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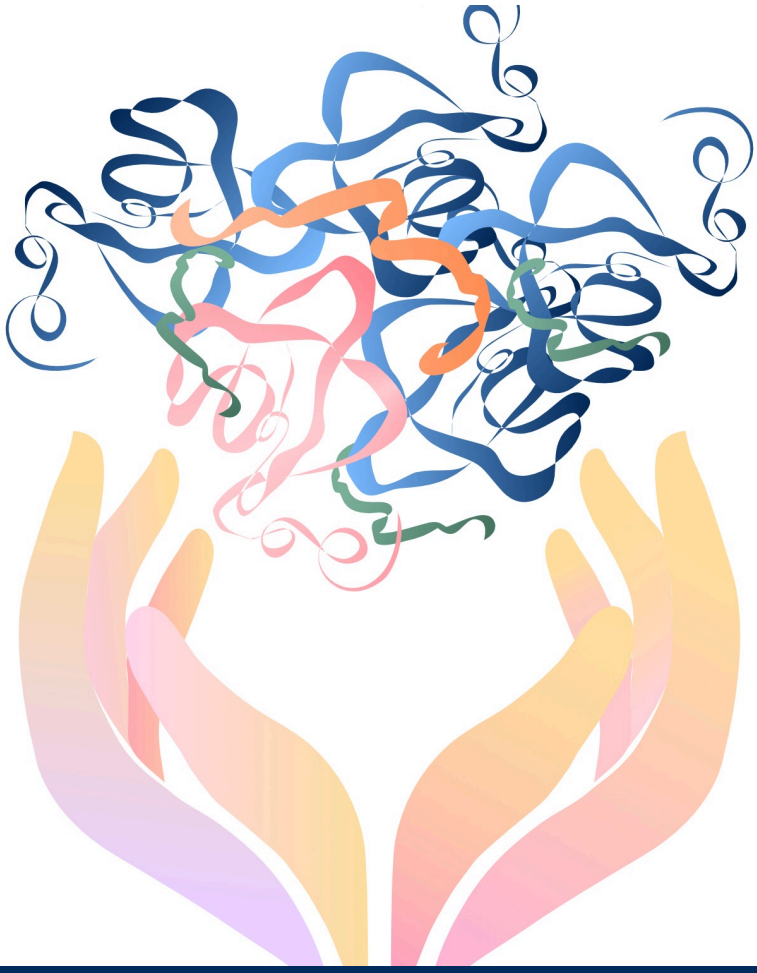
Safeguarding Proteostasis Through Cotranslational Protein Folding

Discovery of a Specialized Ribosome-Associated Chaperone for eEF1A

Jany Quintana Cordero



Doctoral Thesis in Molecular Bioscience at Stockholm University, Sweden 2025

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Academic dissertation for the Degree of Doctor of Philosophy in Molecular Bioscience at Stockholm University to be publicly defended on Friday 14 March 2025 at 09.30 in Vivi Täckholmsalen (Q211) NPQ-huset, Svante Arrhenius väg 20.

Abstract

Newly synthesized proteins are born as unfolded polypeptides emerging from the ribosomal exit tunnel. Folding these nascent chains into native conformations is crucial for protein functionality and preventing off-pathway interactions that trigger misfolding and jeopardize proteome stability. However, achieving the correct 3D structure is a major challenge for nascent chains exposed to high concentrations of molecules in the cytosol. General ribosome-associated chaperones assist co-translational folding of a wide variety of nascent peptides. It is unclear whether this "one-size-fits-all" system ensures the expression of proteins with challenging folding paths or if specialized ribosome-associated chaperones manage the folding of such demanding clients. In **Study I**, we investigated how the Hsp70 chaperone regulates Hsf1, a transcription factor that mediates the cellular response to proteotoxic stress. We demonstrated that Hsp70 directly binds to Hsf1, keeping it in a latent state under non-stress conditions. Protein misfolding, particularly of newly synthesized proteins, titrates Hsp70 away, activating Hsf1 and inducing the stress response. Thus, Hsp70 availability in response to misfolded proteins is a key regulatory mechanism of Hsf1 activity. In **Study II**, we identified a specialized ribosome-associated chaperone, Chp1, that assists in the co-translational folding of eEF1A, a highly abundant multidomain GTPase critical for mRNA translation into proteins. Deleting Chp1 leads to rapid proteolysis of eEF1A, widespread protein aggregation, and activation of the Hsf1-mediated stress response. Finally, in **Study III**, we elucidated how Chp1 assists in eEF1A folding and the ordered sequence of chaperone actions in the eEF1A folding pathway. We found that Chp1 binds to the α3 helix in the switch I region of the eEF1A G-domain, crucial for nucleotide binding, delaying the nucleotide-guided folding of the G-domain. As eEF1A domain II synthesis begins, the substrate is transferred to the downstream chaperone Zpr1 for final maturation. Our results provide insight into the molecular mechanisms of co-translational protein folding and its impact on proteome stability, as well as on the regulation of Hsf1, the central mediator of the response to proteotoxic stress in eukaryotic cells.

Keywords: *proteostasis, Hsf1, Hsp70, Chp1, specialized ribosome-associated chaperone, eEF1A, G-protein folding.*

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A mi familia.

Research summary

Newly synthesized proteins are born as unfolded polypeptides emerging from the ribosomal exit tunnel. Folding these nascent chains into native conformations is crucial for protein functionality and preventing off-pathway interactions that trigger misfolding and jeopardize proteome stability. However, achieving the correct 3D structure is a major challenge for nascent chains exposed to high concentrations of molecules in the cytosol. General ribosome-associated chaperones assist co-translational folding of a wide variety of nascent peptides. It is unclear whether this "one-size-fits-all" system ensures the expression of proteins with challenging folding paths or if specialized ribosome-associated chaperones manage the folding of such demanding clients.
In **Study I**, we investigated how the Hsp70 chaperone regulates Hsf1, a transcription factor that mediates the cellular response to proteotoxic stress. We demonstrated that Hsp70 directly binds to Hsf1, keeping it in a latent state under non-stress conditions. Protein misfolding, particularly of newly synthesized proteins, titrates Hsp70 away, activating Hsf1 and inducing the stress response. Thus, Hsp70 availability in response to misfolded proteins is a key regulatory mechanism of Hsf1 activity. In **Study II**, we identified a specialized ribosome-associated chaperone, Chp1, that assists in the co-translational folding of eEF1A, a highly abundant multidomain GTPase critical for mRNA translation into proteins. Deleting Chp1 leads to rapid proteolysis of eEF1A, widespread protein aggregation, and activation of the Hsf1-mediated stress response. Finally, in **Study III**, we elucidated how Chp1 assists in eEF1A folding and the ordered sequence of chaperone actions in the eEF1A folding pathway. We found that Chp1 binds to the α3 helix in the switch I region of the eEF1A G-domain, crucial for nucleotide binding, delaying the nucleotide-guided folding of the G-domain. As eEF1A domain II synthesis begins, the substrate is transferred to the downstream chaperone Zpr1 for final maturation. Our results provide insight into the molecular mechanisms of co-translational protein folding and its impact on proteome stability, as well as on the regulation of Hsf1, the central mediator of the response to proteotoxic stress in eukaryotic cells.

Populärvetenskaplig sammanfattning

Nybildade proteiner syntetiseras som oveckade kedjor av aminosyror som lämnar ribosomens tunnelmynning. För att dessa kedjor ska få rätt tredimensionell struktur, vilket är avgörande för deras funktion, måste de veckas korrekt. Om detta misslyckas kan det leda till felveckning och påverka proteinets stabilitet. Cellens komplexa miljö som är tätt packad med olika molekyler i cytosolen är en utmanande miljö för proteinveckning. Ribosom-associerade chaperoner spelar en viktig roll för att hjälpa till att veckla nysyntetiserade proteiner. Det är dock oklart om dessa chaperoner som fungerar som generalister är de enda faktorer som krävs för att hantera nysyntetiserade proteiner med komplexa veckningsvägar, eller om specialiserade chaperoner behövs för mer krävande proteiner. I Studie I undersökte vi hur chaperonen Hsp70 reglerar aktiviteten hos Hsf1, en central transkriptionsfaktor som svarar på cellstress. Vi visade att Hsp70 binder direkt till Hsf1 och håller den inaktiv under normala förhållanden. När proteiner veckas fel, särskilt nysyntetiserade proteiner, frigörs Hsp70 från Hsf1, vilket aktiverar Hsf1 och resulterar i ett stressvar. Detta visar att tillgången på Hsp70 i relation till felveckade proteiner är en nyckelfaktor för att reglera Hsf1:s aktivitet. I Studie II identifierade vi en specialiserad ribosomassocierad chaperon, Chp1, som hjälper till med veckning av eEF1A, ett extremt höguttryckt protein som är avgörande för att koda mRNA och därmed syntetisera protein. När Chp1 inaktiverades spjälkades nysyntetiserad eEF1A omedelbart, vilket ledde till att proteiner aggregerade och ett Hsf1-bundet stresssvar aktiverades. Slutligen, i Studie III, förklarade vi hur Chp1 assisterar vid veckningen av eEF1A och hur olika chaperoner arbetar i en bestämd ordning för att veckla denna GTPas. Vi fann att Chp1 binder till en specifik del av eEF1A, vilket fördröjer veckningen tills en annan chaperon, Zpr1, tar över när en ny domän syntetiseras. Våra resultat ger insikt i de molekylära mekanismerna bakom kotranslationell proteinveckning och dess påverkan på proteinstabiliteten, samt hur aktiviteten hos Hsf1, den centrala faktorn för cellens svar på proteotoxisk stress, regleras.

Resumen de divulgación científica

Las proteínas son inicialmente sintetizadas como cadenas de aminoácidos (polipéptidos) extendidas que emergen del túnel de salida del ribosoma. El plegamiento de estas cadenas en su estructura tridimensional correcta es esencial para su funcionamiento, así como para garantizar la estabilidad del proteoma en la célula. Sin embargo, lograr la estructura correcta puede constituir un desafío para estas cadenas nacientes. Chaperonas generales asociadas al ribosoma ayudan en el plegamiento co-traduccional de muchos polipéptidos, pero no está claro si estos sistemas genéricos son eficientes asistiendo la producción de proteínas con rutas de plegamiento complejas, o si se requieren chaperonas especializadas. En el Estudio I, investigamos cómo la chaperona Hsp70 regula la actividad de Hsf1, un factor de transcripción que media la respuesta celular al estrés. Descubrimos que Hsp70 se une directamente a Hsf1, manteniéndolo inactivo bajo condiciones normales. En casos de acumulación de proteínas mal plegadas, Hsp70 libera a Hsf1 para unirse a estas proteínas mal plegadas, lo que activa Hsf1 e inicia una respuesta al estrés. Este hallazgo demuestra que la disponibilidad de Hsp70 en respuesta al mal plegamiento de las proteínas es clave para regular la actividad de Hsf1. En el Estudio II, identificamos una chaperona especializada asociada al ribosoma, Chp1, que ayuda en el plegamiento co-traduccional de eEF1A, una proteína crítica para la traducción de mRNA a proteínas. La ausencia de Chp1 en las células resulta en degradación rápida de eEF1A, agregación generalizada de proteínas y activación de la respuesta al estrés mediada por Hsf1. Finalmente, en el Estudio III, detallamos cómo Chp1 asiste en el plegamiento de eEF1A y el orden en el que las chaperonas de la vía de plegamiento de eEF1A actúan. Encontramos que Chp1 se une a una región específica de eEF1A, importante para la interacción de esta GTPasa con nucleótidos, lo que retrasa el plegamiento de eEF1A hasta que otra chaperona, Zpr1, toma el relevo cuando comienza la síntesis de un nuevo dominio. Nuestros resultados proporcionan una comprensión más profunda de los mecanismos moleculares del plegamiento co-traduccional de proteínas y su impacto en la estabilidad del proteoma celular, así como de la regulación de la actividad de Hsf1, el principal mediador de la respuesta celular al estrés proteotóxico.

List of publications

This thesis is based on the following studies:

I. Masser AE., Kang W., Roy J., Mohanakrishnan Kaimal J., **Quintana-Cordero J**., Friedländer MR., Andréasson C. Cytoplasmic protein misfolding titrates Hsp70 to activate nuclear Hsf1. *Elife*, 8:e47791 (2019).

II. Minoia, M.*, **Quintana-Cordero, J**.*, Jetzinger, K., Eser Kotan, I., Turnbull, KJ., Ciccarelli, M., Masser, AE., Libers, D., Gouarin, E., Czech, M., Hauryliuk, V., Bukau, B., Kramer, G., Andréasson, C. Chp1 is a dedicated chaperone at the ribosome that safeguards eEF1A biogenesis. *Nature Communications*, 15:1382 (2024).

*Contributed equally to the study

III. **Quintana-Cordero, J**. and Andréasson, C. Early folding of eEF1A: G-domain maturation and ordered chaperone interactions. Manuscript, 2025.

Abbreviations

3′UTR	3' Untranslated region
ADP	Adenosine diphosphate
A-site	Aminoacyl site
ATP	Adenosine triphosphate
Aim29	Altered inheritance of mitochondria 29
aa-tRNA	Aminoacylated transfer RNA
Chp1	Chaperone 1 for eEF1A
CHX	Cycloheximide
DNA	Deoxyribonucleic acid
eEF1	Eukaryotic elongation factor 1
eEF1A	Eukaryotic translation elongation factor 1A
eEF1A1	Eukaryotic translation elongation factor 1A isoform 1
eEF1A2	Eukaryotic translation elongation factor 1A isoform 2
eEF1B	Eukaryotic elongation factor 1B
eEF1Bα	Eukaryotic elongation factor 1B alpha
eEF1Bγ	Eukaryotic elongation factor 1B gamma
eEF2	Eukaryotic translation elongation factor 2
eEF3	Eukaryotic elongation factor 3
EF-TU	Elongation factor thermal unstable
EF-TS	Elongation factor thermo stable
eIF2	Eukaryotic initiation factor 2
eIF2B	Eukaryotic initiation factor 2B
eIF4F	Eukaryotic initiation factor 4F
eIF5	Eukaryotic initiation factor 5
eIF5B	Eukaryotic initiation factor 5B
EMSA	Electrophoretic mobility shift assay
eRF1	Eukaryotic release factor 1
eRF3	Eukaryotic release factor 3
ER	Endoplasmic reticulum
GAPs	GTPase-activating proteins
GEFs	Guanine nucleotide exchange factors

GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
GPCRs	G protein-coupled receptors
HDX-MS	Hydrogen-deuterium exchange mass spectrometry
HSE	Heat shock element
HSF1	Heat shock factor 1
HSR	Heat shock response
Hsp	Heat shock protein
JDP	J-domain protein
NAC	Nascent polypeptide-associated complex
NBD	Nucleotide-binding domain
NEFs	Nucleotide exchange factors
PIC	Pre-initiation complex
P-loop	Phosphate-binding loop
P-site	Peptidyl site
PN	Proteostasis network
PTM	Post-translational modifications
RAC	Ribosome-associated complex
RAFs	Ribosome-associated factors
SBD	Substrate-binding domain
SEC	Size exclusion chromatography
SeRP	Selective ribosome profiling
SRP	Signal recognition particle
SUMO	Small ubiquitin-like modifier
trGTPases	Translational GTPases
UPS	Ubiquitin–proteasome system
UPR	Unfolded protein response
Zpr1	Zinc finger protein 1

Contents

Introduction

Proteostasis

Proteostasis (protein homeostasis) refers to the state that the cell upholds to protect the integrity and functionality of its proteome. It operates through a rigorous molecular network termed the proteostasis network (PN). The PN looks after each individual protein in the cell, from its synthesis to its degradation, preventing and correcting off-pathway interactions that risk resulting in accumulation of toxic non-natively folded polypeptides. The wide range of effectors of the PN includes the translational machinery, molecular chaperones and cochaperones, the ubiquitin–proteasome system (UPS) and the autophagy machinery[1].

The PN exerts its protective function at early stages during protein biosynthesis by ensuring that newly synthesized polypeptides fold into their native tertiary structures[2]. However, intrinsic and external factors such as genetic variability, misfidelity during the processes of transcription and translation, abnormal protein modifications, subcellular mistargeting and the influence of different environmental stressors can result in misfolding of newly synthesized polypeptides[3–5]. Spontaneous and chaperone-assisted protein refolding helps the cell to cope with co- and post-translational protein misfolding.

Aberrantly folded polypeptides in the cytosol are cleared by quality control mechanisms of the PN which include degradation by the UPS[6,7], organellar import[8–10] and sequestration into protein aggregates[11]. These aggregates can be degraded via autophagy or serve as a cellular reservoir of already synthesized proteins upon disaggregation and refolding[12,13].

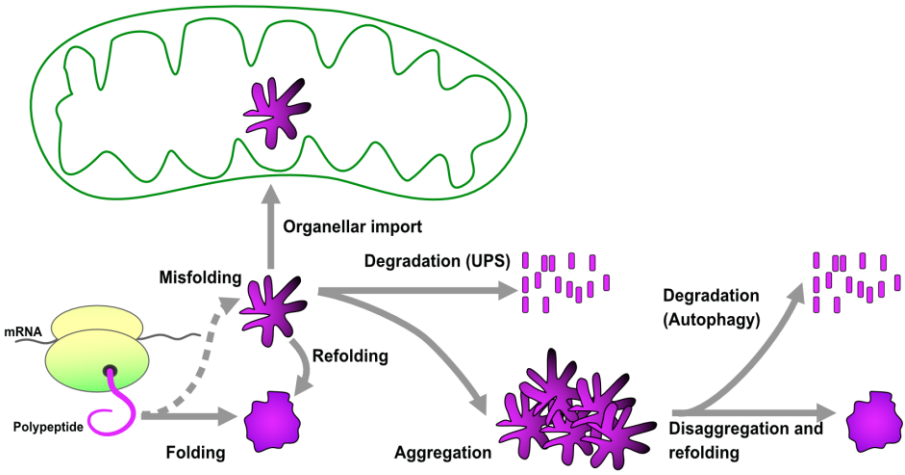

Figure 1. Model illustrating the flow of newly synthesized proteins through the proteostasis system. *Protein synthesis represents the primary influx of substrates into the proteostasis system. Newly synthesized polypeptides can fold into their native structures or misfold due to intrinsic or extrinsic factors. Misfolded proteins may be refolded or cleared through organellar import, degradation via the UPS system, or aggregation. Aggregated proteins can be disaggregated and refolded or degraded through autophagy.*

Despite the enormous cellular effort to maintain a functional proteome, acute and chronic stresses risk resulting in overload of the proteostasis system and consequently in protein imbalances (proteotoxic stress) and may jeopardize cellular fitness. Stress-induced transcriptional programs in the cell dynamically regulate the availability of the PN components to meet the demand of a proteotoxic cellular state. Among the most widely studied transcriptional programs that tune the levels of PN-effectors in response to the proteome stability are the unfolded protein response (UPR) and the heat shock response (HSR). The UPR senses the proteome stability in the endoplasmic reticulum (ER) and accordingly regulates the expression of genes involved in maintenance of the proteostasis in this organelle[14,15]. On the other hand, the HSR, counteracts the accumulation of cytosolic misfolded proteins by activation of the heat shock factor 1 (Hsf1) transcription factor which regulates the expression of genes in charge of restoring protein homeostasis in the cytosol[16–18].

Molecular chaperones

Molecular chaperones are crucial components of the PN, universally present in all cell types. They interact with proteins to assist in proper folding without becoming part of the substrate's final functional structure. Often, their mechanism of action involves binding to exposed hydrophobic or unstructured segments of an unfolded substrate, followed by its release, resulting in new opportunities for folding into the native conformation[19,20]. In the cell, the activity of an elaborate chaperone network prevents premature inter- and intramolecular interactions that would otherwise lead to protein misfolding and aggregation.

Chaperones assist protein folding starting from the moment the nascent polypeptide emerges from the ribosome by the action of ribosome-associated chaperones. In eukaryotes two examples of ribosome-associated chaperones are the ribosome-associated complex (RAC) and the nascent polypeptide-associated complex (NAC)[21]. Additionally, cytosolic chaperones that interact with a wide range of newly synthesized proteins but do not directly bind ribosomes, such as the Hsp70 chaperones, play a major role in *de novo* protein folding[22]. Hsp70 aids folding via adenosine triphosphate (ATP)-regulated cycles of substrate binding and release which necessitate the action of cochaperones such as nucleotide exchange factors (NEFs) and J-domain proteins (JDPs)/Hsp40s. Canonical Hsp70 chaperones are composed of two main functional domains: the N-terminal nucleotide-binding domain (NBD) and the substrate-binding domain (SBD). The SBD is further subdivided into two regions: the SBDα, which is rich in alpha-helical structures, and the SBDβ, composed of beta sheets[23–25]. Conformational changes driven by ATP binding and hydrolysis of Hsp70 regulate the interaction of misfolded protein substrates with the SBD. In the ATP-bound state, Hsp70 adopts an open conformation, exposing the substrate-binding site within the SBDβ for efficient substrate access. ATP hydrolysis triggers a conformational change that causes the SBDα to clamp onto the peptide-binding cleft of the SBDβ, effectively trapping the substrate[26–28].

The cochaperone Hsp40 interacts with both Hsp70 and its substrates, stimulating Hsp70's ATP hydrolysis. This hydrolysis drives the transition to the adenosine diphosphate (ADP)-bound state stabilizing the SBD closed conformation with high substrate affinity[29,30]. The recycling of Hsp70 to its ATP-

bound state is facilitated by NEFs, which accelerate the dissociation of ADP, enabling the process to restart[31–33].

The name "Hsp" stands for heat shock protein since the production of these chaperones and cochaperones is induced under stress conditions that result in an accumulation of protein misfolding such as heat shock. Over the years, numerous Hsps have been named according to their molecular weight, for example: Hsp40s (JDPs), Hsp60s (chaperonins), Hsp70s, Hsp90s, Hsp100s (AAA+ ATPases) and the small Hsps (alpha-crystallin-like proteins).

While the general chaperone systems mentioned above are sufficient to ensure the folding of a wide range of substrates, the efficient production of certain challenging clients with central roles in cellular processes, such as histones and ribosomal proteins, requires additional assistance from dedicated chaperones[34–36]. Both histones and ribosomal proteins contain basic regions that render them prone to detrimental interactions. Specialized chaperones bind to histones, shielding them from nonspecific interactions, assisting in their deposition onto DNA during nucleosome assembly, and regulating their degradation[35]. Similarly, specialized chaperones for ribosomal proteins bind to these substrates, preventing nonspecific interactions and facilitating their incorporation into pre-ribosomal particles during ribosome biogenesis[36,37]. Moreover, the binding of dedicated chaperones to ribosomal proteins regulates client production at the mRNA expression level. In this way, dedicated chaperones coordinate the expression rates of ribosomal proteins with the cell's ribosome assembly capacity safeguarding proteostasis[38]. Recent studies have further uncovered specialized chaperones involved in the *de novo* folding of guanosine triphosphatases (GTPases) of the translational machinery, such as eukaryotic translation elongation factor 1A (eEF1A) and eukaryotic translation elongation factor 2 (eEF2)[39–42]. These findings highlight the essential role of specialized chaperones in facilitating the proper folding, production, and function of complex client proteins that are central to cellular activity.

In addition to their role in folding newly synthesized proteins, chaperones play a critical role in refolding misfolded substrates and in other cellular processes that safeguard proteome stability, including protein transport, degradation of misfolded proteins, disaggregation of protein aggregates, and the assembly of macromolecular complexes[43].

Hsf1 transcription factor

Hsf1 is a conserved transcription factor that acts as the main mediator of the cellular response to proteotoxic stress in eukaryotes. The accumulation of misfolded proteins promotes activation of Hsf1, which in turn induces expression of genes involved in attenuating the proteotoxic state most notably chaperones, cochaperones, aggregation and disaggregation factors[44]. Once proteostasis is restored, Hsf1 becomes inactive, demonstrating a negative feedback loop of regulation. Environmental stressors that negatively affect protein stability including heat, oxidative stress and glucose starvation trigger activation of Hsf1 and unleash the Hsf1-mediated cellular response[45–48]. Thus, Hsf1 is the master regulator that adapts the PN to protein misfolding stress load in the cell.

Hsf1 structure and function

Homologous genes of Hsf1 across different organisms possess conserved key-functional domains. Hsf1 orthologues encoded in human and yeast, both contain a DNA-binding domain (DBD) followed by a three leucine zipper motif (HR-A/B), a regulatory domain (RD) and a C-terminal transactivation (CTA) domain that contains an additional leucine zipper motif known as HR-C[49–55]. Additionally, the yeast-encoded Hsf1 contains two distinctive structural features which are not present in the human homolog: A N-terminal transactivation (NTA)[56] domain and a sequence known as the control element 2 (CE2) in its RD[57]. While monomeric Hsf1 has a very low affinity for DNA, the trimeric conformation of the protein shows gain of DNA binding capacity[58]. Hsf1 trimerization into an active triple-stranded α-helical coiled-coil structure is achieved via hydrophobic interactions between the HR-A/B regions of different monomers[59,60]. The trimeric nuclear form of Hsf1 binds the promoter of target genes via its DBD. DNA binding specifically takes place on conserved DNA sequences of three inverted repeats of nGAAn known as heat shock elements (HSEs)[61,62]. The CTA domain is required for transcriptional activation of the Hsf1 target genes and for regulation of the scope of the Hsf1 activity[52,55]. The RD domain is rich in serine and threonine and can undergo a series of inducible post-translational modifications (PTM) such as phosphorylation, sumoylation, and acetylation which has been associated with regulation of the

Hsf1 activity[63]. The unique features of the yeast-encoded Hsf1, the CE2 sequence and the NTA domain, have been associated with negative and positive regulation of the Hsf1 basal activity respectively[56,57,64].

Hsf1 regulation

Hsf1 is a highly conserved eukaryotic transcription factor with a central role in the response to protein imbalance in the cell. Dysregulation of Hsf1 activity in humans has been associated with proteostasis disorders such as cancer and neurodegenerative diseases highlighting the importance of understanding the molecular mechanisms underlying Hsf1 activation and regulation[65–67].

In the cell, Hsf1 is constitutively expressed independently of the cellular proteostatic state which ensures that already made Hsf1 is always available to provide an instant response to stressors. The constitutive expression of Hsf1 rules out a mechanism of transcriptional regulation. Instead, Hsf1 activity is regulated through multiple layers of post-translational mechanisms including oligomerization, spatial sequestration, PTM and chaperone mediated repression[64].

Hsf1 activation requires oligomerization into an active trimeric form with high affinity for DNA[50,51,58]. In addition, binding to the DNA promoter sequence of target genes requires nuclear localization of the trimeric Hsf1[58,68]. In yeast, Hsf1 is constitutively trimeric and nuclear, which accounts for a basal level of Hsf1 activity that ensures expression of target chaperones such as Hsp70 and Hsp90 at all times[69]. In mammals, on the other hand, under normal growth conditions, Hsf1 exists in its inactive monomeric form in the cytoplasm and proteotoxic stress drives its relocalization to the nucleus and its trimerization. Thus, in mammalian cells Hsf1 activity has been considered to be regulated by readjusting its quaternary structure between inactive monomers and active trimers in a stress-responsive manner as well as by shuttling of the protein between the cytoplasm and the nucleus[58]. Conformational studies of the human Hsf1 trimerization process by hydrogen-deuterium exchange mass spectrometry suggest that heat stress induces unfolding of the HR-C region within Hsf1[70]. The HR-C region can form intramolecular interactions with the oligomerization motif HR-A/B preventing Hsf1 from trimerizing and acting in this way as a negative regulator of the Hsf1 activity. The observed temperature-dependent conformational changes in HR-C are thought to impair

its repressive interaction with the HR-A/B motif and thus, facilitate Hsf1 trimerization and activation. The same study shows that HR-C unfolding and the temperature-induced Hsf1 trimerization are dependent on the Hsf1 concentration. This last finding supports the notion of nuclear accumulation of Hsf1 in mammalian cells as a cellular regulatory mechanism of its activity.

While trimerization and spatial distribution constitute Hsf1 regulatory mechanisms that are specific to mammalian cells, other regulatory tools such as PTM and regulation of Hsf1 activity via chaperone titration are common among all organisms.

PTM such as phosphorylation, sumoylation, acetylation and ubiquitination have been associated with regulation of Hsf1 activity[71]. Phosphorylation is the most extensively studied PTM of Hsf1 and has been linked to both up and downregulation of its activity. For example, phosphorylation of the residues S326 or S230 within the RD is associated with activation of Hsf1 while phosphorylation of residues S303 or S307 is associated with decreased Hsf1 transactivation capacity[64,72–76]. The majority of known phosphorylation sites of the human Hsf1 are located in the RD, suggesting that PTM-signatures of this domain could be associated with its regulatory function. Phosphorylation also drives an indirect PTM-regulatory mechanism by inducing sumoylation of Hsf1. More specifically, Hsf1 phosphorylation can promote SUMO conjugation to phosphorylation-dependent sumoylation motifs (PDSMs) in the protein[77,78]. Sumoylation, acetylation and ubiquitination of Hsf1 take place on lysine residues. Lysine-ubiquitination has been linked to targeting of the protein for degradation via the UPS and thus, competitive lysine-sumoylation and acetylation has been proposed to prevent Hsf1 from proteasomal turnover[79]. Additionally, Hsf1 lysine-sumoylation and acetylation have been suggested to attenuate the activity of the transcription factor under non-stressful conditions[64]. Although all the above mentioned PTM seem to play a role in the dynamic regulation of Hsf1 activity, they do not appear to be essential for the functionality of the transcription factor[80].

Several studies suggest that Hsf1 activity is additionally regulated by a negative feedback mechanism that involves titration of its own target chaperones[80–83]. The chaperone titration model proposes that chaperones bind Hsf1 under basal conditions forming a complex that inhibits its activity. According to this hypothesis, proteotoxic stress results in the accumulation of misfolded proteins that titrate away the limited pool of chaperones, freeing Hsf1 from

the repressive binding. This allows expression of the Hsf1 target genes which include the repressor chaperones themselve. Once cellular proteostasis is restored, the lower levels of misfolded proteins result in substrate-free chaperones that bind Hsf1 again and decrease its activity, thus attenuating the HSR.

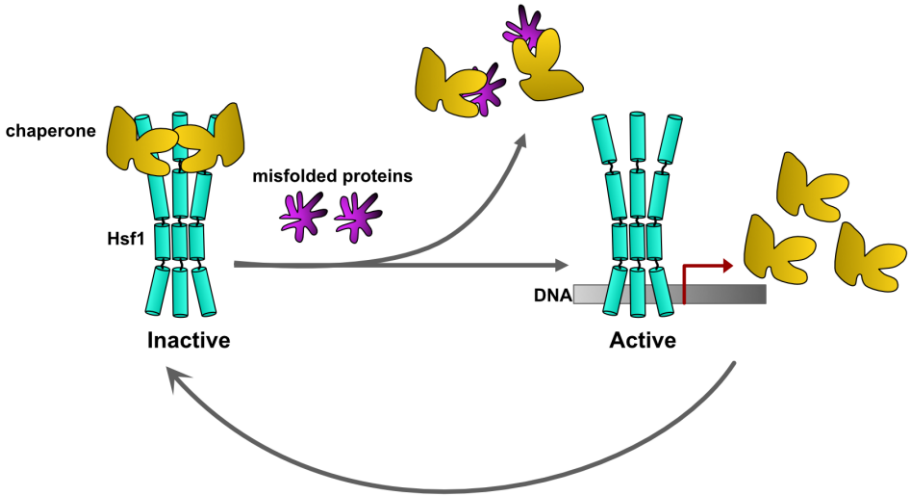

Figure 2. Model illustrating Hsf1 regulation through misfolded protein-mediated chaperone titration. *Under basal conditions, chaperones bind to Hsf1, keeping it inactive. Upon the accumulation of misfolded proteins as a result of proteotoxic stress, the limited pool of chaperones is titrated away through interactions with the misfolded proteins, thereby releasing Hsf1. This release activates Hsf1, leading to increased transcription of its target genes, including those encoding its repressor chaperones. The resulting elevated chaperone levels restore binding to Hsf1, attenuating its activity.*

Identifying the titratable chaperones that regulate the activity of Hsf1 has been the focus of attention of numerous studies over the years. Although both Hsp90 and Hsp70 have been involved in the regulation of Hsf1, growing evidence favors the notion that specifically titration of Hsp70 in response to cellular levels of misfolded proteins regulates the availability of active Hsf1. For example, Hsp70 has been found to interact with Hsf1 in a wide range of organisms including plants, yeast and human cell lines[80,81,84,85]. The interaction between Hsp70 and Hsf1 appears to be hampered upon proteotoxic stress. Additionally, the Hsp70-Hsp40 chaperone-cochaperone system has been linked to repression of Hsf1 activity[86]. The above mentioned observations are some examples in support of a model in which Hsp70 acts as a stress-dependent

titrable repressor of Hsf1 activity. When this study was initiated convincing evidence for Hsp70 as the key regulator of Hsf1 were pending.

In summary, a set of post-translational regulatory mechanisms fine-tunes Hsf1 activity in response to the cell's proteostasis state, enabling Hsf1 to function as an efficient stress sensor and responder.

Ribosome-associated chaperones

Biological activity of newly synthesized proteins is dependent on their folding into the functional native conformation and on their targeting and migration to the appropriate subcellular compartment. These processes are initiated at an early stage during protein translation[87]. In both, prokaryotes and eukaryotes, the architecture of the ribosomal tunnel which traverses the large subunit of the ribosome allows accommodation of peptides in their extended conformation or in simple secondary structures without long distance interactions, for example, α-helix arrangements[88–90]. Once the nascent chain has exited the ribosomal tunnel, a set of factors that directly bind the ribosome, interact with signature segments of the emerging peptide typically based on hydrophobicity patterns[91]. This multifunctional group of factors in eukaryotes includes for example, N-terminal processing enzymes such as peptidases and acetyltransferases that help in early steps of protein maturation[92–94] as well as targeting factors with a central role for the proper cellular functioning. For instance, the targeting factor known as signal recognition particle (SRP) ensures targeting of membrane and secretory proteins to the ER for further sorting to the functional subcellular localizations[95]. Additionally, molecular chaperones that bind the ribosome near the ribosomal tunnel exit also form transient interactions with the nascent polypeptides and are referred to as ribosome-associated chaperones[21].

The ribosome-associated complex

In eukaryotes two main ribosome-associated chaperone complexes support the early stage of *de novo* folding of proteins. One of them is known as the Hsp70/40 system which includes the ribosome-associated complex, RAC, as a conserved element of the system which is present in all eukaryotic cells.

RAC itself is a heterodimeric complex, which in yeast is formed by the non-canonical Hsp70 chaperone Ssz1 and the Hsp40 cochaperone Zuo1[96]. In contrast to canonical Hsp70s, Ssz1 does not engage in productive ATP-regulated cycles of substrate binding and release. This functional distinction is attributed to Ssz1's limited ATP hydrolysis activity, despite its ability to bind ATP. Furthermore, Ssz1 lacks the SBDα subdomain, which is critical for the regulation of substrate interactions[97,98]. The function of RAC in *de novo* folding of proteins in fungal species, such as yeasts, is complemented by interaction with the ribosome-associated Hsp70 chaperones of the Ssb class[99]. ATP-bound Ssb interacts with the NBD of Ssz1 positioning the canonical chaperone in proximity to the J-domain of Zuo1 and to nascent chains. This spatial arrangement enables Zuo1's J-domain to stimulate ATP hydrolysis on Ssb, promoting the trapping of nascent polypeptide substrates. As a result, Ssb directly interacts with the ribosome and dissociates from Ssz1, allowing Ssz1 to bind a new ATP-bound Ssb and continue the folding cycle[100]. Interestingly, Zuo1 has been shown to interact with both the 40S and 60S ribosomal subunits. Its interaction with the 40S subunit occurs near the decoding center, while its interaction with the 60S subunit is near the polypeptide exit site. This suggests an important role for Zuo1 in coordinating mRNA translation and nascent chain folding[101].

The nascent polypeptide-associated complex

The second system that assists the early stages of protein folding on the ribosome in eukaryotes is NAC. NAC is a highly conserved protein complex that directly interacts with the ribosome in a stoichiometric manner and has been attributed chaperone functions[102–104]. This system forms transient interactions with a wide range of structurally diverse emerging peptides[105]. In archaea NAC is present as a homodimer formed by two α-subunits[106]. However, the most widespread NAC system architecture found in yeast and higher eukaryotes is a heterodimeric complex formed by the association of an α-subunit and a β-subunit[21,107,108]. Structural studies of the NAC complexes suggest that homo- or heterodimers are formed by interaction between the six-stranded β-barrel-like NAC domains present in both subunits[106,108]. Both α and β have been found to interact with the nascent polypeptides while the β subunit has been reported to be the main responsible for anchoring NAC to the ribosome via its N-terminal RRK(Xn)KK ribosome binding motif[109,110]. Such interac-

tion with the ribosome appears to be crucial for substrate binding. The presence of an ubiquitin-associated (UBA) domain covering approximately 40 amino acids of the C-terminal sequence of α-NAC defines a structural difference with respect to β-NAC and suggests some specialization of function[106]. Deletion of the domain has been associated with lower levels of protein aggregates, suggesting a role for the UBA domain in negatively regulating the chaperone capacity of the complex[111]. Additionally, it has been shown that the UBA domain of α-NAC plays an important role in coordinating the recruitment of SRP to nascent chains harboring ER-targeting signal sequences[112].

From a general perspective, NAC has been proposed to carry out a broad range of functions interconnecting protein synthesis, protein folding and organellar targeting. Several studies support the role of NAC in folding of the nascent polypeptide chains into the appropriate conformation while protecting them against proteolysis and premature, deleterious folding[103–105]. NAC has also been reported to regulate the interaction of nascent polypeptides with other ribosome-associated factors (RAFs) such as N-terminal processing enzymes and the SRP, modulating protein maturation and the route of membrane and secretory proteins export[107,112,113]. In addition, there is evidence supporting NAC involvement in targeting of mitochondrial proteins into the mitochondria[114,115]. Interestingly NAC has also been reported to be involved in ribosome biogenesis which suggests a potential function in coordinating the activity of the translation machinery and the co-translational folding capacity in the cell[116]. Although NAC plays a role in maintaining proteostasis, its exact functioning is not fully understood, as the mechanism by which NAC assists in protein folding and its interplay with other RAFs beyond the SRP remain unclear.

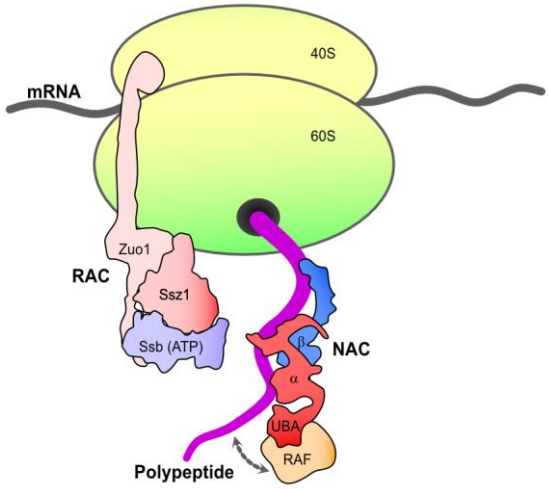

Figure 3. Model illustrating the general ribosome-associated chaperone systems in eukaryotes. *Two general ribosome-associated chaperone systems, RAC and NAC, assist in the de novo folding of most nascent polypeptides in eukaryotes. RAC consists of the non-canonical Hsp70 Ssz1 and the Hsp40 cochaperone Zuo1. In yeast, RAC helps to position the canonical Hsp70 ribosome-associated chaperone Ssb near nascent polypeptide substrates. NAC is a heterodimer composed of the subunits α-NAC and β-NAC. The α-NAC subunit contains a specialized UBA domain at its C-terminus, which regulates access of RAFs to the nascent polypeptides.*

Both RAC and NAC are general chaperones that assist in the folding of a wide range of nascent substrates at the ribosome. However, the proteostasis system also employs specialized chaperones to facilitate the *de novo* folding of essential and abundant proteins that are challenging to fold. Recently, it has been found that specialized cytosolic chaperones play a role in the *de novo* folding of the highly abundant multidomain GTPases eEF1A and eEF2[39–42]. It is reasonable to hypothesize that the proteostasis system has evolved substrate-specific pathways to fold demanding clients, such as these GTPases of the translational machinery. Perhaps these dedicated folding pathways act at early co-translational steps of protein synthesis through specialized ribosome-associated chaperones?

GTPases

GTPases are ubiquitous hydrolytic enzymes that catalyze the hydrolysis of guanosine triphosphate (GTP) to guanosine diphosphate (GDP)[117–119]. They act as molecular switches, mediating essential cellular processes such as intracellular signal transduction, cell division and differentiation, and protein synthesis[117,118,120–122]. Despite the functional versatility of GTPases, their underlying switching mechanism relies on a common principle: alternating between two stable, ligand-induced conformational states. These states include an active GTP-bound form, which enables interaction with partner effectors, and an inactive GDP-bound form, which leads to effector dissociation[123–125]. The transition between these states is typically regulated by accessory factors that stimulate activation or inactivation. For instance, guanine nucleotide exchange factors (GEFs) accelerate the exchange of GDP for GTP, thereby regenerating the active state[126,127]. Conversely, GTPase-activating proteins (GAPs) promote GTP hydrolysis, driving the GTPase into its inactive state[128,129].

Common to the structure of all GTPases is the evolutionarily conserved G-domain, where the catalytic site is located. Within the G-domain, five conserved motifs, named G1 to G5, are essential for nucleotide binding and hydrolysis[123,130–134]. The G1 motif, characterized by the presence of the consensus sequence GXXXXGKS/T and also known as the phosphate-binding loop (P-loop), interacts with the phosphates of the guanine nucleotides[130–134]. The G2 and G3 motifs, or so-called switch I and switch II regions, respectively, are flexible regions that undergo structural rearrangements when switching between the GTP- and GDP-bound states[123,134]. Switch I and II play a crucial role in guanine nucleotide loading as they interact with the γ-phosphate and participate in coordinating the Mg^{2+} ion, which is an essential cofactor for guanine nucleotide binding[123,131,134]. Additionally, the switch I region is important for hydrolysis, as it can serve as an interaction target for GAPs[135]. Furthermore, a conserved glycine or histidine residue in the switch II contacts the catalytic water molecule playing a critical role in GTP hydrolysis[136]. The mechanism leading to structural differences on the switch regions in the presence of GTP compared to GDP is hypothesized to be common among GTPases, based on the structural similarities of their G-domains. In the presence of GTP, hydrogen bonds are formed between the γ-phosphate oxygens

and the main chain NH groups of a conserved threonine in switch I and a glycine in the conserved DXXG motif of switch II. Hydrolysis of GTP to GDP and the consequent loss of the γ-phosphate are believed to result in the relaxation of the switch regions into the GDP-bound specific conformation[133,134,136]. While specific guanine nucleotide-induced structural rearrangements of the G-domain, particularly at the switch regions, are common to all G-proteins, the extent of these modifications can vary depending on the specific protein. Furthermore, the extension of the switch regions also differs among various GTPases. The G4 (containing the conserved NKXD sequence) and G5 (containing the conserved SAK sequence) elements are involved in guanine base recognition and binding contributing to guanine nucleotide specificity[133,134,136,137].

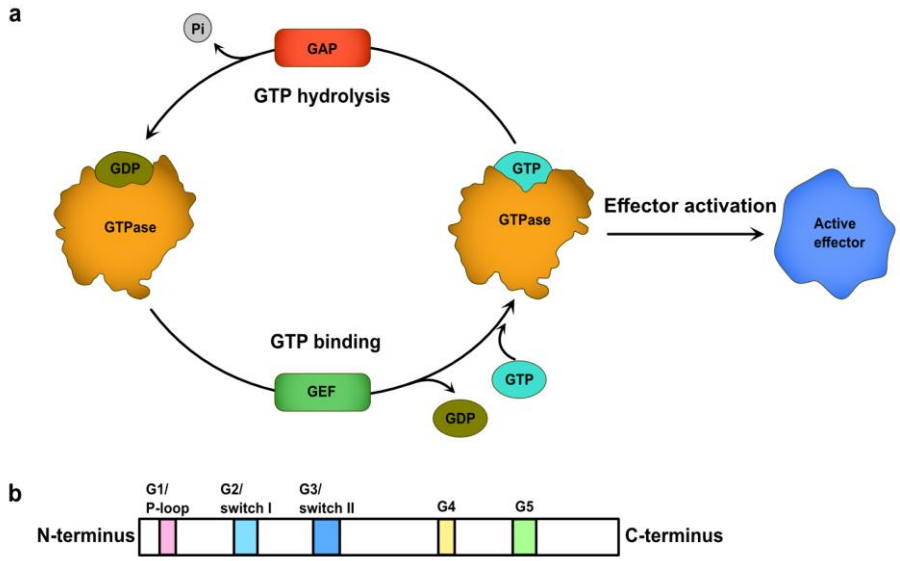

Figure 4. GTPase cycle and conserved G-domain motifs. a *GTPases cycle between the inactive GDP-bound state and the active GTP-bound state, which activates a partner effector. GAPs promote GTP hydrolysis, facilitating the GDP-bound inactive state, while GEFs facilitate GDP dissociation, promoting the active GTP-bound state.* ***b*** *Five conserved motifs (G1 to G5) in the G-domain of GTPases are critical for nucleotide binding and hydrolysis.*

In conclusion, GTPases are highly versatile molecular switches that regulate a wide range of essential cellular processes through a conserved mechanism of alternating between active (GTP-bound) and inactive (GDP-bound) states. Their mechanism of action relies on common structural elements within the conserved G-domain, which is critical for GTP binding and hydrolysis.

GTPase superfamilies

Heterotrimeric G-proteins

Eukaryotic cells express a wide variety of GTPases, among which three major superfamilies have been identified: the heterotrimeric G proteins, the small Ras-like GTPases, and protein-synthesizing or translational GTPases (trGTPases). Heterotrimeric G proteins are involved in sensing extracellular signals and converting them into intracellular responses by coupling receptor activation to downstream effectors in signaling pathways[138]. Heterotrimeric G proteins consist of three subunits: α, β, and γ where the Gα subunit is responsible for guanine nucleotide binding and exhibits the GTPase activity[138–140]. In their inactive state, these proteins are GDP-bound and form a complex with G protein-coupled receptors (GPCRs). When GPCRs detect extracellular stimuli, they act as GEFs, facilitating the exchange of GDP for GTP in the Gα subunit. This activation results in the dissociation of the heterotrimer into a GTP-bound Gα subunit and a βγ dimer, both of which interact with downstream effector proteins[141–144]. Hydrolysis of GTP to GDP by the Gα subunit restores the heterotrimeric complex, terminating the signaling event and resetting the system for subsequent activation.

Small Ras-like GTPases

The small Ras-like GTPase superfamily comprises a large group of structurally homologous GTPases, typically ranging in size from 21 to 30 kDa [131]. The superfamily is further classified into functional subfamilies, with some of the most studied being Ras, Rho, Rab, Arf, Ran, Miro, and BGR. Collectively, these subfamilies contribute to a wide range of cellular processes, including morphogenesis, cell polarity, membrane trafficking, oxidative stress response, apoptosis, secretion of metabolites, nuclear envelope assembly, mitochondrial integrity, and many others[145,146]. Like all GTPases, their function relies on the alternation between the active GTP-bound and inactive GDP-bound conformations[123]. The intrinsic GDP-to-GTP exchange rate and the GTPase activity of these small GTPases are very low and incompatible with their roles in rapid signal propagation. Hence, and not surprisingly, the GTP binding and hydrolysis cycles of many Ras-like GTPases are tightly regulated by GEFs, which

promote GDP dissociation, and GAPs, which enhance GTP hydrolysis[128,147,148]. Thus, small Ras-like GTPases represent a heterogeneous group of structurally related proteins that are essential for cellular function, regulating nearly all cellular processes through their roles in mediating signal transduction.

Translational GTPases

The trGTPase superfamily comprises a group of GTPases that play essential roles in protein biosynthesis, a process in which mRNA is translated into protein at the ribosome[120,137,149,150]. This process unfolds in four distinct phases: initiation, elongation, termination, and recycling. trGTPases collaborate with the ribosome to regulate each of these steps, maintain translational fidelity, coordinate cellular resource availability with protein production, and ensure optimal rates of protein synthesis[137].

trGTPases are typically multidomain proteins. Like all GTPases, they contain a G-domain responsible for nucleotide binding and hydrolysis. However, the structural homology of trGTPases extends beyond the G-domain, as many possess at least one β-barrel domain downstream of the G-domain[151–155]. For some trGTPases, the relative positioning of these β-barrel domains with respect to the G-domain shifts significantly between the GTP- and GDP-bound conformations.

Most trGTPases, with the exception of eIF2 (which only binds to the small ribosomal subunit), interact with the large ribosomal subunit at the GTPase-associated center to perform their functions during the process of mRNA translation[137]. A notable feature of many trGTPases is that the ribosome itself often acts as their GAP, thereby controlling the timing of GTP hydrolysis during protein synthesis[137,156].

trGTPases in translation initiation

During translation initiation, the small and large ribosomal subunits must assemble on the target mRNA and the initiator Met-tRNA (Met-tRNA$_i$) must pair with the mRNA start codon in the peptidyl site (P-site) of the ribosome for accurate decoding. The entire process is aided by initiation factors[157,158]. In eukaryotic cells, canonical translation initiation begins with the activation of the mRNA to be translated. This involves the binding of the eukaryotic initi-

ation factor 4F (eIF4F) complex and other associated initiation factors to signature elements of the mRNA molecule, such as the 5′ cap and the poly(A) tail[157–159]. The trGTPase eIF2, in its active GTP-bound form, binds the Met-$tRNA_i$ to form a ternary complex (eIF2-GTP-Met-$tRNA_i$). This ternary complex assembles with the 40S ribosomal subunit and additional initiation factors to form the 43S pre-initiation complex (PIC)[157,158]. The 43S PIC then associates with the activated mRNA, resulting in the formation of the 48S PIC. This complex scans along the mRNA until an AUG start codon is encountered. Positioning the AUG start codon in the P-site of the 40S subunit triggers GTP hydrolysis by eIF2, facilitated by the initiation factor eIF5 which acts as a GAP[160,161]. Following GTP hydrolysis, GDP-bound eIF2 loses affinity for the Met-$tRNA_i$ and dissociates from the preinitiation complex[162]. The GEF, eIF2B subsequently promotes the dissociation of GDP from eIF2, restoring its active GTP-bound state for another round of initiation[163]. Ultimately, the AUG codon- Met-$tRNA_i$ anticodon complementation induces a major conformational change in the preinitiation complex[164]. This conformational shift halts the scanning process and stabilizes the binding of Met-$tRNA_i$ at the P-site, ensuring that translation initiation begins at the correct codon. Another GTP-bound trGTPase, eIF5B-GTP, binds to the preinitiation complex, triggering the release of initiation factors and the recruitment of the 60S ribosomal subunit, thereby forming the 80S initiation complex[165]. The formation of this complex leads to hydrolysis of the eIF5B-associated GTP and the release of eIF5B-GDP. With the release of this last initiation factor the 80 S elongation complex is fully assembled and the elongation cycle is now primed to begin.

trGTPases in translation elongation

Translation elongation refers to the multistep process that follows translation initiation, during which new amino acids are incorporated at the C-terminus of a growing polypeptide chain during protein biosynthesis. This process can be divided into three main stages: mRNA decoding, peptide bond formation, and ribosomal translocation along the mRNA[158].

During mRNA decoding a new aminoacylated-tRNA (aa-tRNA) is selected based on codon-anticodon complementarity and introduced into the ribosome's A-site. In eukaryotes the delivery of aa-tRNAs to the A-site of the ribosome during the decoding phase of elongation is catalyzed by the eukaryotic elongation factor 1 (eEF1) complex[166,167]. The eEF1 complex includes the

trGTPase eEF1A which is the homolog of the bacterial EF-TU and the eukaryotic translation elongation factor 1B (eEF1B), homolog of the bacterial protein EF-TS[167,168]. Correct pairing between mRNA codon and aa-tRNA anticodon leads to peptide bond formation between the newly introduced amino acid and the one located in the ribosome's P-site. This reaction is catalyzed by the peptidyl transferase center in the the ribosome's large subunit[169].

After the addition of an amino acid to the nascent chain, another trGTPase, eEF2, catalyzes the ribosome's downstream one-codon translocation along the mRNA, positioning the next codon in the A-site and preparing the translational machinery for the next elongation cycle[170–172]. In yeast, the translocation step also requires the essential eukaryotic elongation factor 3 (eEF3), which promotes dissociation of the deacyl-tRNA from the ribosomal E-site[173]. Cycles of amino acid incorporation and ribosome translocation extend the growing polypeptide one amino acid at a time until the ribosome encounters a stop codon, signaling the end of elongation and the onset of translation termination and ribosome recycling.

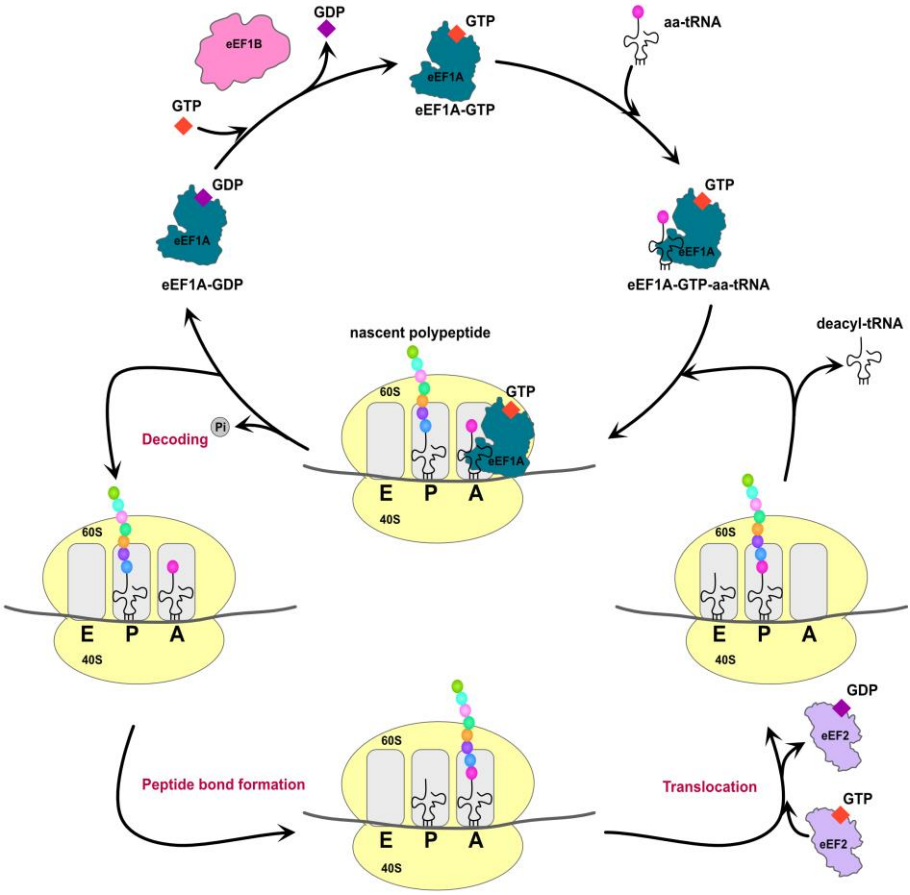

Figure 5. Model illustrating the translation elongation phase of protein synthesis. *During translation elongation, GTP-bound eEF1A forms a complex with aa-tRNA and delivers it to the A site of the ribosome. Recognition of the correct codon-anticodon complementation induces ribosomal conformational changes that stimulate eEF1A GTPase activity. Upon GTP hydrolysis, eEF1A-GDP leaves the ribosome, releasing the aa-tRNA into the A site, a process known as decoding. This is followed by peptide bond formation, catalyzed by the ribosome. Subsequently, the eEF2 GTPase facilitates ribosomal translocation along the mRNA, resetting the translational machinery for the next elongation round. eEF1A-GDP is recycled to its GTP-bound form by the action of the GEF, eEF1B.*

trGTPases in translation termination and recycling

Upon positioning of a stop codon in the ribosome's A site, the translation termination process and recycling begin. In eukaryotes, this process is mediated

by eukaryotic release factor 1 (eRF1), the trGTPase eukaryotic release factor 3 (eRF3), and the ribosome recycling factor ABCE1[158,174]. eRF1 is a tRNA-shaped protein that can enter the ribosome's A site when a stop codon is positioned there[175]. eRF1 forms a complex with GTP-bound eRF3. The eRF1-eRF3-GTP ternary complex recognizes the stop codon, which leads to GTP hydrolysis on eRF3 and dissociation of eRF3-GDP from the complex[176]. Following this, a domain rearrangement of eRF1 enables catalytic motifs of the protein to contact the peptidyl transferase center, resulting in peptide release[158,174]. Alternatively, ABCE1 can bind to eRF1 after dissociation of eRF3 and stimulate eRF1-mediated peptide release[174]. Finally, both eRF1 and ABCE1 collaborate to achieve dissociation of the ribosome into its small and large subunits[158,174].

A set of abundant soluble factors, including trGTPases, are crucial components of the complex translational machinery, alongside the ribosome, mRNAs, and aa-tRNAs. These factors catalyze essential reactions at every stage of protein synthesis. Thus, tight regulation of the level and quality of these translation factors from the very beginning of their production is essential to ensure proteome integrity in the cell.

Table1: Eukaryotic trGTPases and their specific roles in mRNA translation.

Translation process	trGTPase	Function
Initiation	eIF2	Delivers Met-tRNA$_i$ to the PIC
	eIF5B	Facilitates the recruitment of the 60S ribosomal subunit
Elongation	eEF1A	Delivers aa-tRNA to the ribosome's A-site for decoding
	eEF2	Catalyzes ribosomal translocation along the mRNA
Termination and recycling	eRF3	Stimulates eRF1-mediated translation termination

eEF1A

The translation factor eEF1A is an essential nucleotide-binding protein consisting of three domains. The largest domain, Domain I (G-domain), possesses guanine nucleotide-binding and GTPase activity. The G-domain is followed by two β-barrel domains[152]. GTP-bound eEF1A binds aa-tRNAs and delivers them to the A site of the ribosome for mRNA decoding during the process of translation elongation[124,177–179]. Upon recognition of a proper codon-anticodon interaction, conformational changes in the ribosome, which acts as a GAP, induce the GTPase activity of eEF1A, leading to the hydrolysis of GTP to GDP. GDP-bound eEF1A is then released from the ribosome[166,170,180]. This coordinated interplay between the ribosome and eEF1A activity, based on codon-anticodon complementarity, ensures a high degree of fidelity during the translation elongation process, which is critical for accurate protein synthesis. The spontaneous dissociation of GDP from eEF1A is rather slow (about 0.1 s−1)[126]. In the cell the GEF, eEF1B, facilitates the exchange of GDP for GTP on eEF1A, hence recycling the active form of eEF1A for a new round of elongation[126]. In yeast, eEF1B is present as a heterodimer composed of an α (eEF1Bα) and a γ (eEF1Bγ) subunit. The C-terminal domain of the eEF1Bα subunit is responsible for the interaction with eEF1A and possesses the nucleotide exchange activity and thus, constitutes the GEF catalytic domain[152,166,168,181].

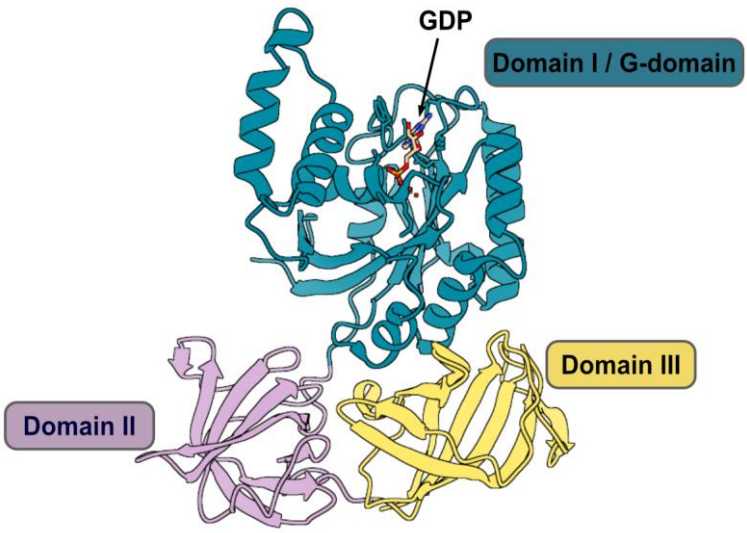

Figure 6. Cartoon 3D structural representation of GDP-bound eEF1A (PDB: 1IJE). *The G-domain (Domain I), Domain II, and Domain III are shown in teal, purple, and yellow, respectively.*

In addition to its canonical role in translation elongation, eEF1A has been linked to a plethora of moonlighting functions, not all very well understood. The translation factor has been, for example, associated with export of both aa-tRNA and proteins from the nucleus[182–184]. Numerous studies suggest that eEF1A binds actin filaments and promotes their bundling *in vitro* and *in vivo* thus, serving as a modulator of the actin cytoskeleton organization[185–189]. A surprising non-canonical function suggested for eEF1A is in quality control of newly synthesized proteins. The first evidence reported was the identification of eEF1A as a factor required for the ubiquitin-dependent degradation of N-α-acetylated proteins[190]. Furthermore, eEF1A was shown to associate with both nascent polypeptide chains and non-ribosome-associated unfolded polypeptides, as well as to aid in the folding of denatured proteins in an *in vitro* setup [191]. Additionally, eEF1A has been found to interact with ubiquitinated proteins and the proteasome upon release of truncated nascent polypeptide chains. This led to the hypothesis that eEF1A is involved in clearance of damaged newly synthesized proteins via the proteasome[192]. A study from 2014 suggests a direct role for eEF1A in the regulation of the Hsf1 activity in mammalian cells[193]. Upon stress, mammalian isoform 1 of eEF1A was found to recruit Hsf1 to the *HSP70* promoter, hence promoting its transcription. eEF1A then, appeared to associate with the 3′UTR region of *HSP70* mRNA and with the elongating RNA polymerase II stabilizing the elongating mRNA and aiding its transport from the nucleus to the ribosome. This study proposed that eEF1A couples chaperone expression to the translation needs of the cell during the HSR.

In summary, the highly abundant eEF1A plays an essential role in mRNA translation elongation and may coordinate protein synthesis with other key cellular processes.

Human eEF1A and its role in health-related conditions

Given the central functions of eEF1A in the cell, it is not surprising that misregulated production and mutations of this translation factor have been associated with human pathologies and disorders. In humans, two predominant

isoforms of the eEF1A protein, eEF1A1 and eEF1A2, are expressed from the paralogous genes *EEF1A1* and *EEF1A2*, located on chromosomes 6q14 and 20q13.3, respectively[194]. These isoforms share over 90% sequence homology at the amino acid level and both function in the canonical role of translation elongation. However, they exhibit distinct expression patterns. During development, eEF1A1 is ubiquitously expressed in all cell types, but its expression in neurons and muscle is replaced by eEF1A2 expression postnatally. Upon maturation, their expression becomes tissue-specific and is typically mutually exclusive, with neurons, skeletal muscle and heart cells exclusively expressing the eEF1A2 isoform[195–199]. Despite the relatively small differences in the primary structures of the eEF1A isoforms, they lead to notable conformational and functional divergences. For instance, eEF1A2 exhibits a higher nucleotide preference ratio (GDP/GTP) compared to eEF1A1[200]. Additionally, properties such as PTMs and self-association capacity differ between the isoforms[201,202].

Deregulated expression of the eEF1A2 isoform, in particular, has been linked to various pathologies. For instance, while eEF1A2 is not expressed in normal breast tissue, it is strongly upregulated in breast tumors. Similarly, eEF1A2 is absent in normal ovarian tissue but is upregulated in ovarian cancer, with the level of upregulation correlating with poor prognosis. High levels of eEF1A2 expression have also been observed in lung adenocarcinoma cell lines derived from lung cancer patients. Furthermore, eEF1A2 is upregulated in liver and pancreatic cancers[203,204]. A study in 2020 revealed that deregulation of the eEF1A2 isoform, specifically at the level of protein stability and turnover, is associated with colorectal cancer. The study demonstrated that the interaction between eEF1A2 and the protein SNX16, which is highly upregulated in colorectal cancer, prevents ubiquitination and degradation of eEF1A2, leading to downstream activation of the tumorigenic c-Myc signaling pathway[205].

The mechanism by which deregulation of eEF1A2 expression contributes to cancer may extend beyond its role in translation elongation. eEF1A2 is also directly involved in activating oncogenic pathways, such as the JAK/STAT and Akt signaling pathways[204,206]. These pathways promote cell survival, proliferation, and the inhibition of apoptosis, thereby driving cellular transformation and oncogenesis.

While upregulation of the eEF1A2 isoform in tissues where it is not normally expressed appears to contribute to cancer, dysregulated expression or

mutations in this muscle- and neuron-specific isoform, without affecting its expression in other tissues, have been linked to neurodevelopmental disorders. The first evidence of this came from studies showing that a severe neurodegenerative phenotype in mice was associated with a deletion in the *EEF1A2* gene, including the promoter and first exon. Mice homozygous for this mutation displayed muscular and neuronal degeneration, leading to postnatal death [197,199]. Heterozygous *de novo* missense single nucleotide mutations in *eEF1A2* have been found in individuals with neurodevelopmental phenotypes such as epilepsy, autism, intellectual disability, dystonia, and choreoathetosis. Some of these mutations have also been associated with neurodegenerative phenotypes, resulting in cerebral and cerebellar atrophy[207–210]. To date, only one homozygous mutation of the isoform has been identified in humans, specifically within siblings in a family where both parents were heterozygous for the same mutation. Mutation homozygosity in these individuals was associated with severe neurodevelopmental and heart conditions, leading to death in early childhood[211].

Table 2: Mutations of eEF1A2 found in humans[207,210,211].

Mutation	Mode of inheritance	Phenotype
D17H G19R A46S G70S I71L D91N A92T D97N F98L F98C M102V E122K E124K A125E D252H R266W N314K R382H G384R	Heterizygous *de novo*	Neurodevelopmental phenotypes (epilepsy, autism, intellectual disability, dystonia and choreoathetosis) Neurodegenerative phenotypes (cerebral and cerebellar atrophy)

E388K R423C T432M Del (457–461) V437F A461V		
P333L	Homozygous	Global developmental delay, epilepsy, dilated cardiomyopathy, death in early childhood

Given the essential role of eEF1A in translation elongation, as well as in a growing number of moonlighting functions, and considering the detrimental effects of its dysregulated expression and mutations, it would not be surprising if significant cellular effort is devoted to the efficient synthesis of functional eEF1A.

eEF1A biogenesis

To date, there are not many studies that focus on elucidating the molecular mechanism that ensures proper biogenesis of eEF1A. This essential multidomain trGTPase is among the fastest synthesized and most abundant proteins in the cell[212,213]. Consequently, it is likely that significant cellular resources are dedicated to the efficient production of this central factor. Insights into eEF1A biogenesis are now unfolding. Just recently, a dedicated cytosolic chaperone-cochaperone system (Zpr1-Aim29) that assists post-translational folding of newly synthesized eEF1A has been identified[39,40]. The Zpr1 chaperone uses zinc-finger and alpha-helical hairpin structures to facilitate folding of eEF1A via a mechanism that requires client GTP binding and hydrolysis. The Aim29 cochaperone senses and binds the Zpr1-eEF1A folding complex in the GTP bound form and dampens eEF1A GTPase activity which promotes release of eEF1A from the folding cycle. Depletion of Zpr1 and Aim29 results in the misfolding of newly synthesized eEF1A which triggers cellular proteotoxicity and activation of Hsf1. Prolonged depletion of Zpr1 and Aim29 deletion has also been associated with decreased levels of eEF1A and translational stress. The identification of Zpr1 and Aim29 as biogenesis factors for eEF1A raises the possibility that the synthesis of eEF1A depends on a complex dedi-

cated folding pathway. Perhaps such dedicated pathway starts exerting its protective action in the early co-translational state of eEF1A production via a dedicated ribosome-associated chaperone yet to be uncovered.

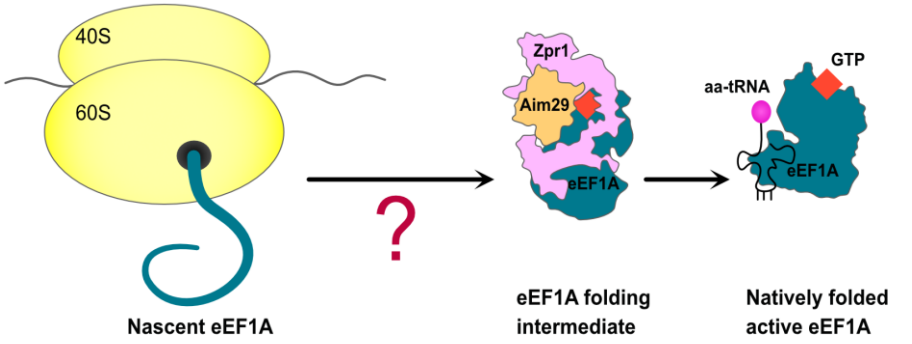

Figure 7. Model illustrating the chaperone-mediated biogenesis of eEF1A. *Newly synthesized eEF1A interacts with the dedicated chaperone-cochaperone system Zpr1-Aim29, which aids the translation factor in attaining its final native structure. This process is presumed to be post-translational. It is currently unknown what steps occur upstream of Zpr1-Aim29 chaperoning, including whether dedicated ribosome-associated chaperones participate in this folding pathway.*

Aims of the thesis

Overall aim:

The overarching aim of this PhD thesis is to advance the understanding of how cells ensure the proper folding of newly synthesized polypeptides, thereby preventing protein misfolding and its associated proteotoxicity.

Specific aims:

Aim I: To elucidate the chaperone-mediated mechanisms regulating Hsf1, the key mediator of the cellular proteotoxic stress response. (**Study I**)

Aim II: To identify novel ribosome-associated chaperones that aid in nascent polypeptide folding and prevent proteotoxic stress. (**Study II**)

Aim III: To uncover how Chp1 facilitates eEF1A folding and to define the chaperones interactions within the eEF1A folding pathway. (**Study III**)

Saccharomyces cerevisiae as a model organism

Saccharomyces cerevisiae, commonly known as baker's yeast, is a unicellular eukaryote that has become a cornerstone of scientific research. Its rapid growth rate, low cost, and ease of cultivation in laboratory conditions make it an attractive model organism. Additionally, a well-established genetic toolbox allows for straightforward manipulation of its genome. For example, gene deletions, additions, and substitutions can be easily introduced via homologous recombination, allowing for precise investigation of gene function and regulation. *S. cerevisiae* was the first eukaryotic organism to have its genome fully sequenced[214], and the majority of its genes are well characterized[215]. Moreover, *S. cerevisiae* shares conserved molecular mechanisms and pathways with higher organisms, including humans, making it a valuable tool for studying fundamental cellular processes. This model organism is widely used in cell and molecular biology research, including the study of protein folding and modeling of diseases associated with proteostasis imbalances, such as cancer and neurodegeneration[216–218]. While its unicellular nature imposes certain limitations in studying multicellular phenomena, *S. cerevisiae*'s simplicity, versatility, and relevance to human biology make it a cornerstone of modern biology, driving groundbreaking discoveries in both basic and applied research. The work presented in this thesis utilizes *S. cerevisiae* to gain insights into the mechanisms employed by the proteostasis system to safeguard the folding of newly synthesized proteins and, consequently, prevent proteotoxic stress.

Summary of individual studies

Study I

Aim I: To elucidate the chaperone-mediated mechanisms regulating Hsf1, the key mediator of the cellular proteotoxic stress response.

Hsf1 is an evolutionarily conserved transcription factor that mediates the cellular response to proteotoxic stress by inducing the expression of genes involved in restoring proteostasis, including chaperones. Increasing evidence suggests that the Hsp70 chaperone negatively regulates Hsf1 activity, though the underlying molecular mechanism remained unclear. In this study, we reconstituted and characterized a complex between Hsp70 (Ssa1) and Hsf1. The isolated complex contained active Hsf1 capable of binding to DNA fragments carrying HSEs. However, we demonstrated that excess Ssa1 leads to the formation of supercomplexes containing both Ssa1 and Hsf1, which inhibits Hsf1's DNA-binding activity. We further showed that Ssa1 binds Hsf1 via its SBD, resulting in competition between substrates and Hsf1 for Ssa1 binding. Additionally, we found that extreme out-titration of Hsp70 by persistent substrates, such as during severe stress conditions, leads to a broader and amplified Hsf1-mediated hyper-stress response. Notably, we identified the misfolding of newly synthesized proteins and the resulting out-titration of Hsp70 to aid in their folding, as a major trigger for Hsf1 activation. Our findings provide new insights into the dynamic regulation of Hsf1 by Hsp70, showing that Hsp70 availability in response to misfolded proteins serves as a critical regulatory mechanism that controls Hsf1 activity and modulates the cellular stress response.

Results

Hsp70 binds Hsf1 via its SBD and regulates its activity in response to proteome stability

To investigate how Hsf1 is regulated by the Hsp70 chaperone (Ssa1), we first isolated an Ssa1-Hsf1 complex by co-purification using a recombinant *E. coli* expression system. We then characterized the complex in terms of its heat shock element (HSE) binding capacity. Electrophoretic mobility shift assay

(EMSA) with an HSE-containing DNA fragment revealed that the purified complex was capable of binding to the HSE, suggesting the presence of active Hsf1 within the complex. Native gel electrophoresis further demonstrated that the intact Ssa1-Hsf1 isolated complex binds to the HSE-DNA fragment. However, the addition of increasing concentrations of Ssa1 to the complex led to a reduction in binding to the DNA fragment carrying the HSE. Size exclusion chromatography (SEC) analysis demonstrated that excess Ssa1 promoted the formation of a large supercomplex containing Ssa1 and Hsf1, likely hindering Hsf1's HSE-binding activity. Site-specific UV crosslinking experiments demonstrated that Ssa1 interacts with Hsf1 via its SBD, and this interaction decreases under conditions that promote protein misfolding. These findings reveal a crucial mechanism by which Ssa1 binds Hsf1 through its SBD, enabling Ssa1 to dynamically regulate Hsf1's DNA-binding activity in response to proteome stability.

Severe stress frees Hsf1 from Hsp70 inhibition and activates a hyper-stress transcriptional program.

RNA sequence (RNA-seq) analysis of heat-shocked *fes1*Δ cells, which are impaired in substrate release from Hsp70, revealed a stronger and broader heat-shock response compared to wild-type cells. Notably, Hsf1 target genes, including *SSA4*, *HSP42*, *HSP104*, *SSE1*, and *HSP82*, exhibited significant hyper-induction in fes1Δ cells. This response was also characterized by the hyper-repression of genes involved in translation and ribosome biogenesis. Importantly, wild-type cells subjected to extreme heat shock conditions could also activate the hyper-stress transcriptional program, as evidenced by the induction of the *CUP1* gene, a marker of the hyper-stress response whose promoter contains a minimal low-affinity HSE. This indicates that under severe stress, the accumulation of persistent Hsp70 substrates leads to heightened Hsf1 activity, resulting in a more pronounced stress response.

Misfolding of newly synthesized proteins drives Hsf1 activation

Taking into account the high propensity of newly synthesized proteins to misfold and their dependence on cytosolic chaperones such as Hsp70 for proper folding, we investigated the role of ongoing protein synthesis in the activation of Hsf1. Induction of newly synthesized protein misfolding by transiently feeding cells with the proline ring analogue AzC triggered Hsf1 activation, with synergistic effects when combined with heat shock, as evidenced by qPCR analysis of the Hsf1 target genes *SSA4* and *HSP104*. We also examined the impact of reduced protein synthesis on heat-shock-induced Hsf1 activation

by using cycloheximide (CHX) to block translation or by starving leu2 auxotrophic cells for leucine prior to heat shock. Both approaches prevented the formation of heat-induced protein aggregates and resulted in decreased Hsf1 activity, as evidenced by reduced *SSA4* transcript levels. Protein aggregation analysis revealed that heat shock induced the migration of soluble Ssa1 to protein aggregates in a protein synthesis-dependent manner. Thus, our results demonstrate that the misfolding of newly synthesized proteins plays a major role in the activation of Hsf1 by titrating Hsp70 away from the Hsf1 inhibitory complex.

Study II

Aim II: To identify novel ribosome-associated chaperones that aid in nascent polypeptide folding and prevent proteotoxic stress.

General ribosome-associated chaperones play a crucial role in supporting the co-translational folding of a wide range of newly synthesized proteins, thereby preventing early misfolding and maintaining proteome stability. However, it remained unclear whether these general proteostasis components are sufficient for the biogenesis of nascent substrates with complex folding pathways. In this study, we identified a novel ribosome-associated chaperone, Chp1, which is specifically dedicated to assisting the co-translational folding of the essential and highly expressed multidomain translational GTPase, eEF1A. Chp1 specifically binds the nascent G-domain of eEF1A, and this interaction is stabilized by binding to the α subunit of the general ribosome-associated chaperone NAC. In the absence of Chp1, misfolded eEF1A is rapidly degraded, accompanied by widespread protein aggregation and activation of the Hsf1-mediated stress response. Beyond its role in eEF1A biogenesis, Chp1 also binds post-translationally to destabilized eEF1A, including mutant variants associated with a neurodevelopmental disorder in humans. Our findings reveal that the essential translational GTPase eEF1A requires specialized folding assistance, beginning at the early stages of protein synthesis at the ribosome, mediated by Chp1.

Results

Chp1 assists in the biogenesis of the eEF1A's GTPase domain, aided by NAC

Comparison of datasets on ribosome-associated proteins and the activity of the proteotoxic stress response mediator Hsf1, upon single gene deletion in yeast cells, allowed us to identify the deletion of the uncharacterized open reading frame YPL225W (*CHP1*) as a potent activator of Hsf1. Using *in vivo* UV-crosslinking, proteomic analysis, and interaction assays, we show that Chp1 directly interacts with the α-subunit of NAC. Additionally, we found that Chp1 binds translating ribosomes in a nascent polypeptide-dependent manner, with this interaction being further facilitated by NAC. Selective ribosome profiling (SeRP) demonstrated that Chp1 specifically binds to the nascent GTPase domain of eEF1A during its biosynthesis. This interaction was further validated through structural modeling, *in vitro* interaction assays, UV-crosslinking, and coexpression experiments in *E. coli*. Thus, Chp1, with the help of NAC, assists in the biogenesis of eEF1A and safeguards proteostasis.

Chp1 safeguards proteostasis by protecting eEF1A biogenesis

We investigated the role of Chp1 in the biogenesis of eEF1A by analyzing the production levels of the protein from an inducible promoter in the presence or absence of Chp1. In the absence of Chp1, the synthesis rate of eEF1A was significantly reduced. Further analysis revealed that Chp1 deletion resulted in extensive polyubiquitination of eEF1A, and proteasome inhibition exacerbated the phenotype. This indicates that Chp1 protects nascent eEF1A from premature proteasomal degradation. Ribosome profiling showed that despite the lower levels of eEF1A in *chp1*Δ cells, these cells did not display major translation defects. Instead, differential expression analysis revealed a proteostasis imbalance, as many of the upregulated genes in *chp1*Δ cells were Hsf1 targets. Additionally, *chp1*Δ cells exhibited increased protein aggregation and temperature sensitivity. The proteostasis imbalance in *chp1*Δ cells was directly linked to the aberrant production of eEF1A in the absence of Chp1, as deletion of one copy of the eEF1A gene suppressed the stress phenotype, while increasing eEF1A dosage exacerbated it. Thus, defective eEF1A biogenesis in the absence of Chp1 places a burden on the proteostasis system.

Chp1 participates in quality control of destabilized eEF1A

UV-crosslinking, *in vitro* interaction studies, and co-expression assays in *E. coli* revealed that, in addition to its role in eEF1A biogenesis, Chp1 binds fully synthesized but misfolded eEF1A. Consistent with this, co-immunoprecipitation analysis showed increased interaction between Chp1 and destabilized eEF1A variants carrying mutations in the switch II region of the GTPase domain (F98C, M102V), which are associated with a neurodevelopmental disorder in humans. For one of these mutants (F98C), we demonstrated that Chp1 not only aids in its biogenesis but also binds to and stabilizes it, protecting it from proteasomal degradation. Thus, Chp1 plays a crucial role in both the biogenesis and quality control of destabilized eEF1A.

Study III

Aim III: To uncover how Chp1 facilitates eEF1A folding and to define the chaperones interactions within the eEF1A folding pathway.

The biogenesis of eEF1A, an essential multidomain GTPase, relies on a dedicated folding pathway involving multiple chaperones. The ribosome-associated chaperone Chp1 plays a pivotal role by binding the nascent G-domain of eEF1A, facilitating its productive synthesis. Subsequent folding is facilitated by the Zpr1 chaperone and its Aim29 cochaperone. However, the precise mechanism by which Chp1 assists eEF1A folding and coordinates with downstream chaperones remained unclear. This study revealed that Chp1 interacts with the switch I region of the G-domain, preventing premature nucleotide binding and maintaining the domain in a flexible state. Nucleotide binding induces G-domain folding and triggers Chp1 dissociation. Early steps in the synthesis of the subsequent domain (domain II) result in accelerated dissociation of Chp1 while enabling engagement of Zpr1. Our findings uncover a sequential chaperone-assisted folding mechanism for eEF1A, where Chp1 delays G-domain folding until the elongation factor is ready to transition to downstream chaperones for maturation.

Results

Chp1 hinders G-domain nucleotide binding and keeps the domain flexible

Hydrogen-deuterium exchange mass spectrometry (HDX-MS) revealed that Chp1 binds the α3 helix within the switch I region of the eEF1A G-domain, locally stabilizing the switch I and II regions while destabilizing the overall domain. Stopped-flow assays were conducted to assess the impact of Chp1 binding on nucleotide association, revealing that Chp1 hinders guanine nucleotide loading onto the G-domain. HDX-MS data showed that nucleotide binding stabilizes the entire G-domain structure, with GTP providing greater stability than GDP, particularly in the switch regions and the vicinity of the guanine ring recognition motifs. These results highlight opposing effects of Chp1 and nucleotide binding on G-domain structural equilibrium: Chp1 prevents nucleotide binding and promotes a flexible, dynamic state, while nucleotide binding induces structural stability.

eEF1A biogenesis involves sequential chaperone handover mechanism from Chp1 to Zpr1.

We used UV-crosslinking experiments and release assays to examine whether nucleotide triggers dissociation of the Chp1-eEF1A G-domain complex. Our data confirmed that nucleotide disrupts this interaction, with GTP being more effective than GDP in promoting dissociation. Notably, increased nucleotide-dependent dissociation was observed with a fragment extending beyond the G-domain, including the β1 and β2 strands of domain II. Interaction assays further revealed that this accelerated dissociation of Chp1 coincided with the engagement of the downstream chaperone Zpr1. Our data reveal a sequential chaperone handover mechanism in which Chp1 initially engages eEF1A, delaying nucleotide-guided folding of the G-domain until the synthesis of the subsequent domain begins, allowing the next chaperone in the folding pathway, Zpr1, to engage the substrate.

Concluding remarks and outlook

The work presented in this thesis investigates the molecular mechanisms that ensure the proper folding of nascent polypeptides to maintain proteome stability and prevent proteotoxicity in the model organism *Saccharomyces cerevisiae*. We have elucidated the role of Hsp70 in regulating Hsf1 activity. Our results demonstrate that the availability of Hsp70, influenced by its interactions with misfolded proteins, primarily arising from the off-pathway folding of newly synthesized proteins, governs the cellular stress response by modulating Hsf1 activity. Moreover, a central finding of this work is the identification of Chp1, a novel ribosome-associated chaperone dedicated to assisting in the biogenesis of the essential multidomain GTPase eEF1A, one of the most abundant proteins in the cell. Chp1-mediated protection of eEF1A synthesis safeguards cellular proteostasis, highlighting the existence of dedicated ctranslational folding mechanisms to prevent proteotoxicity arising from the misfolding of highly expressed, challenging substrates. Finally, we identified the molecular mechanisms by which Chp1 aids in eEF1A biogenesis, carefully timing the folding of the eEF1A G-domain to ensure it occurs only when the domain is fully synthesized and the substrate is ready for subsequent chaperone engagement. Together, these findings provide new insights into the interplay between the folding machinery, protein synthesis, and cellular stress responses, contributing to our understanding of how cells maintain proteome integrity.

In the first study, we established a comprehensive model of Hsp70-mediated regulation of Hsf1 activity. Our findings reveal that, under non-stress conditions, Hsp70 (Ssa1) binds to Hsf1 in the nucleus, maintaining the transcription factor in an inactive state with limited DNA-binding capacity. Misfolded protein substrates and Hsf1 directly compete for Hsp70 binding at its SBD. Upon exposure to stress, the accumulation of misfolded proteins, primarily resulting from faulty maturation of newly synthesized polypeptides, sequesters Hsp70, triggering the release of Hsf1 from its latent complex. This allows the Hsf1-mediated activation of genes critical for restoring proteostasis. Under hyper-stress conditions, the severe buildup of misfolded proteins

and protein aggregates leads to extensive Hsp70 titration, fully unleashing Hsf1 from the chaperone inhibition. This culminates in the activation of an emergency hyper-stress transcriptional program. Collectively, this chaperone-titration model underscores the delicate balance required between protein synthesis, folding, and stress response activation, providing valuable insights into the cellular mechanisms that maintain proteome stability.

Our model is based on the subcellular compartmentalization of Hsp70 between the cytosol and the nucleus, where it regulates Hsf1-DNA binding activity. Protein synthesis, identified as the primary source of Hsp70 substrates, occurs predominantly in the cytosol. This raises an open question: how do misfolded proteins originating in the cytosol titrate nuclear Hsp70 to activate Hsf1? While some misfolded proteins are known to translocate from the cytosol to the nucleus for proteasomal degradation[219], further studies are needed to uncover the precise subcellular localization of the substrates responsible for titrating Hsp70 and releasing nuclear Hsf1 from its latency. Moreover, the exact compositions of the Hsp70-Hsf1 activation and latency complexes remain incompletely characterized. Our findings do not exclude the possibility of additional proteins participating in these complexes. Similarly, further research is required to uncover the mechanism by which excess Hsp70 inhibits Hsf1 DNA binding. Our results emphasize the role of newly synthesized protein misfolding in titrating Hsp70 and activating Hsf1. Identifying the specific proteins involved in this titration process, along with characterizing their key properties, such as folding kinetics, aggregation tendencies, and interactions with co-translational acting chaperones, will be essential for advancing our understanding of cellular stress sensing mechanisms. Finally, it is crucial to investigate whether the regulatory mechanism of Hsf1 via Hsp70-mediated inhibition observed in yeast is conserved in humans. Such studies could provide valuable insights into proteostasis across species and open new avenues for therapeutic strategies targeting protein misfolding and aggregation diseases in humans.

In the second and third studies, we uncovered the role of a highly conserved yet previously uncharacterized eukaryotic protein, which we named Chp1. Study I allowed us to identify Chp1 as a ribosome-associated chaperone specialized in the co-translational folding of nascent eEF1A. Chp1 directly binds the G-domain of eEF1A, assisting in its synthesis. The interaction between Chp1 and the ribosome-eEF1A nascent chain is stabilized by its binding to the

UBA domain of the α-subunit of NAC. Chp1 binding to nascent eEF1A prevents improper folding and premature proteasomal degradation of the substrate, while its absence leads to decreased eEF1A synthesis, widespread protein aggregation, and activation of the Hsf1-mediated stress response. In addition to its role in biogenesis, we found that Chp1 can bind and prevent the degradation of destabilized, fully synthesized eEF1A, such as a mutant variant associated with neurodevelopmental disorders in humans. Study II allowed us to delve deeper into the mechanism by which this novel ribosome-associated chaperone aids in the biogenesis of eEF1A. Chp1 specifically binds to the α3 helix of the switch I region on the G-domain of eEF1A, a region crucial for nucleotide binding. In this way, Chp1 prevents nucleotide association, delaying nucleotide-mediated folding and maintaining the G-domain in a flexible state. The initiation of domain II synthesis serves as a critical signal, accelerating nucleotide binding to the G-domain, promoting Chp1 dissociation, and facilitating the seamless transfer of the nascent polypeptide to the downstream chaperone Zpr1. These steps ensure precise timing for the productive folding of the G-domain and prevent misfolding during translation. These findings highlight how the misproduction of certain vulnerable and highly expressed proteins, such as eEF1A, can disrupt cellular proteostasis to the extent that their proper synthesis depends on specialized chaperones like Chp1, emphasizing the critical role of dedicated folding mechanisms in preventing widespread proteotoxicity and maintaining cellular integrity.

As we begin to uncover the intricate mechanism of eEF1A chaperone-mediated biogenesis and its impact on cellular proteostasis, much remains to be investigated. For instance, in the absence of Chp1, eEF1A is rapidly degraded via the proteasome without accumulating in protein aggregates. This raises the question: is eEF1A a preferred proteasomal substrate? Additionally, further investigation is needed to understand how the aberrant production of eEF1A in the absence of Chp1 causes widespread protein aggregation and activation of Hsf1. One hypothesis is that misfolded eEF1A, being abundantly expressed, might saturate the proteasome, preventing the clearance of other misfolded substrates and triggering their aggregation, Hsp70 recruitment, and Hsf1 activation. Alternatively, it is possible that misfolded eEF1A itself constitutes a primary substrate of Hsp70, preventing assistance to other misfolded substrates while simultaneously triggering Hsf1 activation. Another intriguing possibility is whether eEF1A itself, which has been shown to possess chaperone activity[190–192], might play a more direct role in protein folding that is impaired when Chp1 is absent. Additionally, the paradox of how Chp1 maintains

the G-domain of eEF1A in an unfolded state while simultaneously protecting it from degradation warrants further investigation. Specifically, does Chp1 prevent degradation by inhibiting ubiquitination of the G-domain, particularly at the α3 helix, or is there another mechanism at play? A fundamental question also remains: why is it advantageous to delay folding until the G-domain is fully synthesized and domain II synthesis begins? Insights from the bacterial GTPase EF-G, which employs a similar co-translational folding mechanism[220], suggest that this could represent a broader strategy for multidomain GTPase biogenesis, deserving further exploration. Lastly, while the components of the *de novo* eEF1A folding pathway are conserved across eukaryotes, including Chp1 (human homolog PBDC1), further research is needed to determine whether the folding mechanism itself is conserved in humans and to explore its implications for cellular proteostasis and human health-related disorders.

Through this work, we have advanced our understanding of the interplay between chaperone-assisted folding of newly synthesized proteins and the Hsf1-mediated stress response in maintaining cellular proteostasis. By elucidating the roles of Hsp70 in regulating Hsf1 activity and identifying Chp1 as a specialized chaperone for eEF1A biogenesis, whose mismanagement can lead to proteotoxicity, we demonstrate how cells deploy finely tuned strategies to protect their proteomes. These findings not only enhance our knowledge of cellular stress sensing and chaperone functions but also pave the way for future research into conserved mechanisms of proteome maintenance and their implications for human health.

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