone H2A and H4. These histones have the same N-terminal sequence motif (MSGRG), which is specifically recognized by NatD after methionine excision by METAPs. The other NATs directly acetylate the N-terminal methionine. Of these, NatB has the largest substrate pool, comprising proteins with D, E, N, or Q in the second position (~30%). The remaining NAT substrates (~20%) are processed by either NatC or NatE, which acetylate the initiator methionine followed by a hydrophobic residue (L, F, I, or Y) or lysine (K) in humans (Figure 1) [1,34–37].

NMTs are a class of enzymes that covalently attach a 14-carbon saturated fatty acid, called myristic acid, to the N terminus of target proteins [38,39]. Two NMT isozymes, NMT1 and NMT2, are ubiquitously expressed in human cells [40]. They cotranslationally transfer a myristoyl group from the donor myristoyl-coenzyme A to proteins that expose a glycine residue at the N terminus after methionine excision via METAPs (Figure 1). N-terminal myristoylation is an essential protein modification that occurs in over 500 different substrates in human cells. Most substrates share a characteristic N-terminal consensus sequence M-G-X-X-X-S/T and have often a positively charged residue at position 7 [2,41–43]. In addition to N-terminal glycine residues, recent studies demonstrated that NMTs can myristoylate the  $\epsilon$ -amino groups of lysine side chains in certain substrates [44,45]. The hydrophobic lipid modification is known to affect the cellular localization of many NMT target proteins and regulate their interaction with cellular membranes [46]. It is not yet clear whether NMTs interact directly with the ribosome. However, NMTs are structurally related to the catalytic NatA subunit Naa10 and many NMT substrates can be acetylated by NatA [39,43]. Therefore, NatA and NMTs may compete for the same binding site at the ribosome tunnel exit.

## Glossary

**Cytonuclear proteins:** proteins localized either in the cytosol or the nucleus of cells.

**ER membrane complex (EMC):** large multisubunit complex residing in the ER membrane. Acts as a membrane insertase for signal anchors with N<sub>exo</sub> topology.

**Membrane proteins:** integral membrane proteins that are inserted into the ER membrane; can be further transported to other endomembrane systems or the plasma membrane.

**Methionine aminopeptidases (METAPs):** zinc-dependent metalloproteases that cotranslationally remove the initiator methionine encoded by the translation start codon.

**N-acetyltransferases (NATs):** enzymes that cotranslationally transfer an acetyl group from acetyl-coenzyme A to the N terminus of proteins.

**Nascent polypeptide-associated complex (NAC):** conserved and abundant heterodimeric complex (NAC $\alpha$  and NAC $\beta$  subunits) that interacts with virtually all ribosomes in the cell. Binds to the ribosome tunnel exit and controls the access of other cotranslational factors.

**N-myristoyltransferases (NMTs):** enzymes that cotranslationally transfer a myristoyl group from myristoyl-coenzyme A to the N terminus of proteins.

**Oligosaccharyltransferase A complex (OST-A):** large, multisubunit complex residing in the ER membrane; interacts with the ribosome during protein translocation through the Sec61 channel; transfers a high-mannose oligosaccharide to asparagine residues in nascent chains.

**PAT complex:** heterodimeric complex (CCDC47 and Asterix subunits) residing in the ER membrane; recognizes semihydrophilic TMDs of multipass proteins; acts as a multipass membrane protein insertase.

**Ribosome-associated complex (RAC):** conserved heterodimeric complex (HSP40 and ATPase-inactive HSP70 subunit) that recruits another ATPase-active HSP70 to nascent polypeptides to facilitate cotranslational protein folding.

**Sec61 translocation pore:** heterotrimeric complex (Sec61 $\alpha$ , Sec61 $\beta$ , and Sec61 $\gamma$  subunits) that resides in the ER membrane; interacts with translating ribosomes; forms a translocation channel through which proteins are cotranslationally

The correct enzymatic modifications of the nascent chain N terminus by METAPs, NATs, and NMTs are essential for proper protein maturation and function. The N-terminal modifications are irreversible and affect a wide range of protein properties including stability, folding, and protein–protein and protein–membrane interactions. Defects in N-terminal processing of proteins have been reported in various human diseases, including cancers and developmental disorders [3,33,47]. To process their substrates effectively, N-terminal-modifying enzymes require free access to the N terminus of a nascent substrate. The ideal time during translation is when the N terminus of a nascent substrate leaves the ribosomal tunnel and before the nascent chain begins to acquire a 3D structure. Therefore, it is generally assumed that these enzymes are the first nascent chain processing factors that bind to translating ribosomes. How the nine competing enzymes gain specific and timely access to the ribosome and its substrates is poorly understood. The recently uncovered ribosome interaction mechanism of METAP1 [19] suggests that ribosome binding of N-terminal-modifying enzymes in eukaryotes is not random but tightly regulated through the recruiting factor NAC (see later). In the lower eukaryote yeast, METAP1 and other N-terminal-modifying enzymes seem to be stabilized on the ribosome by flexible rRNA expansion segments (ESs) that protrude from the core structure of the ribosome [48,49]. How these ES elements are regulated in yeast to allow specific recruitment of enzymes to translating ribosomes is unknown. Whether such ES elements also play a role in enzyme recruitment in higher eukaryotes is unclear. Other N-terminal modifications of proteins have been observed, including propionylation, methylation, palmitoylation, and ubiquitylation [3]. These modifications can be co- or post-translational and are much rarer, and their functions are less well understood.

#### Nascent chain folding

In addition to the N-terminal-modifying enzymes, many cytonuclear proteins associate cotranslationally with chaperones that assist in the folding of the nascent chain. Molecular chaperones usually bind to exposed hydrophobic peptide segments in unfolded proteins that are normally buried in the hydrophobic core of a natively folded protein [50]. Chaperones protect these ‘sticky’ surfaces from unspecific interactions thereby preventing protein misfolding and aggregation. A nascent polypeptide exiting the ribosome, in particular, is prone to premature misfolding due to the constantly changing and incomplete polypeptide sequence due to the vectorial synthesis of a protein from its N to C terminus [51]. Eukaryotes possess one conserved chaperone system that directly interacts with the translating ribosome, the **ribosome-associated complex (RAC)**, a specialized atypical chaperone complex comprising chaperones of the HSP70 and HSP40 families (Figure 2) [4–7].

HSP70s are generally ATP-driven chaperones that bind to hydrophobic peptide stretches in substrates. They comprise an N-terminal nucleotide-binding domain (NBD) and a C-terminal substrate-binding domain (SBD) connected by a flexible linker [52]. The domains are allosterically coupled, with ATP hydrolysis in the NBD causing tight binding of substrates in the SBD. Substrate binding is usually triggered by transient interactions with HSP40 family co-chaperones, which have a conserved J-domain to stimulate ATP hydrolysis in HSP70s [53]. However, RAC is an atypical stable heterodimeric HSP70–HSP40 complex comprising a ribosome-binding HSP40 (ZUO1/DNAJC2) and an ATPase-inactive truncated version of HSP70 (SSZ1/HSPA14). Recent cryoelectron microscopy (cryo-EM) structures of yeast RAC bound to ribosomes revealed surprising features. RAC has an extended structure and binds to both the large 60S and the small 40S subunit of ribosomes (Figure 2) [4,6,8]. The contact on the 60S subunit is located near the polypeptide exit tunnel and is mediated by both subunits of RAC. This contact brings the J-domain of the HSP40 and the SBD of the HSP70 subunit in close proximity to the ribosomal tunnel exit. The contact on the 40S subunit is mediated by the HSP40 subunit and involves an rRNA ES (ES12) that is connected to the decoding and peptidyl transferase center (PTC) of the ribosome (Figure 2) [4,6,8,54].

transported into the ER lumen or the ER membrane.

**Secretory proteins:** soluble proteins that are transported into the ER lumen; can be further transported via the secretory pathway to other destinations or secreted into the extracellular space.

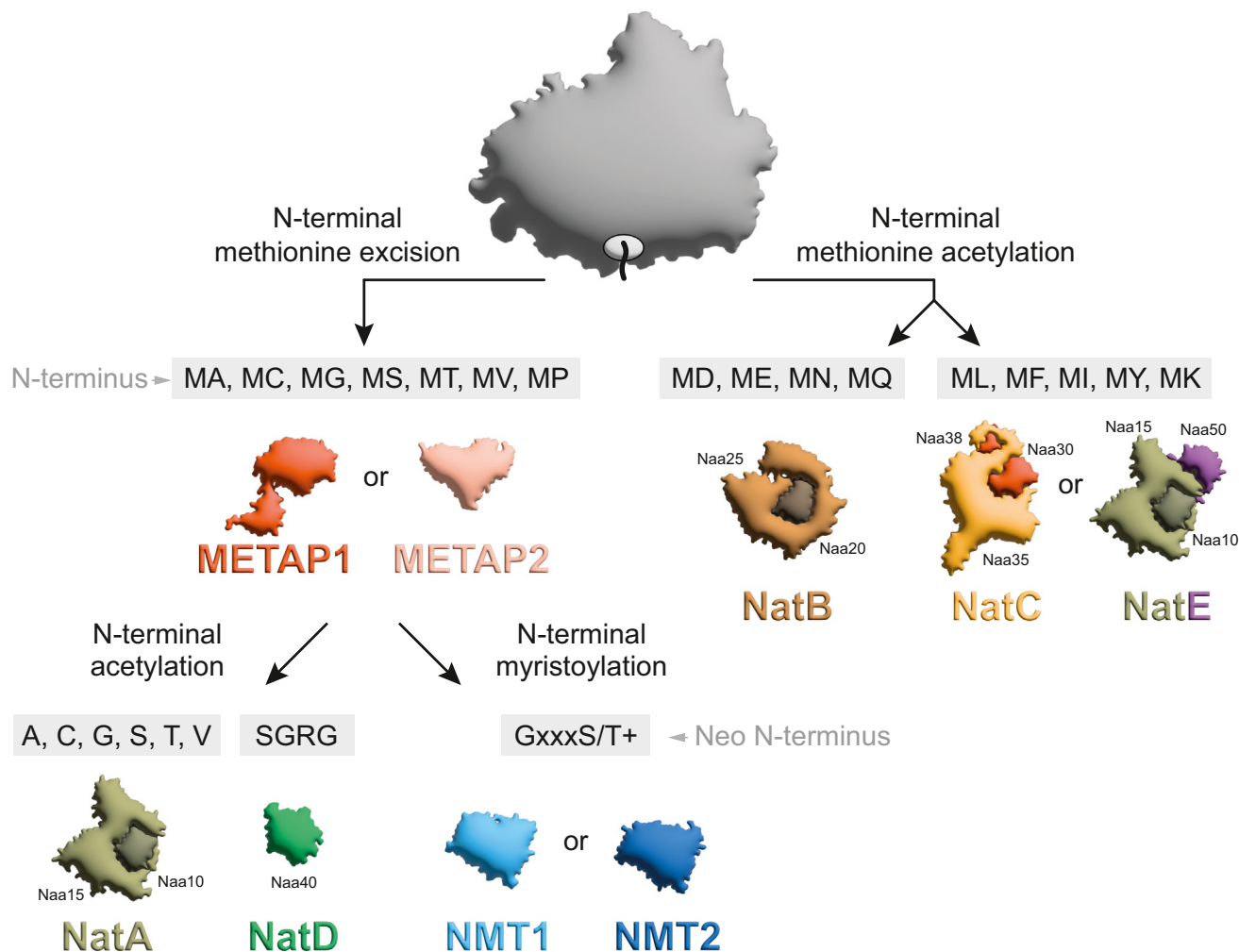
**Signal anchors:** TMDs of integral membrane proteins that serve as ER-targeting signals; recognized by SRP.

**Signal recognition particle (SRP):** GTP-dependent ribonucleoprotein complex that transports translating ribosomes to the ER membrane.

**Signal sequences:** hydrophobic peptide segments in the N termini of proteins that serve as ER-targeting signals; recognized by SRP and removed after successful ER transport.

**SRP receptor (SR):** heterodimeric complex (SR $\alpha$  and SR $\beta$  subunits) anchored in the ER membrane; interacts with SRP in a GTP-dependent manner to transfer translating ribosomes to the translocon pore Sec61.

**Translocon-associated complex (TRAP):** abundant heterotetrameric complex (TRAP $\alpha$ , TRAP $\beta$ , TRAP $\gamma$ , and TRAP $\delta$  subunits) residing in the ER membrane; interacts with translating ribosomes and the Sec61 translocon during protein translocation. Its function is unknown.

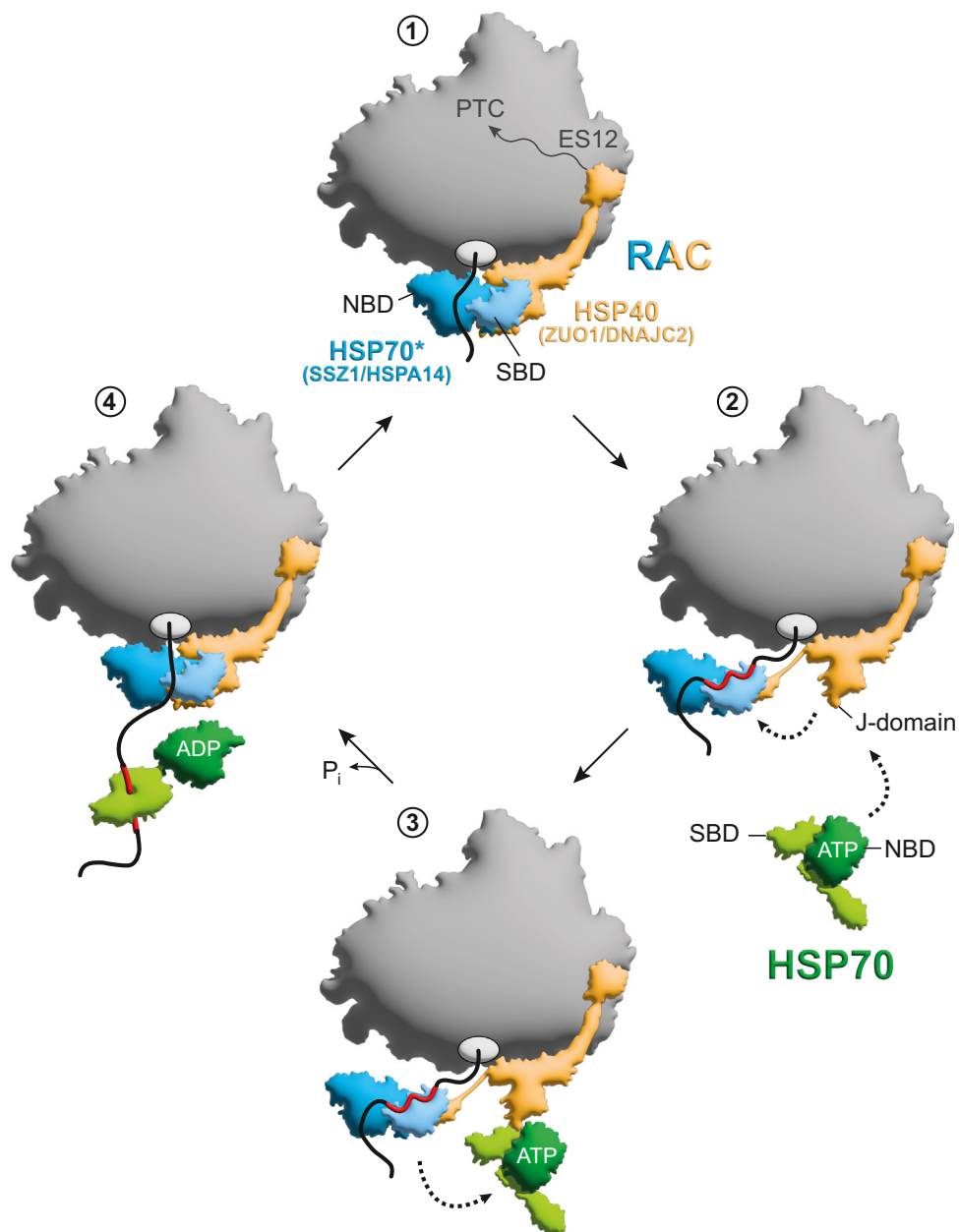


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**Figure 1. Cotranslational N-terminal-modifying enzymes in eukaryotes.** Overview of ribosome-associated enzymes and their substrate specificity in humans. Depending on the second amino acid following the N-terminal methionine (highlighted in gray), cytosolic proteins are processed by methionine aminopeptidases (METAPs), N-acetyltransferases (NATs), or N-myristoyltransferases (NMTs).

Despite decades of research, the chaperone cycle of RAC is still not fully understood mechanistically. It is well established that, with its J-domain, RAC recruits another ATPase-active HSP70 to nascent substrates leaving the ribosomal exit tunnel [55,56]. This suggests that nascent chain folding is triggered by a conventional HSP70 chaperone interaction with ribosome-bound RAC as the ATPase-stimulating HSP40 cofactor. However, the role of the ATPase-inactive HSP70 subunit in RAC remains enigmatic. This HSP70 has a rudimentary SBD that lacks the lid domain required for tight interaction with substrates. However, a recent study suggests that the rudimentary SBD binds to nascent chains before an ATPase-active HSP70 is recruited [7]. Moreover, nascent chain binding in the SBD displaces a flexible N-terminal tail of the HSP40 subunit, which acts as a pseudo-peptide substrate [7]. These findings suggest that substrate binding in the rudimentary SBD causes structural remodeling of RAC.

Consistent with this, recent cryo-EM structures show that the HSP70 subunit of yeast RAC is flexibly tethered to its HSP40 partner and undergoes major structural rearrangements in



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**Figure 2. Cotranslational chaperone function of the ribosome-associated complex (RAC).** RAC comprises an ATPase-inactive HSP70 (blue/HSP70\*; yeast: SSZ1, humans: HSPA14) and an HSP40 subunit (orange; yeast: ZUO1, humans: DNAJC2). The nucleotide-binding domain (NBD) and the substrate-binding domain (SBD) of the HSP70\* subunit bind near the ribosomal tunnel exit. The HSP40 subunit interacts with the large ribosomal subunit near the tunnel exit and the small ribosomal subunit involving an rRNA expansion segment (ES12) that is connected to the peptidyl transferase center (PTC) of the ribosome (1). The SBD of the HSP70\* subunit interacts with sequence motifs in nascent substrates (red). Substrate binding in the SBD is assumed to remodel the complex, leading to the exposure of the J-domain of the HSP40 subunit (2). This leads to the recruitment of a catalytically active HSP70 in the ATP-bound open conformation (green) and the transfer of the nascent substrate (3). Interaction of the HSP70 with the J-domain of RAC induces ATP hydrolysis in the NBD, leading to lid closure and tight binding of the nascent substrate in the SBD of the HSP70 chaperone (4).

response to a translated substrate [4]. In the absence of a nascent substrate, the SBD of the HSP70 seems to block the J-domain of the HSP40 subunit. Conversely, in the presence of a substrate, the HSP70 subunit of RAC detaches from the ribosome and the J-domain is fully exposed (Figure 2) [4]. Taken together, these observations suggest that the HSP70 subunit of RAC serves as a substrate-sensing domain that regulates the J-domain activity of the HSP40 subunit. This mode of action would explain how RAC specifically recruits catalytic HSP70 chaperones to nascent substrates that require chaperone activity for cotranslational folding. While this model is certainly plausible, it is speculative based on the current data and further experiments are needed to confirm this mechanism.

The substrate spectrum of RAC seems to be very broad, including ~80% of all cytonuclear proteins in yeast [55,56]. However, RAC is not stably associated with ribosomes. It is believed that it shuttles on and off the ribosome depending on the folding requirements of the translated substrate. How the binding of RAC to ribosomes is regulated in a temporal and substrate-specific manner is unclear. Furthermore, the ribosome contact of RAC with ES12 at the small 40S subunit is functionally not understood. Since ES12 is structurally coupled to the decoding center of the ribosome, it was hypothesized that RAC could coordinate the decoding speed with the folding process of the nascent chain at the tunnel exit [8]. Supporting this, it has been shown that RAC affects the decoding fidelity and the recognition of stop codons [57]. However, whether RAC indeed mechanistically couples the folding process of the nascent chain at the tunnel exit with the translation process in the decoding center of the ribosome remains to be elucidated. Moreover, the structure and function of RAC have been studied so far mostly in yeast and there is little mechanistic insight into mammalian RAC (mRAC). While the overall architecture of RAC seems to be conserved between yeast and mammals, mRAC possesses two additional protein domains (SANT domains) in the C terminus of the HSP40 subunit with unknown function [58,59]. Whether mRAC serves a substrate pool similar to that in yeast remains to be investigated.

In addition to RAC, general chaperones like Prefoldin, TRiC/CCT, HSP90, and HSP70 also assist in the folding of newly synthesized proteins. Although these chaperones do not bind directly to ribosomes, they interact with nascent polypeptides and continue to act on them after their release from the ribosome to facilitate *de novo* protein folding. These chaperones have an essential role in the folding of newly synthesized proteins and are believed to act downstream of RAC [23,60,61].

## Cotranslational processing of secretory proteins

### Targeting of nascent chains to the ER

Like cytonuclear proteins, secretory and **membrane proteins** are synthesized in the cytosol of cells. However, instead of N-terminal-modifying enzymes, these substrates must initially be processed by the cotranslational ER-targeting factor **signal recognition particle (SRP)** [62]. Eukaryotic SRP is a ribonucleoprotein complex comprising an RNA scaffold and six different proteins (SRP9, SRP14, SRP19, SRP54, SRP68, and SRP72). SRP contacts the large ribosomal 60S subunit near the tunnel exit as well as the ribosomal 40S–60S subunit interface [10]. The main interactions with the ribosome and the nascent substrate are mediated by SRP54, a multidomain protein comprising a ribosome- and GTP-binding NG-domain and a substrate-binding M-domain [9–11]. A large hydrophobic groove in the M-domain interacts tightly with hydrophobic peptide stretches in nascent chains that serve as ER-targeting signals. Such hydrophobic ER **signal sequences** are often located in the N termini of **secretory proteins** and are removed after successful transport by ER-resident signal peptidases [63,64]. Internal transmembrane domains (TMDs) of membrane proteins are also recognized by SRP and thus serve as ER transport signals (referred to as **signal anchors**) [65].

On recognition of an ER-targeting signal by the M-domain, SRP shuttles the translating ribosome to the **SRP receptor (SR)** at the ER membrane (Figure 3; [1]). The SR is a heterodimeric complex comprising a SRP-interacting subunit (SR $\alpha$ ) and an integral ER membrane anchor (SR $\beta$ ) [62]. SR $\alpha$  contains a GTP-binding NG-domain homologous to that of SRP54 and a SRX domain interacting with SR $\beta$ . The NG-domains of SRP54 and SR $\alpha$  interact in a GTP-dependent manner and form a stable heterodimeric complex [9,66]. Formation of the NG complex then promotes GTP hydrolysis and transfer of the ER signal sequence and ribosome to the protein translocation pore Sec61 (Figure 3; [2]).

#### Membrane translocation and insertion of nascent chains

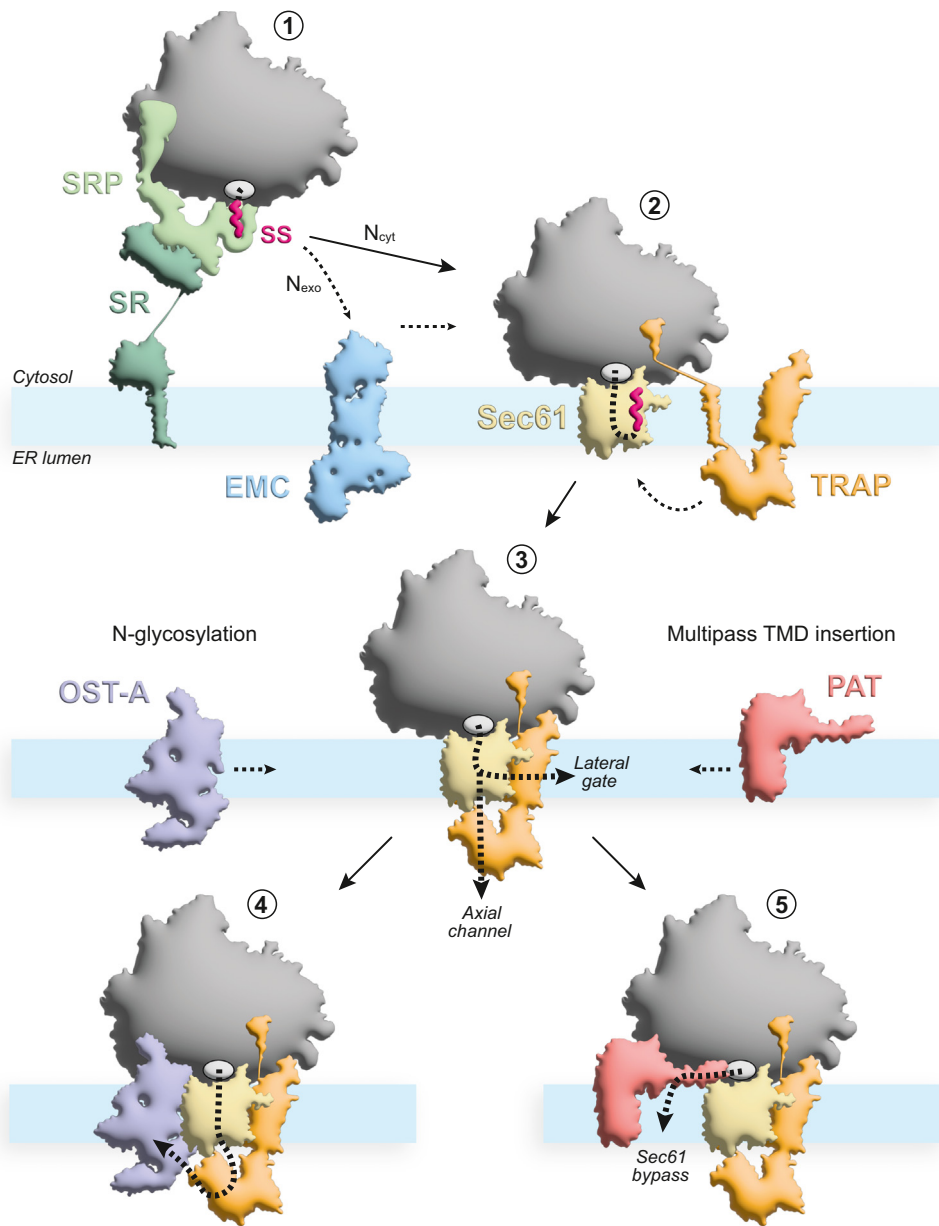
At the ER membrane, ribosomes tightly interact with the **Sec61 translocation pore**, a large, hourglass-shaped, membrane-embedded complex comprising three subunits (Sec61 $\alpha$ – $\gamma$ ) [12–14]. Sec61 forms a membrane-spanning channel that aligns with the ribosomal tunnel to cotranslationally transport proteins into the ER lumen [12,13,67]. Sec61 also possesses a lateral gate that opens towards the lipid bilayer through which substrate TMDs are inserted into the ER membrane (Figure 3; [3]) [12,68]. The prevailing view is that this dual function enables Sec61 to process virtually all of the secretory and membrane proteins in a cell. However, recent studies revealed that proper membrane insertion of a large fraction of substrates requires the assistance of two additional ER membrane insertases that cooperate with Sec61, termed **ER membrane complex (EMC)** and PAT(-GEL-BOS) [15,16,65,69–72].

EMC is a large, membrane-embedded complex comprising ten subunits in mammals (EMC1–10) [71]. EMC samples SRP-targeted ribosomes prior to docking to Sec61 for the presence of signal anchors with N<sub>exo</sub> topology (in which the N terminus faces the exoplasmic side of the membrane) [65]. EMC then inserts these signal anchors into the ER membrane before the ribosome docks onto Sec61 for translocation of the remaining part of the protein. Conversely, signal anchors with N<sub>cyt</sub> topology are directly relayed to Sec61 for membrane integration through the Sec61 lateral gate (Figure 3) [65,69,71].

The **PAT complex**, a heterodimer comprising CCDC47 and Asterix, is specifically recruited to ribosomes translating proteins with multiple TMDs (multipass membrane proteins) [15,16,72]. TMDs of multipass proteins have a characteristic high content of hydrophilic residues. After insertion of the first TMD (via EMC or the Sec61 lateral gate), PAT recognizes these semihydrophilic substrate features in the membrane via the Asterix subunit [15,72]. Substrate binding then recruits the cytoplasmic domain of CCDC47 to the Sec61-docked ribosome, where CCDC47 binds to the mouth of the ribosome exit tunnel to redirect the nascent chain to the ‘back’ of Sec61 opposite to the lateral gate (Figure 3; [5]). There, the PAT complex forms a ‘multipass translocon’ (together with the GEL and BOS complexes [16]), a lipid-filled cavity into which the following TMDs can be inserted independent of the Sec61 lateral gate [15]. The membrane cavity formed at the back of Sec61 likely provides a protected environment in which TMD helix bundles of multipass proteins can properly form.

#### Processing of nascent chains in the ER lumen

Depending on the nature of the translated substrate, additional ER protein biogenesis factors are recruited to membrane-bound ribosomes. One abundant factor that broadly interacts with translating ribosomes is the tetrameric **translocon-associated complex (TRAP)**, a membrane-spanning complex with a large ER luminal domain (Figure 3) [13,17,73,74]. TRAP is essential for the proper biogenesis of many secretory proteins, among them peptide hormones such as insulin and angiotensin [13,75–77]. In addition to secretory proteins, TRAP is likely to play a role in multipass protein biogenesis, as TRAP is often found associated with Sec61-bound ribosomes



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**Figure 3. Cotranslational processing of secretory proteins.** Ribosomes displaying a signal sequence (SS) are targeted to the endoplasmic reticulum (ER) membrane by the signal recognition particle (SRP) and its receptor (SR) (1). Signal anchors with  $N_{exo}$  topology are inserted by the ER membrane complex (EMC), while signal anchors with  $N_{cyt}$  topology and N-terminal signal sequences are directly transferred to the ER translocon complex (Sec61) (2). The ribosome-Sec61 complex then associates with the translocon-associated complex (TRAP), whose function is poorly understood. The ribosome-Sec61-TRAP complex can facilitate the translocation of nascent chains into the ER lumen as well as transmembrane domain (TMD) insertion through a lateral gate of Sec61 (3). The ribosome-Sec61-TRAP complex can be joined by the oligosaccharyltransferase A complex (OST-A), which glycosylates asparagine residues in nascent chains in the ER lumen (4). Multipass substrates are specifically recognized by the PAT complex, which binds at the mouth of the ribosomal tunnel exit and redirects the nascent chain to bypass the Sec61 lateral gate for multipass TMD insertion (in conjunction with the GEL and BOS complex) (5).

together with the PAT complex [15–17]. Knockout of TRAP in animal and cell models results in a strong and ubiquitous ER stress response, suggesting that TRAP has a fundamental function in maintaining ER protein homeostasis [13,76,78]. However, the molecular function of TRAP during ER protein biogenesis is mechanistically not understood. A recent cryo-EM study demonstrated that TRAP engages translating ribosomes via a flexibly attached anchor domain of the TRAP $\alpha$  subunit [13]. This anchor is tethered to the ER membrane by a long flexible linker and could therefore interact with ribosomes early in the ER targeting process, theoretically even before the ribosome is docked onto Sec61 (Figure 3; [2]). TRAP could therefore stabilize SRP-targeted ribosomes near the ER membrane and promote the transfer of the ribosome to the Sec61 translocon. Consistent with such a function, TRAP was found to be critical for the translocation of proteins bearing weak ER-targeting signal sequences (low hydrophobicity and higher glycine/proline content) that engage the Sec61 translocon less efficiently [77,79,80]. However, a role of TRAP in ER targeting is speculative and requires further investigation. A second ribosome contact of TRAP is mediated by TRAP $\gamma$  via a short, protruding finger-like domain that contacts the ribosome near the ribosomal tunnel exit. This contact is likely to ensure proper positioning of the TRAP ER luminal domain underneath the Sec61 axial channel exit (Figure 3; [3]) [13,17,74]. The luminal domain of TRAP has the shape of a cradle and contains many hydrophobic surface patches. It was therefore suggested that TRAP might interact with hydrophobic peptide segments in nascent chains entering the ER lumen to protect them from misfolding and aggregation, similar to molecular chaperones such as bacterial trigger factor [13]. Whether TRAP indeed acts as a cotranslational ER chaperone remains to be established.

The TRAP-Sec61 translocon complex is often joined by another large multisubunit complex, the **oligosaccharyltransferase A complex (OST-A)** (Figure 3; [4]) [17]. OST-A is a membrane-spanning complex that simultaneously interacts with the ribosome on the cytosolic side and Sec61 in the ER lumen [81,82]. It catalyzes the transfer of a high-mannose oligosaccharide to asparagine residues (called N-glycosylation) in nascent chains with specific sequence motifs (N-x-T/S). N-glycosylation is an essential cotranslational protein modification that occurs only in the ER lumen. N-glycans are attached to ~10% of all proteins in humans and are crucial for proper protein folding and transport [83]. The co-association of OST-A and TRAP with Sec61 suggest that the two factors can simultaneously engage nascent chains in the ER lumen. Consistent with this, it has been reported that TRAP is important for proper N-glycosylation of proteins [78].

### Cotranslational sorting of cytonuclear and secretory proteins

The cotranslational processing steps for cytonuclear and secretory proteins differ substantially. Secretory proteins must be kept unfolded and cotranslationally transported to the ER membrane, whereas cytonuclear proteins need to be cotranslationally modified at the N terminus and folded to their native 3D structure in the cytosol. Since cytonuclear and secretory proteins are synthesized in the same cellular compartment, it must be ensured that the dedicated nascent chain processing factors bind specifically to the ribosome exit site. Incorrect binding of SRP to ribosomes translating cytonuclear or mitochondrial proteins would result in mislocalization of proteins in the ER [18]. Conversely, the enzymatic modification of N-terminal ER signal sequences or premature folding of the nascent chain in the cytosol would inhibit protein translocation across the ER membrane [84,85]. Thus, aberrant interactions of cotranslational factors with translating ribosomes would have fatal consequences for cell function and viability. Because cotranslational factors have an intrinsic binding affinity to the ribosome and compete for access to the tunnel exit, it has long been a mystery how they gain substrate-specific and timely access to translating ribosomes. Two recent studies have shed more light on this problem, showing that the binding of cotranslational factors is tightly controlled in eukaryotes by a higher-level ribosome-associated factor, NAC [19,21].

NAC is an abundant heterodimeric complex (NAC $\alpha$  and NAC $\beta$ ) that binds to virtually all translating ribosomes in the cytosol of a cell [20,86,87]. NAC comprises a central globular dimerization domain from which four long flexible arms (N and C termini) protrude (Figure 4). The N-terminal arm of the NAC $\beta$  subunit serves as a ribosomal anchor that flexibly tethers NAC to the periphery of the ribosomal tunnel exit. A second ribosome contact is mediated by the central globular domain that binds directly at the tunnel exit via two amphipathic antiparallel  $\alpha$ -helices [19,21]. *In vitro* studies suggest that NAC already interacts with ribosomes during translation initiation when the ribosomal tunnel is not yet occupied by a nascent polypeptide [20,88]. At this stage, the N-terminal arm of NAC $\beta$  may insert deeply into the empty ribosomal tunnel, while the globular domain of NAC is positioned in front of the tunnel exit (Figure 4). In this conformation, NAC is believed to block the access of virtually all nascent chain processing factors to prevent them from unproductive interactions with inactive ribosomes [20]. When translation proceeds, the elongating nascent chain displaces the NAC $\beta$  arm from the tunnel, which then relocates to another binding site on the surface of the ribosome, while the globular domain of NAC binds adjacent to the tunnel exit, ready to scan the nascent chain leaving the ribosomal tunnel (Figure 4) [19–21]. This unique ribosome interaction mechanism is likely to ensure that NAC is always bound to the ribosome when the N terminus of a new polypeptide exits the tunnel.

On ribosomes translating cytonuclear proteins, NAC retains this position, with the globular domain attached to the ribosomal tunnel exit [19,21]. In this conformation, the globular domain of NAC has two important effects: it blocks the access of SRP while at the same time it provides a binding platform for METAP1 [19,21]. This mechanism ensures that cytonuclear proteins are not mistargeted to the ER but efficiently processed by the N-terminal-modifying enzyme. METAP1 is actively recruited to this binding platform by the long and flexible C-terminal arm of NAC $\beta$  (Figure 4, left). Structural analyses revealed that a conserved hydrophobic motif (<sup>146</sup>VPDLV<sup>150</sup> in human NAC) at the end of the NAC $\beta$  arm specifically interacts with the N-terminal zinc-finger domain of METAP1 [19]. *In vivo* experiments demonstrated that this interaction is essential for ribosome binding of the enzyme and N-terminal methionine excision by METAP1 in cells [19]. Moreover, tight binding of the globular domain of NAC to the ribosome tunnel exit is also critical for functional docking of the enzyme. Thus, with its globular domain NAC inhibits SRP while at the same time it promotes METAP1 binding, ensuring that cytonuclear proteins are efficiently processed by METAP1 and retained in the cytoplasm of cells [19]. Whether NAC also facilitates the ribosome binding of other N-terminal-modifying enzymes is unknown.

Remarkably, the situation is exactly reversed on ribosomes translating secretory proteins; here, NAC inhibits METAP1 while promoting SRP binding [19,21]. How is this achieved? Central to this mechanism is the globular domain of NAC. When bound to the ribosome, it provides a METAP1-binding platform while blocking a critical SRP-binding site at the tunnel exit [19]. By contrast, detachment of the globular domain disrupts the METAP1-binding platform and releases the SRP-binding site on the ribosome [21]. Thus, on ribosomes translating secretory proteins, the globular domain of NAC needs to be released from the tunnel exit. This release is triggered by the nascent substrate itself. Hydrophobic ER-targeting signals in nascent chains invade a hydrophobic pocket below the two  $\alpha$ -helices that attach the globular domain of NAC to the ribosome exit site (Figure 4, right) [21]. This disrupts the ribosome-binding platform of the NAC globular domain, which detaches and thus abrogates the SRP antagonism. However, NAC remains bound to the ribosome via the N-terminal NAC $\beta$  anchor, which binds outside the SRP-binding region [19,21].

The conformational change in NAC induced by ER-targeting signals thus allows binding of SRP while preventing docking of METAP1. Moreover, in this specific NAC conformation SRP is even actively recruited to the ribosome through an interaction between the C-terminal arm of NAC $\alpha$

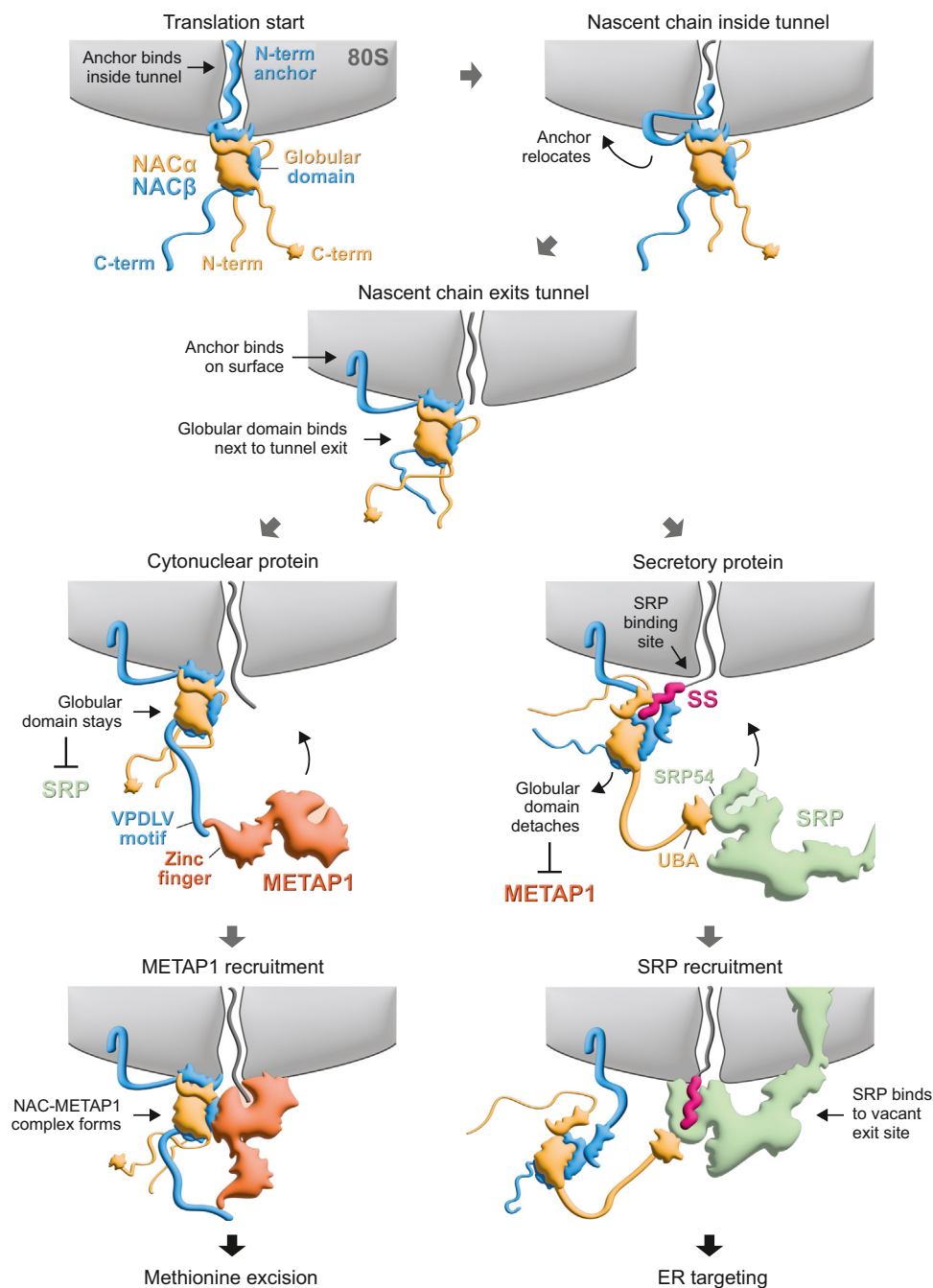
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Figure 4. Cotranslational sorting of cytonuclear and secretory proteins by the nascent polypeptide-associated complex (NAC). Overview showing the ribosome-interaction mechanisms of NAC depending on the length and nature of the translated substrate. In the absence of an endoplasmic reticulum (ER)-targeting signal (SS), NAC blocks signal recognition particle (SRP) binding with its globular domain while specifically recruiting methionine aminopeptidase 1 (METAP1) to ribosomes via the NAC $\beta$  C-terminal arm (left). By contrast, in the presence of an ER-targeting signal the globular domain of NAC detaches, which disrupts the METAP1 binding platform while allowing the recruitment of SRP via the NAC $\alpha$  C-terminal arm (right).

and the SRP54 subunit of SRP (Figure 4, right). Structural and biochemical experiments revealed that the conserved ubiquitin-associated domain (UBA) at the end of the NAC $\alpha$  arm interacts specifically with the NG-domain of SRP54 [21]. Thus, during ER targeting a stable ternary NAC-SRP-ribosome complex is formed. Whether NAC plays a role in the ER-targeting process after signal sequence handover to SRP is unclear. Interestingly, the NAC-UBA binding site on SRP54 overlaps with the binding site of the NG-domain of SR $\alpha$ , suggesting that the NAC-UBA is finally displaced by the SR at the ER membrane [21].

*In vivo* data demonstrate that abrogation of the SRP regulatory functions by NAC has detrimental effects on ER protein homeostasis and cell viability. Disruption of the SRP antagonism by mutation of the ribosome-binding platform of the NAC globular domain results in nonspecific binding of SRP and thus mislocalization of proteins in the ER. By contrast, disruption of the NAC-UBA-SRP54 interaction leads to inefficient binding of SRP to nascent ER substrates and thus impairs the fidelity of ER protein transport in cells. These studies show that both the specificity and the efficiency of ER protein transport in cells depend on NAC [19–21].

### Concluding remarks

The functional interactions of NAC with SRP and METAP1 are two examples of how the precise and efficient processing of nascent polypeptides by ribosome-associated factors is ensured in eukaryotic cells. NAC is an abundant factor that is expressed equimolar to ribosomes, allowing it to associate with all translating ribosomes in the cell. Thus, NAC appears to be an integral structural component of the ribosome exit site able to adopt substrate-specific conformations to allow or block the access of other protein biogenesis factors. By altering the position of its globular domain at the tunnel exit, NAC determines the binding specificity of nascent chain processing factors. At the same time, NAC also increases the efficiency of factor binding through long-range interactions mediated by its C-terminal ‘grabbing arms’.

These findings suggest that, in eukaryotes, substrate binding by cotranslational factors is actively managed and not only triggered by random collisions of the factors with translating ribosomes. This mechanism explains how low-abundance factors like SRP and METAP1 gain efficient access to their specific nascent substrates. Whether similar recruitment mechanisms exist for other cotranslational protein biogenesis factors remains to be investigated in future studies (see Outstanding questions).

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### Declaration of interests

No interests are declared.

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### Outstanding questions

The most recent advances in understanding the mechanisms of cotranslationally acting protein biogenesis factors have provided captivating insights into how they coordinate spatially and temporally. However, these discoveries have also ignited curiosity, as they have introduced intriguing new questions that warrant further exploration.

- How is the access of the different N-terminal modifying enzymes (METAPs, NATs, and NMTs) to the ribosome coordinated to ensure efficient and specific processing of nascent chains?
- How does the nascent chain folding chaperone RAC gain timely and specific access to ribosomes translating cytonuclear proteins? What is the signal that recruits RAC to translating ribosomes?
- What is the molecular function of the abundant Sec61-associated protein biogenesis factor TRAP?
- What is the purpose and functionality of the NAC’s tunnel insertion and how does it work in the context of its function as a molecular control hub at the ribosome tunnel exit?
- Like ER proteins, most mitochondrial proteins also have N-terminal-targeting sequences. Are these mitochondrial proteins also cotranslationally sorted by NAC or another factor and shuttled into the proper transport pathway?

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