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Ribosomes and cryo-EM: a duet

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Ribosomes and electron cryomicroscopy (cryo-EM) share a long, intertwined history. However, cryo-EM only recently usurped X-ray crystallography as the predominant structural method to study ribosomes in atomic detail. The main, but not only, reason for this succession was the introduction of direct-electron detectors enabling cryo-EM to achieve equally high resolutions. Here, we describe how cryo-EM sample preparation and data processing allows new types of structural analyses not possible by X-ray crystallography. Taking advantage of these approaches, cryo-EM structures have revealed unprecedented insights into the function of ribosomes from a wide range of biological sources and in numerous physiological contexts. These include the discovery of a new mechanism of polypeptide synthesis and the identification of the roles of ribosomes in functional supercomplexes.

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Introduction

Electron microscopy (EM) and ribosomes are inextricably linked. As James Lake noted in his seminal paper describing the first three-dimensional structure of a ribosome solved using single-particle methods [1], EM is 'an extremely useful technique for studying the gross structure of ribosomes'. Indeed, ribosomes were seen by EM before they were even known to exist [2], and now make up nearly 15% of the EM Data Bank (EMDB) [3]. Conversely, as ribosomes are large, dense, and almost perfect molecules to image, they have aided the advance of EM methods, including the development of negative and positive stains [4] and random conical tilt [5]. More recently, ribosomes have been instrumental in the development of the hardware and software that led to the modern era of high-resolution cryo-EM, including the introduction of direct-electron detectors [6••,7••].

Cryo-EM now supersedes X-ray crystallography as the method of choice for studying the structure and function of ribosomes. Previously, X-ray crystallography provided the data for the first atomic models of individual ribosomal subunits [8,9] and resolutions that cryo-EM could not rival. Now, the resolutions that can be achieved by both techniques are comparable: the best resolution of a ribosome structure solved by X-ray crystallography [10] is only 0.2 Å better than by cryo-EM [11]. This review highlights how the numerous advantages cryo-EM offers over X-ray crystallography enable new types of experiments and structural analyses in the study of ribosomes.

Throwing off the shackles: visualizing structural diversity and dynamics

Cryo-EM removes the need for crystals. This is beneficial for several reasons. First, the choice of ribosome to be studied is not dictated by its ability to crystallize. Second, cryo-EM requires less sample at lower concentrations than crystallization (often by more than an order of magnitude). Third, while crystallization requires a homogeneous sample, cryo-EM can tolerate some impurities and structural hetereogeneity. These considerations permit the study of diverse ribosomes and ribosomal complexes.

Recent cryo-EM structures include ribosomes from a variety of organisms ranging from pathogenic bacteria [12] to humans [13], as well as organellar ribosomes of chloroplasts [14–16] and mitochondria [17–19]. Indeed, the large subunit of the yeast mitochondrial ribosome was the first ribosome to be visualized at sub-4 Å resolution by cryo-EM [7••]. The increased diversity of ribosome structures is revealing species-specific mechanisms of translation, insights into the evolution of ribosomes, and how ribosomes adapt to different host environments.

Without the need for crystals, cryo-EM is immune to potential artefacts caused by crystal contacts. For example, the cryo-EM structure of the *E. coli* ribosome shows that ribosomal protein bL9 is dynamic and can adopt a closed conformation in which it contacts the small subunit [20]. In contrast, in the crystal structure, bL9 is held only in an extended conformation by contacts with the neighboring ribosome in the crystal lattice [21]. This interaction occludes the GTPase-binding site of the neighboring ribosome [22] and is not thought to be physiologically relevant.

Without the constraints of crystal contacts, cryo-EM has also revealed new ribosomal movements. These include previously undetected global changes in the relative conformations of the ribosomal subunits. Notably, the small subunits of eukaryotic cytoplasmic and human mitochondrial ribosomes were seen to rotate around their long axes [17,23•]. This 'subunit rolling' changes the shape of the intersubunit cavity, but unlike the 'ratcheting' motion of ribosomal subunits that accompanies mRNA translocation, the exact function of this motion remains unknown.

Out of one, many: computationally sorting ribosome structures

An increasingly powerful feature of cryo-EM is the ability to computationally sort particles into different structural classes following data collection — a form of *in silico* purification. These classes may have gross morphological differences, as exploited to separate mitochondrial ribosomes from contaminating cytoplasmic ribosomes [7••], or only small conformational differences. Two recent papers that examine the relationship between codon recognition and the activation of translational GTPases on ribosomes illustrate beautifully the ability of cryo-EM to isolate microheterogeneity [24,25•]. In both studies, extensive computational classification of large datasets (between 500 000 and 1 million particles) facilitated the structure determination of distinct conformations of tRNAs sampling the ribosomal A site, even those that were present as a small percentage and probably occur transiently in the cell. By comparing these structures, the authors could describe near-complete mechanistic models linking tRNA recognition and ribosome-dependent GTPase activation [24,25•].

An equally remarkable earlier study [26••] imaged translating ribosomes from *ex vivo*-derived human polysomes rather than reconstituting an individual stage of translation as in the studies above. From these data, a variety of native translation intermediates could be identified at different stages of translation (Figure 1). The ratio of these states to one another potentially provides information about the population of ribosomes in the cell and even which steps are likely to be rate-limiting. However, such correlations must be treated cautiously until further work determines how well populations seen by single-particle cryo-EM reflect cellular populations.

The chosen ones: isolating specific ribosomal complexes for cryo-EM

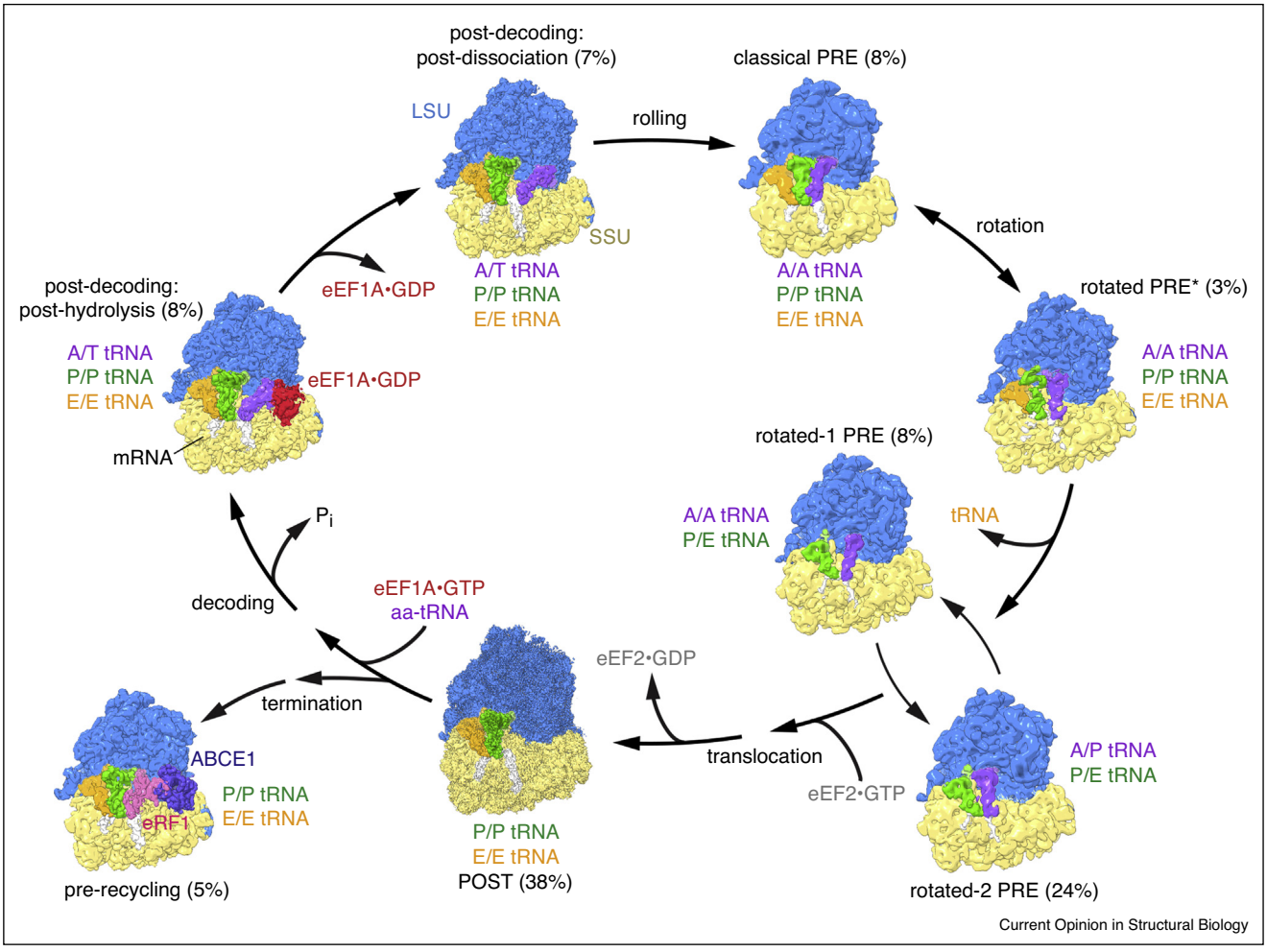
The ability to classify different structural classes *in silico* removes some need for biochemically pure samples. However, as large numbers of homogeneous particles facilitate high-resolution structure determination, biochemical strategies are often required to enrich for the species under study. These strategies include epitope-tagging ribosomal binding partners, substrates, and/or products. Affinity purifying these tagged components can isolate specific ribosomal complexes directly from cellular lysates or compartments. These approaches are not new — the use of epitope-tagged nascent proteins to purify ribosomes stalled during translation for cryo-EM analysis was developed almost 20 years ago [27]. However, the small yields and heterogeneity of the samples generated by these approaches were generally not compatible with X-ray crystallography, and the ability to use these approaches with cryo-EM to gain molecular-level insights is only just beginning to be exploited.

Two different biochemical strategies recently led to cryo-EM structures of the ribosome-associated quality control (RQC) complex [28,29••,30]. This complex ubiquitinates nascent proteins trapped in stalled ribosomes for degradation. One strategy used epitope-tagged nascent proteins to purify programmed ribosome-nascent protein complexes that were subsequently used to reconstitute RQC complexes *in vitro* [30]. The second approach affinity purified RQC complexes from yeast cells via epitope-tagged RQC ribosome-binding partners [28,29••]. In addition to revealing how RQC factors specifically recognize stalled ribosomes, one of these structures [29••] identified a surprising mechanism for a mRNA-independent mode of polypeptide synthesis, which was only possible due to the direct purification of RQC complexes from whole-cell lysates.

Ribosomal samples for cryo-EM analysis can also be prepared using translational inhibitors. These include dominant-negative translation factors (for example, in the use of mutant release factors to trap ribosomes during stop-codon recognition [31]) and small-molecule drugs. Traditionally, to study the binding site and mechanism of ribosome-targeting drugs using X-ray crystallography, high drug concentrations are added to ribosomes during crystallization, or soaked into ribosome crystals [32]. This reconstitution approach is also compatible with cryo-EM: the structure of the ribosome of a human malaria parasite bound to the drug emetine was obtained by mixing 1 mM emetine with 160 nM of ribosomes [33•] (Figure 2). Cryo-EM also enables alternative approaches for studying translational inhibitors. For example, it is now possible to add inhibitors directly to cells or lysates at physiologic concentrations and subsequently purify the inhibited ribosomes for cryo-EM studies. This approach was used to elucidate the molecular basis for erythromycin-dependent ribosome stalling in bacteria [34] and the mechanism by which didemnin B inhibits translation in mammals [35] (Figure 2).

Thus, the combination of different biochemical and *in silico* purification strategies offers many routes to obtain cryo-EM structures of ribosomal complexes.

Figure 1



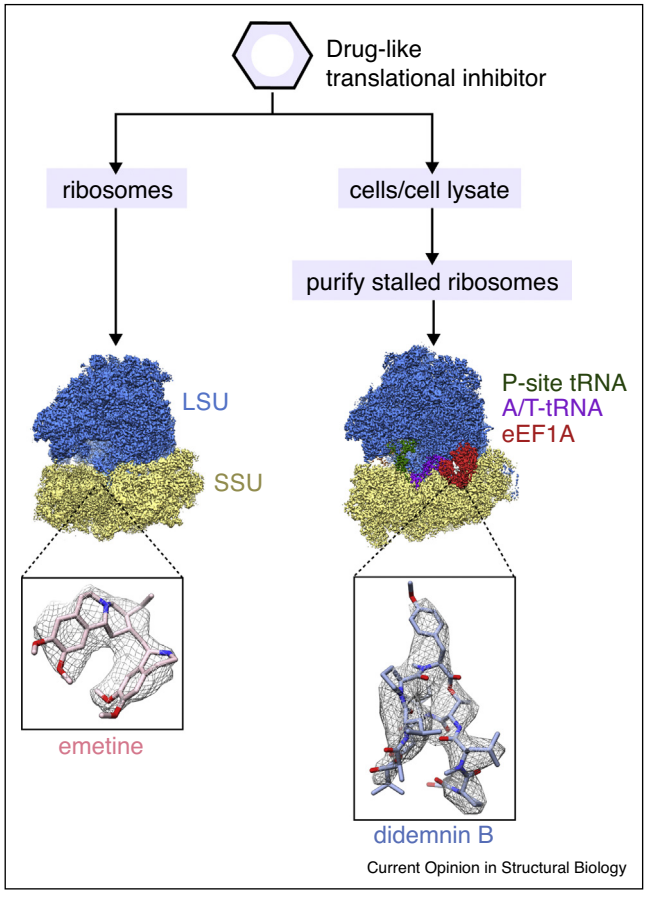
Cryo-EM of dynamic ribosomal processes. Cryo-EM maps of different ribosome conformations sorted computationally from a single dataset of polysomes isolated directly from cultured human cells (EMD-2875; EMD-2902-11) [26••]. Out of over 1.8 million ribosome particles, various intermediate states of translational elongation and termination could be distinguished at different abundances (percentages in parentheses). These ribosomal structures differed in the position of tRNAs, rotation state of subunits, and the presence or absence of elongation and termination factors.

Bigger and more complex: ribosomal supercomplexes in isolation and native contexts

These new tools make possible the visualization of ribosomes as part of supercomplexes, which would have been unthinkably ambitious by X-ray crystallography. Recent cryo-EM structures show the role of ribosomal supercomplexes in the birth and death of mRNA [36••,37]. In bacteria, transcription and translation are functionally coupled by a direct interaction between the RNA polymerase (RNAP) and the ribosome. This complex was recently visualized by cryo-EM to subnanometer resolution by using a stalled transcription elongation complex as a template for *in vitro* translation [36••] (Figure 3a). This remarkable structure shows that the nascent mRNA exits directly from the RNAP into the entrance of the ribosomal mRNA channel. Translocation of the mRNA by the ribosome presumably pulls on the nascent mRNA to prevent RNAP backtracking and maintain forward momentum. The complex also prevents premature termination of transcription as the ribosome physically shields the mRNA that is recognized by the Rho transcriptional termination factor. Shortly afterwards, an independent structure of a complex between RNAP and the 30S ribosomal subunit was reported at a similar resolution [38]. In this complex, formed by mixing the two species directly *in vitro*, the RNAP binds near the exit, rather than the entrance, of the ribosomal mRNA channel (Figure 3a, right panel). Whether both complexes can form *in vivo* at different stages of translation remains unclear.

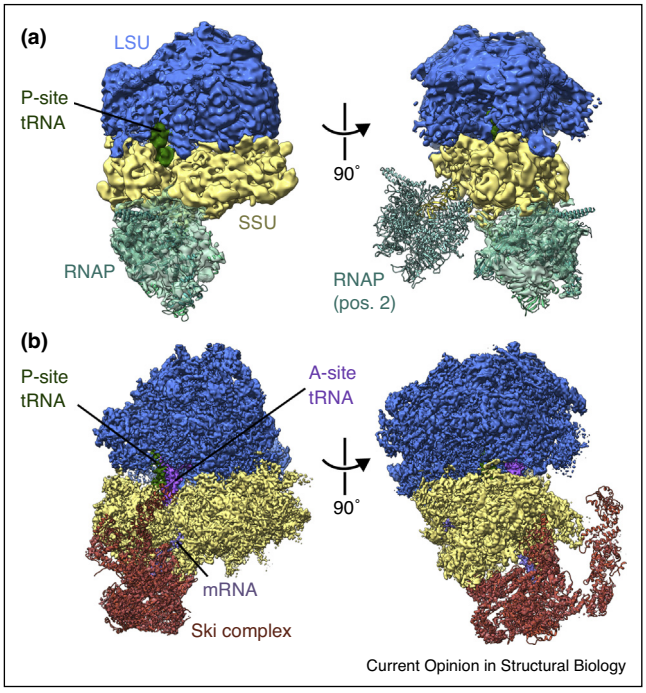
In eukaryotes, translation is not coupled with transcription but is closely linked to mRNA-decay pathways. The Ski complex (formed by Ski2, Ski3, and Ski8) can bind directly to the ribosome and functions with the RNA-

Figure 2



Cryo-EM structures of drugged ribosomes. Schematic showing the two main approaches to prepare ribosomal samples for cryo-EM using small-molecule inhibitors. In the first approach (left), drugs are added directly to purified ribosomes. This approach was used to solve the structure of emetine bound to the ribosome from *Plasmodium falciparum* [33•]. In the second approach (right) the drug is added to cells or during *in vitro* translations in lysate, and the ribosomes purified after exposure. For example, didemnin B was added to mammalian *in vitro* translation reactions and the resultant stalled ribosomes isolated for cryo-EM via an epitope-tagged nascent protein [35]. The structure revealed that didemnin B inhibits translation by preventing eukaryotic elongation factor 1A (eEF1A) dissociation from ribosomes.

Figure 3

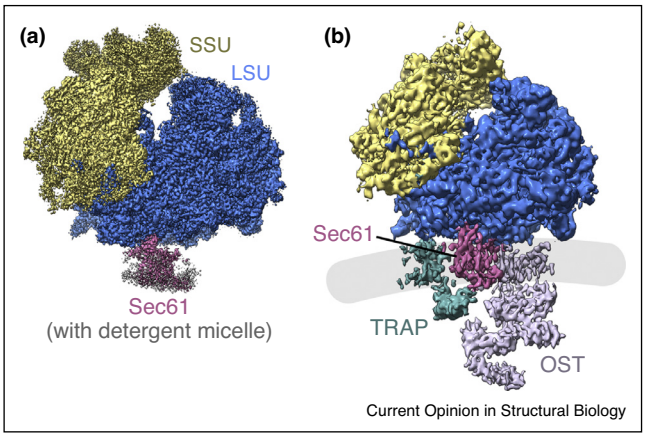


Cryo-EM of ribosomal supercomplexes. **(a)** Two views of the complex between RNA polymerase (RNAP) and the ribosome of *Escherichia coli.* The map corresponds to the complex formed between transcribing RNAP and translating ribosome (EMD-3580) with a fitted model for RNAP [36••]. The model without density (pos. 2) corresponds to the alternative position of RNAP observed when RNAP was mixed with isolated 30S subunits in the absence of DNA or mRNA (EMD-7014) [38]. **(b)** Two views of the complex between the Ski complex involved in mRNA decay and the ribosome of *Saccharomyces cerevisiae* (EMD-3461) [37].

degrading exosome to mediate 3′-5′ mRNA decay in turnover and quality-control pathways. The cryo-EM structure of a ribosome-Ski supercomplex shows that the Ski complex binds to the entrance to the ribosomal mRNA channel, allowing the 3′ end of the mRNA to thread directly into the helicase channel of Ski2 [37] (Figure 3b). The Ski complex likely functions to extract mRNA from the ribosome and transfer it to the exosome for degradation. Whether the exosome binds to this complex to form an even larger supercomplex remains to be seen. A similar complex may form in bacteria, where the multisubunit RNA degradosome has been shown to form stable complexes with ribosomes and translating polysomes [39].

Ribosomes also associate with complexes at cellular membranes during secretory, organelle, and membrane protein biosynthesis. At the eukaryotic endoplasmic reticulum (ER), ribosomes dock at the Sec61 complex, a conserved protein-conducting channel that provides a conduit to translocate hydrophilic polypeptides across the membrane, as well as a lateral gate to release hydrophobic transmembrane domains into the lipid bilayer. Single-particle cryo-EM of detergent-solubilized ribosome-Sec61 complexes from crude ER membranes has revealed how the Sec61 complex interacts with the ribosome (Figure 4a) and the conformational changes associated with protein translocation [27,40,41]. Although this approach is compatible with *in vitro* manipulations to isolate specific steps of protein translocation, it is not known how closely detergent solubilization replicates native membrane environments. Electron cryotomography (cryo-ET) addresses this limitation by observing macromolecules in physiological environments. Cryo-ET combined with subtomogram averaging has visualized ribosome-Sec61 complexes in ER-derived vesicles at subnanometer resolution [42••] (Figure 4b). Sec61 in the

Figure 4



Cryo-EM and Cryo-ET membrane-bound ribosomes. **(a)** Single-particle cryo-EM reconstruction of detergent-solubilized ribosome-Sec61 complexes from crude endoplasmic reticulum (ER) membranes (EMD-2649) [41]. The detergent molecules are shown tightly packed around the Sec61 complex. **(b)** Subtomogram averaging reconstruction of ribosome-Sec61 translocon complexes in ER vesicles (EMD-3245), with the position of the lipid bilayer indicated [42••].

cryo-ET structure adopts a different conformation from that seen after detergent solubilization and single-particle analysis [41,45], which may reflect an opening of the lateral gate, although the basis for this difference remains unclear. The cryo-ET structure also revealed the architectural arrangement of the ribosome with the Sec61 translocon and two physiological accessory factors, the translocon-associated protein (TRAP) and oligosaccharyltransferase (OST) complexes, within intact ER membranes. Supplementing this subtomogram average with molecular models derived from recent cryo-EM structures of detergent-solubilized yeast OST complex [43,44] and the mammalian ribosome-Sec61-OST complex [45] has provided new insights into the interactions of accessory factors around the translocon and the co-translational glycosylation of secretory and membrane proteins.

Conclusions

The number of ribosome structures deposited annually to the EMDB continues to accelerate (there were 36 more structures in 2016 than in 2015) [3]. In part, the reason for this acceleration is the increase in the types of samples that can be analyzed, permitting the determination of ribosomal structures of increasing complexity and transience. Given the numbers of biological questions that remain unanswered, we anticipate that the boom will continue, producing new structural insights into the roles of ribosomes in numerous processes including mRNA surveillance, protein homeostasis, and coordinating translation across cellular compartments. In addition, the recent ribosome interactome [46] hints at many more binding partners whose functions await discovery.

To accompany and facilitate these biological advances, we will likely see the continued use of ribosomes as benchmarks for the development of new cryo-EM methods. These advances will hopefully address some of the limitations of cryo-EM for studying ribosomes, such as resolving flexible ribosome-binding partners, and achieving the truly atomic resolutions required for drug development. One area that will likely see rapid growth is the development of better mathematical models to handle conformational and dynamical heterogeneity [47], including the movement away from the isolation of discrete but approximate states towards dynamic ensembles of maps and models.

Of great excitement is the ability to see ribosomes *in situ* at subnanometer resolution by cryo-ET. This technique is improving rapidly as new technologies are developed. It has become possible to view the topological arrangement of ribosomes within a cell [48], to distinguish ribosomes at different stages of translation [49], and to observe ribosomes within larger complexes [42••]. Collectively, these structures have provided new insights into the specialization of the translation apparatus for localized protein synthesis. As resolutions improve, it will be possible to validate the *in vitro* reconstituted ribosomes seen by single-particle cryo-EM and potentially identify new ribosomal interactions. Hinting at this being possible is the observation of unidentified density bound to the human mitochondrial ribosome [50] not seen by single-particle methods [17].

In summary, cryo-EM has made it an exciting time to work in the ribosome field, but the future is potentially even more exciting as we start to explore ribosomes in ever more physiological contexts.

Conflict of interest

None declared.

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