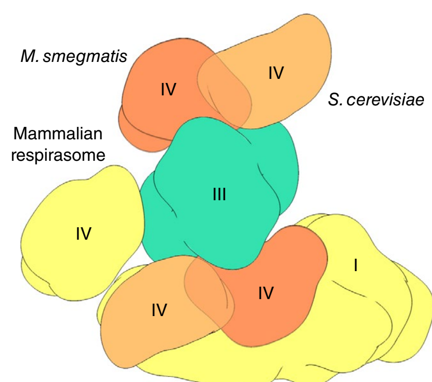

Fig. 3 | Interaction of complex III and IV in supercomplexes. Complex IV occupies different positions relative to complex III (aquamarine) in *S. cerevisiae* (light orange), *M. smegmatis* (dark orange) and the mammalian respirasome (yellow). The structures were aligned on the cytochrome *b*/QcrB subunit of complex III from PDB 6HU9, 6HWH and 5J4Z and are shown from the IMS/periplasmic side.

to the stabilization of the supercomplex by establishing several contacts with both complex III and complex IV.

In both mycobacterial structures[9,10], several menaquinone molecules are modeled. Of particular interest is the menaquinone observed for the first time at the Q_o site. The binding site previously predicted by use of inhibitors suggests that the quinone binds between the iron-sulfur cluster and the b_L heme; however, the menaquinone in these new structures is not located as deep in the pocket. It is unclear at the time if this location is the true Q_o site to which menaquinol binds or if it is specific only to the oxidized product. This site is unlikely to be conserved in the canonical complex III due to the structural differences observed in this region.

The number of respiratory supercomplex structures is rapidly growing. Curiously, the interactions between complex III and complex IV are not conserved, and the bacterial and mitochondrial III_2IV_2 supercomplexes described here and the respirasome all have a different architecture (Fig. 3). Structures of two bacterial supercomplexes of the alternative complex III (ACIII), a quinol:cytochrome *c* oxidoreductase unrelated to complex III, with a aa_3- or a caa_3-type terminal oxidase, have also been described recently[22,23]. Although both of these supercomplexes allow direct inter-complex electron transfer, their architectures also differ.

The ubiquity of respiratory-chain supercomplexes suggests evolutionary benefits from such assemblies. The bacterial supercomplexes discussed[9,10,22,23] clearly serve efficiency of electron transfer by bypassing electron carriers; however, similar advantages in eukaryotes are still highly debated. The distance between the cytochrome *c* binding sites in complex III and complex IV in mitochondrial supercomplexes is 70–100 Å (ref. [7]), showing that the electron carrier must diffuse. Respiratory supercomplexes have also been proposed to have a role in reducing the production of reactive oxygen species[24]. The presence of SOD in the mycobacterial supercomplex[9,10] shows a new strategy for achieving this goal that might also be relevant in mitochondria, as association of SOD with the respirasome has been reported[25].

The revolution in cryo-EM has finally made it possible to determine structures of large, flexible or dynamic membrane protein complexes that have long been a challenge to structural biologists. Structures as reported here will continue to expand the spectrum of respiratory supercomplexes and help shed light on their diverse functions. ❐

Joana S. Sousa and Janet Vonck *
Department of Structural Biology, Max Planck Institute of Biophysics, Frankfurt am Main, Germany.
e-mail: janet.vonck@biophys.mpg.de

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Competing interests

The authors declare no competing interests.

COTRANSLATIONAL FOLDING

Assembly en route

A growing body of evidence suggests that cotranslational folding occurs from bacteria to mammalian cells, in particular for multi-domain proteins. In the assembly of yeast proteasomes, the initial interaction of Rpt1 and Rpt2 subunits has been found to take place on the translating ribosomes, coordinated by elongation pausing and involving the formation of Not1-containing compartments.

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Proteins must achieve proper folding to acquire their designated properties and perform their biological functions. Our current understanding of protein folding is based predominantly on in vitro refolding of denatured full-length proteins[1]. However, in cells, protein folding could occur concurrently with the synthesis of the polypeptide chain on the ribosome[2–4].

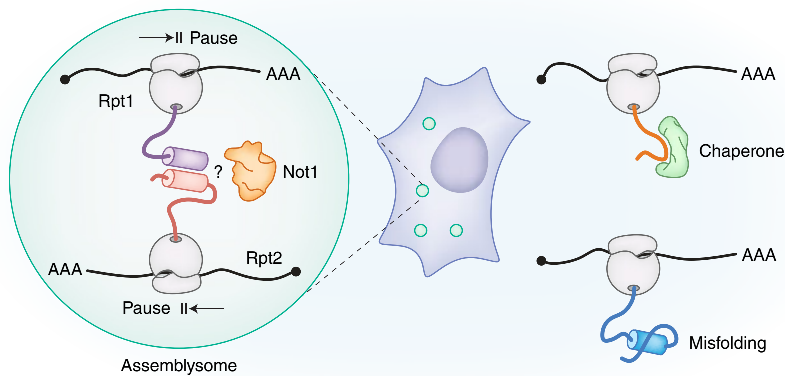

Fig. 1 | Multiple layers of regulatory mechanisms operate during cotranslational folding and assembly of protein complexes. Top right, the nascent polypeptide chain (orange) attached to the elongating ribosomes may undergo folding, aided by molecular chaperones. For oligomeric protein complexes such as the proteasome, translation of different subunits is coordinated within the assemblysome (center; green circles), to facilitate cotranslational assembly (left). Ribosomes translating *Rpt1* and *Rpt2* mRNAs undergo pausing at DP codons. The N-terminal nascent chains of Rpt1 and Rpt2, already emerged from the ribosome tunnel exit, are able to associate with each other, promoting correct folding of the heterodimer and stabilizing Rpt subunits. This cotranslational assembly event occurs in a dense compartment in the cytosol containing Not1. Bottom right, in the absence of partner subunits or if assemblysome formation fails, the nascent chain misfolds (blue) are targeted for degradation.

Cotranslational folding of partially synthesized nascent chains differs from the refolding of full-length polypeptides due to the vectorial nature of polypeptides emerging from the ribosome. Given that protein folding (on the microsecond scale) happens much faster than does translation elongation (~5 amino acids per second in eukaryotic cells), cells must protect nascent chains that have not yet folded completely. Complicating the situation further, the assembly of heterooligomeric protein complexes requires that individual subunits be available at the right place and moment. Cells cope with this challenge by coordinating ribosomes synthesizing different polypeptides; cotranslational folding and assembly minimizes the possibility of generating premature subunits with exposed interface that may lead to aggregation and degradation[5].

It is challenging for researchers to monitor the cotranslational folding and assembly events of nascent chains that are still attached to the translating ribosomes. A major hurdle is the heterogeneous nature of newly synthesized polypeptides. One approach to overcoming this obstacle is to isolate ribosomes bearing recognizable structures and determine the positions of the ribosomes on mRNAs. Since the length of newly synthesized polypeptides can be inferred precisely from the ribosomes' positions on mRNAs, it is possible to link cotranslational folding to stages of ribosome elongation. Taking into account the ~30-amino-acid occlusion within the ribosome tunnel, the minimal length of nascent chains capable of folding can be estimated accordingly. Ribosome profiling, based on deep sequencing of ribosome-protected mRNA fragments, provides a wealth of information about ribosome positions and densities across the entire transcriptome[6]. Through harnessing of the power of ribosome profiling, cotranslational chaperone interaction[7], domain recognition[8] and subunit assembly[9] have been demonstrated from *Escherichia coli* to mammalian cells. A recent study by Bukau and colleagues has further revealed the early onset of cotranslational assembly for heteromeric complex subunits as they emerge from the ribosomal tunnel exit; intriguingly, molecular chaperones actively engage with those nascent subunits, especially the one lacking partner subunit association[10].

The 26S proteasome is a large ATP-dependent protease complex that acts as a primary contributor to the degradation of polyubiquitinated proteins[11,12]. It is composed of two distinct subcomplexes: the core particle (CP; 20S) and the regulatory particle (RP; 19S). The RP itself comprises two distinct regions: the base and the lid. The base consists of six AAA (ATPases associated with diverse cellular activities) subunits, Rpt1–Rpt6, that form a ring structure that is crucial for gating substrate entry. Given such complexity, the assembly of functional proteasomes is a formidable task, requiring the assistance of several general and dedicated chaperones. Collart and colleagues[13] now demonstrate that the assembly of Rpt1 and Rpt2 subunits initiates in the translating ribosomes in a spatially confined region in the cytosol (Fig. 1).

By analyzing ribosome footprints on *Rpt1* and *Rpt2* mRNAs, Panasenko et al. observed elevated ribosome density around the aspartate and proline (DP) codons for both transcripts[13]. This was not entirely surprising, because the proline codon is decoded relatively slowly by the ribosome, even with the assistance of eIF5A[14,15]. But the positions of the DP codon–associated ribosome pausing sites (codon 135 for *Rpt1* and codon 165 for *Rpt2*) were of particular interest, as they are located right before the ATPase domains of both Rpt subunits. Local discontinuous translation has been suggested to facilitate cotranslational folding of distinct polypeptide segments[16]. It is possible that ribosome pausing at DP sites offers temporal flexibility for cotranslational association of the N-terminal domains of Rpt1 and Rpt2. To test this possibility, the authors created a ribosome-associated nascent chain (RNC) by replacing the DP condon–associated pausing sequence with a cluster of lysine codons (K12), followed by a V5 epitope[13]. Prolonged ribosome stalling at K12 should permit evaluation of interaction between Rpt1-RNC and Rpt2-RNC. Indeed, the authors not only detected stable expression of Rpt1-RNC and Rpt2-RNC but also found enrichment for these in polysome fractions. Previous structural studies have suggested that the N-terminal regions of Rpt subunits are necessary for the Rpt ring assembly[17]. Consistent with this notion, N-terminally truncated versions of Rpt-RNC are either less soluble (ΔN-Rpt2-RNC) or found in the free fractions of sucrose gradient (ΔN-Rpt1-RNC). In further support of the critical role of ribosome pausing in the maturation of Rpt proteins, a variant of Rpt1 with the DP site mutated to AA no longer stabilizes the coexpressed Rpt2. Intriguingly, cotranslational interaction of the N-terminal domains of Rpt1 and Rpt2 appears to alleviate ribosome pausing[13].

Perhaps the most surprising finding by Panasenko et al.[13] is that cotranslational assembly of Rpt1 and Rpt2 occurs in a spatially confined region, the so-called 'Not1-containing assemblysomes'. The first clue

was the observation that the distribution of Rpt1-RNC in heavy polysome fractions is resistant to treatment with EDTA or RNase, suggestive of distinct features for the dense compartments in which Rpt1-RNC and Rpt2-RNC reside. Second, the Ccr4–Not complex appears to localize in those discernible granules, in line with the crucial role of Not1 in proteasome assembly, as previously reported by the authors[18]. Third, immunofluorescence staining of human cells reveals that Rpt assemblysomes are distinct from stress granules, GW-bodies or P-bodies. CNOT1, the ortholog of yeast Not1, colocalizes with *Rpt1* and *Rpt2* mRNAs and is required for the targeting of both transcripts to that particular granule. Although many details remain to be hammered out, a spatially confined assemblysome can explain the apparent dilemma of how ribosome pausing can facilitate cotranslational subunit association while escaping the cellular quality-control system.

In summary, the study by Panasenko et al.[13] reveals the cotranslational assembly of yeast proteasome subunits Rpt1 and Rpt2, a process that is organized in a spatial and temporal manner. The coincident ribosome pausing after the ATPase domain is translated suggests that the elongation rate is coordinated with cotranslational assembly. The involvement of Not1-containing assemblysomes implies a physical separation of ribosomes translating oligomeric subunits from ribosomes undergoing general protein synthesis (Fig. 1). The high misfolding propensities of unpaired subunits underscores the importance of this mechanism. While Panasenko et al.[13] showed a specific case for the yeast proteasome, the principle is likely widespread in the assembly of other macromolecular complexes. Clearly, further investigation will be required to understand the assembly of assemblysomes. For instance, how are *Rpt1* and *Rpt2* mRNAs recruited to this unique compartment? What are the other components of the assembly granule? Will cotranslational assembly of different oligomer complexes share the same assemblysome? Given the broad function of the Ccr4–Not complex[19,20], it will be interesting to elucidate the relationship between Not1 granules and other cellular processes, such as transcription and the mRNA quality-control pathway. ❒

Xiao-Min Liu and Shu-Bing Qian *
Division of Nutritional Sciences, Cornell University, Ithaca, NY, USA.
e-mail: sq38@cornell.edu

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Competing interests

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