Protein Folding on the Ribosome

or

Nascent Chains Berlin

15-18 December, 2024

Support from:

Dr. John Vitullo, CEO, Omega Laboratories, Inc
Boehringer Ingelheim Stiftung

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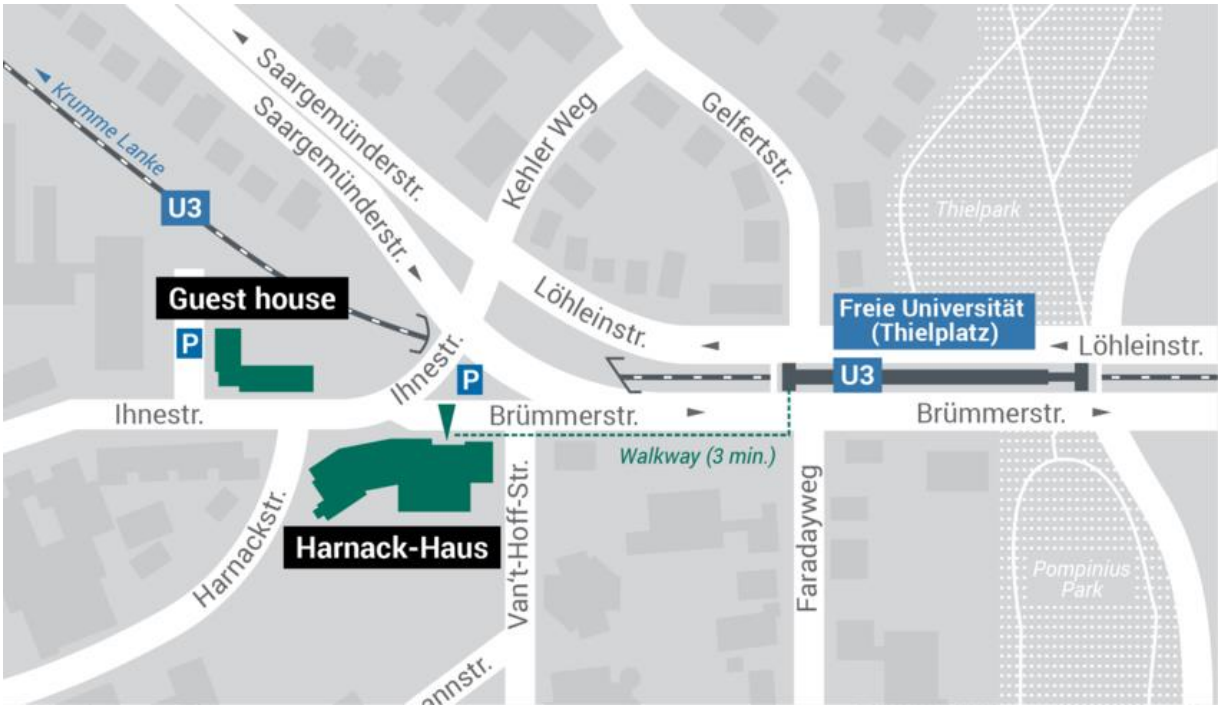
How to get to the Harnack-Haus

Harnack House and its accompanying guest house are located on Ihnestrasse, close to Clayallee, in the Dahlem district of Berlin. The two buildings are just 50 meters apart and offer ample parking spaces for visitors arriving by car.



Start at airport BER (1 h 35 min)

From BER Airport, take the S-Bahn Line S9 in the direction of Spandau. Get off at the station "Warschauer Straße" and change to the subway line U3 in the direction of Krumme Lanke. Get off at the stop "Freie Universität". Then leave the station in the direction of travel through the left exit. The Harnack-Haus is located on the right in about 100 m distance.

Start at Berlin´s Central Station Hauptbahnhof (15 km)

...take S-Bahn line S7 towards Potsdam Hauptbahnhof. At Zoologischer Garten, change onto the U9 towards Rathaus Steglitz. At Spichernstrasse, change onto the U3 towards Krumme Lanke and alight at Freie Universität (Thielplatz). To exit the station, follow the train's direction of travel and take the left exit. From there, bear right; you will reach Harnack House after approximately 100 meters.

Start at train station Südkreuz (9 km)

...take the S41 (Ringbahn) and alight at Heidelberger Platz. Change onto the U3 towards Krumme Lanke and alight at Freie Universität (Thielplatz). To exit the station, follow the train's direction of travel and take the left exit. From there, bear right; you will reach Harnack House after approximately 100 meters.

From Spandau train station (17 km)

...take U-Bahn line U7 towards Rudow and alight at Fehrbelliner Platz. Change onto the U3 towards Krumme Lanke and alight at Freie Universität (Thielplatz). To exit the station, follow the train's direction of travel and take the left exit. From there, bear right; you will reach Harnack House after approximately 100 meters.

Arrival by car

...when approaching on the A 115, take Hüttenweg No. 2 exit. Then bear right towards Dahlem and continue until you reach the junction with Clayallee. Turn right. At the next junction, turn left into Saargemünder Strasse. Harnack House is on the right shortly after at the corner of Ihnestrasse.

Contact

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December 15

16:00-17:00 REGISTRATION

17:00-17:15 OPENING: Marina Rodnina (Welcome)

SESSION 1 FOLDING and ASSEMBLY Chair: Marina Rodnina

17:15-18:00 KEYNOTE: Ulrich Hartl

Molecular chaperone functions in protein folding in situ

18:00-18:30 TALK: Paul Fox

Co-translational assembly of the multi-tRNA synthetase complex

18:30-20:00 DINNER

20:00-23:00 RECEPTION: Informal discussions at Harnack House

December 16

SESSION 2 INSIDE THE TUNNEL Chair: Gunnar von Heijne

9:00-9:40 FOCUS TALK: John Christodoulou

The ribosome lowers the entropic penalty of protein folding

9:40-10:10 TALK: Alexander Mankin

Flip-flop binding in the exit tunnel of the ribosome-targeting antimicrobial peptides

10:10-10:25 Short TALK: Amir Bitran

Cotranslational protein folding through non-native structural intermediates 1

10:25-10:40 Short TALK: Siyu Wang

Cotranslational protein folding through non-native structural intermediates 2

10:40-11:10 Break

11:10-11:40 TALK: Ayala Shiber

Deciphering the dynamics of divergent co-translational assembly pathways

11:40-12:10 TALK: Marina Rodnina

A translation ruler to probe early cotranslational folding

12:10-12:40 TALK: Susan Marqusee

The importance of the N-terminus in nascent chain folding

13:00-14:00 LUNCH

SESSION 3 DOMAIN FOLDING Chair: Zoya Ignatova

14:00-14:30 TALK: Eugene Shakhnovich

Cotranslational folding of disulfide rich proteins – an RBD domain story

14:30-15:00 TALK: Ineke Braakman

Early domain folding and interactions in ABC transporter CFTR

15:00-15:30 TALK: Silvia Cavagnero

Mapping protein-protein interactions at birth

15:30-16:00 BREAK

16:00-16:15 Short TALK: Alzbeta Roeselova

Coordination of molecular chaperones during cotranslational folding of a multidomain protein

16:15-16:30 Short TALK: Ane Metola

Following the co-translational folding of a multidomain protein

16:30-17:00 TALK: Rebecca Voorhees

When protein folding and assembly fails: membrane protein quality control at the ER

17:00-17:30 TALK: Lisa Cabrita

Cotranslational protein misfolding and mis-assembly on translating ribosomes

17:30-18:00 TALK: David Balchin

The ribosome synchronizes folding and assembly to promote oligomeric protein biogenesis

18:30-20:00 Dinner

20:00-23:00 POSTER SESSION 1

December 17

SESSION 4 EMERGING METHODS Chair: Susan Marqusee

9:00-9:30 TALK: Juliette Fedry

Visualisation of protein biogenesis in mammalian cells

9:30-10:00 TALK: Stephen Fried

Protein folding: Queerer than we suppose, or queerer than we can suppose

10:00-10:30 TALK: Sina Ghaemmaghami

Stabilities of ribosome-bound and soluble proteomes measured by rates of methionine oxidation

10:30-11:00 Break

SESSION 5 NC INTERACTIONS Chair: Susan Marqusee

11:00-11:30 TALK: Ning Zhao

Tracking protein co-translational folding with single mRNA resolution in living cells

11:30-12:00 TALK: Christian Kaiser

AP Profiling resolves co-translational folding pathways and chaperone interactions in cells

12:00-12:40 FOCUS TALK: Shu-ou Shan

Principle of cotranslational protein targeting to mitochondria

13:00-14:00 LUNCH

SESSION 6 TRANSLATIONAL TUNING Chair: Anton Komar

14:00-14:30 TALK: Patricia Clark

Vectorial appearance induces conformational biases that alter protein folding yield

14:30-15:00 TALK: Chava Kimchi-Sarfaty

Uncovering model protein characteristics that are codon and codon-pair optimized

15:00-15:30 TALK: Yi Liu

Synonymous but not silent: The codon usage code for gene expression and protein folding

15:30-16:00 Break

16:00-16:30 TALK: Martine Collart

Dwelling of ribosomes at rare arginine codons and production of chaperones connect the Puf3 pumilio family RNA binding protein and Not4 to protein homeostasis

16:30-16:45 Short TALK: Martino Morici

Short-circuiting the bacterial ribosome: RAPPing about hydrogens 1

16:45-17:00 Short TALK: Felix Gersteuer

Short-circuiting the bacterial ribosome: RAPPing about hydrogens 2

17:00-17:15 Short TALK: Lars Bock

Mechanisms of translational stalling and recovery in bacterial ribosomes studied by MD simulations

17:15-17:45 TALK: Guenter Kramer

New functional insights into SRP-mediated protein targeting to the endoplasmic reticulum

17:45-18:25 FOCUS TALK: Judith Frydman

Impaired biogenesis of basic proteins impacts multiple hallmarks of the aging brain

18:30-20:00 Dinner

20:00-23:00 POSTER SESSION 2

December 18

SESSION 7 TRANSLATIONAL TUNING Chair: John Christodoulou

9:00-9:30 TALK: Olivia Rissland

Systematic identification and characterization of eukaryotic and viral 2A peptide-bond-skipping sequences

9:30-10:00 TALK: Sabine Rospert

The yeast E3 ligase Hel2 monitors the duration of ribosome stalling

10:00-10:30 TALK: Jonathan Schlebach

Nascent chain-mediated ribosomal frameshifting during membrane protein translation

10:30-11:00 TALK: Ed O'Brien

How amino acid chemistry at the ribosome A- and P-sites modulates translation elongation speed

11:00-11:45 KEYNOTE TALK: Bernd Bukau

Profiling analysis of co-translational folding and assembly of newly synthesized proteins

11:45-12:00 CLOSING REMARKS: Closing remarks and poster awards

12:30-14:00 LUNCH

1

Molecular chaperone functions in protein folding in situ

Presenter: Ulrich Hartl

In the crowded cellular environment, numerous newly-synthesized proteins depend on molecular chaperones to fold efficiently and at a biologically relevant time scale. Extensive biochemical and structural studies revealed the elaborate architecture and reaction mechanisms of major chaperone systems, including the essential ATP-driven multi-subunit complexes of Hsp70, Hsp60 (chaperonins) and Hsp90. Studies with model proteins in vitro show how these chaperones recognize non-native substrates, reverse misfolded states, and promote productive folding through ATP and cofactor-regulated cycles of binding and release. However, little is known about the dynamics of chaperone functions and the cooperation of different chaperone systems in vivo. What are the lifetimes of co- and post-translational chaperone-client interactions? Do chaperone-substrate complexes diffuse freely in the bulk cytosol or are these interactions organized in a “protected zone”, facilitating chaperone re-binding during cycling? We are utilizing cryo-electron tomography and single particle tracking in live cells to visualize the functions of chaperones *in situ* in the intact cellular environment.

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Co-translational assembly of the multi-tRNA synthetase complex

Presenter: Paul Fox

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The ribosome lowers the entropic penalty of protein folding

Presenter: John Christodoulou

4

Flip-flop binding in the exit tunnel of the ribosome-targeting antimicrobial peptides

Weiping Huang, Max J. Berger, Chetana Baliga, Daniel N. Wilson, Nora Vázquez-Laslop, Alexander S. Mankin

Presenter: Alexander Mankin

Proline-rich Antimicrobial Peptides (PrAMPs) inhibit bacterial growth by interfering with translation. Class I PrAMPs bind in the vacant nascent peptide exit tunnel of the initiating ribosome in the orientation opposite to that of the growing protein chain. By extending their N-terminal residues into the peptidyl transferase center, class I PrAMPs hinder formation of the first peptide bond. Class II PrAMPs, such as drosocin (Dro) encoded in the fruit fly genome, enter the empty tunnel of the post-release ribosome. In contrast to the class I peptides, class II PRAMPs bind in the orientation matching that of the growing protein. C-terminal amino acids of class I PrAMPs establish contacts with the GGQ motif of the release factor and trap the factor on the ribosome. We found that some PrAMPs bind to the ribosome like Class I PrAMPs despite their sequence similarity with Dro. By analyzing modes of binding and action of chimeras of sequence-similar Class I and Class II PrAMPs, we observed that the properties of the N-terminal segment define their mechanism of binding and action and that flipping of the PrAMP's orientation in the ribosomal exit tunnel can be achieved by a single amino acid substitution.

5-6

Cotranslational protein folding through non-native structural intermediates

Siyu Wang, Amir Bitran, Ekaterina Samatova, Eugene I. Shakhnovich, Marina V. Rodnina

Presenters: Amir Bitran / Siyu Wang

Many proteins start to fold co-translationally through the formation of compact intermediates inside the peptide exit tunnel of the ribosome. The nature and physical properties of these intermediates remain largely unknown. In this work, we probed the dynamics of the nascent peptide on the ribosome using a combination of all-atom Monte-Carlo (MC) simulations and PET-FCS. MC simulations identified hydrophobic residues that, despite being solvent-exposed in the native state, participate in the compaction of short polypeptide chains. To test the role of these putative non-native contacts during protein synthesis on the ribosome, we used a reconstructed E. coli in vitro translation system to generate stalled ribosome-nascent chain complexes of HemK NTD at different steps of translation. Further PET-FCS measurement allowed us to estimate the frequency of intermolecular quenching events in the nascent peptide, thereby probing its dynamics on the ribosome. We found that substitutions of the residues predicted to participate in non-native contacts affect the dynamics of nascent peptide at intermediate length, but not when the domain fully appears from the ribosome. Our work demonstrates that cotranslational folding intermediates could be stabilized by very different contacts than the final native state. We also show that MC simulations of truncated N-terminal peptides folding in solution can be used to predict intermediate polypeptide structures on the ribosome after the polypeptide exits the ribosome tunnel.

7

Deciphering the dynamics of divergent co-translational assembly pathways

Johannes Venezian, Qamar Shade, Junyi He, Hagit Bar-Yosef, Oded Kleifeld, Juan Fernandez-Recio, Fabian Glaser, Ayala Shiber

Presenter: Ayala Shiber

Protein-protein interactions are central to cellular functions, with the ribosome playing a key role in coordinating nascent-chain interactions. To uncover the conformational, energetic, and kinetic parameters influencing folding and assembly, we employ a combination of selective ribosome profiling, super-resolution imaging, ribosome-directed N`-degradomics, and Molecular Dynamics (MD) simulations. By investigating divergent co-translational assembly pathways, we identified critical "hotspots"—key nascent-chains residues that anchor the entire interface. Mutations in these hotspots in vivo disrupt co-translational assembly, resulting in misfolding and proteasome recruitment during translation. Deep structural prediction using AlphaFold-multimer revealed a correlation between protein stability, flexibility, and the initiation of co-translational complex assembly. We propose that the energy contribution of nascent-chain interfaces is a strong predictor of co-translational assembly onset. Single-molecule FISH analysis of mRNA cellular distribution shows that the exposure of interface hotspots at the ribosome exit tunnel can predict the co-localization of mRNAs encoding for interacting subunits. Interestingly, analysis of 5' UTR, 3' UTR, and ORF regions encoding for co-translational interfaces suggests that upstream and downstream ORF elements play a major role in mRNA co-localization within translation hubs dedicated to specific complexes. Altogether, our findings suggest that nascent-chain interface assembly is a tightly regulated process in eukaryotic cells, mediated by various factors, including non-coding regions. The process may have evolved to protect misfolding-prone proteins during synthesis. When co-translational assembly interactions are disrupted, we discover new quality-control factors that regulate the degradation of isolated subunits during translation.

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A translation ruler to probe early cotranslational folding

Presenter: Marina Rodnina

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The importance of the N-terminus in nascent chain folding

Presenter: Susan Marqusee

10

Cotranslational folding of disulfide rich proteins – an RBD domain story

Presenter: Eugene Shakhnovich

Many proteins refold very slowly from a denatured state in vitro owing to nonnative misfolding traps that are often linked to disease. It has long been known that co-translational folding increases the folding rate and efficiency for such proteins in vivo. This is especially crucial for disulfide rich proteins which may not be able to fold in vitro when disulfides are reduced and where concurrent cotranslational folding and oxidation might be the only plausible mechanism to form folded functionally active proteins. However, the molecular mechanism underlying this benefit is not well understood. Here, we explore folding of disulfide rich protein RBD domain of SARS-Cov2 at the atomistic level with a unique combination of theory and experiment. First, we show that in vitro refolding of reduced proteins results in a nonnative molten globule like state where disulfide bonds are only partially formed and are scrambled. Using a novel simulation-based algorithm and kinetic modeling, we show that cotranslational folding helps guide RBD to a native oxidation state preventing formation of a specific non-native disulfide that locks the protein in a deeply trapped nonnative conformation that is prone to aggregation guiding the whole process to final native conformation which is metastable in the absence of native disulfides. In the second part of the talk, we discuss how a dedicated cotranslational chaperone prevents elongation factor eF1A from falling into cotranslational kinetic trap and how cotranslational landscapes of orthologous proteins evolve in the genomic context.

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Early domain folding and interactions in ABC transporter CFTR

Presenter: Ineke Braakman

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Mapping protein-protein interactions at birth

Silvia Cavagnero, Meranda M. Masse, Neomi Millan, Rachel B. Hutchinson, Ummay Mahfuza Shapla and Jinoh Jang

Presenter: Silvia Cavagnero

In order to become bioactive, proteins must be translated and protected from aggregation during biosynthesis. The ribosome and molecular chaperones play a key role in this process. In our study, we employed a combination of single-particle cryo-EM and time-resolved fluorescence- anisotropy, low-pH gels and Western blotting to analyze protein-protein interactions experienced by nascent chains in bacteria. We found that ribosome-bound nascent chains (RNCs) of single- domain proteins establish and expand noncovalent contacts with selected ribosomal proteins and chaperones, as they get longer. On the outer surface of the ribosome, RNCs interact specifically with a highly conserved nonpolar patch of the L23 r-protein. Some of the examined RNCs also comprise an independently compact and dynamic N-terminal region lacking contacts with the ribosome. In all, nascent proteins traverse the ribosome and interact with it via their C- terminal regions, while N-terminal residues tend to sample conformational space and form a compact subdomain. The data reveal a peculiar interplay between RNC independent conformational sampling and interactions with the ribosomal surface and with chaperones, at birth. Strategies to further favor co- and immediately post-translational folding and discourage aggregation will also be discussed.

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Coordination of molecular chaperones during cotranslational folding of a multidomain protein

Alžběta Roeselová, Grant Pellowe, Santosh Shivakumaraswamy, Sarah Maslen, Mark Skehel, David Balchin

Presenter: Alžběta Roeselová

Protein folding in the cell begins during protein synthesis at the ribosome in the presence of multiple molecular chaperone systems. What role the ribosome and molecular chaperones play during cotranslational folding of nascent chains remains poorly understood. We optimised a strategy to study protein synthesis intermediates - ribosome nascent chain complexes (RNCs) - with hydrogen-deuterium exchange mass spectrometry, allowing us to obtain a detailed structural description of the cotranslational folding pathway of a multi-domain protein, β-galactosidase (β-gal). The ribosome affects β-gal maturation in multiple ways. It directly interacts with the nascent chain (NC) near the exit tunnel, has a far-reaching destabilising effect on some distant regions, and prevents premature unproductive cotranslational assembly late into translation. Additionally, multiple molecular chaperones engage β-gal during synthesis. Near the ribosome surface, Trigger factor recognises compact folding intermediates exposing extensive non-native regions and dictates Hsp70 access to the nascent chain. Neither of the chaperone systems destabilise cotranslational folding intermediates, but instead collaborate to create a protected space for protein maturation. We also show that the GroEL/ES chaperonin can bind and partially encapsulate destabilised RNCs in competition with Hsp70. By studying a complex model protein which engages multiple chaperone systems, our observations contribute to the general understanding of how protein mature during translation, and how the chaperone network modulates cotranslational folding intermediates.

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Following the co-translational folding of a multidomain protein

Ane Metola, Gunnar von Heijne, Marcelo E. Guerin

Presenter: Ane Metola

Tandem repeat proteins composed of multiple copies of similar domains have a high risk of forming non-native inter-domain contacts that can lead to misfolded states. Therefore, a strong selective pressure may exist to minimize misfolding interaction between adjacent domains during co-translational folding. To explore how this occurs we monitored variations on pulling force generated by the PimA nascent chain as it emerges from the ribosomal exit tunnel during vectorial appearance of one foldon after the other. The protein PimA is an essential enzyme of 386 residues responsible for the initial mannosylation of phosphatidylinositol in Mycobacterium smegmatis. The structure of PimA consists of two Rossmann-fold domains with a deep fissure at the interface forming the catalytic center, followed by a long □-helix which connects back the C-terminal domain with the N-terminal domain. By the use of a set of gradually longer fragments of PimA fused to a translational arrest peptide in the E. coli-based PURE in vitro translation system we generated a series of co-translational force-profiles. The force profile analysis reveals that while the C-terminal domain is able to fold co-translationally as soon as it emerges from the ribosome, the N-terminal domain requires the complementation of the central B-sheet by the very C-terminal portion.

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When protein folding and assembly fails: membrane protein quality control at the ER

Presenter: Rebecca Voorhees

All cellular processes, from DNA replication to cell signalling, depend on multisubunit protein complexes. Assembly of these complexes is highly regulated to ensure production of functional, stoichiometric macromolecules. Any orphan subunits, that are synthesized in excess or remain unassembled, must be recognized and degraded to maintain protein homeostasis.One class of proteins for which quaternary assembly poses a particular challenge is integral membrane proteins. This family includes hundreds of oligomeric ion channels, receptors, and transporters essential for all aspects of cellular physiology. Many membrane protein subunits contain regions that, while necessary for function or oligomerization, would be thermodynamically unfavorable in isolation within the lipid bilayer. However, very little is known about the molecular chaperones that therefore must stabilize unassembled subunits in the membrane, or how these factors triage clients towards a biosynthetic or degradative fate. Towards this goal, we started by dissecting the assembly of the model complex GET1/2. We chose this complex because GET1/2 are essential, themselves playing a critical role in membrane protein biogenesis at the ER and form an obligate hetero-oligomer: if GET1 cannot assemble, it is rapidly degraded by the ubiquitin proteasome pathway. We made an unanticipated finding that GET2 does not adopt the correct topology when initially synthesized, but instead requires GET1 for its folding and insertion. This coupling of insertion (and folding) to subunit assembly represents a novel strategy for the regulation of complex assembly, and seems to be broadly utilized by other membrane protein complexes. However, because unassembled GET1 is rapidly degraded when unassembled, we reasoned that specific quality control factors in the ER must be recognizing the orphan subunit as aberrant. Therefore, we used GET1/2 as a pilot system to identify factors responsible for membrane protein assembly and quality control using two complementary strategies: (i) proteomic analysis of the interactome of a series of orphan subunits, and (ii) genome-wide genetic screens using reporters for membrane protein assembly. Here I will present our ongoing work towards characterizing the suite of factors that regulate protein complex assembly in the ER, with the goal of understanding how a network of biogenesis and quality control machinery works in concert to regulate and maintain cellular proteostasis.

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Cotranslational protein misfolding and mis-assembly on translating ribosomes

Ilgın Eser Kotan, Sabrina Sartori, Rudra Shekhar Bose, Bernd Bukau, Günter Kramer

Presenter: Lisa Cabrita

Protein misfolding is hazardous to cell and it is a process that is liked to an ever-increasing range of human conformational diseases. Neurodegeneration, respiratory diseases, and cancer are linked to proteins which readily form ordered or amorphous structures that are toxic to cells. Our understanding of protein folding is that it begins co-translationally on ribosomes, and is a process that is supported by molecular chaperones and other forms of quality control. Despite these measures, protein folding remains an imperfect process, and far less is understood of the mechanisms that may drive these processes. Our research thus aims to uncover the earliest origins of protein misfolding and mis-assembly in disease by tracing these events back to their earliest point: during biosynthesis on the ribosome. We have shown previously (1) with alpha-1-antitrypsin, an archetype of conformational disease, that ribosome-bound, translating nascent chains form co-translational folding intermediates that influence post-translational folding outcomes. Our emerging biochemical, cellular and structural studies of both alpha-1-antitrypsin and huntingtin are beginning to reveal that protein misfolding and mis-assembly are processes that can also begin on translating, but paused ribosomes. (1) Nascent chains can form co-translational folding intermediates that promote post-translational folding outcomes in a disease-causing protein.

Plessa E et al, Nat Communications (2021)

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The ribosome synchronizes folding and assembly to promote oligomeric protein biogenesis

Alžběta Roeselová, Santosh Shivakumaraswamy, Jessica Zhiyun He, Gabija Jurkeviciute, Radoslav I Enchev, David Balchin

Presenter: David Balchin

Multidomain oligomeric proteins constitute a substantial fraction of proteomes, but are typically poorly refoldable in vitro. To understand how cells optimize protein folding pathways, we explored the molecular basis for efficient cotranslational maturation of the 5-domain homotetramer β-galactosidase (β-gal). During refolding from denaturant, maturation of the central TIM barrel domain housing the active site is frustrated. Assembly outpaces monomer folding, and non-native oligomers accumulate. The ribosome alters the order of folding events. Folding of the catalytic domain is separated into multiple independent steps, and shaped by a novel interaction with L23 which stabilises and sequesters a nascent amphipathic helix. By modulating monomer folding and sterically impeding oligomerisation, the ribosome also dictates the pathway of homomer assembly. Cotranslational assembly initiates asymmetrically via at least one fully-folded subunit, and the failure to do so results in misassembly. Our findings reveal how the ribosome can modulate the timing of folding and assembly to ensure efficient biogenesis of a topologically complex protein.

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Visualisation of protein biogenesis in mammalian cells

Presenter: Juliette Fedry

Structural studies have addressed eukaryotic ribosomes at atomic resolution but the techniques available so far required their isolation, which abolishes spatial relationships in the cell and affects labile complexes. Cryo electron tomography now enables the molecular visualisation of protein biogenesis in the native cellular context. In our recent work, we visualised the mRNA translation process in situ, in intact mammalian cells and analysed its reorganisation under persistent collision stress, indicating how perturbations in initiation, elongation and quality control processes contribute to an overall reduced protein synthesis (Fedry#, ..., Faller, Förster, Mol. Cell 2024). We further identified and quantified the distinct populations of ribosome translocon complexes assembled in native ER membrane, revealing their clustering in polysomes translating different types of nascent chains (Gemmer, ..., Fedry#, Forster#, Nature 2023). Our work provides a global view of the translation machinery and a framework for quantitative analysis of translation dynamics *in situ*.

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Protein folding: Queerer than we suppose, or queerer than we can suppose

Presenter: Stephen Fried

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Stabilities of ribosome-bound and soluble proteomes measured by rates of methionine oxidation

Presenter: Sina Ghaemmaghami

Folding stabilities determine the propensity of proteins to aggregate, degrade, or become modified in cells. However, despite their significance for understanding protein folding and function, quantitative analyses of folding stabilities have been largely limited to soluble proteins in purified systems. To allow analyses of protein stabilities in complex mixtures, we developed a mass spectrometry-based approach to quantify folding stabilities based on rates of oxidation of core methionine residues. We used this proteomic methodology to conduct surveys of folding stabilities of soluble and ribosome-bound nascent proteins. The data indicate that the ribosome can significantly alter the stability of nascent polypeptides. Ribosome-induced stability modulations were variable among different folding domains and were dependent on localized charge distributions within nascent polypeptides. We also demonstrate that ribosome-mediated destabilization can facilitate co-translational enzymatic modifications of nascent polypeptides. Our work establishes a robust proteomic methodology for analyzing localized stabilities within ribosome-bound proteins and sheds light on how the ribosome influences the folding and processing of nascent polypeptides.

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Tracking protein co-translational folding with single mRNA resolution in living cells

Rhiannon Sears, Luis Aguilera, Jake Yarbro, Ning Zhao

Presenter: Ning Zhao

The folding of many proteins occurs co-translationally and involves the orchestration of numerous cellular factors, including a large set of ribosome-associated proteins and chaperones. In co-translational folding, the folding is intimately coupled to translation. If the ribosome elongates too fast or stalls for too long at the wrong place and time, folding kinetics can be severely perturbed, leading to misfolding and/or aggregation. In extreme cases, this can lead to diseases. A better understanding of protein co-translational folding kinetics in the native context of translation is therefore critical to human health. The major challenge in the field is the lack of spatiotemporal resolution needed to track and quantify co-translational folding in a living intracellular environment. To address this challenge, we have developed a novel technology that enables directly visualizing co-translational folding with single mRNA resolution in living cells. With this technology, we will investigate co-translational folding kinetics in living cells and further investigate an ongoing central question in the field – how alterations in translation elongation rate affect folding. The study will help us to further understand co-translational folding process and investigate its regulatory mechanism, which has the potential to lead to new therapeutics that have never been explored before for protein misfolding-related diseases.

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AP Profiling resolves co-translational folding pathways and chaperone interactions in cells

Xiuqi Chen, Christian Kaiser

Presenter: Christian Kaiser

Co-translational steps are crucial for overall folding of newly synthesized proteins. These early steps are guided by an intricate network of interactions with molecular chaperones and other cellular machinery. Because co-translational folding in the cell is challenging to detect, timing and progression of co-translational folding remain largely elusive. We developed a scalable method to quantify co-translational folding in live cells that we term “Arrest Peptide profiling” (AP Profiling). We employed AP Profiling to delineate co-translational folding for a set of GTPase domains with similar structures, defining how topology shapes folding pathways. Genetic ablation of nascent chain-binding chaperones resulted in discrete and localized folding changes, suggesting how functional redundancy is achieved by distinct interactions with the nascent protein. Collectively, our studies provide a window into cellular folding pathways of complex proteins and pave the way for systematic studies on nascent protein folding at unprecedented resolution and throughput.

23

Principle of cotranslational protein targeting to mitochondria

Zikun Zhu, Saurav Mallik, Taylor A. Stevens, Emmanuel D Levy, Shu-ou Shan

Presenter: Shu-ou Shan

The biogenesis of nearly all mitochondrial proteins begins with translation on cytosolic ribosomes. How these proteins are subsequently delivered to mitochondria remains poorly understood. In this work, we investigated the coupling of mitochondrial protein translation and import using selective ribosome profiling in human cells. Cotranslational targeting requires an N-terminal presequence on the nascent protein and contributes to mRNA localization at the mitochondrial surface. This pathway does not favor membrane proteins, but is predominantly used by large, multi-domain and topologically complex proteins, suggesting that the cotranslational mode of import serves to minimize irreversible protein folding in the cytosol. In contrast to the early onset of protein targeting to the endoplasmic reticulum (ER), cotranslational mitochondrial import initiates late during translation, specifically upon the emergence of a large globular domain from the ribosome. Our findings reveal a multi-layered protein sorting system that recognizes both the targeting signal and tertiary folding information in the nascent protein.

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Vectorial appearance induces conformational biases that alter protein folding yield

Iker F. Soto Santarriaga, Patricia L. Clark

Presenter: Patricia Clark

Every protein in every organism is synthesized from N- to C-terminus on a ribosome and can start to fold during synthesis. Folding during vectorial appearance is therefore the physiological environment under which amino acid sequence evolution has occurred, yet we still know very little about the conformations adopted by partially synthesized, N-terminal ribosome-bound nascent chains while they await their C-terminal interaction partners, and to what extent co-translational folding influences subsequent post-translational protein folding, binding, degradation and other events. The complexities of co-translational folding reactions can make it difficult to unambiguously assign how folding is impacted by vectorial appearance of the nascent polypeptide chain, versus (as examples) interactions with the ribosome surface or a chaperone. Can vectorial appearance lead to conformational differences that affect protein folding mechanisms, including the partitioning between native folding and misfolding? We have developed a novel, simple experimental system to explicitly and unambiguously test the impact of vectorial protein appearance on protein folding rate and yield.

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Uncovering model protein characteristics that are codon and codon-pair optimized

Presenter: Chava Kimchi-Sarfaty

Synonymous gene recoding is frequently used to enhance yields of therapeutic proteins. However, introduction of synonymous variants can affect protein folding and function. Here, using ADAMTS13, which can be used to treat Thrombotic thrombocytopenic purpura as model protein, as well as a monoclonal antibody and we evaluated several independent gene recoding strategies, codon, and codon-pair optimization. We demonstrate that gene recoding alters the translational rates and its key characteristics (substrate binding affinity, conformation, stability, post- translational modifications, and immunogenicity). We demonstrate that the protein biogenesis is determined by host cell characteristics and innate differences in gene integrations systems. Our results suggest that the routine use of gene recoding for developing protein therapeutics and in gene therapy could affect the safety and efficacy of the medications.

26

Synonymous but not silent: The codon usage code for gene expression and protein folding

Presenter: Yi Liu

Codon usage bias, the preference for certain synonymous codons, is found in all genomes. Although synonymous mutations were previously thought to be silent, a large body of evidence has demonstrated that codon usage can play major roles in determining gene expression levels and protein structures. Codon usage influences translation elongation speed and regulates translation efficiency and accuracy. Adaptation of codon usage to tRNA expression determines the proteome landscape. In addition, codon usage biases result in nonuniform ribosome decoding rates on mRNAs, which in turn influence the cotranslational protein folding process that is critical for protein function in diverse biological processes. Conserved genome-wide correlations have also been found between codon usage and protein folding. Furthermore, codon usage is a major determinant of gene expression levels through translation-dependent effects and translation-independent effects on transcriptional and posttranscriptional processes. Surprisingly, the nuclear translation-independent effects play a major role in determining codon usage effect on gene expression levels.

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Dwelling of ribosomes at rare arginine codons and production of chaperones connect the Puf3 pumilio family RNA binding protein and Not4 to protein homeostasis

Léna Audebert, George Allen, Siyu Chen, Olesya Panasenko, Suzanne Hugh, Afaf Boulkroune, Vicent Pelechano and Martine A. Collart

Presenter: Martine Collart

Tight regulation of translation elongation dynamics ensures proper folding and assembly of new proteins. Translation elongation dynamics are dependent upon the Ccr4-Not complex, notably through the activity of the Not proteins, consistent with the recently published structure of Not5 binding to post-translocation ribosomes dwelling with empty A-sites. Such ribosomes accumulate in cells lacking individual Not proteins, that also show defects in co-translational assembly and aggregation of new proteins. Rapid depletion of Not1 and Not4 inversely regulate changes in mRNA solubility correlating with codon-specific changes in A-site ribosome dwelling occupancies. mRNAs less soluble upon Not4 depletion are enriched for targets of the yeast Puf3 pumilio family RNA binding protein. Deletion of Puf3 in not4 suppresses temperature sensitivity and protein aggregation. Moreover, it inverses solubility changes for the pool of mRNAs inversely regulated upon Not1 and Not4 depletion, and it suppresses differences in codon-specific changes of A-site ribosome dwelling occupancies upon Not1 and Not4 depletion. Notably, puf3 shows reduced dwelling at rare arginine codons. The more an mRNA has rare arginine codons, the more likely the encoded protein is to be more soluble (less aggregated) and the mRNA less soluble in the double mutant. In addition, production of chaperones disrupted in not4 is partially restored in the double mutant. One such chaperone is Zuo1 whose overexpression in not4 partially suppresses temperature sensitivity.

28-29

Short-circuiting the bacterial ribosome: RAPPing about hydrogens

Martino Morici & Felix Gersteuer, Sara Gabrielli, Keigo Fujiwara, Haaris A. Safdari, Helge Paternoga, Bertrand Beckert, Lars V. Bock, Shinobu Chiba, Daniel N. Wilson

Presenters: Martino Morici / Felix Gersteuer

Programmed translational stalling at specific sequence motifs is critical for the post-transcriptional regulation of specific genes in both prokaryotes and eukaryotes (1). A well-characterized example is the Escherichia coli arrest peptide SecM, which contains a RAGP motif that stalls translation to regulate expression of SecA. Recently, novel arrest peptides containing RAPP motifs were discovered in both Gram-positive and -negative bacteria, where they likely regulate expression of important protein localization machinery components (2), however, the mechanism by which the RAPP motif induces translation arrest is unknown. Here, we present cryo-EM structures of ribosomes stalled on the RAPP arrest motifs in both Bacillus subtilis and E. coli, revealing a conserved and novel mechanism of translation inhibition. Together with molecular dynamics simulations, our results indicate that the RAPP motif allows full accommodation of the A-site tRNA, but prevents the subsequent peptide bond from forming. Our data support a model where the RAP motif in the P-site interacts and stabilizes a single hydrogen atom on the Pro-tRNA in the A-site, thereby preventing an optimal geometry for the nucleophilic attack required for peptide bond formation to occur. Surprisingly, the mechanism of RAPP-mediated arrest is completely distinct from that proposed previously for the RAGP motif of SecM (3,4), therefore, we also determined the structure of a ribosome stalled during translation of the full-length E. coli SecM arrest peptide at 2.0 Å resolution. This revealed a compacted conformation of the stalling peptide inside the exit tunnel, establishing many contacts with the components of the ribosome, better reflecting the pre-existing biochemistry on SecM than previous structures (3,4). Moreover, the arrangement at the peptidyl-transferase center suggests a stalling mechanism of SecM is analogous to that determined for RAPP-motifs. This mechanism to short circuit the ribosomal peptidyltransferase activity is likely to operate for the majority of other RAPP-like arrest peptides found across diverse bacterial phylogenies.

1. Ito K, Chiba S. Arrest peptides: cis-acting modulators of translation. Annu Rev Biochem. 2013; 82:171-202. 2. Sakiyama K, Shimokawa-Chiba N, Fujiwara K, Chiba S. Search for translation arrest peptides encoded upstream of genes for components of protein localization pathways. Nucleic Acids Res. 2021; 49:1550-1566. 3. Zhang J, Pan X, Yan K, Sun S, Gao N, Sui SF. Mechanisms of ribosome stalling by SecM at multiple elongation steps. Elife 2015; 4:e09684. 4. Bhushan S, Hoffmann T, Seidelt B, Frauenfeld J, Mielke T, Berninghausen O, Wilson DN, Beckmann R. SecM-stalled ribosomes adopt an altered geometry at the peptidyl transferase center. PLoS Biol. 2011; 9:e1000581.

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Mechanisms of translational stalling and recovery in bacterial ribosomes studied by MD simulations

Sara Gabrielli, Lars V. Bock

Presenter: Lars Bock

Gene regulation in bacteria can occur through programmed translational stalling, where specific arrest peptides pause the ribosome under certain conditions while being translated. For example, the SecM arrest peptide upregulates protein secretion through the cell membrane by inducing ribosomal stalling. A mechanical pulling force on the N-terminus terminates the stalling, allowing translation to resume. Recently, novel arrest peptides, such as ApdP, have been discovered, which share a conserved Arg-Ala-Pro-Pro motif with SecM. Cryo-EM structures of the stalled E. coli ribosome with a peptide-Arg-Ala-Pro-tRNA in the P site and a Pro-tRNA in the A site suggest that peptide bond formation is impaired during the translation of both ApdP and SecM. In all current models of peptide bond formation, the nucleophilic attack of the aminoacyl-tRNA's α-amino group on the carbonyl-carbon of the peptidyl-tRNA is facilitated by the extraction of a proton from the attacking α-amino group. Using molecular dynamics (MD) simulations of the E. coli ribosome in complex with wild-type ApdP and non-stalling variants, we determined the protonation state of the A-site Pro. We found that specific hydrogen bonds between the Arg-Ala-Pro peptide and the A-site Pro hinder conformations that enable proton extraction and the subsequent nucleophilic attack needed for peptide bond formation. Additionally, through unbiased and pulling simulations, we explored how mechanical pulling on the SecM N-terminus relieves stalling. We identified the sequence of events that disrupt the stalling conformation of the Arg-Ala-Pro-Pro motif, allowing translation to continue.

31

New functional insights into SRP-mediated protein targeting to the endoplasmic reticulum

Ilgın Eser Kotan, Sabrina Sartori, Rudra Shekhar Bose, Bernd Bukau, Günter Kramer

Presenter: Günter Kramer

The Signal Recognition Particle (SRP) is a key player in protein trafficking to the endoplasmic reticulum (ER). Despite its central importance, fundamental questions regarding substrates and mechanism of action remain open. Here, we provide a proteome-wide interaction map of SRP with nascent proteins, determine the onset and efficiency of ER targeting and describe an additional role of SRP during membrane insertion of multipass membrane proteins in Saccharomyces cerevisiae. Contrasting previous data, SRP is not pre-recruited to mRNAs but only binds ribosomes exposing targeting signals, most prominently transmembrane domains (TMDs). Surprisingly, proteins with cleavable signal peptides are often not recognized by SRP but employ alternative systems mediating co- or post-translational targeting. We furthermore show that ribosomes translating long cytosolic domains often detach from the membrane, allowing the chaperone Ssb to engage and support folding. Ribosomes translating multi-pass membrane proteins often cycle on and off the membrane, supported by multiple rounds of SRP retargeting. Taken together, our study clarifies mechanistic aspects of SRP protein triaging and reveals an interplay of SRP and Ssb, coupling membrane insertion with co-translational protein folding.

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Impaired biogenesis of basic proteins impacts multiple hallmarks of the aging brain

Presenter: Judith Frydman

33

Systematic identification and characterization of eukaryotic and viral 2A peptide-bond-skipping sequences

Deviyani M. Rao, Emma R. Horton, Chloe L. Barrington, Chloe A. Briney, Jesslyn C. Henriksen, Federico Martinez-Seidel, Evan J. Morrison, Erica M. Sterling, Edgar D. Provencio, Mia Harris, Emily Allen, Jay R. Hesselberth, Olivia S. Rissland

Presenter: Olivia Rissland

2A peptides are 18-22 amino acid sequences that cause an unusual co-translational peptide-bond skipping event. Initially discovered in viruses, 2A peptides have become useful biotechnical tools because they enable production of multiple separate proteins from a single open reading frame. Despite their utility, little is known about their prevalence throughout the evolutionary tree of life or the range of their sequence diversity. Our computational analyses predicted ~2,200 new 2A peptide instances, significantly expanding the known class of 2A peptides (Class A), and identified a second, previously unrecognized class (Class B). These results also revealed that predicted 2A peptides are widespread in both RNA viruses and eukaryotes. We tested a subset of these new 2A peptides in human cell, and found most had skipping activity, suggesting there are likely thousands of active 2A peptides. Consistent with previous reports, mutational analysis of both classes identified residues near the skipped peptide bond that are necessary for activity, as are those in the upstream region. Notably, Class B 2A peptides uniquely contain a conserved N-terminal tryptophan whose register and identity within the peptide are critical for activity. Together, our results demonstrate that 2A peptides are widespread throughout eukaryotes and viruses, and that they are more diverse than previously appreciated.

34

The yeast E3 ligase Hel2 monitors the duration of ribosome stalling

Ying Zhang, Mario Scazzari, Sabine Rospert

Presenter: Sabine Rospert

Stalled ribosomes lead to collisions, disrupt protein synthesis, and produce potentially harmful, truncated polypeptides. To counteract these deleterious effects, eukaryotic cells employ the ribosome-associated quality control (RQC) and no-go mRNA decay (NGD) pathways. In yeast, the E3 ubiquitin ligase Hel2 is the initial component of the RQC/NGD pathways. Hel2 targets disomes and trisomes and polyubiquitinates the 40S ribosomal protein Rps20. Ubiquitination of Rps20 is required for subsequent steps in the RQC/NGD pathways. Recent research has uncovered high concentrations of disomes and trisomes in unstressed cells. This prompts the question of whether and how Hel2 selects long-term stalled disomes and trisomes for RQC/NGD. To explore this topic, we conducted a comprehensive analysis of in vivo-formed Hel2●ribosome complexes and the in vivo dynamics of Rps20 ubiquitination and deubiquitination. Our results reveal how Hel2 enables the downstream RQC machinery to preferentially target non-productively stalled ribosomes for rescue over temporarily paused elongating ribosomes.

35

Nascent chain-mediated ribosomal frameshifting during membrane protein translation

Patrick J. Carmody, Dyotima, Caden R. Sillman, Rohan Bhardwaj, Wesley D. Penn, Charles P. Kuntz, Jonathan P. Schlebach

Presenter: Jonathan Schlebach

Though the ribosome possesses several features that help maintain its translational reading frame, certain transcripts contain RNA structures that override these mechanisms to promote ribosomal frameshifting. We recently found that the mechanical tensions generated by cotranslational folding enhance the activity of a viral RNA structure that stimulates -1 programmed ribosomal frameshifting (-1PRF). However, it remains unclear whether the nascent chain plays a more general role in translational recoding. Through modifications to the transcript of a model membrane protein LepB, we find that the translocation of a nascent transmembrane domain (TMD) is sufficient to induce efficient -1PRF during the decoding of a slippery sequence. Based on the constraints of this coupling observed both in vitro and in a human cell line, we identify hundreds of comparable “TMD-slip” motifs within the transcripts of human membrane proteins. Using a fluorescent -1PRF reporter, we surveyed the activity of select TMD-slip motifs within the transcripts of a GPCR and two ion channels. Our results suggest the nascent chain potentially enhances translational recoding at a contentious -1PRF site within the CCR5 transcript. Additionally, we identify an active -1PRF site within the transcript encoding the KCNQ1 potassium channel and show that its activity is enhanced by topological rearrangements of the nascent chain that arise from alternative splicing. We outline how this dynamic frameshifting may allow the translation machinery to suppress the production of non-native channels. Together, our findings suggest this mode of feedback between the nascent chain and ribosome may play a general role in translational regulation.

36

How amino acid chemistry at the ribosome A- and P-sites modulate translation elongation speed

Yang Jiang, Justin Pettuci, Vasant Honavar, Ed O'Brien

Presenter: Ed O'Brien

We previously demonstrated that pairs of amino acids, when located at the P- and A-sites of the ribosome, can causally modulate translation-elongation rates (JMB 2020). What we didn't know at the time was why particular pairs of amino acids tend to speed up the ribosome's translation speed, while others slow it down. Here, we explore this question through a combination of interpretable machine learning and biophysical modeling, showing that several physicochemical properties of amino acids contribute to this phenomenon. These include secondary structure preferences, hydrophobicity, electrostatic interactions, and pKa values. We then use simple models to explain why these features modulate speed in the way they do. Finally, we demonstrate that a biophysical equation encompassing these amino acid properties correlates well with the variation in ribosome profiling data from S. cerevisiae, indicating that the model captures the essence of the phenomenon.

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Profiling analysis of co-translational folding and assembly of newly synthesized proteins

Manuel Günnigmann, Jaime Santos Suarez, Frank Tippmann, Günter Kramer, Bernd Bukau

Presenter: Bernd Bukau

Profiling analysis of co-translational folding and assembly of newly synthesized proteins Manuel Günnigmann, Jaime Santos Suarez, Frank

Tippmann, Günter Kramer and Bernd Bukau Center for Molecular Biology of Heidelberg University (ZMBH), DKFZ-ZMBH Alliance, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany. Many of the critical steps of enzymatic processing, membrane targeting, native folding and assembly of newly synthesized proteins occur co-translationally, implying that protein maturation and decoding of genetic information by the ribosome are coupled processes. We are using ribosome profiling technology to dissect the flux of the cellular nascent chain proteome through the system of co-translationally engaged chaperones and targeting factors. This allowed us to reveal principles of functional networking of the co-translational chaperone machineries that promote the formation of correctly folded proteins. We furthermore show that the assembly of oligomers frequently occurs co-translationally, involving the engagement of nascent subunits once the interaction domains have emerged at the ribosomal tunnel exit. We found that translation speed frequently changes at the onset of co-translational assembly, implying coordination of protein synthesis and assembly.

1

Doublet decoding of tRNASer3 demonstrates plasticity of ribosomal decoding center

Shruthi Krishnaswamy, Shirin Akbar, Yang Chen, Daniel Larsson, Maria Selmer

Presenter: Shirin Akbar

Frameshifts can be caused by specific combinations of tRNA and mRNA. The doublet decoding hypothesis was proposed in the 1980s to explain how the AGC-decoding E. coli tRNASer3 with low frequency could induce -1 frameshifts on GCA alanine codons with only two base pairs between the mRNA codon and the tRNA anticodon. It has remained unclear whether this type of non-standard decoding can be accommodated by the ribosome. To test the doublet-decoding hypothesis, we performed single-particle cryo-EM reconstructions on E. coli 70S ribosomes with the frameshift-inducing tRNASer3 bound to the non-cognate GCA codon or the cognate AGC codon in the ribosomal A site. Our structures confirm that doublet decoding occurs when the GCU anticodon of tRNASer3 forms only two Watson-Crick base pairs between the first two anticodon bases G34 and C35 and the two first bases of the GCA alanine codon. This interaction is made possible by U36 from the anticodon forming a Hoogsteen base pair with A1493, the conserved monitoring base in 16S rRNA, pushing the first base of the A-site codon in position for interaction with C35 of the anticodon. The second monitoring base, A1492, is displaced from the decoding center and stacks with 1913 from 23S rRNA. G530 is unable to interact with the mRNA-tRNA pair, leading to a near-cognate-like conformation of the 30S subunit. Nitrocellulose filter-binding experiments show that the anticodon stem-loop (ASL) of tRNASer3 binds to the GCA codon in the ribosomal A site with eight-fold lower affinity compared to the cognate ASL from tRNAAla1, a similar difference as between ASLPhe binding to a fully complementary UUC codon or a UUU codon with a third-position wobble base pair. The results demonstrate how the plasticity of the decoding center allows a doublet-decoding tRNA to bind to the ribosomal A site.

2

Direct observation of folding process of membrane protein during cell free expression by Surface Enhanced Infrared Absorption Spectroscopy

Kenichi Ataka, Ramona Schlesinger, Joachim Heberle

Presenter: Kenichi Ataka

The translocon-unassisted folding processes of various microbial rhodopsins during cell-free expression have been investigated using Surface Enhanced Infrared Absorption Spectroscopy (SEIRAS). Attempts were made to express the transmembrane domains of bacteriorhodopsin (bR), sensory rhodopsins I (SRI) and II (SRII), and channel rhodopsin II (ChR2). However, only bR achieved a fully functional structure, while the other rhodopsins failed to form the relevant chromophore, as indicated by the absence of corresponding absorption bands. Results from SEIRA spectroscopy demonstrate that all proteins successfully produced nascent polypeptides that inserted into the lipid bilayer during the co-translational period (within the first hour of expression). Secondary structural analysis of the SEIRA spectra reveals that each protein incorporated a comparable amount of α-helical structure, suggesting proper insertion into the lipid bilayer and formation of secondary structures during co-translation. However, only bR exhibited a continuous increase in helical content beyond one hour, indicating the formation of tertiary structure through helix association, while the helical content of the other proteins plateaued. This suggests that the unsuccessful cell-free expression of SRI, SRII, and ChR2 may stem from their inability to form tertiary structures after co-translation. Given that SRI and ChR2 fail to form the retinal chromophore, while bR also struggles with tertiary structure formation in the absence of retinal, it is likely that the association of the nascent polypeptide with the retinal molecule is crucial for the formation of a fully functional protein.

3

Identification and characterization of novel quality control factors on the ribosome

Hagit Bar Josef, Junyi He, Fabian Glaser, Johann Venezian, Ayala; Shiber

Presenter: Hagit Bar-Yosef

The proper folding of nascent proteins is a major challenge within the crowded eukaryotic cell. At this essential intersection of translation and folding, the ribosome functions as a hub, coordinating the activities of diverse factors that guide the emerging polypeptide-chain maturation. These various factors include modifying enzymes, targeting factors and folding chaperones. Even the assembly into high-order oligomeric complexes, the final step of folding, was recently discovered to occur during protein synthesis. Co-translational assembly mechanism was found to be a wide spread mechanism, conserved from yeast to human. Misfolding-prone subunits are directed for degradation in a co-translational manner. Leading to the question: How does the cell recognize protein misfolding even before the end of synthesis? Here we identify and characterize assembly-specific degradation and sequestration factors that act in a co-translational manner, maintained throughout evolution from yeast to humans. Utilizing a combination of ribosome profiling, super-resolution imaging and targeted ribosome nascent-chain proteomics approaches, we were able to discover a novel quality-control pathway for complex subunits that fail to assemble into functional complexes. This provides us, direct data on a proteome-wide scale, of factors safeguarding the cellular proteome during synthesis. This research can pave the way for development of potential healthcare strategies of disease conditions characterized by protein misfolding, such as Parkinson's and Alzheimer's.

4

Co-translational ISGylation regulates translation suppression during early innate immune response

Hila Ben-Arie Zilberman, Rawad Hanna, Fabian Glaser, Ayala Shiber

Presenter: Hila Ben-Arie Zilberman

The innate immune response induces the ubiquitin-like protein ISG15 (Interferon-Stimulated Gene 15), which modifies various cellular and viral proteins. The primary ISG15-ligase, HERC5, has been linked to translating ribosomes, yet the effects of ISG15 on cellular translation responses, co-translational folding, and ribosomal interactions remain largely unknown. Combining polysome profiling, ribosome profiling, and co-translational proteomics we analyzed interferon-I dynamic response in ISG15 knockout, and in HERC5 depleted cell lines. Inhibiting ISGylation resulted in decreased ribosome association with initiation and elongation factors, as well as canonical molecular chaperones. In contrast, association with various translation suppression factors including mRNA-modifying enzymes was increased. Ribosome assembly levels were also decreased. TRIM25 ISG15/Ub ligase was identified as a novel ribosome-associated factor, binding during early interferon response. TRIM25 was found to recruit HERC5 ISG15 ligase, resulting in widespread co-translational ISGylation. Structural modeling of the ISG15-ligases, followed by prediction of putative ribosome interaction sites predicted several motifs, which are currently undergoing validation. Analysis of these interactions can promote our understanding of how ISG15 and its ligases dynamically influence co-translational protein folding pathways during the innate immune response. Together, these findings highlight co-translational ISGylation, as well as early ISG15-E3 ligases ribosome association as critical regulators of ribosome function in early stress responses and innate immunity.

5

Structural dynamics of ribosome-stalling peptides in the ribosomal exit tunnel uncovered by MD simulations

Ole Berendes, Sara Gabrielli, Helmut Grubmüller, Lars V. Bock

Presenter: Ole Berendes

The ribosome is a macromolecular complex responsible for synthesizing proteins in all living cells. The growing nascent protein leaves the catalytic center of the ribosome through a tunnel with walls formed by ribosomal RNA and proteins. The tunnel is not a passive pathway but plays a key role in protein synthesis and translation regulation. Specific nascent protein chain sequences can interact with the tunnel walls leading to translational arrest. These stalling mechanisms regulate gene expression during protein synthesis. Induction of translational stalling in the exit tunnel is not limited to nascent proteins. Several small-molecule antibiotics and antimicrobial peptides target the tunnel to inhibit translation at the bacterial ribosome. To elucidate the mechanisms of arrest-inducing nascent proteins and antimicrobial peptides at an atomic level, we employ molecular dynamics (MD) simulations. These offer the possibility to gain insights into the structural dynamics of the peptides and tunnel walls. Here, we present an MD study of two tunnel-binding antimicrobial peptides. Antimicrobial peptides present a promising avenue of research for the development of urgently needed novel antibiotics. We studied the conformational dynamics of two synthetic lead compounds Api137 and Api88 at their primary binding sites in the exit tunnel. The peptides are structurally similar and only differ in their C-terminal functional group. Our MD simulations showed marked differences in the conformational dynamics of the two peptides. We found that Api88 occupies a wider range of conformations than Api137, in agreement with cryo-EM experiments. Our results suggest two different modes of action for Api88 and Api137, entirely determined by the chemical difference at the C-terminus. The detailed understanding of their binding dynamics might prove useful for further sequence optimizations of Api88 and Api137.

6

Mapping the cotranslational folding of DHFR by deep mutational scanning

Rohan Bhardwaj, Jonathan P. Schlebach

Presenter: Rohan Bhardwaj

Though protein folding begins during the early stages of protein biosynthesis on ribosome, relatively little is known about how ribosomes may alter these initial folding pathways. Several recent investigations of cotranslational folding have largely relied upon arrest peptides that interact within the ribosomal exit tunnel in a manner that requires mechanical folding forces to prevent translational stalling. Applications of arrest peptides as cotranslational force sensors are beginning to reveal when proteins begin to form structure. While this approach identifies points during elongation in which structures form, the nature of these conformational transitions often remains unclear. To identify regions of nascent polypeptide chain involved in these transitions, we are developing a deep mutational scanning approach to explore the sequence constraints of Escherichia coli dihydrofolate reductase (DHFR) folding on ribosome. To this end, we have developed an arrest peptide-based fluorescence reporter for cotranslational DHFR folding in live-cells. To probe the profile of amino acids that play key roles in the late-stage cotranslational folding of DHFR, we have generated a diverse codon-saturated library of ~9,500 DHFR variants in the context of this biosensor. Using fluorescence activated cell sorting and deep sequencing, we are currently identifying which of these variants perturb this conformational transition. Moreover, by profiling the same library in the context of a variant of the sensor bearing a defective arrest peptide, we will determine how these mutations specifically disrupt cotranslational folding reaction. This generalizable approach could potentially be used to map cotranslational folding intermediates of a wide variety of other proteins.

7

FP-iTP-Seq: a novel high-throughput method to study co-translational protein folding

Arunima Bhattacharya, Ane Metola, Justin M. Westerfield, Mélanie Gillard, Gunnar von Heijne, C. Axel Innis

Presenter: Arunima Bhattacharya

Force Profile Analysis (FPA) is a widely used technique for studying co-translational protein folding. It is based on the principle that the free energy released by protein folding on the ribosome exerts a pulling force on a nascent polypeptide sufficient to resolve translational arrest induced by a force-sensing arrest sequence fused to its C-terminus. A map of such co-translational force-generating events for multiple N-terminal truncations of a protein of interest is called a force profile (FP) and can reach single-amino acid resolution if sufficient constructs of such truncations are analyzed. In its current form, FPA relies on the low-throughput generation of up to ~100 individual constructs and the subsequent quantification of their translation products on polyacrylamide gels. Here, we present a novel high-throughput technique called FP-iTP-Seq (Force Profile coupled to iTP-Seq), which multiplexes FPA of protein fragments translated in vitro using inverse toeprinting coupled to next-generation sequencing (iTP-Seq). A typical DNA library for FP-iTP-Seq contains one or several protein-coding ORFs fragmented to generate every possible N-terminal truncation, followed by one of several arrest peptides of varying strengths. Ultimately, this approach should make it possible to study the in vitro co-translational folding of hundreds of soluble proteins in a highly parallel fashion.

8

Towards a detailed molecular picture of co-translational folding in real time

Amir Bitran, Carlos Bustamante, Susan Marqusee

Presenter: Amir Bitran

The non-equilibrium process of protein folding on the ribosome is critical for ensuring high folding yield of certain proteins that cannot refold efficiently at equilibrium. However, the precise molecular mechanisms underlying co-translational folding remain poorly understood. We address this question by using hydrogen-deuterium exchange mass spectrometry (HDX-MS) to probe the co-translational folding of Halotag—a protein previously shown to achieve a higher folding yield on the ribosome as compared to in solution. Equilibrium HDX-MS experiments on stalled nascent chains reveal that the protein's alpha/beta core acquires structure in a length-dependent manner; in contrast, the helical lid domain remains largely unfolded until it has fully emerged from the ribosome. These results suggest that the Halotag core folds co-translationally via a vectorial mechanism that differs from its solution refolding pathway, which proceeds through an intermediate involving both termini. To test this hypothesis, we have developed a synchronized translation assay in which we use pulse HDX to monitor nascent Halotag folding at different translation times. We will present results from this experiment and compare the observed real-time co-translational folding pathway to the mechanism predicted by studying stalled nascent chains at equilibrium.

9

Translocon remodeling modulates -1 programmed ribosomal frameshifting

Antonio Bonifasi, Suchetana Mukhopadhyay, Jonathan Schlebach

Presenter: Antonio Bonifasi

Membrane proteins are cotranslationally folded into cellular membranes by way of protein conducting channels known as translocons. Engagement of nascent polypeptides by translocons at various points in the folding process generates fluctuations in the mechanical tension between the ribosome and nascent chain. We recently found that these translocation forces enhance the efficiency of a translational recoding mechanism known as -1 programmed ribosomal frameshifting (-1PRF), which is essential for the translational regulation of various viral polyproteins. Since these initial discoveries, it has been found that translocon complexes are dynamically remodeled throughout the course of membrane protein biogenesis. To determine whether variations in translocon usage impact this feedback between the nascent chain and ribosome, we compared the efficiency of -1PRF during the translation of the Sindbis virus structural polyprotein in the context of a series of CRISPR knockout cell lines lacking various translocon complexes. Our initial findings suggest the PAT and EMC complexes typically suppress -1PRF during polyprotein biogenesis. These results potentially suggest the dynamic association of certain translocon subunits modulates the mechanical feedback that dynamically regulates polyprotein translation in the cell.

10

Towards a structural insight into the delicate interplay between translation and misfolding on the ribosome

Ivana V. Bukvin, Juliana Abramovich, Judith Frydman

Presenter: Ivana Bukvin

The ribosome is increasingly recognised as an active participant in de novo protein folding, not only through the vectorial nature of synthesis but by modulating the energetics of co-translational protein folding, stabilising partially folded states, and acting as a hub for co-translational chaperone engagement. While intrinsically disordered proteins (IDPs) form the molecular basis of most neurodegenerative diseases, all proteins face their earliest decision between folding and misfolding during biosynthesis on the ribosome. Here, we explore whether the ribosome promotes huntingtin misfolding by modulating its conformational landscape during biosynthesis. Huntingtin exon 1 (Httex1) is an IDP and is considered the causal agent of Huntington's disease due to an expansion of CAG repeats encoding its polyglutamine domain (>35). This domain is flanked by a C-terminal polyproline domain, implicated in ribosome stalling and collisions, and an amphipathic N-terminal domain, which is suggested to initiate huntingtin misfolding and oligomerisation and emerges first during its biosynthesis on the ribosome. We are developing a single-molecule fluorescence approach that will allow us to describe the translation kinetics, conformational landscape, and co-translational engagement of huntingtin during its biosynthesis on the ribosome, thereby providing unique insights into the delicate interplay between translation and the earliest steps in protein misfolding.

[1] Streit, J.O., Bukvin, I.V., Chan, S.H.S. et al. The ribosome lowers the entropic penalty of protein folding. Nature 633, 232–239 (2024)

[2] Aviner, R., Lee, TT., Masto, V.B. et al. Polyglutamine-mediated ribotoxicity disrupts proteostasis and stress responses in Huntington's disease. Nat Cell Biol 26, 892–902 (2024).

11

Unveiling the dynamics of protein folding: Insights from single-molecule tracking of chaperone-client interactions in live cells

Niko Dalheimer, Rongqin Li, Ulrich Hartl

Presenter: Niko Dalheimer

The proteome of mammalian cells consists of 10,000-20,000 different proteins. Most of these must fold into unique three-dimensional structures to fulfil their biological functions. However, in the crowded cellular environment, nascent, unfolded polypeptide chains are highly prone to misfolding and aggregation. To ensure efficient folding and prevent such off-pathway reactions, proteins interact with several classes of molecular chaperones. Among these, the chaperonin TRiC/CCT complex and its co-chaperone Prefoldin have been extensively studied due to their specific roles in the cellular proteostasis network. However, mechanistic studies have mainly been performed in vitro using ensemble measurements. To understand the dynamics of chaperone-client interactions in living cells, we use single-molecule tracking to follow the de novo folding process on and off the ribosome. Our experiments revealed the dynamics of the co- and post-translational interaction between TRiC/CCT, Prefoldin and its client protein actin in the intact cellular environment. Our results shed light on how TRiC/CCT and Prefoldin assist substrate proteins throughout their cellular lifetime for de novo folding and conformational maintenance.

12

Probing general principles underlying biogenesis of proteins with a common fold

Karim Elbouri, Sarah Maslen, Marca Mora Hortal, Grant Pellowe, Sergi Garcia-Manyes, Mark Skehel, David Balchin

Presenter: Karim Tarik Wahbi Elbouri

Inside cells, protein folding begins and proceeds cotranslationally, coupled to vectorial N- to C-terminal polypeptide chain extension. Despite being essential for proteostasis and biogenesis, our understanding of cotranslational folding (CTF) pathways and chaperone action remains limited. In particular, it is unclear whether mechanisms of CTF are generalisable across domains that share a common fold. To mimic snapshots of protein synthesis, we prepare a series of precisely stalled E.coli ribosome:nascent chain complexes (RNCs), which represent progressive CTF intermediates and co-purify with endogenous chaperones in a chain-length specific manner. As model nascent chains (NCs), we selected several (βα)8 Triosephosphate Isomerase-barrels (TIM-barrels), which often require chaperones to fold, and adopt a modular fold, enabling comparisons between different NCs that are topologically equivalent. Deploying structural proteomics tools, we study the dynamics, topology and chaperone interactions of these chain length-specific CTF intermediates from sequence-diverse TIM-barrels. The ribosome-associated chaperone TF co-purifies at stoichiometric levels with RNCs of all TIM-barrels studied. Using XLMS and HDX-MS, we characterised NC local folding and stability in structured CTF intermediates. XLMS showed that TIM-barrels studied form long-range intra-NC crosslinks during translation, consistent with structured intermediates resembling the native topology, and that TF forms extensive interfaces with all NC lengths probed, establishing contacts that encompass the entire length of the NC. Alongside monitoring NC conformational dynamics throughout elongation in the presence and absence of TF, HDX-MS sheds light on changes in the chaperone surfaces that bind NCs throughout elongation and how TF differentially engages TIM-barrels to modulate their folding equilibria.

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Exploring the impact of nascent peptide and mRNA sequences on the non-uniform rate of translation

Julia Fricke, Ekaterina Samatova, Tamara Senyushkina, Simon Christ, Nadin Haase, Sophia Rudorf, Marina Rodnina

Presenter: Julia Fricke

Translation of codons along mRNA occurs at a non-uniform rate, with various factors contributing to pauses that are crucial for the efficient production of active, correctly folded proteins. Our study focused on how three primary factors - nascent peptide charge, co-translational folding, and codon usage - modulate translation kinetics. To explore their impact, we engineered variants of the HemK N-terminal domain (NTD) mRNA with synonymous and non-synonymous codon substitutions. We then analyzed ribosome translation of these mRNA variants in vitro using a reconstituted E. coli translation system. Computational analysis provided insights into local kinetics, while smFRET was employed to examine ribosomal conformations during pauses. Our findings demonstrate how changes in nascent peptide and mRNA influence translation efficiency, ribosome pausing patterns, processivity, and drop-off, allowing us to identify the factors that most strongly affect the ribosome's translation rate.

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Kinetics of translation elongation in humans

Vaishali Goyal, Ekaterina Samatova, Frank Peske, Marina V. Rodnina

Presenter: Vaishali Goyal

Non-uniform local rates of mRNA decoding by ribosomes is a hallmark of translation. Transient ribosome pausing during translation elongation aids in proper folding and maturation of proteins. Several factors, such as codon usage bias, composition of aa-tRNA pool, nascent peptide sequence, or mRNA structure modulate the local translation rate. We aim at unraveling the effect of codon usage on co-translational folding mechanisms in humans. We established a fully reconstituted translation system consisting of purified 40S and 60S ribosomal subunits, initiation and elongation factors and human aminoacyl-tRNA capable of translating peptides up to a length of 200 amino acids with a close to in vivo rate of translation. It uniquely equips us to study the kinetic mechanisms of human translation elongation unaffected by cellular conditions. By combining our in vitro experiments with mRNA mutations, we aim to shed a light on how ribosomal pausing during elongation modulates co-translational folding ultimately affecting protein structure and function.

15

Studying co translational folding of membrane proteins in lipid nanodiscs

E.C. Johnston, G. Pellowe, C. Bisson, H.E. Findlay, P. Booth

Presenter: Erin Johnston

In the cell many membrane proteins fold co-translationally, where the protein begins to fold from the N-terminus while translation continues on the ribosome. Co-translational folding is particularly common in membrane proteins however this is challenging to study due to the importance of the membrane lipid environment for correct protein folding. Previous work has shown that enhanced secM facilitated nascent chain stalling and nanodisc technology can be utilised together to capture in vivo generated membrane protein ribosome nascent chain complexes (RNCs) in a native lipid environment. In this work we use RNCs purified in native nanodiscs combined with cryo-EM to study the co translational folding of a polytopic membrane protein. Constructs of the rhomboid protease GlpG stalled at transmembrane (TM) helix 2, 4 and 6 were designed and purified in sulpho styrene-maleic acid co-polymer native nanodiscs. Using this system we aim to use Cryo-EM to gain insight in to co-translational folding mechanisms of membrane proteins, however there are several challenges in this sample including complex dissociation, sample flexibility and the relatively small size of the stalled protein with respect to the ribosome.

16

Visualising nascent chain interactions with the ribosome by cryo-electron microscopy

Gabija Jurkeviciute, Alžběta Roeselová, Jessica He, Radoslav Enchev, David Balchin

Presenter: Gabija Jurkeviciute

Protein folding often begins as the nascent chain (NC) emerges from the ribosome, where it is synthesised. To investigate the origin of ribosome-induced NC destabilisation and implications on conformation, we solved a high-resolution cryo-EM structure of a ribosome-nascent chain complex (RNC) containing two and a half domains of β-galactosidase. The structure revealed density for the NC outside the exit tunnel, offset from the vestibule and proximal to a native amphipathic β-galactosidase helix tethered to ribosomal protein L23. Within the exit tunnel, the NC follows a defined path, initially biased towards L22 past the constriction site, then shifting past the L23 loop extension towards H59, continuing to L29, finally reaching L23 outside the vestibule. Here, the NC forms a stable interaction with L23 displacing its flexible C-terminus and exposing a hydrophobic groove that stabilises the amphipathic β-galactosidase helix (466-478), the isolated peptide of which has been shown to be disordered in solution. The hydrophobic interaction with L23 disrupts domain D3 folding, delaying its docking against domains D1/2 until synthesis is complete. Additionally, the structure revealed conformational changes throughout the ribosome's exit tunnel, particularly L24, H59, L29, and L23, suggesting that the exit tunnel's architecture is more responsive to NC conformation than previously thought. Overall, our study highlights the role of the ribosome surface in local NC folding, leading to co-translational destabilisation and delayed domain assembly.

17

Tuning the SDD1 arrest peptide region in Saccharomyces cerevisiae

Kellogg, M.K., Andréasson, C., von Heijne, G

Presenter: Morgana Kellogg

Arrest peptides are short tracts of amino residues that stall translation at a specific codon or dicodon region in the messenger RNA1. Stalling can be overcome if sufficient force is exerted on the nascent chain, pulling the arrest peptide away from the peptidyl transferase center. The strength of the force required to overcome the arrest can inform about cotranslational events like the formation of protein secondary structure, protein translocation and targeting, and membrane insertion. The best characterized arrest peptide comes from the Escherichia coli SecM protein. The SecM arrest peptide has been used to study the cotranslational protein folding of both soluble and membrane proteins in E. coli. Recently, the uncharacterized yeast protein SDD1 was found to be an endogenous ribosome quality control (RQC) substrate4. RQC ensures that stalled or collided ribosomes are rescued when triggered. This presents an opportunity to characterize the region of SDD1 that promotes arrest (and triggers RQC) as a bona fide arrest peptide and create a suite of stronger and weaker variants as a tool to study cotranslational protein folding. This project uses Saccharomyces cerevisiae's versatility as a model cellular organism to determine whether the SDD1 arrest peptide region can be tuned for future applications in studying the behavior of nascent polypeptide chains at the yeast ribosome.

18

Ribosomal protein eL39: sequence and structural variability and its role in protein co-translational folding.

Oktawia Korcz, Tomasz Włodarski

Presenter: Oktawia Korcz

The ribosomal exit tunnel, which allows nascent proteins to leave the ribosome during synthesis, spans through the large ribosomal subunit and in eukaryotes is shaped by ribosomal RNA and three proteins: uL4, uL22, and eL39. While the internal loops of uL4 and uL22 form constriction sites essential for ribosome assembly and function, eL39, which is specific to archaea and eukaryotes, is located near the tunnel's exit; however, its specific role is unclear. Ribosomes lacking eL39 exhibit reduced translation accuracy and hypersensitivity to paromomycin due to faster aminoacyl-tRNA binding at the A-site. Mutations in eL39 can impair mitochondrial protein synthesis, leading to mitochondrial dysfunction, which is linked to neurodevelopmental disorders. Interestingly, RPL39L, a variant of eL39 found only in mammals, shows a heterogeneous expression across tissues and cells. Additional studies suggest that its overexpression may reduce protein aggregation, indicating its potential role in co-translational protein folding. To study eL39 in more detail and understand its role in protein synthesis, we conducted an extensive bioinformatics analysis of its homologs using sequences from UniProt and structural data from PDB and AlphaFold DB. We identified conserved regions in the eL39 sequence that are positively charged, suggesting their role in RNA binding. Based on the ribosome structures, we identified other important eL39 binding sites to the ribosome. This bioinformatics study is part of our ongoing effort to characterise ribosomal proteins involved in co-translational protein folding, which will provide novel insights into this process from an evolutionary perspective.

19

Unraveling unresolved aspects of SRP-mediated protein targeting to the endoplasmic reticulum

Ilgın Eser Kotan, Sabrina Sartori, Rudra Shekhar Bose, Bernd Bukau, Günter Kramer

Presenter: Ilgın Kotan

The Signal Recognition Particle (SRP) is a key player in protein trafficking to the endoplasmic reticulum (ER). Despite its central importance, fundamental questions regarding substrates and mechanism of action remain open. Here, we provide a proteome-wide interaction map of SRP with nascent proteins, determine the onset and efficiency of ER targeting and describe an additional role of SRP during membrane insertion of multipass membrane proteins in Saccharomyces cerevisiae. Contrasting previous data, SRP is not pre-recruited to mRNAs but only binds ribosomes exposing targeting signals, most prominently transmembrane domains (TMDs). Surprisingly, proteins with cleavable signal peptides are often not recognized by SRP but employ alternative systems mediating co- or post-translational targeting. We furthermore show that ribosomes translating long cytosolic domains often detach from the membrane, allowing the chaperone Ssb to engage and support folding. Ribosomes translating multi-pass membrane proteins often cycle on and off the membrane, supported by multiple rounds of SRP retargeting. Taken together, our study clarifies mechanistic aspects of SRP protein triaging and reveals an interplay of SRP and Ssb, coupling membrane insertion with co-translational protein folding.

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Insights into the dynamics of RNC-SecYEG complex by FCS

Justas Kvietkauskas, Damir Sakhapov, Xiaolin Wang, Jörg Enderlein, Marina Rodnina

Presenter: Justas Kvietkauskas

Membrane proteins are inserted cotranslationally into the membrane via the translocon (SecYEG in bacteria), which opens laterally to allow the protein to enter the lipid bilayer and adopt its final fold. Current models suggests that membrane proteins form α- helices inside the ribosome exit tunnel, which then later assemble into the full protein. SecYEG dynamics can attenuate membrane protein targeting and folding [1] [2]. To study the influence of the ribosome and SecYEG on membrane protein folding, we have used an in vitro translation system to prepare ribosome nascent chain complexes (RNCs) carrying different SecYEG client proteins. To carry out fluorescence spectroscopy experiments SecYEG was fluorescently labeled and reconstituted into nanodiscs. To monitor the dynamics of the RNC-SecYEG complex on a timescale from nanoseconds to milliseconds, we employ fluorescence correlation spectroscopy (FCS)-based methods. Our current data indicate three different modes in the dynamics of the RNC-SecYEG complex, depending on the client protein as well as the nascent chain length. Overall, our findings provide potential insight on how the RNC-SecYEG complex accommodates its large number of client proteins

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Unveiling the dynamics of protein folding: Insights from single-molecule tracking of chaperone-client interactions in live cells

Rongqin Li, Niko Dalheimer, Ulrich Hartl

Presenter: Rongqin Li

The proteome of mammalian cells consists of 10,000-20,000 different proteins. Most of these must fold into unique three-dimensional structures to fulfil their biological functions. However, in the crowded cellular environment, nascent, unfolded polypeptide chains are highly prone to misfolding and aggregation. To ensure efficient folding and prevent such off-pathway reactions, proteins interact with several classes of molecular chaperones. Among these, the chaperonin TRiC/CCT complex and its co-chaperone Prefoldin have been extensively studied due to their specific roles in the cellular proteostasis network. However, mechanistic studies have mainly been performed in vitro using ensemble measurements. To understand the dynamics of chaperone-client interactions in living cells, we use single-molecule tracking to follow the de novo folding process on and off the ribosome. Our experiments revealed the dynamics of the co- and post-translational interaction between TRiC/CCT, Prefoldin and its client protein actin in the intact cellular environment. Our results shed light on how TRiC/CCT and Prefoldin assist substrate proteins throughout their cellular lifetime for de novo folding and conformational maintenance.

22

Measuring homomeric protein assembly in vivo

McKenze J. Moss, Patricia L. Clark

Presenter: McKenze Moss

Department of Chemistry & Biochemistry, University of Notre Dame, Notre Dame, IN, 46556 USA Proper folding is a prerequisite for protein function. Most proteins are multimeric, hence folding includes assembling individual subunits together to form the native structure. Although little is known about multimeric protein folding, failing to form proper interactions can lead to loss of protein function, aggregation and/or degradation. During protein synthesis, subunits of homomeric proteins are close together on neighboring ribosomes and may start to assemble co-translationally ("co-co" assembly), enhancing the efficiency of folding and assembly (Bertolini et al., 2021). Alternatively, nascent chains may interact co-translationally with a full-length subunit ("co-post" assembly). As an initial model to measure the extent of co-translational assembly and its impact on protein folding efficiency, we used E. coli chloramphenicol acetyltransferase (CAT). The native CAT homotrimer is thermostable and shows no evidence of subunit exchange. CAT does not refold to its native structure after dilution from a chemical denaturant. Collectively, these results indicate that the "pioneer round" of CAT folding, potentially including co-translational assembly, is important for achieving the native structure. Consistent with this model, CAT folding is sensitive to synonymous codon substitutions (Walsh et al., 2020). However, we and others have shown that CAT C-terminal residues are essential for proper folding (Van de Schueren et al., 1996). To resolve this conundrum, we designed a novel fluorescence reporter assay to identify co-translational assembly mechanisms and are using arrest peptide force profiling analysis to identify co-translational folding intermediates. Together, these assays should prove useful for defining the CAT in vivo folding and assembly pathway.

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The human ribosome modulates the stability of cotranslational folding intermediates

Grant A. Pellowe, Tomas B. Voisin, Laura Karpauskaite, Sarah L. Maslen, Alzbeta Roeselova, Steven Howell, Chloe Roustan, Mark Skehel, David Balchin

Presenter: Grant Pellowe

Protein biogenesis begins on the ribosome where nascent chains are expressed vectorially from their N- to C-terminus, and start to fold as mRNA is translated. How the context of protein synthesis influences protein folding is poorly understood, especially in eukaryotes. Here we describe novel methods for the generation, purification and analysis of stable, homogeneous ribosome-bound nascent chain complexes from human cells, with the goal of defining the co-translational folding pathway of the nascent polypeptide. As a model for eukaryotic protein biogenesis, we focused on Firefly luciferase, a conformationally labile multidomain protein. Luciferase refolds slowly from denaturant, but folds rapidly in the context of translation on 80S ribosomes. Using hydrogen-deuterium exchange mass spectrometry, crosslinking-mass spectrometry and cryo-electron microscopy, we show that the human ribosome holds nascent chains in a partially unfolded state during synthesis, avoiding interdomain misfolding. This is in contrast to orthogonal bacterial RNCs, which show an overall more folded structure during synthesis, perhaps alluding to the differences observed in productive folding between the two ribosomes which are likely mediated through eukaryote specific interactions with proteins surrounding the exit tunnel, or rRNA. Surprisingly, cotranslational folding is not strictly unidirectional. On both ribosomes, C-domain synthesis partially reverses prior folding in the N-domain. Our work facilitates a detailed mechanistic understanding of how multidomain proteins fold efficiently in human cells.

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Cotranslational complex assembly in E. coli is regulated by a proofreading activity of the chaperone trigger factor

Jaro Schmitt, Carla Galmozzi, Günter Kramer, Bernd Bukau

Presenter: Jaro Leonard Schmitt

The majority of proteins must assemble into multiprotein complexes to become fully functional. Recent evidence suggests that complex assembly is often directly coupled with protein synthesis, raising the question of how the formation of protein complexes during ongoing translation is coordinated with other steps in nascent chain maturation, such as chaperone-assisted folding. Here, we present a ribosome profiling-based study that investigates the prevalence and mechanisms governing co-translational complex assembly in E. coli. We find that the dimerization of two nascent chains, referred to as co-co assembly, is widespread and regulated by the co-translationally acting bacterial chaperone Trigger Factor (TF). TF typically binds nascent subunits prior to their dimerization, thereby preventing premature interactions. Additionally, TF actively separates prematurely formed nascent dimers. Excessive TF concentrations suppress co-co assembly in cells, suggesting competition between TF binding and nascent chain dimerization. We also observe that TF may proofread the quaternary structure of nascent dimers, presumably correcting misassembled states. Consistently, TF-mediated splitting of nascent dimers in vivo is associated with enhanced fidelity of protein synthesis. Our findings suggest that TF temporally orchestrates nascent chain interactions during translation and proofreads nascent chain dimers to maximize the efficiency of protein complex assembly.

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Surveying the impacts of the nascent chain on viral recoding by deep mutational scanning

Caden Sillman, Jonathan Schlebach

Presenter: Caden Sillman

Many viruses rely on a translational recoding mechanism known as -1- programmed ribosomal frameshifting (-1PRF) for translational regulation. -1 PRF involves a cotranslational shift in reading frame that results in the translation of an alternative polypeptide. Viruses rely on several factors to control -1PRF efficiency, including co-translational folding of the nascent chain. Recent findings suggest SARS CoV2 relies on the cotranslational folding of a zinc finger domain within the ribosomal exit tunnel to modulate its -1PRF efficiency. Interestingly, we find that the HIV-1 genome encodes two zinc fingers within the nucleocapsid protein 7 gene (Ncp7) that lie in a similar position relative to its -1PRF site. The two zinc fingers of Ncp7 lie 7 and 28 amino acids upstream of heptanucleotide slip-site, the later of which is at an optimal distance to potentially fold inside the ribosome exit tunnel at the point of frameshifting. To explore the impact of these nascent chain features on -1PRF, we are using deep mutational scanning to map both the positions within the nascent chain and within the RNA structure that modulate recoding at the -1PRF site. We have generated a library of 1,628 mutants spanning the region surrounding the frameshift site in the context of a fluorogenic -1PRF reporter. We are currently mapping the effects of these mutations using fluorescence-activated cell sorting (FACS) in conjunction with deep sequencing. Our results are providing a comprehensive overview of the sequence elements within the transcript and nascent chain that collectively modulate -1PRF in HIV-1.

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The ribosome lowers the entropic penalty of protein folding

Julian O. Streit, Ivana V. Bukvin, Sammy H.S. Chan, Shahzad Bashir, Lauren F. Woodburn, Tomasz Włodarski, Angelo Miguel Figueiredo, Gabija Jurkeviciute, Haneesh K. Sidhu, Charity R. Hornby, Christopher A. Waudby, Lisa D. Cabrita, Anaïs M.E. Cassaignau & John Christodoulou

Presenter: Julian Streit

Most proteins fold during biosynthesis on the ribosome, and co-translational folding energetics, pathways, and outcomes of many proteins have been found to differ considerably from those in refolding studies. The origin of this folding modulation by the ribosome has remained elusive. Here, we have determined atomistic structures of the unfolded state of a model protein on and off the ribosome, which reveal that the ribosome structurally expands the unfolded nascent chain and increases its solvation, resulting in its entropic destabilisation relative to in isolation. Quantitative 19F NMR experiments confirm that this destabilisation reduces the entropic penalty of folding by up to 30 kcal mol-1 and promotes formation of partially folded intermediates on the ribosome, an observation that extends to other protein domains and is obligate for some proteins to acquire their active conformation. The thermodynamic effects also contribute to the ribosome protecting the nascent chain from mutation-induced unfolding, which suggests a crucial role of the ribosome in supporting protein evolution. By correlating nascent chain structure and dynamics to their folding energetics and post-translational outcomes, our findings establish the physical basis of the distinct thermodynamics of co translational protein folding.

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Rescue of bacterial large ribosomal subunits with peptidyl-tRNA jammed in the tunnel

Maxim S. Svetlov, Clémence F. Dunand, Jose Nakamoto, Gemma C. Atkinson, Haaris A. Safdari, Daniel N. Wilson, Nora Vázquez-Laslop, Alexander S. Mankin

Presenter: Maxim Svetlov

Translation of bacterial ORFs is sometimes prematurely interrupted and results in splitting of the ribosome into its constituent small and large subunits. After such splitting, the cell needs to somehow recycle the large subunit whose tunnel is jammed with an incompletely synthesized peptide still attached to tRNA. We have found that peptidyl-tRNA hydrolase (PTH) – a protein whose known function is to hydrolyzes ribosome-free peptidyl-tRNA – is capable of cleaving the faulty nascent protein off tRNA on the split large subunit. Remarkably, in contrast to the hydrolysis of ribosome-free peptidyl-tRNA, the PTH-mediated peptidyl-tRNA cleavage on the large ribosomal subunit crucially depends on the folding status of the peptide jamming the exit tunnel. Our data reveal that specific conformations of peptides in the tunnel are necessary to recycle problematic large subunits and suggest a critical role of PTH in ribosome-associated quality control in bacteria.

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Biochemical characterization of the biogenesis of G-protein coupled receptors

Austin Tedman, Muskan Goel, Sohan Shah, Kobbie Danku, Haritha Manoharan, Jacklyn Gallagher, Charles Kuntz, Jonathan Schlebach

Presenter: Austin Tedman

G-Protein Coupled Receptors (GPCRs) represent the largest class of drug targets in the human genome. Traditional pharmacology has focused on activation or inhibition of select receptors. However, this focus on function ignores that the receptor must be translated, folded and trafficked to the biologically relevant location before it can be targeted in this manner. For most GPCRs, this is the plasma membrane. We therefore endeavored to determine GPCR surface expression by performing a deep receptor scan of a synthesized plasmid library containing the entire GPCRome. Surprisingly, a majority of the >700 GPCRs in the human genome express poorly in HEK 293T cells under physiological conditions, including almost all olfactory receptors. Furthermore, we identified several receptors that experienced significant expression rescue when the temperature was dropped. These receptors may exist on a folding thermodynamic equilibrium near zero, which may be promising candidates for novel pharmacochaperones. One such receptor is NPSR1, in which polymorphisms have been implicated in asthma, among other diseases. We will further probe the cellular basis for these differences in expression by immunostaining for internal as well as external expression of select receptors. To better characterize GPCR expression we will employ unsupervised machine learning to cluster receptors based on expression patterns and chemical features to devise a structural basis for GPCR biogenesis and develop improved pharmacotherapies.

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Diverging co-translational protein complex assembly pathways are governed by interface energy distribution

Johannes Venezian, Hagit Bar-Yosef, Hila Ben-Arie Zilberman, Noam Cohen, Oded Kleifeld, Juan Fernandez-Recio, Fabian Glaser, Ayala Shiber

Presenter: Johannes Venezian

Protein-protein interactions are at the heart of all cellular processes, with the ribosome emerging as a platform, orchestrating the nascent-chain interplay dynamics. Here, to study the characteristics governing co-translational protein folding and complex assembly, we combine selective ribosome profiling, imaging, and N-terminomics with all-atoms molecular dynamics. Focusing on conserved N-terminal acetyltransferases (NATs), we uncover diverging co-translational assembly pathways, where highly homologous subunits serve opposite functions. We find that only a few residues serve as “hotspots,” initiating co-translational assembly interactions upon exposure at the ribosome exit tunnel. These hotspots are characterized by high binding energy, anchoring the entire interface assembly. Alpha-helices harboring hotspots are highly thermolabile, folding and unfolding during simulations, depending on their partner subunit to avoid misfolding. In vivo hotspot mutations disrupted co-translational complexation, leading to aggregation. Accordingly, conservation analysis reveals that missense NATs variants, causing neurodevelopmental and neurodegenerative diseases, disrupt putative hotspot clusters. Expanding our study to include phosphofructokinase, anthranilate synthase, and nucleoporin subcomplex, we employ AlphaFold-Multimer to model the complexes’ complete structures. Computing MD-derived interface energy profiles, we find similar trends. Here, we propose a model based on the distribution of interface energy as a strong predictor of co-translational assembly.

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Membrane insertion of YidC

Xiaolin Wang, Marina V Rodnina

Presenter: Xiaolin Wang

Integral membrane proteins insert into the bacterial plasma membrane in a co-translational fashion through the lateral gate of the SecYEG translocon, a protein-conducting channel in the plasma membrane. Together with the core translocon SecYEG the accessory proteins YidC, SecDF and YajC form the holotranslocon. Membrane insertion of YidC requires SRP, SecYEG and the SecA translocase. YidC consists of an N-terminal TM1 (aa residues 6-23) which upon insertion into the membrane adopts an N-in topology; a large periplasmic domain (aa 24-342); and five TMs (aa 343–370, 417–446, 464–481, 494–509, and 513–535). SRP is expected to be involved in initial targeting of TM1 and the translocation of the periplasmic domain likely requires SecA. To understand the sequence of events and the compositional dynamics of the complexes during YidC insertion, we have reconstituted the membrane insertion process in real time using RNCs synthesizing YidC, SecYEG translocon embedded into E. coli membrane phospholipids, and additional factors SecA and SecB. To make sure that the insertion of YidC occurs on a physiologically relevant time scale, we optimized the translation rate to YidC to the near-in-vivo rate of approximately 3 aa/s. We used a fluorescence-based stopped-flow assay to monitor the insertion using FRET between fluorophores attached to the N-terminus of YidC and the cytoplasmic face of the SecYEG translocon.

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Exploration of ribosomal exit tunnel heterogeneity across all domains of life

Tomek Włodarski

Presenter: Tomek Włodarski

Ribosomes are molecular machines that translate genomic information into protein sequences. During this process, a newly synthesised polypeptide chain travels through the long but narrow ribosomal exit tunnel, which only permits the formation of small secondary structural elements. A growing body of evidence demonstrates that the ribosome alone significantly modulates the nascent chain emergence and the folding process; however, the extent of this modulation and its variability across species remains largely unexplored. Our study aims to apply computational methods to understand the structural and sequence heterogeneity of ribosomal tunnels across all domains of life, including those within mitochondria and chloroplasts. We developed a computational approach based on molecular dynamics simulations to examine in detail the geometric features of ribosomal exit tunnels in 60 distinct ribosome structures. We characterised the nascent chain's main path and how it changes during different stages of biosynthesis and explored potential alternative routes within the tunnel. Additionally, we identified and characterised interaction sites along the tunnel surface, assessing their compositional and spatial diversity across ribosomes. Furthermore, we extended our research beyond the structural characterisation of the whole tunnels to comprehensive bioinformatics analysis of ribosomal protein sequences associated with exit tunnels, including five proteins from bacteria and six from archaea and eukaryotes. This genome-wide survey, accompanied by corresponding AlphaFold models, uncovered significant variability and novel structural features, improving our understanding of ribosomal heterogeneity and its role in co-translational protein folding.

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The yeast E3 ligase Hel2 monitors the duration of ribosome stalling

Ying Zhang, Mario Scazzari, Sabine Rospert

Presenter: Ying Zhang

Stalled ribosomes lead to collisions, disrupt protein synthesis, and produce potentially harmful, truncated polypeptides. To counteract these deleterious effects, eukaryotic cells employ the ribosome-associated quality control (RQC) and no-go mRNA decay (NGD) pathways. In yeast, the E3 ubiquitin ligase Hel2 is the initial component of the RQC/NGD pathways. Hel2 targets disomes and trisomes and polyubiquitinates the 40S ribosomal protein Rps20. Ubiquitination of Rps20 is required for subsequent steps in the RQC/NGD pathways. Recent research has uncovered high concentrations of disomes and trisomes in unstressed cells. This prompts the question of whether and how Hel2 selects long-term stalled disomes and trisomes for RQC/NGD. To explore this topic, we conducted a comprehensive analysis of in vivo-formed Hel2●ribosome complexes and the in vivo dynamics of Rps20 ubiquitination and deubiquitination. Our results reveal how Hel2 enables the downstream RQC machinery to preferentially target non-productively stalled ribosomes for rescue over temporarily paused elongating ribosomes.

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