

# Chapter 2

## Functions of Ribosome-Associated Chaperones and their Interaction Network

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**Abstract** Cells coordinate chaperones at the exit site of the ribosome. Albeit the types and mechanisms of ribosome-associated chaperones differ in the three kingdoms of life, they all share the ability to protect nascent polypeptides from off pathways such as aggregation and degradation and, at least in some cases, support initial folding steps of newly synthesized proteins. Recent progress was made in understanding the nascent interactome of these ribosome-associated chaperones. While the bacteria-specific chaperone trigger factor (TF) binds to almost every nascent polypeptide made by ribosomes except for membrane proteins, the substrate pool of the two eukaryotic ribosome-associated chaperone systems, nascent polypeptide-associated complex (NAC) and ribosome-associated complex (RAC), is more distinct.

Interestingly, there is culminating evidence that these chaperones also display important functions off the ribosome, e.g., in the biogenesis of ribosomal subunits and in protein aggregation under proteotoxic stress conditions. In this chapter, we will discuss the functions of these chaperones with regard to their broad substrate pools.

### 1 Introduction: Ribosome-Associated Chaperones in De novo Folding

Directly upon their synthesis by the ribosome, proteins have to fold into their unique three-dimensional structure in order to become biologically active. The folding of proteins is problematic since hydrophobic residues of the unfolded polypeptide chain are accessible, which enhances the probability that the newly synthesized protein follows an unproductive off pathway leading to misfolding and aggregation [1]. As protein misfolding and aggregation represent the hallmarks of several neurodegenerative diseases, it is of particular importance to understand the mechanisms by which proteins acquire and maintain their structure under cellular conditions.

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To accomplish folding and prevent off pathways, newly synthesized polypeptides interact with at least one cytosolic chaperone that prevents inappropriate inter- and intramolecular interactions, and thus promotes the folding into the native state. Among these chaperones, the ribosome-associated ones interact with nascent polypeptides while they are still attached to the peptidyl-transferase center of the ribosome (Figs. 2.1 and 2.2a) [2–4].

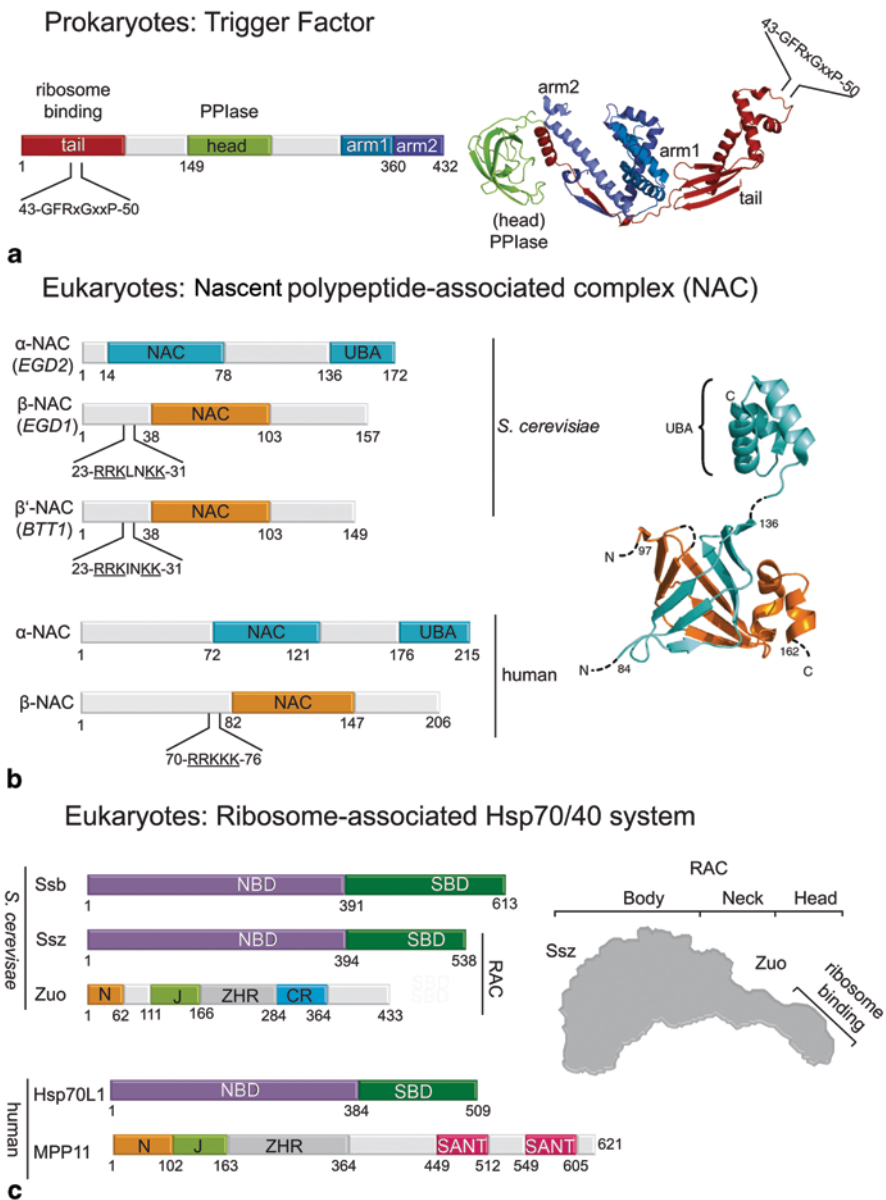
Binding of chaperones to nascent polypeptides can have several effects. The unfolded polypeptide is protected from being degraded or from incorrect contact with other molecules that may lead to aggregation. Moreover, for polypeptides that start their folding program co-translationally, chaperone binding might support very early folding. Beyond the interaction of newly synthesized proteins with ribosome-attached chaperones, additionally other cytosolic chaperones can act during later stages of translation on elongated nascent chains or after release of the polypeptides from the ribosomes. The latter mainly belong to the heat shock protein (Hsp)70/40 and the Hsp60/10 chaperone families. Examples are the DnaK/DnaJ (Hsp70/Hsp40) and GroEL/GroES (Hsp60/Hsp10) systems in the cytosol of *E. coli* cells [3, 5]. Together with ribosome-associated chaperones they form a robust network that promotes the de novo folding of newly synthesized polypeptides and prevents off pathways such as aggregation or degradation early in the life of a new protein [4].

Ribosome-associated chaperones are found in every cell but differ significantly among the different kingdoms of life with regard to their number and type. Whereas prokaryotes have only one ribosome-associated chaperone which is called trigger factor (TF) [6–9], eukaryotic ribosomes coordinate two TF-unrelated chaperone systems at the ribosome (Fig. 2.2a). These systems are the highly conserved nascent polypeptide-associated complex (NAC) and an arrangement of specialized Hsp70 and Hsp40 chaperones that includes a heterodimer called ribosome-associated complex (RAC; Fig. 2.2a) [4, 10]. In yeast, the RAC system has a third ribosome-associated partner, a Hsp70 chaperone called Ssb [11] (Fig. 2.2a).

In the following section, the functions and structures of these ribosome-associated chaperones will be discussed together with their respective substrates.

## 2 The Prokaryotic Ribosome-Associated Chaperone Trigger Factor

The only known chaperone of bacterial cells with a direct binding site on ribosomes is TF. In *E. coli*, TF consists of 432 amino acids and has a molecular weight of 48 kDa. It is a three-domain protein with an N-terminal ribosome-binding domain, a middle domain that displays peptidyl-prolyl *cis/trans* isomerase (PPIase) activity and a C-terminal domain [7, 12] (Fig. 2.1a). Its crystal structure revealed that TF adopts an extended three-dimensional conformation with an unusual domain arrangement [9]. Although distant in the amino acid sequence, the N- and C-terminal domains are located adjacent to each other in the three-dimensional structure to form a cradle-like structure (Fig. 2.1a). The C-terminal domain of TF is located in



**Fig. 2.1 a** *E. coli* trigger factor. The N-domain (red) contains the ribosome-binding motif (40-GFRxGxxP-49). It is located in a loop region between two  $\alpha$ -helices, the N-domain is connected to the PPIase domain (green) via an extended linker. The C-domain (blue) is located in the center of the folded molecule and forms two arm-like protrusions. The N-domain and both arms of the C-domain together form a cavity for nascent polypeptide chains. **b** The nascent polypeptide-associated complex (NAC). The  $\alpha$ -subunit of NAC (encoded in yeast by the *EGD2* gene) consists of a NAC-domain in the N-terminal region and an ubiquitin-associated (UBA) domain at its C-terminus. In yeast two alternative  $\beta$ -subunits ( $\beta$  and  $\beta'$ ) exist, which are either encoded by the

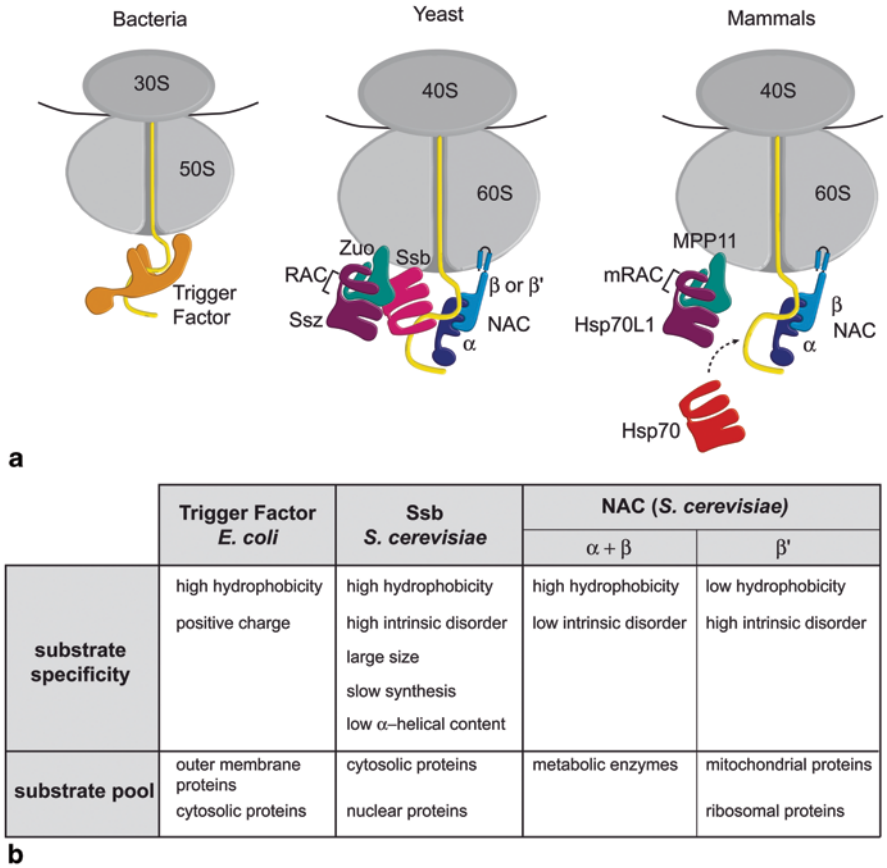
the center of the molecule and forms two arm-like protrusions [9]. The N-terminal domain contains a signature motif (42-GFRxGxxP-50) that is located in an exposed loop region [9] and binds to the ribosomal protein L23 (Fig. 2.1a). Mutation of either the signature motif or a conserved surface-exposed residue within the ribosomal protein L23 strongly impairs ribosome binding of TF and its activity on nascent polypeptides [13]. The N-terminus is connected via a long linker to the second domain, the PPIase domain, which is located at the opposite end of the molecule [9] (Fig. 2.1a). This domain catalyzes prolyl *cis/trans* isomerization in peptides in vitro and presumably slows down folding processes or acts as auxiliary site to assist the folding of substrate proteins [14–16]. However, the in vivo relevance of this domain and its enzymatic activity is still not well understood [17].

Crystallization of the N-terminal fragment of *E. coli* TF together with the 50S large ribosomal subunit from *Haloarcula marismortui* allowed the superposition of full-length TF with ribosomes and paved the way for understanding how this chaperone functions on ribosomes [9]. TF binds to the ribosomal protein L23 and hunches with its extended cradle-like structure over the ribosomal tunnel exit site (Fig. 2.2a). It was suggested that the cradle-like structure provides a shielded environment for the emerging nascent polypeptides and thus supports co-translational folding events [9]. The interior of the cavity formed by the TF N- and C-terminal domains contains hydrophobic as well as hydrophilic areas. Indeed, multiple potential substrate-binding sites within this cradle have been suggested [18, 19]. This structure might enable TF to bind a large variety of substrates.

The ribosome-binding activity of TF has been extensively characterized. It cycles on and off the ribosomes and binds as a monomer to the translation machinery [14, 20, 21]. TF is present in a two- to threefold molar excess over ribosomes and

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genes *EGD1* or *BTT1*, respectively. The  $\beta$ -subunits of NAC contain a conserved ribosome-binding motif in the N-terminus and a central NAC domain. Dimerization of the NAC subunits involves the NAC domains of both subunits.  $\alpha$ -NAC (*blue*) and  $\beta$ -NAC (*orange*) form a stable heterodimer. The UBA domain is derived from the crystal structure of archaeal NAC (PDB 1TR8) and was modeled on the human NAC domain heterodimer PDB 3LKX, aa 84–136 of  $\alpha$ -NAC (NACA) and aa 97–162 of  $\beta$ -NAC (BTF3 isoform). Broken lines indicate unresolved parts of the molecule. **c** The ribosome-associated heat shock protein (Hsp)70/40 system in eukaryotes. The yeast ribosome-associated chaperone system consists of the Hsp70s Ssb, Ssz, and the Hsp40 Zuo. Ssb1 and Ssz contain an N-terminal nucleotide-binding domain (NBD, *violet*) and a C-terminal substrate-binding domain (SBD, *dark green*). The SBD of Ssz is shorter compared to canonical Hsp70s. Like all Hsp40 co-chaperones Zuo contains a J-domain (*light green*) required for stimulation of the adenosine triphosphatase (ATPase) activity of its Hsp70 partner. In addition, Zuo contains a charged region (*blue*) in its C-terminus, which is involved in ribosome binding. The N-terminus (N, *orange*) of Zuo is predicted to be unstructured. Ssz and Zuo form the stable heterodimeric ribosome-associated complex (RAC). A schematic model of RAC based on small-angle X-ray scattering (SAXS) analysis of yeast RAC (on the *right* side, shown in *light grey*) reveals that the complex forms an elongated structure that was divided into body, neck and head. Most of the body is composed of Ssz, whereas Zuo forms the neck and the head. The head region of Zuo was shown to contact the ribosome near the tunnel exit. *Lower panel*: In contrast, in human, no Ssb homolog is found while RAC is conserved. The RAC complex is formed by the Hsp40 MPP11 and Hsp70L1. MPP11 contains two SANT domains (*pink*) at its C-terminus. The functions of the SANT domains are still unknown



**Fig. 2.2 a** Ribosome-associated chaperones bind to ribosomes (grey) in close proximity to the ribosomal tunnel exit and interact with nascent polypeptide chains (yellow). In bacteria, only trigger factor (orange) binds to the ribosome and interacts with the growing nascent polypeptide. In eukaryotes, two chaperone systems are found in association with the ribosome. On the one hand, the heterodimeric nascent polypeptide-associated complex (NAC) complex that consists of a  $\alpha$ - (dark blue) and  $\beta$ - (light blue) subunit binds to the ribosome via its  $\beta$ -subunit. Additionally, a system consisting of heat shock protein (Hsp)70 and Hsp40 family members binds to ribosomes and interacts with nascent chains. In yeast this system comprises the Hsp70 Ssb (pink) as well as the ribosome-associated complex (RAC), which is formed by the Hsp40 Zuo (green) and the Hsp70 Ssz (violet). Mammalian RAC (mRAC) is formed by the Hsp40 MPP11 and Hsp70L1. As Ssb is restricted to fungi, cytosolic Hsp70s (red) are recruited to the nascent chain by mRAC. **b** The table summarizes the co-translational substrate specificities and the substrate pool of trigger factor from *E. coli* as well as Ssb and NAC from *S. cerevisiae*. The substrate specificities of trigger factor were taken from: [36]; the substrate pool from: [27]. The co-translational interactome of Ssb was analyzed in [92] and that of NAC in [79]

thus is bound to most cytosolic ribosomes. Unbound TF diffuses freely in the cytosol, probably as a homodimer. The in vivo relevance of this dimerization is unclear but dimer formation may represent a storage form of this chaperone to prevent its

cradle from unspecific associations [22, 23]. Although TF binds to non-translating ribosomes with a  $K_D$  of approximately 1  $\mu$ M and a mean residence time of 10–15 s, the presence of a nascent chain increases the affinity of TF for ribosomes up to 30-fold [20, 24–26]. It could be shown by cross-linking studies combined with cryo-electron microscopy structures that TF interacts with very short nascent chains as soon as they emerge from the ribosomal exit site in vitro [18]. In vivo, however, recruitment of TF to ribosome-nascent chain complexes is delayed until the polypeptides reach a length of 100 amino acids [27]. TF binding to nascent substrates is controlled by processing enzymes that act first on the N-terminus of the newly synthesized protein. It was demonstrated recently that peptide deformylase associates with nascent chains as soon as they emerge from the ribosomal exit tunnel, followed by methionine aminopeptidase [28]. Both enzymes prevent the premature recruitment of TF [27, 29]. Most likely TF can also stay bound to a subset of nascent polypeptides after their release from the ribosome [30].

Initially, TF was discovered as a cytosolic protein involved in the translocation of the outer membrane protein pro-OmpA across the plasma membrane as it has the ability to promote the insertion of chemically denatured pro-OmpA into membrane vesicles [31]. However, TF is not essential for viability of *E. coli* and deletion of its gene *tig* does not cause any growth defect albeit the heat shock response is induced leading to enhanced levels of chaperones and proteases [32, 33]. First evidence that TF fulfills chaperone function in vivo came from the finding that the simultaneous deletion of TF and the cytosolic Hsp70 chaperone DnaK is synthetically lethal at temperatures above 30 °C [32, 34]. Cells with low levels of DnaK and without TF are viable but show slow growth and massive aggregation of several hundreds of cytosolic proteins, especially large-sized proteins and components of protein complexes. Interestingly, TF and DnaK show some overlap in their substrate specificity. Both chaperones interact with peptide segments of an unfolded protein that has a high mean hydrophobicity and an overall positive net charge [35, 36] (Fig. 2.2b). Taken together, these findings suggest that the cytosolic Hsp70 DnaK and the ribosome-associated chaperone TF act on a similar substrate spectrum and thus are able to cooperate in the de novo folding of newly synthesized proteins.

The mechanism by which the non-adenosine triphosphate (non-ATP)-consuming TF promotes de novo folding of newly synthesized proteins is still not fully understood. On the one hand, the cavity formed by the N- and C-terminal domains could provide a folding chamber that protects the emerging nascent chain from unfavorable interactions while translation proceeds. In agreement with these findings it could be demonstrated that TF protects nascent proteins from proteolytic digestion in vitro [37, 38]. Additionally, it was shown recently that TF stimulated native folding in constructs of repeated maltose-binding protein (MBP) domains by protecting partially folded domains from distant interactions that produce stable misfolded states [39]. On the other hand, it was proposed that TF delays the folding of a nascent polypeptide until sufficient sequence information (encoded in the C-terminal area of the synthesized polypeptide chain) is available outside of the ribosome to allow productive folding [35, 38]. Indeed, recent data suggest that TF can unfold preexisting folded states to prevent and revert premature folding, thus limiting the



formation of misfolded intermediate states during protein synthesis [8]. In the current model, first the ribosome itself limits the conformational freedom of the newly synthesized polypeptide chains. As translation proceeds and the chain lengthens the influence of the ribosome, in particular on the N-terminal regions, decreases and the risk to form misfolded intermediates increases. Therefore, TF binds to nascent polypeptides of a length of 100 amino acids in repeated binding and release cycles. It limits conformational sampling and folding more efficiently than the ribosome and thereby prevents folding intermediates [8]. TF is not only able to block folding but also unfolds preformed segments in order to give the nascent chain a new opportunity for productive folding. After release from the ribosome, some nascent chains that do not need further assistance acquire their native structure after dissociation of TF. In contrast, other proteins stay associated with TF and are transferred to downstream chaperone systems, in particular to the DnaK/DnaJ/GrpE system or to GroEL/ES.

Very recently, the nascent interactome of ribosome-bound TF was identified using an elegant approach: selective ribosome profiling [27, 40]. For this technique, actively translating ribosomes are isolated; the messenger itochondrial ribonucleic acid (mRNA) bound to them is converted into deoxyribonucleic acid (DNA) and subsequently analyzed by high-throughput sequencing. To gain insights into which nascent polypeptides are bound by TF, only those ribosomes with bound TF were isolated and the mRNA was analyzed. TF was fused with an affinity purification tag, ribosomes were isolated, TF binding to the nascent polypeptides was stabilized by cross-linking, and thus specifically the ribosome-nascent-chain complexes containing TF could be isolated by affinity purification [27].

The data revealed that the medium average length at which TF engaged a polypeptide was 112 amino acids, half of all nascent chains being bound within  $\pm 20$  amino acids of this position. Nascent chains are generally not engaged by more than one TF molecule. Furthermore, the data revealed that TF interacts with all nascent chains in *E. coli* except for those that localize to the plasma membrane (Fig. 2.2b). Membrane proteins are poorly bound by TF. Interestingly, outer membrane  $\beta$ -barrel proteins (OMPs) represent the strongest TF interactors (Fig. 2.2b). For example, the five best-characterized OMPs (LamB, LptD, OmpA, OmpC, and OmpF) were found among the 25 strongest TF-interacting nascent polypeptides. This finding is in agreement with very early data, as TF was initially discovered by its ability to promote insertion of pro-OmpA into membranes [31]. To prove the *in vivo* relevance of this finding the authors analyzed the protein content of the outer membrane fraction of wild type *E. coli* cells and compared it with those lacking TF using stable isotope labeling with amino acids in cell culture (SILAC) combined with mass spectrometry. The data revealed that in cells lacking TF, 60% of all detected proteins were found in lower amounts than in the wild type, whereas no proteins were found in higher amounts. In agreement with this result, it was shown that loss of TF causes enhanced sensitivity of *E. coli* to SDS/EDTA (sodium dodecyl sulfate/ethylenediamine tetraacetic acid) treatment or vancomycin, which suggests defects in the biogenesis of outer membrane proteins. In sum, these data clearly suggest that TF not only plays an important role in the folding of cytosolic proteins but is also crucial for the folding-competent conformation of outer membrane proteins during their biogenesis [27].

### 3 The Nascent Polypeptide-Associated Complex (NAC)

NAC is a heterodimeric complex that associates with ribosomes likely in a 1:1 stoichiometry [41, 42] (Fig. 2.2a). It is highly conserved among eukaryotes and consists of one  $\alpha$ - and one  $\beta$ -subunit, referred to as  $\alpha$ -NAC and  $\beta$ -NAC (Fig. 2.1b). NAC is not found in bacteria but in archaea, where it is built by an  $\alpha$ -NAC homodimer [43, 44].

Both subunits of heterodimeric NAC were shown to contact nascent polypeptide chains [42, 45] while only  $\beta$ -NAC interacts with the ribosome and mediates ribosome binding of the complex [43, 46] (Fig. 2.2a). NAC is present in equimolar concentration relative to ribosomes and binds to them irrespective of their translation status [41]. In yeast, NAC is encoded by three genes. The  $\alpha$ -NAC subunit is encoded by *EGD2* while *EGD1* as well as *BTT1* both encode for  $\beta$ -subunits. The gene product of *EGD1* is referred to as  $\beta$ -NAC and the one of *BTT1* as  $\beta'$ -NAC (Fig. 2.1b). Both  $\beta$ -subunits are able to form a complex with the  $\alpha$ -subunit. However, the  $\beta'$ -NAC version is approximately 100 times less expressed than the  $\beta$ -version [44, 47]. Thus, the most abundant NAC type is formed by  $\alpha\beta$  in yeast cells.

Both subunits of NAC share a homologous structural element, the so-called NAC-domain. These domains are responsible for the dimerization of NAC into the heterodimeric complex (Fig. 2.1b). The NAC-domain is located in the N-terminal part of  $\alpha$ -NAC and in the central domain of  $\beta$ -NAC. Crystal structures of parts of the human complex give insights into the molecular interactions governing NAC complex formation [48, 49]. They reveal a handshake interaction of the two NAC-domains consisting of a six-stranded  $\beta$ -barrel that is stabilized by hydrophobic contacts between conserved residues [49, 50]. Additionally,  $\alpha$ -NAC contains an ubiquitin-associated (UBA) domain [50] (Fig. 2.1b). UBA domains were described to bind mono- and polyubiquitin but also mediate other protein-protein interactions [51]. They are commonly found in factors dedicated to ubiquitin-dependent protein degradation or in components of signal transduction pathways [51–54]. However, the function of the UBA domain of  $\alpha$ -NAC remains elusive.

There is no structure available of NAC in complex with the ribosome and thus its orientation on the ribosome and relative to the exit site for nascent polypeptides is unknown. Different physical contact points for NAC are under debate. Based on cross-linking data various anchor points have been suggested: either Rpl25, the L23 homolog that coordinates TF in bacteria [55] or Rpl31 [56, 57] which is also the main interaction site for RAC (see below). Both Rpl25 and Rpl31 are located directly at the ribosomal exit site and would position NAC in a way that favors its interaction with nascent polypeptides. Previous work suggested that NAC binds to Rpl25 via a conserved motif (RRK-(X)<sub>n</sub>-KK, amino acids 23–31 located in a loop region between two  $\alpha$ -helices in the N-terminus of  $\beta$ -NAC (Fig. 2.1b). Upon mutation of the amino acid residues arginine arginine lysine (RRK) to alanine alanine alanine (AAA) ribosome binding of NAC was abolished both in vivo and in vitro [55].

Despite the evolutionary conservation of NAC in eukaryotes, its in vivo function is still rather obscure. Originally, NAC was discovered in 1994 by Wiedman and



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