Role of GTPases in Ribosome Assembly

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ABSTRACT:

GTPases are a universally conserved class of regulatory proteins involved in such diverse cellular functions as signal transduction, translation, cytoskeleton formation, and intracellular transport. GTPases are also required for ribosome assembly in eukaryotes and bacteria, where they present themselves as possible regulatory molecules. Strikingly, in bacteria they represent the largest class of essential assembly factors. A review of their common structural, biochemical and genetic interactions is presented and integrated with models for their function in ribosome assembly. © *2007 Wiley Periodicals, Inc. Biopolymers 87: 1–11, 2007.*

Keywords: GTPase; ribosome; ribosome assembly

This article was originally published online as an accepted preprint. The "Published Online" date corresponds to the preprint version. You can request a copy of the preprint by emailing the Biopolymers editorial office at biopolymers@wiley.com

INTRODUCTION

Ribosomes catalyze protein synthesis and are therefore central components of all cells. Consequently, their mechanism of action and their assembly pathway is of outstanding interest both from a biological perspective as well as from a pharmacological point of view, where ribosomes represent important drug targets.[1,2] Recent breathtaking advances in crystallography have revealed the atomic coordinates for bacterial ribosomes (e.g., Refs. 3–5), providing us with a point of reference for the product of the ribosome assembly pathway. These structures have rationalized and extended the ground-breaking work from the Nomura and Nierhaus labs,[6–9] which have revealed years ago, in which order ribosomal proteins are assembled onto ribosomal RNAs (rRNA). Additionally, it was shown that functional bacterial ribosomes can be reconstituted from rRNA and ribosomal proteins in vitro.[10–13]

In vivo bacteria transcribe an rRNA precursor that includes the 16S, 23S, and 5S rRNAs. In addition, a tRNA is typically encoded between the rRNAs. Primary processing occurs via RNase III sites and the mature 5′- and 3′-ends of 16S and 25S, and 5S rRNA are formed by subsequent endonucleolytic steps catalyzed by RNases E, G, and T (Figure 1A). There is also evidence that binding of some ribosomal proteins occurs cotranscriptionally (for a review see Ref. 14). Given this wealth of information, and despite some hints that additional factors might be involved (e.g., Refs. 15–19), one might have taken the ribosome assembly problem to be understood.

However, work over the last decades, accelerated in the last several years in large part because of the advent of large-scale affinity purification of ribosome precursors coupled with mass spectrometry, has revealed that in yeast ribosome assembly requires the concerted action of well over 170 proteins as well as more than 70 RNAs (for a review see Ref. 20). These proteins orchestrate modification and processing of the initial 35S precursor rRNA transcript into the mature 18S, 5.8S, and 25S rRNAs, folding of the rRNA, and binding of ribosomal proteins and 5S rRNA. During or shortly after transcription certain conserved residues are methylated at their 2′-hydroxyl residue, while specific uridines are converted into pseudouridines. Once transcription is completed, at least 11 endonucleolytic and exonucleolytic cleavage steps are required to generate the mature 5′ and 3′-ends of 18S, 5.8S, and 25S rRNA (Figure 1B). However, how these processing steps are integrated with rRNA folding, and binding of ribosomal proteins as well as insights into the role of the ribosome assembly factors remain forthcoming. Sequence analysis provides clues to the function of some of these proteins, which include RNA binding proteins, DEAD box

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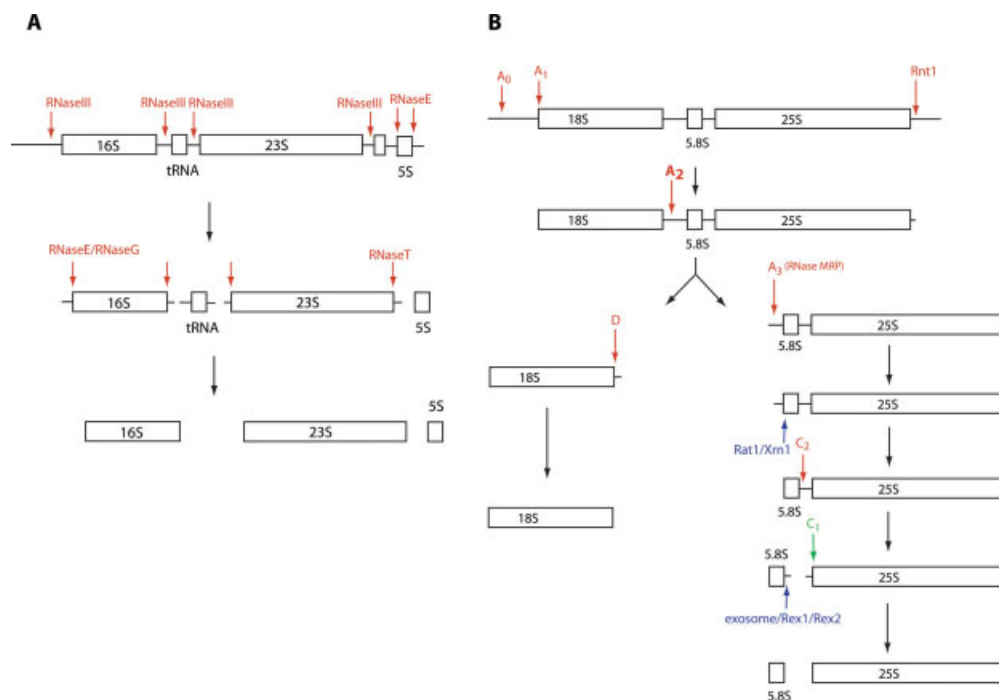
FIGURE 1   rRNA processing in (A) bacteria and (B) yeast. (A) The rRNA operon in bacteria contains all three rRNAs, separated by noncoding sequences and tRNAs. Primary processing occurs at RNaseIII cleavage sites, formed by base pairing between sequences 5′ and 3′ to the *rRNA* sequence. In *E. coli* cleavage by RNase E (aided by RNase G) generates the mature 5′ end of 16S and 5S rRNA and cleavage by RNase T generates the mature 3′ end of rRNA. The endonucleases for maturation of the 5′ end of 23 S rRNA and the 3′ end of 16S and 5S rRNA remain unknown. (B) The initial transcript in yeast contains 18S, 5.8S, and 25S rRNA. 5S rRNA is transcribed separately. Processing is initiated by RntI cleavage at the 3′ end. RntI is the yeast RNaseIII. Endonucleases for most cleavage steps remain unknown (red arrows). The 5′ and 3′ ends of 5.8S rRNA (blue arrows) are generated via exonucleolytic cleavage by Rat1/Xrn1 and the exosome/Rex1/Rex2, respectively. It is unclear whether the processing sites C1 and C2 (green arrows) are generated via endo- or exonucleolytic cleavage.

proteins (also dubbed "RNA helicases"), PIN-domain containing putative nucleases, AAA-type ATPases, as well as the machinery for modification of rRNA. Surprisingly, among the proteins required for ribosome assembly were also proteins with a possible regulatory function, such as protein kinases and GTPases.

Recently, it has also become clear that ribosome assembly in bacteria is not as straightforward as previously thought. For example, it was shown that the DnaK chaperone system promoted a conformational rearrangement in assembling ribosomes, which otherwise requires a heating step.[21] Furthermore, a combination of genetic, genomic, and biochemical work has revealed that assembly of bacterial ribosomes in vivo was promoted by the action of several accessory proteins, some of which are essential (e.g., Refs. 15–19,22,23). Interestingly, GTPases comprise the largest class of essential ribosome assembly factors in bacteria, suggesting that the requirement for regulation is evolutionarily conserved. Given

the importance of ribosomes for cellular growth and the importance of growth regulation this result may not be surprising, but was nevertheless not anticipated. This review will give an overview about the GTPases known to be involved in ribosome assembly, describe common features, and present model studies that give examples for their possible roles.

CASE STUDIES

While the GTPases involved in ribosome assembly have been studied biochemically, genetically, and structurally with fruitful results (see later), surprisingly little is known about the exact function of these GTPases in ribosome assembly. Below is a review of what is known about the molecular function of the best-studied of these GTPases, as an example of what GTPases might do during ribosome assembly. These case studies provide examples of functions for GTPases in ribosome assembly and also illustrate how their biochemical and
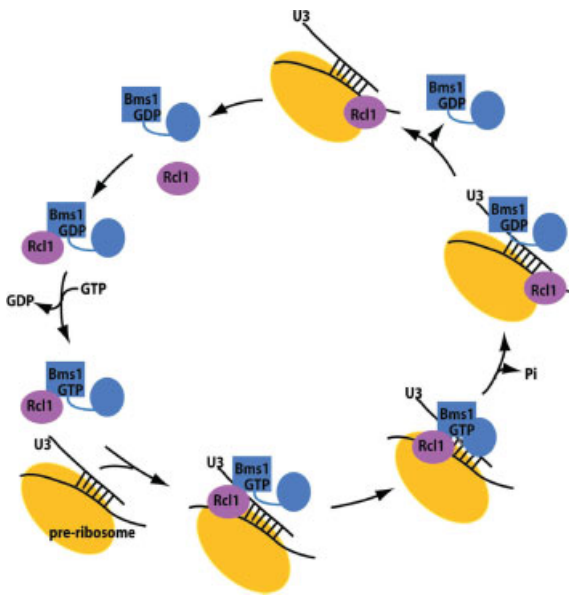
FIGURE 2 Model for the function of Bms1 in ribosome biogenesis adapted from.[26,27] Two of Bms1's domains are shown explicitly, an N-terminal GTPase domain (square) and a C-terminal GAP domain (oval). These domains are linked by a flexible tether. Pre-ribosomes are shown with pre-18S rRNA in a duplex with U3 snoRNA. Rcl1 binds to GDP-bound Bms1 and increases the affinity for GTP, thereby leading to GDP/GTP exchange. The active ternary complex of Bms1•GTP and Rcl1 binds tightly to U3 snoRNA. Because U3 snoRNA is bound to pre-rRNA the complex is located to pre-ribosomes. A conformational change within Bms1 may activate its GTPase activity by promoting interaction between the GTPase and the GAP domain. Dissociation of Rcl1 from GDP-bound Bms1 in turn leads to dissociation of Bms1•GDP from U3 snoRNA because of the weak U3 snoRNA affinity of Bms1•GDP.

cell biological characteristics reflect their biological function, giving us a reference point for interpretation of these data from other GTPases.

Bms1

Bms1 is an essential yeast protein that was identified in parallel in a synthetic lethal screen with depletion of one of the 14-3-3 proteins and as a protein interacting with the known ribosome assembly factor Rcl1.[24,25] Initial experiments provided strong evidence that Bms1 was involved in assembly of the 40S subunit.[24,25] Using purified recombinant proteins, it was subsequently shown that Bms1 hydrolyzes GTP, binds directly to Rcl1, a putative endonuclease, and the essential snoRNA U3.[26,27] Analysis of the interdependence of these activities showed that Rcl1 binding leads to exchange of bound GDP for GTP via a thermodynamic effect, thereby activating the protein.[27] The Bms1•GTP•Rcl1 complex binds with high affinity to U3 snoRNA and thus localizes to pre-ribosomes which contain U3 snoRNA base paired to pre-18S

rRNA.[26,27] Activation of the GTPase activity involves interaction of an internal GTPase activating protein (GAP)-domain with the GTPase domain.[26] Because these domains are bound via a flexible linker, activation might involve a conformational change, which could be modulated by factors in the nascent ribosome. Because GDP-bound Bms1 binds Rcl1 more weakly, GTP hydrolysis leads to dissociation of Rcl1, which is rendered irreversible when Bms1•GDP dissociates from pre-ribosomes.[26] In this model, Bms1 uses its GTPase switch to promote binding of Rcl1 to nascent ribosomes (Figure 2). This model, which is based on biochemical data obtained with purified components, is supported by in vivo data, showing that in strains carrying Bms1 alleles that bind Rcl1 poorly, Rcl1 binding to pre-ribosomes is disrupted.[26]

Lsg1

The yeast protein Lsg1 was identified as a GTPase associated with the 60S export adaptor Nmd3.[28] Immunofluorescence experiments indicated that the essential Lsg1 is a cytosolic protein, which does not shuttle between nucleus and cytosol. Nevertheless, depletion of Lsg1 results in defective export of 60S subunits from the nucleolus.[28] Further experiments show that this export defect is due to a failure to release Nmd3 from 60S subunits in the cytoplasm when Lsg1 is absent or nonfunctional.[29] Additional genetic and biochemical data provide evidence that the ribosomal protein L10 and Sqt1 form a complex that is located on ribosomes even when
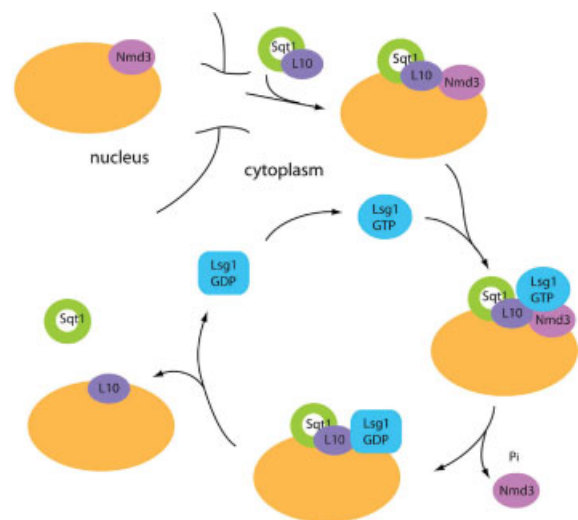
FIGURE 3 Model for the function of Lsg1 in ribosome biogenesis adapted from.[28–30] 60S precursors with Nmd3 bound exit from the nucleus and bind the Sqt1-bound ribosomal protein L10 in the cytosol. Lsg1 then binds to this 60S precursor and promotes release of Nmd3 and incorporation of L10 into ribosomes. Lsg1•GDP is then recycled.

L10 cannot be incorporated, suggesting that L10 initially binds pre-ribosomes in a complex with Sqt1.[30] Furthermore, genetic data indicate that stable incorporation of L10 occurs in the cytoplasm.[29] L10 binding is required for Nmd3 release either because it provides a binding site for Lsg1 or because it affects GTP hydrolysis on Lsg1 (Figure 3). These possibilities can be distinguished by testing whether Lsg1 is bound to 60S ribosomes in the absence of L10. While the first model would predict reduced binding of Lsg1 in the absence of L10, the second model makes the opposite prediction, as Lsg1 would not be released from on 60S ribosomes.

Strikingly, a similar model has been proposed for function of the GTPase Ria1.[31] It has been shown that depletion of Ria1 leads to accumulation of Tif6 in the cytosol, leading to an assembly defect in the nucleus, where Tif6 acts. Additionally, it has been shown that GTP hydrolysis by Ria1 is stimulated by 60S ribosomes. Lastly, GTP hydrolysis by Ria1 promotes release of Tif6 from 60S ribosomes.[31] These data suggest that Ria1 uses the energy from GTP hydrolysis to promote release of Tif6 from nascent 60S subunits. Interestingly, Ria2 has very strong homology to elongation factor G, which promotes translocation of peptidyl-tRNA and dissociation of 70S ribosomes after termination, likely by promoting a conformational change in 50S subunits dubbed "unlocking."[32,33] A similar conformational change might be required for Tif6 release.

GENERAL FEATURES OF GTPASES

GTPases are a subfamily of P-loop NTPases[34] and have been widely studied as regulators of cellular signaling, transport, cytoskeleton organization, and as translation factors. On the sequence level GTPases are characterized by the presence of five conserved motifs, G1–G5 (for reviews see: Refs. 34,35). G1 [GXXXXGK(S/T)], also referred to as the Walker A motif, is shared with other NTPases and is responsible for binding of the α- and β-phosphates. G2 regions contain a conserved threonine, but otherwise differ between GTPases of different subfamilies and are responsible for coordination of a Mg^{2+} ion that binds to the β- and γ-phosphates. Because in ras-like GTPases, this region shows often large structural differences between the GTP and GDP bound states, it is also referred to as the switch I region. The DXXG motif of the G3 region (Walker B motif) is involved in Mg^{2+} coordination and binding to the γ-phosphate. The G4 region [(N/T)KXD] senses the identity of the bound nucleotide by forming hydrogen bonds with the guanine ring. Lastly, the G5 region [SA(K/L)] interacts with the guanine via water-mediated hydrogen bonds. However, this region is often poorly conserved in GTPases associated with ribosome assembly. In addition to the GTPase domain, the ribosome associated GTPases have additional domains as described later (Figure 5 and "GTPases in Ribosome Assembly Are RNA-Binding Proteins").

GTPases have been well characterized structurally as well as biochemically. From these studies, performed largely with small GTPases from the ras superfamily, it has become clear that the GTP- and GDP-bound forms often differ largely in two exposed loops (the switch I and II regions), which are located on the surface of the molecule and provide the interface with the effector. These structural differences are triggered by the presence of the γ-phosphate and explain why the GTP bound forms bind more strongly to their effectors than the GDP bound form (for examples see Table III in Ref. 27). [The word "effector" is used herein to describe another molecule that affects the GTPase's function, e.g., one that promotes nucleotide binding or GTPase activity, etc.; in this definition ribosomes are effectors for several of the ribosome associated GTPases (see later).]

GTPASES CONSTITUTE A LARGE AND DIVERSE CLASS OF RIBOSOME ASSEMBLY FACTORS

While Bms1 is the only known GTPase involved in assembly of the 40S small subunit in the yeast *Saccharomyces cerevisiae* (see "Case Studies" above for a more thorough description of Bms1's function), work in *E. coli* and *B. subtilis* has identified Era, RsgA (YjeQ/YloQ) and YqeH as proteins critical for assembly of the 30S small subunit.[36–39] Depletion of Era and RsgA leads to accumulation of a 17S precursor to 16S rRNA and depletion of all three GTPases leads to loss of 30S subunits.[37–40] However, how these GTPases function in 30S assembly remains unknown.

Assembly of the 60S large subunit in yeast requires five essential GTPases: Nog1, Nug1, Nug2 (Nog2), Ria1, and Lsg1.[28,41–44] Nog1, Nug1, Nug2 are nuclear and nucleolar proteins.[41,43,44] Ribosome assembly is interrupted when Nog1 is trapped in the nucleolus, suggesting that Nog1 acts in the nucleus.[28,45] Lsg1 and Ria1 are cytoplasmic proteins.[28,42] Only depletion of Nog1 and Nug2 results in an rRNA processing phenotype where 25S accumulation is reduced,[28,43] suggesting that the other GTPases act after processing of 25S is complete (Figure 4A). Depletion of Nog1 results in accumulation of the 27SA, intermediate, while depletion of Nug2 results in accumulation of the later 27SB intermediate, suggesting that Nog1 acts prior to Nug2.[28,43] In addition, proteomic analysis of the 60S precursors, to which these GTPases bind, show that both the Nog1 and Nug2 associated particles lack the ribosomal proteins Rpp0 and L12.[43]
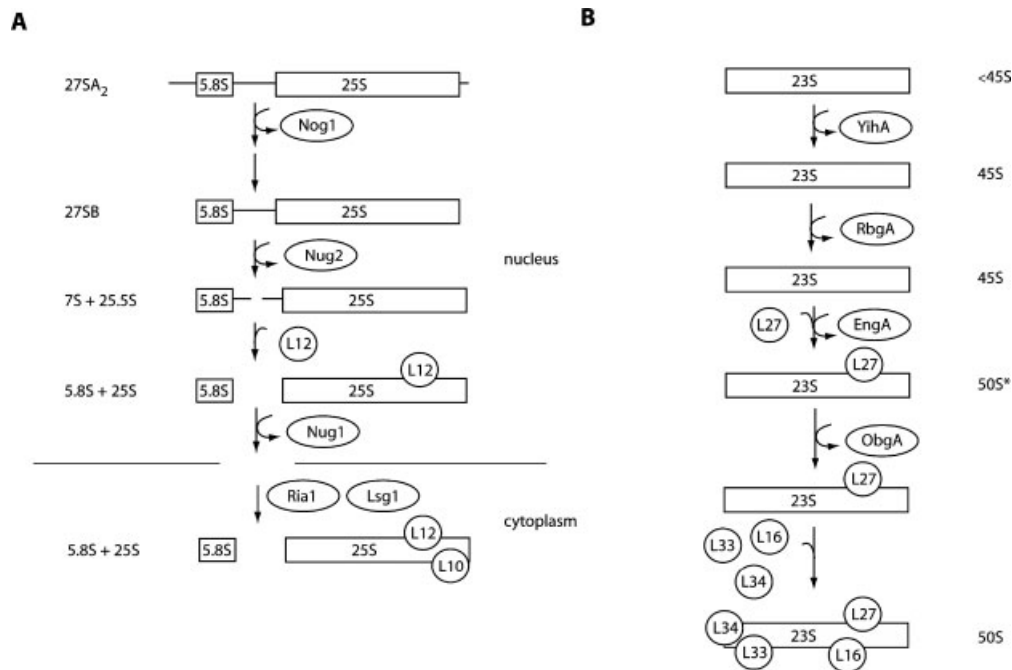
FIGURE 4 GTPases in assembly of the large ribosomal subunit in (A) *S. cerevisiae* and (B) bacteria. Boxes outline the rRNA incorporated into mature ribosomes and lines precursor rRNA that will be removed during maturation. GTPases are shown as ovals and ribosomal proteins as circles. (A) Preliminary order in which GTPases act to assemble the yeast 60S subunit based on Northern analysis of rRNA processing intermediates accumulating in the absence of the GTPase as well as on proteomic analysis of particles associated with the GTPase.[28,42–44] rRNA processing intermediates are described by their sedimentation coefficient (27S, 25.5S, 7S) as well as the cleavage site that generated their ends (A$_2$, B). (B) Preliminary order in which GTPases act to assemble the bacterial 50S subunit based on sedimentation (45S, 50S) and proteomic analysis of ribosome precursors.[46,47–49] 50S* describes a particle that sediments as 50S, but is salt-labile.[49]

In contrast, the Nug1 associated precursor does have L12 but not Rpp0 bound.[44] Taken together these data indicate that Nog1 is the first GTPase to act on the 27SA-containing 60S precursors in the nucleus, followed by Nug2, which acts on 27SB-containing precursors. Nug1 then acts on a nuclear particle that contains 25S rRNA and has L12 incorporated (Figure 4A). Ordering the function of Ria1 and Lsg1 is not possible based on the available data. It is also possible that there is no stringent order but diverging pathways.

In contrast to GTPases involved in small subunit assembly, some of the GTPases involved in 60S assembly have homologs in bacteria, where Obg (CtgA) is the Nog1 homolog and RbgA may be the Nug1 homolog, but also shares the circularly permuted GTPase domain with Lsg1 and Nug2. In addition to these GTPases, bacteria also encode additional GTPases involved in 50S assembly: YihA and EngA (Der).[46,50,51]

In bacteria, depletion of these GTPases leads to the accumulation of distinct intermediates, which are stable in sucrose gradients and can be purified and analyzed for their protein content. The intermediate observed upon YihA depletion has a slightly slower sedimentation rate than the 45S intermediates observed when RbgA and EngA are depleted.[46–48] In contrast, depletion of Obg leads to accumulation of a 50S pre-ribosome that is unstable at low Mg^{2+} concentrations.[49] The increasing sedimentation rate observed with intermediates accumulating when YihA, RbgA, EngA, and Obg are depleted, suggest a preliminary order to the action of these GTPases with YihA acting before RbgA and EngA, and Obg being the last GTPase to act on assembling 50S subunits (Figure 4B). Furthermore the YihA, RbgA, and EngA-depletion intermediates lack the ribosomal proteins L16, L27, and L36[46–48] (note that the gel-electrophoretic separation procedure may not have allowed detection of additional missing proteins). The Obg-depletion intermediate lacks L16, but has L27 bound. L36 was not analyzed.[49] These data suggest that L27 is being incorporated in going from the 45S intermediate that is the substrate for RbgA and EngA to the 50S intermediate that binds Obg. L16 is incorporated after Obg's function. In addition L33, L34, and possibly L23
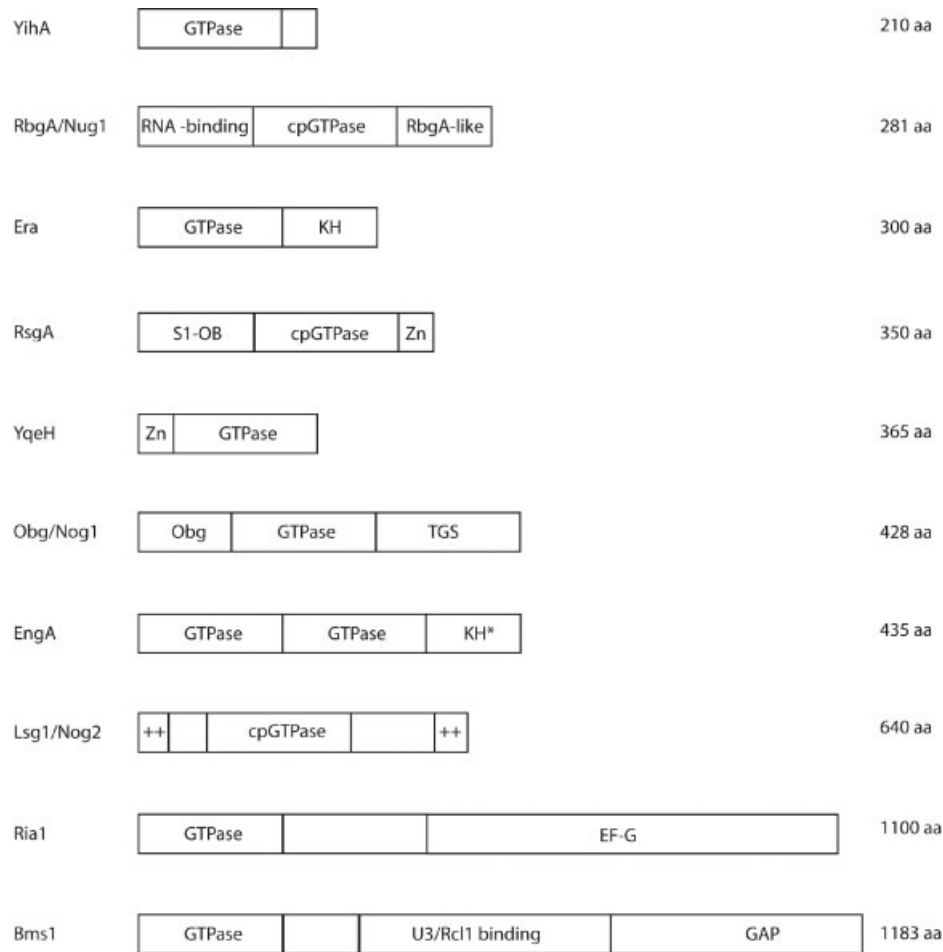
FIGURE 5 Domain organization of GTPases involved in ribosome assembly. GTPase refers to canonical GTPase domains where the G-motifs are arranged in the order G1-G2-G3-G4-G5, while cpGTPase refers to circularly permuted GTPase domains, where the G motifs assume the order G4-G5-G1-G2-G3. KH refers to a KH RNA binding domain, while the KH* domain in EngA lacks the residues typically associated with RNA binding[51] and is therefore likely not involved in interactions with RNA. RsgA contains an OB fold as found in the ribosomal protein S1. Both RsgA and YqeH contain Zn^{2+}-binding loops that are likely RNA binding elements.[52,53] Obg has glycine-rich N-terminal domain found in all Obg proteins[54] as well as a less conserved TGS domain also found in threonine tRNA synthetases, GTPases, and SpoT.[54] Bms1 has binding motifs for U3 snoRNA as well as Rcl1 protein and contains an internal GAP domain.[26] Bacterial proteins typically refer to the *B. subtilis* homolog, while yeast proteins refer to the *S. cerevisiae* homolog.

are missing in the Obg-depletion intermediate. This result is consistent with these proteins being late-binding proteins according to the assembly map.[7,9]

GTPASES IN RIBOSOME ASSEMBLY ARE RNA BINDING PROTEINS

GTPases involved in ribosome assembly contain at least one domain in addition to the GTPase domain (Figure 5). While there is large diversity in the size and sequence of these domains a common thread appears to be that ribosome assembly GTPases contains RNA binding domains. For *E. coli* Era and Obg, and *S. cerevisiae* Bms1 and Nug1 RNA binding activity has been demonstrated biochemically[26,55–57] suggesting that they are intimately bound to nascent ribosomes instead of being localized via interactions with ribosomal proteins or other ribosome assembly factors. YihA and EngA, whose KH domain does not have the residues associated with RNA binding, may not directly bind RNA, but may instead interact with ribosomes via protein–protein interactions.

WEAK NUCLEOTIDE BINDING

With the possible exception of RsgA, all characterized GTPases involved in ribosome assembly bind nucleotide weakly (Table I), suggesting that GDP release is fast. For RsgA presteady state kinetic analysis has shown that GTP hydrolysis is fast and followed by a slow step that limits turnover. The simplest model is that turnover is limited by GDP release. If this model were true the steady-state rate constant for GTP hydrolysis of 0.17 min^{-1} would reflect the dissociation rate constant for GDP. Consistent with this proposal, RsgA copurifies with GDP bound, suggesting strong affinity. Bound GDP will result in an increased apparent binding constant, since exogenous GTP has to compete with bound GDP. Assuming an association rate constant of 10^6 M^{-1} s^{-1}, which is typically observed for GTPases,[58,59] and assuming that the steady-state rate constant of 0.17 min^{-1} represents the dissociation rate constant, one can estimate the GDP binding affinity of RsgA to be 3 n*M*. Interestingly, there are two reports that 30S ribosome stimulate the turnover rate for GTP hydrolysis over 100-fold.[38,60] If the turnover rate does reflect GDP release, this would provide evidence that the nascent 30S subunit acts as a guanine exchange factor (GEF) for RsgA, ensuring that RsgA is only activated upon substrate binding]. For Era and Obg, rapid nucleotide release has been directly demonstrated.[58,59] This observation has important biological ramifications, since it suggests that these GTPases, in contrast to the small GTPases from the ras-superfamily, do not require assistance from GEFs to promote release of bound GDP and binding of GTP. Furthermore, the "timer-model" of GTPase function, developed based on these small GTPases, requires that both the activation of the GTPase (via binding of GTP) as well as the deactivation (via hydrolysis of GTP) be regulated via the function of GEFs and GAPs.[61] If these GTPases can bind GTP without the assistance of a GEF, it suggests that their function does not involve a timing mode.

For Bms1, Era, and Obg the affinities for both GTP and GDP have been determined. In these three cases, it has been shown that GDP binding is moderately stronger than GTP binding (Table I). Better competition of GDP than GTP for binding of radiolabeled GTP to EngA, YqeH, RbgA, and YihA qualitatively demonstrated that these proteins have higher affinity for GDP than GTP.[22] In the case of Bms1 and Era the binding differential is so large that, even when taking into account that the GTP concentration exceeds the GDP concentration by a factor of ~6, one must assume that in vivo the free GTPase is in the inactive, GDP-bound state (Table I). For Bms1 it has been shown that binding of Rcl1 increases the affinity for GTP while not affecting the affinity for GDP. Thus, Rcl1 binding leads to GDP/GTP exchange

Table I GTP and GDP Affinities for GTPases Involved in Ribosome Assembly

Name	K_d^{GTP} (μM)	K_d^{GDP}	Reference
Bms1	182	22	27
Era	3.6	0.6	58
RsgA	120; 30.5[a,b]		38,61
Nug1	200[a]		57
Obg	1.2	0.5	59
EngA	143; 110[a,c]		50,51

[a] K_m value was obtained in steady-state measurements and may thus not report on binding affinity.

[b] RsgA purifies with GDP bound. Thus, in any binding experiment GTP has to compete with bound GDP and affinities may well be large underestimates (see "Weak Nucleotide Binding").

[c] Although both GTPase domains are essential, the bulk of the GTPase activity apparently resides in the first GTPase domain.[41,42] The second domain may bind nucleotide more strongly since it co-purifies with GDP.[61]

and consequently activation of the protein.[27] Similarly, RbgA, EngA, Obg, and RsgA preferentially bind to the ribosomal subunit they help assemble in their GTP-bound form.[38,46,47,49,60,63] These results show that ribosomes must strengthen binding of GTP for these proteins. This is because the energy of binding is conserved regardless of the order of GTP and ribosome binding (see Ref. 26 for a more detailed explanation). Thus, ribosomes strengthen GTP binding for EngA, RsgA, Obg, and RbgA and shift the nucleotide preference from GDP to GTP, leading to binding of GTP. Assuming that the GTP-bound state is the active state, the ribosome appears to activate many of the GTPases involved in its assembly.

INTEGRATION OF RIBOSOME ASSEMBLY AND GROWTH

Cell cycle progression requires the ability to synthesize ribosomes.[64–66] Conversely, because protein synthesis and growth is only desirable under favorable cellular conditions, ribosome synthesis is highly responsive to growth conditions, including nutrient availability, heat and cold shock, as well as oxidative stress.[67–71] The mechanisms underlying these regulatory steps are poorly understood.

Genetic and cytological analysis has provided strong evidence that depletion of Era leads to a block in cell cycle progression, with a defect after DNA replication and nucleoid separation, but before cell division.[72] Era mutants were able to suppress defects in chromosome partitioning but not in septum formation. Correspondingly, the cells have four (or more) nucleoids and become elongated.[72] Flow cytometry also shows increased DNA content of cells grown in the

absence of Era. Similarly, it was shown that depletion of the chicken Era protein leads to cell cycle arrest in G1 and subsequent apoptosis.[73] Depletion of YqeH, another GTPase involved in 30S assembly has a similar morphological phenotype.[22]

Recent work has also provided strong evidence for a role of Obg in replication.[74] It was shown that Obg mutants are sensitive to replication fork inhibitors. Additionally, genetic interactions with RecA and RecB (which repair stalled replication forks) and SeqA (which inhibits reinitiation of replication) were uncovered. These chemical–genetic interactions strongly argue for a role of Obg in initiation of replication and stabilization of replication forks. Obg mutants show filamentous cell morphology, higher DNA content and condensed nucleoids. This phenotype is also shared with depletion of RsgA, involved in 30S assembly, as well as RbgA, EngA, and YihA, all of which are involved in 50S assembly.[22,40] It is worth noting that the same phenotype is elicited when translation is repressed via the addition of antibiotics inhibiting ribosome function. Thus, this phenotype might be a nonspecific response to a stall in protein synthesis and not reflect a cell division phenotype. It has not been investigated however, whether these compounds also inhibit ribosome assembly.

In addition to the role Obg plays in regulating DNA replication, its function is required for activation of the environmental stress response in *B. subtilis.*[75] Furthermore, Obg binds directly to the ppGpp synthase/hydrolase SpoT[76] and crystallizes with ppGpp bound,[54] suggesting that it may modulate the stringent response. While these activities are poorly understood, they suggest that Obg integrates (a stall in) ribosome assembly into the stress response in bacteria. Finally, it has been shown in streptomyces that overexpression of Obg suppresses spore formation. Because this effect is enhanced in an Obg mutant predicted to be in the GTP-bound form, it has been suggested that this effect is associated with GTP-bound Obg.[77] Consequently, Obg expression is shut off during spore formation.[77] Taken together these data indicate that the GTPase Obg integrates a number of different cellular functions with ribosome assembly in response to nutrient availability.

Recent work in the yeast *S. cerevisiae* has shown that assembly of the large 60S subunit is regulated via the target of rapamycin pathway, which is inactivated upon nitrogen starvation.[45] Nitrogen starvation can be mimicked by addition of the drug rapamycin. Interestingly, it has been shown that the subcellular localization of the GTPase Nog1 is directly affected by rapamycin addition. It was shown that Nog1 (and likely the pre-ribosomal particle it is associated with) remains trapped in the nucleolus, instead of being released into the nucleoplasm. This effect was abolished in the presence of a Nog1 allele.[45] Since the mutation was not identified it is not possible to determine whether Nog1 is a substrate for phosphorylation. However, in trypanosomes Nog1 interacts with the kinase CK2α,[78] suggesting that this kinase might phosphorylate Nog1.

Bms1, the yeast GTPase involved in 40S assembly, also appears to be the target of regulation via phosphorylation. Previous work has shown that Bms1 genetically interacts with the yeast 14-3-3 proteins Bmh1 and Bmh2.[24] 14-3-3 proteins recognize phosphorylated proteins and serve to interpret the information contained in phosphorylation. Inspection of the Bms1 protein sequence reveals the existence of a 14-3-3 recognition site, suggesting that the genetic interaction between Bms1 and Bmh1/2 reflects a direct physical interaction. Mutation of the threonine, the putative target of phosphorylation, to alanine does not affect protein function, while mutation to aspartate completely abolishes growth. This effect is due to an interruption of ribosome assembly (J. A. Doudna and K.K., unpublished results). Because the negative charge on the aspartate mimics the negative charge on a phosphothreonine, these results suggest that phosphorylation of Bms1 at the 14-3-3 recognition site turns off protein function. Detailed investigations into the mechanism of this down-regulation, as well as the cellular conditions leading to down-regulation, are currently under way.

In summary, data in the literature provide strong evidence that the GTPases involved in ribosome assembly in bacteria also serve to regulate growth via an impact on DNA replication and/or cell division, possibly providing checkpoints for cell growth in response to ribosome assembly states. Furthermore, Obg (and Era) apparently also function in the opposite direction by being responsive to changes in the levels of nutrients and thus regulating ribosome assembly and sporulation in response to stress and starvation.[75,77] Similarly, there is good evidence that two GTPases in yeast, Bms1, and Nog1, are also targets of regulation. In the case of these GTPases however, regulation apparently occurs via phosphorylation instead of a change in the bound nucleotide. This may reflect the higher sophistication of signaling cascades in eukaryotes, as well as the additional possibilities afforded by compartmentalization.

POSSIBLE ROLES FOR GTPASES IN RIBOSOME ASSEMBLY

The available data in the literature suggest that many GTPases are essential factors involved in assembling ribosomes in bacteria and eukaryotes. Extensive biochemical and genetic work has provided insight into the function of some

GTPases as well as into the biochemical and functional properties of most of them. Here I will generalize and consider possible functions for GTPases in ribosome assembly.

Energy from GTP Hydrolysis Can Be Used to Regulate Delivery or Removal of Proteins to Nascent Ribosomes

Ribosome assembly, akin to splicing, requires the successive binding and removal of many components. If protein binding is thermodynamically stable, removal requires energy input or vice versa. GTPases are a means to provide such energy input into binding or dissociation processes.

Biochemical and genetic data indicate that Bms1 uses the energy from GTP hydrolysis to promote binding of Rcl1 to nascent ribosomes.[26] Similarly, it has been suggested, although not shown, that Lsg1 might use the energy from GTP hydrolysis to incorporate L10 into nascent 60S subunits.[29,30] The same suggestion has been made for incorporation of L16 by RbgA.[48] Three different mechanisms can be envisioned that would allow the energy of GTP hydrolysis to be used to *promote protein binding* to nascent ribosomes.

(1) GTP hydrolysis can drive protein binding, if the GTPase in the GTP-bound state, but not the GDP-bound state, binds to the protein it delivers and ribosomes. GTP hydrolysis on nascent ribosomes could then promote dissociation of the GTPase. The transiently bound factor might then remain bound long enough to function. This model would allow for transient binding of an assembly factor such as Rcl1.

(2) Alternatively, the additional binding interactions from the GTPase might stabilize an encounter complex long enough to allow for conformational changes that result in a stable interaction between ribosomes and a ribosomal protein. In the absence of the GTPase such an encounter complex would not be long-lived enough to allow for conformational rearrangements.

(3) Finally, the energy from GTP hydrolysis might be used to accelerate a conformational change required for protein insertion into nascent ribosomes. In the absence of GTP hydrolysis the rearrangement would be too slow to occur from an unstable encounter complex.

GTPases might *promote release* of transiently bound proteins, as described for Ria1 and Lsg1.[29,31] This could be done by one of two mechanisms: (1) GTPases could accelerate a conformational change within the ribosome that results in destabilization of the protein. In this model dissociation from the original conformation is much slower than dissociation from the new conformation, with interconversion being slow in the absence of the GTPase, but accelerated by GTP hydrolysis. This role would be similar to the role EF-G

plays during translocation of tRNA, where it "unlocks" a ribosomal conformation as described earlier.[32,33] Interestingly, Ria1 has very close sequence homology to EF-G, not only within the G-domain but also the other domains, required by EF-G to unlock ribosomes. This observation suggests that Ria1 might displace Tif6 from nascent ribosomes by promoting a conformational rearrangement.

(2) Alternatively, the GTPase could act as a mechanical device and directly displace the bound protein, similar to myosin. Interestingly, kinesin and myosin are a part of the GTPase family even though they hydrolyze ATP and not GTP and RbgA, RsgA, and YqeH as well as their yeast homologs Lsg1, Nug1, and Nog2 are part of the myosin/kinesin superfamily of GTPases.[34]

Energy from GTP Hydrolysis Could Be Used to Promote a Conformational Rearrangement Within Nascent Ribosomes

Ribosome assembly likely involves numerous conformational rearrangements. Some of these undoubtedly involve dissociation of RNA duplexes and/or RNA protein interactions and are likely catalyzed by DEAD-box proteins required for ribosome assembly. However, more extensive rearrangements could also be facilitated by GTP-binding proteins, as shown for EF-G. There is currently no evidence that one of the GTPases involved in ribosome assembly functions in this way.

GTPases Could Act As a Reversible Placeholder, Preventing Premature Protein Binding

A major challenge in assembling ribosomes is likely to prevent formation of structure that occurs prematurely. While there is no such instance reported for ribosomes, in the case of the much smaller group I ribozyme, premature formation of a native tertiary structure element can slow down folding to the native conformation.[79] In the case of ribosomes it is known that proteins stabilize tertiary structure, thereby possibly locking in not just native but also nonnative contacts. GTPases could prevent this problem by acting as placeholders, thereby sterically preventing protein binding and the ensuing formation of native tertiary contacts, until folding traps are avoided. In this model the GTP-bound protein binds to nascent ribosomes, while GTP hydrolysis weakens the affinity resulting in release of the GTPase and thus allowing for subsequent conformational rearrangements. These rearrangements must occur faster than rebinding of the GTP-bound form. This model would thus be facilitated if GDP/GTP exchange was slow and required a GEF, as is the case for RsgA.

GTPases Could Sense the Nutritional State of the Cell Reflected in the GTP/GDP Ratio

Above it is shown that the ribosome assembly GTPases that have been analyzed moderately prefer binding of GDP to binding of GTP (Table I and "Weak Nucleotide Binding" above). Because GTPases are typically active in their GTP but not their GDP bound state, this finding suggests that their function might be regulated by a change in the GTP/GDP ratio, e.g., at a GTP/GDP ratio of six, present in normally growing cells, 50% of the Era pool is in the GTP and 50% is in the GDP bound form. If the GTP/GDP ratio drops to three under starvation conditions, GTP occupancy would fall to 33%, while it would increase to 60% for a GTP/GDP ratio of nine under optimal growth conditions. For Era, Obg and RbgA a nucleotide sensing role has been suggested.[48,58,59] While it has been shown that induction of the stress response, one of Obg's functions, does not result from a change in GTP/GDP ratio,[80] strong evidence for a role in GTP/GDP ratio has been presented in induction of sporulation, another suggested role of Obg.[81]

SUMMARY AND FUTURE DIRECTIONS

Biochemical analysis indicates that many of the GTPases involved in ribosome assembly function differently from the well-studied ras-like GTPases in that they bind nucleotide weakly. This suggests that they are not regulated by GEFs and likely do not act as "molecular timers." Instead, functional analysis of these proteins suggests that they may use the energy from GTP hydrolysis to provide directionality to otherwise equilibrated processes, such as protein binding and removal. Additionally, previous work and discussions herein outline additional roles for GTPases as mechanical devices or nucleotide sensors. While genetic data have been fruitful in revealing the involvement of GTPases in ribosome assembly and in uncovering interacting partners, future progress in determining the function of the GTPases involved in ribosome assembly will require recombinant, purified proteins as well as new assays to study the interaction with nascent ribosomes, e.g., Lsg1 has been suggested to both incorporate L10 and remove Nmd3 from nascent 60S subunits. Using recombinant protein, and purified 60S precursors, one could show that Nmd3 is removed from pre-ribosomal particles and that L10 becomes incorporated stably (as it might become stable to changes in salt and/or result in new protections from DMS footprinting). Furthermore, the effect of GTP hydrolysis on these processes could be studied using GMPPNP, a nonhydrolyzable GTP-analog, and determining whether it inhibits one or both of these processes. Such work might be particularly fruitful in the case of the bacterial GTPases, since most of them have been successfully over expressed and purified and because ribosome assembly intermediates are also readily available by purification from bacteria. The next several years should therefore reveal additional new functions for the GTPases involved in ribosome assembly.

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Reviewing Editor: Sarah Woodson