Functional analysis of the nascent polypeptide-associated complex using an in vitro crosslinking approach

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Abstract

The sorting of proteins into appropriate compartments is fundamental for proper cell function. Newly synthesized proteins must be transported to their subcellular destination with high specificity and in a timely manner to prevent protein misfolding, unproductive interactions with incorrect factors, and premature degradation. In eukaryotes, the transport of secretory and integral membrane proteins generally occurs in a co-translational manner. Ribosomes translating these proteins are targeted to the Sec61 translocation pore of the endoplasmic reticulum (ER) by the signal recognition particle (SRP). Transport specificity is achieved by SRP and Sec61 by recognizing hydrophobic targeting signals in nascent substrates. However, both factors can interact indiscriminately with ribosomes via high-affinity binding sites near the ribosomal tunnel exit. Thus, to ensure fidelity of transport, aberrant SRP and Sec61 interactions must be antagonized on ribosomes translating non-secretory proteins. This antagonistic function in ER targeting is carried out in eukaryotes by the nascent polypeptide-associated complex (NAC), a ubiquitous and abundant ribosome interacting factor with a central globular domain and flexible C- and N-terminal tails. The mechanism of how NAC controls SRP and Sec61 access to translating ribosomes is unknown.

To better understand the antagonistic function of NAC in ER targeting, a series of site-specific photo-crosslinking experiments were performed to map direct interactions of NAC with the ribosome and nascent substrates. The crosslinking experiments revealed an unusual mechanistic feature of NAC: the flexible and positively charged N-terminus of βNAC (N-βNAC) inserts deep into the empty ribosomal tunnel before translation starts. Cryogenic electron microscopy (cryo-EM) data showed that the globular domain of NAC outside the tunnel occupies a critical binding platform on ribosomes that is used by Sec61 and other factors. This suggests that NAC antagonizes Sec61 upon tunnel insertion. Indeed, follow-up studies in vivo in the nematode Caenorhabditis elegans showed that tunnel insertion by NAC is important to prevent aberrant Sec61-ribosome interactions. Thus, NAC resets ribosomes after translation termination and protects the tunnel exit site from unproductive interactions with the ER translocation pore to maintain ER targeting specificity. Furthermore, crosslinking experiments with ribosome nascent chain complexes
(RNCs) revealed that N-βNAC contacts very short nascent chains (10 amino acids) inside the tunnel. As the length of the nascent chain increases, N-βNAC is displaced from the tunnel and binds to the ribosomal protein eL22 on the surface near the tunnel exit. A conserved negatively charged motif (α-DSD) present in the flexible N-terminus of αNAC (N-αNAC) was found to be critical for regulating N-βNAC binding dynamics. A NAC variant lacking this motif strongly inhibited in vitro translation of a model substrate partially by clogging the ribosomal tunnel.

The identified alternate binding site of N-βNAC at eL22 on the surface of ribosomes suggests that NAC also regulates the access to the ribosomal exit site in the presence of longer polypeptide chains. This was confirmed by a cryo-EM structure of NAC bound to ribosomes translating a cytosolic substrate. In addition to N-βNAC at eL22, a second ribosome interaction was revealed that attaches the globular domain of NAC directly to the tunnel exit, where it occupies a binding site of SRP. This second binding site is essential to antagonize SRP both in vitro and in vivo. Moreover, binding of the globular domain is facilitated by two flexible amphipathic helices covering a conserved hydrophobic pocket. Photo-crosslinking experiments showed that this pocket specifically interacts with hydrophobic targeting signals in nascent chains. Engagement of the pocket with signal peptides leads to the detachment of the NAC globular domain from the ribosome, releasing the SRP binding site. Interference with signal peptide binding in the pocket results in protein secretion defects and ER stress in vivo. Therefore, efficient protein transport depends on the recognition of signal sequences in the hydrophobic pocket of NAC, which reverses the antagonistic effect on SRP. Interestingly, while the globular domain is released from ribosomes translating ER substrates, N-βNAC stays bound at eL22. These data suggest that NAC binds targeting sequences before SRP and that a coordinated substrate transfer occurs, which involves a ternary NAC-SRP-RNC complex.

In sum, this study reveals the molecular mechanism of how NAC sustains ER targeting specificity. The structural insights obtained by photo-crosslinking and cryo-EM data point out how NAC interacts with inactive and translating ribosomes and explain how NAC antagonizes Sec61 and SRP. Moreover, the study reveals the mechanism of how the ER targeting inhibition is reversed on ribosomes translating ER substrates, through a signal peptide induces a conformational change of NAC on ribosomes that releases the SRP binding site. These findings lay the groundwork for a deeper understanding of how newly synthesized proteins are sorted and directed into specific protein biogenesis pathways in eukaryotes.
Zusammenfassung


Der Mechanismus, wie NAC den Zugang von SRP und Sec61 zu translatierenden Ribosomen kontrolliert, ist unbekannt. Um die antagonistische Funktion von NAC beim ER-Targeting besser zu verstehen, wurde eine Reihe von positionsspezifischen Crosslinking-Experimenten durchgeführt, um direkte Interaktionen von NAC mit dem Ribosom und naszierenden Substraten aufzuzeigen. Die Crosslinking-Experimente enthüllten ein ungewöhnliches mechanistisches Merkmal von NAC: Der flexible und positiv geladene N-Terminus von βNAC (N-βNAC) inseriert tief in den leeren ribosomalen Tunnel, bevor die Translation beginnt. Die Kryoelektronenmikroskopie (Kryo-EM) zeigte, dass die globuläre Domäne von NAC außerhalb des Tunnels eine kritische Bindungsplattform auf Ribosomen besetzt, die von Sec61 und anderen Faktoren genutzt wird. Dies deutet darauf hin, dass NAC durch die Tunnelinsertion die Sec61 Bindung inhibiert. Tatsächlich konnte *in vivo* in dem Fadenwurm *Caenorhabditis elegans* gezeigt werden, dass die Tunnelinsertion von NAC wichtig ist, um unerwünschte Sec61-Ribosomen-Interaktionen zu verhindern. Demnach kann
Zusammenfassung

NAC durch die Tunnelinsertion Ribosomen nach der Translation in den Ausgangszustand zurückversetzen und von der ER Membran lösen. Zudem schützt es den Tunnelausgang vor unproduktiven Interaktionen mit der ER-Translokationspore, um die ER-Targeting-Spezifität aufrechtzuerhalten.


Zusammenfassend zeigt diese Studie den molekularen Mechanismus auf, wie NAC die ER-Targeting-Spezifität aufrechterhält. Die strukturellen Einblicke, die durch
Crosslinking-Experimente und Kryo-EM-Daten gewonnen wurden zeigen, wie NAC mit inaktiven und translatierenden Ribosomen interagiert und erklären, wie NAC Sec61 und SRP antagonisiert. Darüber hinaus enthüllt die Studie den Mechanismus, wie diese antagonistische Funktion spezifisch an Ribosomen, welche ER-Substrate translatieren umgekehrt wird. Ein Signalpeptid bewirkt eine Konformationsänderung von NAC auf diesen Ribosomen, was die SRP-Bindestelle freisetzt. Diese Erkenntnisse legen den Grundstein für ein tieferes Verständnis dafür, wie neu synthetisierte Proteine in Eukaryoten gezielt in spezifische Wege der Proteinbiogenese geleitet werden.
1 Introduction

The present work deals with processes of protein biogenesis at the ribosome that occur near the tunnel exit. The timely and accurate recognition of nascent polypeptide chains by specific ribosome-associated factors is fundamental to ensure correct protein folding and transport in cells. As a crucial binding platform, the ribosomal exit site allows various protein biogenesis factors to initiate specific protein maturation and transport processes in a co-translational manner. The structure and function of relevant factors involved in co-translational protein biogenesis are briefly discussed in the following.

Figure 1: The ribosome as a binding platform for protein biogenesis factors. A selection of protein biogenesis factors that compete for binding at the ribosomal exit tunnel. The cross-section through the large ribosomal subunit shows the ribosomal exit tunnel. A ribosome-bound aminoacyl-tRNA is depicted in blue, peptide bond formation to the growing nascent chain (NC) takes place at the peptidyl transferase center (PTC). As modifying and folding factors MAP (methionine aminopeptidase), NAT (N-acetyltransferase) and RAC (ribosome-associated complex) are mentioned. Targeting and transport are mediated by SRP (signal recognition particle), SR (signal recognition particle receptor), and Sec61 (a major component of the ER translocon complex). The nascent polypeptide-associated complex (NAC) is not assigned to either of the two groups, as it is a multifunctional complex that has various functions on the ribosome. Figure was modified from (Gense and Gamerdinger 2020) under a CC BY 4.0 license.
1 Introduction

1.1 The ribosome

Protein biosynthesis is defined by the central dogma of molecular biology introduced by Francis Crick, who first described the connection of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and proteins (Crick 1970). DNA is transcribed into messenger RNA (mRNA), which is a template by ribosomes to translate proteins. Ribosomes were first described in 1955 as “small, rounded bodies” observed under the electron microscope (Palade 1955). Since then, technical advances in X-ray crystallography and cryo-electron microscopy (cryo-EM) have provided detailed molecular insights into the structure and function of ribosomes (Ban et al. 2000, Schluenzen et al. 2000, Wimberly et al. 2000).

Decoding of mRNA and catalysis of peptide bond formation are carried out by the small and large ribosomal subunit, respectively, which dimerize during translation forming an 80S ribosomal complex in eukaryotes (S, Svedberg sedimentation coefficient). The small ribosomal subunit (40S in eukaryotes) enables pairing of an mRNA codon with the anticodon of a transfer RNA (tRNA) charged with a specific amino acid (aa). It consists of one rRNA (ribosomal RNA) chain (18S) and 33 proteins (Figure 2). Connection of the individual peptides is catalyzed in the peptidyl-transferase-center (PTC) of the large ribosomal subunit (60S in eukaryotic cells), which consists of 46 proteins and three different rRNAs (25S, 5.8S, and 5S) (Ben-Shem et al. 2011) (Figure 2). The 40S subunit and the 60S subunit combined make up the 80S ribosomal complex, active in translation.

At the interface of both subunits lay three tRNA-binding sites termed “A” (aminoacylation), “P” (peptidyl), and “E” (exit) site, where the synthesis of a polypeptide chain proceeds from the N- to the C-terminus of a protein. A peptide bond is then formed upon binding of an amino-acylated tRNA at the A-site. Subsequently, the A- and P-site tRNAs are translocated to the P- and E-site, respectively, along with the mRNA, resulting in an empty A-site that is bound by the next decoding tRNA. By repeating this cycle, an mRNA-encoded polypeptide chain is formed that exits the ribosome through a narrow ribosomal tunnel in the large subunit (see chapter 1.2). Although the structural core and mechanism of action are largely conserved, eukaryotic ribosomes are larger and more complex than those of prokaryotes. This is mainly due to the higher content of rRNA, which increases with organism complexity (Petrov et al. 2014). Large expansions of rRNA, which protrude from the ribosomal surface, are called expansion segments (ES). ES vary greatly regarding sequence and length among different species and can be highly mobile and flexible. They serve diverse functions in ribosome biogenesis (Ramesh and Woolford 2016), translational...
regulation (Fujii et al. 2018), and recruitment of ribosome-associated factors (Lee et al. 2016, Zhang et al. 2014, Knorr et al. 2019).

**Figure 2: The ribosome.** An overall structure of the human 80S ribosome obtained by high-resolution single-particle cryo-EM in 2015. Ribosomal proteins of the small (S) and the large (L) subunit are indicated. The L1 stalk (mobile domain of the large ribosomal subunit), the P stalk (part of the GTPase center), and the central protuberance (CP) describe the three protrusions at the large ribosomal subunit. Figure was modified from (Khatter et al. 2015). ©2015, Nature Publishing Group, a division of Macmillan Publishers Limited. License received via the copyright clearance center, Inc.
1.2 The ribosomal tunnel

The ribosomal tunnel has a length of 100 Å and an average width of 15 Å. The nascent polypeptide can interact with the tunnel interior, which is predominantly lined by 28S rRNA characterized by a negative electrostatic potential (Lu and Deutsch 2008). A narrow tunnel constriction site is found adjacent to the PTC, which is formed by loops of rRNA and the r-proteins uL4 and uL22 (Figure 3A) (Nissen et al. 2000). In general, the tunnel forms a predominantly hydrophilic environment, is quite flexible, and can adapt to the properties of various nascent chains regarding charge and conformation (Lu et al. 2007). This allows the first steps of protein folding already to take place in the narrow tunnel, whereby the more negative electrostatic potential near the tunnel exit could promote the formation of α helices. The occurrence of more significant folding events is hindered by the tunnel properties, favoring a stretched conformation of the nascent polypeptide (Lange et al. 2016). If the nascent chain adopts a linear conformation, about 30-35 aa are covered by the tunnel walls (Malkin and Rich 1967, Bernabeu and Lake 1982). There are extensive interactions between nascent chains and ribosomes over the entire length of the exit tunnel (Figure 3B) (Seidelt et al. 2009).

Figure 3: The ribosomal tunnel. (A) A schematic cross-section of the 80S ribosome is shown. The P-site tRNA-linked nascent chain (dark yellow) and the tunnel constriction site formed by the ribosomal proteins uL4 (green) and uL22 (purple) are highlighted. Nascent chains also contact the ribosomal protein eL39 (magenta) directly at the tunnel exit. (B) Schematic cross-section showing the dimensions of the ribosomal tunnel. The nascent chain is shown in yellow. Only in the lower tunnel region nascent chains can form first secondary structures like α helices. PTC, peptidyl-transferase-center. Figure was modified from (Bhushan et al. 2010). ©2015, Nature Publishing Group, a division of Macmillan Publishers Limited. License received via the copyright clearance center, Inc.
1.3 The ribosomal tunnel exit

Encircled by a ring of ribosomal proteins, the tunnel exit is the docking point of many co-translational protein biogenesis factors, including chaperones, N-terminal-modifying enzymes and targeting factors. Therefore, binding of the different factors must be well-orchestrated to prevent incorrect or inappropriate nascent chain interactions, which could lead to protein misfolding or mistargeting.

In eukaryotic ribosomes, the docking platform consists of several r-proteins, including eL39, uL24, uL29, eL22, eL19, uL22, eL31, and uL23, as well as rRNA segments surrounding the exit site (Figure 4) (Ban et al. 2000, Nissen et al. 2000). The ribosomal proteins uL23 (Kramer et al. 2002) and uL29 have been defined as universal adaptor site for multiple nascent chain interaction factors on the ribosomal surface (Nyathi and Pool 2015, Halic and Beckmann 2005). Amongst them are the signal recognition particle (SRP) (Voorhees and Hegde 2015), methionine aminopeptidases (MAP) (Wild et al. 2020), NAC (Wegrzyń et al. 2006), and many others. Some important exit site binding factors are briefly discussed in the following chapters.

Figure 4: View on the exit of the ribosomal tunnel. Shown is the 60S subunit of a yeast ribosome. The tunnel exit is marked as a white circle. The ribosomal proteins eL39 (light pink), uL29 (green), uL23 (dark blue) eL19 (orange), eL22 (light blue), eL31 (yellow), uL22 (turquoise), and uL24 (magenta) are highlighted (PDB ID: 4V88).
1.4 Co-translational enzymatic modifications

The correct co-translational enzymatic processing of nascent chains has a critical impact on protein stability and functionality (Sandikci et al. 2013). Figure 5 provides an overview of the most common modifications of nascent chains in eukaryotes. However, space and time for binding are limited at the ribosomal tunnel. Thus, there is a rising interest in how binding at the exit site is coordinated (Raue et al. 2007). The very first enzymatic process in eukaryotic cells is methionine excision from 70% to 90% of cytosolic proteins (Kramer et al. 2019). The corresponding enzymes are the MAPs. They are essential in all kingdoms of life, but in bacteria, the first enzymatic process is the deformylation of the N-formylated initiator methionine, followed by MAP cleavage. In eukaryotes, two types of cytosolic MAPs are known. In yeast, the two enzymes seem to have at least partially overlapping functions, as the deletion of one of the genes leads to slow growth, but the deletion of both genes is lethal (Li and Chang 1995). Additionally, there are organelle-specific MAPs (Kramer et al. 2019). Ribosome binding in bacteria is mediated via a charged loop that is crucial for nascent chain processing to bL17 and uL23, which is in close proximity of the tunnel (Sandikci et al. 2013, Figure 4). Also in yeast uL23 was described as a docking site of Map1, as well as uL29 (Nyathi and Pool 2015). Other studies identify the ES ES27 as an interaction partner at the ribosomal surface (Fujii et al. 2018, Wild et al. 2020). However, a high-resolution structure of MAP bound to ribosomes is not available, and the ribosome binding of MAPs is therefore not sufficiently understood.

Another important N-terminal (Nt) modification is the Nt-acetylation occurring at approximately 80% of cytosolic mammalian proteins (Arnesen et al. 2009). This modification is carried out by N-acetyltransferases (NATs) when 25-50 aa of a nascent chain are exposed outside the ribosomal tunnel (Strous et al. 1974, Varland et al. 2015). NATs attach an acetyl group to the free amino group at the N-terminus of a polypeptide chain. In yeast, five different N-acetyltransferases are expressed (NatA-NatE), while the system of higher eukaryotes is even more complex with six or more NATs (NatA-NatG and NAA80) (Starheim et al. 2012, Drazic et al. 2018). A recent cryo-EM structure of a native ribosome-NatA complex from *Saccharomyces cerevisiae* revealed that the main ribosome contact of NatA is mediated via the rRNA ES ES27a, ES7a, and ES39a. The ES place NatA near the ribosomal exit site in an ideal position to process nascent chains. In this position, NatA competes with other ribosome-associated protein biogenesis factors, including the chaperone RAC as well as the targeting factor SRP (Knorr et al. 2019). How
NatA gains regulated access to its nascent substrates is therefore unclear. Other Nt modifications, like Nt-propionylation, Nt-myristoylation, Nt-palmitoylation, and Nt-ubiquitylation are far less understood and rare in comparison to Nt-acetylation. Nt-propionylation might also be carried out by NATs (Foy et al. 2013), but the substrates and functional importance remain largely unknown (Varland et al. 2015). Nt-myristoylation describes the irreversible transfer of fatty acids to an N-terminal glycine residue of the target protein mediating protein-membrane and protein-protein interactions (Peitzsch and McLaughlin 1993, Murray et al. 1997). The enzymes catalyzing the reactions are called Nt-myristoyltransferases (NMTs) (Farazi et al. 2001), which interact with nascent chains of 100 aa in length (Deichaite et al. 1988). The N-terminus of NMTs seems to be important for ribosome binding (Glover et al. 1997), but their exact localization on ribosomes is unknown. N-terminal palmitoyltransferases (PATs) catalyze the rare Nt-palmitoylation. The addition of palmitate to an N-terminal cysteine increases the lipophilicity of the modified protein and often alters its subcellular distribution (Planey 2013). The rare attachment of ubiquitin, a small 9 kDa protein, to the free amino group of the first N-terminal residue is termed Nt-ubiquitylation. Its physiological relevance has not been fully elucidated (Ciechanover and Ben-Saadon 2004, Bloom et al. 2003). In general, ubiquitin serves as a proteasome targeting signal when added to internal lysine residues of a substrate (Hershko et al. 1982). Whether Nt-ubiquitylation also subjects substrates to degradation is unknown.

![Diagram of common eukaryotic co-translational modifications of the nascent chain.](image)

**Figure 5:** The most common eukaryotic co-translational modifications of the nascent chain. The N-terminal modifications are listed in bold, the corresponding enzyme in brackets. Abbreviations: MAP: methionine aminopeptidase; NAT: N-acetyltransferase; NMT: N-myristoyltransferase. Figure was modified from (Kramer et al. 2019). ©2019 by Annual Reviews. License received via the copyright clearance center, Inc.
1.5 Co-translational protein folding

The folding of newly synthesized cytosolic proteins is assisted by specialized ribosome-associated chaperone systems that bind to the ribosome exit site, including Trigger factor in bacteria and RAC in eukaryotes. These chaperones shield hydrophobic regions within a nascent chain to prevent aberrant interactions with other macromolecules, which can lead to protein misfolding and aggregation (Cassaignau et al. 2020).

1.5.1 The ribosome-associated complex (RAC)

RAC is a stable heterodimeric complex composed of an Hsp40 (heat shock protein 40 kDa) and an adenosine 5’-triphosphatase (ATPase)-inactive Hsp70 (heat shock protein 70 kDa) (Gautschi et al. 2002, Deuerling et al. 2019, Huang et al. 2005, Conz et al. 2007). To be fully functional as a chaperone complex, RAC acts together with an additional, catalytically active Hsp70, which is ribosome-bound in yeast (Ssb) and recruited from the cytosol in humans (Preissler and Deuerling 2012, Zhang et al. 2017). Regarding co-translational folding, Hsp70 recruited by the RAC complex can transiently bind exposed hydrophobic patches of nascent chains in repeated cycles of association and dissociation (Döring et al. 2017). However, these processes have only been analyzed in detail for yeast. Likewise, nearly all structural information of ribosome binding was generated with the yeast RAC complex: Zuo1 (Hsp40) spans over both ribosomal subunits, contacting the 60S subunit via its zuotin homology region (ZHR), close to eL31 and eL22 at the tunnel exit, whereas the C-terminus contacts the 40S subunit at ES12 (Lee et al. 2016, Zhang et al. 2014, Zhang et al. 2017). These unique structural data imply that RAC might affect conformational dynamics of the ribosome by connecting the tunnel exit with the decoding center of the ribosome. Indeed, it could be shown that a disruption of the Zuo1-40S contact leads to diminished translational fidelity (Lee et al. 2016).

1.6 Ribosome-associated protein quality control (RQC)

Ribosome-associated protein quality control (RQC) is based on sensing the status of translation to detect irregularities. It is therefore independent of the folding state of the nascent chains. Other co-translational quality control mechanisms are available to degrade misfolded or erroneous nascent chains (Wang et al. 2015, Duttler et al. 2013).

The recognition and degradation of RNCs was described for three conserved path-
ways in eukaryotes. These are non-sense mRNA decay (NMD), non-stop mRNA decay (NSD), and no-go decay (NGD) (Doma and Parker 2006, Frischmeyer et al. 2002, Graille and Séraphin 2012, Isken and Maquat 2007, Shoemaker and Green 2012, Joazeiro 2017). The names of the pathways indicate the type of mRNA that causes erroneous translation. NMD is activated by mRNAs with premature stop codons, NSD by those without stop codons, and NGD by those that cause elongation problems (Joazeiro 2017). The signal for activating the quality control mechanisms for NSD and NSG is the stalled ribosome. These stalled translation complexes have no termination codon in the A-site of the ribosome. Therefore, no translation release factors can be recruited. Two homologous factors, Hbs1 and Pelota, recognize these stalled RNCs (Figure 6). The subunits are separated from each other with the help of the ATPase ABCE1 (Pisareva et al. 2011, Shoemaker et al. 2010). The non-stop mRNA is degraded by the exoribonuclease Xrn1 and the exosome (Shoemaker and Green 2012, Graille and Séraphin 2012). However, the tRNA ester bond cannot be dissolved, and a complex of the 60S subunit with exposed peptidyl tRNA remains (Pisarev et al. 2010, Shoemaker et al. 2010). These aberrant complexes are recognized by the RQC component NEMF (Rqc2 in yeast), which binds to both the 60S subunit and the peptidyl-tRNA (Lyumkis et al. 2014, Shen et al. 2015, Shao et al. 2015). NEMF subsequently recruits and stabilizes the E3 ligase Listerin (Ltn1 in yeast) (Defenouillère et al. 2013, Shao et al. 2015). Listerin ubiquitinates the stalled nascent chains (Bengtson and Joazeiro 2010), which recruits VCP (Cdc48 in yeast) and its co-factors (Brandman et al. 2012, Defenouillère et al. 2013). This recruitment is also dependent on TCF25 (Rqc1 in yeast), whose exact function is not yet known (Joazeiro 2019). VCP enables the degradation of the nascent chain in the proteasome by extracting it and delivering it there. This extraction requires ANKZF1, which can detach the nascent chain from the tRNA (Verma et al. 2018, Rendón et al. 2018).
1 Introduction

Figure 6: Simplified overview about detection and splitting of aberrant ribosome-nascent chain complexes (RNCs) followed by ribosome quality control (RQC). After splitting of the 60S and 40S subunit the exposed tRNA in the 60S subunit triggers the RQC assembly and ubiquitination of the nascent chain. After polyubiquitination, the complex is disassembled and the components recycled. The nascent chain gets degraded. The components of the RQC shown in this figure are named according to yeast nomenclature. Rqc2 (green, in mammals NEMF). Ltn1 (yellow, in mammals Listerin). Figure was modified from (Brandman and Hegde 2016). ©2016, Nature Publishing Group, a division of Macmillan Publishers Limited. License received via the copyright clearance center, Inc.

1.7 Co-translational protein targeting

A critical step in protein biogenesis is the correct sorting and transport of proteins to appropriate cell organelles. Proteins can only be transported across a membrane in an unfolded state through narrow translocation pores. This requires that nascent transport substrates are detected in time during translation to prevent premature misfolding. The decision where to transport a newly synthesized protein is made at the ribosomal tunnel exit by targeting factors like SRP (see 1.7.2). In eukaryotes, most secretory and integral membrane proteins are transported cotranslationally. Proteins of the inner mitochondrial membrane have also been shown to be transported in a co-translational manner (Eliyahu et al. 2010, Lesnik et al. 2014, Chen et al. 2019). In addition, it is common in bacteria for plasma membrane proteins (Rapoport et al. 1996). Critical for the co-translational recognition of transport substrates are signal sequences in nascent chains that are often located in the N-terminus, as outlined in the following paragraph.
1.7.1 N-terminal signal sequences

N-terminal signal peptides are found on mitochondrial and ER proteins. They serve as sorting signals for targeting the corresponding protein to the appropriate cell organelle (Blobel and Dobberstein 1975). The signal peptides are essential for protein segregation in cells and bound by both targeting factors and translocation pores (Martoglio and Dobberstein 1998).

Signals for the transport to the secretory pathway vary a lot in their aa sequence as well as in length (15 to 50 aa), though there are some common characteristic features, which are depicted in Figure 7A (Von Heijne 1985, Martoglio and Dobberstein 1998). The essential part for protein targeting and membrane insertion is the hydrophobic core region (h-region) (Von Heijne 1985). In yeast, the hydrophobicity of the h-domain is critical in determining whether a protein is co-translationally or post-translationally transported. A highly hydrophobic h-domain marks the nascent protein for SRP-dependent, co-translational transport (Ng et al. 1996, Ast et al. 2013). Also in mammalian in vitro translation approaches, the deletion of hydrophobic aa in the signal sequence led to reduced interaction of SRP with the RNCs (Voorhees and Hegde 2015). At the N-terminus, the h-region is flanked by the so-called n-region, characterized by a net positive charge. The C-terminal region contains the cleavage site for signal peptidases, which cleave signal sequences after successful translocation (Martoglio and Dobberstein 1998).

Canonical mitochondrial targeting sequences (MTS) are short peptides consisting of basic and hydroxyl-containing residues (serine and threonine) (Figure 7B). Acidic aa residues are mainly absent in MTS. Like ER signal peptides, mitochondrial signals differ significantly in sequence and length. Necessary for mitochondrial transport is the formation of an amphipathic α-helical conformation of the signal sequence (von Heijne 1986, Bedwell et al. 1989).
Figure 7: Different N-terminal signal sequences. (A) ER transport signal. The core of the signal peptide is the hydrophobic h-region (green, about 5-16 residues long). Flanked by the N-terminal, positively charged n-region (grey) and the c-region (blue), containing the cleavage site for the signal peptidase (SPase). Figure was modified from (Martoglio and Dobberstein 1998). ©1998 Elsevier Science Ltd. (B) Cleavable mitochondrial targeting sequence (MTS). These sequences are enriched in positively charged, hydroxylated, and hydrophobic aa and have a high tendency to form an amphipathic α-helix with one positively charged and one hydrophobic surface. Modified from (Pfanner and Geissler 2001). ©1969, Nature Publishing Group. Licenses received via the copyright clearance center, Inc.
1.7.2 The signal recognition particle (SRP) pathway

The co-translational targeting of ribosomes to the translocation pore in the ER membrane is mediated by the SRP pathway. The SRP pathway is evolutionarily conserved in all kingdoms of life (Keenan et al. 2001). SRP recognizes signal sequences in the nascent chain, binds to the RNC, and targets the translating complex to the ER membrane by interacting with the membrane-associated SRP receptor (SR). The SR then transfers the ribosome to the translocation pore in the ER membrane, the Sec61 complex, through which proteins are channeled into the membrane and the ER lumen (Rapoport 2007, Driessen and Nouwen 2008).

SRP is a ribonucleoprotein complex consisting of six proteins named according to their apparent molecular weight in kilodalton (SRP9, SRP14, SRP19, SRP54, SRP68, and SRP72) and one RNA (7SL RNA) in mammals (Walter and Blobel 1980, Walter and Blobel 1982) (Figure 8). The prokaryotic complex consists of only one protein, the SRP54 homologue, Ffh (Fifty-four homologue), and prokaryotic SRP RNA (termed 4.5S RNA in *Escherichia coli*) (Ribes et al. 1990, Luirink et al. 1992). SRP54 comprises an N-terminal domain that associates with a central guanosine triphosphate (GTP)ase domain (NG) and a methionine-rich C-terminal domain (M) (Figure 8). SRP54 recognizes signal sequences in nascent chains through the M domain. The NG domain mediates ribosome and SR interactions (Halic et al. 2004, Lütcke et al. 1992).
In detail, the eukaryotic SRP-dependent targeting process can be divided into several steps. The first step is the recognition of RNCs with signal sequences, followed by the binding of SRP to these RNCs. In the next step, the NG domain detaches from the vicinity of the ribosomal tunnel to allow binding of the SR, finally a conformational change of SRP takes place at the ribosome to allow docking to the membrane and recruitment of the translocon with subsequent transfer of the signal sequence to Sec61 (Jomaa et al. 2021, Kobayashi et al. 2018). How exactly SRP recognizes ribosomes translating a signal sequence is still unclear. A scanning mechanism as proposed mainly in the prokaryotic system (Bornemann et al. 2008, Holtkamp et al. 2012) seems unlikely since SRP is by far not as abundant as the number of RNCs in a cell (Jensen and Pedersen 1994, Chartron et al. 2016), and it might therefore be inefficient to scan all ribosomes individually for signal sequences (Elvekrog and Walter 2015). In addition, studies show specific recruitment of SRP to ribosomes that do not yet expose the signal peptide, which is still inside the ribosomal tunnel. The recruitment of SRP to these ribosomes has not yet been clarified (Voorhees and
Hegde 2015, Flanagan et al. 2003, Berndt et al. 2009, Mariappan et al. 2010, Zhang et al. 2012). The Alu domain of SRP binds at the interface of 40S and 60S and is localized near the GTPase center of the ribosome (Figure 9). At this position, the Alu domain would compete with elongation factors for ribosome binding. This might explain the slowdown of translation described for SRP, which could extend the time for transport of the RNC-SRP complexes to the ER membrane (Wolin and Walter 1989, Halic et al. 2004, Voorhees and Hegde 2015).

Figure 9: Mammalian SRP bound to 80S ribosomes (cryo-EM map with 12.0 Å resolution). The 40S subunit (yellow), the 80S subunit (blue), and SRP (red) are shown as molecular models in the transparent Cryo-EM densities. Labeled are the ss (signal sequence), the different SRP proteins, as well as rRNA helix 8. Figure was modified from (Halic et al. 2004). ©2004, Macmillan Magazines Ltd. Licenses received via the copyright clearance center, Inc.
The binding of mammalian SRP to RNCs with signal sequences was recently structurally resolved in molecular detail (Jomaa et al. 2021, Voorhees and Hegde 2015). In this early SRP-RNC targeting complex, the M domain of SRP54 binds to the signal sequence, and the NG domain associates with uL23 and uL29 at the tunnel exit (Voorhees and Hegde 2015, Halic et al. 2006, Jomaa et al. 2016). The structure from Jomaa et al. (Figure 10) shows that in eukaryotes specific parts of the C-terminal region of the M domain (more precisely the helices αhM5 and αhM6) interact directly with the hydrophobic signal peptide. The binding pocket formed by the M domain is extended by a GM linker connecting the NG domain to the M domain (Jomaa et al. 2021).

Figure 10: Close up of the ribosome bound M domain of SRP. The high-resolution model is shown as a cartoon with uL23 (dark green) and uL29 (wheat) as docking points at the ribosomal tunnel exit. The signal sequence is depicted in magenta, bound by helices αhM5 and αhM6 of the M domain (cyan). This is better visible in the enlarged section on the right. The binding pocket is extended over the GM linker, which connects the NG domain (dark purple) with the M domain. SRP19 is shown in pink, the SRP RNA in white and yellow, SRP68, and the RNA binding domain (RBD) in purple. © 2021 The Authors. Figure taken from (Jomaa et al. 2021) under a CC BY-NC-ND 4.0 license.

Delivery of the SRP-RNC to the ER membrane is facilitated by the SR. The eukaryotic SR is a heterodimer of SRα and SRβ, in which SRβ anchors the complex in the ER membrane (Miller et al. 1995). Eukaryotic SRα consists of a universally conserved NG domain and a SRX domain connected via a flexible linker. The SRX domain associates with SRβ (Young et al. 1995). Interaction of the SRP-bound
RNC with SR is mediated via the evolutionary conserved N and GTPase (NG) domains in SRα and SRP54, which interact in a GTP-dependent manner to form a heterodimer (Figure 11A) (Rapiejko and Gilmore 1997, Wild et al. 2016). A conformational change in the NG domain of SRP54 leads to detachment from ribosomes (Figure 11B). The NG heterodimer translocates to the SRP RNA distal site. The M domain at the tunnel exit is also detached from the ribosomal surface, exposing the signal sequence, which is then captured by Sec61 (Figure 11C). In addition, GTP hydrolysis in the NG/NG heterodimer is delayed in this position by a conserved residue of SRP72 and one of the SRP RNA to provide a time window for the delivery of the signal sequence (Jomaa et al. 2021, Kobayashi et al. 2018). After signal handover, the GTPases are activated, and the GTP-hydrolysis dissociates SRP from SR to complete the targeting cycle (Wild et al. 2019).
Figure 11: Schematic overview of the mammalian SRP targeting process at the ER membrane. The factors involved are shown as a cartoon. (A) Shown is the binding of SRP to a ribosome (dark grey) with signal sequence (SS; red). The SRP54 NG domain (blue) occupies the tunnel exit, the SRP54 M domain (light blue) is bound to the signal sequence. The black arrow implies the impending contact of the NG domain of SRP54 and the NG domain of the receptor subunit SRα. SRα NG is connected via a long linker to the SRX domain (yellow) bound to SRβ (brown) in the ER membrane. (B) The NG heterodimer is formed in the presence of GTP, leading to detachment of the SRP54 NG domain from the ribosomal exit tunnel and reorientation to the distal side of the SRP RNA. (C) Shown is the "Pre-handover" complex. The NG heterodimer is bound by SRX-SRβ and SRP68 RBD, which frees the tunnel exit for Sec61p binding. The GTPase active site of the NG heterodimer is marked with a red arrowhead. A schematic enlargement of this state is shown in the figure on the right. GTP hydrolysis is prevented by SRX-SRβ, SRP72 RBD and SRP RNA. G232 is a universally conserved flipped-out base of SRP RNA. PBD, protein binding domain. Figure adapted from (Kobayashi et al. 2018). ©2018, The American Association for the Advancement of Science. Licenses received via the copyright clearance center, Inc.
1.7.3 The Sec61 translocon complex

Sec61 is a highly conserved heterotrimeric protein complex consisting in eukaryotes of Sec61α, Sec61β, and Sec61γ (Görliech and Rapoport 1993, Osborne et al. 2005). Sec61α spans the membrane ten times and forms the translocation channel with a narrow pore that allows the transport of unfolded proteins across the membrane. In addition, the pore complex has an inbuilt lateral gate that allows transmembrane domains (TMDs) of substrates to enter the membrane (Van den Berg et al. 2004). Sec61γ is a tail-anchored protein and wraps around Sec61α like a clip, whereas Sec61β, also a tail-anchored protein, might serve as a regulator of the transport process (Lang et al. 2019). In yeast and bacteria, only Sec61α and Sec61γ seem to be essential for protein translocation, and they also share a high sequence homology (Lang et al. 2019, Nishiyama et al. 1994, Brundage et al. 1990, Görliech and Rapoport 1993, Matlack et al. 1998).

Sec61 interacts tightly with the ribosome near the tunnel exit. The ribosomal tunnel and the Sec61 channel form a functional conduit that allows translocation of the nascent chains without compromising the ion impermeability of the membrane. The main contact is mediated by Sec61α and Sec61γ binding to 28S rRNA, uL23, and uL29 adjacent to the tunnel exit (Figure 12). Additional contacts are made to eL19 and eL39 (Voorhees et al. 2014). In the inactive state, the Sec61 channel is closed by a plug located in the center of the hourglass-shaped channel, maintaining the membrane permeability barrier during translocation. Polypeptide chains enter this channel through a lateral gate. These two regulation points allow the opening of the channel for translocation in the ER lumen or transmembrane domain insertion in the lipid bilayer. Binding of the ribosome activates the translocon by opening the lateral gate to the cytosolic side, which lowers the energetic barrier for translocation. Access to the membrane and the ER lumen is still restricted at this stage. To fully open the channel, a signal sequence must bind to and activate the Sec61α lateral gate. Binding of signal peptides also destabilizes the plug, allowing substrates to pass through the channel into the ER lumen (Voorhees et al. 2014). Reading the signal peptide by SRP and Sec61 reduces the likelihood of protein mistargeting in cells (Jungnickel and Rapoport 1995, Voorhees et al. 2014). However, both factors have a high binding affinity to ribosomes independent of signal peptides (Borgese et al. 1974, Jungnickel and Rapoport 1995). This suggests the existence of an antagonist in cells that prevents inappropriate interactions of the translating ribosome with the ER transport machinery. This antagonistic function is exerted by NAC, as described in the next section.
**Figure 12: Structural details of the mammalian ribosome-translocon complex.** (A) An empty ribosome (40S (brown) and 60S subunit (blue)) is bound to the Sec61 translocon (red) in the ER membrane. The peptidyl transferase center (PTC) is indicated in the model. (B) Close-up of Sec61 surrounded by ribosomal proteins. Sec61α is shown in red, γ in tan, and β in light purple. The main contact is mediated by Sec61α and Sec61γ binding to 28S rRNA, uL23, and eL29. (C) Magnified view of two loops formed by Sec61α, showing additional contacts to the ribosomal proteins eL19 and eL39. Figure was modified from (Voorhees et al. 2014) under a CC BY 3.0 license. ©1969, Elsevier.
1.8 The nascent polypeptide-associated complex (NAC)

NAC is an eukaryotic ribosome-associated heterodimeric complex consisting of an α and β subunit (αNAC and βNAC) (Figure 13). NAC is highly conserved and ubiquitously expressed in cells at levels similar to ribosomes (Möller et al. 1998a, Raue et al. 2007, Preissler and Deuerling 2012). This distinguishes NAC from other ribosome-associated factors like SRP that are expressed in lower concentrations (Akopian et al. 2013). Moreover, unlike other factors in protein biogenesis, the binding of NAC to ribosomes is broad, interacting with all types of RNCs in the cell, including cytosolic, mitochondrial, and ER substrates (Wiedmann et al. 1994). This suggests a more general function of NAC in protein biogenesis. Deletion of NAC in animals is embryonically lethal, and knockdown of NAC in human cells leads to apoptosis, indicating an essential function in protein biogenesis (Deng and Behringer 1995, Markesich et al. 2000, Bloss et al. 2003, Brockstedt et al. 1999).

Both NAC subunits contain a homologous domain that dimerizes by forming a β-barrel-like structure with a hydrophobic core (Figure 13) (Liu et al. 2010, Wang et al. 2010). The β-barrel-like fold of the NAC domain is conserved in archaeal NAC, which consists of an αNAC homodimer. Structural analysis of archaeal NAC also revealed a conserved three helix bundle, a so-called ubiquitin associated (UBA) domain, at the C-terminus of αNAC (Spreter et al. 2005). UBA domains have a characteristic hydrophobic patch on their surface used for a range of protein-protein interactions (Mueller and Feigon 2002). However, the function of the NAC UBA domain is unknown. The N-terminal domains of αNAC and βNAC, as well as the C-terminal domain of βNAC, have not yet been structurally resolved. The N- and C-terminal tails of NAC include intrinsically disordered regions, indicating a high degree of flexibility (Deuerling et al. 2019).

Ribosome binding of NAC is mainly mediated by the βNAC N-terminus (N-βNAC). Deletion of the first eleven N-terminal residues or mutation of a conserved positively charged motif (RRKHK) in the N-βNAC abolishes ribosome binding of the complex (Wegrzyn et al. 2006, Pech et al. 2010, Reimann et al. 1999). The exact position of NAC on the ribosome is unknown. Both NAC subunits could be crosslinked to short nascent chains, indicating that NAC binds in the vicinity of the ribosomal tunnel exit (Wiedmann et al. 1994). Consistent with this assumption, crosslinks between NAC and several r-proteins surrounding the exit site, including uL23, eL31, and uL22, were detected (Wegrzyn et al. 2006, Pech et al. 2010, Zhang et al. 2012). These ribosomal proteins are located on opposite sides of the tunnel exit (Figure 4), suggesting that the binding of NAC to ribosomes is very flexible. Furthermore, also
αNAC crosslinks to r-proteins were reported suggesting a possible multimodal ribosome interaction mechanism of NAC (Nyathi and Pool 2015). Thus, based on the crosslinking data, NAC likely binds in close vicinity to the ribosomal tunnel exit similar to other ribosome-associated factors like RAC (Lee et al. 2016, Zhang et al. 2014, Zhang et al. 2017), SRP (Voorhees and Hegde 2015, Halic et al. 2006, Jomaa et al. 2016) and the Sec61 translocon (Voorhees et al. 2014).

The high concentration of NAC and its broad interaction with RNCs in cells suggest an interplay with other protein biogenesis factors at the tunnel exit site. Indeed, a critical function of NAC is to regulate the access of SRP and Sec61 to translating ribosomes (Zhang et al. 2012, Wiedmann et al. 1994, Lauring et al. 1995). NAC antagonizes inappropriate binding of these factors to signal-less RNCs, thereby preventing erroneous transport of proteins to the ER (Gamerdinger et al. 2015). ER stress phenotypes caused by protein mistargeting were observed in different model organisms upon NAC depletion, including nematodes (Gamerdinger et al. 2015), zebrafish (Murayama et al. 2015) and human cells (Hotokezaka et al. 2009). Chronic ER stress is known to induce cell apoptosis (Tabas and Ron 2011, Walter and Ron 2011), which might explain the lethal phenotypes observed in NAC knockout animals (Deng and Behringer 1995, Markesich et al. 2000, Bloss et al. 2003). The finding that protein transport via SRP and Sec61 in eukaryotes must be antagonized to ensure protein transport specificity was predicted by in vitro studies. These studies revealed that SRP and Sec61 have a high intrinsic binding affinity to empty ribosomes and signal-less RNCs in the low nanomolar range (Borgese et al. 1974, Jungnickel and Rapoport 1995). The addition of NAC at physiological concentrations prevented these nonspecific interactions (Möller et al. 1998b), suggesting that NAC somehow blocks access of SRP and Sec61 to their binding sites near the tunnel exit. Moreover, in vitro studies have shown that ribosomes do not readily detach from the ER translocon after nascent chain release (Adelman et al. 1973). Dissociation of the empty 60S ribosomal subunit after completion of translation is important to prevent possible translocation of incorrect substrates in the following translation cycle. In vitro studies showed that NAC detaches ribosomes from ER membranes after puromycin-induced nascent chain release (Gamerdinger et al. 2015). This suggests that NAC also acts as a ribosome release factor in cells, preventing erroneous transport of proteins across the ER membrane.
Furthermore, several studies suggest a role for NAC in mitochondrial protein transport. Yeast mutants lacking NAC exhibit a mitochondrial targeting defect (George et al. 1998). In vitro studies showed that NAC promotes targeting of RNCs that translate mitochondrial proteins (Funfschilling and Rospert 1999), and a yeast-specific potential NAC receptor (OM14) on the mitochondrial outer membrane has been described (Lesnik et al. 2014). In addition, mitochondrial stress was observed in cells deprived of NAC (Hotokezaka et al. 2009). These results suggest a possible function of NAC as an RNC targeting factor for mitochondria, like SRP for the ER. However, whether co-translational protein transport to mitochondria occurs in
cells is controversial, and the role of NAC in this process is highly speculative. NAC critically controls the access of the ER transport machinery to the ribosome exit site as outlined above. However, the mechanistic basis of how NAC inhibits Sec61 and SRP binding on signal-less RNCs is not understood (Gamerding et al. 2015). How this inhibition is overcome when an ER signal sequence is translated remains enigmatic. It is also unclear how NAC recognizes inactive ribosomes on the ER membrane to release them. To act as a molecular router at the ribosome exit site, NAC should be able to distinguish between different types of nascent chains. However, potential substrate-specific interacting domains have not yet been identified in NAC. Therefore, further structural and functional analyses are required to understand the mechanism of action of this crucial ribosome interactor.
2 Aims and objectives

The eukaryotic nascent polypeptide-associated complex (NAC) interacts with newly synthesized proteins at the ribosomal tunnel exit. NAC prevents inappropriate cotranslational transport of cytosolic and mitochondrial proteins to the ER by antagonizing non-specific ribosome interactions with the targeting factor SRP and the ER translocon Sec61. The mechanism of how NAC antagonizes the ER targeting machinery and how this inhibition is overcome on ribosomes translating ER proteins is unknown.

The main goal of this work was to uncover the precise interaction mechanisms of NAC with ribosomes translating cytosolic, mitochondrial, and ER proteins. High-resolution information on the interaction of NAC with the ribosome and nascent chains should be obtained by a site-specific in vitro photo-crosslinking approach. This approach was based on the incorporation of the unnatural aa p-benzoyl-phenylalanine (Bpa) at different positions in NAC, which allows zero-space cross linking to interaction partners. The Bpa-NAC variants should be photo-crosslinked with stalled, radioactively labeled RNCs carrying specific types and lengths of nascent chains generated by in vitro translation of non-stop mRNAs in rabbit reticulocyte lysate. Specific crosslinks between NAC and the ribosome and nascent chain should then be detected by gel-shift analyses using immunoblotting and autoradiography detection techniques.

The interaction information obtained from the site-specific crosslinking experiments should then be combined with cryo-EM data obtained by external collaborators to derive the mechanistic basis of the antagonistic function of NAC on SRP and Sec61. The predicted structure-function relationships should then be tested by mutational analyses of NAC using in vitro RNC binding studies and by in vivo analyses in the model organism *C. elegans*.
3 Results

3.1 Interaction mechanism of NAC with ribosomes and nascent chains

Key results presented in the following chapter have been published in:

Early Scanning of Nascent Polypeptides inside the Ribosomal Tunnel by NAC

Important findings from collaborators that are essential for understanding and interpreting the results have been included, and their work is explicitly mentioned in the corresponding figure legends.

The mechanistic basis of how NAC antagonizes ER protein targeting to prevent inappropriate protein transport into the ER is not understood. As outlined in the introduction, NAC can be crosslinked to short nascent chains and r-proteins surrounding the tunnel exit site. This suggests that NAC binds near the tunnel exit and may occupy critical binding sites of SRP and Sec61 to antagonize them on signal-less ribosomes. However, the exact interaction mechanism of NAC with ribosomes and nascent chains is unknown. To gain a better understanding, a series of biochemical and structural analyses were performed that revealed new molecular details of the co-translational interaction mode of NAC with ribosomes, which are presented in the following subchapters.

3.1.1 Conserved charged motifs in N-αNAC are crucial for ribosome binding of NAC

Previous studies suggest that the N-βNAC is essential for ribosome binding of NAC (Wegrzyn et al. 2006, Pech et al. 2010, Reimann et al. 1999). The first eleven aa and a conserved charged motif (RRKKK) in the center of the N-βNAC domain was
found to be critical for ribosome binding. Crosslinking studies in yeast identified the ribosomal proteins uL23 and eL31 near the tunnel exit as potential binding sites for βNAC (Wegrzyn et al. 2006, Pech et al. 2010). Interestingly, a previous study also reported a specific crosslink between the αNAC subunit and uL29 at the tunnel exit site (Nyathi and Pool 2015). However, the region in αNAC that crosslinks to uL29 and the importance of this interaction for ribosome binding are unknown. Sequence alignments of αNAC from different species revealed two conserved charged motifs in N-αNAC: a negatively charged motif (α-DSD) in the N-terminal region and a positively charged motif (α-KKAR) in the C-terminal region (Figure 14A). The two motifs are separated by a poorly conserved ~35 aa linker sequence that is predicted to be unstructured and flexible. To test whether these motifs contribute to ribosome binding, different NAC variants with mutations in N-αNAC were generated for *in vitro* binding studies (Figure 14B). Interestingly, removing the positive charge in the α-KKAR motif (KKAR/AAAA) or deletion of N-αNAC (∆N1-64) decreases ribosome binding of NAC (Figure 14C). Strikingly, however, a deletion variant lacking only the region upstream of α-KKAR (∆N1-53) binds stronger to ribosomes than WT NAC (Figure 14C). These data suggest that the α-KKAR motif promotes ribosome binding of NAC, whereas the upstream α-DSD motif weakens it.
Figure 14: Conserved motifs in N-αNAC regulate ribosome binding of NAC in vitro. (A) Sequence alignments of N-αNAC from *Caenorhabditis elegans* (CAEEL), *Drosophila melanogaster* (DROME), *Xenopus laevis* (XENLA) and *Homo sapiens* (HUMAN). The conserved positively and negatively charged motifs are colored in blue and red, respectively. (B) Schematic representation of *C. elegans* αNAC mutants used in panel C. WT, wildtype (C) Indicated purified NAC variants were incubated with 60S ribosomes (ratio 1:1) and ribosomes pelleted by sucrose cushion centrifugation. Proteins in the pellet and supernatant fraction were analyzed by immunoblotting. Analysis was performed by Martin Gamerdinger and Renate Schlömer. Figure was modified from (Gamerdinger et al. 2019). ©2019 Elsevier Inc.

Our data suggest that in addition to the previously reported N-βNAC also a motif in N-αNAC (α-KKAR) contributes to ribosome binding. Moreover, the negatively charged motif α-DSD upstream of α-KKAR seems to down-regulate ribosome binding of NAC by an unknown mechanism. To investigate the interaction mechanism of α-DSD, the photo-crosslinking probe Bpa was incorporated into human αNAC at position V35 (α-V35) directly downstream of α-DSD (Figure 14A). Ultraviolet (UV) crosslinking of this NAC variant in solution revealed two crosslinks: an αNAC-βNAC inter-crosslink and an αNAC intra-crosslink. Analysis of the crosslinked protein species using mass spectrometry showed that Bpa at α-V35 directly crosslinks to the positively charged ribosome binding motifs, α-KKAR (intra-crosslink) and β-
RRKKK (inter-crosslink), in the N-termini of NAC (Figure 15A). Thus, the α-DSD motif seems to down-regulate ribosome binding of NAC through direct interaction with the positively charged ribosome binding motifs (Figure 15B).

Figure 15: The α-DSD motif interacts with the positively charged ribosome binding motifs of NAC. (A) Photo-crosslinking of a NAC variant containing the UV-activatable crosslinking probe Bpa at position α-V35, located three aa downstream of the negatively charged α-DSD motif (Figure 14A). UV-induced αβNAC inter-crosslink and αNAC intra-crosslinks are indicated. Immunoblots and Coomassie gel are shown. Mass spectrometry (MS) identified the crosslinks as indicated. (B) Model of the NAC heterodimer showing N-αNAC and N-βNAC with the conserved charged motifs that regulate ribosome binding of NAC. The negatively charged DSD motif in N-αNAC binds and antagonizes the two positively charged ribosome binding motifs α-KKAR and β-RRKKK. NAC domain structure PDB: 3LKX (Liu et al. 2010). Crosslinking experiments were performed by Renate Schlömer and Martin Gamerdinger, and mass spectrometry (MS) analysis by Carolin Sailer (Florian Stengel Group, University of Konstanz). Figure was modified from (Gamerdinger et al. 2019). ©2019 Elsevier Inc.
3.1.2 Cryo-EM reconstruction of NAC bound to empty 60S ribosomal subunits

The data obtained so far suggest that NAC binds ribosomes in a multivalent manner involving both N-termini of NAC. To gain deeper insights into the structure of NAC bound to ribosomes, *C. elegans* NAC-60S ribosomal complexes were investigated by cryo-EM in collaboration with the group of Nenad Ban (ETH Zurich, Switzerland). NAC was found in the cryo-EM reconstruction as an additional globular density at the tunnel exit with an overall resolution of 8-9 Å (Figure 16). Although reliable docking of the NAC crystal structures (PDB: 3MCB and PDB: 3LKX; (Liu et al. 2010; Wang et al. 2010)) was not possible due to low resolution, an unexpected discovery was made. An extra density of NAC protrudes deep into the ribosomal tunnel up to the tunnel constriction site adjacent to the PTC (Figure 16). Nascent chains reach this constriction site with a length of about 10 aa (Zhang et al. 2013). It has been hypothesized that the properties of nascent chains are sensed as they pass through the tunnel constriction, transmitting a signal from inside of the tunnel to the surface of the ribosome (Wilson and Beckmann 2011). The finding that NAC extends to the constriction site suggests that NAC may be involved in the preemptive recognition of nascent substrates before they reach the cytoplasm.
Figure 16: Cryo-EM map of NAC-60S. NAC and the 60S ribosome are colored light green and gray, respectively. In (A), a cross-section of the ribosome is shown to better visualize the tunnel insertion of NAC. In (B), the yeast 60S ribosome structure (PDB: 5APO, Greber et al. 2016) was docked into the density map. Ribosomal proteins, as well as rRNA H59 around the exit tunnel, are highlighted. Cryo-EM was performed by Kan Kobayashi and Ahmad Jomaa of the Ban Lab in Zurich, Switzerland. Figure was modified from (Gamerdinger et al. 2019). ©2019 Elsevier Inc.

3.1.3 N-βNAC contacts nascent chains deep inside the ribosomal tunnel

The low-resolution cryo-EM structure did not reveal the identity of the tunnel inserting NAC domain. To investigate in detail which domain of NAC inserts deeply into the ribosomal tunnel, ribosomes were engineered in yeast containing the photoactivatable crosslinker Bpa at the tunnel constriction in a loop region of uL22 (R126) facing the tunnel interior (Figure 17A). Empty 60S ribosomes containing the photocrosslinking probe were purified and incubated with human or C. elegans wildtype (WT) NAC. In addition, NAC variants with a deletion of the flexible N-αNAC con-
taining the α-DSD motif, which down-regulates ribosome binding of NAC (outlined in 3.1.1), were used. After UV irradiation, uL22-NAC crosslinks appeared with a molecular weight of ~45 kDa, indicating the βNAC subunit as the crosslinking partner of uL22, which have an apparent SDS-PAGE molecular weight of 20 and 24 kDa, respectively (Figure 17B). Consistent with this assumption, no molecular weight shift of the crosslinks were observed with the αNAC N-terminus deletion variants, which crosslinked stronger than the wildtype NAC variants ((Figure 17B) see below for further interpretation of these results). Moreover, the crosslinks could also be detected with an antibody against human βNAC (Figure 17B, lower panel). Overall, these data suggest that an extended domain of the βNAC subunit inserts deeply into the tunnel of inactive ribosomes.

Figure 17: In vitro crosslinking identifies βNAC as the tunnel inserting subunit of NAC. (A) Shown is a close-up of the NAC density inside the ribosomal tunnel extending from the mouth (at eL39) to the tunnel constriction site (at uL22). The residue R126 of the ribosomal tunnel lining protein uL22 (blue) at the tunnel constriction was substituted with the photo-crosslinking probe Bpa for the in vitro crosslinking experiments shown in panel (B). (B) Site-specific photo-crosslinking of engineered FLAG-tagged Bpa-(R126)-uL22 yeast ribosomes with human or C. elegans (C. e.) NAC. Wildtype (WT) and NAC variants lacking the flexible αNAC N-terminus (C. e. ∆N1-53 or human ∆N1-67) were used. Crosslinks were detected by immunoblot analysis using indicated antibodies. Red arrowhead indicates crosslinks between FLAG-uL22 and βNAC. Asterisk indicates NAC-independent intra-ribosomal crosslink. Cryo-EM reconstruction was performed by Kan Kobayashi and Ahmad Jomaa of the Ban Lab in Zurich. Crosslinking experiments were performed by Stefan Kreft. Figure edited from (Gamerdinge et al. 2019). ©2019 Elsevier Inc.
In the next step, site-specific photo-crosslinking experiments with Bpa-NAC variants were performed to identify the tunnel-inserting βNAC domain. Because the narrow ribosomal tunnel dimensions (Figure 3) allow only unstructured or secondary-structured protein elements like α-helices to enter, the globular domain of NAC was excluded from the analysis. The focus was particularly on the flexible N-terminal domain of βNAC, which has been shown in previous studies to be essential for ribosome binding (Wegrzyn et al. 2006; Pech et al. 2010; Reimann et al. 1999). N-βNAC is highly conserved, has a net positive charge and a length of 40 aa, which would fit very well in the negatively charged ribosomal tunnel. The photo-crosslinking probe Bpa was incorporated in N-βNAC at two positions close to the N-terminus (position 2 and 14, Figure 18A). As a control, Bpa was incorporated at different positions in the flexible N-αNAC, which contains the positive and negative ribosome binding elements described in Chapter 3.1.1 (Figure 18A). The purified Bpa-NAC variants were then probed with stalled RNCs carrying radioactive labeled nascent chains of different lengths. The RNCs were generated by in vitro translation of non-stop mRNAs encoding a cytosolic (Human glucose-6-phosphate isomerase (GPI)) or a mitochondrial (human heat shock protein 60 (HSPD1)) protein using rabbit reticulocyte lysate (RRL). The length of the nascent chains ranged from 10 to 50 aa, thus reaching from the tunnel constriction site to the mouth of the exit tunnel. Crosslinks between radioactive nascent chains and Bpa-NAC variants were then detected by an UV-induced molecular size shift of the NC-tRNA complex on an autoradiography gel.

Interestingly, the 10 aa nascent chain whose N-terminus just passes the ribosomal tunnel constriction crosslinked exclusively with the Bpa-NAC variant containing the crosslinking probe at position two (β-X2) in N-βNAC (Figure 18B). This strongly suggests that the tunnel-inserting domain of NAC indicated by the cryo-EM structure (Figure 16A) and the yeast ribosome crosslinking assay (Figure 17) is N-βNAC. Moreover, the crosslinking data reveal that NAC can interact with nascent chains deep within the tunnel, which is unique among ribosome-associated protein biogenesis factors. Thus, NAC is most likely the first factor in the cell that interacts with newly synthesized proteins. Robust β-X2 crosslinks to nascent chains were detected up to a length of 25 aa, but then became weaker, suggesting that the growing nascent chain eventually displaces N-βNAC from the tunnel (Figure 18B, Figure 19). Thus, tunnel insertion appears to be highly dynamic, preventing N-βNAC from blocking the path of nascent chains through the narrow ribosomal tunnel.
Figure 18: N-βNAC crosslinks to short nascent chains inside the ribosomal tunnel. (A) Overview of positions in the N-termini of αNAC and βNAC in which the photo-crosslinking probe Bpa was incorporated. The highlighted residues were substituted with Bpa, except β-X2, which is an insertion between the first and second residue of βNAC. The negatively and positively charged ribosome-binding regulatory motives are colored in red and blue, respectively. (B) NAC variants containing Bpa at indicated positions were crosslinked to stalled RNCs harboring S35-labeled nascent chains of different lengths (10-50 aa). Shown is an autoradiography gel. Crosslinks between Bpa-NAC variants and NC-tRNAs are marked with arrowheads. Human glucose-6-phosphate isomerase (GPI) was translated as a model substrate. NC-tRNA; tRNA-attached nascent chain. Figure was modified from (Gamerdinge et al. 2019). ©2019 Elsevier Inc.
Figure 19: N-βNAC crosslinks to short nascent chains inside the ribosomal tunnel. NAC variants containing Bpa at indicated positions were crosslinked to stalled RNCs harboring S35-labeled nascent chains of different lengths (10-50 aa). Shown is an autoradiography gel. Crosslinks between Bpa-NAC variants and NC-tRNAs are marked with arrowheads. Human heat shock protein 60 (HSPD1) was translated as a model substrate. NC-tRNA; tRNA-attached nascent chain. Figure was modified from (Gamerdinger et al. 2019). ©2019 Elsevier Inc.

Furthermore, starting with a length of 30 aa, weak but specific crosslinks appeared with a NAC variant containing Bpa at position α-V64 upstream of the positively charged ribosome binding motif α-KKAR (Figure 18 and Figure 19). This suggests that the α-KKAR motif binds near the mouth of the ribosomal tunnel, where the tunnel widens, and first secondary structures of nascent chains can form (Nilsson et al. 2015; Farías-Rico et al. 2018). The flexible N-terminal region upstream of the α-KKAR motif seems to loop into the opening of the tunnel and contact nascent
chains as short as 30 aa. A weak but specific crosslink of $\alpha$-V64 to the ribosomal protein eL39 located at the mouth of the exit tunnel (Figure 17A) strengthens this assumption (Figure 20).

Figure 20: Position $\alpha$-V64 in N-$\alpha$NAC is located near the mouth of the ribosomal tunnel. NAC variants with Bpa incorporated at the indicated positions were incubated with purified 60S ribosomes. Shown is an eL39 immunoblot, and a specific crosslink between $\alpha$-V64-NAC and eL39 is indicated by a red arrowhead. Figure was modified from (Gamerdinger et al. 2019). © 2019 Elsevier Inc.

Interestingly, the crosslinking pattern to longer nascent chains that exited the ribosomal tunnel with the N-terminus (45-50 aa) differed between the translated substrates. The cytosolic substrate GPI is predominantly crosslinked with $\beta$-L14, whereas the mitochondrial protein is crosslinked with $\alpha$-I59 and $\alpha$-V64 (Figure 18 and Figure 19). This suggests that NAC can adopt different conformations on the ribosome depending on the substrate being translated. Indeed, a substrate-specific conformation of NAC on ribosomes translating ER proteins was later revealed in molecular detail as outlined in Chapter 3.2.

3.1.4 N-$\beta$NAC binds an alternative site on the ribosome surface upon tunnel extrusion

The crosslinks of NAC to longer nascent chains indicate that NAC remains bound to ribosomes even though N-$\beta$NAC is pushed out of the tunnel. Previous crosslinking studies suggested that N-$\beta$NAC binds to the surface of ribosomes near the tunnel exit at eL31 (Pech et al. 2010; Nyathi and Pool 2015; Zhang et al. 2012). Therefore, it was assumed that N-$\beta$NAC might bind to an alternative site on the ribosomal surface when it is displaced from the tunnel by a growing nascent chain. Interestingly, the cryo-EM structure with inactive 60S ribosomes showed an additional density of
NAC at eL22 adjacent to eL31 (Figure 21A). To test whether this density represents N-βNAC, the β-X2 NAC variant was crosslinked with active or inactive 80S ribosomes from human HEK cells. Active ribosomes were purified in the presence of cycloheximide, an elongation inhibitor that stabilizes nascent chains (Sisler and Siegel 1967). Inactive ribosomes were obtained by treating ribosomes with puromycin, a tRNA analog that releases nascent chains (Nathans 1967). Strikingly, β-X2 NAC crosslinked to the surface-located eL22 with both active and inactive ribosomes, whereas crosslinks to uL22, which is located inside the tunnel at the constriction site, only occurred with puromycin-treated, empty ribosomes (Figure 21B). Thus, as suspected, N-βNAC binds both within the empty tunnel and at the surface of ribosomes near eL22 after being forced out of the tunnel by longer nascent chains.

**Figure 21:** N-βNAC binds to an alternative site on the ribosome surface at eL22. (A) Cryo-EM structure of *C. elegans* empty 60S ribosomes without and with NAC. R-proteins surrounding the exit site are highlighted and shown as a cartoon. NAC density is shown in green. Arrow indicates protruding extra density of NAC at eL22 and eL31. (B) Site-specific photo-crosslinking of a NAC variant containing Bpa at position two of N-βNAC (β-X2) with active and inactive 80S ribosomes from human HEK cells. Inactive ribosomes were treated with puromycin (Puro) to release nascent chains. Immunoblots of eL22, which is located on the surface, and uL22, which lines the tunnel of ribosomes, are shown. Specific UV-induced crosslinks are indicated (eL22 x β-X2 and uL22 x β-X2). Cryo-EM was performed by Kan Kobayashi and Ahmad Jomaa of the Ban Lab in Zurich. The experiment in (B) was performed by Martin Gamerdinger and Renate Schlömer. Figure was modified from (Gamerdinger et al. 2019). ©2019 Elsevier Inc.
3.1.5 The α-DSD motif in N-αNAC critically regulates RNC binding dynamics of NAC

The interaction of NAC with the tunnel interior and the surface region of ribosomes seems to be very dynamic. This is important to allow a growing polypeptide chain to traverse the tunnel unhindered. As shown above, the dynamic binding of NAC is based on the negatively charged α-DSD motif, which interacts with the positively charged ribosome binding motifs in NAC (Figure 15B). Too strong interactions inside or at the mouth of the tunnel by NAC lacking the α-DSD motif would partially clog the path of nascent chains through the narrow tunnel and thus inhibit translation elongation, with serious consequences for protein biogenesis. Indeed, *in vitro* translation of the model substrate luciferase was strongly inhibited by a NAC deletion variant lacking the α-DSD motif (ΔN1-53, (Figure 22A)). Inhibition of translation was also observed *in vivo* in *C. elegans* upon overexpression of this NAC variant using a puromycin labeling method to monitor protein synthesis (Figure 22B) (Schmidt et al. 2009).

![Luciferase activity plots](image)

**Figure 22:** Deletion of α-DSD motif in NAC induces translational defects *in vitro* and *in vivo*. (A) *In vitro* translation of the model substrate firefly luciferase in reticulocyte lysate (RRL). Wildtype (WT) NAC or a NAC variant lacking the N-terminus of αNAC containing the α-DSD motif (ΔN1-53) were added at indicated concentrations before the start of translation. After translation for 15 min, luciferase activity was measured. The luciferase activity is shown in percent relative to the control condition (without the addition of NAC). Error bars represent SD; n = 4. A related autoradiographic gel image showing the amount of translated S\(^{35}\)-labeled luciferase is depicted below. (B) *C. elegans* worms overexpressing the indicated FLAG-tagged αNAC variants were treated with puromycin for 4 h. An immunoblot of the total lysates probed with puromycin antibody is shown. Experiments, besides the autoradiographic image were performed by Martin Gamerdinger. Figure was modified from (Gamerdinger et al. 2019). ©2019 Elsevier Inc.
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These data underline the importance of the α-DSD motif in regulating the dynamics of ribosome binding of NAC. The inhibition of translation caused by ∆N1-53 NAC might be due to partial clogging of the nascent peptide exit tunnel because a stronger tunnel insertion of this variant is indicated by the stronger crosslinks of βNAC inside the tunnel of engineered Bpa-60S yeast ribosomes (Figure 17B). Thus, to investigate the possible tunnel blockade by ∆N1-53 NAC, a small globular protein (GFP) was fused to the N-terminus of βNAC to prevent tunnel insertion. In vitro binding studies with 60S ribosomes showed that the ribosome binding strength of GFP-βNAC per se was unchanged compared with that of WT NAC (Figure 23A). However, the fusion of the GFP tag to Bpa-β-X2 NAC prevented crosslinks to short nascent chains (25 aa) buried in the ribosomal tunnel (Figure 23B). In addition, site-specific photocrosslinking experiments suggested that ribosome binding of GFP-βNAC only occurred on the ribosome surface but not inside the tunnel, as Bpa at β-X2 crosslinked to the surface exposed eL22, but no longer to the tunnel lining r-proteins, uL22 and eL39 (Figure 23C). Overall, these data demonstrate that NAC variants carrying a GFP at the N-terminus of βNAC still bind efficiently to the ribosomal surface but can no longer interact with the interior of the tunnel. Thus, GFP was fused to the N-terminus of βNAC from ∆N1-53 NAC to test if preventing tunnel insertion rescues the translation defect. Indeed, in vitro translation of luciferase was significantly less inhibited (Figure 23D). However, at higher concentrations, also this variant caused a translational defect (Figure 23D), suggesting that deletion of the ribosome binding regulatory element, including the α-DSD motif, impairs translation through unproductive RNC interactions by NAC that occur both inside but also outside the ribosomal tunnel.
Figure 23: ΔN1-53 NAC partially blocks the ribosomal tunnel. (A) In vitro ribosome binding analysis of wildtype NAC (WT) and a NAC variant containing GFP fused to the N-terminus of βNAC (GFP). Purified NAC was incubated with empty 60S (1:1) and ribosomes pelleted by sucrose cushion centrifugation. Shown is an immunoblot of the pellet fraction. (B) Crosslinking of purified human NAC variants as in (A) containing Bpa at position two of βNAC (β-X2) with RNCs carrying S\textsuperscript{35}-labeled 25 aa nascent chains (substrate GPI). Shown is an autoradiographic gel. (C) Bpa-NAC variants as in (B) were incubated with purified empty 60S ribosomes. Indicated proteins were detected by immunoblotting. Arrowheads mark specific crosslinks. *, unspecific band. (D) In vitro translation of the model substrate firefly luciferase in reticulocyte lysate. NAC variants consisting of wildtype or GFP-tagged βNAC (WT-β or GFP-β) and αNAC lacking the N-terminus (ΔN1-53-α) were added at indicated concentrations before the start of translation. After translation for 15 min, luciferase activity was measured using a luminescence reader. The luciferase activity is shown in percent relative to the control condition (without added NAC). Error bars = s. d.; **/*** p<0.01/0.001 vs. WT-β/ΔN1-53-α-NAC (two-tailed t-test); n = 3. (A), (C) and (D) were performed by Renate Renate Schlömer and Martin Gamerdinger. Figure was modified from (Gamerdinger et al. 2019). ©2019 Elsevier Inc.
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3.1.6 NAC antagonizes SRP and Sec61 in the tunnel-inserted conformation

The data presented so far provide a better understanding of the interactions between NAC and active and inactive ribosomes. The interaction mechanism suggests important structure-function relationships that are critical for NAC to antagonize the ER targeting machinery. The globular domain of NAC in the tunnel-inserted conformation occupies critical ribosome binding sites of SRP and Sec61 (Figure 24). It collides with the ribosome-bound NG domain of SRP54 as well as with Sec61, both of which bind in the vicinity of uL23 and uL29 (Halic et al. 2004; Voorhees and Hegde 2015; Becker et al. 2009). This suggests that NAC senses the empty tunnel of ribosomes after translation termination by inserting N-βNAC deep into the tunnel, thereby placing the globular domain in an antagonistic conformation to SRP and Sec61. The ability to insert a flexible domain into the empty ribosomal tunnel would allow NAC to act as a specific factor that recognizes and releases 60S ribosomes from the ER translocon upon completion of translation, which, as previously shown, is critical to prevent erroneous protein transport across the ER membrane (Gamerdinger et al. 2015).

Figure 24: The globular domain of NAC antagonizes SRP and Sec61. Cryo-EM reconstruction of C. elegans NAC bound to empty 60S in comparison to SRP and Sec61 bound to active ribosomes (PDB ID: 3JAJ, 3J7R). Ribosomal proteins uL23 and uL29 are colored blue and green, respectively. Cryo-EM reconstruction was done by Kan Kobayashi and Ahmad Jomaa of the Ban Lab in Zürich. Figure was modified from (Gamerdinger et al. 2019).
Therefore, tunnel insertion of N-βNAC may serve to recognize empty ribosomes and protect the tunnel exit from unproductive interaction with other protein biogenesis factors. To test the functional importance of the tunnel insertion in vivo, the tunnel sensing-deficient GFP-βNAC fusion variant described in 3.1.5 was used. Interestingly, *C. elegans* worms expressing GFP-βNAC showed reduced embryo viability and an ER stress phenotype, indicating that tunnel insertion by NAC is critical to maintain ER protein homeostasis (Figure 25A, B). A previous *C. elegans* study demonstrated that NAC directly blocks ribosomes from binding promiscuously to Sec61, thereby preventing incorrect transport of proteins across the ER membrane (Gamerding et al. 2015). Thus, the observed ER stress in GFP-βNAC expressing worms may be due to failed Sec61 antagonism. To test this possibility directly, native rough microsomes from *C. elegans* were purified and puromycin was added to release nascent chains from Sec61-bound ribosomes to mimic translation termination experimentally. As reported previously, puromycin alone did not release ribosomes from the ER membrane (Figure 25C) (Adelman et al. 1973, Gamerding et al. 2015). However, the addition of wildtype NAC significantly enhanced the ribosome detachment, whereas the GFP-βNAC mutant showed little effect (Figure 25C). These findings suggest that tunnel insertion by N-βNAC is critical to release inactive ribosomes from the ER translocon. Hence, without its ability to bind within the empty ribosomal tunnel, NAC is no longer able to recognize inactive ribosomes and move its globular domain into an antagonistic conformation to Sec61.
Figure 25: N-βNAC tunnel insertion regulates ribosome Sec61 interactions. (A) Quantification of progeny in *C. elegans* worms expressing either wildtype (WT) or GFP-tagged βNAC (GFP). The Diagram shows the number of progeny in percent compared to N2 wildtype animals. Error bars represent SD; **p < 0.01 versus WT (two-tailed t-test); n = 3. (B) Quantitative RT-PCR analysis of indicated ER stress-regulated genes in worms as in (A). Diagram shows mean log2 ratio of mRNA levels compared to N2 wildtype animals. Error bars represent SD; n = 3. (C) Rough ER microsomes from *C. elegans* were incubated with NAC variants as in (A) at indicated concentrations without or with puromycin to release nascent chains from ribosomes. The supernatant and the pellet fraction were analyzed via immunoblotting. (A) and (B) were performed by Martin Gamerdinger. Figure was modified from (Gamerdinger et al. 2019). ©2019 Elsevier Inc.
3.2 Mechanistic basis of SRP antagonism by NAC

The main results presented in the following chapter are submitted for publication in:

**Mechanism of signal sequence handover from NAC to SRP on ribosomes during ER protein targeting**

Ahmad Jomaa, Martin Gamerdinger, Hao-Hsuan Hsieh, **Annalena Wallisch**, Viswanathan Chandrasekaran, Zeynel Ulusoy, Alain Scaiola, Ramanujan S. Hegde, Shu-ou Shan, Nenad Ban, Elke Deuerling

Submitted to Science (December 2021)

The main findings of collaborators that are important for understanding and interpreting the results are included in the following chapter. Their contributions are mentioned in the corresponding figure legends.

In the first part of this thesis, new molecular details about the interaction of NAC with ribosomes and short nascent polypeptides were revealed at a very early stage of translation. The finding that NAC inserts N-βNAC into the empty ribosomal tunnel and protects the tunnel exit site with its globular domain from nonspecific interactions with Sec61 explains how NAC releases inactive ribosomes from the ER membrane to prevent co-translational translocation of non-ER proteins. However, it is still unclear how NAC antagonizes the ER targeting machinery at later stages of translation when N-βNAC is entirely displaced from the tunnel by nascent chains. Moreover, it is not clear how the antagonistic function of NAC is overcome when ribosomes translate an ER protein with a hydrophobic signal sequence. For a better molecular understanding of the specific antagonism on signal-lacking ribosomes, biochemical and structural analyses were performed that addressed the cotranslational interaction mechanism of NAC with active ribosomes carrying longer nascent chains that completely displace N-βNAC from the ribosomal tunnel. Potential substrate-specific responses of NAC were investigated using different RNCs translating cytosolic, mitochondrial, and ER model proteins. The data obtained are presented in the following subchapters.
3.2.1 Structure of NAC bound to translating 80S ribosome

A recent cryo-EM structure of a β-tubulin-translating ribosome in complex with the tubulin-interacting factor TTC5 contained an additional density at the tunnel exit that was derived from NAC (Lin et al. 2020). Because β-tubulin is a cytosolic protein, this structure would likely show NAC in its antagonistic conformation to the ER targeting machinery. Refinement of the NAC density model in collaboration with the group of Ramanujan Hegde (MRC Laboratory of Molecular Biology; Cambridge, UK) provided new molecular insights into the interaction of NAC with ribosomes after displacement of N-βNAC from the ribosomal tunnel (Figure 26A). Consistent with the photo-crosslinking data shown in Figure 21, the tunnel discharged N-βNAC was found at the alternative binding site on the ribosome surface near the tunnel exit at eL22 (Figure 26B). Surface bound N-βNAC consists of an α-helix followed by a loop in an anchor-shaped turn wrapping around eL22 and also contacting eL19 and rRNA (Figure 26B and Appendix 7.8.4, page 165, Figure 1A, B, C). The structural data show that N-βNAC makes the main ribosomal contact of NAC in both the tunnel-inserted as well as the surface-bound conformation, explaining why this domain is essential for ribosome binding (Pech et al. 2010).

In addition to N-βNAC, the structure revealed a second ribosome contact of NAC that attaches the globular domain directly to the tunnel exit of the ribosome (Figure 26A). The binding of the globular domain is mediated by two antiparallel helices, one each of αNAC and βNAC (Figure 26C). The helices interact with the ribosome exit site mainly via salt bridges to ribosomal rRNA. Consistent with the structural model, charge reversal mutation of one positively charged aa in the helices (K43E in βNAC or K78E in αNAC) strongly decreased ribosome binding of NAC in vitro (Figure 26D). Interestingly, the ribosome-binding helix of αNAC contains the α-KKAR motif (Figure 14), which contribute in addition to K78 to an overall positive net charge of the helix side that is oriented towards the rRNA at the tunnel exit, explaining its crucial role for ribosome binding (see Appendix 7.8.4, page 179, Figure S5).

Interestingly, the two ribosome interacting domains (N-βNAC and helices/globular domain) are separated by a flexible linker (Figure 26B). This suggests the possibility that NAC can adopt different conformations by having only one domain bound to the ribosome, which might be regulated by translated substrates.
Figure 26: Cryo-EM structure of NAC-TTC5-80S complex reveals two independent ribosome binding sites of NAC. (A) Shown is the structure of the β-tubulin translating ribosome with bound NAC and TTC5 (purple). αNAC is depicted in yellow and βNAC in magenta. The nascent chain is colored red. (B) A close-up of the tunnel exit site is shown with a structural model of NAC fitted into the electron density. Colors are the same as in (A). The ribosomal proteins eL19 and eL22, which interact with the N-terminus of βNAC are shown in light blue. (C) Close-up of the ribosome contact of the NAC globular domain at the tunnel exit. Ribosome binding is mediated by two antiparallel helices making two salt bridges to the 28S rRNA backbone via K78 (in αNAC) and K43 (in βNAC). Colors are the same as in (A). (D) In vitro ribosome binding of wildtype NAC (WT) or variants carrying a charge reversal mutation in the ribosome-interacting lysine residues shown in panel (C) (αNAC K78E and βNAC K43E). NAC variants were incubated with human 80S (ratio 2:1) followed by sucrose cushion centrifugation of ribosomes. Proteins in the supernatant (sup) and pellet fraction were detected by immunoblotting. Cryo-EM analysis were performed by Viswanathan Chandrasekaran in cooperation with the Hegde Lab (Cambridge, UK).
3.2.2 Ribosome binding of the globular domain antagonizes SRP

A comparison of the ribosome interaction of NAC with that of SRP (Voorhees and Hegde 2015; Jomaa et al. 2021) suggests that specifically the globular domain of NAC is positioned antagonistically to SRP (Figure 27A). In contrast, the N-βNAC domain binds at some distance to the exit site outside the SRP binding region (Figure 27A). This suggests that mainly the ribosome binding helices that attach the globular domain to the tunnel exit site (Figure 26C) mediate the SRP antagonism by NAC. To investigate this, an ex vivo ribosome binding competition experiment was performed using a NAC variant carrying charge reversal mutations in the two lysine residues that are critical for globular domain binding, K43E in βNAC and K78E in αNAC (KK-EE, Figure 26C). SRP was efficiently displaced from ribosomes by addition of purified wildtype NAC, whereas KK-EE NAC had no effect (Figure 27B). Even when an 8-fold molar excess was added, KK-EE NAC did not displace SRP from ribosomes, although ribosome association under these conditions was comparable to that of wildtype NAC (Figure 27B). Thus, loss of the globular domain interaction with ribosomes in KK-EE NAC results in loss of SRP antagonism. The remaining ribosome interaction of KK-EE is most likely mediated by N-βNAC, which binds non-competitively with SRP (Figure 27A).

Next, the effects of the KK-EE mutation in NAC on SRP were examined in vivo in C. elegans. As expected from the in vitro studies, ribosome association of KK-EE NAC was significantly reduced in vivo compared to wildtype NAC (Figure 27C). Consistent with the loss of SRP antagonism, overall SRP binding to ribosomes was increased in KK-EE NAC-expressing worms (Figure 27C). These data suggest that ribosome binding of SRP is no longer regulated in these worms. Indeed, SRP pull-down studies demonstrated an increased association of SRP with ribosomes translating cytosolic and mitochondrial proteins in KK-EE NAC animals (Appendix 7.8.4, page 180, Figure S6).
Figure 27: Ribosome binding of the globular domain of NAC is critical to antagonize SRP. (A) Schematic representation showing the overlapping binding sites of NAC (magenta) and SRP (blue) at the exit tunnel of ribosomes. Exclusively the globular domain competes with SRP, whereas N-βNAC binds outside the SRP-binding region. (B) Crude *C. elegans* RNCs were purified and incubated with either wildtype NAC (WT) or a NAC variant with charge reversal mutations in the lysine residues shown in (Figure 26C) (K43E in βNAC and K78E in αNAC = KK-EE). The immunoblot shows proteins in the ribosome pellet fraction after sucrose cushion centrifugation. (C) Sucrose cushion centrifugation of ribosomes in worms expressing the NAC variants as in panel (B). In the worm strains, SRP72 and αNAC were tagged with GFP and FLAG, respectively. Proteins in the pellet fraction were detected by immunoblotting. Experiments in (C) were performed by Martin Gamerdinger.

The faulty interactions of SRP with ribosomes in KK-EE NAC animals likely leads to incorrect targeting of cytosolic and mitochondrial proteins to the ER. The potential mislocalization of those proteins in the oxidizing environment of the ER lumen leads to protein misfolding, which could provoke the unfolded protein response of the ER (UPR\textsubscript{ER}). Indeed, using a GFP-based UPR\textsuperscript{ER} reporter strain (hsp-4p::GFP, Calfon et al. 2002), revealed that KK-EE animals suffer from ER stress. Worms expressing αNAC K64E or βNAC K45E (charge reversal mutations analogous to the human
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variant shown in Figure 26D) showed higher GFP fluorescence, particularly in the gut, indicating ER stress in this highly secretory tissue (Figure 28A) (Calfon et al. 2002). In addition to increased ER stress levels, worms expressing NAC with the KK-EE double mutation showed a shortened lifespan compared to wildtype NAC animals (Figure 28B). However, worms expressing no NAC at all were even more short-lived, suggesting that KK-EE NAC still exerts a residual function in vivo (Figure 28B). In summary, these data show that the ribosome contact of the NAC globular domain is crucial for SRP antagonism, protein targeting specificity, organelle homeostasis, and longevity in vivo.

![Figure 28: Characterization of NAC globular domain ribosome binding mutants in vivo (A). Fluorescence microscope images of C. elegans ER stress reporter worms. GFP is expressed under control of the ER stress-regulated hsp-4 promoter (hsp-4p::GFP). Worms expressing indicated NAC genes (charge reversal mutations analogous to human variants in (Figure 26D)) were analyzed on day 1 of adulthood. (B) Lifespan analysis of worms expressing the indicated NAC variants. KK-EE refers to charge reversal mutant of NAC as in Figure 26D. Diagram shows the percentage of live worms at the indicated time points. WT, wildtype NAC expressing C. elegans strain. Experiment in panel (A) was performed by Martin Gamerdinger.](image-url)
3.2.3 ER signal sequences induce a conformational change in NAC

The data presented so far suggest a mechanistic basis of SRP antagonism by NAC. On ribosomes translating cytosolic (and probably mitochondrial) proteins, NAC occupies a critical SRP binding site adjacent to the tunnel exit via its globular domain. On ribosomes translating ER proteins, only the globular domain of NAC needs to be displaced to allow SRP binding while N-βNAC can remain bound (Figure 27A). Thus, one mechanism to overcome the antagonism would be an ER signal sequence-induced conformational change in NAC that releases the globular domain.

To detect a possible conformational change by NAC on RNCs, site-specific photo-crosslinking experiments were performed in vitro. To analyze the ribosome interaction of N-βNAC with eL22, the NAC variant carrying the photo-crosslinking probe Bpa at position two of βNAC (β-X2) was used (Figure 18A and Figure 29A). The ribosome contacts of the NAC globular domain cannot be tested directly by protein-protein crosslinking approaches because the interaction is only mediated through rRNA at the exit site (Figure 26C). However, release of the globular domain likely involves repositioning of N-αNAC, the regulatory arm of NAC containing the α-DSD motif that critically controls ribosome binding dynamics of NAC (Chapter 3.1.5). On cytosolic RNCs, when the globular domain is bound to the exit site (as seen with the β-tubulin RNC, Figure 26A), N-αNAC is localized separately from N-βNAC on the opposite side of the tunnel exit (Figure 29A, left panel). Release of the globular domain might bring the flexible and negatively charged N-terminus of αNAC into proximity with N-βNAC and the β-RRKKK motif at eL19 (Figure 29A, right panel). To test this possibility, NAC variants carrying Bpa at multiple positions in N-αNAC were first photo-crosslinked with inactive ribosomes where NAC has conformational freedom. Interestingly, robust and highly specific crosslinks to eL19 occurred exclusively with the NAC variant carrying Bpa at α-V35 adjacent to the negatively charged α-DSD motif (Figure 29B), which is known to interact with the β-RRKKK motif (Figure 15A). Thus, NAC can adopt a conformation on inactive ribosomes in which N-αNAC and N-βNAC are no longer separated, which most likely involves the detachment of the globular domain (Figure 29A, right panel).

To investigate a possible reorientation of the NAC globular domain on ribosomes exposing a signal sequence, stalled RNCs carrying 55 aa nascent chains of a cytosolic (GPI), mitochondrial (HSPD1), or ER (HSPA5) substrate were generated. These RNCs have only short N-terminal substrate sequences (~25 aa) exposed at the tunnel exit, while the remaining part of the nascent chain is buried inside the ribosomal tunnel. Thus, for the ER and mitochondrial proteins, the exposed sequences mainly consist of N-terminal targeting peptides (aa 1-18 of HSPA5 and aa 1-26 of HSPD1).
3 Results

(Figure 29C). Photo-crosslinking of β-X2 and α-V35 Bpa-NAC with the different substrate RNCs revealed a conformational change of NAC specifically on the ribosome translating the ER substrate. The β-X2 crosslink to eL22 was comparable with all three RNCs suggesting that the substrates do not alter ribosome binding of N-βNAC (Figure 29D). However, the α-V35 crosslink to eL19 only occurred with the RNC translating the ER protein (Figure 29E). This suggests that on ribosomes translating cytosolic or mitochondrial proteins, the globular domain of NAC is bound to the exit site, separating N-αNAC from eL19 (consistent with the structure of NAC bound to the β-tubulin RNC) (Figure 26A). However, when ribosomes translate an ER protein with a hydrophobic signal sequence, the globular domain of NAC detaches, changing the position of N-αNAC towards eL19. In addition, the data suggest that although the globular domain is released by signal sequences, NAC remains bound to the ribosome via N-βNAC, similar to cytosolic and mitochondrial RNCs.
Figure 29: NAC adopts a different conformation on ribosomes translating ER proteins. (A) Schematic drawing of possible NAC (αNAC, yellow; βNAC, red) conformations on ribosomes. Bpa positions in NAC used in panel (D) and (E) are indicated. Binding of the NAC globular domain to the tunnel exit separates the flexible N-αNAC from eL19, preventing crosslinking between α-V35 and eL19 (left). Release of the globular domain brings N-αNAC close to eL19, enabling crosslinking between α-V35 and eL19 (right). N-βNAC stays bound in both conformations enabling β-X2 crosslinking with eL22. (B) Photo-crosslinking of inactive 60S ribosomes with NAC variants carrying Bpa at indicated positions within N-αNAC. Red arrowhead marks a specific eL19 crosslink revealed by immunoblotting. Figure was modified from (Gamerdinger et al. 2019). (C) Stalled 55 aa RNC constructs used in (D) and (E) translating HSPA5, HSPD1 or GPI. Light grey background indicates part of the nascent chain that is buried in the ribosomal tunnel (~30 aa). SS, signal sequence; MTS, mitochondrial targeting sequence. (D) Photo-crosslinking of Bpa-NAC variant β-X2 to stalled RNCs as in (C). Immunoblot shows crosslink between β-X2 and eL22 (β-X2 x eL22). High exposure of the crosslink band is shown on the right. (E) Similar analysis as in (D) but with Bpa-NAC variant α-V35. Immunoblot shows HSPA5-RNC-specific crosslink between α-V35 and eL19.
3.2.4 ER signal sequences are sensed in conserved hydrophobic pocket of NAC

The described conformational change of NAC on ribosomes translating ER proteins suggests that NAC senses the presence of a hydrophobic signal sequence (Figure 29). The sensing mechanism is apparently coupled to the release of the globular NAC domain and therefore most likely involves the ribosome binding helices that connect this domain to the tunnel exit. In previous crystal structures of NAC, these helices were not found in the same position as in the ribosome-bound conformation, suggesting that the helices are flexibly attached (Spreter von Kreudenstein 2006, Liu et al. 2010, Wang et al. 2010). Interestingly, the ribosome binding helices are amphipathic and orient the positively charged side toward the ribosome surface, while the hydrophobic side contribute to a buried hydrophobic pocket (Figure 30, Appendix 7.8.4, page 179, Figure S5). When the helices become flexible upon ribosome detachment, the hydrophobic pocket would be exposed, allowing it to interact with hydrophobic signaling peptides (Figure 30A). Interaction of signal peptides in the pocket would then prevent pairing of helices and thus ribosome binding of the globular domain, explaining the observed conformational change on HSPA5-RNCs (Figure 29E).
Figure 30: Signal sequence-sensing mechanism of NAC. (A) Model showing the amphipathic ribosome binding helices that orient their charged side toward the ribosome. In the ribosome-bound conformation, the helices are paired and cover an underlying hydrophobic pocket (green). The helices become flexible after release from the ribosome leading to exposure of the hydrophobic pocket. αNAC is shown in yellow; βNAC in purple. (B) Structure of the hydrophobic pocket in the ribosome-bound conformation. Indicated residues between the two helices of NAC were substituted with the photo-crosslinking probe Bpa in panel (C). (C) Photo-crosslinking of indicated Bpa-NAC variants to stalled RNCs carrying 50 aa S\(^{35}\)-labeled nascent chains of cytosolic GPI (left) or a GPI fusion protein containing the N-terminal signal peptide of HSPA5 (right). Autoradiograph images are shown. The positions of the tRNA-attached nascent chain (NC-tRNA) and its crosslinks to αNAC and βNAC are indicated. Asterisk indicates a position outside the hydrophobic region not displayed in panel (B).

To test this hypothesis, several hydrophobic aa within the hydrophobic pocket were replaced by the photo-crosslinking probe Bpa (Figure 30B). As negative control, Bpa was incorporated at α-L53 in the flexible N-terminus upstream of the αNAC ribosome binding helix. The Bpa-NAC variants were then photo-crosslinked with RNCs carrying S\(^{35}\)-labeled nascent chains with a length of 50 aa. No crosslinks...
could be detected with the cytosolic model substrate GPI (Figure 30C). However, after an exchange of the N-terminus of GPI with the N-terminus of the ER substrate HSPA5, which contains an ER signal sequence, strong and specific crosslinks to residues within the hydrophobic pocket occurred (Figure 30C). This suggests that the hydrophobic pocket of NAC specifically interacts with hydrophobic signal sequences consistent with the proposed sensing mechanism. To investigate this further, the Bpa-NAC variants were photo-crosslinked with stalled RNCs carrying 45-60 aa long $\text{S}^{35}$-labeled nascent chains of cytosolic (GPI), mitochondrial (HSPD1), or ER (HSPA5) substrates. Again, strong crosslinks to pocket residues were only detected with the ER substrate containing a hydrophobic signal sequence (Figure 31). The crosslinks became stronger with increasing length of the ER nascent chain, indicating that only fully exposed signal sequences interact efficiently with the NAC pocket (Figure 31). The mitochondrial chain, which has an amphipathic mitochondrial targeting sequence (MTS) at the N-terminus (Figure 7B), showed weak crosslinks to two pocket residues (α-M80 and α-L83). These residues are located in a less buried position of the pocket just beyond the αNAC helix (Figure 30B). However, no crosslinks could be detected between the mitochondrial substrate and residues located deep within the pocket (such as α-I121), suggesting that an MTS does not fully engage the pocket despite its partial hydrophobic character.
Figure 31: Co-translational interaction of NAC with signal sequences. Photocrosslinking of indicated Bpa-NAC variants with stalled S\textsuperscript{35}-labeled nascent chains with a length between 45 aa and 60 aa. Three different proteins were translated: a cytosolic substrate without a signal sequence (GPI), a mitochondrial substrate harboring an amphipathic mitochondrial targeting sequence (HSPD1), and an ER substrate containing a hydrophobic signal sequence (HSPA5). Autoradiograph images are shown. The positions of the tRNA-attached nascent chain (NC-tRNA) and its crosslinks to αNAC and βNAC are indicated. Asterisk indicates a control position outside the hydrophobic pocket.

The finding that the NAC pocket interacts efficiently with hydrophobic ER signal sequences but not with amphipathic MTS suggests that the hydrophobicity of the peptide sequences is a critical factor for pocket engagement. To test this, the hydrophobicity in the ER signal sequence of the model substrate preprolactin (PRL) was altered to be either more or less hydrophobic. Stalled 55 aa RNCs with the modified signal sequences at the N-terminus were then probed with Bpa-NAC variants carrying the crosslinking probe in positions either deep inside the pocket (α-I\textsubscript{121} and β-L\textsubscript{48}) or less deeply buried (α-M\textsubscript{80}) (Figure 30B). Interestingly, the crosslinking efficiency at the more buried positions, α-I\textsubscript{121} and β-L\textsubscript{48}, increased with higher
hydrophobicity of the signal sequence, while the less buried α-M80 showed the opposite effect (Figure 32A). This suggests that α-M80 makes initial contact with nascent chains and may serve as a “gatekeeper” that allows only substrates with sufficiently high hydrophobicity to access the pocket interior where α-I121 and β-L48 are located. This mechanism would also explain the weak α-M80 crosslink to the mitochondrial substrate (Figure 31). To investigate this possibility, α-M80 was replaced with a polar aa (M80S) and the crosslinking efficiency of signal sequences within the pocket at α-I121 was examined. Consistent with a gatekeeper function of α-M80, the crosslinking efficiency between α-I121 and signal sequences was significantly reduced (Figure 32B). The observed difference was specific for the interaction with the nascent chain, since the intramolecular photo-crosslink between α-I121 and βNAC, which also occurred, was unchanged in the M80S-NAC variant (Figure 32B, lower panel).

Figure 32: Pocket engagement depends on substrate hydrophobicity. (A) Photocrosslinking of Bpa-NAC variants to stalled RNCs carrying S^{35}-labeled nascent chains (55 aa) with preprolactin (PRL) signal sequences (SS) of variable hydrophobicity (hy.). Bpa was incorporated either deep inside the hydrophobic pocket (α-I121 and β-L48) or less deeply buried (α-M80) (Figure 30B). An autoradiograph image is shown. The positions of the tRNA-attached nascent chain (NC-tRNA) and its crosslinks to αβNAC are indicated. The grand average of hydropathy (GRAVY) value for the SS are indicated below (Kyte and Doolittle 1982). (B) In vitro crosslinking of M80S NAC variant containing Bpa at α-I121 to stalled RNCs with S^{35}-labeled nascent chains (55 aa, HSPA5). An autoradiograph image (top) and βNAC immunoblot (bottom) are shown. It should be noted that Bpa at α-I121 also partially crosslinks to βNAC leading to the formation of a covalently linked αNAC::βNAC dimer, as indicated.
The exact structure of the exposed hydrophobic pocket upon release of the ribosome binding helices from the ribosome is unknown. The above data suggest that α-M80 is in an exposed site and initially senses the hydrophobic properties of the nascent chain. In the ribosome-bound conformation, α-M80 is located between the two helices (Figure 30B), suggesting that the helices must separate to allow substrate binding at this site and eventually reach the pocket interior. Therefore, an in vitro experiment was designed to test whether helices separation is a prerequisite for binding of the signal sequence deep in the pocket at α-I121. A cysteine pair (αC75::βC51) was introduced into the helices at sites close enough to form a disulfide bridge under oxidizing conditions (Figure 33A). SDS-PAGE analysis showed that the protein runs mostly as an αNAC::βNAC dimer under nonreducing conditions, suggesting that the helices are covalently linked (Figure 33B, lower panel). Strikingly, crosslinking of signal sequences at α-I121 within the pocket occurred only when the cysteine pair NAC variant was reduced (Figure 33B, top panel). In the oxidized state, only minor crosslinking occurred, which was likely due to the remaining amount of reduced protein indicated by the αNAC monomer levels (Figure 33B, lower panel). These data strongly suggest that the helices must move apart to allow binding of signal sequences in the pocket. This, in turn, suggests that the signal sequences prevent the helices from adopting the paired conformation compatible with ribosome binding, explaining why the globular domain of NAC detaches on ER-RNCs (Figure 29).

Figure 33: Substrate binding in the pocket depends on separation of the ribosome binding helices. (A) Structure of the ribosome binding helices of NAC showing the position of the cysteine pair used in panel (B). (B) In vitro photo-crosslinking of cysteine pair NAC variant (αC75::βC51) carrying Bpa at α-I121 with stalled 35S-labeled RNCs (55 aa, HSPA5). Crosslinking was performed in the oxidized (ox.) or reduced (red.) state of the protein. Autoradiograph image (top) and αNAC immunoblot (bottom) are shown. It should be noted that Bpa at α-I121 also partially crosslinks to βNAC leading to the formation of a disulfide-independent, covalently linked αNAC::βNAC dimer. NC-tRNA; tRNA-attached nascent chain.
3.2.5 Functional relevance of the signal sequence-sensing mechanism by NAC in vivo

The data obtained suggest that the antagonism of NAC toward SRP is overcome by ER signal sequences that displace the inhibitory globular domain from the SRP-binding site at the exit of the ribosomal tunnel. Thus, recognition of the signal sequence by NAC appears to be the first mechanism of action that initiates protein targeting to the ER before SRP engages the ribosome. However, elucidating the importance of the signal-sensing mechanism by NAC for ER protein transport in vivo is difficult. The approach with the cysteine pair variant (Figure 33) is not applicable in the reducing environment of a cell. Moreover, any mutation of the hydrophobic pocket that destabilizes the pairing of the ribosome binding helices would impair the SRP antagonism via the globular domain. Therefore, mutational variants that affect only the sensing mechanism but not SRP antagonism need to be carefully designed and tested. The replacement of hydrophobic residues deep within the pocket by hydrophilic aa likely destabilizes the ribosome binding platform of the globular domain. Therefore, the focus was on the less deeply buried α-M80 residue, which appears to act as a gatekeeper allowing only hydrophobic peptides to access the interior of the pocket (Figure 32). Thus, a C. elegans strain expressing a FLAG-tagged αNAC variant with a F66S mutation (analogous to human M80S) was generated. Should the F66S mutation affect ribosome binding of the globular domain, a lower overall binding of NAC to ribosomes would be expected, as observed with KK-EE NAC (Figure 27C). However, control experiments showed that heterodimerization with βNAC and overall ribosome binding of α-F66S NAC was comparable to wildtype NAC in vivo (Figure 34A). This suggests that the polar residue at the less buried position of the hydrophobic pocket does not destabilize the pairing of the ribosome binding helices and thus should not affect SRP antagonism on signalless ribosomes. Further analysis of the α-F66S mutant strain in vivo revealed a significantly lower number of progeny, suggesting that embryo viability is severely impaired in the signal-sensing mutant strain (Figure 34C). Moreover, α-F66S NAC worms showed higher GFP fluorescence in the hsp-4p::GFP reporter background, especially in highly secretory tissues such as the intestine, indicating ER stress (Figure 34D). The disruption of ER protein homeostasis could result from impaired ER protein transport. To investigate this, a secretion reporter strain expressing GFP fused to a signal sequence (ssGFP) was used (Fares and Greenwald 2001). In this strain, ssGFP is expressed and secreted by muscle cells and accumulates in the body cavity of worms, which can be measured by worm flow cytometry. Worm fluorescence-activated cell sorting (FACS) analysis showed that secretion of ssGFP
was reduced in α-F66S NAC expressing animals, despite unchanged ssGFP mRNA levels, indicating a protein targeting defect (Figure 34E, F). Collectively, these data suggest that recognition of ER signal sequences in the hydrophobic pocket of NAC is important for the maintenance of organellar homeostasis, cell viability, and efficient protein secretion.

Figure 34: In vivo analysis of the signal sequence-sensing mechanism by NAC. (A) Anti-FLAG co-immunoprecipitation (co-IP) analysis of C. elegans worms expressing indicated FLAG-tagged αNAC and untagged βNAC constructs. Proteins in FLAG immunoprecipitated fractions were detected by immunoblotting. (B) Sucrose cushion centrifugation of ribosomes in worms as in (A). Proteins in the ribosomal pellet fraction were detected by immunoblotting. (C) The diagram shows the number of progeny of worms as in (A) in percent compared to N2 wildtype animals. It should be noted that animals expressing no NAC (-) produce no progeny. Error bars represent SD; **p < 0.01 vs. WT (two-tailed t-test); n=3. (D) Fluorescence microscope images of worms as in (A) in the hsp-4p::GFP genetic background (ER stress reporter). Analysis was performed on day 1 of adulthood. BF, bright field. (E) C. elegans worms as in (A) were mated to protein secretion reporter strain, expressing GFP fused to a signal sequence (ssGFP). GFP fluorescence was assessed by worm flow cytometry on day 1 of adulthood. The box plot shows the ratio of ssGFP to the axial length of worms (time-of-flight; TOF). Dots indicate individual data points. Box plot center line = median; box length = upper + lower quartile; whiskers = minimum/maximum quartile. RFU, relative fluorescence units. (F) Quantitative RT-PCR analysis of ssGFP mRNA levels in worms as in (E). Diagram shows the fold-change of mRNA levels (WT set to 1). Error bars represent SD. Experiments were performed by Martin Gamerdinger.
In conclusion, the data presented in this work provide new mechanistic insights into the function of NAC as an ER targeting inhibitor. The work unravels the mechanism of how NAC specifically releases inactive ribosomes from the ER translocon by inserting a flexible domain into the empty ribosomal tunnel. This resets the ribosome after translation is complete, preventing translocation of non-ER proteins in the next round of translation. Moreover, the globular domain of NAC was found to be crucial to protect the ribosome exit site from unproductive interaction with other protein biogenesis factors like SRP. Molecular details of its interaction with the exit site of ribosomes were uncovered, and a mechanism of how ER signal sequences relieve the SRP antagonism by NAC was discovered. The results are of great importance for understanding the accuracy in protein transport to cellular organelles and will be discussed in detail in the next chapter with reference to the literature.
4 Discussion

This study provides detailed insights into the molecular mechanism of NAC, which acts as a gatekeeper at the ribosome exit site and directs newly synthesized proteins into the correct protein biogenesis pathway. The unique structure of NAC, consisting of a central globular domain and four flexible terminal arms (Figure 35), allows NAC to bind ribosomes in a flexible manner and to adopt specific substrate-dependent conformations that determine the access of other protein biogenesis factors to their specific nascent substrates. In the course of this work, two important sensing mechanisms by NAC were discovered. NAC can discriminate between active and inactive ribosomes through its unique ability to insert a flexible domain deep into an empty ribosomal tunnel. Moreover, NAC can discriminate between ER proteins and cytosolic or mitochondrial substrates through specific interactions with the hydrophobic ER signal sequence in a conserved hydrophobic pocket (Figure 36). Both sensing mechanisms are coupled to an effector domain, the central globular domain of NAC, which is positioned antagonistically to other ribosome-associated factors depending on the translation state. The obtained data reveal the molecular mechanism of how NAC antagonizes SRP and Sec61 to sustain ER protein targeting accuracy. The underlying mechanisms are discussed in detail in the following chapters and cross-referenced with existing literature. Possible additional functions of NAC at the ribosomal tunnel derived from the new findings and from the literature are also discussed.
4 Discussion

Figure 35: Prediction of the entire NAC structure. Representation of the unrelaxed entire NAC generated with alphafold (Jumper et al. 2021). The orientation is the same as in Figure 26. βNAC is depicted in magenta, αNAC in yellow.

4.1 NAC resets ribosomes after translation termination

After translation is complete, the 80S ribosome is split into the 40S and 60S subunits, which are then recycled for the next round of translation (Hellen 2018). Translationally inactive ribosomes represent a substantial fraction of total ribosomes in cells at any given time depending on the cell type and growth conditions (up to 70% in nondividing cells) (Cooper et al. 1976). Moreover, the pool of inactive ribosomes in cells can increase sharply under stress conditions that lead to global translation repression (Martinez-Pastor and Estruch 1996, Van Den Elzen et al. 2014, Yamamoto and Izawa 2013, Starosta et al. 2014, Hinnebusch 2005). Thus, inactive ribosomes in cells are also subject to regulation by ribosome-binding factors that cover critical functional sites of ribosomes to prevent unproductive interactions. One example is IFRD2, which binds directly to inactive 80S ribosomes to prevent uncontrolled binding of mRNAs and P-site tRNAs to the 40S subunit (Brown et al. 2018). Inactive 60S ribosomes and active 80S ribosomes have the same binding platform at the tunnel exit, which is recognized by various protein biogenesis factors (Figure 1). An
unprotected binding platform on inactive ribosomes would therefore attract factors that pause unproductively at the tunnel exit in the absence of a specific nascent substrate. This would be detrimental for protein biogenesis, since most ribosome-associated factors, like SRP and RAC, are much less abundant in the cell than ribosomes (Akopian et al. 2013, Gamerdinger 2016). Thus, these factors would be functionally depleted from translationally active ribosomes in cells with severe consequences for co-translational protein transport and folding. The data obtained in this work suggests that NAC is the critical factor in cells that generally protects the binding platform at the exit of the ribosomal tunnel from unproductive interactions with other factors. Consistent with this hypothesis, NAC is the only exit site binding factor expressed in at least equimolar concentration to ribosomes, sufficient to regulate all inactive, as well as active, ribosomes in a cell (Del Alamo et al. 2011, Raue et al. 2007).

The platform protective action by NAC is shown as an example for Sec61. The translocation channel of the ER membrane binds with a very high affinity in the low nanomolar range to ribosomes (Borgese et al. 1974, Jungnickel and Rapoport 1995). While efficient opening of the channel is triggered by hydrophobic signal sequences (Hessa et al. 2005, Voorhees and Hegde 2016), the Sec61-ribosome interaction per se is not. Early in vitro studies showed that inactive ribosomes bind to purified ER microsomes with a similar affinity than ribosomes translating an ER protein (Adelman et al. 1973, Rolleston 1972, Borgese et al. 1974, Kalies et al. 1994). Moreover, in vitro data demonstrated that after successful translocation of a substrate, ribosomes remain bound on the ER membrane (Adelman et al. 1973). High-resolution structures of the Sec61-ribosome complex explain these observations, revealing several high-affinity Sec61 binding sites adjacent to the ribosomal tunnel exit (Voorhees et al. 2014). It is hypothesized that strong binding is required to maintain the membrane ion permeability barrier during co-translational translocation of proteins through the channel (Ménétret et al. 2000, Beckmann et al. 2001, Beckmann et al. 2018). However, the inherently strong binding of the two factors requires, in part, the action of a release factor following successful protein translocation mediated by NAC (Gamerdinger et al. 2015). It was clear that the release factor must act specifically on inactive ribosomes to prevent premature release of ribosomes from the ER channel. The unexpected finding that NAC inserts the flexible N-βNAC deep into the empty tunnel of an inactive ribosome explains this specificity. After successful translocation, N-βNAC recognizes the empty tunnel which gives NAC, through direct interactions of N-βNAC inside the tunnel, a selective advantage over Sec61 for ribosome binding. After tunnel insertion, the globular domain of NAC is positioned
outside the tunnel in an antagonistic manner toward Sec61, preventing the ribosome from rebinding to the ER membrane, which eventually leads to ribosome release.

It is apparent that NAC must release ribosomes as soon as translation is complete to prevent the initiation of translation of non-ER proteins by Sec61-bound 60S ribosomes. Therefore, in order to act in a timely manner, it would be beneficial if NAC is already present at the surface of the tunnel exit during protein translocation. Interestingly, our structural data suggest that this is possible due to the alternative binding site of N-βNAC on the surface of ribosomes at eL22, which is in some distance to the exit site (Figure 21). This NAC binding site lies outside the binding region of Sec61, which binds on the opposite side of the tunnel exit at uL29 and uL23 (Figure 27A). This indicates that NAC can also bind ribosomes in a non-competitive manner to Sec61 through the surface contact of N-βNAC and when the globular domain is detached. It is thus reasonable that N-βNAC is continuously bound to the exit site, sensing for the presence of an empty ribosomal tunnel. Sensing the tunnel properties during translocation is possible because the conduit formed by the Sec61-ribosome complex is not tightly sealed. On one side of the tunnel exit, the complex is less tightly associated thereby allowing exit of cytosolic loop regions of transmembrane proteins during translocation (Ménétret et al. 2000, Beckmann et al. 2001, Beckmann et al. 2018). This side faces the N-βNAC binding site at eL22, which explains how NAC can rapidly recognize an empty tunnel after complete translocation to release the ribosome from the ER membrane.

Protection of the exit site from non-specific interactions between ribosomes and Sec61 is particularly important to prevent translocation of non-ER proteins across the ER membrane (Gamerdinger et al. 2015). However, in the absence of the release factor NAC, also the specific protein transport of ER proteins to the ER is likely impaired, as there are less vacant Sec61 translocons to be targeted by the SRP machinery. As mentioned above, most tunnel exit site binding protein biogenesis factors are low abundant in cells. Thus, unproductive interactions of any factor with inactive ribosomes would significantly reduce the protein biogenesis capacity of cells. It is likely that NAC generally blocks the exit site binding platform of inactive ribosomes and antagonizes various factors in addition to Sec61. In the tunnel-inserted conformation, the globular domain of NAC occupies the entire tunnel exit site (Figure 21), where critical binding sites of most factors are located. The globular domain seemingly also competes with SRP, MAPs, NATs, and RAC (Jomaa et al. 2021, Nyathi and Pool 2015, Nyathi and Pool 2015, Knorr et al. 2019, Lee et al. 2016). Consistent with this assumption, a recent yeast study suggests competition between NAC and MAPs on ribosomes (Nyathi and Pool 2015). Furthermore, an in vitro ribosome
binding competition experiment showed that RAC is displaced from ribosomes by wildtype NAC but not by a ribosome binding deficient variant with a mutation in the β-RRKKG motif (Appendix 7.8.4, page 146, Figure 2). Moreover, the antagonism of SRP binding by NAC on active ribosomes described in this work (discussed in detail below) strongly indicates that NAC also antagonizes SRP in the tunnel-inserted conformation on inactive ribosomes. Overall, these data suggest that NAC is a broad antagonist that displaces various factors from non-translating ribosomes, thereby resetting ribosomes for the next round of translation. Future studies are required to test this hypothesis.

4.2 Does NAC sense nascent chain features inside the tunnel?

Early co-translational recognition and discrimination of substrates are essential for proper protein biogenesis. This is especially true for ER transport substrates such as transmembrane proteins, which must be co-translationally inserted into a membrane. If timely targeting of ribosomes fails, these substrates will expose their highly hydrophobic transmembrane domains (TMDs) in the cytoplasm, leading to protein aggregation (Nyathi et al. 2013). Early recognition of ER substrates and specific recruitment of SRP to translating ribosomes are therefore essential for successful protein transport. However, protein biogenesis factors like SRP can bind nascent polypeptides not before they exit the ribosomal tunnel (Krieg et al. 1986, Kurzchalia et al. 1986). The ability of NAC to insert a flexible domain deep into the ribosomal tunnel to the constriction site near the PTC suggests the possibility that NAC could recognize specific features in the N-termini of nascent chains, such as hydrophobic signal sequences before they reach the cytoplasm.

The photo-crosslinking experiments performed in this study show that NAC directly contacts nascent substrates inside the tunnel starting at a length of 10 aa. Nascent chain contacts are made deep in the tunnel by the N-terminus of βNAC, and at the mouth of the tunnel by the N-terminus of αNAC (Figure 19). It is unclear whether these interactions are of functional significance for protein biogenesis or transport. Interestingly, however, SRP binds ribosomes that carry a not yet exposed signal anchor (SA) inside the tunnel with significantly higher affinity than ribosomes translating a cytosolic substrate (Berndt et al. 2009). This is remarkable because the SA cannot yet be bound by SRP in this case. Thus, the feature of the nascent chain is sensed inside the tunnel and a signal transmitted to the ribosome
surface that initiates SRP binding. A previous \textit{in vitro} study demonstrated that the early SRP binding to ribosomes displaying a SA inside the tunnel depends on NAC (Zhang et al. 2012). This indeed suggests that NAC recognizes N-terminal signal sequences already inside the tunnel and actively recruits SRP (likely via the C-terminal UBA domain as discussed below). However, it is difficult to imagine how NAC could sense the physicochemical properties of substrates in the narrow tunnel that restricts large-scale three-dimensional substrate interactions.

An alternative hypothesis is that the ribosomal tunnel itself discriminates nascent substrates leading to subtle conformational changes that are perceived by NAC within or at the mouth of the tunnel. Previous studies suggest that the ribosomal tunnel is not a passive conduit. Rather, the tunnel appears to be a functional compartment that monitors the structure and properties of the nascent polypeptide (Lu et al. 2007, Seidelt et al. 2009). Nascent substrate segments such as TMDs interact with the tunnel lining wall at the constriction site proximal to the PTC (Woolhead et al. 2004). Upon sensing of a TMD at the tunnel constriction, an unknown signal can be transmitted to the surface leading to specific recruitment of protein biogenesis factors such as the translocon-associated protein RAMP4 (Schröder et al. 1999, Johnson 2009). Thus, it can be speculated that NAC may play a role as a mediator that relays the signals from the ribosomal tunnel to specific protein biogenesis factors such as SRP. Further studies are needed to decipher this potential mechanism.

In addition to NAC two other proteins are known to insert a flexible domain deep into the ribosomal tunnel. The ribosome biogenesis factor Rei1 probes the tunnel in late steps of ribosome biogenesis during 60S maturation (Greber et al. 2016). Rei1 inserts its C-terminus into the tunnel, which even passes the constriction site and makes specific contacts with the tunnel wall along its entire length. Failure to insert the C-terminus of Rei1 impairs subsequent maturation steps of 60S ribosomes suggesting that Rei1 proofreads the integrity of the ribosomal exit tunnel (Greber et al. 2016). Rei1 is required to release the nuclear export factor Arx1 from the 60S subunit (Hung and Johnson 2006).

The other tunnel inserting factor is the mitochondrial ribosome (mitoribosome) associated protein mL45, a transport factor that docks mitoribosomes to the membrane insertase OXA1L (Kummer et al. 2018, Itoh et al. 2021). Interestingly, mL45 shares some characteristic features with NAC. Like NAC, mL45 inserts its flexible positively charged N-terminal tail deep into the ribosomal tunnel up to the constriction site. The tail is pushed out by a growing nascent chain and realigns at the mouth of the ribosomal tunnel, forming a narrow opening (less than 8 Å) through which a polypeptide chain can migrate to the membrane insertase (Itoh et al. 2021). How-
ever, the function of mL45’s tunnel sensing is not understood. Deletion of the tunnel sensing domain of mL45 is associated with membrane insertion defects in yeast, suggesting a crucial role in protein translocation (Bauerschmitt et al. 2010). Mitoribosomes are thought to be constantly linked to the membrane translocon, being specialized in the synthesis of only highly hydrophobic membrane proteins (13 proteins in humans) of the oxidative phosphorylation (OxPhos) system (Englmeier et al. 2017, Anderson et al. 1981, Anderson et al. 1982). Thus, a role of mL45 in ribosome targeting seems unlikely. Rather, mL45 may regulate the activity of the membrane insertase OXA1L and associated proteins. Like NAC, mL45 can distinguish active ribosomes from inactive ribosomes through its tunnel insertion domain and thus signal the translational state to the insertase (Bauerschmitt et al. 2010). It can be speculated that mL45 may also discriminate specific substrates and relay signals to the insertase for specific recruitment of required insertase-associated factors, similar to the RAMP4 recruitment to the ER translocon described above. However, whether mL45 or NAC indeed recognize and transmit signals, other than the translation activity state of ribosomes, from inside the tunnel to other factors remains to be investigated.

4.3 NAC functions as a molecular router at the ribosomal tunnel exit

With the exception of a few proteins translated in mitochondria (see 4.2), all other proteins in a cell are translated by cytosolic ribosomes (Roberts et al. 2002, Anderson et al. 1981, Anderson et al. 1982, Hansen and Herrmann 2019). The eukaryotic cell can be divided into three main compartments: the cytoplasm, the ER, and the mitochondria. The newly synthesized proteins must be specifically transported into the latter two during or after translation by cytosolic ribosomes (Blobel 1980). Both the ER and mitochondria are endomembrane compartments that contain many transmembrane proteins harboring TMDs of similar hydrophobicity (Hegde and Keenan 2021). Therefore, sorting transmembrane proteins into the correct intracellular compartment is quite a challenge for eukaryotic cells that is not present in organelle-less bacteria. This likely explains why eukaryotic cells evolved an additional specificity factor, NAC, that antagonizes the ER targeting machinery on ribosomes translating cytosolic and mitochondrial proteins. Consistent with this assumption, mitochondrial proteins are translocated to the ER when NAC is removed (Gamerdinger et al. 2015), whereas ER proteins are transported to mitochondria when SRP is removed (Costa et al. 2018).
The results obtained in this work reveal the mechanistic basis of how NAC antagonizes SRP on ribosomes translating cytosolic and mitochondrial proteins (Figure 36). By placing its globular domain to the tunnel exit site, NAC simply sterically blocks the access of SRP to these ribosomes. That NAC and SRP compete for ribosome binding sites at the tunnel exit has been suggested by several in vitro binding studies (Wiedmann et al. 1994, Wiedmann and Prehn 1999, Powers and Walter 1996). However, a recent study showed that NAC forms a ternary complex with SRP on ribosomes translating an ER protein (Hsieh et al. 2020). This was inconsistent with the assumption that NAC and SRP use mutually exclusive ribosome binding sites. This discrepancy could be resolved by the fact that NAC has two independent ribosome binding domains, one of which is antagonistic to SRP (globular domain) and the other is not (N-βNAC).

Installation of an SRP antagonist at the ribosome exit site in eukaryotes requires an intelligent evolutionary design that ensures relief of the antagonism on ribosomes translating an ER substrate. Our data demonstrate that this problem was solved by NAC itself recognizing hydrophobic signal sequences even before SRP and releasing the SRP binding site at the ribosomal tunnel exit by a conformational change (Figure 36). The sensing domain is a hydrophobic pocket that is buried in the ribosome bound conformation. A prerequisite for signal sequence binding is the opening of this pocket. Our data suggest that the paired antiparallel helices covering the pocket separate when the globular domain is detached from the ribosome, resulting in the exposure of the hydrophobic residues in the pocket. This is consistent with two published crystal structures of the globular NAC domain, in which the helices were not found in the same position as in the ribosome-bound conformation (Wang et al. 2010, Liu et al. 2010). In the crystal structures, the helices were detached from the globular domain and partially fragmented, suggesting that they were flexible and partially hydrolyzed during the crystallization process. Moreover, in a third crystal structure of the globular NAC domain (Spreter von Kreudenstein 2006), the entire helices were resolved, protruding widely from the globular domain. The two protruding helices expose their hydrophobic side to the solvent and, together with the hydrophobic surface of the β-barrel domain, form an exposed hydrophobic pocket. This crystal structure likely shows the ribosome unbound conformation of NAC. Binding of a signal sequence in the exposed pocket would prevent pairing of the helices and thus the formation of a platform competent in ribosome binding, which explains why the globular domain is detached on ribosomes translating ER proteins. The proposed sensing mechanism requires the initial detachment of the globular domain to prime the pocket for substrate binding. This is very likely a
substrate-independent process and due to rapid dissociation and association kinetics of the globular domain. Therefore, the signal sequences likely interact with NAC when the globular domain is detached, the pocket is open, and then prevent reclosure of the hydrophobic pocket and thus globular domain reattachment.

Signal sequences specifically release the ribosome contact that is antagonistic to SRP (globular domain) while NAC remains bound to ribosomes with its non-competitive anchor domain (NβNAC) (Figure 36). The fact that NAC is not fully released suggests that NAC also serves an additional, probably proactive targeting function on ribosomes translating ER substrates. Indeed, work from collaborators show that NAC directly contacts SRP via the C-terminal UBA domain of αNAC (Appendix II 7.8.4). The UBA domain is flexibly bound to the globular domain via a long linker (~55 aa), suggesting that NAC can use it to capture factors in far distances and bring them to the tunnel exit. Indeed, the UBA-SRP interaction was found to be critical for the efficient binding of SRP to ribosomes translating ER proteins. Thus, NAC antagonizes SRP on signal-less RNCs but promotes SRP binding on signal-containing RNCs. Only the combination of both, signal sequence-induced NAC globular domain detachment and UBA-mediated SRP recruitment results in efficient reversal of the NAC antagonism on ER targeting. It is not yet clear whether the UBA-mediated recruitment of SRP is functionally coupled to the ER signal sequence-sensing mechanism. This would be beneficial for ER targeting efficiency by preventing attempts to recruit SRP to signal-less RNCs. However, it is difficult to imagine how the UBA could be regulated, since it is flexibly bound to the globular domain of NAC. Interestingly, the UBA binds to the SRP54 subunit in a region where also the SR binds (Appendix 7.8.4, page 191, Figure S15). This suggests that the UBA recruits SRP and then interacts with SRP until the ribosome is targeted to the ER membrane, where SRP-SR binding occurs. Thus, the UBA, which seems not to regulate SRP-SR interactions (Appendix 7.8.4, page 192, Figure S16), is displaced from SRP specifically at the ER membrane by the SRP receptor. This evolutionary design, the transient silencing of the UBA during targeting and its release at the ER membrane, could predict an additional function of the UBA on ER membrane-bound ribosomes. Consistent with an additional function, the UBA deletion in C. elegans is lethal, whereas the UBA-SRP interaction is not (Martin Gamerdinger, personal communication). Thus, it is possible that the UBA binds and recruits another unknown factor to ribosomes before and after the ER targeting process, which is not desired during the SRP-mediated targeting reaction. It is known that SRP mediates translational arrest while bound to ribosomes to increase the time window for the targeting reaction (Wolin and Walter 1989). Interestingly,
Discussion

Preliminary data suggest that the UBA also interacts with eEF1α (Stefan Kreft, personal communication), an essential translation elongation factor (Carvalho et al. 1984, Sasikumar et al. 2012). In sum, these data may suggest that the UBA recruits SRP to signal-containing ribosomes and SRP subsequently transiently silence the UBA to mediate translational arrest until the ribosome is successfully targeted to the ER membrane. Future studies should investigate this interesting hypothesis.

While the router function of NAC on ribosomes for ER substrates is now better understood, very little is known about the function of NAC on ribosomes translating mitochondrial or cytosolic proteins, apart from the fact that NAC blocks the access of SRP with its globular domain. An important question that remains to be answered is whether NAC can discriminate mitochondrial from cytosolic substrates. A role for NAC in mitochondrial protein transport is indicated by several studies (George et al. 1998, Funfschilling and Rospert 1999, Hotokezaka et al. 2009). A study in yeast suggests that NAC targets ribosomes translating mitochondrial substrates to mitochondria, where NAC binds to the mitochondrial outer membrane (MOM) protein OM14 (Lesnik et al. 2014). This suggests that OM14 acts as a targeting receptor at the MOM, similar to the SRP receptor at the ER membrane. However, the existence of a co-translational targeting pathway to mitochondria with NAC as the ribosome-associated targeting factor is controversial. In contrast to ER substrates, mitochondrial proteins are efficiently translocated into mitochondria in a post-translational manner in vitro (Gasser and Schatz 1983, Zimmermann and Neupert 1980).

Transport in vivo also appears to be predominantly post-translational, as “rough mitochondria” with ribosomes attached to the MOM have only rarely been observed in electron microscopy analyses, in contrast to rough ER, which is studded with ribosomes (Perrotta 2020, Roberts et al. 2002). This was to be expected because, unlike Sec61 (see above), the TOM complex, the translocase of the MOM, does not appear to interact directly with ribosomes (Abe et al. 2000, Eilers and Schatz 1986, Van Wilpe et al. 1999). However, a recent in situ electron cryo-tomography study of yeast cells detected some loosely attached cytosolic ribosomes on the surface of mitochondria (Gold et al. 2017). The number of ribosomes at the MOM increased significantly after cycloheximide treatment, indicating that under physiological conditions, most ribosomes terminate translation before reaching the MOM. Consistent with this, a ribosome profiling study of MOM-associated ribosomes in yeast suggests that co-translational mitochondrial import is low but increases after cycloheximide treatment (Williams et al. 2014). Overall, these data show that while some co-translational import into mitochondria occurs in yeast, the vast major-
ity of substrates are transported post-translationally. The reason for this could be that translocation is faster than translation, which means that many more proteins can be imported in a post-translational manner by fewer TOM complexes (Neupert 1997). However, the ribosome profiling study identified a pool of mitochondrial substrates that are substantially co-translationally transported under normal conditions: mitochondrial inner membrane (MIM) proteins (Williams et al. 2014). MIM substrates have highly hydrophobic TMDs, which are aggregation-prone when exposed in the cytosol, similar to the integral membrane proteins of the ER (Williams et al. 2014, Englmeier et al. 2017, Brandman et al. 2012). Thus, it seems that ribosomes translating MIM proteins are specifically targeted by an unknown mechanism to the TOM complex at the MOM. Whether NAC plays a role in this targeting pathway remains to be investigated, but this is plausible given that NAC is the first factor to associate with all translating ribosomes. NAC could act either as a router similar to the ER pathway by recruiting a specific transport factor or as a targeting factor itself by binding to a MOM receptor, as proposed in a previous yeast study (Lesnik et al. 2014). However, whether a NAC receptor at the MOM exists in higher eukaryotes is unclear, as OM14 is not conserved.

The discrimination between mitochondrial and cytosolic substrates by NAC could be based on the N-terminal mitochondrial targeting sequence and/or the MIM TMDs. The latter are not known to serve as signal anchors as in ER substrates, making it more likely that NAC perceives the MTS. Interestingly, our photo-crosslinking data indicated a weak but specific interaction of NAC with mitochondrial targeting sequences mediated by the ribosome binding helix of αNAC (α-M80 and α-L83, Figure 31). Moreover, the crosslinking data show that an MTS does not fully penetrate the hydrophobic pocket of NAC underlying the two helices, in contrast to ER signal sequence. This suggests that the MTS is bound while the pocket is closed, whereas the pocket is open in the presence of an ER signal sequence. Interestingly, there is a striking similarity in the structure of an MTS and the ribosome binding helices of NAC. They consist of a short amphipathic α-helix that is hydrophobic on one side and positively charged on the other (von Heijne 1986, Bedwell et al. 1989). Considering that the NAC helices are flexible and continuously sample nascent chains, it can be speculated that an MTS may exchange the position with one of the NAC helices. Thus, the pocket would be closed, but one NAC helix would be replaced by the MTS. The liberated NAC helix and its attached domain (for example, the αNAC helix with the upstream located flexible arm containing the α-DSD motif) could then bind a specific targeting factor or receptor at the MOM. Future studies will investigate this or other possible MTS-sensing mechanisms by NAC in more
Another important question that remains to be addressed is whether NAC also directs cytosolic nascent substrates into the proper biogenesis pathway. Most cytosolic substrates need to be co-translationally processed by enzymes modifying the nascent chain N-terminus, including MAPs and NATs (Kramer et al. 2019). It is important to note that ER and mitochondrial substrates might not be processed by these enzymes. Otherwise the modification of the N-terminus would interfere with the translocation of the protein across the membrane (Forte et al. 2011, Linster and Wirtz 2018). Thus, as the N-termini of ER and mitochondrial nascent chains can be matching substrates for these enzymes, their binding to ribosomes must be actively prevented. Therefore, it is likely that NAC sorts all types of nascent chains and specifically recruits MAPs and NATs to ribosomes translating cytosolic proteins. It is likely that in the absence of an ER SS and an MTS, NAC generally adopts the conformation seen on the β-tubulin-translating ribosome (Figure 26). This is a conformation in which NAC does not interact with the nascent chain. Since cytosolic proteins have no specific recognition signal, it is likely that NAC simply remains in this basic position on ribosomes in the absence of an ER SS and an MTS. Thus, NAC likely recognizes cytosolic substrates indirectly by exclusion of mitochondrial and ER targeting signals. In this basic “cytosolic conformation”, NAC may attract MAPs and NATs, possibly by releasing binding sites on the ribosome for these enzymes, or by acting as a binding partner itself. MAPs and NATs are not expected to be present on the β-tubulin RNC because the nascent chain is bound by the tubulin-specific factor TTC5, and tubulin itself is not a substrate of the enzymes (Lin et al. 2020, Charpilloz et al. 2014). The exact ribosome binding sites of these enzymes in higher eukaryotes have not yet been elucidated by structural studies, suggesting that they bind very dynamically (Giglione et al. 2015, Vetro and Chang 2002, Lyon 2013). A previous yeast study showed that deletion of NAC decreases MAP ribosome binding due to displacement by overexpressed SRP (Nyathi and Pool 2015). This study suggests that NAC plays a critical role in coordinating the binding of MAP as well as SRP to translating ribosomes. Whether NAC actively recruits MAP in a similar manner as SRP via a flexibly attached domain needs to be investigated. Further studies are required to decipher the molecular mechanism of the differential router functions of NAC for cytosolic, mitochondrial, and ER proteins.
Figure 36: Mechanistic model of SRP antagonism by NAC. (A) Binding of NAC to an empty ribosome. NAC inserts N-βNAC into the empty ribosomal tunnel and places its globular domain in an antagonistic conformation to other factors, including SRP and Sec61. (B) Binding of NAC to translating ribosomes without exposed signal peptides. N-βNAC binds to the ribosome surface at eL22 and eL19. The globular domain is attached to the tunnel exit by two antiparallel helices antagonistically to SRP (blue). (C) A hydrophobic ER signal sequence (green) binds in a hydrophobic pocket formed between the two helices and the globular domain. This prevents the pairing of the helices and rebinding of the globular domain to the tunnel exit reversing the SRP antagonism. (D) The flexibly tethered C-terminal UBA domain of αNAC directly binds and recruits SRP to the tunnel exit site facilitating signal sequence handover to SRP.
5 Outlook

The results of this study reveal the function of NAC as a molecular router at the ribosome exit site that directs newly synthesized proteins into the correct cotranslational biogenesis pathway. The unique ability to enter the empty ribosomal tunnel with a flexible domain gives NAC a selective advantage in binding to the ribosome over other biogenesis factors, allowing it to reset the tunnel exit site after translation is complete. This also ensures that NAC is the first factor to interact with the nascent chain in the next round of translation. On ribosomes translating mitochondrial or cytosolic proteins, NAC adopts a conformation that opposes the ER targeting factor SRP to prevent misdirection of these substrates into the ER. Upon translation of a secretory protein, the hydrophobic signal sequence is recognized by NAC, which then undergoes a conformational change that allows the SRP targeting machinery to capture the substrate. This study thus reveals the molecular basis of a substrate triage mechanism at the tunnel exit of eukaryotic ribosomes, which is essential for accurate protein localization in cell organelles.

Future studies need to investigate whether NAC can also discriminate between mitochondrial and cytosolic proteins and direct these substrates into specific transport or folding pathways. A sorting mechanism based on the recognition of N-terminal mitochondrial targeting sequences that induces a conformational change in NAC similar to ER signal sequences is conceivable. In this context, the unknown function of two flexible domains of NAC could be investigated, the C-terminal domain of βNAC and the N-terminal domain of αNAC (Figure 35). Of particular interest is the N-terminal domain of αNAC, which contains the conserved α-DSD motif that controls ribosome binding dynamics of NAC. It is possible that this negatively charged tail controls the formation of a substrate-specific conformation of NAC on ribosomes by modulating the properties of the positively charged ribosome binding motifs. Furthermore, the exact function of the C-terminal UBA domain in αNAC is not well understood. Preliminary data suggest that UBA binds another unknown factor in addition to SRP and that this interaction is essential for cell viability. Future studies should identify this factor and investigate how the binding of the UBA to different factors is regulated depending on a translated substrate.

The high-resolution structural information and mechanistic details of NAC revealed
in this study lay the foundation for a deeper understanding of how newly synthesized proteins are sorted and directed to specific protein biogenesis pathways, which is essential for maintaining protein homeostasis in cells.
6 Materials

6.1 Chemicals

All chemicals used in this study were purchased from Sigma-Aldrich, Merck, VWR or Carl Roth, unless indicated otherwise. Enzymes and corresponding buffers were purchased from New England Biolabs. Molecular weight standards for DNA and proteins were purchased from Thermo Fisher Scientific.

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<tr>
<th>Chemical</th>
<th>Source</th>
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<tr>
<td>[S(^{35})] Met-label</td>
<td>Hartmann Analytic GmbH, Braunschweig</td>
<td>SRIS-103</td>
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<tr>
<td>Aprotinin</td>
<td>Genaxxon, BioScience, Ulm</td>
<td>Cat#M6361</td>
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<td>ATP</td>
<td>Sigma</td>
<td>Item-Nr.: A7699</td>
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<tr>
<td>CoA</td>
<td>Sigma</td>
<td>Item-Nr.: C4282</td>
</tr>
<tr>
<td>Complete EDTA-free protease</td>
<td>Roche</td>
<td>Cat#505648900</td>
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<tr>
<td>inhibitor cocktail</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deoxyribonucleotide triphosphate (dNTPs)</td>
<td>New England Biolabs, USA</td>
<td>Cat#N0447S</td>
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<td>DNase I</td>
<td>Sigma-Aldrich</td>
<td>Cat#DN25</td>
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<tr>
<td>DTT (1,4-Dithiothreit)</td>
<td>Carl Roth</td>
<td>Cat#6908.4</td>
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<td>Gen agarose LE</td>
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<td>IPTG</td>
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<td>CN08.3</td>
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<td>Luciferin</td>
<td>Synchem</td>
<td>BC219</td>
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### Chemicals

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<td>N,N,N’,N’-Tetramethylethylendiamine (TEMED)</td>
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<tr>
<td>Nonidet P-40</td>
<td>Fluka Biochemica</td>
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<td>para-benzoylphenylalanine (pBpa)</td>
<td>Bachem</td>
<td>Cat#4017646.005</td>
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<td>Protino® Ni-NTA/IDA Agarose</td>
<td>Machery-Nagel GmbH &amp; Co. KG</td>
<td>Item-Nr.: 12718702</td>
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<tr>
<td>Puromycin</td>
<td>InvivoGen, San Diego, USA</td>
<td>Cat#ant-pr</td>
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<tr>
<td>RNAsin Ribonuclease inhibitor</td>
<td>Promega</td>
<td>N2515</td>
</tr>
<tr>
<td>Ulp</td>
<td>Deuerling Lab</td>
<td>N/A</td>
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**Table 1:** Additional chemicals used in this study.
### Critical Commercial Assays

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<th>Supplier</th>
<th>Identifier</th>
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<tbody>
<tr>
<td>DNA-spin&lt;sup&gt;TM&lt;/sup&gt;</td>
<td>iNtRON Biotechnology,</td>
<td>Cat#17096</td>
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<tr>
<td>Plasmid DNA Purification Kit</td>
<td>Korea</td>
<td></td>
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<tr>
<td>MEGAquick-spin&lt;sup&gt;TM&lt;/sup&gt;</td>
<td>iNtRON Biotechnology,</td>
<td>Cat#17289</td>
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<tr>
<td>Total Fragment DNA Purification Kit</td>
<td>Korea</td>
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</tr>
<tr>
<td>mMESSAGE mMACHINE T7 Transcription Kit</td>
<td>Thermo Scientific</td>
<td>Cat#AM1344</td>
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<tr>
<td>NEBuilder HiFi DNA Assembly Cloning Kit</td>
<td>New England Biolabs</td>
<td>Cat#E5520S</td>
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<tr>
<td>Q5&lt;sup&gt;®&lt;/sup&gt; Mutagenesis Kit</td>
<td>New England Biolabs</td>
<td>Cat#E0554S</td>
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<tr>
<td>QIAprep&lt;sup&gt;®&lt;/sup&gt; Spin Miniprep Kit</td>
<td>Qiagen</td>
<td>Cat#27104</td>
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<tr>
<td>QIAquick&lt;sup&gt;®&lt;/sup&gt; Gel Extraction Kit (50)</td>
<td>Qiagen</td>
<td>Cat#28704</td>
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<tr>
<td>QuantiTect&lt;sup&gt;®&lt;/sup&gt; Reverse Transcription Kit</td>
<td>Qiagen</td>
<td>Cat#205311</td>
</tr>
<tr>
<td>Rabbit Reticulocyte Lysate</td>
<td>Promega, USA</td>
<td>Cat#L4960</td>
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<tr>
<td>RNA Clean Concentrator Kit</td>
<td>Zymo</td>
<td>Cat#R1017</td>
</tr>
<tr>
<td>T7 RiboMAX&lt;sup&gt;™&lt;/sup&gt; Large Scale RNA Production Systems</td>
<td>Promega</td>
<td>Cat#P1300</td>
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**Table 2:** Commercially available Kits, used in this study.
### 6.2 Buffers

#### 6.2.1 General Buffers

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<th>Composition</th>
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<tr>
<td>BisTris gel buffer (3.5x)</td>
<td>1.25 M Bis-Tris HCl pH 6.5</td>
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<tr>
<td>BisTris running buffer (5x) (MOPS)</td>
<td>250 mM MOPS</td>
</tr>
<tr>
<td></td>
<td>250 mM Tris-Base</td>
</tr>
<tr>
<td></td>
<td>5 mM EDTA</td>
</tr>
<tr>
<td></td>
<td>0.5% (v/v) SDS</td>
</tr>
<tr>
<td>Coomassie blue staining solution</td>
<td>0.6% (w/v) Coomassie R250</td>
</tr>
<tr>
<td></td>
<td>50% (v/v) ethanol</td>
</tr>
<tr>
<td></td>
<td>10% (v/v) acetic acid</td>
</tr>
<tr>
<td>Destaining solution</td>
<td>50% (v/v) ethanol</td>
</tr>
<tr>
<td></td>
<td>10% (v/v) acetic acid</td>
</tr>
<tr>
<td>DNA loading dye (6x)</td>
<td>30% (v/v) glycerol</td>
</tr>
<tr>
<td></td>
<td>60 mM EDTA</td>
</tr>
<tr>
<td></td>
<td>1.2 mg/mL xylene cyanol</td>
</tr>
<tr>
<td></td>
<td>1.2 mg/mL bromophenol blue</td>
</tr>
<tr>
<td>ECL</td>
<td>(A) 0.1 M Tris-HCl pH 8.6</td>
</tr>
<tr>
<td></td>
<td>0.25 mg/mL luminol</td>
</tr>
<tr>
<td></td>
<td>(B) 1.1 mg/mL p-cumaric acid in DMSO</td>
</tr>
<tr>
<td></td>
<td>(C) 30% (v/v) H₂O₂</td>
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<td></td>
<td>Working solution: A:B:C = 1000:100:1</td>
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<tr>
<td>Laemmli buffer (5x) (sample buffer)</td>
<td>250 mM Tris-HCl pH 6.8</td>
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<td></td>
<td>12.5 mM EDTA</td>
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<tr>
<td></td>
<td>5% (v/v) SDS</td>
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<td></td>
<td>5% (v/v) β-mercaptoethanol</td>
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<td>50% (v/v) glycerol</td>
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<td></td>
<td>5 mg/mL bromophenol blue</td>
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<tr>
<td>Phusion polymerase buffer</td>
<td>10 mM Tris-HCl pH 8.8</td>
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<td></td>
<td>50 mM KCl</td>
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<td></td>
<td>2 mM MgCl₂</td>
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<td></td>
<td>0.1% (v/v) Triton X-100</td>
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### Buffer Composition

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<td>2 mM EGTA pH 8.0</td>
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<td></td>
<td>4% (v/v) SDS</td>
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<tr>
<td></td>
<td>20% (w/v) sucrose</td>
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<td>Add directly before use:</td>
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<tr>
<td></td>
<td>1x Tm complete</td>
</tr>
<tr>
<td>SDS running buffer (10x)</td>
<td>25 mM Tris</td>
</tr>
<tr>
<td></td>
<td>20 mM glycine</td>
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<td>1% (w/v) SDS</td>
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<td>SDS separation gel buffer (4x)</td>
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<td>0.4% (v/v) SDS</td>
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<td>0.5 M Tris-HCl pH 6.8</td>
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<td>0.4% (v/v) SDS</td>
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<td>TAE buffer (50x)</td>
<td>2 M Tris base</td>
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<tr>
<td></td>
<td>5.71% (v/v) acetic acid</td>
</tr>
<tr>
<td></td>
<td>50 mM EDTA pH 8.0</td>
</tr>
<tr>
<td>TBS (10x)</td>
<td>50 mM Tris-HCl pH 7.5</td>
</tr>
<tr>
<td></td>
<td>150 mM NaCl</td>
</tr>
<tr>
<td>TBS-T (10x)</td>
<td>75 mM Tris-HCl pH 8.0</td>
</tr>
<tr>
<td></td>
<td>150 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>1% (v/v) Tween-20</td>
</tr>
<tr>
<td>Western Blot transfer buffer (10x)</td>
<td>25 mM Tris</td>
</tr>
<tr>
<td></td>
<td>192 mM glycine</td>
</tr>
<tr>
<td></td>
<td>0.02% (w/v) SDS</td>
</tr>
<tr>
<td></td>
<td>20% (v/v) methanol</td>
</tr>
</tbody>
</table>

**Table 3:** Buffer compositions of general buffers used.
6.2.2 RNC-Purification Buffers

### Table 4: Buffers for RNC purification and their composition.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNC Buffer</td>
<td>50 mM Hepes pH 7.4</td>
</tr>
<tr>
<td></td>
<td>100 mM KOAc</td>
</tr>
<tr>
<td></td>
<td>5 mM Mg(OAc)$_2$</td>
</tr>
<tr>
<td></td>
<td>1 mM DTT</td>
</tr>
<tr>
<td></td>
<td>1x Tm complete</td>
</tr>
<tr>
<td>RNC Wash Buffer I</td>
<td>RNC Buffer</td>
</tr>
<tr>
<td></td>
<td>+ 0.1% Triton-X</td>
</tr>
<tr>
<td>RNC Wash Buffer II</td>
<td>RNC Buffer</td>
</tr>
<tr>
<td></td>
<td>+150 mM KOAc</td>
</tr>
<tr>
<td></td>
<td>0.5% Triton-X</td>
</tr>
<tr>
<td>RNC Elution Buffer</td>
<td>RNC Buffer (w/o Tm complete)</td>
</tr>
<tr>
<td></td>
<td>10% Glycerol</td>
</tr>
<tr>
<td></td>
<td>0.2 mg/ml 3xFLAG peptide (Sigma)</td>
</tr>
</tbody>
</table>
### 6.2.3 Protein Purification Buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>20 mM sodium phosphate (pH 7.5)</td>
</tr>
<tr>
<td></td>
<td>300 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>6 mM MgCl$_2$</td>
</tr>
<tr>
<td></td>
<td>2 mM $\beta$-mercaptoethanol</td>
</tr>
<tr>
<td></td>
<td>2 mM PMSF</td>
</tr>
<tr>
<td></td>
<td>10 µg/ml Aprotinin</td>
</tr>
<tr>
<td></td>
<td>5 µg/ml Leupeptin</td>
</tr>
<tr>
<td></td>
<td>8 µg/ml Pepstatin A</td>
</tr>
<tr>
<td></td>
<td>10 µg/ml DNase I</td>
</tr>
<tr>
<td></td>
<td>10% (v/v) glycerol</td>
</tr>
<tr>
<td>High salt wash buffer</td>
<td>50 mM sodium phosphate (pH 8.0)</td>
</tr>
<tr>
<td></td>
<td>750 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>6 mM MgCl$_2$</td>
</tr>
<tr>
<td></td>
<td>10% (v/v) glycerol</td>
</tr>
<tr>
<td></td>
<td>2 mM $\beta$-mercaptoethanol</td>
</tr>
<tr>
<td>Low salt wash buffer</td>
<td>High salt Buffer with 25 mM NaCl</td>
</tr>
<tr>
<td>Elution buffer</td>
<td>Low salt buffer with 250 mM imidazole</td>
</tr>
<tr>
<td>Dialysis buffer</td>
<td>Low salt buffer with 5% glycerol and 8 µg Ulp-1 per mg protein</td>
</tr>
<tr>
<td>NAC buffer</td>
<td>20 mM sodium phosphate (pH 7.4)</td>
</tr>
<tr>
<td></td>
<td>25 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>6 mM MgCl$_2$</td>
</tr>
<tr>
<td></td>
<td>2 mM $\beta$-mercaptoethanol</td>
</tr>
<tr>
<td></td>
<td>5% (v/v) glycerol</td>
</tr>
</tbody>
</table>

**Table 5:** Buffers for protein purification and their composition.
6.3 Growth Medium

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luria-Bertani-Broth (LB medium)</td>
<td>1% (w/v) Bacto\textsuperscript{TM} tryptone</td>
</tr>
<tr>
<td></td>
<td>0.5% (w/v) Bacto\textsuperscript{TM} yeast extract</td>
</tr>
<tr>
<td></td>
<td>0.5% (w/v) NaCl</td>
</tr>
<tr>
<td></td>
<td>1.5% (w/v) Bacto\textsuperscript{TM} agar</td>
</tr>
<tr>
<td>Nematode Growth Medium (NGM)</td>
<td>2% (w/v) Bacto\textsuperscript{TM} agar</td>
</tr>
<tr>
<td></td>
<td>0.3% (w/v) NaCl</td>
</tr>
<tr>
<td></td>
<td>Add after autoclaving:</td>
</tr>
<tr>
<td></td>
<td>1 mM CaCl\textsubscript{2}</td>
</tr>
<tr>
<td></td>
<td>13 µM cholesterol</td>
</tr>
<tr>
<td></td>
<td>25 mM KHPO\textsubscript{4} pH 6.0</td>
</tr>
<tr>
<td></td>
<td>1 mM MgSO\textsubscript{4}</td>
</tr>
<tr>
<td>RNAi medium</td>
<td>2% (w/v) Bacto\textsuperscript{TM} agar</td>
</tr>
<tr>
<td></td>
<td>0.3% (w/v) NaCl</td>
</tr>
<tr>
<td></td>
<td>Add after autoclaving:</td>
</tr>
<tr>
<td></td>
<td>1 mM CaCl\textsubscript{2}</td>
</tr>
<tr>
<td></td>
<td>13 µM cholesterol</td>
</tr>
<tr>
<td></td>
<td>25 mM KHPO\textsubscript{4} pH 6.0</td>
</tr>
<tr>
<td></td>
<td>1 mM MgSO\textsubscript{4}</td>
</tr>
<tr>
<td></td>
<td>100 mg/ml amicillin</td>
</tr>
<tr>
<td></td>
<td>1 mM IPTG</td>
</tr>
</tbody>
</table>

Table 6: Composition of growth media for *E. coli* and *C. elegans*. 
### 6.4 Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Identifier</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP-anti-mouse IgG</td>
<td>Jackson</td>
<td>RRID:AB_2340771</td>
</tr>
<tr>
<td>HRP-anti-rabbit IgG</td>
<td>Jackson</td>
<td>RRID:AB_2340585</td>
</tr>
<tr>
<td>Mouse monoclonal anti-eL19</td>
<td>Santa Cruz</td>
<td>RRID:AB_2181588</td>
</tr>
<tr>
<td>Mouse monoclonal anti-eL19</td>
<td>Santa Cruz</td>
<td>RRID:AB_2181588</td>
</tr>
<tr>
<td>Mouse monoclonal anti-uL4</td>
<td>Santa Cruz</td>
<td>RRID:AB_2181910</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-eL22</td>
<td>Proteintech</td>
<td>Cat#25002-1-AP</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-eL22</td>
<td>Proteintech</td>
<td>RRID:AB_287984</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-eL39</td>
<td>Proteintech</td>
<td>RRID:AB_223867090</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-uL22</td>
<td>Proteintech</td>
<td>RRID:AB_2253985</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-α-NAC (NACA, Human)</td>
<td>Invitrogen</td>
<td>RRID:AB_2533448</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-β-NAC</td>
<td>Abcam</td>
<td>Cat#ab203517</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-NACAL</td>
<td>Biorbyt</td>
<td>Cat#orb411671</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-SRP54 (C. elegans)</td>
<td>In house</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 7:** Antibodies used in this study.
## 6.5 Antibiotics

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Working solution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (Amp)</td>
<td>100 µg/mL (stock: 100 mg/mL in ddH₂O)</td>
<td>AppliChem</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>550 µg/mL (stock: 50 mg/mL in ddH₂O)</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>25 µg/mL (stock: 25 mg/mL in 70% ethanol)</td>
<td>Serva</td>
</tr>
<tr>
<td>Neomycin (G418)</td>
<td>62.5 µL/g plate (stock: 25 mg/mL in ddH₂O)</td>
<td>InvivoGen</td>
</tr>
</tbody>
</table>

**Table 8:** Antibiotics used in this study.
6.6 Primer

6.6.1 Constructs for *in vitro* translation

The DNA fragment for the SUMO tag was amplified via PCR from the 6xHis-SUMO NAC expression plasmid (Deuerling Lab Stock). The sequence for the 3xFLAG tag was included in the forward primer. The plasmids (pPD61_125_GPI, pPD61_125_HSP, pPD61_125_GRP78) were linearized by PCR and each assembled with the 3xFLAG-SUMO inserts via NEBuilder® HiFi DNA Assembly. To generate the templates containing 3xFLAG-SUMO tag for *in vitro* translation and subsequent RNC purification seqM13rev was used as a forward primer (see Table 11). The reverse primers to generate specific lengths were the same as for the templates without a tag (see Table 11).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRP78-F</td>
<td>cggtggcggccgctctagataataatcactcactataggagaccatgaagctctctcctggtggc</td>
</tr>
<tr>
<td>GRP78-R</td>
<td>agagcttgacgggaagccacacatcgaaggttcc</td>
</tr>
<tr>
<td>HSP60-F</td>
<td>cggtggcggccgctctagataataatcactcactataggagaccacatgttctcgttaccacagtc</td>
</tr>
<tr>
<td>HSP60-R</td>
<td>agagcttgacgggaagcccttttgtattataaaatgtatggg</td>
</tr>
<tr>
<td>GPI-F</td>
<td>cggtggcggccgctctagataataatcactcactataggagaccatggccgctctcacccgggac</td>
</tr>
<tr>
<td>GPI-R</td>
<td>agagcttgacgggaagccctgtgttagtagacagggc</td>
</tr>
<tr>
<td>sumo_fwd</td>
<td>cgactcactataggagaccatggattataaagatcatgatggagattataaagatcatgattataaaatgtattataaagatgatggagattataaagatgatggag</td>
</tr>
<tr>
<td>sumo_GPI_rev</td>
<td>tccgggtgagagcggccataccaccaactctgttctctg</td>
</tr>
<tr>
<td>sumo_HSP60_rev</td>
<td>actgtgggttaacccgaagcataccaccaactctgttctctg</td>
</tr>
<tr>
<td>sumo_GRP78_rev</td>
<td>gccaccaggagagcttcataccaccaactctgttctctg</td>
</tr>
<tr>
<td>GRP78_tag_fwd</td>
<td>atgaagctctccctgtg</td>
</tr>
<tr>
<td>GRP78_tag_rev</td>
<td>tttatcatcatcattttataaatcaaatatcatgatettatatataatctcctcatgatett</td>
</tr>
<tr>
<td></td>
<td>tataatccaggggtctctatttagtggtc</td>
</tr>
</tbody>
</table>
Table 9: Primers used to generate plasmids with cytosolic (GPI), mitochondrial (HSP60) and ER (GRP78) containing genes for \textit{in vitro} translation. With or without 3xFLAG/SUMO tag. Sumo tag was amplified out of 6xHis-SUMO expression constructs (see Table 14). The PD61_125 plasmids encoding the respective genes were linearized via PCR and fragments were assembled with NEBbuilder®.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPI_{10aa_{annealed}}_{F}</td>
<td>tggcggccgctctagaataataacgactcactataggagaccatggccgctctcatgatgatgatgagtt</td>
</tr>
<tr>
<td>GPI_{10aa_{annealed}}_{R}</td>
<td>aaccatcatcattagagagcggccatggtctccatatagtgagtcgtattattctagcgcgcggccatggtctccctatagtgatgatgatgagtt</td>
</tr>
<tr>
<td>GPI_{15aa_{annealed}}_{F}</td>
<td>taatagctcactataggagaccatggccgctctcacccegggacccccagatgatgatgatgatggtt</td>
</tr>
<tr>
<td>GPI_{15aa_{annealed}}_{R}</td>
<td>aaccatcatcattagagagcggccatggtctccatatagtgagtcgtattattctagcgcgcggccatggtctccctatagtgatgatgatgagtt</td>
</tr>
<tr>
<td>GPI_{20aa_{annealed}}_{F}</td>
<td>taatagctcactataggagaccatggccgctctcacccegggacccccagatgatgatgatgatggtt</td>
</tr>
<tr>
<td>GPI_{20aa_{annealed}}_{R}</td>
<td>aaccatcatcattagagagcggccatggtctccatatagtgagtcgtattattctagcgcgcggccatggtctccctatagtgatgatgatgagtt</td>
</tr>
<tr>
<td>HSP_{10aa_{annealed}}_{F}</td>
<td>gccgctcctagaataataacgactcactataggagaccatgtctccctatagtgagtcgtattattctagcgcgcggccctatagtgatgatgagtt</td>
</tr>
<tr>
<td>HSP_{10aa_{annealed}}_{R}</td>
<td>aaccatcatcattagagagcggccatggtctccatatagtgagtcgtattattctagcgcgcggccctatagtgatgatgatgagtt</td>
</tr>
<tr>
<td>HSP_{15aa_{annealed}}_{F}</td>
<td>cggtggcggccgctctagaataataacgactcactataggagaccatgtctccctatagtgagtcgtattattctagcgcgcggccctatagtgatgatgagtt</td>
</tr>
<tr>
<td>HSP_{15aa_{annealed}}_{R}</td>
<td>aaccatcatcattagagagcggccatggtctccatatagtgagtcgtattattctagcgcgcggccctatagtgatgatgatgagtt</td>
</tr>
<tr>
<td>HSP_{20aa_{annealed}}_{F}</td>
<td>cggtggcggccgctctagaataataacgactcactataggagaccatgtctccctatagtgagtcgtattattctagcgcgcggccctatagtgatgatgagtt</td>
</tr>
<tr>
<td>HSP_{20aa_{annealed}}_{R}</td>
<td>aaccatcatcattagagagcggccatggtctccatatagtgagtcgtattattctagcgcgcggccctatagtgatgatgatgagtt</td>
</tr>
<tr>
<td>GRP_{10aa_{annealed}}_{F}</td>
<td>cggtggcggccgctctagaataataacgactcactataggagaccatgtctccctatagtgagtcgtattattctagcgcgcggccctatagtgatgatgagtt</td>
</tr>
<tr>
<td>GRP_{10aa_{annealed}}_{R}</td>
<td>aaccatcatcattagagagcggccatggtctccatatagtgagtcgtattattctagcgcgcggccctatagtgatgatgatgagtt</td>
</tr>
</tbody>
</table>
Table 10: Primer for short *in vitro* translation constructs. This primers were directly annealed to get respective constructs.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>seqM13rev</td>
<td>caggaacagctatgac</td>
</tr>
<tr>
<td>GPI_F</td>
<td>tggcggcgcgctctagaataataacgactcactatagggagaccatgg</td>
</tr>
<tr>
<td>GPI_25aa_R</td>
<td>aaccatcatcatcatctcgggtaccattggtgcag</td>
</tr>
<tr>
<td>GPI_30aa_R</td>
<td>aaccatcatcatcatcagctegggaggtggtcgcggttaccatg</td>
</tr>
<tr>
<td>GPI_35aa_R</td>
<td>aaccatcatcatcagcgggcgegcaggtttcagct</td>
</tr>
<tr>
<td>GPI_40aa_R</td>
<td>aaccatcatcatcatctttgtggcagtaagagggggcg</td>
</tr>
<tr>
<td>GPI_45aa_R</td>
<td>aaccatcatcatcatctgggttaagcgggtctggggca</td>
</tr>
<tr>
<td>GPI_50aa_R</td>
<td>aaccatcatcatcatagggtcaactgaagttggtgagacgc</td>
</tr>
<tr>
<td>GPI_55aa_R</td>
<td>aaccatcatcatcatccccatggtgtggtgtagggtcaag</td>
</tr>
<tr>
<td>GPI_60aa_R</td>
<td>aaccatcatcatcatatccaggatagcccatgtggttg</td>
</tr>
<tr>
<td>HSP60_F</td>
<td>eggtggeggcgcgctctagaataataacgactcactatagggagaccatgtggttgttcaccacagtc</td>
</tr>
<tr>
<td>HSP_25aa_R</td>
<td>aaccatcatcatcactgcagctaccctggcacccgggtctcactctg</td>
</tr>
<tr>
<td>HSP_30aa_R</td>
<td>aaccatcatcatcactccgagttgagtggagggcaggctaccctggacccgggtctt</td>
</tr>
<tr>
<td>HSP_35aa_R</td>
<td>aaccatcatcatcatctttggtgcataagcccgaggtgaga</td>
</tr>
<tr>
<td>HSP_40aa_R</td>
<td>aaccatcatcatcattgcacaaatttcattttgctgaacag</td>
</tr>
<tr>
<td>HSP_45aa_R</td>
<td>aaccatcatcatcattttagctgggctctgcacccatattttgccataagcctg</td>
</tr>
<tr>
<td>HSP_50aa_R</td>
<td>aaccatcatcatcatattacacctgaagcatattagggtcgggc</td>
</tr>
<tr>
<td>HSP_55aa_R</td>
<td>aaccatcatcatcactctgggtetaaaggtcattacacttgaga</td>
</tr>
<tr>
<td>HSP_60aa_R</td>
<td>aaccatcatcatcattgtaaagggccacaacgcatctggtaaa</td>
</tr>
<tr>
<td>GRP78_F</td>
<td>eggtggeggcgcgctctagaataataacgactcactatagggagaccatgtggttgtgcgc</td>
</tr>
<tr>
<td>GRP_25aa_R</td>
<td>aaccatcatcatcatctggggcgcgccggggtgagagcagcag</td>
</tr>
<tr>
<td>GRP_30aa_R</td>
<td>aaccatcatcatcatctttttgtcctcctgggccccggegcgctga</td>
</tr>
<tr>
<td>GRP_35aa_R</td>
<td>aaccatcatcatcatctgggctcctcctcctgggccccggegcgcttc</td>
</tr>
<tr>
<td>GRP_40aa_R</td>
<td>aaccatcatcatcatattgtggagcaccacccctg</td>
</tr>
<tr>
<td>GRP_45aa_R</td>
<td>aaccatcatcatcatattgtggagcaccaccccttcgccc</td>
</tr>
</tbody>
</table>
Table 11: Primers to generate constructs for \textit{in vitro} translation. The reverse primers encodes for 5 methionines for radioactive labeling and one valine for stabilization of the RNCs. Primers were used for constructs with and without 3xFLAG-SUMO tag.
Insertion of the GRP signal sequence (ss) into the GPI encoding plasmids was enabled via Q5 mutagenesis. The signal sequence of human preprolactin (PRL) and variants thereof were encoded in the forward primer and fused to the cytosolic GPI via PCR. The reverse primers specified the length of the constructs and the proportion of methionines for radioactive labeling.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPL_GPI</td>
<td>ctctagaataatcagactcactatatagggagaccatgaacatcaaggagcgcctctctctgctgtgtgtcacaaccctgtggtgtggtcaccatccagaggtgcgtgccatggcctctcacccgggac</td>
</tr>
</tbody>
</table>
| PPL_4L_GPI       | 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6.6.2 Primer for Bpa-NAC variants

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6 Materials

Table 13: Primers used for insertion of amber stop codons at desired positions in the human His-SUMO-NAC expression construct using the Q5® Site-Directed Mutagenesis Kit (New England Biolabs).

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6.7 Plasmids

Table 14: Plasmids used in this study. All plasmids contain the T7 promotor sequence.
6.8 *E. coli* strains

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*Table 15: E. coli strains used in this study.*
7 Methods

7.1 Molecular Cloning

Primers for molecular cloning and sequencing were purchased from Biomers. Plasmid DNA was purified out of *E. coli* cells with the QIAprep® Spin Miniprep Kit (Qiagen), subsequent sequencing of DNA constructs was carried out by Eurofins Genomics (former GATC Biotech AG).

7.1.1 cDNA synthesis

Human cDNA was prepared by total RNA extraction (RNeasy Mini Kit®), following reverse transcription. HEK293 cell powder was solved in 600 µL RLT buffer and sonicated six times (Output control level 2; 50% DutyCycle). Cell debris was pelleted by full speed centrifugation for 2 min. The supernatant was applied on a column and RNA was purified according to the manufacturer’s protocol. Finally, the RNA was eluted in 30 µL ddH₂O and 1000 ng total RNA was used in the QuantiTect® Reverse Transcription Kit according the manufacturer’s protocol.

7.1.2 Polymerase chain reaction (PCR)

PCR was used to generate DNA fragments of the respective human gene with cDNA as a template and to generate DNA templates of different lengths for *in vitro* transcription reactions. Primers for amplification were generated with the NEBuilder® Assembly tool. For each PCR reaction 25 ng template DNA was mixed with 1x Phusion reaction buffer, 0.2 mM dNTP mix (stock: 10 mM), 2 µM of each primer (stock: 100 µM) and 1 µl Phusion polymerase and adjusted to 50 µl with ddH₂O. Amplification was performed in the thermal cycler Biometra TRIO (Analytik Jena) using the conditions shown in Table 1. Annealing temperatures, as well as elongation time were adjusted according to the melting point of the primers and the size of the template. Samples were mixed with 6x DNA loading dye and loaded onto a 2% agarose gel.
### 7 Methods

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<tr>
<td>Hold</td>
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Table 16: Thermocycling conditions for PCR.

#### 7.1.3 Agarose gel electrophoresis

DNA fragments or PCR products were analyzed and purified by agarose gel electrophoresis using 1% or 2% (w/v) agarose gels in 1x TAE buffer, supplemented with 2 µL Midori Green (NIPPON Genetics) per 50 mL gel solution. DNA samples and 6 µL of 1 kb or 100 bp Gene Ruler Marker® were loaded on the gel. Fragments were visualized by UV illumination for evaluation and extracted by using the QIAquick® Gel Extraction Kit (Qiagen).

#### 7.1.4 NEBuilder® HiFI DNA Assembly

NEBuilder® HiFI DNA Assembly Cloning Kit was used to insert the respective PCR fragment into the empty vector backbone of pPD61.125. Therefore, pPD61.125 was digested with SpeI and NaeI, or linearized via PCR. For standard digestion, 3 µg of DNA were cut in the suitable reaction buffer in a final reaction volume of 20 µL and incubated overnight at 37°C. The resulting DNA fragment was purified by preparative agarose gel electrophoresis. Vector and insert were assembled in a 1:2 ratio according to the manufacturers protocol.

#### 7.1.5 Heat shock transformation of chemically competent E. coli

Competent *E. coli* DH5α cells were used for heat shock transformation of assembly products. DNA was added to ice-cold 50 µL bacterial cells and the mixture was incubated for 20 min on ice. After heat shock for 40 s at 42°C the cells were shortly cooled down on ice and provided with 950 µL LB medium. For phenotypic expression, *E. coli* cells were incubated at 37°C for 60 min under constant agitation. Cells
were pelleted by centrifugation and plated on LB-plates containing the respective antibiotic(s). The plates were incubated overnight at 38°C.

7.1.6 Generation of non-stop templates

All non-stop transcript templates were generated by PCR as previously described (Sharma et al. 2010). The T7 promoter (TAATACGACTCACTATAGGGAGA) needed for in vitro transcription was included in the 5' end of the forward primer. The reverse primer encoded five C-terminal methionines for radioactive S\textsuperscript{35}-labeling and a terminal valine residue to stabilize the tRNA-nascent chain complex (Table 11). PCR was performed on pPD61_125 containing the respective human genes and PCR products were cleaned-up by gel extraction (Qiagen). For very short transcript templates the forward and reverse primer were directly annealed (Table 10). Therefore, the two primers were mixed with annealing buffer in a reaction volume of 50 µl, denatured for 3 min at 95°C and cooled down in a PCR cycler to 37°C with 0.1°C/per sec. DNA templates were in vitro transcribed using the mMESSAGE mMACHINE T7 Transcription Kit or the T7 RiboMAX\textsuperscript{TM} Large Scale RNA Production System (Promega) according to manufacturer’s protocol (Thermo Scientific). RNA was purified with the RNA Clean&Concentrator Kit (Zymo).

7.2 Bpa containing NAC mutants

7.2.1 Amber stop codon insertion

For incorporation of the photo-activatable aa analog p-benzoylphenylalanine (Bpa) into human NAC, amber stop codons were inserted at desired positions in the human His-SUMO-NAC expression construct using Q5\textsuperscript{®} Site-Directed Mutagenesis Kit (New England Biolabs) (Table 13). Constructs were co-transformed into E. coli BL21*(DE) cells (Novagen) together with Bpa/amber suppression-Rosetta plasmid (pSup-Bpa-6TRN), as previously described (Ryu and Schultz 2006).

7.2.2 Protein purification

Human NAC variants containing Bpa were recombinantly expressed in E. coli BL21* Rosetta (DE3) cells (Novagen) as His-SUMO fusion constructs, as previously described (Gamerdinger et al. 2015). Cultures were induced with 1 mM IPTG over night at 20°C. Cell pellets were resuspended in lysis buffer and lysed by French Press. Proteins were captured using Ni-IDA matrix (Protino; Macherey-Nagel),
washed with high salt buffer and low salt buffer and eluted with elution buffer. Elution fractions were dialyzed overnight against low salt buffer with 5% (v/v) glycerol in the presence of 8 µg Ulp-1/ mg protein for proteolytic cleavage of the His-SUMO tag. Ion exchange chromatography with a Resource Q column (GE Healthcare) was used for further purification. Elution fractions containing αNAC and βNAC in a 1:1 ratio were pooled and dialyzed against NAC buffer overnight. Amicon® Ultra-4 10K centrifugal filter devices (Merck KGaA, Darmstadt) were used to further concentrate protein samples if needed.

**Bradford assay**

The protein concentrations were determined with the Bradford assay. The assay is based on the absorbance shift of Coomassie brilliant blue G-250 with an absorbance maximum of 470 nm as soluble dye in its cationic (red) form. After binding to proteins the absorbance maximum changes to 595 nm of the anionic form (blue). The increase of absorbance is dependent on the amount of bound protein (Bradford 1976). 1 ml Bradford solution was mixed with 1 µl of the respective protein and vortexed. After 5 min of incubation the samples were measured against a buffer control as reference at A595. The amount of protein was calculated with the help of the absorbance curve of a standard protein of known concentration.

### 7.3 In vitro translation

#### 7.3.1 RNC generation

Non-stop mRNAs were *in vitro* translated using rabbit reticulocyte lysate (Promega) (0.5 µg RNA in a 25 µl translation reaction; for 10 aa nascent chains: 0.8 µg RNA /25 µl reaction). Translation reactions were performed at 26°C for 20 min with/or without 1 mCi/ml S<sup>35</sup>-methionine (Hartmann Analytics). Translation of 3xFLAG-SUMO tagged constructs was conducted in the presence of 1x protease inhibitor cocktail (Roche) to prevent cleavage of the tag.

#### 7.3.2 Luciferase

Luciferase mRNA (Promega) was *in vitro* translated using rabbit reticulocyte lysate (Promega) for 15 min at 30°C (200 ng RNA/5 ml translation reaction). Purified *C.elegans* NAC (WT- and ΔN1-53-NAC) was added on ice to a final concentration of 2-8 µM before starting the translation reaction at 30°C. Activity was measured in
luminescence buffer (15 mM KHPO₄ pH 8, 4 mM EGTA, 2 mM ATP, 1 mM DTT, 15 mM MgSO₄, 0.1 mM CoA, 75 µM luciferin).

7.4 Purification of RNCs

7.4.1 Bulk purification of RNCs without 3xFLAG-SUMO tagged nascent chains

Translation reactions were layered on a 25% (w/v) sucrose cushion in RNC wash buffer (50 mM HEPES (pH 7.5), 500 mM KOAc, 5 mM Mg(OAc)₂) and centrifuged at 200,000 x g for 90 min at 4°C. The ribosomal pellets were resuspended in resuspension buffer (20 mM HEPES (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 1x protease inhibitor cocktail (Roche)) by constant agitation for 1 h on ice.

7.4.2 Purification of RNCs via 3xFLAG-SUMO tagged nascent chains

To remove peripheral factors, 1 mL of translation reaction was adjusted to 750 mM KOAc/0.5% NP-40 and layered onto a 1.6 ml sucrose cushion (0.5 M) in high-salt RNC buffer (50 mM HEPES pH 7.4, 750 mM KOAc, 15 mM Mg(OAc)₂, and 1 mM DTT) and centrifuged in a TLA110 Beckmann rotor at 100,000 rpm for 1 hour. Ribosome pellets were resuspended in RNC buffer (50 mM HEPES pH 7.4, 100 mM KOAc, 5 mM Mg(OAc)₂, 1 mM DTT, 1x protease inhibitor cocktail (Roche)) with constant shaking for 1 h on ice. Affinity purification was performed using ANTI-FLAG M2 affinity agarose gel (Sigma-Aldrich) using gravity flow chromatography columns (BIO-RAD). Columns were washed with wash buffer I (RNC buffer + 0.1% Triton X-100), wash buffer II (RNC buffer + 150 mM KOAc + 0.5% Triton X100) and RNC buffer. To elute bound RNCs, the resin was dried via a short centrifugation step and subsequently incubated with one bead volume of elution buffer (RNC buffer + 10% glycerol + 0.2 mg/ml 3xFLAG peptide) for 30 min at 20°C. Eluted RNCs were concentrated by sucrose cushion centrifugation in RNC buffer and resuspended in elution buffer (without FLAG peptide and protease inhibitor cocktail). Buffer composition is also described in (Table 4). Affinity purified RNCs were used in (Figure 32B; Figure 33B, Figure 29D, E).

7.4.3 ULP cleavage

Eluted RNCs were diluted 1:2 with RNC buffer (w/o Tm complete) and incubated with 0.45 µg/µl ULP protease for 30 min at 23°C on a thermomixer (500 rpm) to cleave the N-terminal 3xFLAG-SUMO tag.
7.5 Crosslinking

7.5.1 Bpa crosslinking

Bpa-NAC variants were added to the resuspended RNCs at a concentration of 2 µM and incubated at 25°C for 1 h. Samples were photo-crosslinked by UV irradiation (365 nm) for 30 min on ice. S\textsuperscript{35}-methionine labeled tRNA-NCs and crosslinked complexes were separated by SDS-PAGE and visualized by autoradiography.

7.5.2 Cysteine crosslinking

Cysteine variant NAC αK75C/βL51C I121 was generated as described for the Bpa containing NAC variants. Since NAC αK75C/βL51C I121 was largely oxidized after purification, it could be kept in this state during the crosslinking assay by not using DTT in the sample. To fully reduce the protein a 50 mM DTT treatment was conducted for 5 min at 25°C. Afterwards the reduced protein was diluted to 2 mM DTT for the crosslinking assay to maintain its reduced state. Subsequent crosslinking was performed as described for the Bpa-NAC variants in the respective buffers with or without DTT.

7.6 SDS/Bis-Tris polyacrylamide gel electrophoresis and Coomassie blue staining

To separate proteins according to their molecular weight, a protein sample was mixed with protein sample buffer and incubated for 5 min at 95°C. Protein samples and a molecular weight standard were loaded on a 10% SDS-gel or 10% bis-tris-gel. The electrophoretic separation was performed in the respective running buffer (SDS or bis-tris/MOPS buffer) at 150 V. To visualize the protein pattern, the gels were stained for 20 min in Coomassie staining solution and subsequently destained in Coomassie destaining solution. The buffer compositions are listed in (Table 3).

7.6.1 Western Blot analysis, Ponceau S staining and immunodetection

Proteins separated by Bis-Tris-PAGE were transferred to a nitrocellulose membrane (GE Healthcare) in a wet blot chamber filled with 1x western blot transfer buffer at 100 V for 30 min. Afterward, the proteins on the membrane could be stained with Ponceau S solution. For immunostaining membrane was blocked for at least 1 h with 5% (w/v) milk in TBS-T followed by overnight incubation with primary antibody. The membrane was washed three times with TBS-T for 5 min and incubated
with secondary antibody for 1-2 h. After washing with TBS-T (around 1 h), HRP-
coupled secondary antibodies were visualized with freshly prepared ECL-solution
and detected with the Fusion SL (Peqlab) imaging system. The buffer compositions
are listed in (Table 3)

7.6.2 Autoradiography

After Western blotting, the nitrocellulose membrane was dried and placed in a cas-
sette with the phosphor screen, which was exposed for 48 hours. Signals were de-
tected with Typhoon FLA9500 (Control Software: Version 1.0; GE Healthcare).

7.7 Protein-ribosome interaction study (add back)

Purified 80S ribosomes (1 mM) were incubated 1:2 with NAC variants (K38/E;
K43/E) in binding buffer (30 mM HEPES (pH 7.4), 100 mM KOAc, 5 mM MgCl₂, 1
mM DTT, 1x protease inhibitor cocktail (Roche)) for 30 minutes at 18°C with gentle
shaking (300 rpm). Samples (30 µl) were loaded onto a 25% (w/v) sucrose cushion
(150 µl) in binding buffer and centrifuged at 220,000 x g for 90 min at 4°C in a S100-
AT3 rotor (Thermo Scientific). Proteins in the supernatant (TCA-precipitated) and
pellet were analyzed by immunoblotting (Gamerdinger et al. 2019).

7.8 C.elegans methods

7.8.1 ER stress reporter analysis

Worm strains carrying RNAi-resistant NAC genes were mated with the ER stress
reporter strain SJ4005 (zcIs4[hsp-4p::GFP]) (Calfon et al. 2002). The endogenous
NAC genes were silenced in the worms after hatching on plates containing RNAi
bacteria. Animals in the adult stage were immobilized with 1% sodium azide, and
GFP fluorescence from 5 animals was measured using a DM6000B-Cs microscope
(Leica) equipped with a DFC 365FX camera (Leica) and a 5x objective.

7.8.2 Lifespan

Lifespan analyses were performed at 20°C. Worms were grown on E. coli HT115(DE3)
RNAi as a food source from hatch. One hundred animals were used per condition
and dead animals were sorted out every second day, starting at day 2 of adulthood.
For this purpose, it was checked whether the worms still reacted to being
poked with the platinum wire worm picker. Animals with vulval protrusion were censored (Gamerdinger et al. 2015).

7.8.3 *Ex vivo* ribosome binding competition

Crude *C. elegans* RNCs were extracted in a physiological extraction buffer (20 mM HEPES (pH 7.4), 100 mM KOAc, 2 mM Mg(OAc)$_2$, 2 mM DTT) containing 100 µg/mL cycloheximide and 1x protease inhibitor mix (Roche). Samples were pre-cleared by centrifugation at 20,000 x g and filtered through a 0.45 mm membrane. Afterwards they were adjusted to 20 A$_{260}$ U/ml with extraction buffer and incubated with recombinant NAC (WT or KK-EE) for 20 min on ice. Ribosomes were pelleted by sucrose-cushion centrifugation in extraction buffer using an S140-AT rotor, and proteins in the pellet fraction were analyzed using standard immunoblotting techniques.

7.8.4 *In vitro* ribosome-ER translocon binding assay

Rough ER microsomes (RMs) were purified from *C. elegans* N2 worms as previously described (Gamerdinger et al. 2015). RMs were resuspended and incubated in a total reaction volume of 35 µL in the presence and absence of puromycin (1 mM) with purified *C. elegans* NAC proteins. For this, either wild-type βNAC (WT) or N-terminal GFP-tagged βNAC (GFP) was incubated with the RMs at a concentration of 0.5 or 2 mM for 2 h at 25°C with constant shaking. Microsomes were then re-isolated by centrifugation and ribosome content in the pellet and supernatant fractions was determined by immunoblotting.
# Abbreviations

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<td>°C</td>
<td>degree celsius</td>
</tr>
<tr>
<td>A</td>
<td>Alanine</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BpA</td>
<td>p-benzoyl-phenylalanine</td>
</tr>
<tr>
<td>C. elegans</td>
<td>Caenorhabditis elegans</td>
</tr>
<tr>
<td>Cryo-EM</td>
<td>Cryogenic electron microscopy</td>
</tr>
<tr>
<td>D</td>
<td>Aspartate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleosid triphosphate</td>
</tr>
<tr>
<td>E</td>
<td>glutamic acid</td>
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<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ES</td>
<td>expansion segment</td>
</tr>
<tr>
<td>et al.</td>
<td>lat. et alii (and others)</td>
</tr>
<tr>
<td>ev</td>
<td>empty vector</td>
</tr>
<tr>
<td>Ffh</td>
<td>Fifty-four homologue</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescence protein</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate (GTP)</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)-piperazine-4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
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<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-Thiogalactopyranoside</td>
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<td>K</td>
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<td>KAc</td>
<td>potassium acetate</td>
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<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>M</td>
<td>Methionine</td>
</tr>
<tr>
<td>MAP</td>
<td>Methionine aminopeptidases</td>
</tr>
<tr>
<td>MIM</td>
<td>Mitochondrial inner membrane</td>
</tr>
<tr>
<td>Mitoribosome</td>
<td>Mitochondrial ribosome</td>
</tr>
<tr>
<td>MOM</td>
<td>Mitochondrial outer membrane</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-morpholinopropane-1-sulfonic acid</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>MTS</td>
<td>Mitochondrial targeting sequence</td>
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<td>NAT</td>
<td>N-acetyltransferases</td>
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<tr>
<td>NGD</td>
<td>No-go decay</td>
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<tr>
<td>NMD</td>
<td>Nonsense mRNA decay</td>
</tr>
<tr>
<td>NMT</td>
<td>Nt-myristoyltransferases</td>
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<tr>
<td>NSD</td>
<td>Non-stop mRNA decay</td>
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<tr>
<td>Nt</td>
<td>N-terminal</td>
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<tr>
<td>N-αNAC</td>
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<tr>
<td>N-βNAC</td>
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<tr>
<td>OD600</td>
<td>Optic density at 600 nm</td>
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<tr>
<td>PAT</td>
<td>Nt-palmitoyltransferases</td>
</tr>
<tr>
<td>PBD</td>
<td>Protein binding domain</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain reaction</td>
</tr>
<tr>
<td>PTC</td>
<td>Peptidyl transferase center</td>
</tr>
<tr>
<td>R</td>
<td>Arginine</td>
</tr>
<tr>
<td>RAC</td>
<td>Ribosome associated complex</td>
</tr>
<tr>
<td>RBD</td>
<td>RNA binding domain</td>
</tr>
<tr>
<td>RM</td>
<td>Rough ER microsomes</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNC</td>
<td>Ribosome-nascent chain complex</td>
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<tr>
<td>rpm</td>
<td>Rounds per minute</td>
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<tr>
<td>r-proteins</td>
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<tr>
<td>RQC</td>
<td>Ribosome-associated quality control</td>
</tr>
<tr>
<td>RRL</td>
<td>Rabbit reticulocyte lysate</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>S</td>
<td>Serine</td>
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<tr>
<td>S. cerevisiae</td>
<td>Saccharomyces cerevisiae</td>
</tr>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>SR</td>
<td>SRP receptor</td>
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<td>SRP</td>
<td>Signal recognition particle</td>
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<td>SS</td>
<td>Signal sequence</td>
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<tr>
<td>Ssz</td>
<td>Stress seventy family Z</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate with EDTA</td>
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<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>TBS with Tween-20</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
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<tr>
<td>TM</td>
<td>Transmembrane helix</td>
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<tr>
<td>TMD</td>
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<tr>
<td>UBA</td>
<td>Ubiquitin-associated</td>
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<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>w/o</td>
<td>Without</td>
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<tr>
<td>w/v</td>
<td>Weight per volume</td>
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<tr>
<td>WT</td>
<td>Wildtype</td>
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<tr>
<td>ZHR</td>
<td>Zuotin homology region</td>
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<tr>
<td>β-ME</td>
<td>β-Mercaptoethanol</td>
</tr>
<tr>
<td>µ</td>
<td>Micro (10^{-6})</td>
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Appendix I

Early Scanning of Nascent Polypeptides inside the Ribosomal Tunnel by NAC
Early Scanning of Nascent Polypeptides inside the Ribosomal Tunnel by NAC

Graphical Abstract

Highlights

- Charged motifs in the flexible N termini of NAC regulate ribosome binding
- NAC scans nascent chains deep inside the ribosomal tunnel
- Tunnel-probing activity of NAC is crucial for correct protein sorting

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In Brief

NAC is an essential ribosome-associated protein biogenesis factor. Gamerdinger et al. demonstrate that NAC deeply inserts into the ribosomal tunnel to sense nascent polypeptide chains directly upon birth. The early scanning of nascent polypeptides inside the ribosomal tunnel is crucial for the correct sorting of proteins in the cell.
Early Scanning of Nascent Polypeptides inside the Ribosomal Tunnel by NAC

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SUMMARY

Cotranslational processing of newly synthesized proteins is fundamental for correct protein maturation. Protein biogenesis factors are thought to bind nascent polypeptides not before they exit the ribosomal tunnel. Here, we identify a nascent chain recognition mechanism deep inside the ribosomal tunnel by an essential eukaryotic cytosolic chaperone. The nascent polypeptide-associated complex (NAC) inserts the N-terminal tail of its β subunit (N-βNAC) into the ribosomal tunnel to sense substrates directly upon synthesis close to the peptidyl-transferase center. N-βNAC escorts the growing polypeptide to the cytosol and relocates to an alternate binding site on the ribosomal surface. Using C. elegans as an in vivo model, we demonstrate that the tunnel-probing activity of NAC is essential for organismal viability and critical to regulate endoplasmic reticulum (ER) protein transport by controlling ribosome-Sec61 translocon interactions. Thus, eukaryotic protein maturation relies on the early sampling of nascent chains inside the ribosomal tunnel.

INTRODUCTION

Newly synthesized polypeptides are cotranslationally processed by various ribosome-associated factors, including chaperones, enzymes, and transport proteins (Kramer et al., 2019; Pechmann et al., 2013; Preisser and Deuerling, 2012). These specialized factors transiently bind in a highly dynamic manner to both the translating ribosome and the emerging nascent substrate. The mechanism of nascent chain interaction is established for some factors, including the chaperone trigger factor (TF), the targeting factor signal recognition particle (SRP), and the translocon Sec61 (Becker et al., 2009; Ferbitz et al., 2004; Halic et al., 2004; Merz et al., 2008; Voorhees et al., 2014; Voorhees and Hegde, 2015). These factors bind in the vicinity of the ribosomal tunnel exit of the large 60S ribosomal subunit and position their nascent chain interaction surfaces near the mouth of the tunnel exit, enabling them to bind nascent chains as soon as they exit the ribosomal tunnel. This interaction mechanism serves as a paradigm for how nascent chains are generally recognized by ribosome-associated protein biogenesis factors.

A major eukaryotic factor that transiently interacts with translating ribosomes is the nascent polypeptide-associated complex (NAC). NAC was discovered 25 years ago as a heterodimeric complex (Wiedmann et al., 1994). It consists of two subunits, αNAC and βNAC, that dimerize via their homologous NAC domains, forming a β-barrel-like structure (Liu et al., 2010; Wang et al., 2010). NAC is essential in higher eukaryotes and assists cotranslational protein folding and transport (del Alamo et al., 2011; Gamerdinger et al., 2015; George et al., 1998; Kirstein-Miles et al., 2013; Lesnik et al., 2014; Wiedmann et al., 1994). This complex is expressed at least equimolar relative to ribosomes and broadly associates with translating ribosomes (del Alamo et al., 2011; Rauer et al., 2007). However, the mechanism of how NAC interacts with the ribosome and with nascent substrates is unknown. Here, we used a combination of biochemical, genetic, and structural analysis to get a more detailed understanding of the cotranslational interaction mechanism of this central ribosomal interactor.

RESULTS

N-αNAC Acts as a Ribosome-Binding Regulatory Device of NAC

How NAC specifically interacts with ribosomes is poorly understood. Studies in yeast demonstrated that the first eleven N-terminal residues as well as a conserved central positively charged motif (β-RRKKK) in the βNAC N terminus (N-βNAC) are essential for ribosome binding (Pech et al., 2010; Wegrzynek et al., 2009). Previous crosslinking data, however, suggest that also αNAC directly contacts the ribosome (Nyathi and Pool, 2015), indicating a complex interaction mode involving multiple ribosomal contacts. To dissect the roles of different features of αNAC and βNAC in ribosome binding, we first focused on a fully conserved...
positively charged motif (α-KKAR) in N-nNAC that was revealed by multiple sequence alignments of eukaryotic nNAC (Figure S1A). C. elegans strains were constructed expressing either C-terminally mono-FLAG-tagged wild-type (WT)-nNAC or mutant variants in which the positively charged residues in the motif were substituted with alanines (KKAR/AAAA) or the motif was deleted by removing the entire N-terminal domain (ΔN1–64; Figure 1A). Expression was driven by an integrated single-copy nNAC transgene that is resistant to RNAi of the endogenous gene by alternate codon usage (Figure S1B). This correlated well with a reduced rescuing activity of the embryonic lethal phenotype caused by knockdown of the endogenous nNAC gene (Figure 1C). Thus, the α-KKAR motif is critical for ribosome binding and function of NAC in vivo. The importance of α-KKAR for ribosome binding was further tested in vitro using purified components, including a third nNAC mutant lacking the N-terminal region upstream of α-KKAR (ΔN1–53; Figure 1A). Consistent with the in vivo results, KKAARKAAA and ΔN1–64-NAC bound weaker to purified 60S ribosomes (Figure 1D). Binding was restored in ΔN1–53-NAC, which remarkably bound ribosomes stronger than WT-NAC (Figure 1D).

To explore the structural basis of the interplay between the positively and negatively charged stretches, we inserted a positively charged ribosome binding motif into the negatively charged stretch of human NAC. The destabilizing effect caused by knockdown of the endogenous nNAC (see Figure S1A). Expression was driven by an integrated single-copy nNAC transgene that is resistant to RNAi of the endogenous gene by alternate codon usage (Figure S1B). This correlated well with a reduced rescuing activity of the embryonic lethal phenotype caused by knockdown of the endogenous nNAC gene (Figure 1C). Thus, the α-KKAR motif is critical for ribosome binding and function of NAC in vivo. The importance of α-KKAR for ribosome binding was further tested in vitro using purified components, including a third nNAC mutant lacking the N-terminal region upstream of α-KKAR (ΔN1–53; Figure 1A). Consistent with the in vivo results, KKAARKAAA and ΔN1–64-NAC bound weaker to purified 60S ribosomes (Figure 1D). Binding was restored in ΔN1–53-NAC, which remarkably bound ribosomes stronger than WT-NAC (Figure 1D).

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This interaction seemed highly specific, as other Bpa-N-sNAC variants did not crosslink to eL19 (Figure S1B). Thus, ribosome binding is accompanied by a conformational switch in N-sNAC that involves an interaction of the negatively charged region with eL19, which releases the autoinhibitory contacts to \( \alpha \)-KKAR and \( \beta \)-RRKKK.

The relevance of the ribosome-binding regulatory sequence for NAC function in de novo protein synthesis was investigated using an in vitro translation system. Strikingly, addition of purified \( \Delta N1-53\text{-NAC} \) strongly inhibited in vitro translation of a model protein, firefly luciferase, whereas WT-NAC had almost no effect (Figure 1K). Thus, the unregulated, abnormally strong ribosome association of \( \Delta N1-53\text{-NAC} \) likely results in tight and unproductive ribosome nascent chain (RNC) interactions that impair translation. Consistent with a translational defect in vivo, \( \Delta N1-53\text{-NAC} \)-overexpressing worms were developmentally delayed by ~24 h (data not shown) and showed a reduced protein synthesis rate compared to WT-NAC worms (Figure 1L). Thus, the cotranslational function of NAC relies on an intrinsic ribosome-binding regulatory system that is based on oppositely charged elements in the flexible N-terminal tails (Figure 1M).

**Cryo-EM Reconstruction of C. elegans NAC-60S Ribosomal Complex**

To better understand how NAC interacts with ribosomes, cryo-electron microscopy (cryo-EM) analysis was performed with in vitro reconstituted C. elegans NAC-60S ribosomal complexes. The reconstruction at an overall 4.2-Å resolution shows the typical appearance of the 60S ribosomal subunit with an additional density at the ribosomal tunnel exit representing NAC (Figures 2A–2C). The local resolution of the NAC density is around 8-9 Å, and its globular shape did not allow for a reliable docking of the NAC heterodimer crystal structure (PDB: 3MCB and PDB: 3LIQ; Liu et al., 2010; Wang et al., 2010). However, despite the limited resolution, the map revealed interesting NAC-ribosome interaction features. The largest density that likely involves the NAC domain heterodimer is located next to uL23 and uL29 directly beneath the ribosomal tunnel exit and forms a direct contact to uL29, consistent with previous crosslinking data (Figure 2A; Nyathi and Pool, 2015; Wegrzyń et al., 2008). Thus, NAC binds to a universal ribosome binding site that is recognized by diverse cotranslational protein biogenesis factors, including trigger factor, SRP, and the Sec61 translocon (Figure S3A; Becker et al., 2009; Ferbitz et al., 2004; Halic et al., 2004; Merz et al., 2008, 2014; Voorhees et al., 2014; Voorhees and Hegde, 2015). The location of the NAC density indicates that it would sterically clash with bound Sec61 (Becker et al., 2009; Voorhees et al., 2014), in agreement with previous functional data that showed that NAC antagonizes incorrect endoplasmic reticulum (ER) protein targeting by interfering with ribosome-Sec61 interactions (Gamerding et al., 2015; Lauring et al., 1995; Möller et al., 1996). Moreover, NAC would also clash with the NG domain of SRP54 (Halic et al., 2004; Voorhees and Hegde, 2015). This implies that ER substrate engagement by SRP is repressed by NAC and coupled to dissociation of NAC from ribosomes, as suggested by earlier binding studies (Powers and Walter, 1996; Wiedmann et al., 1994). Furthermore, our structural data also indicate that NAC competes with the ribosome-associated complex (RAC) for binding to the ribosome, in particular with the RAC binding site at H59, eL22, and eL31 (Lee et al., 2016; Zhang et al., 2014), where an additional NAC density was identified (Figure 2B). In support of this, we found that purified WT-NAC, added ex vivo to worm lysates, but not a ribosome-binding-deficient NAC variant with a mutation in the \( \beta \)-RRKKK motif, RRK/AAA-NAC, displaces a component of RAC, the C. elegans Zuo1 homolog DNA1-11, from ribosomes (Figure 2C).

**N-\( \alpha \)-NAC Probes Nascent Chains inside the Ribosomal Tunnel**

The most striking and unexpected structural feature, however, is a protruding arm of the NAC density reaching far into the
ribosomal tunnel (Figures 3A and S3B). The density fills the tunnel up to the constriction point formed by the extended loops of uL22 and uL4 just close to the peptidyl-transferase center (Klinge et al., 2011). To identify the tunnel-inserting NAC domain, we conducted a series of site-specific crosslinking experiments. Therefore, we introduced Bpa at several positions in N-αNAC and N-βNAC, the known ribosomal interacting domains (Figure S4A). The Bpa-NAC proteins were photo-crosslinked to in vitro translated stalled RNCs harboring radiolabeled nascent chains (NCs) with a defined length between 10 and 50 aas in the tunnel. We found that only Bpa at position two of N-βNAC (β-X2) crosslinked to very short NCs (10–25 aas; Figure 3B). These data suggest that the conserved ~40-aa flexible N-βNAC tail inserts deeply into the ribosomal tunnel to contact NCs early during synthesis. This interaction mode is consistent with the previous report that NAC forms a protective environment for very short nascent chains on ribosomes (Wang et al., 1995). With increasing NC length (30–40 aas), the β-X2 crosslinks got weaker, indicating that the tail is pushed out during polypeptide elongation. At a length of 45–50 aas, when the NC N terminus exits the tunnel, the crosslink switched to position β-L14 (Figure 3B). A similar β-X2-specific interaction pattern with short tunnel-embedded NCs (20–25 aas) was also observed with another translated substrate (HSP60; Figure S4B), indicating a general NC sensing inside the tunnel by N-βNAC. Interestingly, however, the crosslinking pattern with exposed 45–50-aa NCs differed between the two model substrates (HSP60: α-V64 and α-I59; GPI: β-L14), suggesting that NAC may adopt variable, substrate-specific conformations on ribosomes once the N-αNAC tail is pushed out of the tunnel. Moreover, starting with a NC length of 30 aas, a weak but consistent crosslink to N-αNAC at position α-V64 appeared (Figures 3B and S4B). This position is directly upstream of the identified α-KKAR ribosome-binding motif (Figure S4A), indicating that it binds very close to the tunnel exit. Consistent with this, α-V64 also specifically crosslinked, albeit weak, to eL39 located at the mouth of
the ribosomal exit tunnel (Figure S4C). These data suggest that the region upstream of a-KKR loops into the distal end of the ribosomal tunnel and senses NCs starting with a length of ~30 aas.

To further investigate the binding of NAC within the ribosomal tunnel, we engineered yeast ribosomes in which the distal end of the ribosomal tunnel and senses NCs starting with a length of ~30 aas.

The data suggest that mutant NAC lacking the ribosome binding regulatory arm in the nNAC N terminus impairs translation through unproductive ribosome nascent chain interactions that occur both inside but also outside the ribosomal tunnel.

**Tunnel Insertion of N-\textit{nNAC} Regulates Ribosome-Sec61 Interactions**

To investigate the functional relevance of the tunnel insertion and NC sensing inside the tunnel by N-\textit{nNAC}, we constructed a NAC mutant containing a globular GFP tag fused to the N terminus of \textit{nNAC} attached via a short flexible linker. Overal ribosome binding in vitro was not inhibited by the GFP tag (Figure S4A), and GFP-\textit{NAC} N1-67-NAC did crosslink to eL31, ex-

Thus, the GFP-NAC mutant binds to ribosomes exclusively outside the tunnel. To analyze whether tunnel insertion is important for NAC function in vivo, we constructed C. elegans strains expressing an \textit{nNAC} RNAi-resistant transgene as well as done before with \textit{nNAC} (Figure S5I). Expression levels of WT- and GFP-\textit{nNAC} in the endogenous \textit{nNAC} RNAi background were comparable (Figure 4C). Interestingly, the rescuing activity of the embryonic lethal phenotype caused by knockdown of the endogenous \textit{nNAC} gene was significantly reduced in worms expressing the tunnel-insertion-deficient GFP-\textit{nNAC} variant (Figure 4D). These data suggest that proper NAC function indeed relies on the early sensing of NCs inside the ribosomal tunnel by N-\textit{nNAC}. A main function of NAC is to regulate ER protein transport specifically by inhibiting the access of the Sec61 translocon to translating ribosomes. In the absence of NCs, ribosomes incorrectly associate with Sec61, leading to erroneous protein transport into the ER lumen, resulting in ER stress, which is strongly potentiated when the SRP pathway is impaired in addi-

Gamerding et al., 2019. As shown previously, we found that simultaneous knockdown of \textit{nNAC} together with the \textit{m} sub-

unit of the SRP receptor (SRRI) strongly induced ER stress already in young worms (larval stage 4), evident by the induced expression of the ER-stress-regulated proteins, HSP4 and PD31 (Figure 4E). Strikingly, although expression of WT-\textit{nNAC} effectively suppressed the ER stress, GFP-\textit{nNAC} had almost no rescuing effect (Figure 4F). Furthermore, qPCR analyses in aged \textit{nNAC} RNAi worms (day 3 adult) revealed significantly up-

regulated transcript levels of several ER-stress-induced genes in GFP-\textit{nNAC} worms, which were repressed in WT-\textit{nNAC} ani-

mals (Figure 4G). These data indicate that the ER protein transport function of NAC relies on the tunnel-sensing activity of N-\textit{nNAC}. Consistently, we found in vitro using purified compo-

nents that tunnel-insertion-deficient GFP-NAC was strongly impaired in displacing puromycin-treated ribosomes from ER microsomes (Figure 4G). Thus, tunnel probing by NAC is critical for the ribosomal exit tunnel (Figure S4C). These data suggest that the region upstream of a-KKR loops into the distal end of the ribosomal tunnel and senses NCs starting with a length of ~30 aas.

To further investigate the binding of NAC within the ribosomal tunnel, we engineered yeast ribosomes in which the distal end of the ribosomal tunnel and senses NCs starting with a length of ~30 aas.
to prevent binding of idle—and likely also early-stage translating—ribosomes to the Sec61 translocon, thereby preventing mistranslocation of proteins into the ER (Figure 5).

**DISCUSSION**

Our study reveals an unexpected mechanism in eukaryotes in which the nascent polypeptides are sensed deep inside the ribosomal tunnel by the ubiquitous cytosolic chaperone NAC. The conservation, abundance, and broad ribosome interaction of NAC (del Alamo et al., 2011; Preissler and Deuerling, 2012; Raue et al., 2007) implicates that the deep tunnel sensing of nascent chains by N-bNAC is a universally conserved process in eukaryotes, and our in vivo analyses in *C. elegans* demonstrate its importance for proper de novo protein biogenesis. The plasticity of interactions between NAC and the ribosome allows for tunnel sensing without interfering with polypeptide elongation and enables NAC to interact with a range of cotranslational substrates with varying properties. The unique interactions are mediated by the flexible N-terminal tails of NAC with distinct oppositely charged motifs, capable of regulating their propensity for ribosome interactions and tunnel insertion. The intrinsically regulated ribosome-binding mode of NAC is critical to ensure productive ribosome nascent chain interactions evident by the strong translation defects induced by mutant NAC lacking the negatively charged regulatory element in N-bNAC.

An emerging question is how ribosomal tunnel probing by NAC regulates de novo protein biogenesis processes. Our study demonstrates one important function: the tunnel sensing allows NAC to discriminate between idle and translating ribosomes. NAC is the only tunnel-exit-site-binding factor expressed equimolar to ribosomes and is thus ideally suited to act as a general ribosome sorting device that prevents other low-abundant nascent chain processing systems to unproductively interact with inactive or early-phase translating ribosomes (Figure 9). An example is our finding that tunnel insertion by NAC is critical to regulate the
Ribosome binding of NAC is mediated by a ribosome-binding regulatory arm (N-\(\text{NAC}\)) and a translation sensor domain (N-\(\text{iNAC}\)). N-\(\text{NAC}\) directly contacts the ribosome close to the tunnel exit but also possesses a ribosome binding inhibitory element that interacts with eL19. The empty tunnel of idle and early-stage translating ribosomes is sensed by N-\(\text{iNAC}\), which inserts deeply into the ribosomal tunnel up to the constriction formed by uL22. In the tunnel-inserted conformation, NAC blocks the premature, unproductive ribosome association of Sec61 and likely of other cotranslational protein biogenesis factors, including RAC and SRP (left, early state). During polypeptide elongation, N-\(\text{iNAC}\) senses short nascent chains and is partially pushed out of the ribosomal tunnel, which likely repositions the NAC domain outside the tunnel (dotted arrows) to facilitate the early recruitment of other protein biogenesis factors, like SRP (middle, intermediate state). Once the N-\(\text{iNAC}\) tail is completely pushed out of the tunnel, it can relocate to an alternate binding site on the ribosome surface involving eL22 and eL31 (right, late stage). At this stage, NAC may orchestrate cotranslational protein folding and targeting processes by regulating the specific binding of other chaperones and targeting factors.

In addition to the translation activity sensor function, tunnel probing by NAC could also be important for the timely recognition and entry of nascent chains into the correct downstream protein biogenesis pathway. An obvious possibility is that NAC can sense N-terminal targeting sequences in the ribosomal tunnel, which direct proteins to either the ER or mitochondria (Keenan et al., 2001; Stojanovski et al., 2012). A function of NAC in promoting the cotranslational transport to these organelles is indicated by several studies (del Alamo et al., 2011; Gamerdinger et al., 2015; George et al., 1998; Lesnik et al., 2014; Wiedmann et al., 1994). Supporting that NAC recognizes N-terminal targeting sequences inside the tunnel, a previous in vitro study showed that the early binding of SRP to ribosomes carrying a not yet exposed signal sequence inside the tunnel depends on the presence of NAC (Zhang et al., 2019). Consistent with an active role of NAC in substrate handover to SRP, an in vivo study in yeast showed that SRP binds a subset of secretory RNCs less efficiently in the absence of NAC (del Alamo et al., 2011). Thus, it is possible that NAC senses a signal sequence in the tunnel and—through a conformational switch—actively helps to recruit SRP to ribosomes. The multiple ribosomal contacts, the highly flexible binding mode, and the fact that both N termini of NAC can sense nascent chains already in the ribosomal tunnel indeed suggest that depending on the nature of a specific substrate in the tunnel NAC can adopt different conformations on ribosomes. Consistent with this, the crosslinking patterns of NAC with the two translated substrates—cytosolic GPI and mitochondrial HSP60—were different, indicating substrate-specific conformations of NAC. Thus, through its high flexibility and conformational versatility, NAC may signal the character of a tunnel-embedded nascent chain to the ribosome surface, which might be critical to regulate the early ribosomal access and timely substrate engagement of other protein biogenesis factors, such as SRP (Figure 5).
Furthermore, the tunnel-sensing activity could be critical for NAC to assist cotranslational protein folding. Because of its localization at the ribosomal tunnel exit and direct nascent chain interactions, NAC has been proposed to act as a cotranslational ATP-independent molecular chaperone. Several lines of evidence indicate such a function for NAC, e.g., NAC ablation increases the ubiquitination of nascent substrates, suggesting that NAC covers newly synthesized proteins and protects them from premature degradation (Dutler et al., 2013; Kirstein-Miles et al., 2013; Martin et al., 2018; Wang et al., 2013). Our finding that N-iNAC is the first domain interacting with nascent chains already inside the ribosomal tunnel indicates a chaperone activity for this domain. Indeed, a recent study identified N-iNAC as a major chaperone entity of NAC that is critical and sufficient to prevent aggregation of polyglutamine-expanded proteins like mutant Huntingtin (Shen et al., 2019). This study showed that N-iNAC exerts this chaperone activity posttranslationally independent from its ribosome association. Considering our finding that N-iNAC is the primary nascent-chain-sensing domain, it is conceivable that N-iNAC senses nascent chain segments that require chaperoning for correct folding at the ribosome. Thus, N-iNAC may bind nascent chains inside but also outside the ribosomal tunnel to assist cotranslational protein folding, and—depending on the physicochemical nature of the substrate—coordinates substrate transfer to other cotranslational chaperone systems, like RAC, to facilitate cotranslational protein folding (Figure 5).

In sum, our structural and functional insights into the activity of ribosome-associated NAC revealed an exciting concept of how nascent chains are sensed upon birth deep within the ribosomal tunnel to facilitate their folding and sorting to appropriate cellular destinations. This new concept will pave the way for further experiments aimed at understanding the early events of protein synthesis in eukaryotes.

**STAR METHODS**

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Supplemental information can be found online at https://doi.org/10.1016/j.molcel.2019.06.030.

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**AUTHOR CONTRIBUTIONS**

Conceptualization, M.G., K.K., N.B., and E.D.; Investigation, M.G. performed genetic and in vivo biochemical experiments, M.G. and R.S. performed in vitro biochemical experiments with contribution from N.S., K.K. prepared the cryo-EM samples and processed the cryo-EM data with contribution from A.J., A.W. performed in vitro translation and crosslinking experiments, S.G.K. performed yeast Bpa-60S crosslinking experiments, and C.S. and F.S. performed XL-MS experiments and processed XL-MS data; Writing – Original Draft, M.G.; Writing – Review & Editing, K.K., N.B., and E.D.; Funding Acquisition, M.G., N.B., and E.D.; Supervision, M.G., N.B., and E.D.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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Appendix I
Structure of monomeric yeast and mammalian Sec61 complexes interacting with the translating ribosome. Science 326, 1528–1533.
Appendix I


Appendix II

Mechanism of signal sequence handover from NAC to SRP on ribosomes during ER protein targeting
Title: Mechanism of signal sequence handover from NAC to SRP on ribosomes during ER-protein targeting

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Abstract:
The nascent polypeptide-associated complex (NAC) interacts with newly synthesized proteins at the ribosomal tunnel exit. NAC competes with the signal recognition particle (SRP) to prevent mistargeting of cytosolic and mitochondrial polypeptides to the endoplasmic reticulum (ER). How NAC antagonizes SRP and how this is overcome by ER targeting signals is unknown. Here, we discover that NAC uses two domains with opposing effects to control SRP access. The core globular domain prevents SRP from binding to signal-lacking ribosomes, whereas a flexibly attached domain transiently captures SRP to permit scanning of nascent chains. The emergence of an ER targeting signal destabilizes NAC’s globular domain and facilitates SRP access to the ribosome. These findings explain how NAC hands over the signal sequence to SRP, impart specificity of protein localization.

One-Sentence Summary: NAC acts as a gatekeeper on the ribosome to regulate SRP function and control ER targeting.
Main Text

Localization of nascent proteins to the appropriate organelle is essential for cell function and homeostasis. The accuracy of co-translational targeting to the ER relies on two ribosome-binding factors. SRP uses a methionine-rich M-domain to engage hydrophobic ER targeting signals as they emerge from the exit tunnel of the ribosome and delivers the ribosome-nascent chain complex (RNC) to the SRP receptor at the ER membrane via its special GTPase, NG-domain (1–4). The abundant heterodimeric factor NAC (composed of NACα and NACβ) prevents SRP from promiscuously targeting ribosomes that do not display an appropriate signal (5–9). Hence, acute depletion of SRP causes failed ER targeting (10), whereas acute depletion of NAC causes inappropriate ER targeting of non-ER proteins (11). Both situations lead to widespread organelle stress and shortened lifespan in model organisms.

Considering that SRP is far less abundant than ribosomes in the cell and has high affinity for ribosomes with or without an ER targeting signal on the nascent chain, it is clear that NAC must control and regulate SRP access so that it only targets ribosomes displaying the ER signal sequence. NAC consists of a central globular domain from which flexible N- and C-terminal tails extend (12–14). Crosslinking studies suggest that the N-terminal tails are used for a range of interactions and participate in ribosome binding (15, 16). The function of the C-terminal tails, which carry a conserved structured ubiquitin-associated domain (UBA) in NACα, are unknown. NAC and SRP share overlapping ribosome binding sites, which may give rise to their antagonism (15). However, biochemical experiments demonstrated that NAC co-binds with SRP to RNCs translating ER proteins (6, 8, 9). These results appear contradictory in the absence of mechanistic and structural information that would explain how NAC binds the ribosome to prevent SRP from binding and how this inhibition is preferentially overcome for ER targeting signals.

Structures of NAC in complex with translating ribosome

To reveal how NAC and SRP interplay on the ribosome to control ER transport of proteins, we set up a reaction with signal-containing RNCSS mixed with both NAC and SRP and analyzed the complexes formed by cryo-electron microscopy (cryo-EM) (fig. S1). We rationalized that this reaction is likely to contain intermediates at critical steps of cargo recognition and handover, which could be deconvoluted in silico by 3D image classification. To this end, we resolved two complexes within the particles, a pre-cargo handover RNCSS•NAC complex, which we will discuss first, and a ternary post-cargo handover RNCSS•NAC•SRP complex, which is discussed later.

Notably, the structure of the RNCSS•NAC complex is very similar to the RNC•NAC structure obtained in parallel from re-analysis of a previous RNC intermediate during translation of the cytosolic protein tubulin (TUBB) (fig. S2 and 3), on which NAC co-purified (17). This suggests that NAC initially engages both signal-containing and signal-lacking RNCs in a similar manner, but is expected to handover to SRP only in the presence of an ER signal sequence.

The structure of the RNCSS•NAC complex (Fig. 1A-D) reveals the interactions between the N-terminal tail of NACβ and the ribosome at 3.5 Å resolution (Fig. 1C, fig. S4). The position of the tail agrees with previous crosslinking studies, whereas the nature of interactions explains why this domain has a key role in ribosome binding (15, 18). The tail is composed of an α-helix followed by a loop in an anchor-shaped turn wrapping around eL22, while also contacting eL19 and the ribosomal RNA through mainly electrostatic contacts (Fig. 1C, fig. S4). Several point mutations in this N-terminus weakened NAC-RNC binding by 10-40-fold (Fig. 1E-F), consistent with previous data showing that mutation of the RRKKK motif abolished ribosome binding of NAC (16). Therefore, the N-terminus of NACβ can be considered as the ribosome anchor for NAC.
The globular domain of NAC was resolved to approximately 8 Å resolution, which allowed rigid body fitting of an Alphafold\(^{(19)}\) predicted structure with minimal adjustments (Fig. 1A-B, fig. S5). Based on this interpretation, and in agreement with the features of the map, two α-helices contributed by both NAC subunits are positioned at the interface with the ribosome (Fig 2A, fig. S5). These helices are positively charged and could potentially interact with the 28S rRNA in the vicinity of the ribosome tunnel exit and with the emerging nascent chain (fig. S5B). Charge reversal mutations of one positively charged residue in the helices (K78E-NAC\(\alpha\) or K43E-NAC\(\beta\)) weakened ribosome binding of NAC in vitro (Fig. 2B and C) and in vivo (fig. S6A).

The binding site of NAC globular domain overlaps with that of SRP M-domain and is mutually exclusive with SRP binding (Fig. 2A and fig. S6B)\(^{(3, 4)}\), consistent with a low-resolution cryo-EM map of NAC in complex with inactive ribosomes\(^{(15)}\), and suggesting that its interaction at the exit site is the basis of SRP inhibition. In agreement with this hypothesis, a ribosome binding mutant in the globular domain (K78E-NAC\(\alpha\) combined with K43E-NAC\(\beta\), termed NAC KK-EE) was impaired in its ability to compete with SRP binding in vitro (Fig. 2D), even when NAC KK-EE was added at 8-fold molar excess (Fig. 2D). Based on the structural and biochemical data, the residual binding of NAC KK-EE to the ribosome is likely mediated by the N-terminus of NAC\(\beta\), the position of which would not interfere with SRP binding (fig. S6B). These observations suggest a model in which NAC can control SRP binding by changing the position of the globular domain while remaining anchored on the surface of the ribosome through one of its tails, rationalizing previous biochemical results\(^{(8, 9)}\).

The corresponding NAC KK-EE mutations in \(C.\) elegans showed reduced competition of SRP binding by NAC as judged by elevated levels of ribosome-bound SRP (Fig. 2E, fig. S6C) as well as increased recovery of mRNAs coding for non-ER proteins in SRP pulldowns (fig. S6D). The reduction in SRP competition correlated with elevated levels of a GFP reporter of ER stress (hsp-4p::GFP)\(^{(20)}\), particularly in highly secretory intestinal cells (Fig. 2F). Moreover, worms expressing mutant NAC showed reduced embryonic viability (fig. S6E) and a shortened adult lifespan (fig. S6F). Thus, the NAC globular domain interaction with the ribosome is crucial for SRP antagonism, protein targeting accuracy, cell homeostasis, and longevity in vivo. The combination of the above described structural, biochemical and in vivo experiments suggest that the central globular domain acts as a gatekeeper of the ribosome tunnel, the binding of which could be subject to regulation.

**NAC is destabilized by the ER signal sequences allowing access of SRP to the ribosome**

SRP antagonism by NAC must be relieved when an ER targeting signal emerges from the ribosome. One possibility is that hydrophobic ER targeting signals somehow weaken the interactions between the globular domain and the ribosome to allow SRP access. To test this, we compared the affinity of NAC for RNCs displaying either an ER signal sequence (RNC\(SS\)) or a mutated signal sequence that inhibits ER targeting (RNC\(SS_{smn}\)) (fig. S7)\(^{(21)}\). The RNC binding affinity of NAC was measured using FRET between a donor dye placed near the signal sequence on the nascent chain and an acceptor dye placed on NAC. Wildtype NAC binds RNC\(SS_{smn}\) ~two-fold more strongly than RNC\(SS\) (Fig. 2G, fig. S8A). While this difference is modest, we reasoned that differential interactions of the globular domain of NAC with RNCs may be masked by high affinity binding of the NAC\(\beta\) anchor to the ribosome. We therefore repeated these measurements with NAC mutants bearing point mutations in the RRKKK motif of the anchor, which increased the difference between RNC\(SS_{smn}\) and RNC\(SS\) to ~3.5-fold and ~5-fold with mutants NAC-R27A and NAC-K29A, respectively (Fig. 2G; fig. S8A). Notably, the binding preference of NAC is opposite of that of SRP, which binds RNC\(SS\) 4-fold more strongly than RNC\(SS_{smn}\)\(^{(9)}\). To further validate this model, we measured NAC binding to purified RNCs bearing ER, cytosolic and
mitochondrial nascent chains (HSPA5, GPI and HSPD1, respectively) stalled at residue 60. These RNCs expose only short N-terminal substrate sequences (~30 aa) at the tunnel exit, which consist mainly of N-terminal targeting peptides in the case of RNC_{HSPA5} (aa 1-18) and RNC_{HSPD1} (aa 1-26) (fig. S7). In agreement with the observations with RNC_{SS} and RNC_{SSmt}, NAC R27A binds 5-fold more weakly to RNC_{HSPA5} exposing an ER signal sequence (Fig. 2H and fig. S8B-C).

To distinguish whether the weaker NAC affinity for RNC_{SS} and RNC_{HSPA5} is due to destabilization by the ER signal or more stable interaction with cytosolic/mitochondrial nascent chains, we repeated the binding measurements with purified RNCs bearing an ER signal sequence at nascent chain lengths of 30, 40 and 60 aa (Fig. 2H, fig. S8B-C). Indeed, NAC showed the strongest interaction with ribosome when the nascent chain is in the tunnel (30 and 40 aa), and binding is weakened more than 10-fold when the ER signal peptide is exposed (60 aa). Thus, the emergence of a hydrophobic signal peptide weakens the interaction of NAC globular domain with the ribosome.

Therefore, we investigated the role of the two ribosome-binding antiparallel helices that dock the globular domain on the ribosome in proximity to the emerging nascent chain. The helices are amphipathic and orient the positively charged side toward the ribosome surface, while the hydrophobic side contribute to a buried hydrophobic pocket (fig. S5). These helices were sensitive to proteolysis when human NAC was subjected to crystallization (13), suggesting that they are flexibly attached. To test this, we engineered two apposed cysteines in the helices that become covalently connected upon disulfide bond formation. As predicted, apposed cysteines can form a disulfide bond after oxidant treatment but, notably, only in the presence of the ribosome (Fig. 3A and fig. S9).

These experiments also suggest that the release of the globular NAC domain from the ribosome likely destabilizes the pairing of the two helices, exposing the underlying hydrophobic residues for potential sensing of hydrophobic signaling peptides (Fig. 3B). We investigated this by incorporating photo-crosslinking probes at six positions on NAC, both inside and outside the two interacting helices (Fig. 3B) and tested their proximity to nascent chains coding for a cytosolic, mitochondrial, or ER protein. NAC variants carrying the probe within the hydrophobic pocket (e.g., NACα-I121) crosslinked to targeting signals (Fig. 3C and fig. S10A-C). This interaction was due to the targeting signal because fusing only this element to the cytosolic nascent chain was sufficient for crosslinking (Fig. 3C). Crosslinking was dependent on nascent chain length and only seen once the targeting signal was fully exposed outside the exit tunnel (fig. S10A). Importantly, crosslinking of the signal peptide to the hydrophobic residues (NACα-I121) was prevented when the helices were covalently linked by disulfide bond formation, demonstrating that ER signal peptide recognition requires separation of the helices (Fig. 3D). Furthermore, crosslinking to NACα-I121 and NACβ-L48 was modulated by changing targeting signal hydrophobicity (fig. S10D). Furthermore, a less buried position (NACα-M80) just beyond the NACα helix was less sensitive to hydrophobicity (fig. S10D), even showing weak crosslinking to non-ER substrates (fig. S10A). Interestingly, mutating this residue to serine substantially impaired nascent chain photo-crosslinking to NACα-I121 inside the pocket (Fig. 3E), which suggests this residue also contributes to nascent chain sensing.

These results indicate that an ER signal sequence destabilizes the NAC globular domain, however the NACβ anchor remains attached to the ribosomal surface regardless of the nascent chain as evidenced by crosslinking between a residue in the NACβ anchor and the ribosomal protein eL22 (fig. S11A). In contrast, a probe in the flexible N-terminus of NACα changed its location only for the ER substrate, moving near eL19 as would be expected if the ER targeting signals facilitate displacement of the NAC globular domain (fig. S11B-C).
Flexibly tethered UBA domain of NAC recruits SRP

The cryo-EM data on the signal-containing RNC\textsubscript{SS} mixed with both NAC and SRP, as discussed above, also allowed us to visualize the complex with NAC and SRP simultaneously bound to the ribosome (RNC\textsubscript{SS}\textbullet{}NAC\textbullet{}SRP) (Fig. 4A, fig. S1 and S12). The conformation of SRP in the ternary complex was similar to previously observed SRP-ribosome complexes, with the M-domain of SRP\textsubscript{54} bound to the signal sequence and the SRP\textsubscript{54} NG-domain interacting with ribosomal proteins uL23 and uL29 \cite{3, 4}. The density for the NAC\textsubscript{B} anchor was still on the ribosome, in a similar position as observed in the RNC\textbullet{}NAC complexes (fig. S12C). However, as expected, the globular domain of NAC is no longer resolved since its binding position at the tunnel exit is occupied by the SRP\textsubscript{54} M-domain, which binds an ER signal sequence (Fig. 4A-D and Fig. 10A).

Interestingly, we observed density for the flexibly tethered C-terminal UBA domain of NAC\textsubscript{\alpha} bound to the N-domain of SRP\textsubscript{54} (Fig. 4B-C, fig. S12 and S13). The interactions occupy two patches of contact points and involve a number of salt bridges and specific hydrogen bonds between highly conserved residues (Fig. 4C-D, fig. S14). Notably, the UBA binding site on SRP\textsubscript{54} overlaps with the binding site of the NG-domain of the SRP receptor (SR) (fig. S15), which suggests that formation of the SRP\textbullet{}SR complex will displace NAC from SRP at the ER membrane \cite{22–24}. The observation that the UBA domain of NAC can directly interact with SRP raises interesting questions regarding its role in ER targeting.

To test whether the NAC UBA helps SRP overcome the antagonism by NAC, we generated a NAC mutant in which the UBA is deleted (dUBA), and NAC or SRP mutants containing charge reversal mutations at contact points between the UBA and the NG-domain of SRP\textsubscript{54} (D205R/N208R-NAC\textsubscript{\alpha}, termed UBAmt, and K50E/R53E-SRP\textsubscript{54}, termed SRP\textsubscript{54}mt based on human sequence numbering). We measured the effects of these mutations on the binding affinity of SRP for NAC engaged RNCs displaying the signal sequence. Although none of the above-described mutations changed the affinity of NAC or SRP to RNCs, they decreased the affinity of SRP for RNC\textsubscript{SS}\textbullet{}NAC complex over 5-fold (Fig. 4E, fig. S17A). The same effect was observed in a control experiment when NAC was titrated to a pre-formed RNC\textsubscript{SS}\textbullet{}SRP complex (fig. S17B-C). Therefore, the contact between NAC UBA and SRP\textsubscript{54} NG-domains is important for stabilizing the binding of SRP on signal sequence displaying ribosomes pre-engaged with NAC.

The flexible tethering of the NAC\textsubscript{\alpha} UBA further suggests that this domain mediates the initial recruitment of SRP to ribosomes pre-bound with NAC. To test this hypothesis, we used total internal reflection fluorescence (TIRF) microscopy to study single molecule events in which SRP binds to surface-immobilized RNC\textsubscript{SS} (Fig. 5A). If SRP is captured by NAC via the UBA domain prior to stable engagement with the ribosome, then the arrival of SRP on NAC-bound RNC\textsubscript{SS} would be synchronous with the onset of FRET between a dye pair engineered on the SRP\textsubscript{54} NG and NAC\textsubscript{\alpha} UBA domains. If, in contrast, this contact is not an obligatory early interaction, the onset of high FRET would occur at stochastic time intervals after the arrival of SRP on the RNC. The results are consistent with the first model: the initiation of co-localized fluorescence signals from NAC and SRP is synchronous with the onset of FRET in every single molecule fluorescence time trace (Fig. 5B, C), even in recruitment events that did not lead to long-lived SRP association with the RNC (example in Fig. 5B). Statistical analysis further supported the synchronization: when the time traces for RNCs prebound with NAC (n = 45) are aligned to the start of the SRP fluorescence signal, peak FRET efficiency is coincident with SRP arrival (Fig. 5D). Once a stable RNC\textbullet{}NAC\textbullet{}SRP ternary complex is formed, NAC\textsubscript{\alpha} UBA dynamically associates with and dissociates from SRP\textsubscript{54}, as shown by the frequent transitions between low and high FRET states on the seconds timescale (Fig. 5E). These results suggest that the contact between UBA and NG
initiates early, before the productive docking of SRP at the exit of the ribosomal tunnel and signal sequence handover.

Finally, we investigated the effects of NAC and SRP mutants in vivo. NAC and SRP54 mutations that impair NAC UBA-SRP54 NG interactions in C. elegans showed elevated levels of a GFP reporter of ER stress, particularly in highly secretory intestinal cells (Fig. 4F and fig. S18A, B). Furthermore, the secretion of a signal sequence-bearing GFP reporter (ssGFP) was significantly lower in both NAC and SRP54 mutant worms compared to wild-type NAC worms (Fig. 4G and fig. S18C-D). The defects observed with SRP54mt is not due to impaired interaction with the SR NG domain, as the SRP54 K50 and R53 mutations did not affect SRP•SR complex assembly (fig. S15). In addition to ER stress, the mutant worms also showed a cytosolic stress response, suggesting a possible accumulation of misfolded ER proteins in the cytosol due to failed targeting (fig. S18E). Together, these results demonstrate that the contacts between SRP and the UBA domain of NAC is critical for the successful SRP targeting of proteins to the ER.

**Mechanism of the NAC and SRP interplay on the ribosome to initiate ER targeting**

Our data allow us to propose a molecular mechanism of the interplay of NAC and SRP at the ribosome that controls and initiates protein targeting to the ER: NAC acts a ‘gatekeeper’ at the exit of the ribosomal tunnel to shield nascent chains from non-physiological interactions with SRP (Fig. 6). Due to its abundance and high affinity for the ribosome (K_d ~ 1 nM), NAC is bound to most ribosomes at early stages of translation via a high affinity anchor, and a globular domain that blocks SRP access to nascent polypeptides. At the same time, the flexibly tethered UBA domain recruits SRP and increases its local concentration at the tunnel exit region to initiate sampling of nascent chains. The emergence of an ER signal sequence weakens the interactions of NAC’s globular domain with the surface of the ribosome, allowing SRP to access the signal sequence and bind to the exit of the ribosomal tunnel displacing the globular domain of NAC. In the resulting signal sequence engaged ternary complex, NAC remains associated with both the ribosome and SRP, via respective anchor and UBA contacts, until it reaches the ER membrane where the SRP receptor will displace the UBA domain from SRP.

This study answers the longstanding question regarding the molecular basis of NAC as a sorting factor for nascent chains on the ribosome and the nature of its spatiotemporal coordination with SRP. It explains how NAC, bound to virtually all ribosomes, prevents sub-stoichiometric SRP from forming tight but unproductive complexes with signal-less ribosomes. It also explains how the UBA domain recruits SRP to quickly scan all NAC bound ribosomes for the presence of the ER signal sequence. These results further open opportunities for dissecting the mechanisms that govern the distribution of substrates to different cellular compartments. Stepwise recognition by NAC followed by SRP (and ultimately, by SR and Sec61) would improve the overall fidelity of protein targeting. It is plausible that degenerate and highly diverse targeting sequences cannot be recognized with sufficiently high specificity in a single step and/or by individual targeting factors, warranting not only multiple filters, but also quality control pathways to degrade the failures that occur nonetheless. Such multiple checkpoints are crucial to maintain proper protein homeostasis in cells.
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Appendix II

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**Supplementary Materials**

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Figures

(A) Cryo-EM structure of the RNCss•NAC complex, boxed region indicates the close-up region shown in panel B. (B) A closeup on the ribosome tunnel exit region. NACβ is colored green and NACα is colored orange. N-terminal domains of NACα and NACβ are indicated. (C) Closeups on the N-terminus of NACβ fitted into cryo-EM densities shown as mesh. Ribosomal proteins eL22 is shown as cartoon ribbon and colored blue. (D) Schematic of the RNC•NAC complex with a domain structure of NAC. (E) Equilibrium titrations to measure the binding of the indicated NAC mutants to RNCss. The fluorescence signal changes were normalized to the end point of each titration for comparison. The lines are fits of the data to Eq 2, and the obtained $K_d$ values are summarized in panel F. (F) Summary of the $K_d$ values from panel E.

Fig. 1. Structure of the ribosome•NAC complex reveals interactions of a high affinity anchor. (A) Cryo-EM structure of the RNCss•NAC complex, boxed region indicates the close-up region shown in panel B. (B) A closeup on the ribosome tunnel exit region. NACβ is colored green and NACα is colored orange. N-terminal domains of NACα and NACβ are indicated. (C) Closeups on the N-terminus of NACβ fitted into cryo-EM densities shown as mesh. Ribosomal proteins eL22 is shown as cartoon ribbon and colored blue. (D) Schematic of the RNC•NAC complex with a domain structure of NAC. (E) Equilibrium titrations to measure the binding of the indicated NAC mutants to RNCss. The fluorescence signal changes were normalized to the end point of each titration for comparison. The lines are fits of the data to Eq 2, and the obtained $K_d$ values are summarized in panel F. (F) Summary of the $K_d$ values from panel E.
Fig. 2. NAC binds RNCs with ER signal sequences with lower affinity. (A) Closeup of the NAC globular domain highlighting the two antiparallel α-helices interactions with the rRNA and nascent chain (NC, magenta). The two residues K78 (NACα) and K43 (NACβ) shown as spheres (blue) interact with the backbone of the rRNA (red). Dashed line indicates flexible nascent chain (NC), as it emerges from the ribosome tunnel. (B) and (C) In vitro sucrose cushion centrifugation of purified 80S ribosomes incubated with indicated NAC variants (ratio 80S to NAC = 1:2). Proteins in the supernatant (Sup) and ribosomal pellet were detected by immunoblotting. (D) Crude cellular RNCs were incubated with indicated purified NAC proteins and ribosomes pelleted by sucrose cushion centrifugation. Proteins in the ribosomal pellet fraction were analyzed by immunoblotting. (E) Sucrose cushion centrifugation of ribosomes in C. elegans worms expressing indicated NAC variants and GFP-tagged SRP72. Proteins in the pellet fraction were detected by immunoblotting. (F) Fluorescence microscope images of hsp-4p::GFP C. elegans worms expressing indicated RNAi-resistant NAC genes. Analysis was performed in the endogenous NAC RNAi background on day 1 of adulthood. (G) Summary of the equilibrium dissociation constants ($K_d$) of wildtype and mutant NAC for RNCss and RNCsSmt (based on the data in fig. S8A). (H) Summary of the $K_d$ values of NAC R27A for RNCs with different nascent chains, GPI (cytosolic), HSPD1 (mitochondrial)and HSPA5 (ER) fitted from (fig. S8B-C) stalled at different nascent chain length. Error bars are covariances of fitted $K_d$ values.
Fig. 3. ER signal sequences are sensed by the ribosome-binding helices of NAC. (A) NAC’s ribosome binding helices showing the positions of pairwise cysteine mutants tested for disulfide bond formation. Side chains shown are based on Alphafold prediction. Dashed lines indicate pairs sufficiently close to form a disulfide bond revealed by immunoblotting (right panel). Disulfide bond formation was induced by oxidation in the presence and absence of inactive 80S ribosomes. (B) Residues contributing to the hydrophobic pocket between the two α-helices of NAC (purple). The right side shows a model where ribosome dissociation leads to separation of the helices thereby exposing a hydrophobic pocket. (C) Photo-crosslinking of indicated Bpa-NAC variants to stalled RNCs carrying 50 aa S35-labeled nascent chains of cytosolic GPI (left) or a GPI fusion protein containing the N-terminal signal peptide of HSPA5 (right). Autoradiograph images are shown. The positions of the tRNA-attached nascent chain (NC-tRNA) and its crosslinks to NACα and NACβ are indicated. Asterisk indicates a position outside the hydrophobic region not displayed in panel B. (D) Photo-crosslinking of αC75-βC51 cysteine variant of panel A carrying Bpa at α-1121 to HSPA5-RNCs (55 aa). Assay was performed in the reduced (red.) and oxidized (ox.) state. Autoradiograph and immunoblotting images are shown. (E) 55 aa HSPA5-RNCs photo-crosslinking of indicated α-1121 Bpa-NAC variants. Autoradiograph and immunoblotting images are shown.
Fig. 4. Structure of the ribosome-SRP-NAC complex. (A) Cryo-EM structure of the RNCss•NAC•SRP complex, boxed region indicates the close-up region shown in panel B. (B) A closeup on the ribosome tunnel exit region depicting SRP54 NG and M domains, NACα UBA domain, and NACβ anchor domain colored slate, cyan, orange, and green, respectively. Underlying EM-density is shown as transparent surface. (C) Closeups on the UBA interactions with SRP54 NG domain shown as cartoon and sticks, fitted into cryo-EM densities shown as mesh. (D) Schematic representation of the ternary complex. Boxed region shows sequence alignment of NACα UBA domain in eukaryotes. (E) $K_d$ summary of SRP and SRP variants for RNCss•NAC complex, based on the fitting in figure S17A. N.D. not determined. (F) Fluorescence microscope images of hsp-4p::GFP C. elegans worms expressing indicated RNAi-resistant NAC or SRP54 genes. Analysis was performed in the endogenous NAC or SRP54 RNAi background on day 3 of adulthood. (G) Worm flow cytometry analysis of ssGFP in worms carrying indicated RNAi-resistant genes in the endogenous RNAi background.
Fig. 5. Interactions between SRP54 and NACα UBA domain delivers SRP to the ribosome harboring an ER signal sequence. (A) Scheme of the single molecule experiment. RNC is immobilized on the glass coverslip surface via 3’ biotinylated mRNA (not shown). NAC labeled with Cy3b (green star) in the UBA domain and SRP labeled with Atto647N (red star) in the SRP54 NG domain were presented to the RNC. In the proposed model, SRP arrival on RNCs is predicted to be synchronous with the onset of FRET signal between SRP54 NG and NAC UBA. (B) and (C) Representative single molecule fluorescence time traces. \( D_{em-Dex} \), donor emission during donor excitation. \( A_{em-Aex} \), acceptor emission during donor excitation. \( A_{em-Aex} \), acceptor emission during acceptor excitation. \( E \), apparent FRET efficiency, calculated from the \( A_{em-Dex} \) and \( D_{em-Dex} \) traces. The region after donor photobleaching is masked. As expected from the reversibility of protein interactions, the initial contact of SRP NG and FRET onset (indicated by an arrows) with NAC
UBA can be followed by SRP dissociation from the transient recruiting complex (B) or by stable SRP engagement with the RNC (C). (D) Time traces of FRET efficiency ($n = 45$) are aligned to the start of the SRP (acceptor) signal. The median FRET value of all traces at each time frame is plotted as solid blue line. The blue shaded area encloses the FRET range that includes the first to third quartile of data at each frame. The onset of FRET signal coincides with SRP arrival at $t = 0$, indicated by an arrow. (E) Representative time trace after a stable NAC•SRP•RNC ternary complex is formed, in which the NAC UBA-SRP54 NG interaction reversibly forms and dissociates on the ribosome.
Appendix II

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1. **“NAC binding”**

2. **“SRP delivery and cargo handover”**

3. **“ternary complex formation”**

4. **“protein targeting to ER”**
Fig. 6. Model of co-translational signal sequence handover from NAC to SRP during ER-protein targeting. NAC interactions at the ribosome exit tunnel are mediated by the globular and anchor domains. The NAC globular domain overlaps with the binding site of SRP54 M-domain, blocking the binding of the latter to the ribosome exit site. The flexibly tethered UBA domain of NACα recruits SRP to the ribosome via interaction with the SRP54 NG domain, generating a transient recruiting complex in which the local concentration of SRP is increased and in which the nascent chain can sample between SRP54 M and NAC globular domains. The emergence of an ER signal sequence weakens NACαβ globular domain interactions with the ribosome and leads to cargo handover and relief of antagonism by NAC. NAC is retained on the targeting complex via interactions of the N-terminal domain of NACβ with the ribosome and the UBA domain of NACα with the N-domain of SRP54. At the ER membrane, the UBA domain of NAC is displaced from SRP by the SRP receptor after NG-heterodimer formation.
Supplementary Materials for

Mechanism of signal sequence handover from NAC to SRP on ribosomes during ER-protein targeting

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Materials and Methods
Figs. S1 to S18
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Supplementary Figures:

**A**
- Dataset 1: 15,876 micrographs, 896,916 particle images
- Dataset 2: 9,274 micrographs, 594-404 particle images
- 2D class averages
- 1st 3D classification: 105,756 particles
- 2nd 3D classification: 51,843 particles
- Full size: 448x448 pixels
- 3D mask
- Binmed 80x80 pixels

**B**
- RNC_{SRP-NAC} and RNC_{NAC}

**C**
- RNC_{SRP-NAC} and RNC_{NAC}

**D**
- Graph showing resolution vs. density distribution for half maps (masked), half maps (unmasked), and model map (RNC_{SRP-NAC})
- Resolution: 3.1 Å, 3.2 Å
Fig. S1
Image classification and 3D refinement scheme of the ternary $\text{RNC}_{\text{ss}}$$\cdot$SRP$\cdot$NAC and of the $\text{RNC}_{\text{ss}}$$\cdot$NAC complexes. (A) After particle picking, an initial 2D classification was performed on binned particles (box size 80 x 80 pixels). The selected particle images were then subjected to 3D refinement followed by a 3D focused classification without alignment on the ribosome tunnel region. To improve NAC and SRP EM-densities, a second round of focused 3D classification focusing on the SRP proximal site was performed. The particles in the selected class were subjected to a 3D refinement using full size images without binning (448 x 448 pixels) and a final pixel size of 1.08 Å in RELION3, which yielded a map with an overall resolution of 2.8 Å for both maps. (B) Local resolution of the determined complexes (right) was calculated in Relion3. (C) Angular distribution of the particles in the two cryo-EM maps after final 3D refinements. (D) Fourier Shell Correlation (FSC) plots for the cryo-EM maps shown in panel A and the model versus map plot of the $\text{RNC}_{\text{ss}}$$\cdot$SRP$\cdot$NAC and $\text{RNC}_{\text{ss}}$$\cdot$NAC complexes, calculated using the gold standard FSC criteria cutoff (FSC=0.143) using independent two half maps as implemented in RELION3, and the cutoff for the resolution of the model is determined based on the FSC cutoff (FSC=0.5).
Fig S2
Summary of cryo-EM workflow for the RNC<sub>tubb</sub>•TTC5•NAC. (A) Cryo-EM data processing schematic. All EM processing steps were performed using Relion 3. Model building was performed in Coot and real space refinement in PHENIX. FCwSS - focussed classification with partial signal subtraction. (B) Fourier Shell Correlation (FSC) plots for the cryo-EM maps shown in panel A and the model versus map plot of the RNC<sub>tubb</sub>•NAC•TTC5 complex, calculated using the gold standard FSC criteria cutoff (FSC=0.143) using independent two half maps as implemented in RELION3, and the cutoff for the resolution of the model is determined based on the FSC cutoff (FSC=0.5). (C) Angular distribution of the particles in the two cryo-EM maps after final 3D refinements. (D) Local resolution of the determined complexes was calculated in Relion.
Fig. S3
Comparison of the RNC\textsubscript{Tub}•TTC5•NAC and of the RNC\textsubscript{ss}•NAC complexes. (A) and (B) Cryo-EM maps of the two complexes depicting densities for NAC (green and orange) and TTC5 (gray). Ribosomal proteins are colored yellow and blue, for small and large subunits, respectively. rRNA is colored gray. (C) and (D) Closeups of the ribosome tunnel exit region resolving densities for NAC globular and anchor domains. Density is shown as transparent surface with underlying atomic coordinate of the RNC•NAC complex. The coordinates of the NAC can be fitted into both maps without further rearrangements. Both maps are filtered to 8 Å resolution.
Fig. S4

Molecular interactions between the N-terminal tail of NACβ with the ribosome. (A) Close-up of the interaction interface mediated by the RRKKK motif of NACβ shown as sticks with overlaid cryo-EM density shown as mesh. NACβ is colored green and ribosome is colored white and orange and is shown as cartoon and sticks. (B)-(D) Close-up of the interaction between NACβ N-terminal domain and rRNA (light orange), eL22 (blue) and eL19 (yellow) shown as cartoon and sticks with overlaid cryo-EM density shown as mesh. (E) Sequence alignments of the NACβ in eukaryotes performed with the Clustal Omega [https://www.ebi.ac.uk/Tools/msa/clustalo/] and displayed with ESPript 3.0 [http://esprit.ibcp.fr/ESPript/ESPript/]. The N-terminal of NACβ domain is highlighted. Asterisks indicate the potential interaction site between NACβ and the ribosome. Asterisks indicate the residues that were targeted for mutational experiments.
Fig. S5

**Interactions of NAC globular domain with the ribosome. (A)** Schematic of the ribosome tunnel exit, with docked NAC shown as cartoon. The anti-parallel helices that form a platform for NAC are shown as ribbons. Dashed area represents a hydrophobic pocket shielded by the two helices. **(B)** Left, surface representation of NAC globular domain colored by hydrophobicity. Residue contributing to the hydrophobic pocket are indicated. Pocket was exposed by removing the platform helice of NACα from the model. Right, surface representation of NAC platform helices colored by surface charge. Residues contributing to the overall positive charge of the platform are labelled. View is the same as in panel A, right.
**Fig. S6**

**Analysis of NAC KK-EE animals.** (A) Sucrose cushion centrifugation of ribosomes in double transgenic *C. elegans* strains expressing indicated RNAi-resistant FLAG-tagged NACα and untagged NACβ variants. Analysis was performed on day 1 of adulthood in the endogenous NACαβ background. Proteins in the supernatant (Sup) and pellet fractions were detected by immunoblotting. (B) Schematic of the tunnel exit with SRP and NAC binding positions indicated. The NAC globular domain, but not the NACβ anchor, competes with SRP. (C) A *C. elegans* strain for SRP pulldown studies as in (D) was generated by inserting a STREP-GFP tag into the native SRP72 (srpra-72) gene locus using CRISPR/Cas9 gene editing. This strain was mated to strains carrying RNAi-resistant NAC WT and NAC KK-EE transgenes. Ribosomes were sedimented through sucrose cushion centrifugation, and protein levels in the input (Total) and ribosomal pellet (Ribo pellet) fractions were detected by immunoblotting. (D) Left panel: Pulldown of SRP-bound RNCs from animals expressing STREP-GFP-SRP72 using Streptactin affinity purification. Immunoblot shows levels of SRP54 and uL16 in the total and pulldown RNC fractions. Right panel: Pulldown of SRP-bound RNCs in animals as in (C). Levels of ribosome-associated mRNAs in total and SRP pulldown fractions were assessed by quantitative RT-PCR. Log2-transformed
pulldown-to-total ratios for select mRNAs coding for proteins with destination in the ER, cytoplasm and mitochondria were calculated. Diagram shows difference of log2 ratios between NACKK-EE and NACWT animals. Data are represented as mean ± SD. (E) Quantification of progeny in animals as in (A). Diagram shows the percentage of progeny in the endogenous NAC RNAi background compared to N2 empty vector (ev) control animals. Error bars = s.d.; *p<0.05 vs. WT (two-tailed t-test); n=3. Note that complementation with WT-NAC is not complete. (F) Life span analysis of C. elegans worms expressing indicated RNAi-resistant NAC genes in the endogenous NAC RNAi background.
Fig. S7
Schematic representation of RNC constructs used for NAC affinity measurements. Light grey background indicates part of the nascent chain that is buried in the ribosomal tunnel (~30 aa). Signal sequences (SS) and mutant signal sequences (SSmt) are indicated. MTS, mitochondrial targeting sequence.
Fig. S8
Interactions of NAC with the ribosome and the nascent chain. (A) Equilibrium titrations to measure the binding of the indicated NAC mutants to RNC\textsubscript{SS}. The fluorescence signal changes were normalized to the end point of each titration for comparison. The lines are fits of the data to Eq 2, and the obtained \(K_d\) values are summarized in figure 2G. (B) and (C) RNC-NAC FRET titration curves with WT and R27A NAC mutants. The RNCs bear the GPI, HSPD1 and HSPA5 nascent chains stalled at 30, 40, and 60 amino acids as indicated. The lines are fits of the data to Eq 2, and the obtained \(K_d\) values are summarized in figure 2H.
Fig. S9
Additional controls related to Figure 3A. (A) Coomassie gel showing protein samples used for analysis in Figure 3A. The NAC cysteine variants were adjusted to the same concentration, and a control gel was run under reducing conditions before performing the assay. (B) Immunoblot (IB) showing reduced NACβ levels under the assay conditions shown in Figure 3A.
Appendix II

A

Bpa NAC variants + stalled RNCs radiolabeled NC (45 - 60 aa) → SDS-PAGE Autoradiography NC-NAC x-links

B

Bpa NAC variants

C

Bpa NAC variants - ribosome binding (50S)

D

Bpa: α-L53, β-L48, γ-M60, δ-L85, α-1121

100 mM KOAc

400 mM KOAc

Pellet

Coomassie

Hydropathy:

WT 0.625
less hy. 0.329
more hy. 1.469

NACα

NACβ

x-NACα

x-NACβ

NC-tRNA

NACα

NACβ

NC-tRNA

x-NACα

x-NACβ

NC-tRNA

x-NACα

x-NACβ

NC-tRNA
Fig. S10 Co-translational interaction of NAC with ER signal sequences. (A) Site-specific photo-crosslinking of indicated Bpa-NAC variants to stalled RNCs carrying 45-60 aa S\textsuperscript{35}-labeled nascent chains of cytosolic (GPI), mitochondrial (HSPD1) or ER substrates (HSPA5). Bpa-NAC variants were added in 10-fold molar excess to RNCs (NAC = 2 µM, RNC = 200 nM). Autoradiograph images are shown. The tRNA-attached nascent chain (NC-tRNA) and its crosslinks to NAC\textalpha and NAC\textbeta are indicated. Asterisk indicates Bpa position in the flexible NAC\textalpha N-terminus outside the hydrophobic pocket. (B) Coomassie gel showing all Bpa-NAC variants used in this study. Protein samples were adjusted to the same concentration (20 µM), and a control gel was run with a 1:10 dilution of the NAC samples before running the photo-crosslinking experiments. (C) Ribosome binding test of Bpa-NAC variants used in this study. Wildtype NAC without Bpa served as control. NAC variants and inactive 60S ribosomes were mixed 1:1 (0.5 µM each) followed by sucrose cushion centrifugation of ribosomes under low (100 mM) and high (400 mM) KOAc conditions. Proteins in the ribosome pellet fractions were detected by immunoblotting. (D) Photo-crosslinking of Bpa-NAC variants to stalled RNCs carrying S\textsuperscript{35}-labeled nascent chains (55 aa) with preprolactin (pPL) signal sequences (SS) of variable hydrophobicity (hy.). Autoradiograph images are shown. The positions of the tRNA-attached nascent chain (NC-tRNA) and its crosslinks to NAC\textalpha\textbeta are indicated.
Fig. S11

Signal sequences induce a conformational change in NAC. (A) Photo-crosslinking of NAC variant carrying Bpa at position 2 of the NACβ anchor (β-X2) to stalled RNCs carrying 55 aa S35-labeled nascent chains of cytosolic (GPI), mitochondrial (HSPD1) or ER substrates (HSPA5). NAC was added in 10-fold molar excess to RNCs (NAC = 2 µM and RNCs = 200 nM). Immunoblot shows crosslink between Bpa-β-X2-NAC and eL22 (β-X2 x eL22). A high exposure of the crosslink band is shown on the right. (B) Similar analysis as in (A) but with NAC variant carrying Bpa in the flexible NACα N terminus (α-V35). Immunoblot shows ER-RNC (HSPA5)-specific crosslink between Bpa-α-V35-NAC and eL19. (C) Schematic drawing of possible NAC conformations on ribosomes. Bpa positions in NAC used in (A) and (B) are indicated. Binding of the NAC globular domain to the tunnel exit site separates the flexible NACα N terminus from eL19, preventing crosslinking between α-V35-NAC and eL19 (left). Detachment of the NAC globular domain brings the NACα N terminus within reach of eL19, enabling crosslinking between α-V35-NAC and eL19 (right). The NACβ anchor stays bound in both conformations consistent with the observed RNC-independent crosslinking between Bpa-β-X2-NAC and eL22 in panel A.
Fig. S12
Representative EM-densities for the RNC$\text{SS}$•SRP•NAC. (A) Closeup views of the SRP54 NG (slate blue) and NAC$\alpha$ UBA (orange) domains. (B) Cryo-EM density of the SRP54 NG and NAC$\alpha$ UBA domain protein helices (termed $\alpha$hN2-3 and $\alpha$hU1-3) with overlaid atomic coordinates. (C) Closeup view of the NAC tail anchor (green) domain in the RNC$\text{SS}$•SRP•NAC shown with interactions with eL19 (yellow) and eL22 (blue). (D) Close-up view of the SRP54 M-domain (cyan) and the bound signal peptide. Cryo-EM densities are shown as mesh. Coordinates are shown as cartoon and colored as in main Figure 4.
Fig. S13
*Alphafold validates SRP NG and NAC UBA domains interaction as resolved in the cryo-EM complex.* Left panel: Structure prediction of the NACα and SRP54 using Alphafold multimer prediction tool, part of the alphafold2 package. Right panel: Overlay of structure prediction (pink) with SRP-NAC structure as resolved in this study.
Fig. S14
Molecular interactions between the UBA of NACα with NG domain of SRP54. (A) and (B) Close-up of the patch1 and patch2 interaction between NG (slate) and UBA (orange), respectively, shown as sticks and cartoon. Coloring is the same as in main Figure 4.
Fig. S15
Recognition mode of UBA overlaps with SRP receptor binding site on SRP. (A) Overview of the SRP54 NG domain and NACα UBA domain interaction interface shown as cartoon, coloring scheme is similar as in main figure 4. (B) Overview of the SRP54 NG-domain and the α subunit of SRP receptor (SRα) NG domain (PDB ID:6FRK) interaction interface shown as cartoon. The NG domain of SRα is colored green. Dotted orange line indicates the binding site of NAC UBA domain.
**Fig. S16**

**SRP variant interactions with SRP receptor.** Kinetics of SRP-SR association is measured by FRET between donor and acceptor dyes labeled in the NG domains of SRP54 and SRα, respectively. SRP 54mt displays similar SR interaction kinetics as SRP WT.
Fig. S17
Equilibrium titration to measure the RNC binding affinity of SRP and NAC variants that disrupt the NAC UBA-SRP54 NG interaction. (A) Equilibrium titrations to measure the binding of wildtype SRP and mutant SRP 54mt to RNC_{SS} with and without the indicated NAC complex pre-bound to the ribosome. The lines are fits of the data to Eq 2, and the obtained $K_d$ values are summarized in Fig. 4E. (B) Equilibrium titrations to measure the binding of NAC WT (black), NAC dUBA (orange), and NAC UBAmt (brown) to the RNC_{SS}-SRP complex (squares and dashed lines) and to RNC_{SS} without SRP present (circles and solid lines). The lines are fits of the data to Eq 2. (C) Summary of the $K_d$ values of WT NAC and indicated NAC variants for RNC_{SS} in the absence (grey bars) and presence (dark bars) of SRP pre-bound to the ribosome, based on the data in panel B. Error bars are the uncertainty in fitting $K_d$ to the titration data.
Fig. S18
Characterization of NAC UBA-SRP54 interactions in C. elegans. (A) Immunoblot analysis showing expression levels of FLAG-NACα variants in the endogenous NACα RNAi background of worms. Expression is driven by an integrated single-copy NACα transgene that is resistant to RNAi of the endogenous gene by alternate codon usage. UBAmt refers to SRP54 binding deficient FLAG-NACα D185R/N188R variant. (B) C. elegans strains carrying single-copy SRP54 transgenes resistant to RNAi of endogenous SRP54 were constructed similarly to NACα approach by alternative codon usage. Transgene expression was driven by the native SRP54 promoter and terminator (3′UTR). Immunoblot shows the expression levels of WT-SRP54 and mutant SRP54 deficient in NACα UBA binding (K50E/K53E, SRP54mt) in the endogenous SRP54 RNAi background. (C) Microscope images of protein secretion reporter worms used in this study. Worms express GFP fused to a signal sequence (ssGFP) under the muscle-specific myo-3 promoter and cytosolic RFP (cytoRFP) under the ubiquitous icd-2 promoter. Additionally, worms carry a loss-of-function mutation in mtm-9 inducing a coelomocyte uptake defect (cup) leading to...
accumulation of secreted ssGFP in the body cavity (see image on the right). This allows quantification of ssGFP secretion by worm flow cytometry. BF, bright field. (D) Knockdown of SRP54 and Sec61α in protein secretion reporter worms as in panel C. RNAi was performed in the young adult stage of worms for 48 hours. Fluorescence of secreted ssGFP was assessed by worm flow cytometry and normalized to cytosolic RFP. Dots indicate individual data points (n ≥ 2000). Box plot center line = median; box length = upper + lower quartile; whiskers = minimum/maximum quartile. Bottom panel: immunoblot showing knockdown efficiencies of SRP54 and Sec61α. (E) QPCR analysis of indicated stress-regulated genes in NAC UBAmt worms. Diagram shows log2-transformed ratios of mRNA levels in NAC UBAmt worms compared to wildtype NAC worms. Actin (act-1) served as housekeeping gene. cyto, cytosol; mito, mitochondria.