Protein Quality Control during Protein Biosynthesis

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1. Summary

1.1 Summary (english version)

Directly upon their synthesis by the ribosome proteins have to fold into their unique three-dimensional structure in order to become biologically active. If the folding process fails, newly synthesized polypeptide chains are highly prone to misfold and aggregate. De novo protein folding thus imposes a major challenge for the cellular protein quality control systems. 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. To accomplish folding, the majority of nascent proteins interacts with ribosome-associated chaperones, which represent the main focus of this thesis. They assist the folding of newly synthesized proteins and prevent the formation of destructive non-native inter- or intramolecular interactions. In eukaryotic cells two different chaperone systems bind to ribosomes and support de novo folding of nascent polypeptide chains directly upon their emergence from the ribosomal tunnel. These are the nascent polypeptide-associated complex (NAC) as well as the ribosome-associated complex (RAC). In yeast, the RAC system forms a functional triad together with the Hsp70 chaperone Ssb (Figure 7).

In addition, nascent polypeptides that result from erroneous translation can be degraded at an early stage by ribosome-associated quality control factors. These include the ribosome-associated quality control complex (RQC) as well as the ubiquitin E3 protein-ligase Not4, which is part of the multifunctional Ccr4-Not assembly (Figures 14 and 15). Whereas the chaperone systems seem to act by supporting the correct folding, the latter two complexes rather prevent synthesis or degrade damaged proteins.

To date, the precise functions, the mechanisms of action and the importance for protein homeostasis of these chaperones and quality control factors remain unclear. The major aim of this study was therefore to investigate the function of ribosome-associated factors in maintaining protein homeostasis. Besides studying this in yeast, a further major task was to establish a new model organism, the nematode Caenorhabditis elegans, for these analyses in the laboratory.
A) Analysis of eukaryotic ribosome-associated protein quality control factors

(i) NAC is a key regulator of proteostasis in C. elegans
Although NAC has been studied extensively and despite its high conservation from yeast to human, the in vivo function of NAC remains largely unclear. To gain insights into the role of NAC in the proteostasis network of higher eukaryotes we used the metazoan animal model C. elegans. We observed that protein synthesis is tightly linked to the protein folding load of the cell through the functional properties of NAC. Under non-stress conditions, NAC associates with ribosomes to promote translation and de novo protein folding. When proteostasis is challenged upon proteotoxic stress, NAC relocalizes from ribosomes to protein aggregates and depletion of NAC from ribosomes reduces translation activity. Relocalization of NAC from ribosomes to aggregates was observed in animals expressing disease-relevant and highly aggregation-prone polyglutamine (PolyQ)-expansion proteins and Aβ-peptides as well as in response to heat shock and during aging. Importantly, our data indicate that NAC is also required for efficient resolubilization of protein aggregates. We were able to demonstrate that NAC has a crucial proteostatic function in metazoans and acts as a modulator of protein synthesis to establish a regulatory feedback mechanism that adjusts translational activity to the cellular protein folding load. This work contributes to this study by establishing C. elegans as model organism in the laboratory, performance and quantification of polysome profiles, isolation and analysis of ribosomes and ribosome-associated factors, preparation and analysis of protein aggregates as well as the expression and purification of NAC.

* equal contribution and shared first authorship

(ii) NAC and the Ssb-RAC system are functionally connected in yeast
In yeast, NAC as well as Ssb-RAC bind to ribosomes and interact with nascent polypeptide chains. However, little is known about the interplay of both ribosome-associated systems in de novo protein folding. We thus applied genetic and biochemical approaches to investigate their functional relationship. Simultaneous deletion of NAC and Ssb encoding genes caused conditional loss of cell viability under protein folding stress conditions. Furthermore, loss of Ssb resulted in aggregation of newly synthesized polypeptides, ribosomal proteins as well as several ribosome biogenesis factors. Likewise, the levels of translating ribosomes and 60S ribosomal subunits were decreased in ssbΔ cells. These defects were aggravated when NAC was absent in addition to Ssb and additionally halfmers, which represent uncomplexed 40S particles, accumulated. These findings indicate that ribosome biogenesis is affected by the
loss of Ssb and NAC. Taken together our data suggest that Ssb-RAC and NAC cooperate in cotranslational folding processes and may play a role in the biogenesis of ribosomes. The present work contributed this study by performance of polysome profiles, demonstrating that the observed defects and the occurrence of halfmers is specific for the loss of Ssb and does not occur by the deletion of cytosolic Hsp70 chaperones.


(iii) The Hsp70 chaperone Ssb contributes to ribosome biogenesis and interacts with aggregated proteins in vivo

The ribosome-associated chaperone triad consisting of the Hsp70 Ssb, as well as the ribosome-associated complex (RAC) binds to ribosomes in yeast. Whereas Ssb interacts with nascent polypeptide chains, RAC acts as co-chaperone for Ssb to stimulate its ATPase activity. In earlier in vitro studies it was shown that Ssb does not bind to classical Hsp70 recognition motifs. As ribosomal proteins aggregate in the absence of Ssb, we assumed that Ssb primarily acts as a chaperone for ribosomal proteins. The finding that Ssb interacts with aggregated ribosomal proteins in trans supports this assumption. Furthermore, we were able to show by size exclusion chromatography combined with mass spectrometry that Ssb binds to small peptides derived from certain ribosomal proteins. As ribosome biogenesis is hampered at an early step in the absence of Ssb, we hypothesize that the interaction between Ssb and newly synthesized ribosomal proteins is crucial for ribosome biogenesis. We also observed that Ssb interacts with aggregates formed upon heat shock and with amyloid-like polyglutamine (PolyQ) aggregates. Thus, beyond its action on ribosomes in supporting de novo folding and ribosome biogenesis, Ssb might fulfill additional proteostatic functions on misfolded proteins. Indeed, the interaction with PolyQ aggregates seems to have physiological relevance, as Ssb modulates the toxic effects of PolyQ protein expression. Taken together we identified new chaperone-substrate interactions of the non-canonical ribosome-associated Hsp70 chaperone Ssb and provide first experimental indications for stress-related functions of Ssb.

Scior A., Hanebuth A., Gümpel M., Bruderek M., Preissler S., Deuerling E. to be submitted

(iv) Formation of RAC requires interactions between the N-terminus of Zuotin with both Ssz-domains

Very little was known about the molecular architecture of RAC, which itself is formed by an unusually stable chaperone-chaperone interaction between the Hsp70 Ssz and the Hsp40
Summary

Zuotin. Using advanced biochemical methods like amide-hydrogen deuteron exchange combined with subsequent analysis by mass spectrometry we were able to demonstrate that the flexible and largely unfolded N-terminal region of Zuotin and the C-terminal part of Ssz are required for complex formation. Furthermore, dynamic changes in the J-domain of Zuotin, which mediates the contact to Ssb, were observed upon complex formation with Ssz. Taken together all data suggest that Ssz induces a conformation of Zuotin that favors the interaction with Ssb and might be crucial for the function of RAC as a co-chaperone. This work contributed to this study by mutational analysis and copurification experiments. The data revealed that the N-terminus of Zuotin is not only required but also sufficient to establish complex formation with Ssz.


(v) Not4 and the Ccr4-Not complex fulfill quality control functions on stalled ribosomes
Nascent chains that are unable to adopt their native state have to be degraded at an early time point to prevent them from aggregation. Therefore, the eukaryotic cotranslational protein quality control system comprises not only molecular chaperones but also additional factors that cooperate with the ubiquitin-proteasome system to initiate the degradation of defective nascent polypeptides. Recently, the ribosome-associated quality control complex (RQC) was identified, which plays an important role in the ubiquitination of arrested nascent polypeptide chains. In addition, the multifunctional Ccr4-Not complex was suggested to function in cotranslational degradation of nascent chains upon ribosome stalling. However, we were able to demonstrate that Not4, the E3 ubiquitin-protein ligase subunit of the Ccr4-Not complex, does not act in the degradation of arrested nascent chains but rather suppresses their expression. Whereas the E3 ligase Ltn1, which is part of the RQC, ubiquitinates arrested polypeptides to target them for degradation, Not4 acts upstream by repressing translation and regulating the levels of mRNAs that cause ribosome stalling. Loss of Not4 function provokes massive protein folding stress and aggregation, demonstrating the importance of translation quality control to sustain proteome integrity. This work complemented the study by in vivo analysis of aggregates accumulating in not4Δ through microscopic analysis using aggregation reporter constructs with fluorescent protein moieties. Furthermore, this work contributed to the analysis of mRNA levels that cause ribosome stalling by Northern Blotting.

B) Development of new in vitro protein aggregation reporters

A new method was developed to generate highly repetitive DNA sequences encoding disease-related polyglutamine (PolyQ) proteins, to investigate their aggregation. Cloning of such repetitive DNA sequences is challenging due to the lack of specific primer annealing sites. Therefore, we designed a PCR-free seamless cloning strategy that enables the assembly of highly repetitive nucleotide sequences. Using this strategy we generated DNA templates to produce proteins containing defined stretches of consecutive glutamine residues in bacteria. These proteins were successfully tested for their applicability to monitor the aggregation behavior of PolyQ proteins in vitro by filter retardation assays. During this thesis all described constructs were cloned. Moreover, expression and purification of PolyQ proteins as well as the filter retardation assay were established and performed.


* shared first authorship
1.2 Zusammenfassung (deutsche Version)

A) Analyse von eukaryotischen ribosomenassozierten Proteinqualitätskontrollfaktoren

(i) NAC ist ein Schlüsselregulator der Proteinhomöostase in C. elegans

* gleicher Beitrag und geteilte Erstautorenschaft

(ii) In Hefen bilden NAC und Ssb-RAC eine funktionelle Einheit


(iii) Das Hsp70 Chaperon Ssb spielt eine Rolle bei der Ribosomenbiogenese und interagiert in vivo mit Proteinaggregaten


Scior A., Hanebuth A., Gümpel M., Bruderek M., Preissler S., Deuerling E. fertiges Manuskript
(iv) Die Formierung von RAC beruht auf Interaktionen zwischen dem N-Terminus von Zuotin mit beiden Domänen von Ssz


(v) Not4 und der Ccr4-Not Komplex erfüllen Qualitätskontrollfunktionen an Ribosomen mit arretierten naszierenden Ketten
Naszierende Polypeptidketten die ihre native Struktur nicht erreichen können, müssen zu einem sehr frühen Zeitpunkt abgebaut werden, um ihre Aggregation zu verhindern. Daher besteht das kotranslationale eukaryotische Qualitätskontrollsystem nicht nur aus Chaperonen, sondern auch aus Faktoren, die mit dem Ubiquitin-Proteasom System kooperieren. Diese initiieren den Abbau beschädigter naszierender Proteine. Kürzlich wurde der ribosome quality control complex (RQC) identifiziert, der eine Rolle bei der Ubiquitinierung von arretierten naszierenden Ketten spielt. Es wurde vorgeschlagen, dass neben dem RQC auch der Ccr4-Not Komplex eine Funktion bei der kotranslationalen Proteindegradation erfüllt. In dieser Arbeit konnte gezeigt werden, dass Not4, die E3 Ligase des Ccr4-Not Komplexes, nicht am Abbau der naszierenden Kette beteiligt ist, sondern die Expression von defekten naszierenden Polypeptiden unterdrückt. Während die E3 Ligase des RQC, Ltn1, arretierte naszierende Ketten ubiquitiniert um sie für den Abbau zu markieren, agiert Not4 schon vorher, indem es die Translation der mRNAs reprimiert die zu arretierten naszierenden Ketten führen. Zusätzlich kontrolliert Not4 die Menge abnormaler mRNAs. Der Verlust von Not4 führt zu massivem Proteinfaltungsstress und Proteinaggregation, was unterstreicht, wie wichtig die Translationsqualitätskontrolle auf mRNA Ebene für die Aufrechterhaltung der
Proteinhomöostase ist. In dieser Arbeit wurde die Proteinaggregation in not4Δ Stämmen durch mikroskopische Analysen in vivo untersucht. Dazu wurden Aggregationsreporter genutzt die unter anderem ein fluoreszierendes Protein enthalten. Weiterhin wurde dazu beigetragen, die Menge an mRNAs, die einen Arrest der Translation verursachen, über Northern Blots zu bestimmen.


B) Entwicklung eines neuen Reporters zur in vitro Untersuchung von Proteinaggregation


* gleicher Beitrag und geteilte Erstautorenschaft
2. Introduction

2.1 Cellular protein homeostasis

Protein homeostasis or proteostasis refers to processes that control the cellular concentration, conformation, and localization of the cellular proteins to keep the proteome in a balanced and functional state (1). The so-called protein homeostasis network includes diverse pathways that control all aspects of a protein's life cycle, such as synthesis, folding, transport, modification and degradation. Therefore, the proteostasis network fulfills essential tasks to ensure cell viability. Moreover, it provides the cell with the ability to adapt to changing environmental conditions. Thereby it allows cells to defend against the fatal consequences of proteotoxic stress, which is also associated with aging and a variety of protein misfolding diseases (Figure 1). The following chapters will describe the basic principles and challenges of protein folding and how cells cope with perturbations in protein homeostasis. Additionally, the role of protein aggregation in numerous neurodegenerative diseases will be discussed.

Figure 1: The cellular proteostasis network
Arrows represent individual proteostasis pathways. All steps underlie cellular regulation and are adjusted according to the cellular conditions.
2.2 Protein synthesis by the ribosome

The central dogma of molecular biology describes the flow of the genetic information from DNA via mRNA to protein. It was first stated by Francis Crick in 1958 (2,3) and summarized by Marshall Nirenberg: “DNA makes RNA makes proteins” (4). The dogma describes that the amino acid sequence of every protein is encoded by the nucleotide sequence of cellular DNA. Upon transcription into messenger RNA (mRNA) the nucleotide sequence is converted into a linear amino acid sequence by ribosomes, in a process called translation. Therefore, the life cycle of each protein starts with its synthesis by the ribosomes (Figure 2).
Figure 2: The central dogma of molecular biology

The central dogma of molecular biology describes the flow of genetic information within a biological system. Genetic information is propagated by replication, whereby DNA molecules are copied. Information on DNA can be converted into mRNA (transcription), which serves as a template for ribosomes to synthesize proteins (translation). In eukaryotic cells replication and transcription take place in the nucleus. The mRNA is subsequently transported into the cytosol where ribosomes convert genetic information into amino acid sequences of polypeptides.

2.2.1 Structure and function of ribosomes

Ribosomes are the macromolecular machineries of the cytosol that catalyze the conversion of genetic information into the linear amino acid sequence of proteins (Figure 2). The overall structural organization and the basic mechanism by which ribosomes mediate the biogenesis of new proteins is conserved throughout all kingdoms of life. Ribosomes are composed of a large and a small subunit, which work together during the translation process. Each subunit consists of ribosomal proteins and ribosomal RNA (rRNA) (Figure 3).

Despite their similarities, prokaryotic and eukaryotic ribosomes differ significantly in size and complexity (5). The prokaryotic 70S ribosome has a molecular weight of approximately 2.5 MDa and consists of a small 30S subunit and a large 50S subunit. In total it is composed of three rRNA molecules (5S, 23S and 16S) and 55 ribosomal proteins (Figure 3). By contrast, the eukaryotic 80S ribosome is substantially larger (MW ~ 4.2 MDa) and contains four rRNAs (5S, 28S, 5.8S and 18S) and approximately 82 ribosomal proteins. It consists of a small 40S subunit and a large 60S subunit (6,7). The number of ribosomes per cell and the translation rates also differ between bacteria and eukaryotes. A bacterial cell contains up to $10^5$ ribosomes. In contrast the number of ribosomes in eukaryotes is dependent on the cell type and synthetic activity and can reach numbers of up to $10^7$ ribosomes in rapidly dividing mammalian cells (6,8,9). Prokaryotic ribosomes can incorporate 15-20 amino acids per second into growing nascent polypeptides whereas translation in higher eukaryotes typically proceeds at rates of 5-7 amino acids per second (8).

Interestingly, in a growing yeast cell, rRNA and ribosomal proteins represent >80% of the total RNA and 30–50% of total protein, respectively (10,11) and ribosomes constitute up to 40% of the dry mass of *Escherichia coli* cells grown under favorable conditions (12).
Introduction

Figure 3: Structure of the bacterial 70S ribosome

A) Surface representation of the *E. coli* ribosome. The rRNA of the small 30S subunit is illustrated in light grey and the rRNA of the 50S large subunit in dark grey, whereas the proteins of the small subunit are shown in yellow and the ones of the large subunit in blue spheres. The figure was generated using PyMOL (composite of PDB entries 2AVY and 2AW4). B) Cryo-EM reconstitution of the translating 70S ribosome. The peptidyltransferase center that catalyzes peptide bond formation is located at the interface between the small (yellow) and large (blue) ribosomal subunit. Three tRNA molecules bind to the A-site (red), P-site (green) and E-site (orange) the mRNA (purple) and deliver the individual amino acids that form the growing nascent polypeptide chain (yellow spheres). A cross section of the 50S subunit (yellow) is shown to illustrate the passage of the nascent chain through the ribosomal tunnel. The mRNA as well as the nascent polypeptide chain were modeled into the structure. Figure modified from (13).

Due to their size and complexity it was for a long time challenging to obtain structural information on the molecular architecture of ribosomes with resolution high enough to reveal the molecular details, which are essential for the catalytic mechanism of protein synthesis. However, in the past decade several crystal structures and electron microscopy reconstructions of pro- and eukaryotic ribosomes in complex with diverse translation factors, have dramatically increased our understanding of the translation process (14-16). The first crystal structures of ribosomal particles were obtained from the 50S subunit of the archaeon *Haloarcula marismortui* (17) and the bacterium *Deinococcus radiodurans* (18), as well as the 30S subunit of the bacterium *Thermus thermophilus* (19) in the years 2000 and 2001, respectively. Meanwhile the number of crystal structures increased and structures for the entire prokaryotic (20,21) as well as eukaryotic (22) ribosome from famous model organisms are available.
The basic principles of translation are conserved throughout all kingdoms of life. During translation the mRNA molecules bind to the small ribosomal subunit, where the decoding by the tRNAs takes place. In contrast, the large subunit harbors the catalytically active site of the ribosome, the peptidyl transferase center (PTC). The PTC catalyzes peptide bond formation between amino acids to form the growing polypeptide chain. The large subunit additionally provides the ribosomal tunnel through which the nascent proteins leave the ribosome into the cytosol (Figure 3). The active sites of the PTC are located close to the interface between the ribosomal subunits and are predominantly composed of RNA. Therefore, the ribosome is often considered as a ribozyme (23).

The entire translation process depends on additional non-ribosomal factors and can be divided into three different steps: initiation, elongation and termination. During initiation the small and the large subunit join at the start codon of the mRNA with the help of translation initiation factors to form a translation competent ribosome. The decoding center of the assembled ribosome contains three distinct tRNA binding sites, the A (aminoacyl), P (peptidyl) and the E (exit) site (Figure 3). The first aminoacylated tRNA binds to the start codon, which is located at the P site. Afterwards the second aminoacyl-tRNA enters the ribosome and binds to subsequent codon at the A-site. While the PTC catalyzes peptide bond formation between the two adjacent amino acids the A-site tRNA is translocated to the P-site and the P-site tRNA is moved to the E-site from which it can leave the ribosome. Thus, the A-site becomes available to allow binding of another aminoacyl-tRNA to the next codon. Repeated cycles of tRNA binding, peptide bond formation and translocation are called elongation and lead to the growth of the nascent polypeptide chain. Each elongation step requires trans-acting elongation factors and both subunits undergo dynamic conformational changes that ensure translocation of the bound mRNA by exactly a single codon. When the ribosome reaches the end of an open reading frame termination factors recognize the stop codon and catalyze the release of the nascent polypeptide chain from the peptidyl-tRNA and the ribosome (14,16,24).

Although the basic steps of translation are universally conserved, the trans-acting factors involved in this process as well as regulatory mechanisms differs significantly between eukaryotes and prokaryotes. Eukaryotic translation requires not only a larger number of initiation and elongation factors compared to their prokaryotic counterparts but also regulation of translation activity and the biogenesis of ribosomal subunits is more complex (5).
2.2.2 The ribosomal tunnel as functional environment for nascent polypeptide chains

After their synthesis at the PTC growing nascent polypeptide chains leave the ribosome through a tunnel that is located in the large ribosomal subunit and connects the PTC with the cytosol (Figure 3). Structural data revealed that the ribosomal tunnel is predominantly composed of rRNA (21,25-27). Additionally, extensions of ribosomal proteins form parts of the tunnel wall. In bacteria, protrusions of the ribosomal proteins L4 and L22 reach into lumen of the tunnel and form so-called “constriction points”, where the tunnel narrows (25). For many years after its discovery, the tunnel was assumed to act only as a passive conduit for nascent polypeptide chains (28). Meanwhile, however, there is substantial evidence that the at least some nascent chains undergo specific interactions with the tunnel on their way out of the ribosome and that these interactions can influence the folding of the growing peptide as well as the translation process. The exit tunnel has a length of about 80-100 Å and its diameter varies between 10 and 20 Å (25). These dimensions allow the accommodation of peptides of a length of 30 amino acids in an extended conformation or α-helices composed of up to 60 amino acids (29-31). As the diameter of the ribosomal tunnel is only 20 Å at its widest point, structure acquisition of nascent proteins inside the ribosome is assumed to be limited to the formation of α-helical elements (25). Indeed, cryo-EM data (32) as well as elaborate biochemical and biophysical analyses (33,34) confirmed the formation of α-helices inside the tunnel. So far, however, α-helical conformations of nascent polypeptide chains were only detected in the proximal and distal parts of the tunnel near the PTC and the exit site, respectively, but not in its constricted central regions (32,33,35-37).

A number of leader peptides encoded by short upstream open reading frames in the 5’ untranslated regions of certain mRNAs have been identified, which interact strongly with the tunnel wall during their synthesis and thereby induce transient ribosome stalling to regulate the protein expression. For example, translation of the bacterial protein SecM causes ribosome stalling that leads to elevated translation of the down-stream secA open reading frame on the same mRNA. In this case, the stalled ribosome induces a conformational change of the mRNA that exposes the shine dalagarno sequence of secA and thereby facilitates translation initiation (38-40). Ribosome stalling also exists in eukaryotes, although the stalling events are much more transient compared to bacteria. For example expression of elongated stretches of consecutive positively charged amino acids (e.g. poly-lysine or poly-arginine) induces transient pausing of eukaryotic ribosomes that might be explained by electrostatic interactions between the positively charged polypeptide and the negatively charged rRNA of the ribosomal tunnel wall (41,42). By using a novel technique called ribosome profiling, a recent study showed that the presence of a sequence encoding at least eight consecutive lysine residues within an open reading frame leads to enhanced
occupancy of the mRNA with ribosomes (43). This suggests that the charge distribution within nascent chains has a strong influence on translation rates. Additionally, it was shown that certain nascent polypeptides communicate with the A-site of the ribosome to restrict its ability to catalyze peptide bond formation and thereby cause translation arrest (44). These examples clearly demonstrate that nascent polypeptides are able to control the translation process by interacting with the ribosomal tunnel wall. Thus, the ribosomal tunnel is a key element for regulation of translation.

2.3. Cotranslational modifications of nascent polypeptides

Already during its synthesis important decisions about the fate of the newly synthesized protein have to be made. The first crucial decision addresses the destination of the protein. A large set of proteins becomes cotranslationally incorporated into or translocated across membranes. This is facilitated by targeting factors such as the signal recognition particle (SRP) (45). Moreover, proteins need to fold into defined three-dimensional structures and often have to be enzymatically modified to become biologically active. All processes start already during the synthesis of the proteins by factors that directly bind to the ribosome (Figure 4). Thus, several factors that support cotranslational folding and catalyze covalent modifications are able to bind directly to ribosomes. Additionally, in eukaryotes, nascent polypeptides can become ubiquitinated and targeted for degradation during their synthesis by ribosome-associated protein quality control systems (Figure 4) (43,46,47).
Introduction

Figure 4: Cotranslational processes on nascent polypeptide chains.
Different proteins bind to translating ribosomes (grey) and interact with nascent polypeptide chains (yellow) to initiate downstream process such as covalent modification, transport, folding or degradation.

# Deformylation occurs only in prokaryotes.
* In contrast, the ubiquitin-proteasome system exists only in eukaryotes and myrisotylation is restricted to some eukaryotic and viral proteins.

The translation of almost all mRNAs starts with the universal start codon AUG, which encodes for the amino acid methionine. As a consequence nascent polypeptides typically contain a methionine residue at their N-terminus. Often the removal of this methionine is the first proteolytic event for a large number of polypeptides. The proteins that catalyze the N-terminal excision of methionine are called methionine aminopeptides (MAPs).

In bacteria translation starts with a specialized initiator tRNA that is charged with formylmethionine. N-terminal formylation is assumed to block the reactive amino group of the methionine residue in order to prevent unfavorable side reactions and to enhance the efficiency of the translation initiation. After initiation the formyl group is removed by the bacterial enzyme peptide deformylase (PDF). This excision step is essential for the subsequent action of MAP that removes the N-terminal methionine (48). The PDF from E. coli was shown to bind to the ribosome via a C-terminal helical extension in close proximity to the ribosomal tunnel exit in a groove between the ribosomal proteins L22 and L32.

| Enzymes                      | Targeting factors | Chaperones | Ubiquitin proteasome system *
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**Figure 4: Cotranslational processes on nascent polypeptide chains.**

Different proteins bind to translating ribosomes (grey) and interact with nascent polypeptide chains (yellow) to initiate downstream process such as covalent modification, transport, folding or degradation.

# Deformylation occurs only in prokaryotes.
* In contrast, the ubiquitin-proteasome system exists only in eukaryotes and myrisotylation is restricted to some eukaryotic and viral proteins.
The third type of covalent modification of nascent polypeptides is N-terminal acetylation (N-acetylation). Whereas the vast majority of eukaryotic proteins become cotranslationally acetylated, this modification is very rare in prokaryotes. It was reported that 80-90% of all mammalian and 50% of the yeast proteins become N-acetylated (49). However, the in vivo function of acetylation is largely unknown (50,51). A recent study suggested that N-terminal acetylation might play a role in protein quality control and creates a specific degradation signal (AcN-degron) for the ubiquitin-proteasome system (52). Finally also myristoylation of N-terminal glycine residues was suggest to occur cotranslationally on a few eukaryotic proteins. This modification is catalyzed by N-myristoyltransferases (NMTs). However, mechanistic details are lacking and no direct evidence for an interaction between NMTs and ribosomes was reported so far (53-56).

2.3.1 Folding of newly synthesized proteins

Newly synthesized proteins need to fold in their native three-dimensional structure in order to become biologically active. Folding is initiated cotranslationally and supported by chaperones that directly interact with the translation machinery and contact nascent chains as soon as they emerge from the ribosomal exit site. As this work focuses on cotranslational protein quality control mechanisms, the following chapters will provide a detailed overview about de novo protein folding and the factors involved in this process.

2.4 Protein folding

The native three-dimensional structure of a protein is based on intramolecular interactions within the peptide backbone and its amino acid side chains. Fundamental insights into protein folding came from pioneering in vitro experiments of Christian Anfinsen showing that fully denatured proteins can fold back spontaneously into their native state upon removal of the denaturant in the absence of an energy source or other components. This indicates that all necessary information that specifies a proteins native structure is contained in its linear amino acid sequence (Anfinsen’s dogma) (57). Although the principles of protein folding have been studied extensively for more than 50 years, the precise physicochemical mechanisms that govern the folding process are not yet fully understood. In the recent past it became also clear that protein folding in complex biological mixtures such as the cytosol is much more complicated than folding of isolated proteins under diluted in vitro conditions. Therefore, a
large fraction of newly synthesized proteins needs the assistance of molecular chaperones to fold correctly.

A polypeptide can adopt a tremendous number of theoretical conformations on its folding pathway. It was calculated that a polypeptide consisting of only 150 amino acids could adopt \(2^{150}\) possible conformations. Under the assumption that such a protein would sample each of these conformations during the folding process and the transition between two conformations would take \(10^{-13}\) seconds, it would take \(10^{24}\) years for the protein to acquire the native conformation. However, proteins fold typically within a few seconds or even microseconds (58-61). This discrepancy between theory and experimental observation is described by the so-called Levinthals paradox. It states that a protein would never reach its native structure if it would explore its entire conformational space and supports the idea that driving forces must exist to facilitate folding of a polypeptide into its native state (62,63).

In most cases the folding of a protein from an unfolded linear polypeptide chain into the native three-dimensional structure is not a one-step transition but rather a sequential process. This means that proteins fold in a funnel-shaped energy landscape along a pathway of defined intermediates until they reach their low energy state in the native structure (Figure 5) (64-66). Even some small proteins that acquire their native state on a subsecond timescale were shown to form folding intermediates (67). Such intermediates can represent either stepping-stones on the way to the native state or kinetically stable off-pathway conformations that require structural reorganization to continue productive folding (68). In aqueous solutions water represents the main driving force for a protein to fold (69,70). Typically, as a first step a fast hydrophobic collapse of the unfolded polypeptide results in a compact intermediate, or molten globule, that harbors native-like secondary structures like α-helices or β-sheets, but lacks well-packed side chains or a defined tertiary structure (71). These intermediates are relatively instable and are especially prone to misfold or aggregate as they often expose hydrophobic and disordered regions, which are mostly buried within the interior of a natively folded protein. Thus, most proteins have to overcome an energetic bottleneck situation to exit partially folded transition states upon which multiple structure elements condense against each other to form the native tertiary structure (72-74).
Introduction

Figure 5: On- and off-pathways during protein folding

Scheme of the funnel-shaped free-energy landscape that proteins explore as they fold into the native state by forming intramolecular contacts. The ruggedness of the free-energy landscape can trap protein conformations that need to overcome free-energy barriers to return to a productive downhill folding path. *In vivo*, these transition steps between on- and off-pathway conformations are accelerated by molecular chaperones. When several molecules fold simultaneously in the same compartment, the free-energy surface of folding may overlap with that of intermolecular aggregation, resulting in the formation of amorphous aggregates, toxic oligomers or ordered amyloid fibrils. Fibrillar aggregation may result from intermediates populated during *de novo* folding or after destabilization of the native state or partially folded states and is prevented by molecular chaperones. Figure taken from: (75)

2.4.1 *De novo* folding under cellular conditions

Our current knowledge about the driving-forces of protein folding is mainly based on results from *in vitro* refolding experiments. Such experiments, however, were usually performed under diluted conditions where protein concentrations are low and which differ substantially from conditions in cells. One fundamental difference is that *in vivo* protein folding occurs in the presence of high concentrations of other proteins and organic molecules such as DNA and RNA (300-400 mg/ml) (76,77). This so-called molecular crowding results in excluded volume effects that strongly enhance the affinities between molecules as well as unfavorable intra- and intermolecular interactions of partially folded proteins (78). Thus, protein folding in
a crowded environment such as the cytosol increases the risk of misfolding and aggregation (79). Additionally, whereas the entire sequence information for correct folding is available during refolding of a mature protein from the denatured state, this holds not true for de novo folding of newly synthesized proteins because translation by the ribosome is a strictly vectorial process that proceeds from the N- towards the C-terminus of a polypeptide chain. Therefore, during synthesis the protein sequence is incomplete, which prevents native folding. Since translation is relatively slow (20-80 seconds for a 400 amino acid protein), nascent polypeptide chains expose hydrophobic elements for a prolonged period of time (80). Such elements are highly aggregation-sensitive as they provide an surface for aberrant interactions. To overcome these constraints, cells have evolved a variety of strategies, including folding catalysts and molecular chaperones, that prevent folding errors and keep newly synthesized proteins on the correct folding pathway (75).

2.4.2 De novo protein folding models

As motioned above, the availability of only limited sequence information is a major problem in the folding process of newly synthesized proteins in vivo. Therefore, it was for a long time under debate whether folding can start cotranslationally and different models for de novo folding have been proposed (Figure 6). In the posttranslational folding model newly synthesized polypeptide chains stay unfolded during their synthesis and start to fold into the native state after release from the ribosome. In contrast, the cotranslational folding model suggests that nascent chains form intermediate folding states on the ribosome as soon as enough sequence information becomes available outside the ribosome. The third model proposes a cotranslational domainwise folding mode. In this case an individual folding unit, or domain, remains unfolded until its entire sequence is exposed for productive structure formation. The subsequent domains would then fold in the same way (Figure 6). This model is especially attractive for large multi-domain proteins with complex architectures (81).
Figure 6: Theoretical models for de novo protein folding

The posttranslational folding model states that polypeptides remain unfolded during their synthesis and fold into their native conformation upon release from the ribosome. In contrast, proteins could fold cotranslationally by forming intermediate structures as soon as sufficient sequence information becomes available outside the ribosome. The third model suggests that cotranslational folding of multidomain proteins occurs in a domainwise manner. In this case, an individual domain remains unfolded until its entire sequence is exposed to allow productive structure formation. The subsequent domain folds likewise. Figure modified from: (82)

It was postulated that protein synthesis without folding is energetically unfavorable as the conformational space and the free energy of the growing polypeptide chain increases continuously with ongoing translation (83,84). Meanwhile there is also increasing experimental evidence that nascent polypeptides start to fold cotranslationally on ribosomes. Different studies, for example, probed the structure acquisition of nascent polypeptides by biochemical methods such as limited proteolysis, analysis of correct disulfide formation or with conformation-specific antibodies, and found that nascent chains can adopt compact folds while still attached to the ribosome (85-88). Additionally, full-length enzymes exposed as nascent polypeptides display enzymatic activity (89,90). This finding underlines that proteins can adopt their native and biologically active conformation while being attached to
the ribosome. For some proteins it could be shown that cotranslational folding is faster (91) and more efficient compared to refolding (92,93). Taken together, all these data suggest a strong preference of many proteins for the cotranslational folding mode. However, it was for a long time challenging to obtain structural insights into the folding of nascent polypeptides due to the lack of adequate methods to detect transient folding intermediates. The development of ultra-fast nuclear magnetic resonance (NMR) techniques allowed recently to study the conformation of nascent chains at the atomic level. With this method the cotranslational folding pathway of the sarc-homology 3 (SH3) domain from ß-spectrin was analyzed and revealed that SH3 folds in a domainwise manner without populating folding intermediates (94).

2.4.3 Cellular strategies to support protein folding

As mentioned above, proteins are confronted with the constant risk to misfold and to aggregate in the crowded environment of the cytosol. The previous chapters mainly focused on the problems of de novo protein folding. However, also mature proteins unfold and refold repeatedly throughout their lifetime. The reason for this is that proteins must retain conformational flexibility in order to perform their functions. Thus proteins were optimized during evolution to be only marginally stable under physiological conditions. As a consequence, the energy barriers between native and non-native conformations are usually small (68,95) and proteins tend to unfold especially when physical parameters deviate from the respective optimum. This supports the idea that factors must be present, which help to maintain the proteome in a native and functional state even under changing conditions (Figure 5) (72,75). Indeed, an arsenal of different proteins, such as folding enzymes and molecular chaperones, have been identified that support folding processes in vivo (Figure 5). Importantly, these factors are neither present in the final native structure of the protein nor do they provide steric information for folding. In contrast, they act in kinetic partitioning between productive folding and aggregation to improve the yield of native protein (75).

Folding enzymes that catalyze rate-limiting steps in the folding pathway of a protein. Examples for folding enzymes are peptidy-prolyl isomerases (PPIases) and protein disulfide isomerases (PDIs). The latter ones act in the secretory pathway and promote formation and remodeling of disulfide bonds between cysteine residues within proteins. This accelerates establishment of the correct intra- and intermolecular disulfide bond arrangement during folding (96).
PPIases are enzymes that interconvert the cis and trans isomers of peptide bonds N-terminal from proline residues. Most amino acids have a strong energetic preference for the trans peptide bond conformation. Due to its unusual cyclic structure, however, proline also stabilizes the cis form so that both isomers are populated under biological conditions, which limits protein folding kinetics. PPIases thus accelerate protein folding by catalyzing the rapid cis-trans isomerization of peptide bonds (97-99).

Folding enzymes only act on a subset of polypeptides. In contrast, all polypeptides are potential substrates of molecular chaperones. A molecular chaperone is defined as a protein that interacts, stabilizes or helps a non-native protein to adopt its native conformation, but that is not part of the final functional structure (100). Cells are equipped with several types of molecular chaperones to maintain protein homeostasis. They play not only essential roles in de novo protein folding and refolding of stress-denatured proteins but also perform a broad range of other cellular functions including intracellular transport, assembly of oligomeric complexes and are involved in the proteolytic degradation of terminally misfolded proteins (101). Therefore, it seems likely that the fundamental requirement for molecular chaperones arose very early during evolution in order to support protein folding and minimize aggregation in the crowded environment of cells and to maintain proteins in a soluble and yet conformationally dynamic state (75).

2.5 Molecular chaperones in protein folding

Laskey et al. used the term molecular chaperone for the first time to describe the nuclear protein nucleoplasmin that is required for the assembly of nucleosomes (102). In 1987, however, John Ellis proposed the term molecular chaperones “to describe a class of cellular proteins whose function is to ensure that the folding of certain other polypeptide chains and their assembly into oligomeric structures occurs correctly” (103).

The majority of chaperones belong to the class of heat shock protein (Hsps). Hsps are transcriptionally upregulated in response to a variety of stress conditions such as elevated temperatures, exposure to toxins, starvation, and hypoxia. The heat shock response is an evolutionary conserved and universal reaction of cells to stress and results in the upregulation of a variety of genes involved in maintenance of protein homeostasis. Amongst the Hsps, however, molecular chaperones are the only members that show high conservation between distantly related species (104,105).

Meanwhile it is clear that the action of chaperones is not only required as reaction to stress but that chaperones also fulfill essential function under physiological conditions (106,107). They are present in all living organisms and in all cellular compartments of eukaryotic cells.
Chaperones can be subdivided into highly conserved families according to their approximate molecular weight in small Hsps (sHsps), Hsp40s, Hsp60s (chaperonins), Hsp70s, Hsp90s and Hsp110 (108,109). In addition, less conserved and species-specific chaperones exist that do not belong to any of these families. Important examples are found amongst chaperones that bind to ribosomes and interact with nascent polypeptide chains, such as the bacterial Trigger Factor (TF) or the nascent polypeptide-associated complex (NAC), which is only present in archaea and eukaryotes (110) (Figure 7). They may originate from evolutionary optimization of ribosome-associated chaperone systems to match the kingdom-specific differences in protein synthesis and the different demands for cotranslational folding support.

Although many chaperones are up-regulated in response to stress, that causes an imbalance of protein homoeostasis such as heat stress, they are also expressed at basal levels under normal conditions (72). Additionally, cells contain a set of chaperones, which are not upregulated upon stress (e.g. Hsc70s and Hsc90s) and fulfill housekeeping functions (110,111). This indicates a constant need for chaperones that support for example de novo folding of proteins or aid in refolding of spontaneously denatured proteins.

A general feature of all chaperones is that they interact with unfolded proteins (75,82). Therefore, the question arises how chaperones are able to discriminate between native and non-native protein species. Unfolded proteins usually expose hydrophobic amino acid stretches that are buried in in the interior of the native structure. Thus, molecular chaperones are supposed to recognize such hydrophobic elements in non-native polypeptides (112). The binding to these aggregation-prone elements prevents aggregation and supports folding into the native state (113). Whereas all chaperones are able to prevent aggregation by shielding aggregation-prone regions of client proteins, only chaperones that consume chemical energy can resolve inappropriate interactions. Therefore, chaperones can be classified according to their mode of action in so-called “foldases” and “holdases” (48,114). Foldases such as the Hsp70s, Hsp90s and the Hsp60s, are multicomponent molecular machines that promote folding by ATP- and cofactor-regulated substrate binding and release cycles (115). In contrast, holdases like sHsps or Hsp40s bind their substrates independent of ATP. Their main function is to prevent aggregation, by shielding aggregation prone elements that are exposed in unfolded proteins. (116,117).

Importantly, in order to maintain protein homeostasis, the different chaperone classes work together in complex chaperone networks (118). For example, the cooperation between Hsp100s, Hsp70s, Hsp40s and sHsp is required for efficient disaggregation of protein aggregates (119,120).
2.6 Ribosome-associated chaperones

During *de novo* protein folding different cytosolic chaperones act at distinct steps of protein biogenesis to promote the folding of newly synthesized polypeptide chains into native and functional protein (110). Among them, the ribosome-associated chaperones interact with nascent polypeptides while they are still attached to the peptidyl-transferase center of the ribosome (Figure 7) (48,107). Thereby, they are assumed to support very early cotranslational folding events. Those chaperones that do not bind to ribosomes, act during late stages of translation on elongated nascent chains or after release of the polypeptides from the ribosomes. They mainly belong to the Hsp70/40 and the Hsp60/10 chaperone families. Examples are DnaK/J (Hsp70/40) and GroEL/ES (Hsp60/10) systems in the cytosol of *E. coli* cells. (Figure 9) (82). Together these two groups of cytosolic chaperones form a chaperone network that enables the *de novo* folding of newly synthesized polypeptides (101). Whereas prokaryotes have only one ribosome-associated chaperone, which is called Trigger Factor (121-124) (Figure 7A) the ribosome-tethered chaperone system of eukaryotes is more versatile (Figure 7B+C). Here, two chaperone complexes are found in association with the ribosome. On the one hand, the highly conserved nascent polypeptide-associated complex (NAC) binds to ribosomes and contacts nascent chains. On the other hand, a chaperon system consisting of specialized Hsp70 and Hsp40 family members associates with ribosomes and supports *de novo* folding of nascent chains (Figure 7 B+C) (110). The latter system varies in its composition amongst different eukaryotic species. In the following section ribosome-associated chaperons of eukaryotic cells will be discussed in more detail.
**Figure 7: Ribosome-associated chaperones in pro- and eukaryotic cells**

Ribosome-associated chaperones bind to ribosomes (grey) in close proximity to the ribosomal tunnel exit site and interact with nascent polypeptide chains (yellow). A) In bacteria only Trigger factor (orange) binds to the ribosome and interacts with the growing nascent polypeptide chain. B) In eukaryotes two chaperone systems are found in association with the ribosome. On the one hand the highly conserved heterodimeric NAC complex, that consists of an α- (dark blue) and β- (light blue) subunit binds to the ribosome via its β-subunit. Additionally, a system consisting of Hsp family members binds to ribosomes and interacts with nascent chains. In yeast this system compromises the Hsp70 Ssb (pink) as well as the RAC complex, which is formed by the Hsp40 Zuotin (light green) and the Hsp70 Ssz (violet). C) 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. Figure modified from: (110)

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**2.6.1 The nascent polypeptide-associated complex (NAC)**

NAC is heterodimeric complex that associates with ribosomes in a 1:1 stoichiometry (125,126). It is highly conserved among eukaryotes and consists of one α- and one β-subunit, referred to as α-NAC and β-NAC (127,128). Whereas both subunits were shown to contact nascent polypeptide chains (125,129), only β-NAC interacts with the ribosome and mediates ribosome-binding of the complex (Figure 7+8) (127,130). NAC is present in equimolar concentration relative to ribosomes and binds to them irrespective of their translation status (126). In yeast NAC is encoded by three genes. The α-NAC subunit is encoded by *EGD2* while *EGD1* as well as *BTT1* encode both for β-subunits (Figure 8). The gene product of the *EGD1* is referred to as β-NAC and the one of *BTT1* as β’-NAC. Both β-subunits are able to form a complex with the α subunit. However, the β’-NAC version is approximately 100 times less expressed compared to the β version (128,131). Thus, the most abundant NAC is formed by α β in yeast cells.
Figure 8: The nascent polypeptide-associated complex (NAC)
Schematic representation of the conserved domains in yeast (S. cerevisiae) and C. elegans NAC. A) The α-subunit of NAC consists of a NAC domain (green) as well as an ubiquitin-associated (UBA) domain (blue) at its C-terminus. In yeast two alternative β-subunits (β and β’) exist, which are either encoded by the genes EGD1 or BTT1, respectively. β-NAC as well as β’-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. B) In C. elegans the β-NAC subunit is encoded by the gene icd-1 and the α-subunit by icd-2. Also here the conserved ribosome-binding motif is found in the N-terminal part of β-NAC. Figure modified from: (110)

Crystal structures of human NAC give insights into the molecular interactions governing NAC complex formation (132,133). They revealed a handshake interaction of the two NAC domains consisting of a six-stranded β-barrel that is stabilized by hydrophobic contacts between conserved residues (Figure 9) (132,134). Additionally, α-NAC contains an ubiquitin-associated (UBA) domain of still unknown function at its C-terminus (Figure 8+9) (134). UBA domains were described to bind mono- and polyubiquitin but also mediate other protein-protein interactions (135). They are commonly found in factors dedicated to ubiquitin-dependent protein degradation or in components of signal transduction pathways (135-138). However, the function of the UBA domain of α-NAC remains elusive.
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Figure 9: Structural model of NAC

α-NAC (blue) and β-NAC (orange) form a stable heterodimer via their NAC domains. The UBA domain is derived from the crystal structure of archaeal NAC (PDB 1TR8) and was modeled on the human NAC domain heterodimer [PDB 3LKK, aa 84–136 of α-NAC (NACA) and aa 97–162 of β-NAC (BTF3) isoform. Broken lines indicate unresolved parts of the molecule. Figure taken from (110)

The ribosomal contact point for NAC is still under debate. Based on crosslinking data different studies suggested either Rpl25 (139) or Rpl31 (140,141) as the main interaction site. However, structural data for NAC bound to the ribosome are still not available. Both Rpl25 as well as Rpl31 are located directly at the ribosomal exit site and would position NAC in a way that favors its interaction with nascent polypeptides (22,142). Previous work suggested that NAC binds to Rpl25 via a conserved motif (RRK-(X)n-KK) located in a loop region between two α-helices in the N-terminus of β-NAC (Figure 8). Upon mutation of the RRK (arginine arginine lysine) residues to AAA (alanine alanine alanine) ribosome-binding of NAC was completely abolished (139). In addition, NAC was suggested to be ubiquitinated by the E3 ubiquitin-protein ligase Not4 and that ubiquitination of NAC is required for the stability of the complex and ribosome binding (143,144). However, whether ubiquitination of NAC is important for its in vivo function remains unclear.

Despite the high conservation of NAC, its in vivo function remains unclear. Originally, NAC was described as cytosolic complex that interacts with nascent polypeptides in order to prevent their mislocalization to the endoplasmic reticulum (ER) (125,145-151). Another study reported a correlation between altered NAC levels and neurodegenerative diseases (152). Additionally, β-NAC was described as a target of caspases (153) and an apoptosis-suppressing activity of NAC was reported (154). Moreover, functions as a transcriptional regulator were described for NAC subunits (155-158). The individual or combined deletion of the NAC encoding genes has no significant effect on
the growth or viability of yeast cells. However, a β-NAC knockout strain (egd1Δbtt1Δ), where only the α-NAC subunit is expressed, shows a growth defect at 37°C (128). Nevertheless, the embryonic lethality of NAC mutants in C. elegans (154), D. melanogaster (159) and mice (160) indicates that this complex fulfills essential functions in higher eukaryotes.

Since NAC binds to ribosomes and interacts with nascent polypeptides, a chaperone-like function of NAC was proposed (8,82,107,110,161). First evidence for a chaperone role for NAC were obtained by a recent study showing that NAC cooperates with the Ssb-RAC system in yeast (162). We were able to demonstrate that sensitivity to several drugs, aggregation of newly synthesized polypeptides and ribosomal proteins as well as defects in translation and ribosome assembly were aggravated when NAC was absent in addition to Ssb ((162), chapter 4.3).

Another study by Del Alamo et al. identified the nascent interactome of NAC in yeast (163). To do so, they expressed individual Tandem-Affinity-Purification (TAP) tagged NAC subunits to purify the ribosome-nascent chain complexes (RNCs) together with the corresponding mRNA that are in association with NAC. The mRNAs were identified by DNA microarray hybridization. Using this strategy, the authors were able to show that the different subunits of NAC interact with distinct sets of nascent polypeptides, but also that virtually every nascent protein contacts at least one NAC subunits. Thus, every polypeptide that is translated in a yeast cell may interact cotranslationally with NAC. According to these data β'-NAC associates with ribosomes translating mRNAs that encode proteins with high intrinsic disorder and low hydrophobicity as well as mitochondrial or ribosomal proteins. The substrates of β-NAC and α-NAC showed a large overlap and were more distinct from those of β'-NAC. For example, hydrophobic proteins and metabolic enzymes were highly enriched among their interactors (163).

As mentioned above a role for NAC in cotranslational protein targeting has been discussed since decades (125,141,145-151,164). Therefore, the authors also analyzed the interplay between targeting factor SRP and NAC. Originally it was proposed that NAC and SRP compete for ribosome binding and that a nascent polypeptide can only interact with either one at the same time. In contrast, the study demonstrates that both factors can bind simultaneously to one ribosome and the presence of NAC does prevent the interaction of nascent chains with SRP in yeast. In the absence of NAC, however, a subset of the nascent secretory proteins was unable to interact with SRP, whereas on the other hand, increased false contacts between SRP and cytosolic proteins were detected, which are no SRP substrates. Taken together these data support a role of NAC as a modulator of SRP specificity during the cotranslational targeting of secretory nascent polypeptides to ER membrane (163).
2.6.2 The ribosome-associated chaperone triad of \textit{S. cerevisiae}

As mentioned above, besides NAC a second ribosome-associated chaperone system consisting of Hsp70 and Hsp40 chaperones is found in association with eukaryotic ribosomes (Figure 7). In \textit{S. cerevisiae} The Hsp70 Ssb binds to the ribosome and contacts the nascent polypeptide chain (165). For efficient stimulation its ATPase activity Ssb requires the interaction with the ribosome associated complex (RAC) that is composed of Ssz (Hsp70) and Zuotin (Hsp40) (166-168). Zuotin anchors RAC at the ribosome and mediates the contact to Ssb (169). RAC seems not bind to substrate proteins itself (Figure 7) (170). The function of Ssz is unclear so far. It might fulfill regulatory functions by inducing structural rearrangements within the J-domain of Zuotin upon complex formation that could enable the contact to Ssb ((171), chapter 4.2). Other data suggest that Ssz might play a role in the recruitment of substrates to Ssb (172).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure10.png}
\caption{The chaperone triad from \textit{S. cerevisiae}.}
\end{figure}

The yeast ribosome associated chaperone triad consists of the Hsp70s Ssb1/2, Ssz1p and the Hsp40 Zuo1. Ssb1/2 and Ssz1 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 Zuo1 contains a J-domain (light green) required for stimulation of the ATPase activity of its Hsp70 partner. In addition, Zuo1 contains a charged region (blue) in its C-terminus, which might be involved in ribosome binding. The N-terminus (N, orange) of Zuo is predicted to be unstructured. Ssz1 and Zuo1 form the stable heterodimeric ribosome associated complex (RAC). Figure modified from: (110)

In the yeast genome two genes (\textit{SSB1} and \textit{SSB2}) encode the alternative Ssb proteins Ssb1 and Ssb2, which differ only in four amino acids. Therefore, in the following they are collectively referred to as Ssb. The functional cooperation between the members of the yeast chaperone triad was discovered by genetic experiments. Yeast cells lacking either one or all three proteins of the triad showed similar phenotypes: sensitivity to high salt concentrations, hypersensitivity to aminoglycosides that increased translational error rates and cold-sensitivity (165,168,169,173,174). The first hint that the triad might be involved in folding of nascent chains came from crosslinking experiments showing that Ssb is able to contact short
nascent polypeptide chains and this interaction was dependent on RAC (165,175,176). This suggests that Ssb acts as a chaperone for nascent polypeptides. In addition, these data indicate that the chaperone triad must bind to ribosomes in close proximity to the ribosomal tunnel exit site. Moreover, the finding that expression of prokaryotic ribosome-associated chaperone trigger factor could partially alleviate the aminoglycoside sensitivity of triad-deficient yeast cells, indicate overlapping functions of the two chaperone systems (177).

Ssb represents a member of the Hsp70 chaperone family with a N-terminal nucleotide binding domain and C-terminal substrate-binding domain. Additionally, it contains a nuclear export sequence (NES) in its C-terminus (Figure 10) (178). Ssb is found primarily in the cytosol, however upon mutation of the NES, it strongly accumulates in the nucleus (178). This suggests that Ssb may rapidly shuttle between the nucleus and cytosol. In contrast to other canonical Hsp70 chaperones Ssb has further non-conventional features. First, its ATPase activity is not stimulated by an Hsp40 co-chaperone but by RAC, which is a stable complex consisting of an Hsp40 and an Hsp70 (168) (Figure 7). Second, although Ssb forms chemical crosslinks to nascent polypeptide chains, indicating that it contacts substrates in vivo, it does not interact with peptides containing classical Hsp70 recognition motifs (179). This suggests that Ssb has a largely different substrate spectrum compared to other Hsp70s (179,180). Therefore, its role as a chaperone is largely uncharacterized.

A recent publication addressed the substrate-specificity of Ssb (181). Thereby a TAP-tagged Ssb2 version was expressed to pull down associated ribosomes and the translated mRNA. Subsequently, the mRNAs were subjected to sequence analysis to determine the cotranslational interactome of Ssb. The data revealed that Ssb preferentially binds to nascent cytosolic and nuclear proteins, but not secretory polypeptides, which are targeted by SRP. Moreover, about 80% of all nascent cytosolic and nuclear proteins interact with Ssb. A common feature amongst the Ssb substrates is that they are large in size, have a low α-helical content and an increased content of hydrophobic elements. Additionally, Ssb interacts with many nascent subunits of oligomeric complexes and proteins that are engaged in a large number of interactions. For example, all subunits of the chaperonin complex TriC, most subunits of the proteasome, and a large set of ribosomal protein associated with Ssb during their synthesis. In contrast, the abundance of a protein had no influence on Ssb binding. Thus, Ssb may assist cotranslational folding of large proteins with complicated structures or that require binding partners for their stability. This also agrees with a potential role for Ssb in stabilizing free subunits of oligomeric complexes, which may expose contact sites that are prone to undergo false interactions. In a next step the authors of the same study analyzed if RAC modulates the substrate specificity of Ssb. Therefore, they compared the substrate interaction of Ssb in a wt yeast strain and in a strain lacking both RAC subunits. They indeed observed an influence of RAC on substrate binding of Ssb. While a subset of Ssb-substrate
interactions was unaffected by the loss of RAC, others significantly altered. For example, the interaction between Ssb and nascent cytosolic proteins was reduced, while the interaction with membrane and mitochondrial proteins was enhanced in cells lacking RAC. Thus RAC influences the cotranslational substrate-specificity of Ssb (181).

We were able to demonstrate recently, that simultaneous deletion of NAC and Ssb encoding genes caused conditional loss of cell viability under protein folding stress conditions. Furthermore, loss of Ssb resulted in aggregation newly synthesized polypeptides, ribosomal proteins as well as several ribosome biogenesis factors. Likewise, the levels of translating ribosomes and 60S ribosomal subunits were decreased in ssbΔ cells. These defects were aggravated when NAC was absent in addition to Ssb and additionally halfmers, which represent uncomplexed 40S particles, accumulated. These findings indicate that ribosome biogenesis is affected by the loss of Ssb and NAC and suggest that ribosomal proteins may represent important substrates of Ssb ((162), chapter 4.3).

Like Ssb, also the subunits of RAC have particular features that differ from their classical Hsp family counterparts. In contrast to other Hsp70 chaperones, for example, Ssz is neither able to hydrolyze ATP nor to bind to substrate proteins (167,170) and its substrate binding domain is shorter compared to that of other Hsp70s (Figure 10) (175). A very recent study suggested that amino acid substitutions at three positions in the ATP binding pocket might be the reason for the inability of Ssz to hydrolyze ATP (172). As Ssz is contentiously in an ATP-bound state its affinity for potential substrates is assumed to be rather low. However, this study suggested that Ssz could act as low-affinity holding chaperone that guides the growing nascent chain from the tunnel to Ssb (172).

The other subunit of RAC, Zuotin, consists of an unstructured N-terminal domain that is followed by a J-domain. The J-domain has a conserved HPD (Histidine, Proline, Aspartic acid) motif and is required to stimulate the ATPase activity of Ssb. A characteristic feature of Zuotin is a highly positively charged region (charged region) that is located within its C-terminus (Figure 10) (166,169,182). This region was for a long time discussed to be involved in ribosome association of Zuotin (169,183).

Recently, the crystal structure of a truncated RAC version from the thermophilic fungi Chaetomium thermophilium could be solved (172). The structure was modeled into Small-angle X-ray scattering (SAXS) densities of RAC bound to the ribosome. The data revealed that RAC forms an elongated complex of 180 Å in length that binds to the ribosome near the ribosomal proteins L22 and L31 (Figure 11). The complex hunches over the ribosomal tunnel exit site and contacts the ribosomal extension segment ES27 that stabilizes a distinct conformation of RAC. The elongated RAC can be dived into a head, neck and a body region. Whereas the large body is mainly composed of Ssz, the head is formed by Zuotin and
mediates the ribosome association. In agreement with previous data, it could be shown that ribosome association is mediated by a helix that is formed by the C-terminal part of the charged region (Figure 11) (172).

**Figure 11: Structure of the ribosome-associated complex (RAC)**

SAXS analysis of yeast RAC 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 Zuotin forms the neck and the head. The crystal structure of the Zuotin head domain was obtained from *Cheatomium thermophilium* and modeled into the SAXS density. The head region is formed by four helices (red, green, blue and yellow) that are located in the C-terminal part of Zuotin. Figure modified from: (172)

While Ssb is found exclusively in fungi, homologs of RAC were identified in mammals as well (184-186). This indicates that the presence of Hsp70/40 chaperones on ribosomes is common in the eukaryotic world (Figure 7). In agreement with this assumption, the human homologs of Ssz (Hsp70L1) and Zuotin (MPP11) can partially substitute for the loss of RAC in yeast (185,186). Additionally, the knock down of human MPP11 in HeLa cells results in growth defects and sensitivity towards drugs similar to what was observed for yeast cells lacking RAC (184). These findings indicate that RAC fulfills the similar functions in yeast as well as in metazoans. Despite these similarities between yeast and mammalian RAC, there are also large structural and functional differences. Most strikingly, complementation of yeast RAC with the mammalian system is independent of Ssb (185). It was thus suggested recently that cytosolic Hsp70, which cannot bind to the ribosome on their own, act as functional partners for RAC in higher eukaryotes (Figure 7) (184).
2.7 Structure and mechanism of action of Hsp70 chaperones

As the ribosome-associated chaperones Ssb and Ssz (as well as the homologs of Ssz in higher eukaryotes) belong to the family of Hsp70 chaperones, this chaperone family will be described in more detail. Members of the Hsp70 chaperone family are very abundant proteins that are found in all three kingdoms of life and they are involved in diverse cellular functions (187). In eukaryotes they are present in the cytosol and all subcellular compartments and were shown to contribute to de novo folding, refolding of stress-denatured proteins, complex assembly and disassembly, as well as to transport processes (188,189). Despite their diverse functions, Hsp70s share all the same molecular architecture. They consist of a N-terminal ATPase domain and a C-terminal substrate-binding domain that are connected by a flexible linker (Figure 12) (190,191). The substrate-binding domain is subdivided in a peptide-binding subdomain and a α-helical lid (Figure 12). Hsp70 chaperones work in concert with co-chaperones that belong to the J-protein chaperone family as well as with nucleotide exchange factors (NEFs) (Figure 13) (192).

The interaction between Hsp70s and their client proteins is controlled by their nucleotide status. In the ATP-bound state, the substrate-binding domain is in an open-state (lid displaced) and the affinity for substrate protein is low (194) (Figure 13). This results in fast
substrate binding and release. However, J-protein stimulated hydrolysis of ATP to ADP induces conformational changes including closure of the lid domain what stabilizes the substrate interaction (Figure 13). Meaning that in the ADP bound state the Hsp70 is in a closed state with high substrate affinity. Afterwards, NEFs stimulate the release of ADP, so that a new ATP molecule can bind (Figure 13). The binding of ATP in turn, leads to release of the bound substrate and allows for binding of a new substrate molecule (195). In addition, J-proteins themselves have been shown to recognize unfolded substrates and to deliver them to Hsp70s (196) (Figure 13). Previous studies suggested that Hsp70 interact preferentially with short hydrophobic peptides that are surrounded by positively charged residues (197,198). As such motifs are present in amino acid sequence of most proteins, Hsp70 are assumed to have a broad client spectrum (and may theoretically be able to interact with the majority of proteins). However, it is likely that many Hsp70s may have a much more defined preference for certain substrates (198,199).

Figure 13: Hsp70 chaperone cycle
(1) The J-proteins (green) bind non-native client proteins (yellow) and deliver them to Hsp70 chaperones (purple). Alternatively, substrate proteins can be bond directly by Hsp70. (2) Hsp40s
interact with Hsp70 via their J-domain and stimulate ATP hydrolysis. ATP hydrolysis induces a conformational change within Hsp70, including lid-closure, what stabilizes substrate interaction. Subsequently Hsp40 dissociates from the Hsp70-ADP-substrate complex. (3) Nucleotide exchange factors (NEF, orange) bind Hsp70-ADP-substrate complexes. (4) NEFs promote the release of ADP, so that ATP can re-bind. (5) The binding of ATP leads to opening of the substrate binding domain of the Hsp70 and the release of substrate proteins. (6) Released substrate proteins may be able to fold productively. If substrates require further folding assistance they can re-enter chaperone cycle.

The co-chaperones of the Hsp70s are considered as J-protein, as common feature they all share the J-domain. This J-domain is essential for the interaction with the Hsp70 and is composed of four α-helices that form a four-helix-bundle. The loop region between helix two and tree harbors a conserved His, Pro, Asp motif (HPD-motif) that is essential for the stimulation of the Hsp70's ATPase activity (193). Most of the J-proteins, however not all of them, belong to the Hsp40 chaperone family. This chaperone family is much more diverse regarding size and structure compared to Hsp70s (200).

2.8 Ribosome-associated protein quality control systems

Besides chaperones, targeting factors and modifying enzymes, also factors that initiate the degradation aberrant nascent polypeptides bind to eukaryotic ribosomes. Several studies reported that degradation of defective polypeptides can be initiated cotranslationally before synthesis of the full-length protein is completed (201-204). In line with this assumption two E3 ubiquitin-protein ligases, Not4 and Ltn1 (Figures 14+15) were identified in yeast to be involved in targeting arrested nascent polypeptides for degradation (46,205).

Recently the group of Jonathan Weissman showed that Ltn1 is part of a larger ribosome-associated complex, which is called ribosome quality control complex (RQC) (43) (Figure 14). By biochemical analysis and electron microscopy the authors showed that the complex stably associates with the 60S ribosomal subunit and triggers the degradation of stalled polypeptides. The complex is composed of the two highly conserved, but poorly characterized proteins Tae2 and Rqc1, the AAA+ ATPase Cdc48 and its co-factors. A central component of the complex is the E3 ubiquitin-protein ligase Ltn1, which catalyzes ubiquitination of the stalled peptides (Figure 14). In addition, the complex also activates the major stress-responsive transcription factor Hsf1 via a pathway that is independent of other stress signals (43).
Figure 14: RQC mediated degradation of arrested nascent polypeptides

Upon ribosome stalling that is caused by expression of polybasic amino acid sequences Asc2 and Hel2 induce translation termination and as a consequence the two ribosomal subunits dissociate. Subsequently Ltn1, Rqc1 and Tae2 bind to the disassembled subunit and Ltn1 ubiquitinates the nascent chain. The ubiquitination serves as a signal to recruit Cdc48 and its cofactors Npl4 and Ufd1 that might extract the ubiquitinated polypeptide from the disassembled subunit. Tae2 additionally signals translation stress to the major stress induced transcription factor Hsf1. The overall activity of the pathway is controlled by a negative feedback loop, as the levels of Rqc1 are downregulated by the action of the RQC. Figure taken from: (43)

Like Ltn1, also Not4 is part a larger assembly, the Ccr4-Not complex. The Ccr4-Not complex of yeast consists of nine core subunits (Ccr4, Caf1, Caf40, Caf130 and Not1-5) and is localized in the nucleus and the cytosol (Figure 15) (206,207). The major function of the Ccr4-Not complex is the regulation mRNA synthesis and turnover at different levels (208,209). On the one hand, the Not proteins play a role in regulation of transcription in the nucleus (210-212), on the other hand the Ccr4 and Caf1 represent the major deadenylases in the yeast cytosol (213,214). By catalyzing the shorting of the polyA (poly-adenylated)-tail of mRNAs they regulate mRNA stability and therefore have an impact on protein expression. The Ccr4-Not complex is also connected to the ubiquitin proteasome system through the function of the RING-type E3 ubiquitin-protein ligase Not4 (215). However, only few substrates are known to be ubiquitinated by Not4. These include the histone demethylase Jhd2 (216), the catalytic subunit of the DNA polymerase-α (217), the ribosomal protein Rps7A (218) and the proteasome assembly factor Ecm29 (219), as well as both subunits of the nascent-polypeptide associated complex (NAC). It was suggested that Not4 mediates the ubiquitination of NAC and that ubiquitination is required for ribosome association and stability of NAC (143,144). However, these results are contradictory to other analysis showing that purified NAC expressed recombinantly in E. coli, where ubiquitination does not exist, is able to bind to ribosomes (139). Thus, whether ubiquitination of NAC is important for its in vivo function remains unclear. Additionally, the ubiquitination of polylysine stalled nascent chains by Not4 was described (205). In contrast, other publications suggested that the ubiquitination
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of arrested nascent chains depends only on Ltn1 (43,46) (Figure 14). Therefore, the function of Not4 in cotranslational quality control remains elusive.

Figure 15: Cryo-EM densities of the Ccr4-Not complex from S. cerevisiae
The Ccr4-Not core complex consists of nine subunits in S. cerevisiae (Ccr4, Caf1, Caf40, Caf130 and Not1-5). Not1 is the only essential protein of the complex and acts as scaffold as it interacts with all other subunits. Figure modified from: (220)

2.9 Protein aggregates

During proteotoxic challenges proteins are at high risk to unfold or misfolding. As a consequence, proteins are prone to aggregate under such conditions. Unfolded proteins and folding intermediates expose hydrophobic surfaces that are normally covered inside a natively folded protein. Such hydrophobic protein stretches tend to associate with each other to form aggregates. Moreover, metastable but otherwise functional proteins may coaggregate with primary aggregating species and become depleted from the pool of soluble proteins (221). Sequestration of such proteins into aggregates may thus disrupt various cellular processes and cause loss-of-function defects. This may be one reason for the toxicity of aggregates that accumulate during neurodegenerative diseases (222). A major function of the cellular protein quality control system is therefore to minimize aggregation and to protect native proteins (223).

The main actors of the cellular protein quality control system are molecular chaperones and proteases, including the ubiquitin-proteasome system (UPS) of eukaryotes (117,224). Whereas chaperones facilitate the folding and refolding of non-native proteins, proteases mediate the proteolytic degradation of irreversibly misfolded polypeptides (225,226). However, the capacity of this system is limited and thus the formation of aggregates is a common consequence of severe stress conditions (227). Scenarios that favor protein aggregation include (i) mutations which affect protein stability (228) (ii) defects during protein
biogenesis by translational errors (229)(iii) environmental stress conditions like heat shock (230), and (iv) a reduced capacity of cells to cope with misfolded proteins as it occurs during aging (231-233).

Protein aggregates differ to a great extend in their structure and morphology (111,234) and can be grouped into two classes: The first class comprises largely disordered and amorphous aggregates, which typically occur during acute proteotoxic stress. In contrast, the second class includes highly ordered aggregates that often consist of amyloid-like fibrillar structures. These aggregates are dominated by β-strand conformations that form so-called cross-β-sheets, which are visible as long fibrils by electron microscopy. Such fibrils are a symptomatic hallmark of several neurodegenerative diseases, like Alzheimer's disease, Huntington's disease, Parkinson’s disease and prion diseases (235-238). However, there is increasing evidence that not the large insoluble fibrils, but rather their soluble oligomeric precursors consisting of misfolded protein are the main toxic species (Figure 20) (239).

A strategy of cells to cope with aggregates once they are formed is their deposition into cellular inclusions. One prominent example is the formation of one or two large “inclusion bodies” at cell poles of bacterial cells (240). Recently, similar deposits - so-called aggresomes - were found also in yeast and mammalian cells (241). In eukaryotes two additional compartments exist for deposition of misfolded proteins: the JUNQ (JUxta Nuclear Quality control compartment) as well as the IPOD (Insoluble Protein Deposit) (234). The JUNQ is located at the nucleus of the cell. The proteins in the JUNQ show a fast exchange with the cytosol, suggesting that the proteins are soluble and that the JUNQ has a dynamic structure. Delivery of proteins to the JUNQ is dependent on the ubiquitination of the misfolded protein. Misfolded proteins that accumulate in the JUNQ may be either refolded by the cellular chaperone machinery, or degraded by the proteasome. Thus, the sequestration of a protein to the JUNQ is reversible. In contrast, proteins in the IPOD are tightly packed, insoluble and don’t exchange with the cytosol. Amyloidogenic proteins, such as the Huntingtin protein, are the IPOD's substrates (234).

Besides deposition, nature has also evolved mechanisms to clear protein aggregates from cells. In unicellular organisms such as yeast and E. coli, it was shown that aggregates are unequally distributed between the mother and the daughter cell during cell division in a way that the new-pole progeny are free of parental inclusions (242,243). This strategy may allow to deplete aggregates from growing cell populations after proteotoxic stress.

One mechanism to prevent cells from the accumulation of aggregates involves the action of the ubiquitin proteasome system (UPS). Although the UPS may contribute to the removal of aggregates, its main task is the degradation of misfolded and damaged proteins to prevent their aggregation (244-246). Therefore, specific E3 ubiquitin-protein ligases attach a chain of
ubiquitin moieties to their substrate proteins. This serves as a signal for targeting to the proteasome by which ubiquitinated proteins are degraded (247-250).

In contrast, pre-existing aggregates are removed by autophagy (251-253). Thereby membrane vesicles form around the aggregates and fuse afterwards with lysosomes where proteolytic degradation takes place (254,255). Additionally, chaperones play an important role in the removal of aggregates. ATP-driven chaperones that mediate disaggregation and subsequent refolding of aggregated proteins were identified in all kingdoms of life. In yeast, plants and bacteria for efficient disaggregation and refolding Hsp70-type chaperones typically act in concert with their Hsp40 partners and AAA+ proteins of the Hsp100 family. In contrast, mammals are lacking chaperones of the Hsp100 family. Very recent data suggest that here Hsp110 might be involved in the clearance of protein aggregates (256).

In addition, another class of chaperones, the smalls Hsps (sHsps), were shown to be involved in re-solubilization of aggregates. sHsps interact tightly with misfolded proteins already during the aggregation process and become themselves incorporated into the aggregates. Thereby sHsps are assumed to modulate the structure of aggregates in a way that facilitates subsequent disaggregation by Hsp70-40-100 system (257,258).

2.10 The cellular response to protein folding stress

Several stress-responsive pathways evolved during evolution to protect the organism from damage caused by folding stress. The induction of these pathways can result in altered gene expression, changes the cellular metabolism and cell cycle arrest. Together, these physiological changes allow for adaptation to the stress situation. The heat shock response pathway, for example, is conserved from bacteria to human and is induced upon exposure to environmental stresses that cause severe protein unfolding (230,259). The heat shock response is an evolutionary conserved protective gene expression program that results in repression of global protein synthesis, whereas the expression of a variety of cytoprotective genes is activated (260,261). The majority of proteins that are expressed during heat shock are molecular chaperones (262,263), which protect proteins from misfolding and aggregation as well as promote refolding or proteolytic degradation.

In eukaryotes, additional organelle-specific stress response pathways exist, such as the unfolded protein response (UPR). It adopts the folding capacity of the endoplasmic reticulum to elevated folding load (105).
2.11 The role of protein quality control in aging and protein misfolding diseases

The integrity of the proteome is essential for the health of individual cells and the organism. As discussed above, the cellular protein homeostasis network monitors the cellular folding status and supports folding processes as well as the maturation of newly-synthesized proteins (Figure 1) (1). However, not only acute stress situations challenge proteome integrity, but also chronic stresses that arise continuously as a byproduct of normal cellular metabolism. In young and healthy cells the proteome is kept in balance by the concerted action of molecular chaperones, the proteolytic degradation machinery and detoxifying enzymes, as well as the adaptive stress responses. In contrast, aged cells fail to induce such protective pathways. As a consequence, the proteostasis network becomes overwhelmed with damaged and misfolded proteins. The accumulation of inactive proteins over time leads to loss-of-function toxicity and interferes with multiple cellular processes (239,264).

2.11.1 Aging

Aging describes the symptomatic changes in physiology and morphology of an organism over time. Aging in metazoans is a complex and poorly understood process and refers to physiological, physical, psychological and social changes and finally results in the death of the organism (265,266). Several theories exist that try to describe the molecular basis of the aging process. The most prominent hypothesis is the so-called “telomere theory” (267,268). Telomeres are regions of repetitive nucleotide sequences at each end of a chromatid. During every cell division the telomeres are shortened. Thus, it was proposed that the telomere-shortening mechanism naturally restricts the number of divisions a cell can undergo. Animal studies supported that the shortening of telomeres is responsible for aging on the cellular level and sets a limit for lifespans (269). Telomeres protect the chromosomes from fusing with each other. When the telomeres are shortened below a minimal length cells undergo programmed cell death by apoptosis (270,271). Indeed, it has been shown that telomere extension by activation of the telomerase enzyme (272) has successfully reversed multiple signs of aging in laboratory mice (273) as well as in C. elegans (274). However, it is likely that other processes besides telomere-shorting contribute to the aging process and although aging is studied extensively its exact biological basis is unknown so far (268).

As mentioned above aging always comes along with accumulation of damaged proteins (275). One sources of protein damage during aging are oxidative protein modifications (276). The “free radical theory” (277-279) postulates that oxygen free radicals as well as reactive
oxygen and nitrogen species (ROS and RNS) accumulate as a byproduct of normal metabolism and damage DNA, lipids and proteins (280-282). Loss of protein function by oxidative modification can be caused by covalent modification of side chains at the active site, by alternations in the protein stability or by restricting conformational flexibility (283). Covalent modifications of proteins are especially deleterious as they are usually irreversible. In addition, free radicals can cause cleavage of the polypeptide backbone (284-286), which promotes the elimination of the damaged protein by proteases. During aging, the concentration of oxidatively damaged proteins is thought to be further increased as a consequence of impaired protein turnover due to lower activity of the ubiquitin-proteasome system (287-289).

Besides oxidative modifications, protein damage also arises from variations in the amino acid sequence of proteins caused by genetic mutations as well as from polymorphism, genomic instability, mistranslation or incorporation of amino-acid analogues. Such mutations affect the folding, intermolecular interactions and structural stability of proteins (290-292).

Aged cells and organisms also seem to have deficiencies in the regulation of chaperone activity. On the one hand, old cells were shown to be unable to respond appropriately to acute stress situation by inducing the protective heat shock response or the unfolded protein response (Figure 17+18) (293-295). The failure to induce stress-responsive pathways upon exposure to proteotoxic conditions increases the concentration of misfolded and aggregated proteins over time (296,297). On the other hand, the steady-state levels of the heat shock transcription factor HSF1 was significantly higher in aged cells compared to young cells (298). In addition, cancer cell proliferation depends on HSF1 activity and increased levels of chaperones. Therefore, it is under debate if the higher steady state levels of HSF1 contribute to increase the risk of developing cancer during aging (299-302). However, it appears that the precise regulation of proteostasis networks (Figure 1) is essential for life, while accumulating evidence points to dysregulation of proteostasis networks during aging (303).

In sum, the loss of the robustness of the proteostasis network and increased chronic protein folding stress has significant implications for aging. First, the aging of an organism is accompanied by the accumulation of damaged proteins e.g. due to oxidative modification, translational errors, genetic mutations or the reduced ability of cells to react to external stress. Second, the general fidelity of protein biosynthesis and stress-protective mechanisms decrease, including the activity of repair-enzymes, the ubiquitin proteasome system and chaperones. Third, protein-quality control processes and stress response pathways work less efficient in aged organisms. All these aspects together favour the accumulation of misfolded and aggregated proteins during aging.
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## 2.11.2 Misfolding diseases

Protein misfolding and subsequent aggregation causes a variety of different protein folding diseases, including Alzheimer’s disease Parkinson’s diseases or various polyglutamine expansion diseases, such as Huntington’s disease (228,253). In contrast to aging or acute stress conditions, where typically many different proteins end up in amorphous aggregates, during protein misfolding diseases initially one individual protein species loses its native structure and forms highly ordered insoluble inclusions (304). Importantly, protein folding diseases share some common features: First, neurons are often most susceptible to protein folding defects and are progressively destroyed during the course of the disease (305). Therefore, protein folding diseases often considered as neurodegenerative diseases. Second, the association of protein folding diseases with aging is one of the most distinctive characteristics as their clinical onset usually not occurs until mid-age or often even later (306). Neurons can sustain the conformationally challenging disease protein in a soluble state for decades until age-associated destabilization of protein homeostasis predisposes the diseases protein for aggregation in different neuronal populations what initiates neurotoxicity (305). Third, the individual disease-causing proteins typically form homotypic fibrillar aggregates with amyloid-like structure. These types of aggregates are dominated by very stable cross-β structures (Figure 20), which are even resistant towards the treatment with detergents (236,238,307). The aggregating species can form intracellular inclusions or extracellular deposits. However, the formation of fibrillar aggregates is considered to be a multistep process involving the self-association of a wide range of intermediates. Such intermediates may include also soluble but misfolded monomeric and oligomeric species. Growing evidence points towards pre-fibrillar oligomers as being the primary toxic species underlying misfolding disease, as they may have a high potential to interact with other proteins and thereby interfere with cellular processes (Figure 20) (239,308). In contrast, the large fibrillar aggregates may be less toxic.

## 2.11.3 Alzheimer’s disease

Alzheimer’s disease (AD) is the most common form of dementia and usually it is diagnosed in people over 65 years of age. The psychiatrist and neuropathologist Alois Alzheimer first described the symptoms of the disease in 1906 (309,310). Alzheimer’s disease manifests in a progressive decline of cognitive and muscle function. Gradually all cognitive and body
functions are lost, which ultimately leads to death of the patient. Alzheimer’s disease is a relatively common neurodegenerative disorder and affects nearly 30% of all individuals older than 85 years of age (311,312). The cause and progression of Alzheimer’s disease are not well understood. However, fibrillar deposits consisting of aggregated proteins are typically found in the brains of Alzheimer’s patients. These so-called plaques are mainly formed by peptides, which are generated by proteolytical cleavage of the amyloid precursor protein (APP) (313) (Figure 16). The disease state results from sequential cleavage of APP by different proteases (314-317). These endoproteolytic events give rise to two different peptides, the so-called-Aβ peptides. They consist of 40 or 42 amino acids referred to as Aβ40 or Aβ42, respectively (318-320). Aβ peptides assemble into characteristic extracellular amyloid-like plaques (Figure 16) during Alzheimer’s diseases (321,322). Growing evidence points towards Aβ42 being the main toxic product, whereas Aβ40 seems to be less important for the disease development (Figure 16) (323,324).

Recent findings raise the possibility that not the plaques might cause neuronal toxicity, but rather the partially soluble Aβ intermediates may be responsible for neuronal cell death (Figure 16). The aggregation process of Aβ starts with the formation of relatively small intracellular inclusions, before the extracellular plaques accumulate. In transgenic mouse models of the disease it was shown, for example, that these intracellular Aβ inclusions caused neurotoxicity before the first extracellular plaques were detectable (325,326). This supports the idea that soluble, oligomeric intermediates are the most toxic species during Aβ aggregation. Moreover, artificially driving the assembly of Aβ intermediates into amyloid fibrils even reduced the cytotoxicity in tissue culture experiments (327), C. elegans (328) and mouse models (327,329).
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Proteolytic cleavage of the amyloid precursor protein (APP) by β-secretase and γ-secretase can produce Aβ42 and shorter Aβ fragments (yellow). Aggregation of Aβ42 aggregates into Aβ42 oligomer. Finally Aβ42 forms amyloid fibrils. The amyloid fibrils are deposited as extracellular plaques. Oligomerization and maybe plaque formation is cytotoxic and causes neuronal death. Additionally, the Aβ42 oligomers induce oxidative stress that initiates a cascade of Tau (green) –hyperphosphorylation (red) by disregulation of kinase (purple) activity. Under non-stress conditions Tau is bound to microtubules (orange) as a structural component. Upon hyperphosphorylation, Tau dissociates from microtubules and aggregates via oligomers into neurofibrillar tangles (NFT). Also the oligomers and fibrils formed by Tau may contribute to neuronal cell death during Alzheimer’s disease.

Besides Aβ plaques additional fibrillar deposits that are formed by the protein Tau represent a major pathogenic marker for Alzheimer’s disease (330,331) (Figure 16). These so-called neurofibrillary tangles are typically located within the cell bodies and dendrites of neurons and their formation goes along with neuronal cell death (332). Under non-disease conditions Tau is phosphorylated at multiple sites and acts as microtubule-stabilizing protein (333,334). However, aberrant hyperphosphorylation of Tau, causes its dissociation from microtubules and aggregation into fibrillar amyloids (Figure 16) (335-338). The collapse of the proteostasis network in aged cells may also cause an imbalance of kinase and phosphatase activities, which in part may account for hyperphosphorylation of Tau. Like in the case Aβ aggregation it is likely that soluble oligomeric forms of Tau represent the most cytotoxic species rather
than the large inclusions (339,340). However, although Aβ and Tau aggregation are the most obvious molecular pathologic symptoms, it is not clear whether they are the basic cause of neuronal dysfunction and cell death in Alzheimer’s disease (Figure 16).

2.11.4 Polyglutamine expansion diseases

At least nine different neurodegenerative diseases, such as Huntington’s disease, spinobular muscular atrophy and the spinocerebellar ataxias are caused by expansions of CAG triplets in coding regions of genes (341,342). CAG codes for the amino acid glutamine and thus the expressed proteins harbour untypically long stretches of consecutive glutamine residues. The by far best studied example for a polyglutamine expansion disease is the Huntington’s disease or Chorea Huntington. Huntington’s disease is a genetic disorder that affects muscle coordination and leads to progressive cognitive decline. The first symptoms of the disease appear usually in mid-adult life. The disease is caused by the expansions of glutamine-encoding CAG triplets within the first exon of the Huntingtin gene. Normally, the Huntingtin gene of healthy individuals codes for up to 35 consecutive glutamines (PolyQ), which results in production of a fully soluble cytoplasmic protein (343). However, if the number of repeated glutamines is increased beyond the critical threshold of 35 glutamine residues Huntingtin starts to form intracellular amyloid fibrils, which causes the disease (344). The length of the PolyQ tract correlates with the aggregation propensity of the protein and long repeats accelerate disease onset (345-348).

Expression of human mutant Huntingtin harbouring a PolyQ stretch of more than 35 consecutive glutamines leads to aggregation and cell death in its natural cellular context, but also in various animal models and in different cell types. Meanwhile reporter constructs are available that allow for the expression of different Huntingtin variants in almost every eukaryotic model organism. To study the aggregation process and toxicity of PolyQ proteins in vivo the first exon of human huntingtin containing PolyQ stretches of different length is commonly fused to a fluorescent protein and subsequently expressed in cells (349-351). Also in the case of PolyQ proteins it seems to be more likely that the soluble intermediates that are formed during the aggregation process are the main toxic species responsible for neuronal cell death rather than the large fibrillar end products (Figure 17) (352). Importantly, the number of inclusions does not correlate with severity of Huntington’s disease (353,354). In addition, amyloid fibril formation of mutant Huntingtin that sequestered the soluble oligomers into aggregates reduced cell death in cultured neurons (355,356). Furthermore the intracellular accumulation of low-molecular weight oligomers of mutant huntingtin correlated with cytotoxicity in cell culture models and in yeast, whereas no correlation could be
observed between the amount of fibrils and cell death (357,358). Overexpression of the eukaryotic chaperonin complex TriC was able to reduce cytotoxicity of PolyQ proteins in yeast. TriC reduces toxicity of PolyQ proteins by driving the aggregation of soluble oligomers into large amyloid-like fibrils. (359). This study was the first example showing that depletion of toxic oligomers through protective aggregation correlates with disease protein detoxification.

**Figure 17: Aggregation and toxicity PolyQ proteins**

Upon expression of proteins containing an elongated PolyQ tract they first lose their native structure and form β-sheet-rich monomers. These monomers assemble into soluble oligomers. Subsequently, the soluble oligomers are converted into amyloid fibrils that finally form large inclusion bodies. Which form of PolyQ oligomers represents the toxic species that causes neurodegeneration is still under debate. Increasing evidence points towards soluble oligomers as being the main toxic species, whereas the large inclusion bodies may be even cytoprotective.

It is still unclear why aggregation of Huntingtin into amyloid-like fibrils is toxic for neuronal cells. On the one hand it is possible that the soluble Huntingtin protein fulfills an essential function in neurons that is lost when aggregation starts and thus aggregation may lead to loss-of-function toxicity. On the other hand, the aggregates per se could be toxic for cells because they may cause co-aggregation of other proteins. This scenario would be an example of gain-of-function toxicity. However, although the pathogenic role of Huntingtin is studied extensively, the cellular function of the wild-type protein is still unclear (346).
Experiments in mice suggest that Huntingtin is essential during embryonic development (360) whereas the onset of the disease does typically not occur before mid-age (361). This may indicate that the Huntingtin-mediated pathogenesis is unrelated to its normal cellular function. Additionally, the overall similarities between the various PolyQ disorders suggested that the PolyQ expansions itself are responsible for the observed pathologies (303). To address this question PolyQ reporters in which solely of the PolyQ tracts without any flanking protein sequences were fused to fluorescence moieties. The data obtained by studying these constructs provided the first *in vivo* evidence that PolyQ tracts themselves have a high propensity to aggregate and are toxic in different animal models and cell types (362-364). The fact that expression of human mutant huntingtin leads to aggregation and cell death in all eukaryotic model organisms tested and even affects viability of unicellular yeast cells, confirms that PolyQ proteins are generally toxic across species (351). This leads to the hypothesis that the highly conserved cellular protein quality control systems are incapable to prevent damage by these PolyQ-containing disease proteins (365,366).
3. Aims of the study

To prevent protein misfolding and aggregation, cells are equipped with a protein quality control network that supports productive folding and initiates the clearance of terminally damaged proteins and aggregates (Figure 1). The activities of this protein homeostasis network are required to maintain the proteome in native and thus functional state. However, especially the synthesis of new polypeptides by the ribosome and their subsequent folding is a challenging and error prone process. Therefore, newly synthesized polypeptide chains interact with several ribosome-associated factors that modify nascent chains and support de novo folding or act in the removal of defective nascent proteins (Figure 7).

The major aim of this study was to investigate the functions of ribosome-associated chaperones and factors and to elucidate their role in the cellular proteostasis network.

3.1 NAC and its role in maintenance of protein homeostasis

Although NAC has been studied extensively and despite its high conservation from yeast to human, the in vivo function of NAC remains largely unclear. Therefore, a major aim of this study was to unravel the function of NAC in yeast as well as to establish C. elegans as model organism the laboratory to analyze the role of NAC in a metazoan animal model.

Important questions were:

What is the impact of NAC on maintenance of protein homeostasis? What are the substrates of NAC? Does NAC fulfill stress-related functions upon proteotoxic stress conditions?

3.2 Characterization of the Ssb-RAC and NAC systems in yeast

An unusual chaperone triad binds to eukaryotic ribosomes and supports de novo folding of nascent polypeptide chains. In yeast the Hsp70 Ssb binds to the ribosome and contacts the emerging nascent polypeptide chain. Its ATPase activity is stimulated by the heterodimeric RAC that consist of the Hsp40 Zuotin and the Hsp70 Ssz. So far, it is not understood why Ssb requires both complex partners and only very little structural information about the atypical Hsp70-Hsp40 dimer could be obtained. However, insights into the molecular basis of the RAC complex are important and will help us to unravel the function and mechanism of this unusual chaperone system on yeast ribosomes.
Aims of the study

Canonical Hsp70 chaperones have a well-defined binding motif, which they recognize for substrate interaction. Ssb seems not to bind to this motif and thus may have a different substrate specificity. So far, the substrate interaction properties of Ssb were not characterized.

Important questions were:
How does the unusual Hsp70-Hsp40 complex formation of RAC occur? How does the complex formation regulate the ability of RAC to act as co-chaperone for Ssb? What are the substrates of Ssb? How does it recognize its substrates? Does Ssb act strictly cotranslationally or does it also bind to misfolded proteins post-translationally? Do the ribosome-associated chaperone system Ssb-RAC and NAC cooperate functionally with each other?

3.3 Analysis of quality control systems on ribosomes

Recently, the ribosome-associated quality control complex (RQC) was identified, which seems to play an important role in the ubiquitination of arrested nascent polypeptides (Figure 14). Besides the RQC, the E3 ubiquitin-protein ligase Not4, a component of the Ccr4-Not complex, was suggested to function in cotranslational protein degradation (Figure 15). Hence, it was a further aim to obtain insights into how translation is coupled to protein degradation by the action of Not4 and the Ccr4-Not complex.

Important questions were:
Do Not4 and the entire Ccr4-Not complex physically interact with the translation machinery? Does Not4 act on arrested nascent polypeptides? Does it act together with the RQC? Is Not4 a component of the cellular protein quality control network? How does it contribute to maintain protein homeostasis in eukaryotes?
4. Results and discussion

4.1 The nascent-polypeptide associated complex is a key regulator of proteostasis

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Contributions:

1. Establishment of *C. elegans* as model organism in the laboratory
2. Isolation and analysis of protein aggregates
3. Preparation and quantification of polysome profiles
4. Isolation of ribosomes
5. Cloning and purification of *C. elegans* NAC
6. Preparation of figures
7. Contribution to manuscript writing

Objective

To prevent protein misfolding and aggregation, cells are equipped with a protein homeostasis network that supports the productive folding and initiates the clearance of terminally damaged proteins and aggregates (Figure 1). The action of this protein homeostasis network is required to maintain the proteome in native and thus functional state (1). However, especially the synthesis of new polypeptides by the ribosome and their subsequent folding is a challenging and error prone process (367). To reduce the load of unfolded proteins during proteotoxic stress it is thus essential to restrict protein synthesis and to adopt translation rates to the reduced folding capacity of the cells. Therefore, the cell responds to stressful environmental conditions by down-regulation of global protein synthesis. An example of such a regulatory circuit that adopts protein synthesis and folding capacity to increased folding load is the unfolded protein response (UPR) of the endoplasmic reticulum. Besides the
induction of ER-specific chaperones, the UPR also results in the attenuation of translation activity via phosphorylation of the translation initiation factor eIF2. Similarly, the activation of the heat shock response increases the levels of cytosolic chaperones and causes transient reduction of protein synthesis. However, in contrast to the UPR the underlying mechanisms that regulate protein synthesis during folding stress in the cytosol of eukaryotes have not been entirely resolved yet. As ribosome-associated chaperones are important components of the proteostasis network and seem to have functions in control of translation (see results chapter 4.3), we asked if these chaperones also play a role in adaption to stress. To answer this question we used the multicellular animal model C. elegans. In contrast to yeast, deletion of the ribosome-associated chaperone complex NAC results in embryonic lethality in higher eukaryotes like C. elegans (154). As the in vivo function of NAC is still unknown and it seems to be essential during early development of higher eukaryotes, C. elegans represents suitable system to study NAC function.

Summary of the experimental data

To determine if NAC exhibits chaperone function in higher eukaryotes such as C. elegans that could account for the embryonic lethality caused by loss of NAC (154), we used an aggregation-prone PolyQ protein folding sensor to examine the proteostastic environment upon depletion of NAC. We used a reporter construct containing 35 consecutive glutamine residues fused to YFP to monitor its folding status in vivo (362,363). This reporter remained soluble when expressed in muscle cells of young animals. Knockdown of α-NAC, β-NAC or both subunits in combination, however, resulted in the aggregation of the construct (Figure 18). These data indicate that the knockdown-mediated reduction of NAC levels affects protein homeostasis and are in agreement with the idea that NAC is a component of the cellular proteostasis network. This assumption was further supported by the finding that NAC interacts with a variety of members of the cytosolic chaperone network in vivo (data not shown).
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Figure 18: NAC is an important component of the cellular proteostasis network in *C. elegans* (A) Depletion of NAC by RNAi leads to a higher aggregation propensity of PolyQ proteins. The Q35-YFP protein was expressed in muscle cells nematodes with RNAi against of α-NAC, β-NAC or α+β-NAC as well as in control animals without RNAi. The aggregation of the PolyQ model protein was monitored at day4 of adulthood. The pictures show representative images of the head region. PolyQ aggregates are indicated by red triangles. The scale bars are 25 µm. (B) Quantification of the number of Q35 aggregates in the whole nematode. Error bars represent mean±s.d. of 50 animals.

Having first indications that NAC has important chaperone functions in *C. elegans* (Figure 18), we next asked whether NAC also interacts with endogenous protein aggregates. One naturally occurring scenario that is associated with widespread protein aggregation is aging (368). To directly test if NAC interacts with aggregates that are formed during aging, we prepared the soluble, insoluble and total cell fractions from age-synchronized animals and probed the levels of NAC in each fraction by SDS-PAGE and subsequent immunodetection (Figure 19A). The overall levels of α- and β-NAC did not change during over the entire lifespan of the animals, however, the localization of both subunits did. Whereas up to 90% of each subunit was found in the soluble fraction in young worms, in aged worms NAC could be detected almost exclusively in the insoluble fraction (Figure 19 A). These findings were confirmed by immunostaining of NAC (Figure 19B). We observed a strong shift from a homogenous distribution of NAC on day 3 to the appearance of foci, which likely represent age-associated aggregates, on day 7 (Figure 19B). Additional data from in vitro experiments showed that NAC does not aggregate by itself during aging, but that it interacts with age-induced aggregates. These findings support the idea that NAC fulfills chaperone functions during aggregation.
Results and discussion

Figure 19: NAC shifts from a soluble state to the insoluble fraction upon aging

The protein content of 100 mg (wet weight) synchronized days 3, 5, 7 and 10 old C. elegans cultures were fractionated according to their solubility. The total, (not fractionated), soluble and insoluble fractions of all samples were subjected to SDS–PAGE and subsequent Western blot analysis using antibodies against NAC and YFP. The quantification of the relative signals for α-NAC (left), β-NAC (middle) and YFP (right) in each of the total (green), soluble (blue) and insoluble (red) fractions reveals that the total amount of NAC stays constant during aging, whereas the amount in the soluble fraction decreases with concomitant increase in the insoluble fraction. YFP serves as a control for a protein that remains soluble throughout the lifespan of the animal. B) NAC localizes to foci during ageing. 3, 7, 10 and 16 day old animals were analyzed using immunohistochemistry for NAC localization. NAC localizes to foci with the progression of aging. The nucleus is highlighted with blue triangles and foci that likely represent protein aggregates are marked with white triangles. The scale bars are 10 μm.

Recent findings suggested that ribosome-associated chaperones might be required to regulate normal translation activity (see chapter 4.3). We asked if this is also the case for NAC in C. elegans and how the re-localization of NAC from ribosomes to aggregates affects translation during aging (Figure 20). Therefore, we first compared the translation activity of wt animals with ones depleted of NAC by polysome profiling. RNAi mediated knock-down of α-NAC or β-NAC resulted in a substantial decline (up to 50% reduction) of polysomes (Figure 20 A+B). Thus, the ribosome-associated chaperone NAC is an important modulator of translation activity in C. elegans.

Our observation that NAC is almost quantitatively interacts with protein aggregates that
accumulate during aging (Figure 19) led us to examine whether protein synthesis was also affected by NAC sequestration into aggregates. The translational capacity during aging was analyzed by comparing polysome profiles of age-synchronized populations of *C. elegans* starting from day 2 of life through to day 14, which corresponds to the period of medium lifespan. The amount of polysomes declined significantly in synchronized animals beginning at a young age of 2 days (Figure 20C). Quantification of the relative abundance of polysomes with respect to total RNA revealed that the polysome fraction declined by 87% during aging between day 2 and day 10 (Figure 20C). This demonstrates a significant decrease in translational activity as *C. elegans* ages that could be linked to NAC sequestration into aggregates.

![Figure 20: NAC modulates translation](image)

A) NAC is required for translational activity. Depicted are the polysome profiles starting with the 80 S peaks of age-synchronized day 3-old *C. elegans* cultures upon RNAi against α-NAC (green), β-NAC (red) and the control culture without RNAi (black). B) The quantification of the relative polysome fractions with respect to the total RNA of α-NAC (green) and β-NAC (red) knockdowns compared to the control (black) is shown on the right. Error bars represent mean ± s.d. of three independent experiments. C) Translation activity declines during aging. Quantification of the relative proportion of polysomes (in %) with respect to the total RNA level of non-synchronized and age-synchronized *C. elegans* cultures from day 2 to day 10. At least three independent analyses were used for each time point to calculate and draw error bars representing mean ± s.d.

Also acute heat shock results in the formation of cytosolic aggregates (111). Like for aging we observed that NAC strongly co-localizes with heat shock aggregates (Figure 21A). Next, we addressed the question if there is direct functional involvement of NAC in modulating protein aggregates. We thus investigated the ability of *C. elegans* to resolve protein aggregates after heat-shock in the presence or absence of NAC. Therefore, day 2 old
nematodes were exposed to heat shock for 1 hour at 35°C and then shifted back to 20°C for a recovery period of 24 hours. Animals fed on β-NAC dsRNA expressing *E. coli* during this recovery period still showed significant protein aggregates after this recovery time compared to control animals without RNAi treatment where almost no insoluble proteins were detected (Figure 21B). These data suggest the recruitment of NAC to protein aggregates as a physiological function as it enables the resolving of the heat shock aggregates. Interestingly, NAC was not only strictly required for resolving of heat shock aggregates, but also for recovery of translation after heat shock (Figure 21C). Our observation that a strong decline in protein synthesis is either caused by a reduced NAC function (by RNAi treatment) (Figure 20A) or under conditions when NAC re-localizes to insoluble protein aggregates, suggests that the sequestration of NAC into aggregates could directly contribute to the proteotoxic-stress dependent inhibition of protein synthesis.
Figure 21: NAC is important for recovery after heat shock.

A) Heat shock causes reversible accumulation of NAC in foci. Shown is the localization of NAC in a muscle cell of C. elegans grown at 20°C (top; left panel) and of C. elegans heat shocked for 30 min at 35°C (middle; left panel) and after 24 hours recovery at 20°C (bottom; left panel). The NAC foci formed upon heat shock and co-localize with the aggregation reporter luciferase (Luc)-YFP. The images of the middle panel show the separate and overlay images of NAC (red) and Luc-YFP (green) at 20°C. The heat shock conditions are depicted on the right, respectively. The scale bars are 10 µm.

B) The gel shows the total (left lanes) and aggregated protein fractions (right lanes) of animals before they were exposed to heat shock at 35°C for 1 hour, directly after heat shock, and after a recovery at 20°C for 24 hours upon knockdown of β-NAC and of control animals, that were allowed to recover without RNAi treatment, respectively. The right panel gives the quantification of aggregates. The quantification of aggregation was normalized to after the heat shock. Three independent experiments...
were used to calculate error bars representing mean±s.d. C) Heat shock of day 2 old C. elegans at 35°C for 1 hour results in a reduction of polysomes (red, left panel). The animals before the heat shock (grey, left panel) and animals recovered for 24 hours (black, left panel) serve as controls. The recovery from heat shock (1 hour, 35°C) is greatly diminished upon knockdown of β-NAC (red, middle panel) during the recovery period of 24 h compared to animals fed with RNAi bacteria expressing the empty vector (black; middle panel). A quantification of the polysome content with respect to the total RNA level is shown in the right panel.

Under non-stress conditions NAC is associated with ribosomes, where it may promote protein synthesis and folding. We found that upon stress that comes along with formation cytosolic protein aggregates, NAC primarily localizes to aggregates. Thus, we considered the possibility that this dual distribution might serve to adjust protein synthesis during cellular proteostasis. Depletion of NAC from ribosomes by sequestration into aggregates causes a decline in polysomes, indicating attenuation of protein synthesis. As a consequence, less proteins are synthesized, which reduces the burden for the protein quality control system and would allow to re-establish protein homeostasis. To address this, we attenuated protein synthesis by RNAi-mediated knock-down of the translation initiation factors, eIF4G (ifg-1) and eIF4E (ife-2) in the Q35-YFP model (Figure 22). Because these factors are essential for development, the RNAi experiments were performed with L4 stage animals. We observed a substantial reduction in Q35-YFP aggregation upon knockdown of the initiation factors. This effect was not due to differences in levels of Q35-YFP. We thus conclude that a reduction in overall protein synthesis is beneficial for cells exposed to protein folding stress (Figure 22).
Figure 22: Reduction of translation initiation enhances proteostasis.
A) The knockdown of translation initiation factors eIF4G (ifg-1) and eIF4E (ife-2) reduces aggregation of Q35-YFP. Images show the head region of day 5 old Q35-YFP nematodes. The control (empty vector) is shown on the left. The scale bars are 10 µm. B) The graph shows a quantification of the number of Q35 aggregates in the whole nematode on day 5 for the control and the knockdown of eIF4G and eIF4E. Error bars represent mean ± standard deviation (s.d.) of 50 animals. C) Western blot analysis of the Q35-YFP protein levels in the lysates of control animals without RNAi and upon knockdown of eIF4G and eIF4E by RNAi.

Taken together our data provide a new model of how an organism reacts to proteotoxic conditions in order to maintain proteostasis by the action of a ribosome-associated chaperone (Figure 23). Under non-stress conditions NAC is bound to the ribosome and supports protein synthesis. Upon proteotoxic stress that leads to the formation of protein aggregates, NAC leaves the ribosomes and interacts with the aggregates, where it is required for resolublization of aggregates (Figure 21 A+B). The sequestration of NAC in leads to a dramatic decrease of translational activity (Figure 21C) However, we also showed that a decrease of translation is beneficial for protein homeostasis as it might decrease the folding load and saves energy that is necessary for remodeling of existing aggregates (Figure 22). After rebalancing of protein homeostasis and clearance of aggregates, NAC is again available for ribosome-binding and translation can be resumed (Figure 23).
Figure 23: Model for the action of NAC in rebalancing of proteostasis upon stress

Under non-stress conditions NAC (blue) binds to ribosomes (grey) and supports translation activity. Upon proteotoxic stress, NAC co-localizes with protein aggregates and, as a consequence, is depleted from ribosomes. Its relocalization reduces translation activity by an unknown mechanism. Reduced translation results in reduced folding load. At the same time NAC is required for refolding the aggregated proteins. Both effects contribute to rebalancing of proteostasis. NAC can cycle back to ribosomes and the translation can be resumed after aggregates have been resolved.
4.2 Structural analysis of the ribosome-associated complex (RAC) reveals an unusual Hsp70/Hsp40 interaction

*these authors contributed equally

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Contributions

1.) Cloning of Zuotin fusion constructs and their analysis in vitro and in vivo
2.) Contribution to preparation of figures
3.) Discussion of experiments
4.) Proofreading the manuscript

Objective

Hsp40s act typically as co-chaperones for Hsp70 chaperones by transiently interacting with them via their J-domain in order to stimulate the ATPase activity of the Hsp70 (Figure 13). Eukaryotic ribosomes, however, associate with an unusual and stable heterodimeric complex consisting of the Hsp40 Zuotin and the Hsp70 Ssz to assist de novo protein folding (166). This complex is called ribosome-associated complex (RAC) and acts as co-chaperone for an additional ribosome-associated Hsp70, Ssb in yeast (168). In contrast to Ssb, RAC does not interact with substrates and its function seems to be restricted to act as co-chaperone for Ssb (Figure 9) (165,175). The RAC complex binds to the ribosome presumably by a positively charged region in the C-terminus of Zuotin (Figure 10+11) (166,167,169). In addition, Zuotin contacts Ssb in order to stimulate ATP hydrolysis of Ssb (169). This function, however, critically depends on the complex formation with Ssz. Thus Ssb and RAC form a functional triad on yeast ribosomes (Figure 7). This assumption is supported by findings that yeast cells lacking either Ssb, Zuo or Ssz show almost identical phenotypic defects (173) and that combined deletions have no additive defects (165,168,169,173,174). So far it is not clear why a the complex between Ssz and Zuotin is required for stimulation of Ssb. Ssz seems neither to bind nor to hydrolyze ATP and no interactions with other proteins except for Zuotin have been identified so far (170). It was suggested that Ssz might be required to facilitate the
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ability of Zuotin to interact with Ssb by inducing structural rearrangements within Zuotin (172). However, data supporting this hypothesis are still lacking. Importantly, homologs of RAC are also found in higher eukaryotes. A stable complex of the Hsp40 Mpp11 and Hsp70L1 was recently discovered to associate with human ribosomes (Figure 7) (184-186). Hence, atypical Hsp70/Hsp40 protein pairs were maintained during evolution. As attempts to obtain structural information about RAC or its homologs failed for a long time, we aimed to investigate its architecture as well as its conformational dynamics by biophysical and biochemical techniques. This data would help to understand the function and structural basis of the unusual Hsp70/40 complex on eukaryotic ribosomes.

Summary of the experimental data

A central aim of this study was to analyze the molecular basis of the RAC complex formation. It is of particular importance to understand the architecture and molecular basis of the complex formation and to identify the binding interface of Zuotin and Ssz in the RAC complex. Therefore, amide hydrogen-deuterium exchange experiments and subsequent mass spectrometry was used to detect conformational changes in the individual subunits upon complex formation. Amide hydrogen/deuterium exchange (H/D exchange) coupled with mass spectrometry has been widely used to analyze the interface of protein-protein interactions, conformational changes, protein dynamics and protein-ligand interactions (369-371). It is based on the exchange of amide protons of proteins with solvent deuterons. The exchange of the protons to a deuteron depends critically on the accessibility of the proton. Whereas exposed protons exchange fast, those protons, which a buried in the inside of a folded protein are highly protected. Thus, conformational changes or the interaction with binding partners results in shifts of the molecular mass in the protein areas due to the changed accessibility of the protons (372-374).

To gain insights into the RAC complex formation either Ssz or Zuotin alone or the RAC complex were expressed and purified from E. coli cells, respectively. The proteins were then diluted into a D2O containing buffer and exchange from hydrogen to deuteron was allowed to occur for different time intervals. Upon quenching the reaction, the samples were analyzed by MS to determine the exchange of hydrogen to deuteron. Zuotin, and even more Ssz, showed a high structural flexibility with 64% or 73% exchange of all amide hydrogen’s within a 2 min time scale, respectively. This suggests that Zuotin as well as Ssz adopt loosely folded conformations in the absence of their complex partner. However, the addition of ATP reduced the flexibility of Ssz, indicating that Ssz has a more compact structure in an ATP bound state (Figure 24A). Next the exchange experiments were performed with the
assembled RAC complex. In comparison to Ssz alone, Ssz in complex with Zuotin showed a pronounced HDX protection (Figure 24A). The protection of Zuotin was less pronounced but still significant (Figure 24B+C). This indicates that complex formation between Ssz and Zuotin stabilizes both proteins and reduces their conformational flexibility (Figure 24).

To map the interaction sites between Zuotin and Ssz after HDX the samples were digested into peptides using pepsin before MS analysis. This allows to analyze which areas of Ssz or Zuotin become protected upon complex formation and thus may reveal the complex interface. The data suggest that Ssz interacts mainly via its C-terminal domain with Zuotin, as the peptides corresponding to aa 396-447 and aa 479-538 showed the strongest protection after complex formation (Figure 24B). These regions are localized within the predicted lid of the substrate-binding domain of Ssz. For Zuotin an extensive protection was observed within the N-terminal region covering aa 1-51 (Figure 24B). Interestingly, this region was identified to be extremely dynamic in the absence of Ssz, indicating that it might be rather unfolded in the uncomplexed state. Taken together the complex formation of RAC likely involves the C-terminal domain of Ssz and the N-terminus of Zuotin (Figure 24).

**Figure 24: Formation of RAC complex protects Ssz C-terminus and Zuotin N-terminus but deprotects Zuotin J-domain**

A) Schematic representation of the domain architecture of Ssz shaded according to the observed HX protection induced by Zuo and ATP binding, respectively. B) Difference of deuteron incorporation between Zuo and Zuo(RAC) after different incubation times (10 seconds to 30 minutes) in D$_2$O. The
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data were resolved to individual peptides as indicated by the start and end residue numbers of the corresponding segments. C) Schematic model of the domain structure of Zuo shaded according to the observed HX protection patterns induced by Ssz binding.

Black, HX protection upon Zuo, ATP or Ssz binding; dashed, HX deprotection upon Zuo, ATP or Ssz binding, gray, no changes in HX properties upon binding, and white, no data available. Protection and deprotection effects were considered that were ≥ 1 D and ≥ 10% of the total exchangeable sites for at least one HX time point measured in all peptides covering a particular region.

Complex formation between Ssz and Zuotin resulted also in deprotection of the Zuotin J-domain (Figure 24B). Therefore, it is tempting to speculate that upon complex formation the increased dynamics in the J-domain might promote the contact of RAC to Ssb. This also supports the hypothesis that Ssz may play a role in the regulation of the activity of the chaperone triad on ribosomes.

To confirm that complex formation between Ssz and Zuotin occurs mainly via the N-terminal domain of Zuotin additional biochemical experiments were performed. First, limited proteolysis revealed that the first 62 amino acids of Zuotin were rapidly cleaved off when Zuotin was incubated with protease. In contrast, almost no cleavage of this fragment occurred when Zuotin was in a complex with Ssz. Additionally, a truncated version of Zuotin lacking the first 62 aa was unable to interact with Ssz in vitro and in vivo. Accordingly, yeast cells expressing the truncated Zuotin variant instead of endogenous full-length Zuotin showed the same phenotype as cells lacking Ssz. These experiments demonstrate that the N-terminus of Zuotin is strictly required for complex formation with Ssz. Moreover, in a reverse experiment N-terminus of Zuotin (either aa 1-62 or 1-102) was fused to the J-domain of another Hsp40 that does not interact with Ssb or Ssz on its own, as well as to a C-terminal His<sub>6</sub>-tag (Figure 25). These two constructs were expressed in zuo<sup>Δ</sup> cells to test if they are able to form a stable complex with the endogenous Ssz in vivo. Therefore, lysates were prepared and the fusion constructs were pulled down via their His<sub>6</sub>-tag. Subsequently the eluates were analysed for the presence of Ssz via immunoblotting (Figure 25).
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Figure 25: The N-terminus of Zuotin is sufficient for complex formation with Ssz in vivo

Endogenous Ssz associates with chimeric fusion proteins carrying the N-terminal region of Zuotin in vivo. Two N-terminal fragments of Zuotin (aa 1-62 and 1-102, upper panel) were fused to the J-domain of Ydj1 and a C-terminal His6-tag, respectively. The constructs were expressed individually in zuo1Δ yeast cells. Afterwards, the fusion constructs were affinity purified via the His6-tag. The eluates were analyzed for copurification of Ssz by Western blotting. The Zuotin fusion constructs were visualized by immunoblotting with antibodies directed against the His6-tag. As a control, the zuo1Δ cells were transformed with an empty plasmid (vector). The totals show that the levels of Ssz were similar in the lysates used for the pulldown experiment. Ssz signals were obtained specifically in the eluate fractions of samples in which the fusion constructs were expressed.

Ssz co-purified with both fusion proteins carrying parts of the N-terminal region of Zuotin, whereas it was absent in the control sample lacking these portions (Figure 25). Taken together the analysis of this work provides evidence that the N-terminus of Zuotin is required and sufficient to mediate the complex formation with Ssz in vivo (Figure 25). We propose a model according to which the unstructured N-terminal extension of Zuotin and the C-terminal part of Ssz provide the structural basis for RAC formation. As RAC formation leads to an enhanced flexibility in the J-domain of Zuotin, which is responsible for stimulation of Ssb, it seems likely that complex formation between Ssz and Zuotin has a regulatory function during de novo protein folding (Figure 26).
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Figure 26: Model for RAC complex formation

Binding of ATP (yellow) stabilizes the ATPase domain of Ssz (blue). However, complex formation with Zuo (pink) is most likely independent of the ATP status of Ssz. The formation of a kinetically stable RAC complex involves the N-terminus of Zuo and the C-terminal domain of Ssz, and leads to significant stabilization of the participating regions in both proteins. The regions in Ssz and Zuo, which show changes in the conformational dynamics upon RAC formation, are indicated in dark blue and purple, respectively. Binding of Zuo to Ssz results in increased dynamics around the HPD motif within the J-domain of Zuo. The increased dynamics of the J-domain may enable RAC to act as a co-chaperone. Binding of ATP to RAC further stabilizes the ATPase domain of Ssz but has no effect on Zuo.
4.3 A dual function for chaperones SSB-RAC and the NAC nascent polypeptide-associated complex on ribosomes

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Contributions

1. Performance and analysis of polysome profiles
2. Discussion of experiments and the manuscript
3. Proofreading the manuscript

Objectives

Two different chaperone systems bind to eukaryotic ribosomes and interact with nascent polypeptides to support their productive de novo folding (110). The first system compromises classical Hsp family members. In yeast, the Hsp70 Ssb binds to the ribosome and interacts with nascent polypeptide chains (165,175). Its ATPase activity is stimulated by its co-chaperone, the ribosome-associated complex (RAC), which consists of the Hsp40 Zuotin and the Hsp70 Ssz (166,168). RAC is anchored to the ribosome via Zuotin that additionally mediated the contact between RAC and Ssb (Figure 7) (169,183). Besides Ssb-RAC, a second complex with chaperone-like features is found in association with eukaryotic ribosomes, the nascent polypeptide-associated complex (NAC) (Figure 7). NAC is a stable heterodimer composed of a α- and a β-NAC subunit (125,129) (Figure 8). Whereas both subunits were shown to interact with nascent polypeptide chains, only the β-subunit contacts the ribosomes and thus mediates ribosome-association of the complex (Figure 7) (127,130). Although NAC is highly conserved in eukaryotes, only very little is known about its in vivo function (128). Several functions, like targeting of nascent polypeptide chains to the ER (125,145-150) suppression of apoptosis (154), or a role as a transcriptional regulator (155-158) were discussed. However, compelling in vivo evidence for each of these functions is still lacking. Due to its localization at the ribosomal tunnel exit and the interaction with unfolded substrates (125,129), a potential chaperone function for NAC in the folding of newly synthesized was proposed (8,110,161). Hence, we aimed to obtain insights into the role of NAC in the cellular chaperone network. We therefore analyzed if it is functionally connected...
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to the second ribosome-associated chaperone system Ssb-RAC in yeast, what are the major substrates of these chaperone systems and how these systems together promote the folding of newly synthesizes polypeptides.

Summary of the experimental data

To investigate if both ribosome-associated systems, NAC and RAC, are functionally interconnected in S. cerevisiae, knockout mutations in which all three genes coding of the NAC subunits (egd1Δ, btt1Δ, and egd2Δ; referred to hereafter as nacΔ) were combined with deletions of the two SSB genes (ssb1Δ and ssb2Δ; referred to hereafter as ssbΔ). The resulting quintuple knockout strain (nacΔssbΔ) was viable but displayed pronounced growth defects and grew significantly slower compared to wt cells or to ssbΔ and nacΔ mutants (Figure 27). The synergistic growth defects of nacΔssbΔ cells revealed a genetic interaction between NAC and Ssb, suggesting that the two eukaryotic ribosome-associated systems work in parallel or partly overlapping pathways.

Figure 27: Ssb and NAC interact genetically

Growth analysis of wt and chaperone mutant cells. Serial dilutions of cells were spotted on synthetic complete media without uracil for plasmid selection and with drugs where indicated. Arginine was omitted when cells were plated on the arginine analogue L-canavanine. Cells were incubated for 3 days at 30°C. NAC-wt indicates cells expressing NAC from plasmid, whereas NAC-RRK/AAA indicates the expression of a NAC variant that is deficient in ribosome binding (139).

To investigate if the folding of newly synthesized proteins is affected by the loss of the two ribosome-associated chaperone systems, wt and chaperone knock out cells were grown to mid-log phase and labeled with radioactive methionine for 1 minute. Subsequently,
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translation was stopped, cells were lysed, and aggregates were quantitatively isolated by differential centrifugation (Figure 28A). Neither wt nor nacΔ contained substantial amounts of insoluble material. In contrast, the absence of Ssb caused aggregation of a variety of newly made polypeptides that was even enhanced when NAC was absent in addition (Figure 28A). Thus, cells lacking NAC and Ssb show a synergistic defect in folding of newly made proteins. To identify the protein species, which are especially prone to aggregation in the absence of Ssb and NAC, aggregates were isolated from wt, nacΔ, ssbΔ and nacΔssbΔ, separated by SDS-PAGE and subsequently visualized by Coomassie staining (Figure 28B). Individual bands were subjected to mass spectrometry (MS) to identify aggregated proteins. In agreement with the findings above, aggregation was only detected in cells lacking Ssb alone or Ssb and NAC (Figure 28). The aggregates mainly consisted of small proteins with a size between 17 and 55 kDa. The pattern of the aggregates was identical in ssbΔ and nacΔssbΔ albeit more pronounced in cells lacking both systems (Figure 28B). This suggests that solubility of a similar set of client proteins is affected by loss of Ssb and NAC. A total of 64 proteins from the aggregates could be unambiguously identified by mass spectrometry, including predominantly ribosomal proteins (52 out of 64) from both ribosomal subunits and several ribosome biogenesis factors.

Figure 28: Newly synthesized ribosomal proteins aggregate upon loss of Ssb and NAC
A) Yeast cells were pulsed for 1 minute with [35S]methionine and after cell lysis, aggregated proteins were isolated by sedimentation. 15 µg total lysate and the entire aggregate fractions were separated by SDS-PAGE for subsequent autoradiography. B) Quantitative preparation of aggregated material from wt and chaperone mutant cells. Cells were grown to logarithmic phase in YPD media at 30°C and
after lysis the isolated aggregated proteins were separated by SDS-PAGE and visualized by Coomassie staining. Black lines indicate that intervening lanes have been spliced out. Western blotting revealed the aggregation of ribosomal Rpl35.

Besides ribosomal proteins also RNA could be identified in the aggregates of ssbΔ and ssbΔnacΔ cells, suggesting that ribosomal particles are specific components of the insoluble fraction. We thus examined next whether loss of Ssb and NAC had any consequences on the cellular levels of ribosomes and translation rates. Therefore, we compared polysome profiles of wt cells with the profiles of cells lacking NAC, Ssb or both (Figure 29). We detected a strong reduction of the 80S and polysome peaks in ssbΔ cells, which was even more pronounced in nacΔssbΔ mutants, indicating that cells lacking Ssb and NAC have a reduced translational activity (Figure 29). Furthermore, in ssbΔ and more distinct in nacΔssbΔ cells the formation of ribosomal halfmers was detectable as a shoulder in the 80S and polysome peaks (Figure 29). Ribosomal halfmers represent uncomplexed 40S ribosomal particles bound to mRNA.

**Figure 29: Polysome profiling reveals an imbalance in the ration of 60S to 40S subunits and the formation of halfmer ribosomes in cells lacking Ssb and NAC**

Lysates of wt and mutant yeast cells were prepared and equal A260 units were loaded on 15–45% linear sucrose gradients to separate ribosomal particles. Ribosomal particles were detected by readout at A254 after centrifugation. The cytosolic (sol.) and ribosomal fractions in the gradient is indicated below the profile of the wt. Arrows in the profiles of nacΔssbΔ cells indicate the shoulder in the 80S and polysomal peaks as a result of the formation of ribosomal halfmers. Profiles are representative for three independent runs.
The appearance of ribosomal halfmers is frequently observed in strains with impairments in ribosome biogenesis. Such strains typically have an imbalanced ratio of 40S to 60S subunits. As a consequence, the uncomplexed 40S subunits accumulate as halfmers on mRNAs. An imbalanced ratio of 40S to 60S subunits in strains lacking Ssb and NAC was further supported by the observation that the amount of 60S subunits was reduced by more than 50% in ssbΔnacΔ cells compared to wt cells. From these results we conclude that Ssb and NAC play a role in production and assembly of ribosomal particles. Loss of these factors results in aggregation of ribosomal proteins, which may consequently reduce the amount of ribosomal particles and the levels of actively translating ribosomes. Thus, although deletion of NAC alone did not result in any phenotypic defect our results suggest that NAC acts synergistically with Ssb to support folding of ribosomal proteins and assembly of ribosomal particles (Figure 30).

Figure 30: Model: NAC and Ssb–RAC link chaperone-assisted de novo protein folding with the production of ribosomes.

NAC (yellow/red) and Ssb–RAC (pink/green) associate with ribosomes and contact nascent polypeptides to assist de novo protein folding. Some ribosome biogenesis factors and ribosomal proteins are among the potential client proteins, which particularly rely on NAC and Ssb to fold into their active conformations and to support the assembly of ribosomal subunits. Moreover, NAC and Ssb–RAC bind to ribosomes in a dynamic manner and may also directly contribute to the maturation of ribosomal subunits during ribosome biogenesis. The dual function of NAC and Ssb–RAC in ribosome production and chaperone-assisted protein folding aligns ribosome production and protein synthesis with the folding capacity of ribosome-associated chaperones.
4.4 The Hsp70 chaperone Ssb contributes to ribosome biogenesis and interacts with aggregated proteins \textit{in vivo}

\textbf{Scior A., Hanebuth A., Gümpel M., Bruderek M., Preissler S., Deuerling E.}

to be submitted

\textbf{Contributions}

1. Analysis of Ssb interaction with aggregates by different lysate fractionation methods
2. Analysis of PolyQ aggregation by fluorescence microscopy
3. Phenotypic analysis of yeast cells
4. Establishment and performance of northern blots to detect rRNA precursors
5. Discussions on experiments and the manuscript
6. Preparation of figures and writing the manuscript

\textbf{Objectives}

In contrast to canonical Hsp70 chaperones, the ribosome-associated Hsp70 Ssb has non-conventional properties. First, its ATPase activity is not stimulated by a single Hsp40 co-chaperone but by a complex of an Hsp40 and an Hsp70, the so-called ribosome-associated complex (RAC) (168. Second, although Ssb forms chemical crosslinks to nascent polypeptide chains (Pfund, 1998 #261), indicating that it is able to bind to substrate proteins, it does not interact with peptides containing classical Hsp70 recognition motifs (179). Typically Hsp chaperones bind to hydrophobic unfolded peptide segments within their client proteins. The binding motif of the bacterial Hsp70 DnaK consists of a hydrophobic core of four to five residues enriched particularly in Leu, but also in Ile, Val, Phe and Tyr, and two flanking regions enriched in basic residues. Acidic residues are excluded from the core and disfavored in flanking regions (197,376). Meanwhile the binding motives of several other Hsp70, have been characterized and most of them recognize similar peptide sequences. The peptide APPY, for example, is bound by several Hsp70 chaperones. Ssb, however, does not bind to APPY suggesting that Ssb has a different substrate spectrum compared to other Hsp70s (179). Therefore, its role as a chaperone is largely uncharacterized. However, in
contrast to NAC, only a small fraction of cellular Ssb interacts with translating ribosomes *in vivo* (126). Thus, it is unclear whether Ssb acts only in *de novo* folding of newly synthesized proteins or if it has additional stress-related functions beyond its action on the ribosome. Here we aimed to gain further insight into the interaction of Ssb with substrate proteins to understand the role of Ssb in the protein homeostasis network of yeast cells.

**Summary of the experimental data**

It was shown that in *ssbΔ* yeast cells, which lack Ssb, a large set of ribosomal proteins aggregates (Figure 28, chapter 4.3). In addition, translation activity was lower compared to wt cells and the pool of 60S subunit was largely reduced (Figure 29, chapter 4.3). These findings suggest that ribosomal proteins are major substrates of Ssb. This assumption is supported by data showing that expression of Ssb is co-regulated with ribosomal proteins and that Ssb associates with ribosomes translating ribosomal proteins (181,377). However, so far no direct binding of Ssb to specific substrate peptides has been shown (179) and it is unclear whether such interactions occur strictly cotranslational. Thus we determined if Ssb binds post-translationally to aggregated ribosomal proteins and developed the following experimental setup: We mixed wt cells with *ssbΔ* mutants, separated soluble and insoluble proteins and tested for the presence of Ssb in the aggregate fractions. While the wt cells contain Ssb but no aggregates, *ssbΔ* cells lack Ssb, which causes aggregation of ribosomal proteins (Figure 31A). Therefore, this setup is based on the rationale that Ssb chaperone derived from the wt strain would bind to aggregates formed in the *ssbΔ* strain after cell lysis. As controls we used unmixed samples from *ssbΔ* cells or wt cells, respectively. Indeed, we observed a signal for Ssb only in the aggregates prepared from the mixed sample (Figure 31B). This is the first indication that Ssb is able to bind post-translationally to aggregated ribosomal proteins and thus supports the assumption that it might act as a chaperone for ribosomal proteins.
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Figure 31: Ssb interacts with aggregates from ssbΔ cells in trans

A) Schematic representation of the experimental setup. Wt and ssbΔ cells were grown to midlog phase. Half of the wt cells were mixed with half of the ssbΔ cells. Subsequently, we performed quantitative isolation of protein aggregates and analyzed them by SDS-PAGE and immunoblotting to determine if Ssb is able to interact with the aggregates consisting of ribosomal proteins. B) Coomassie staining (upper panel) and immunodetection of Ssb (lower panel) in the totals and aggregate fraction prepared from wt, ssbΔ or mixed cells. Ssb can only be detected in the aggregate fraction of the mixed sample indicating that it binds aggregates formed in ssbΔ mutants.

In contrast to other canonical Hsp70 chaperones no substrate peptides have been identified so far for Ssb (179). The strong aggregation of ribosomal proteins in ssbΔ cells and the interaction of Ssb with aggregates containing ribosomal proteins led us to hypothesise that ribosomal proteins might be substrates of Ssb. We thus aimed to investigate if Ssb is able to bind to peptides derived from ribosomal proteins. To do so, we extracted ribosomal proteins from purified yeast ribosomes by glacial acid treatment (378) and subsequently digested these proteins with the protease trypsin to generate ribosomal peptides. The peptides were then labeled with biotin for subsequent detection by immunoblotting. Afterwards, the labeled peptides were incubated with purified recombinant Ssb and applied to size exclusion chromatography. The collected fractions were spotted on membranes and Ssb and the peptides were detected by Western blotting (Figure 32). Samples containing only the peptides but no Ssb served as a further control. In the absence of Ssb, the peptides eluted mainly in fractions 18-26 (Figure 32 A). In contrast, Ssb eluted at much earlier fractions...
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According to its larger molecular weight (fraction 4-16). When both, Ssb and biotin-labeled peptides, were mixed, signals of the peptides were also detected higher molecular weight fractions that contain Ssb (Figure 32 A, red box). These data indicate that Ssb binds at least to a subset of peptides derived from ribosomal proteins.

Figure 32: Ribosomal peptides interact with Ssb
A) Upon gelfiltration of ribosomal peptides with or without Ssb the individual fractions were spotted on nitrocellulose membranes. Ssb was detected by immunodetection using Ssb specific antibodies (upper panel). The biotinylated ribosomal peptides were detected with Streptactin-AP (lower panel). Upon addition of Ssb signals of ribosomal peptides shifted to higher molecular weight fractions corresponding to those fractions in which also Ssb elutes, indicating an interaction between Ssb and peptides derived from ribosomal proteins. The red box shows the shift of the rPeptides in the Ssb containing fractions when Ssb is present. B) Ribosomal peptides or the peptide APPY, which was identified to not interact with Ssb (179), were labeled with biotin, incubated with or without Ssb and subjected to gelfiltration. The blot shows the signal for the peptides eluting in the fractions corresponding to 70 kDa (Ssb containing fractions). When Ssb is present rPeptides shift to much greater extent to the 70KDa fractions compared to the APPY peptides.

In control experiments we could show that it is not the biotin label of the peptides that is responsible for the interaction with Ssb (Figure 32B) and that the peptides interact specifically with Ssb rather than with other proteins. We were further interested if the Ssb-interacting peptides were derived from a specific set of ribosomal proteins and if they share any common feature. To analyze this, we identified the interacting peptides by mass spectrometry (MS) analysis. We obtained hits for 59 different peptides that interacted with Ssb, which were derived from 23 ribosomal proteins and seven non-ribosomal proteins.
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(Figure 3). Interestingly, almost all ribosomal proteins have a high isoelectric point (pI) value and thus exhibit a positive net charge at physiological pH as a common feature. Only five out of all 79 ribosomal proteins have a low pI (379-381). Interestingly, peptides from all this acidic ribosomal proteins were found among the Ssb interactors (Figure 3). Therefore, the positive charge of ribosomal proteins alone seems not to be the critical feature that marks them as substrates for Ssb. When we used peptides generated from total lysate we got eleven peptides from nine different proteins that showed an interaction with Ssb. Among the nine different proteins we identified six ribosomal proteins and again peptides from four acidic ribosomal proteins were found among the Ssb-interactors. These findings indicate that Ssb has a strong preference to interact with a specific subset of ribosomal proteins. However, it might be possible that we did not identify the entire set of Ssb-bound peptides, as especially highly charged peptides may be difficult to be analyzed using mass spectrometry.

<table>
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<tr>
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**Figure 33: Ssb interacts with peptides from different ribosomal proteins of both subunits**

Trypsin digested ribosomal peptides were incubated with Ssb and subjected to gel filtration. The fractions corresponding to 70kDa were applied to SDS-PAGE and Coomassie staining. The part of the gel containing the interacting peptides was cut out and the peptides were identified by mass spectrometry. The table shows the proteins from which the interacting peptides were derived. The colour-code marks acidic ribosomal proteins (orange), ribosomal proteins of the large subunit (green), ribosomal proteins of the small subunit (blue) and non-ribosomal proteins (white). The black asterisk marks proteins that could be identified in the aggregates of ssbΔ cells and the red asterisk marks peptides that contain to the ribosomal protein(s), which are part of the potential binding site of Ssb on the ribosome.

Based on the finding that Ssb interacts with ribosomal proteins and is required for their solubility, we asked for consequences of loss of Ssb on ribosome biogenesis. During ribosome maturation in the nucleus the ribosomal proteins need to be incorporated in the precursors in the right order and at the correct position (382). We wondered if the aggregation of ribosomal proteins might lead to a lack of new ribosomal proteins in the nucleus where ribosomes are assembled and thereby causes defects in ribosome biogenesis. To characterize the dependence of the ribosome-associated chaperones on ribosome biogenesis we isolated total RNA from wt, nacΔ, ssbΔ, zuoΔ as well as from ssbΔnacΔ and zuoΔnacΔ. Afterwards, we performed a Northern blot analysis using a probe that specifically detects the 35S, 33S, 32s, 27S and 7S rRNA precursors but not the mature rRNAs (Figure 34A). Under physiological conditions the rRNA precursors are immediately further processed into the mature rRNAs. Thus, an accumulation of the precursors is a sign for impaired ribosome biogenesis (18408888). We observed the accumulation of 35S/33S/32S rRNA only in ssbΔ and ssbΔnacΔ strains (Figure 34B). This indicates that upon loss of Ssb ribosome biogenesis is hampered at an early step.
Figure 34: Loss of Ssb leads to impairments in ribosome biogenesis at an early step
A) Schematic representation of rRNA processing in *S. cerevisiae*. Upon transcription of the rDNA by RNA polymerase I, one 35S rRNA molecule is synthesized that contains the regions that form 25S rRNA the 18S rRNA as well as the 5.8S rRNA in the mature ribosome, but also additional nucleotides sequences that are removed during ribosome biogenesis. To detect impairments in ribosome biogenesis we used a probe (red) that binds to such a region, the ITS2. Thereby the probe specifically detects the accumulation of 35S, 33S, 32S, 27S and 7S precursors, whereas the mature rRNAs are not recognized. As precursors accumulate only when ribosome biogenesis is hampered, the probe allows the detection of impairments in ribosome biogenesis. B) Northern blots from different yeast knockout strains. Ethidium bromid stained agarose gel as well as Methylene blue staining of the membrane served as loading control. The ITS2 probe was used to detect rRNA precursors. Probes against the mature 25S and 18S rRNA were used as controls. The accumulation of the early precursor is only detectable upon loss of Ssb (red box).

When we mixed wt with *ssbΔ* cells we observed that Ssb binds to the aggregates that were formed in its absence (Figure 31). However, these aggregates differ from other naturally occurring aggregates, as they are almost exclusively composed of ribosomal proteins and RNA. We thus asked if it is a general property of Ssb and RAC to interact with insoluble
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proteins, such as aggregates formed during heat shock. To distinguish whether Ssb coaggregates during heat heat shock or binds to aggregates in trans, we grew ssbΔ cells at 30°C and lysed them. In a subsequent step aggregates were removed by centrifugation and the cleared lysate was incubated at 42°C for one hour to form heat shock aggregates in the absence of Ssb. We also grew wild type cells at 30°C in parallel and prepared non-heat shocked lysates that containing Ssb but no aggregates. The two lysates were then mixed to allow Ssb to interact with the preformed heat shock aggregates in the ssbΔ lysate. Afterwards, we isolated the aggregates and analyzed the presence of Ssb in the aggregate fraction (Figure 35A). Although Ssb itself was not exposed to heat shock in this experimental set-up, we detected Ssb signals in the aggregate fraction of the mixed sample (Figure 35B). This indicates that Ssb is able to interact with heat shock aggregates in trans.

![Diagram](https://via.placeholder.com/150)

**Figure 35: Ssb interacts with heat shock aggregates**
A) Schematic representation of the experimental setup. Wt and ssbΔ yeast cells were grown at 30°C to midlog phase. Cells were lysed and aggregates were removed by centrifugation. Cleared ssbΔ lysate was incubated at 42°C for one hour to form heat shock aggregates in the absence of Ssb. After heat shock ssbΔ lysate was incubated with non-heat shocked wt lysate to allow interaction of Ssb derived from the non-heat shocked wt lysate with the heat shock aggregates in the ssbΔ lysate. Non-heat shocked ssbΔ lysate, wt lysate incubated with buffer and heat-shocked ssbΔ lysate incubated with buffer severed as control. Upon incubation aggregates were re-isolated from all samples and the presence of Ssb in the aggregate fraction was analyzed by immunoblotting. B) Coomassie staining (upper panel) and immunodetection of Ssb (lower panel) in the totals and aggregate fraction prepared.
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from the samples as shown in A. Ssb can only be detected in the aggregate fraction of heat shocked ssbΔ lysate that was incubated with non-heat shocked wt lysate, indicating that Ssb interacts with heat shock aggregates.

We wondered whether Ssb fulfills any function in the heat shock aggregates and analyzed the disaggregation of heat shock aggregates. To do so we exposed wt and ssbΔ cells to heat shock for one hour shifted them back to 30°C stopped further cell division by addition of cycloheximide and analyzed the formation of aggregates at 42°C and the disaggregation of the heat shock aggregates after 10 and 45 minutes of recovery after heat shock. We were not able to detect any difference in formation or dissolving of the aggregates between the wt and ssbΔ strain (data not shown). Therefore the physiological consequence of the interaction between Ssb and the heat shock aggregates is still unclear.

To further proof that Ssb has a general affinity to aggregated proteins, we additionally analyzed its interaction with PolyQ aggregates. The PolyQ aggregates were formed by expression of the first exon of the mammalian protein Huntingtin. The constructs we used coded for 25 or 97 consecutive glutamines (25Q and 97Q). We used the 97Q construct as well as the 25Q construct either with or without the adjacent proline-rich domain (PRD). The expression of these constructs is controlled by a galactose-inducible promoter. Importantly, all constructs differ in their aggregation behavior and cytotoxicity. The construct with 97Q and the PRD (referred to as 97QP) forms commonly one single aggregate per cell (aggresome) that is not toxic (383). In contrast, the 97Q construct without the PRD forms multiple toxic aggregates (383). The 25Q and 25QP constructs both are soluble and do not form toxic aggregates and thus they severed as controls. In contrast to heat shock aggregates, which form unstructured, amorphous aggregates, the PolyQ aggregates form highly ordered, β-sheet-rich amyloid-like fibrils. Therefore, we were interested if Ssb is also able to interact with such type of aggregates. Using CsCl density gradients we observed that Ssb interacts with 97Q as well as with 97QP aggregates. Thus, Ssb interacts with aggregated PolyQ proteins (Figure 36 A+B).
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Figure 36: Ssb interacts with PolyQ aggregates and modulates their toxicity

A) Cells expressing 25Q, 25QP, 97Q or 97QP were grown in -URA galactose media to midlog phase. Upon lysis all aggregated material was pelleted by centrifugation. The pellet was resuspended and loaded onto CsCl gradients to separate amyloid-like PolyQ fibrils from other insoluble material. Upon density gradient centrifugation the CsCl gradients were fractionated from top to bottom. Samples from each fraction were spotted onto nitrocellulose membranes and PolyQ containing fractions were detected by immunoblotting using Flag-specific antibodies. Shown are the immunoblots of the membranes containing the fractions from cells expressing 97Q or 97QP. The PolyQ aggregates are found mainly in fraction 13 to 20 (indicated by the red box) B) Samples of the PolyQ containing fractions (13 to 20) from each gradient were applied to SDS-PAGE and the presence of Ssb in each fraction was analyzed by immunoblotting using Ssb1-specific antibodies. Ssb could be detected in the same fractions like 97Q or 97QP aggregates or aggresomes. However, it could not be detected in these fractions when non-aggregating control constructs 25Q or 25QP were expressed. C) Wt and chaperone mutant cells were transformed with a plasmid either encoding the galactose-inducible 97Q or 97QP constructs were grown overnight in SCD-URA medium. Subsequently, the cells were adjusted to an OD<sub>600</sub> 0.5 and 5-fold serial dilutions were spotted onto SCD-Ura plates containing either glucose or galactose as carbon source. Plates were incubated for 3 days at 30°C. Whereas the expression of the Q-constructs is repressed in the presence of glucose its strongly induced by galactose.
As Ssb binds to PolyQ aggregates we wondered whether it also modulates PolyQ toxicity. As described earlier, expression of 97QP is not toxic in wt cells (383), however expression in ssbΔ led to a strong growth defect (Figure 36C). Toxicity was even more pronounced when NAC was absent in addition to Ssb (Figure 36C). Thus, Ssb does not only interact with PolyQ aggregates but also modulates their toxicity. Although the mechanism by which Ssb influences the toxicity of PolyQ aggregates is still unclear, our data suggest that besides its functions on the ribosome, Ssb may have additional chaperone activities in the cytosol.
4.5 Directed PCR-free engineering of highly repetitive DNA sequences

Scior A*, Preissler S*, Koch M., Deuerling E.
*these authors contributed equally

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Contributions

1. Cloning of all constructs
2. Contribution to the development of the strategy
3. Contribution to designing of primers and oligonucleotides
4. Establishment of expression and purification conditions for PolyQ fusion proteins
5. Purification of PolyQ fusion proteins
6. Biochemical characterization of the PolyQ constructs by SDS-PAGE and establishment of the filter retardation assay
7. Writing the manuscript and preparation of figures

Objective

During the course of this thesis we aimed to generate reporter proteins that are highly aggregation-prone, in order to study the effects of molecular chaperones on the protein aggregates. We decided to use reporter constructs that contain a stretch of consecutive poly glutamine (PolyQ) residues. PolyQ proteins have been used previously to study protein aggregation in vitro and they are of medical relevance as their expression leads to toxic protein aggregation in vivo. For example, the neurological disorder Huntington’s chorea is caused by the accumulation of CAG triplets (coding for glutamine) within the first exon of the gene encoding the protein Huntingtin (Htt). More than 35 consecutive glutamine residues have been shown to be pathogenic, as they promote Htt aggregation into amyloid-like fibrils (384,385). The translation product of the first exon of the htt gene was previously used to study the aggregation behavior of PolyQ protein in vitro (386).

Highly repetitive nucleotide sequences are frequently found in nature (e.g. in telomeres (387), in microsatellite DNA (388) or in poly-adenylated (polyA)-tails of eukaryotic messenger RNA (389)) and are of particular interest in biopolymer technology for the design of artificial
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proteins with unique physical properties (390-392). Additionally, besides Chorea Huntington, expansions of DNA repeat sequences are associated with several other inherited neurodegenerative diseases (393-395). Thus, due to their numerous occurrences in nature, their aggregation propensity and characteristic features, studying proteins encoded by repetitive DNA sequences is of biological, biotechnological and medical relevance (396,397). However, cloning of such repetitive DNA sequences is challenging because PCR-based amplification is hampered by the lack of unique primer binding sites resulting in unspecific products (398-400). We therefore aimed to develop a cheap and simple cloning strategy that allows the PCR-free generation of repetitive sequences of defined lengths. This technique was then used to generate new PolyQ containing reporter constructs that are suitable to study the aggregation of PolyQ proteins in vitro.

Summary of the experimental data

Several methods have been described for cloning of repetitive DNA sequences. However, most of them include PCR-based amplification, which often results in imperfect repeats or generate a pool of clones that differ in the number of repeats. Hence we developed an approach that is PCR-free and generates exclusively clones carrying the desired length of repeat sequences. For the PCR-free generation of repetitive DNA sequences we used antiparallel oligonucleotides flanked by restriction sites of Type IIS and Type IIP endonucleases (401,402) (Figure 37A). The arrangement of recognition sites allowed for stepwise and seamless elongation of repetitive sequences (Figure 37B). This facilitated the assembly of highly repetitive DNA segments and open reading frames coding for polypeptides with periodic amino acid sequences of any desired length (Figure 37C). By this strategy we cloned a series of PolyQ-proteins containing stretches of glutamine residues with defined lengths between 11 and 218 consecutive glutamine residues (Figure 38A).
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Figure 37: Cloning strategy for generation of PolyQ constructs

A) Annealed oligonucleotides used for cloning of repetitive DNA sequences. The recognition sequences and the corresponding cleavage sites of the restriction endonucleases are indicated by different colors. The orientation of the two inward facing Type IIS restriction sites (BsaI and BsmBI) results in cleavage within the repetitive sequence (red) and allows for seamless elongation of the repetitive nucleotide stretch. The length and sequence of the repetitive region is fully variable, allowing for the generation of any desired sequence. Other combinations of Type IIS and Type IIP restriction sites can be used instead of the indicated ones. B) Detailed representation of the seamless elongation of repetitive DNA sequences. Digestion of the doublestranded oligonucleotides (BsaI/Sacl) and the target vector (BsmBI/Sacl) resulted in compatible overhangs allowing for seamless elongation. Used restriction sites are underlined. C) Overview of the cloning strategy. The annealed oligonucleotides are inserted via BsaI and Sacl into the target vector. For elongation of the repetitive sequence, the BsaI/Sacl digested oligonucleotides can be ligated into the vector digested with BsmBI and Sacl. This elongation cycle can be repeated for several rounds.

These PolyQ sequences were then used to generate new PolyQ aggregation reporters for filter retardation assays. This assay is based on the characteristic of PolyQ fibrils to remain insoluble in solutions containing 2% sodium dodecyl sulfate (SDS). Such fibrils can therefore be specifically retained on a cellulose-acetate filter, whereas soluble and monomeric species become denatured by SDS and are filtered through the membrane. The captured aggregates can then be visualized by immunodetection. Typically PolyQ aggregation reporter constructs used for in vitro studies contain a well-folded globular domain that can be cleaved off by protease treatment. The globular domain keeps the entire protein in a soluble state and aggregation can be initiated by the release of the domain (386). In our final aggregation reporter constructs the PolyQ regions were flanked by the 17 N-terminal amino acids of Htt (N17) and contained a His6-SUMO moiety at their N-terminus. A Flag-tag was fused to the C-terminus of the constructs for immunodetection giving rise to a construct: His6-SUMO-N17-Qstretch-Flag. The His6-tag of the constructs allows the affinity purification of the constructs via Ni2+ matrices. The SUMO domain keeps the protein in a soluble state and allows the controlled induction of aggregation by addition of the protease Ulp1, which cleaves behind the folded SUMO domain (403) and thereby releases N17-Q-Flag (Figure 38 D). To test the functionality of our constructs we purified a construct containing 47 consecutive glutamine residues from E. coli (Figure 38C) and subsequently performed filter retardation assays with the purified fusion protein (Figure 38). In the control reaction, where Ulp1 was omitted, no aggregation was observed beyond marginal background signals (Figure 38 E). By contrast, significant amount of SDS resistant aggregates were formed one hour upon induction of SUMO cleavage (Figure 38E). Thus, our His6-SUMO fusion strategy provides a useful tool to study kinetics of PolyQ aggregation in vitro.
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Figure 38: Analysis of repetitive sequences generated in this study.

A) Different length of PolyQ encoding sequences cloned by the described strategy were cut out of the vectors. The DNA fragments were separated on a 2 % agarose gel and visualized by ethidium bromide (EtBr) staining. B) Plasmids containing the indicated number of adenines were digested to release poly(A) sequences. The DNA fragments were separated on a 10 % polyacrylamide gel and visualized by EtBr staining. C) Three different PolyQ constructs were successfully produced in E. coli and purified. The proteins consisting of His6-SUMO-N17-Qn-Flag were applied to SDS-PAGE and visualized by immunoblotting against the Flag-tag. D) Ulp1 cleavage induces aggregation of the PolyQ proteins. Purified His6-SUMO-N17-47Q-Flag fusion proteins were treated with Ulp1 for 1 minute and analyzed by SDS-PAGE and α-Flag immunoblotting. Cleavage was almost complete after 1 minute, indicating that the method allows for the fast induction of PolyQ aggregation. E) Filter retardation assay using His6-SUMO-N17-47Q-Flag fusion proteins to detect the kinetics of PolyQ protein aggregation. Membrane bound PolyQ fibrils were visualized by α-Flag immunodetection.

Importantly, our cloning strategy is not limited to generate PolyQ encoding sequences but can be also used for other nucleotide repeats. To demonstrate this, we adopted the strategy to generate poly adenosine (poly(A)) stretches of defined length (Figure 38B). These sequences can be used e.g. as DNA templates for the in vitro production of poly-adenylated
messenger RNA (Figure 38B) (389). Taken together, our PCR-free seamless cloning strategy is based on simple standard cloning procedures and can be used to assemble highly repetitive DNA sequences. The method is cheap and fast and can be easily adopted to produce any desired nucleotide sequence for a wide range of applications.
4.6 Not4 and the Ccr4-Not complex function in quality control of protein synthesis


**Contributions**

1. Cloning of constructs
2. Microscopy of yeast cells
3. Analysis of enolase aggregation by fluorescence microscopy
4. Establishment and performance of Northern Blotting
5. Discussions on experiments and the manuscript

**Objectives**

The synthesis of proteins by ribosomes is a fundamental process that is controlled on multiple levels to maintain the integrity of the cellular proteome. Thereby it is an important aspect to prevent production of defective proteins that can be encoded by aberrant messenger RNAs (mRNAs). Translation of truncated mRNAs can result in ribosome stalling which is recognized by quality control systems that prevent further synthesis of defective polypeptides. These systems include mRNA surveillance pathways that cotranslationally induce degradation of aberrant mRNA molecules and recycle stalled ribosomes (404). Recently a ribosome-bound quality control system was discovered that facilitates the degradation of such arrested nascent polypeptides (43,46) (Figure 14). This system consists of the so-called ribosome-quality-control complex (RQC), which ubiquitinates arrested chains and releases them from ribosomes. The key component of the RQC is the E3 ubiquitin-protein ligase Ltn1. Ltn1 binds to disassembled 60S ribosomal subunits and ubiquitinates arrested polypeptides to target them for degradation (Figure 14) (46).

In yeast, also the E3 ligase Not4 has been suggested to be involved in ubiquitination of arrested nascent chains (205). Not4 is part of a large molecular assembly, the Ccr4-Not complex, which consists of at least nine core subunits (Ccr4, Caf1, Caf40, Caf130, Not1-5) (Figure 15) (405). The complex is evolutionary conserved in eukaryotes and localizes partly to the nucleus while the majority resides in the cytosol. In the nucleus, the Ccr4-Not complex
has been implicated in the regulation of transcription. In contrast, an important cytosolic function involves the Ccr4 and Caf1 subunits, which constitute the major deadenylases in the cytosol of yeast cells and catalyze poly(A)-tail shortening of mRNAs to initiate their degradation (406). Although Not4 was suggested to target arrested nascent polypeptides for proteasomal degradation, no Not4-dependent ubiquitination of arrested chains has been demonstrated that far. In addition, more recent data showed that the deletion of NOT4 alone had no effect on the levels of translation arrest products (46).

Despite the identification of multiple components that are involved in cotranslational quality control processes, a holistic understanding of their precise roles and the functional interplay of the individual factors is lacking. In particular, it is unclear whether Not4 acts in cotranslational protein quality control and how its function relates to the second E3 ligase in this process, Ltn1.

**Summary of the experimental data**

To understand if Not4 plays any role in cotranslational protein quality control we first analyzed its interaction with the translation machinery by density gradient centrifugation. We could observe that different members of the Ccr4-Not complex interacted specifically with late polysomes (Figure 39). Polysomal fractions contain translating ribosomes and assemblies that become jammed due to ribosome stalling on defective mRNAs (407).
Figure 39: Not4 and the Ccr4-Not complex interact with polysomes

Ribosomal particles from lysate treated with RNase A to convert polysomes into 80S monosomes and an untreated control lysate were separated by density gradient centrifugation. Top: Absorbance profile at 254nm. Bottom: Protein fractions were precipitated and analyzed by Western blotting using antibodies directed against proteins as indicated.

As Not4 and the Ccr4-Not complex have a strong preference to interact with polysomes (Figure 39) it is possible that they contribute to quality control processes on stalled ribosomes. We next compared the functions of Not4 and Ltn1, which ubiquitinates arrested nascent chains for proteasomal degradation (43,46), by analyzing the expression of reporter proteins that stall ribosomes during translation. These reporters consisted of an N-terminal GFP moiety fused to a Flag-tag and the His3 protein (Figure 40A). To mediate ribosome stalling, twelve consecutive lysine residues (K12) were inserted between GFP and the Flag-tag (GFP-K12-Flag-His3; called hereafter K12-M). The same protein without a lysine stretch (K0) served as a non-arrested reporter (Figure 40A). We performed immunoblotting to analyze the levels of the K0 and K12 polypeptides in wt and mutant cells (Figure 40B). While the K0 reporter was produced in all strains at similar levels, no or only weak signals for both K12 arrest products were detected in wt and not4Δ cells (Figure 40B). As observed earlier (43,46), K12 arrest protein levels were increased in cells lacking Ltn1. However, the signals of K12-arrest products were strongly enhanced up to the level of non-arrested K0 proteins when Not4 and Ltn1 were both absent (not4Δltn1Δ) (Figure 40B).
Results and discussion

Figure 40: Not4 represses synthesis of polybasic reporter proteins

A) Schematic diagram of mRNA encoding the non-stalling GFP-Flag-HIS3 (K0) control construct or ribosome-stalling constructs where 12 consecutive lysine residues were inserted between GFP and Flag (GFP-K12-Flag-HIS3; K12-M) B) Yeast cells transformed with plasmids expressing either the non-stalling K0 construct or the ribosome-stalling K12-M construct were grown in SCD-His to an OD_{600} of 0.8 and normalized lysates were analyzed by Western blotting. Full-length proteins and arrested products were detected with GFP-specific (α-GFP) and Flag-specific (α-Flag) antibodies. Rpl25 was detected as a loading control. The asterisk marks degradation products. Arrest product levels were quantified, normalized to the loading control and expressed relative to the levels in ltn1 mutants.

The simultaneous deletion of LTN1 and NOT4 has thus a synergistic effect on K12 arrest product levels and suggests an inhibitory effect of Not4 on K12 protein expression (Figure 40B). Next, we immunoprecipitated K12 proteins to analyze their ubiquitination in cells lacking Not4, Ltn1, or both (Figure 41A). As shown previously, K12 proteins were ubiquitinated in wt cells but not in ltn1Δ mutants (43,46). In contrast, ubiquitination of K12 proteins was strongly enhanced in not4Δ cells. Furthermore, the simultaneous deletion of LTN1 and NOT4 reduced ubiquitination of K12 proteins again to the level of ltn1Δ cells (Figure 41A). Based on these results, we conclude that Ltn1 is the major E3 ligase for arrested K12 proteins, whereas Not4 is not required for the ubiquitination of K12 proteins.
polypeptides. Thus, Not4 contributes to the quality control process on ribosomes by a mechanism distinct from Ltn1.

**Figure 41: Not4 influences the levels of mRNAs that cause ribosome stalling**

A) Wt and mutant yeast cells were transformed with a plasmid expressing the ribosome-stalling construct GFP-2A-Flag-HIS3-K12. Cells were grown in SCD-His medium to the mid-log phase and treated with the proteasome inhibitor MG132. Lysates were prepared and the fusion proteins were immunoprecipitated with Flag-specific antibodies. Samples of the lysates and the precipitated proteins were analyzed by Western blotting. Proteins were detected with Flag-specific antibodies and ubiquitination was detected with ubiquitin-specific (α-Ubiquitin) antibodies B) Northern blot analysis of wt and ccr4-not mutant cells expressing K12-M. The membrane was stained with methylene blue (MB) to detect the 18S ribosomal RNA (rRNA) as a loading control. The K12-M mRNA signals were quantified and normalized to the loading control

Since Not4 is a member of the Ccr4-Not complex that regulates mRNA turnover, we investigated if Not4 influences the levels of mRNAs that cause ribosome stalling. Therefore, we determined the steady-state levels of K12 mRNA in wt and mutant yeast cells by Northern blots (Figure 41B). We focused on the K12-M reporter as we observed for this construct the strongest increase of arrest protein levels in not4Δltn1Δ mutants (Figure 40B). Cells lacking either Not4 alone or in combination with Ltn1 showed ~2-3 fold enhanced K12-M mRNA levels (Figure 41B). Thus, while Ltn1 targets K12 polypeptides for degradation, Not4 specifically regulates K12 mRNA levels. However, we noticed a quantitative discrepancy in the K12-M arrest product levels, which were ~12-fold higher in not4Δltn1Δ mutants compared to ltn1Δ cells (Figure 40B), and the corresponding K12-M mRNA levels, which were only 2-3-fold increased in the double mutants (Figure 41 B). It is thus unlikely that the elevated K12 mRNA levels alone account for the strongly increased K12 protein levels in not4Δltn1Δ mutants and we speculated that Not4 reduces the expression of K12 polypeptides by an additional mechanism.
Results and discussion

Another mechanism to prevent the accumulation of defective proteins might involve repression of their synthesis. Eukaryotic cells inhibit overall translation in response to a variety of stresses to allow for adaptation. For example, depletion of glucose or amino acids from the growth medium of yeast cells leads to rapid inhibition of protein synthesis, which is reflected by loss of polysomes and accumulation of 80S monosomes (408). To test if Not4 is involved in translation repression, we performed polysome profiling with wt or mutant cells shortly upon removal of glucose or amino acids from the medium (Figure 42A+B). In contrast to wt cells, polysome peaks were still detected in not4Δ mutants after glucose depletion, or amino acid withdrawal indicating that translation inhibition was less efficient (Figure 42A+B). Accordingly, the relative rate of protein synthesis upon glucose withdrawal was higher in not4Δ mutants than in wt cells (Figure 42C). We note that fast amino acid depletion likely affects the cellular concentration of aminoacyl-tRNAs and thereby promotes ribosome stalling. Thus our data suggest an important role of Not4 in translation repression, especially under conditions that may favor stalling of ribosomes.

Figure 42: The Ccr4-Not complex is required for general translation repression in response to nutrient withdrawal

A) Polysome profiling with wt or mutant yeast cells. Absorbance traces at 254 nm (A254) are shown. Cells were grown to OD_{600} 0.5 in YPD medium, pelleted, resuspended in YP medium with or without 2% glucose and incubated for 10 min. Translation was stopped by the addition of cycloheximide and cells were collected for polysome profiling on 15-45% sucrose gradients. B) Polysome profiling of wt and mutant yeast cells as in (A). Cells were grown in SCD medium to OD_{600} 0.5 and transferred to SCD or yeast nitrogen base (YNB) containing 2% glucose without amino acids. Cells were incubated for 10 minutes and collected for polysome analysis. C) 35S-methionine incorporation into proteins after
glucose depletion. Wt and not4Δ cells were grown in SCD medium to OD_{600} 0.5 and transferred to SC labeling medium without glucose containing radioactive $^{35}$S-methionine. Cells were incubated for 10 minutes and samples were taken. TCA-precipitable radioactivity was measured by liquid scintillation counting. Translation activity is given as incorporated radioactivity relative to t=0. Best-fit trendlines are shown in grey.

Based on our discovery that Not4 controls protein synthesis we reasoned that deregulated translation in not4Δ cells might interfere with protein homeostasis and cause folding stress. Indeed we could observe that the heat shock response, which responds to protein folding stress in the cytosol, was turned in not4Δ cells. We thus investigated whether cells lacking Not4 accumulate insoluble proteins. We could observe severe protein aggregation in cells lacking Not4 (Figure 43B+C). SDS-PAGE analysis revealed that the aggregated species isolated, from not4Δ cells were distributed over a broad molecular weight range (Figure 43B). We thus analyzed the aggregated proteins by mass spectrometry. More than 500 proteins were unambiguously identified in the insoluble fractions of not4Δ mutants. The insoluble proteins comprise mainly cytosolic factors that are connected to diverse cellular processes.

The turnover of newly made proteins was similar in not4Δ and wt cells and no enhanced accumulation of insoluble polypeptides was observed in ltn1Δ cells excluding that aggregation was caused by defects in cotranslational protein degradation.

In summary, the data show that loss of translation control by Not4 causes severe protein folding stress and disturbs protein homeostasis (Figure 43).
Results and discussion

Figure 4: Not4 is required for cellular protein homeostasis
A) Wt and not4Δ cells with a complementation plasmid (pNOT4) or empty vector (pEV) were adjusted to an OD_{600} 0.5 and 5-fold serial dilutions were spotted onto SCD-Ura plates. Plates were incubated for 3 days at 30°C or 37°C B) Protein aggregates were quantitatively isolated from equal volumes of normalized lysates of not4 mutant cells either grown at 22°C or 30°C to mid log phase, respectively. Subsequently the isolated aggregates were applied to SDS-PAGE and Coomassie staining. C) Eno2-Flag-mCherry was expressed from a centromeric plasmid in wt and not4Δ cells at 22°C or 30°C and analyzed by fluorescence microscopy. Numbers give the percentage of cells that showed mCherry foci. Bars: 5 µm
5. Outlook

5.1 Function of Ssb-RAC and NAC in protein homeostasis of eukaryotes

The main focus of this study was the analysis of the molecular architecture, the functional interplay and the substrate interactions of the ribosome-associated chaperone systems NAC and Ssb-RAC (Figure 7). This work provides insights into the molecular basis of RAC formation (chapter 4.2) and we demonstrated that Ssb-RAC and NAC cooperate in the de novo folding of newly synthesized proteins (chapter 4.3). The data suggest that Ssb is especially important to prevent the aggregation of ribosomal proteins and thus supports the biogenesis of new ribosomes (chapters 4.3 and 4.4). Furthermore, we showed that NAC fulfills chaperone function in C. elegans and is an important player in the proteostasis network of higher eukaryotes (chapter 4.1). A key function thereby seems to be the adaption of protein synthesis according to the cellular folding load in order to rebalance proteostasis upon proteotoxic stress conditions. Although we gained new insights into the function of ribosome-associated chaperones many open questions remain regarding the role of these chaperones in maintenance of protein homeostasis.

5.2 Influence of Ssb on ribosome biogenesis

Although, we have strong evidence that Ssb is important to support ribosome biogenesis in yeast, so far it is unclear how it contributes mechanistically to this process. The facts that (i) Ssb expression is coregulated with ribosomal proteins (377), (ii) ribosomal proteins aggregate in the absence of Ssb (Figure 28), (iii) Ssb binds to ssbΔ aggregates in trans and to several peptides derived from ribosomal protein (Figures 31-33) (iv) and finally the fact that Ssb with ribosomes translating ribosomal proteins (181) support the idea that ribosomal proteins represent important chaperone substrates of Ssb. Ribosome biogenesis is a highly complex and energy consuming process and thus has to be tightly regulated. In eukaryotes the ribosomal proteins are synthesized in the cytosol and subsequently imported into the nucleus where the assembly of the pre-ribosomal particle takes place. With the help of a variety of ribosome biogenesis factors the pre-ribosomes undergo several processing steps and are transported out of the nucleus into the cytosol. Here, the final maturation steps occur to form a translation competent ribosome (382).
We could observe that ribosomal proteins aggregate in the absence of Ssb (Figure 28) and that ribosome biogenesis is hampered at an early step (Figure 34). In addition, Ssb interacts with peptides derived from a specific set of ribosomal proteins in vitro (Figure 32+33). Therefore, it is possible that Ssb acts cotranslationally as a chaperone for ribosomal proteins to promote their de novo folding. This idea is support by the fact that ribosomal proteins are very abundant that make up a large portion of the cellular proteome. In addition, ribosomal proteins have a high propensity to misfold and aggregate as many of them are largely unfolded and became stabilized only upon their incorporation into the ribosomal particle (379-381). The spatial separation of the synthesis of ribosomal proteins in the cytosol and their assembly into ribosomal precursors in the nucleus may account for the requirement of particular mechanisms that support folding and prevent aggregation during their synthesis. Thus, Ssb-RAC may represent key factors that ensure the solubility of newly-synthesized ribosomal proteins.

Another possibility is that Ssb is not only required for the folding of the ribosomal proteins but also for their transport into the nucleus where the ribosomal proteins become incorporated into pre-ribosomal particles. In this scenario, Ssb would stay bound to newly synthesized ribosomal proteins even after their release from the ribosome and accompany them during the transport to the nucleus. Thus, Ssb could act by preventing the aggregation of ribosomal proteins after their synthesis but before they become stabilized within the ribosome.

Alternatively, Ssb could act by directly modulating ribosome biogenesis in nucleus, for example by supporting the processing of rRNA or by modulating the activity of certain ribosome biogenesis factors. To dissect the function of Ssb in ribosome biogenesis, it is of particular interest to further analyze the substrates and interaction partners of Ssb. Furthermore, the role of Ssb during nuclear transport of ribosomal proteins needs to be investigated carefully.

The finding that Ssb accumulates in the nucleus upon mutation of its NES supports the idea that Ssb is not only engaged in the folding of ribosomal proteins in the cytosol but additionally fulfills functions in the nucleus (377).

5.3 Regulation of translation by NAC in C. elegans

Also regarding the function of NAC in higher eukaryotes several open questions remain. Our data provide a new model of how a metazoan organism reacts on proteotoxic conditions in order to maintain proteostasis by the action of a ribosome-associated chaperone (Figure 23). Under non-stress conditions NAC is bound to ribosomes and ensures a high rate of protein
Outlook

Upon proteotoxic stress that leads to the formation of protein aggregates, NAC preferentially interacts with the aggregates (Figure 19+21A) and thereby ensures aggregate clearance or protein refolding (Figure 21B). As a consequence less NAC is present on ribosomes to support protein synthesis. A decrease of translation however, seems to be beneficial for protein homeostasis under stress conditions (Figure 22). Possibly, because it reduces the folding load for the protein homeostasis network and saves energy that is required for the remodeling of misfolded proteins and aggregates. After rebalancing of protein homeostasis and clearance of aggregates, NAC is again available for binding to ribosomes and translation can be resumed. However, it is still unclear how translation is reduced upon re-localization of NAC and by which regulatory mechanism NAC controls protein synthesis.

Translation is a complex and multistep process that can be regulated on multiple levels, including ribosome biogenesis, initiation as well as elongation of translation (409,410). To gain a deeper understanding how NAC is able to slow down translation in response to different proteotoxic stress conditions it is of particular interest to analyze which of these steps is affected by NAC. Interestingly, NAC was initially described to be involved in regulation of ER-transport in yeast (125,145-151) and some recent data provide evidence that also in *C. elegans* the depletion of NAC seems to induce the ER stress response (164). Importantly, induction of the ER-stress response reduces overall protein synthesis by a mechanism that transiently inactivates the translation initiation factor eIF2α by phosphorylation (411). Thus it is of especial interest if the ER stress response might be involved in NAC-mediated down-regulation of translation upon folding stress.

Additionally, the involvement of the second ribosome-associated chaperone system RAC in maintenance of cellular protein homeostasis in *C. elegans* was not thoroughly analyzed so far and needs to be investigated.

5.4 The role of ribosome-associated chaperones connected to protein aggregation

The function of ribosome-associated chaperones was initially thought to be limited to assistance in *de novo* folding of newly-synthesized proteins (101). We have strong evidence that ribosome-associated chaperones fulfill additional functions during misfolding and aggregation of cytosolic and nuclear proteins beyond their action on ribosomes. We showed that *C. elegans* NAC interacts with different types of amorphous aggregates, like aggregates
that accumulate during aging (Figure 19) or upon heat shock (Figure 21A), as well as with amyloid-like Aβ fibrils (data not shown). These findings are supported by a publication that identified NAC to associate with artificial β-sheet fibrils (412). The physical interaction between NAC and aggregates has also physiological consequences, as disaggregation of heat shock aggregates was dependent on the presence of NAC (Figure 21B). However, it is not clear how NAC is able to remodel aggregates. As NAC has no ATPase activity and thus cannot apply mechanical force its mechanism must be different from the one of classical disaggregases such as Hsp70-Hsp40-Hsp100 system (117). One possibility is that NAC in *C. elegans* works similar to small heat shock proteins (sHsps). They tightly interact with non-native proteins already during the aggregation process and thereby become themselves incorporated into the aggregates. sHsps are assumed to remodel the aggregates in a way that disaggregation by Hsp70-40-100 system is favored (258). However, additional experiments are required to determine if NAC works in a similar manner to assist in the resolubilization of aggregates.

Like NAC in *C. elegans* also Ssb was identified in association with different types of aggregates in yeast. We found Ssb in the aggregates of cells lacking the E3 ligase Not4 (data not shown) as well as in association with heat shock aggregates (Figure 35) and in PolyQ aggregates and aggresomes (Figure 36 A+B). These data suggest that Ssb might recognize and interact with various types of protein aggregates. To determine if Ssb might modulate the aggregates in its role as a chaperone, we asked if the formation and resolubilization of heat shock aggregates is different in the presence or absence of Ssb. However, we did not observe any influence of Ssb, neither on the formation nor on the disaggregation of heat shock aggregates (data not shown). Therefore, the function of Ssb in the prevention of aggregation or disaggregation of heat-damaged proteins is still unclear and requires further investigation. For example, it needs to be determined whether cells lacking Ssb are more sensitive towards high temperature compared to wt yeast cells.

In addition, we observed an interaction between Ssb and the large aggresomes formed by PolyQ aggregates as well as with the smaller PolyQ aggregates (Figure 36 A+B). Whereas the smaller PolyQ aggregates are toxic, the formation of the larger aggresomes does not impair growth in wt yeast cells (383). Aggresome formation is dependent on the presence of the proline-rich domain that is located directly behind the PolyQ stretch in exon one of huntingtin (383). In contrast to wt cells, expression of a PolyQP protein that contains the proline-rich domain was toxic in the absence of Ssb (Figure 36 C). Therefore, we assumed that Ssb might contribute to aggresome formation and thereby detoxification of PolyQ proteins. However, the aggregates and aggresomes formed in wt or ssbΔ cells were morphological indistinguishable from each other (data not shown). Thus, although PolyQ aggresomes are formed PolyQ expression in ssbΔ cells is toxic. Taken together, we could
demonstrate that Ssb interacts and detoxifies PolyQ aggregates by an unknown mechanism (Figure 36).

To investigate the influence of chaperones on the aggregation of PolyQ proteins in vitro we can make now use of our novel PolyQ aggregation reporters (chapter 4.5). We developed aggregation reporters that are suitable to study the aggregation process of PolyQ proteins by filter retardation assays (Figures 37+38). This assay is based on the characteristic of PolyQ fibrils to remain insoluble in solutions containing low concentrations of the ionic detergent sodium dodecyl sulfate (SDS). Such SDS-resistant fibrils are specifically retained on a cellulose-acetate filter, whereas soluble and monomeric species are denatured by SDS and are filtered though the membrane. The captured aggregates can then be visualized by immunodetection (386). In our constructs (His₆-SUMO-N17-Qstrecth-Flag) the aggregation process is initiated by cleavage with the Ulp1 protease that leads to a release of the folded SUMO domain. We demonstrated that the constructs are in a soluble state as long as the SUMO domain is present, however, as soon as it is cleaved off the construct, aggregation into SDS-resistant fibrils is initiated (Figure 38). A decisive advantage compared to previously developed in vitro aggregation reporters is that Ulp1-mediated release of the SUMO domain is much faster than the aggregation process of the PolyQ segment and therefore does not significantly influence aggregation kinetics. In order to determine the influence of chaperones on the aggregation of PolyQ proteins it is possible to add chaperones before addition of the Ulp1 to test if they can modulate the kinetics of aggregation. Such experiments can be done with every chaperone or chaperone system of interest to study if they have any function in prevention of aggregation of PolyQ protein. In addition, it is also possible to first start the fibril formation and subsequently add chaperones. Thereby the disaggregation activity of the chaperones is studied. Thus, our PolyQ reporters represent a useful tool to determine the direct action of ribosome-associated and other chaperones during the aggregation of metastable proteins.

5.5 Impact of Not4 on protein homeostasis

Not4 and Ltn1 have been suggested to target arrested nascent polypeptides for proteasomal degradation (46,205). Whereas Ltn1 was shown to directly ubiquitinate arrested nascent chains (46) the precise function of Not4 remained ambiguous. We thus thought to dissect the role of Not4 in this process. First, we analyzed the association of Not4 with ribosomal particles and found that it accumulated predominantly in the late polysomal fractions (Figure 39). We thus assumed the strong interaction of Not4 with polysomes to be functionally relevant. One possibility is that Not4 binds to translated mRNAs rather than to ribosomes.
However, upon mRNA digestion we still detected significant Not4 signals in 80S ribosome fractions (Figure 39), suggesting that Not4 and the Ccr4-Not complex bind at least in part directly to ribosomal particles or in their close vicinity. It is also possible that Not4 is recruited to stalled ribosomes. In fact, ribosome stalling is supposed to occur frequently in vivo during the translation of aberrant mRNAs that result from splicing errors or premature polyadenylation. When a ribosome stalls the subsequent ribosomes become jammed on the mRNA and therefore form large polysomes. We thus designed polybasic reporter constructs similar to the ones that have been used before to arrest translation (Figure 40A) (46,205,413), and directly compared the influence of Not4 and Ltn1 on their expression. The synthesis of the arrested reporters was severely repressed in wt cells. Deletion of LTN1 resulted in increased levels of arrested products, whereas deletion of NOT4 alone had no effect (Figure 40B). The combined deletion of LTN1 and NOT4, however, synergistically increases the levels of polybasic translation arrest products (Figure 40B). We also confirmed that ubiquitination of translation arrest products strictly depends on Ltn1 (Figure 41A). Unexpectedly, ubiquitination of translation arrest products was severely enhanced in the absence of Not4 (Figure 41A). These results clearly indicate that Not4 is dispensable for the ubiquitination of arrested nascent polypeptides and rather suggest that Not4 acts upstream of Ltn1 by a mechanism that restricts the synthesis of arrested nascent polypeptides. Thus, we aimed to identify the mechanism by which Not4 regulates protein synthesis. One possibility is that Not4 might contribute to the turnover of mRNAs that encode polybasic proteins and cause ribosome stalling. We indeed observed that the mRNA levels of our K12-M reporter were specifically elevated in not4Δ cells (Figure 41B).

Interestingly, biochemical and structural data suggested that Not4 might locate adjacent to the deadenylases Ccr4 and Caf1 in the Ccr4-Not complex (414-416). This suggests that Not4 may direct deadenylation activity of the complex to mRNAs that cause ribosome stalling to promote their turnover. However, we noticed a quantitative discrepancy between the moderate increase of K12-M mRNA levels (Figure 41B) and the strong increase of K12-M translation arrest product levels in not4Δltn1Δ cells (Figure 40B). Not4 may therefore not only promote mRNA decay but may also regulate expression of arrested polypeptides by an additional mechanism. In agreement with this assumption, deletion of genes encoding the deadenylase subunits of the Ccr4-Not complex increased the reporter mRNA levels but not translation arrest product levels. These results are consistent with previous studies suggesting that the expression of arrested polypeptides is restricted by accelerated mRNA decay and translation repression (202,413).

The finding that Not4 is required to repress translation of mRNAs that cause ribosome stalling is based on the analysis of our polybasic translation reporters. Polybasic sequences as used in our reporters might be rare in vivo, however, recent ribosome profiling data
confirmed that translation is not homogenous and ribosomes stall transiently on certain transcripts due to various reasons, such as clusters of rare codons or secondary structures (417,418). These transient stalling events may represent signals for translational control to fine-tune protein expression on the post-transcriptional level. The identification of mRNAs, which are targets of Not4-dependent translational regulation, will be an important task for future studies. It is of particular importance to understand how Not4 recognizes and localizes to such mRNAs causing ribosome stalling. Ribosome stalling does not only occur by the translation of polybasic amino acid stretches but also during the translation of mRNAs harboring stretches of rare codons or that form mRNA structures, like stem loops. Therefore it will be of interest to generate reporter constructs with such elements instead of the polylysine encoding stretch and analyze if Not4 also functions in translational repression of such reporter constructs. Furthermore, it would be possible to isolate polysomes, pulldown Not4 together with the ribosome and the mRNA that it translated by the ribosome and to subsequently identify the mRNA by a deep sequencing approach (419). By this strategy the mRNAs that undergo Not4-dependant translation repression could be unambiguously identified.

The integrity of the cellular proteome depends on the maintenance of the correct stoichiometric ratios of protein species. We reasoned that translational misregulation under normal growth conditions should affect protein homeostasis and cause folding stress. Indeed, we observed severe accumulation of aggregated proteins in cells lacking Not4 (Figure 43B+C). In addition, not4Δ cells were sensitive to folding stress and showed elevated levels of stress-responsive reporters. Different scenarios may explain protein aggregation in translation repressor mutants. One possibility is that the aggregating proteins are directly affected by translation de-repression. For example, proteins that are part of multimeric assemblies require their binding partners to stay soluble. Misregulated protein synthesis may interfere with the correct stoichiometric ratios of proteins and thereby increase the concentration of proteins that lack an appropriate binding partner. Alternatively, the elevated synthesis of proteins that require folding assistance may challenge the cellular chaperone system and reduce the folding capacity of the mutant cells. As a consequence, the solubility of proteins with labile native structures would be affected indirectly.

In summary, our results present a functional link between Not protein-dependent translation repression and cotranlational protein quality control. Moreover, we provide for the first time evidence that factors, which are not directly involved in protein quality control, are crucial for cellular protein homeostasis. Mutations that interfere with translation repression cause severe protein aggregation.
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<th>Abbreviation</th>
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<tr>
<td>aa</td>
<td>Amino acid</td>
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<tr>
<td>AAA+</td>
<td>ATPases associated with diverse cellular activities</td>
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<td>ADP</td>
<td>Adenosine diphosphate</td>
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<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>CHX</td>
<td>Cycloheximide</td>
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<td>Cryo-EM</td>
<td>Electron cryomicroscopy</td>
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<td>DNA</td>
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<td>ER</td>
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<td>FRET</td>
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<td>Hydrogen-deuterium exchange</td>
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<td>IPOD</td>
<td>Insoluble protein deposit</td>
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<td>JUNQ</td>
<td>Juxta nuclear quality control compartment</td>
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<td>MAP</td>
<td>Methionine aminopeptidase</td>
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<td>MCS</td>
<td>Multiple cloning site</td>
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<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>MS</td>
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<td>NAC</td>
<td>Nascent polypeptide-associated complex</td>
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<td>NAT</td>
<td>N-acetyltransferase</td>
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<td>PPIase</td>
<td>Peptidyl-propyl isomerase</td>
</tr>
<tr>
<td>PTC</td>
<td>Peptidyltransferase center</td>
</tr>
<tr>
<td>RAC</td>
<td>Ribosome-associated complex</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase A</td>
<td>Ribonuclease A</td>
</tr>
<tr>
<td>RNC</td>
<td>Ribosome nascent-chain complex</td>
</tr>
<tr>
<td>RQC</td>
<td>Ribosome quality control complex</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>SAXS</td>
<td>Small-angle X-ray scattering</td>
</tr>
<tr>
<td>SBD</td>
<td>Substrate-binding domain</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SecM</td>
<td>Secretion monitor</td>
</tr>
<tr>
<td>sHsp</td>
<td>Small heat shock protein</td>
</tr>
<tr>
<td>SRP</td>
<td>Signal recognition particle</td>
</tr>
<tr>
<td>TAP</td>
<td>Tandem affinity purification</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
</tr>
<tr>
<td>UBA</td>
<td>Ubiquitin associated</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin proteasome system</td>
</tr>
<tr>
<td>Wt</td>
<td>Wild type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
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<td>Zuo</td>
<td>Zuotin</td>
</tr>
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</table>
7. Literature

7.1 Cited references

65. Tsai, C. J., Kumar, S., Ma, B., and Nussinov, R. (1999) Protein science : a publication of the Protein Society 8, 1181-1190

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Literature


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Literature


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7.2 Publications and manuscripts from this thesis

7.2.1 Accepted publications

Kirstein-Miles A*, Scior A*, Deuerling E, Morimoto R;
The nascent polypeptide associated complex is a key regulator of proteostasis
EMBO Journal (2013)
* these authors contributed equally

Scior A*, Preissler S*, Koch M, Deuerling E;
Directed PCR-free engineering of highly repetitive DNA sequences
BMC Biotechnol. (2011)
* these authors contributed equally

A dual function for chaperones SSB-RAC and the NAC nascent polypeptide-associated complex on ribosomes;

Structural analysis of the ribosome-associated complex RAC reveals an unusual Hsp70/Hsp40 interaction
J. Biol. Chem. (2009)
* these authors contributed equally

7.2.2 Written manuscripts

Scior A, Hanebuth A, Gümpel M, Bruderek M, Preissler S, Deuerling E,
The Hsp70 chaperone Ssb contributes to ribosome biogenesis and interacts with aggregated proteins in vivo

Preissler S, Koch M, Reuther J, Scior A, Bruderek M, Deuerling E;
The Ccr4-Not complex functions in translation repression and is important for cellular protein homeostasis
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Danksagung

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9. Appendix