# Single-step in vitro ribosome reconstitution mediated by two GTPase factors, EngA and ObgE

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**Abstract**

When bacterial ribosomes are assembled in vitro, manipulation of incubation temperature and magnesium ion concentration have been an essential procedure, which is a crucial step for the assembly of active large subunits. The present study tackles with this issue to develop a single-step procedure, which can be performed in a near-physiological condition, where cell-free protein synthesis is active. We found that GTPase factors EngA and ObgE can complement the changes in temperature and magnesium ion concentrations. In the presence of these factors, both the ribosome assembly and translation processes were successfully integrated in the reconstituted cell-free protein synthesis system. Furthermore, we found that these GTPase factors can reassemble the ribosomes to an active state, whose structure was disrupted by EDTA chelation of magnesium ions, indicating that these two factors can reversibly induce the ribosome structure to an intact state. The findings are essential for the bottom-up construction of synthetic cells.

## Introduction

Ribosomes are large intracellular complexes that play a central role in a protein translation system. They consist of two subunits, each composed of one or a few rRNAs and dozens of ribosomal proteins. Maturation processes of these complex assemblies have been studied for decades. For the bacterial ribosomes, the construction of assembly maps of both subunits from *Escherichia coli*, describing the order of ribosomal proteins bind to rRNA, is well known as milestone achievements[1,2]. However, many aspects have yet to be elucidated, including differences between the comparatively slow in vitro reconstitution processes and the more efficient and refined cellular assembly processes, accompanied inclusion of various assembly factors, and effects of a variety of rRNA modifications on these maturation processes. Various in vivo and in vitro studies are still being performed, aiming for a deeper understanding of these processes, by incorporating advanced technologies, including quantitative mass spectrometry (qMS), cryo-electron microscopy (cryo-EM), and single molecule imaging[3-8].

Recently, with the increasing focus on constructing synthetic cells from the bottom-up viewpoint[9,10], the importance of the studies on assembling ribosomes from individual parts in vitro has been highlighted. The integrated synthesis, assembly, and translation (iSAT) method developed by Jewett *et al.* demonstrated that in the presence of cell extracts, the assembly of both subunits proceeds using transcribed rRNAs and ribosomal proteins purified from native ribosomes, termed total proteins of the 30S subunit (TP30) and total proteins of the 50S subunit (TP50)[11]. Cell extracts-free ribosome assembly methods have been developed by the integration with the reconstituted cell-free protein synthesis system (PURE system)[12], enabling the in vitro evolution of 16S rRNA[13]. Also, TP-free methods have been constructed by preparing individually purified recombinant ribosomal proteins for both subunits[14-17]. The use of cell-free expressed ribosomal proteins is also addressed[8,16,18-20], aiming for the development of self-replicating synthetic cells in the future[21].

These cell-free synthetic biology studies can help us understand the minimally required building blocks for producing functional assemblies. For the small subunit, functionally active subunits have been shown to be assembled by merely mixing synthetic parts, transcribed rRNA and recombinant ribosomal proteins with minimal post-translational modifications[16]. The functional large subunit has also been shown to be assembled with recombinant proteins with minimal post-translational modifications[17]. However, two major issues exist with the large subunit reconstitution method.

The first issue is the requirement of posttranscriptional modifications in 23S rRNA. According to Green and Noller, modified nucleotides in certain regions of rRNA are

necessary for the peptidyl transferase activity of the reconstituted large subunit from *E. coli*[22]. For *Bacillus stearothermophilus*, it has been shown that the subunit with the peptidyl transferase activity can be reconstituted even with the transcribed 23S rRNA[23]. The addition of some chemical substances into the assembly mixtures for *E. coli* ribosomes has also been reported to be effective for the peptidyl transferase activity of the reconstituted subunit using transcribed 23S rRNA[24]. All of these experiments have focused on the peptidyl transferase activity of the reconstituted subunit. Whether or not the assembled subunits are competent for the whole translation processes, including initiation, elongation, and termination steps, remains unclear. By contrast, protein synthesis activity of the reconstituted large subunit was observed if the iSAT method is applied[11]. It can be considered that some factors in the cell extract, including rRNA modification enzymes, are involved in the functional assembly of the large subunit.

The second issue is the need for a two-step procedure that involves temperature and ion concentration changes in the reaction solution. Nierhaus and Dohme revealed that two significant steps are required for the successful assembly of the large subunit[25,26]. In the first step, 5S rRNA, 23S rRNA, and ribosomal proteins are incubated at 44 °C to form 41S or 48S intermediates. Then, by raising the magnesium ion concentration from 4 mM to 20 mM and the temperature to 50 °C, active subunits formation is completed by further incubation (**Fig. 1a**). The need to raise the temperature to a non-physiological value and to manipulate the ion concentrations can be a major barrier when attempting the bottom-up construction of synthetic cells. It is necessary to develop the assembly methods under physiological conditions without temperature changes or ion concentration manipulations. It is noteworthy that the second issue also does not apply to the iSAT method. In the method, the ribosome assembly and the translation processes are seamlessly linked in the same reaction solution, without manipulation of the temperature and ion concentrations[11]. It is highly likely that specific factors in the cell extracts complement this issue.

The present study aimed to tackle with the second issue by using ribosome biogenesis factors. It was found that changes in temperatures are effective and changes in magnesium ion concentration are essential for the assembly of translation competent ribosomes. We found that the presence of GTPase factors EngA[27] and ObgE[28,29] is necessary to complement these changes. In the presence of these factors, ribosome assembly can proceed under near-physiological conditions, with magnesium ion concentrations below 10 mM, potassium concentrations around 100 mM, and temperatures of 37 °C, where cell-free protein synthesis is active. Both the ribosome assembly and translation processes were successfully linked together in the PURE system, demonstrating that the ribosome assembly proceeds in the same solution conditions as the translation reaction, and the

assembled ribosomes are competent for the whole translation processes. Furthermore, it was revealed that unfolded ribosomes, whose structure was disrupted by EDTA chelation of magnesium ions[30], could be restored to an active state with both the supplementation of magnesium ions and the addition of EngA and ObgE, indicating that these two factors can reversibly induce the ribosome structure to an intact state.

## Results

*Effect of changes in temperature and ion concentration on the ribosome assembly*

In our previous study on small subunits, we have found that the assembly proceeds autonomously without the support of any factors[16], whereas temperature and ion concentration changes are necessary for the assembly of large subunits[25]. To evaluate the impact of these changes on the reconstitution of entire ribosomes, we performed in vitro ribosome assembly experiment using total ribosomal RNAs (5S, 16S, and 23S rRNA) and total proteins of the 70S ribosome (TP70) prepared from the purified native ribosomes[31]. Four patterns of temperature and magnesium ion concentration changes were applied to clarify each effect (**Fig. 1b**). The reconstituted ribosomes were purified from the assembly mixtures and then they were added into the PURE system without the ribosome to test superfolder green fluorescent protein (sfGFP) synthesis. When no magnesium ion concentration changes were applied, sfGFP synthesis by the assembled ribosomes was not detected, and when no temperature changes were applied, sfGFP synthesis decreased by about one-third (**Fig. 1b**). The results showed that the changes in magnesium ions are particularly essential, indicating that the two-step procedure established for the reconstitution of large subunits[25] (**Fig. 1a**) is also an essential key process for the assembly of entire ribosomes with translation activity, consistent with the previous statement[31].

*Single-step ribosome assembly using ribosome biogenesis factors*

Based on the differences between the iSAT assembly experiments[11] and those using purified parts, including this study (**Fig. 1b**), we hypothesized that some factors in the cell extracts used in iSAT, particularly ribosome biogenesis factors, may complement the changes in temperature and divalent ion concentration required in the two-step procedure, and facilitate a single-step assembly in the cell-free reaction mixtures (**Fig. 2a**). Furthermore, in previous experiments examining the effects of ribosome biogenesis factors on small subunit reconstitution, we found that the factors Era and RsgA (YjeQ) were effective in promoting subunit assembly, both being GTPase factors[15,16]. Therefore, we focused on GTPase factors, which have been suggested to be involved in large subunit

147 biogenesis. Based on literature surveys, we selected BipA[32], EngA (Der)[27], EngB
148 (YihA/YsxC)[33], HflX[34], LepA[35], and ObgE[28,29], which were prepared as recombinant
149 proteins. Surprisingly, when these six factors were mixed with total ribosomal RNAs (5S,
150 16S, and 23S rRNA) and TP70, along with an energy regeneration system to maintain
151 GTPase activity, we found that it was possible to assemble the ribosomes with sfGFP
152 synthesis activity at a constant but relatively low magnesium concentration of 8 mM at
153 37 °C (**Fig. 2b**). The sfGFP synthesis activity significantly dropped at a magnesium
154 concentration of 17 mM in the presence of these six factors. Almost no sfGFP synthesis
155 activity was observed in the absence of six factors, even with 8 mM magnesium,
156 suggesting the selected factors exhibit some activity that compensates for the magnesium
157 ion concentration changes in the two-step procedure. The optimal magnesium
158 concentration for this reaction was 8 mM (**Fig. 3a**), and the optimal monovalent salt
159 concentration for ribosome assembly was several hundred millimolar, consistent with
160 previous assembly experiments[25] (**Fig. 3b**).
161
162 *Coupling of the assembly and translation reactions*
163 The results with GTPase factors suggested that the factors were able to complement the
164 temperature and ion concentration changes in the two-step procedure, realizing a single-
165 step ribosome assembly. Because the required monovalent and divalent ion
166 concentrations are within the range for the optimum conditions of the translation reactions
167 (**Fig. 3**), it appeared probable to integrate the ribosome assembly and translation processes
168 in a single reaction mixture. To this end, we attempted to construct a coupled reaction, by
169 adding 3 rRNAs, total proteins of the 70S ribosome (TP70), and selected GTPases into
170 the ribosome-omitted PURE system (**Fig. 4a**). The result showed that after a lag period
171 that likely corresponds to the time required for the ribosome assembly, increase of sfGFP
172 fluorescence was observed (**Fig. 4b**). An optimal magnesium concentration was 8 mM
173 (**Fig. 4b, c**), consistent with the large subunit assembly experiment (**Fig. 3a**). The
174 synthesis of sfGFP was unaffected by a monovalent salt concentration above 100 mM
175 (**Fig. 4d**). While high salt concentrations were effective for large subunit assembly (**Fig.
176 3b**), they may have negatively impacted translation reactions. An empirically translation-
177 competent salt concentration of 100-250 mM[36-38] was found to be optimal. The results
178 demonstrated that under near-physiological conditions suitable for translation reactions,
179 a single-step ribosome assembly has proceeded in the solution, where the assembled
180 ribosomes are competent for the whole translation processes, resulting in sfGFP synthesis.
181
182 *EngA and ObgE are responsible for the single-step ribosome assembly process*

We examined which of the selected six factors plays a role in facilitating the single-step ribosome assembly. Since more than one factor could be involved in the process, we addressed the activity of ribosomes assembled using GTPase mixtures with each factor removed one by one. When tested in the coupled reaction system, sfGFP synthesis activity significantly decreased in the absence of ObgE and was not detected in the absence of EngA (**Fig. 5a**). Similar results were obtained in an uncoupled system (**Fig. 5b**), suggesting that EngA and ObgE are the responsible factors which may complement the changes in temperature and ion concentration in the two-step protocol for large subunit assembly. Furthermore, when only ObgE was added to the coupled system, sfGFP synthesis was not detected, similar to the case without any factors, and when only EngA was added, comparatively slow sfGFP synthesis was observed (**Fig. 5c**). When both factors were added, sfGFP synthesis was highly active, indicating that ribosome assembly was achieved with the presence of EngA and ObgE.

Characteristics of the ribosomes assembled using the two factors were analyzed. When comparing the rate of increase in fluorescence of synthesized sfGFP with those of native ribosomes, it was approximately 0.6-fold implying 60% of the input rRNA was assembled into functional ribosomes (**Fig. 6**). SDS-PAGE analysis showed products of the expected size (**Fig. 7a**), and MS analysis allowed assignment of nearly all fragments (**Fig. 7b** and **Supplementary Data 2**), with no apparent issues in fidelity. These results suggest that, although the reconstitution efficiency is around 60%, the ribosomes are reconstituted in a nearly complete form comparable to native ribosomes. In addition to sfGFP, we also targeted dihydrofolate reductase (DHFR) synthesis and confirmed that this protein was produced in an active form (**Fig. 7c**). We note that these results do not indicate that the two factors are necessary and sufficient for ribosome reconstitution. The TP70 used in this study was derived from native ribosomes, and the possibility remains that additional factors are involved. MS analysis of TP70 identified various biogenesis factors, including RsfS, which has been previously reported to function with ObgE in cryo-EM studies[28] (**Supplementary Data 3**). Further studies will be required to elucidate the detailed mechanisms, including the roles of these additional factors.

*EngA and ObgE are required for the reassembly of unfolded ribosomes*

It has been shown that adding EDTA to ribosomes collapses their structure to a level detectable by electron microscopy[30]. The rRNA used in this study was extracted from native ribosomes with phenol, and it is possible that the partial structure, formed depending on magnesium ion, was still retained, potentially reducing ObgE dependency. We added EDTA to native ribosomes to collapse their structure and then restored the

environment by adding magnesium ions. We tested whether ribosome activity would be restored in the presence of ObgE and EngA. The results showed that active ribosomes were formed only in the presence of both factors (**Fig. 8**), suggesting that both ObgE and EngA are equally necessary for reassembling ribosomes.

## Discussion

In the present study, the change in divalent ion concentration was crucial for the translationally active ribosome assembly (**Fig. 1**). In contrast, in the presence of GTPase factors EngA and ObgE, ribosome assembly and translation reactions could be coupled under constant magnesium ion concentrations (8 mM), indicating the significant role of these two factors under near-physiological conditions (**Fig. 2-7**). Although the dependency on ObgE was slightly mitigated in these experiments, both factors were indispensable for the reversible unfolding and regeneration of ribosomes by manipulation of EDTA and magnesium ion concentrations (**Fig. 8**), suggesting that both of two factors are essential in ribosome biogenesis, compensating for the divalent ion concentration and temperature changes in the conventional in vitro assembly conditions. It is possible that some tertiary structures completed by ObgE catalysis might have been retained in phenol-extracted rRNA prepared from native ribosomes.

The two GTPases identified in this study belong to the 13 highly conserved GTPases in bacteria, implying their significant importance for cell viability[39]. GTPases EngB, HflX, and LepA, examined in this study, also belong to the 13 members and have been suggested to be involved in ribosome assembly[33-35], whereas no significant activity was detected in the present study. This does not deny their involvement in ribosome assembly under different assembly conditions, and further verification is necessary. We note that an important finding regarding HflX function, which is different from ribosome assembly pathway, has been found very recently[40]. BipA is not included in the 13 members but is reported as a homolog of EF-G and LepA and reported to be involved in the ribosome assembly process[32], and so it was also tested in this study.

The finding that the two GTPases compensate for the changes in temperature and divalent ion concentration provides new insights into the 50S subunit assembly process. These changes are known to be required for late-stage assembly processes[26] and therefore, it is suggested that the two factors catalyze the late-stage assembly processes. Indeed, cryo-EM based studies have suggested the involvement of these two factors in the late-stage assembly processes[5]. The structures of *E. coli* 50S subunits with EngA[27] and ObgE[29] have been individually elucidated, and structural analysis of 50S subunits in the assembly process obtained by affinity purification with tagged ObgE has been performed[28].

Additionally, in the cryo-EM study on the assembly process of the 50S subunit of *Bacillus subtilis*, a deep relationship with YphC, a homolog of EngA, was demonstrated[33]. These reports suggest the involvement of the two factors in the maturation of critical structures in the late-stage process, including functional core (FC) and peptidyl transferase center (PTC), and the binding of ribosomal proteins involved in the late-stage processes like uL16 and bL36. The present study clarifies the deep relationship between these critical maturation steps in the late-stage processes and the essential operations in the in vitro assembly process identified by Nierhaus and Dohme[25].

In addition to the structural analyses, genetic studies have suggested the involvement of both proteins in the 50S assembly process[41,42]. Interestingly, both proteins have been reported as suppressor proteins of cold-sensitive RlmE (RrmJ) knockout mutants[43]. RlmE is an enzyme that methylates U2552 in domain V of 23S rRNA, where PTC is located. The 50S subunits obtained from the RlmE-knockout mutants show some translational defects and structural differences, particularly in the FC and PTC regions, compared to the wild-type subunits[44]. Since the cell growth of RlmE knockout mutants restores with increased expression of EngA and ObgE[43], the structural differences in ribosomes may also be recovered by these factors. This suggests that one of the roles of these two factors includes the maturation of these regions, and their roles might overlap.

However, the present analysis shows that active ribosomes are not assembled with ObgE alone, and the activity of ribosomes assembled with EngA alone is significantly lower than when both factors are used (**Fig. 5**). Furthermore, the regeneration experiment of ribosome structure by EDTA chelation of magnesium ions and subsequent restoration with magnesium ions revealed that the presence of both factors is essential (**Fig. 8**). This suggests that these two factors individually play different roles in the late-stage assembly process. There has been no structural study focusing on both EngA and ObgE at the same time. According to the individual cryo-EM studies, a C-terminal GTPase domain of ObgE binds to the GTPase-associated center (GAC), and its N-terminal domain binds to the interface region towards the PTC from the A site space[28,29]. In contrast, an N-terminal GTPase domain (GD1) of EngA binds near the L1 stalk, its C-terminal GTPase domain (GD2) binds to the P site space, and its C-terminal KH domain binds to the interface region towards the PTC[27]. The N-terminal domain of ObgE and the C-terminal region of EngA, which is not fully observed with electron density maps, are very close, suggesting that they might not bind to the subunit simultaneously. Moreover, the complex obtained by affinity purification with tagged ObgE shows additional electron density maps corresponding to YjgA and RluD, which may prevent EngA binding[28]. Therefore, ObgE and EngA might independently support the late-stage assembly processes in different

291 steps, suggesting that at least two critical maturation steps in the late-stage assembly
292 processes exist. Further structural analysis will elucidate the details of these processes.
293 Unlike gram-negative bacteria, represented by *E. coli*, gram-positive bacteria,
294 represented by *B. subtilis,* might require a different set of GTPases. Previous reports
295 showed that intermediate 45S or 44.5S subunits accumulate in cells lacking YphC (a
296 homolog of EngA), RbgA, and YsxC (a homolog of EngB)[33,45]. These intermediates lack
297 ribosomal proteins binding in the late-stage processes, such as uL16 and bL36, suggesting
298 stalling before critical maturation steps. Although *B. subtilis* contains a gene annotated as
299 *obgE*, this protein does not appear in a series of studies, while RbgA, which has no
300 corresponding protein in *E. coli*, and YsxC, a homolog of EngB, are essential. This
301 suggests that diversity in support systems for critical maturation steps has arisen during
302 evolution.
303 The cryo-EM structure of 50S subunits during the assembly process, which are
304 affinity-purified with tagged ObgE, suggested the presence of three factors that may work
305 cooperatively with ObgE, including RsfS, YjgA, and RluD[28]. By contrast, the assembly
306 process progresses well only with ObgE and EngA in the present study. Several scenarios
307 can explain these differences. First, it can be considered that the factors identified in the
308 cryo-EM analysis might be supportive but not essential, consistent with the fact that the
309 genes for these factors are not essential for the cell viability. As another possibility, their
310 involvement in rRNA folding can be considered. In the present study, we use rRNA
311 extracted from native ribosomes (**Fig. 1-7**) or unfolded ribosomes with EDTA (**Fig. 8**).
312 The requirement of ObgE is slightly different between these experiments, suggesting
313 different degrees of rRNA folding in both preparations, which might dependent on the
314 presence of magnesium ions. Moreover, it can be considered that secondary structures
315 formed by simple complementary duplex formation are still preserved in both
316 preparations. In contrast, newly transcribed rRNA is produced without any secondary and
317 tertiary structures. We have attempted the 50S subunit assembly with transcribed rRNAs
318 but failed to achieve active subunits with the two factors (data not shown), suggesting
319 further multifaceted mechanisms might be required for the formation of the 50S subunits.
320 They might include rRNA modifications and associated rRNA folding, and the protein
321 group identified by the cryo-EM study may function in such processes. Further in-depth
322 analysis based on the developed assay system will clarify the overall assembly process of
323 the 50S subunits.
324 The present study reveals that the process corresponding to the changes in temperature
325 and magnesium concentration, identified by Nierhaus and Dohme[25], is catalyzed by two
326 GTPases. In reactions using cell extracts like iSAT[11], the presence of these two factors in

the extracts may have facilitated the ribosome assembly without any changes in temperature and ion concentration. While improvement of the iSAT is extensively performed[46-48], the addition of two factors identified in this study may contribute to such studies. The two GTPases may also be one of the key factors for the coupling systems using cell-free systems for expressing ribosomal proteins[18,20], as well as the future developments towards self-replicating synthetic cells[9,10]. At the same time, investigation of the mechanisms of actions of the two GTPases, through structural analysis using cryo-EM, protein composition analysis using qMS, and dynamic analysis using imaging techniques will lead to a deeper understanding of ribosome assembly mechanisms.

It should be emphasized that the two factors presented here are not necessary and sufficient factors for the total ribosome assembly. To achieve true bottom-up construction of life represented by creating self-replicating synthetic cells[9,10], it will be necessary to move beyond components derived from native ribosomes, as used in this study, and instead, employ individually prepared synthetic parts. For example, it will be important to integrate approaches such as ribosome reconstitution from transcribed 23S rRNA[22,23] and reconstitution using synthetic ribosomal proteins[14-17], which represents a key future perspective of this study. To accomplish this, continuing efforts using the PURE system-based approaches may help identify additional essential factors, such as rRNA modification enzymes and other biogenesis factors beyond the two factors described here. Through these processes, we also expect to gain a deeper understanding of ribosome assembly mechanisms.

## Methods

**Sample preparation for in vitro experiments.** Native ribosomes and the PURE system components were prepared as described previously[49]. Preparation of TP70 was performed according to the conventional methods[31], as follows: First, 3.5 mL of Buffer 3 (20 mM Hepes-KOH, pH 7.6, 4 mM $MgCl_2$, 30 mM $NH_4Cl$, 2 mM spermidine, 0.2 mM spermine, 7 mM 2-mercaptoethanol) was added to 1 mL of 24 μM ribosome solution, followed by the addition of 450 μL (0.1 volume) of 1 M $Mg(OAc)_2$. Then, 10 mL of glacial acetic acid was added and the resultant solution was stirred at 4°C for 45 minutes. The solution was centrifugated at 10,000 *g* for 30 minutes and the supernatant was recovered. Five volumes of ice-cold acetone (e.g., 75 mL to ~14.95 mL of solution) was added and the solution was kept at -30°C overnight. The solution was centrifuged at 10,000 *g* for 30 minutes, and then the pellet was vacuum-dried and dissolved in 2 mL of Buffer 5 (20 mM Tris-HCl, pH 7.6, 4 mM $Mg(OAc)_2$, 400 mM $NH_4Cl$, 6 M urea, 0.2 mM EDTA, 7 mM 2-mercaptoethanol). The solution was dialyzed overnight against 500 mL of Buffer 5 using

363 3,500 MWCO dialysis membrane. It was further dialyzed against 500 mL of Buffer 4 (20
364 mM Tris-HCl, pH 7.6, 4 mM $Mg(OAc)_2$, 400 mM $NH_4Cl$, 0.2 mM EDTA, 7 mM 2-
365 mercaptoethanol) for 45 minutes, three times, using the same membrane. Finally, the
366 concentration was measured and stored in small aliquots at -80°C. Total ribosomal RNAs
367 containing 5S, 16S, and 23S rRNAs Ribosomal RNAs were prepared from the native
368 ribosomes via water-saturated phenol extraction followed by 2-propanol precipitation,
369 according to the same literature[31]. Ribosome biogenesis factors used in this study were
370 prepared as follows. *E. coli* genes for BipA, EngB, HflX, and LepA were cloned into
371 pET15b (Merck Millipore, USA) as His-tag fusion protein. The genes for EngA and ObgE
372 were cloned into pET15b (Merck Millipore, USA) as small ubiquitin-like modifier
373 (SUMO) protein-fusion proteins where His-tag, SUMO protein, and EngA or ObgE were
374 tandemly arranged. Detailed sequences are described in the supplementary data
375 (**Supplementary Data 1**). BipA, EngB, HflX, and LepA proteins were purified in a same
376 way as His-tagged translation factors[49]. EngA and ObgE were purified in a same way as
377 uS2 according to our previous study[15]. DNA templates for the sfGFP synthesis were
378 prepared as previously described[16].
379
380 **Ribosome assembly with a two-step procedure.** Reaction mixtures (25 μL) contained
381 20 mM Tris-HCl, pH 7.4, 4 mM $Mg(OAc)_2$, 400 mM $NH_4Cl$, 7 mM 2-mercaptoethanol,
382 22.5 pmol total rRNAs, and 27 pmol TP70. Reactions were carried out at 44 °C for 20
383 min and then 1 μL of 400 mM $Mg(OAc)_2$ solution or water was added to change the
384 magnesium ion concentration. The mixtures were further incubated at 44 °C or 50 °C for
385 90 min. The mixtures were centrifugated at 5,000 *g* for 5 min at 25 °C and then
386 supernatants were recovered. The assembled ribosomes were concentrated and buffer
387 exchanged using 0.5 mL centrifugal filter units MWCO 30 kDa with ribosome buffer (10
388 mM Hepes-KOH, pH 7.6, 10 mM $Mg(OAc)_2$, 30 mM K(OAc), and 1 mM DTT). The
389 activity of ribosomes was monitored in the home-made PURE system[49], where reaction
390 mixtures (20 μL) containing the assembled ribosomes from 25 μL mixtures and 5 nM
391 DNA template encoding sfGFP were incubated at 37 °C in Mx3005P (Agilent
392 Technologies, USA), and sfGFP fluorescence was monitored during incubation.
393
394 **Ribosome assembly with ribosome biogenesis factors.** The assembly reactions were
395 performed based on the PURE system reaction mixtures[49], in anticipation of future
396 integration with a cell-free translation system. The reaction mixtures (25 μL) contained
397 50 mM Hepes-KOH, pH7.6, 2 mM spermidine, 1 mM DTT, 2 mM ATP, 2 mM GTP, 1
398 mM UTP, 1 mM CTP, 10 mM creatine phosphate, 0.1 mM each 20 amino acids, 0.25 μg

399 each of creatine kinase, nucleotide diphosphate kinase, BipA, EngA, EngB, HflX, LepA,
400 and ObgE, varying concentrations of $Mg(OAc)_2$ and potassium glutamate, 22.5 pmol total
401 rRNAs, and 27 pmol TP70. The mixtures were incubated at 37 °C for 110 min and they
402 were centrifugated at 5,000 *g* for 5 min at 25 °C, followed by the recovery of supernatants.
403 The assembled ribosomes were concentrated and buffer exchanged using 0.5 mL
404 centrifugal filter units MWCO 30 kDa with ribosome buffer. The activity of ribosomes
405 was monitored in the home-made PURE system[49], where reaction mixtures (20 μL)
406 containing the assembled ribosomes from 25 μL mixtures and 5 nM DNA template
407 encoding sfGFP were incubated at 37 °C in Mx3005P (Agilent Technologies, USA), and
408 sfGFP fluorescence was monitored during incubation.
409
410 **Integration of ribosome assembly and translation systems.** Reaction mixtures (20 μL)
411 were based on the home-made PURE system[49] without ribosomes. In addition to the
412 standard components, 0.25 μg each of BipA, EngA, EngB, HflX, LepA, and ObgE, 22.5
413 pmol total rRNAs, and 27 pmol TP70 were added in to the mixtures. Concentrations of
414 $Mg(OAc)_2$ and potassium glutamate are varied according to the experiments and
415 designated in the figure legends. After the addition of 5 nM DNA template encoding
416 sfGFP, the mixtures were incubated at 37 °C in Mx3005P (Agilent Technologies, USA),
417 and sfGFP fluorescence was monitored during incubation.
418
419 **Characterization of the translation products.** To compare the protein synthesis
420 efficiency of reconstituted ribosome, 10 μl reactions with varying concentrations of native
421 ribosome (1 μM, 0.5 μM, 0.25 μM, 0.125 μM, 0.0625 μM and 0.03125 μM) and 10 μl
422 ribosome reconstitution reaction (0.72 μM of TP70 with 0.6 μM total rRNAs) were
423 prepared. Reactions were carried out at 37 °C for in Mx3005P (Agilent Technologies,
424 USA) and sfGFP fluorescence was monitored during incubation. For the SDS-PAGE
425 analysis and mass spectrometry analysis of the synthesized sfGFP, Strep-tag II was fused
426 at the N-terminus of sfGFP for the purification[50]. Tagged sfGFP was purified using
427 MagStrep-type 2HC beads (IBA Lifesciences, Germany) according to the manufacturer's
428 protocol. Elution was performed with the solution containing 2.5 mM D-desthiobiotin and
429 analyzed. SDS-PAGE gel was stained with SYPRO Ruby Protein Gel Stain (Thermo
430 scientfific, USA) according to the manufacturer's instructions. The activity of synthesized
431 DHFR was measured as follows: DHFR assay reaction mixtures (1 mL) contained 50 mM
432 MES-KOH, pH 7.0, 25 mM Tris-HCl, pH 7.0, 25 mM ethanolamine, 100 mM NaCl,
433 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 100 μM dihydrofolic acid, and a 5 μL aliquot
434 of the PURE reaction mixture, and they were incubated at 37°C for 15 min. Next, 20 mM

435 NADPH was added to a final concentration of 200 μM. Reaction was aliquoted to a
436 volume of 300 μl on the 96-well titer plate and the decrease in absorbance at 340 nm was
437 measured at 37 °C with Multiskan Go (Thermo scientific, USA).
438
439 **Mass spectrometry analysis.** The proteins were reduced with 10 mM TCEP at 37 °C for
440 30 min, alkylated with 20 mM iodoacetamide at 37 °C for 30 min, and quenched with 20
441 mM L-cysteine. Protein digestion was performed by adding 100 ng of Trypsin/Lys-C mix
442 (Promega, USA) and incubating overnight at 37 °C. Detergents were precipitated by
443 adding a final concentration of 0.5% TFA, after which the mixture was centrifuged at
444 15,000 *g* for 5 min. The resulting supernatant was purified using GL-Tip SDB (GL
445 Sciences) according to the manufacturer's instructions and then dried under reduced
446 pressure. The dried peptides were dissolved in 20 μL of 0.1% TFA, and 4 μL of the sample
447 was analyzed by LC-MS. The peptides were concentrated and separated using a nano-LC
448 system (UltiMate 3000, Thermo Scientific, Germany) equipped with a trap column (C18,
449 0.075 × 20 mm, 3 μm, Acclaim PepMap 100, Thermo Scientific) and a nanocapillary
450 analytical column (C18, 0.075 × 150 mm, 3 μm, Nikkyo Technos, Tokyo, Japan) at a flow
451 rate of 200 nL/min. Mobile phases A (0.1% formic acid) and B (acetonitrile and 0.1%
452 formic acid) were combined in a gradient as follows: 5% B for 5 min, 5–40% B for 75 min,
453 40–90% B for 1 min, and 90% B for 4 min. MS analysis was performed using an Orbitrap
454 mass spectrometer (Q Exactive, Thermo Scientific) equipped with a nanospray ion source
455 (Nanospray Flex, Thermo Scientific) with the following parameters: spray voltage of
456 2.2 kV, positive mode, scan range of m/z 310–2000, and 70,000 resolution. The ten most
457 intense multiply charged ions (z = 2–4) were fragmented in the collision cell by collision-
458 induced dissociation (CID). Raw data was processed using Proteome Discoverer 3.2
459 (Thermo Scientific).
460
461 **Reassembly of unfolded ribosomes.** First, 0.5 μL of 5 μM ribosome (0.5 pmol) was
462 mixed with 1 μL of 100 mM EDTA (final concentration was 67 mM) and incubated on
463 ice for 30 min. Then, PURE translation mixtures were prepared. In this experiment, we
464 used solution II (Enzyme Mix) of PURExpress 2.0 (GeneFrontier Corporation) instead of
465 using home-made translation factors and enzymes, whereas other components were based
466 on the home-made PURE system[49]. In addition to the standard components, the mixtures
467 (10 μL) contained 1.5 μL ribosome solution (0.5 pmol in 67 mM EDTA), 0.5 μL of
468 solution II of PURExpress 2.0, 0.2 μg each of EngA and ObgE. The concentration of
469 potassium glutamate was 150 mM and the concentration of $Mg(OAc)_2$ was 32 mM to
470 restore the actual magnesium ion concentration. After the addition of 5 nM DNA template

encoding sfGFP, the mixtures were incubated at 37 °C in Mx3005P (Agilent Technologies, USA), and sfGFP fluorescence was monitored during incubation.

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**Author Contributions**

Y.Shimizu. designed the study. A.S., W.Y.L., Y.Sakai. and Y.Shimizu. wrote the manuscript. A.S., W.Y.L., Y.Sakai, and K.M. performed the experiments. All authors discussed the results and commented on the manuscript.

**Competing interests**

The authors declare no competing interests.

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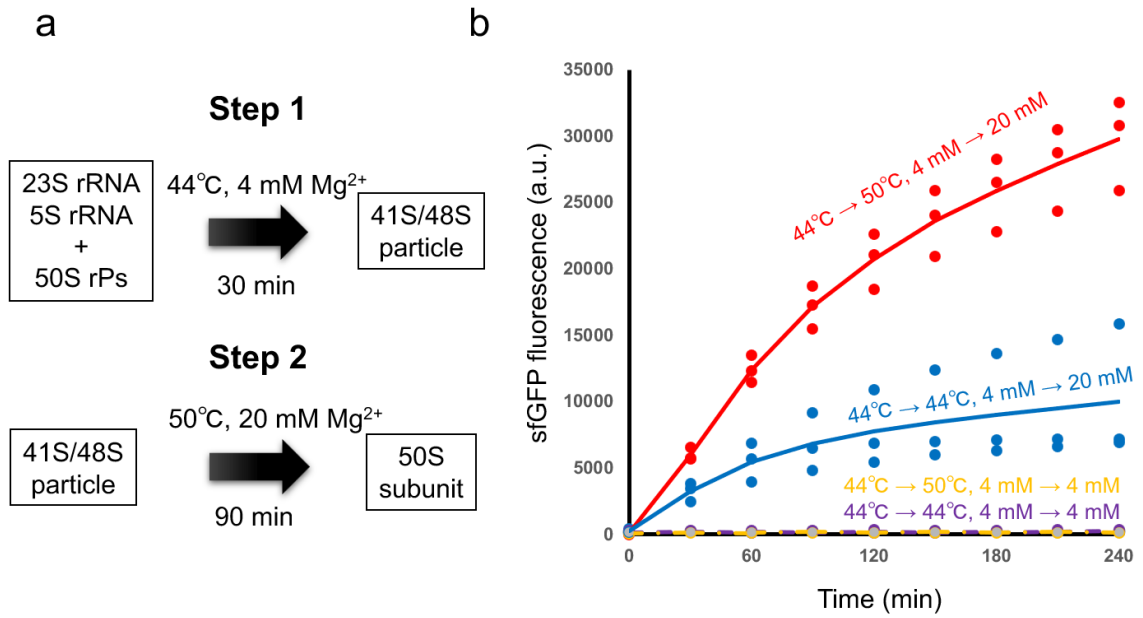
615

616 **Figures**

617

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619

620 **Figure 1. Ribosome assembly with two-step procedure.** (a) Schematic of the two-step
621 procedure identified by Nierhaus and Dohme. In the step 1, comparatively low
622 magnesium ion concentration and low incubation temperature are required to assemble
623 the intermediate state of the 50S subunit (41S/48S particle), whereas in the step 2,
624 increasing the incubation temperature and magnesium ion concentration are essential for
625 the assembly of translationally active 50S subunit. (b) Individual effect of temperature
626 and magnesium concentration changes on the activity of assembled ribosomes.

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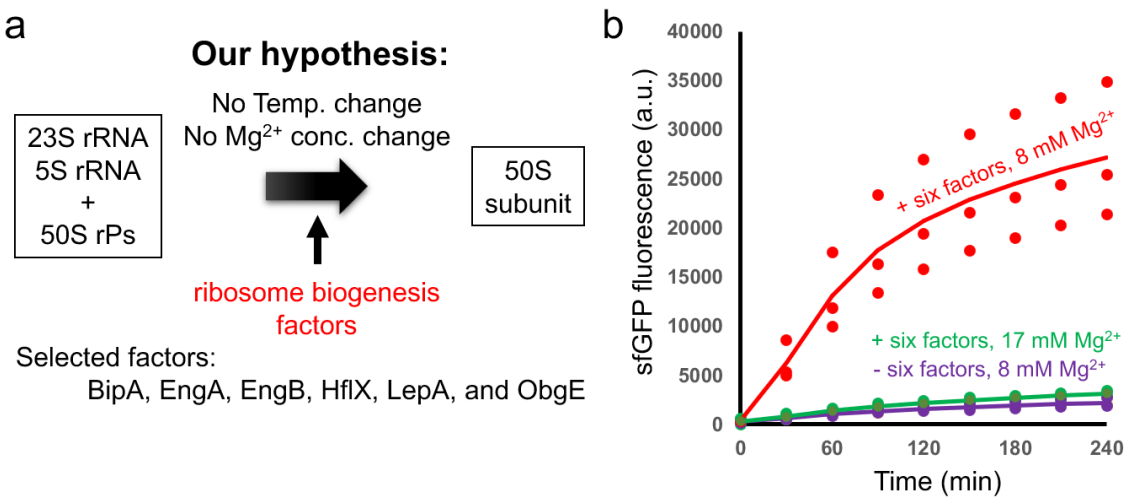


**Figure 2. Ribosome assembly with ribosome biogenesis factors.** (a) Working hypothesis of this study. We hypothesized that some of the ribosome biogenesis factors take over the role of temperature and magnesium concentration changes in the two-step procedure. We selected six GTPase factors as candidates of these factors. (b) Single-step ribosome assembly by selected ribosome biogenesis factors. Activity of assembled ribosomes in the presence or absence of six factors at 8 or 17 mM magnesium ion concentration at 37 °C. Concentration of potassium glutamate for the ribosome assembly experiment was fixed at 200 mM.

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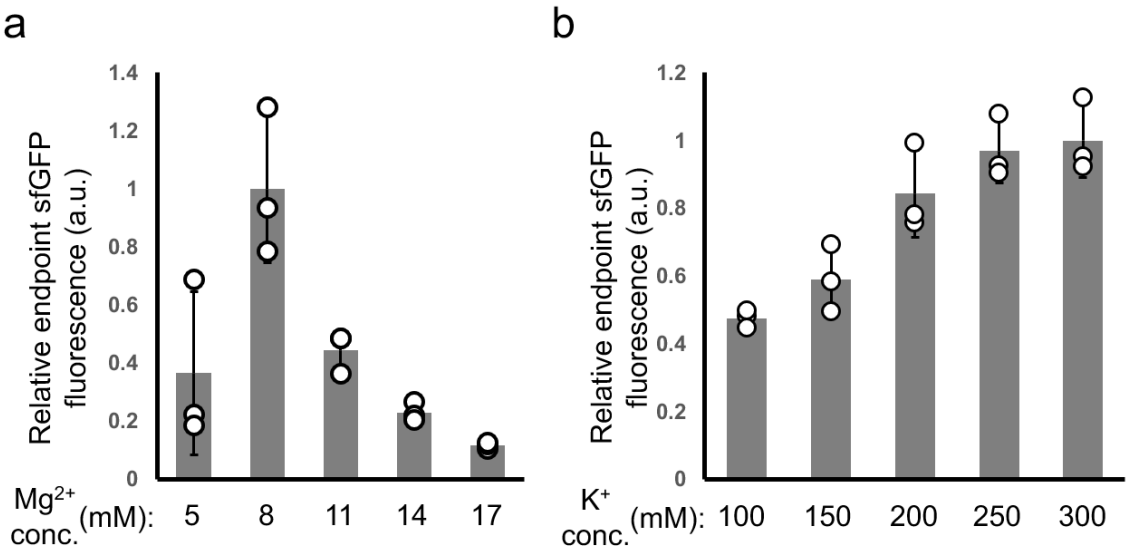


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643

644 **Figure 3. Effect of magnesium and potassium concentrations on the single-step**
645 **ribosome assembly using six GTPase factors.** (a) Effect of magnesium concentration
646 on the assembled ribosome activity. (b) Effect of potassium concentration on the
647 assembled ribosome activity. Relative endpoint sfGFP fluorescence at 4 h timepoint,
648 compared to 8 mM magnesium or 300 mM potassium, respectively, is shown. Potassium
649 concentration was fixed at 200 mM (a) and magnesium concentration was fixed at 8 mM
650 (b).

651

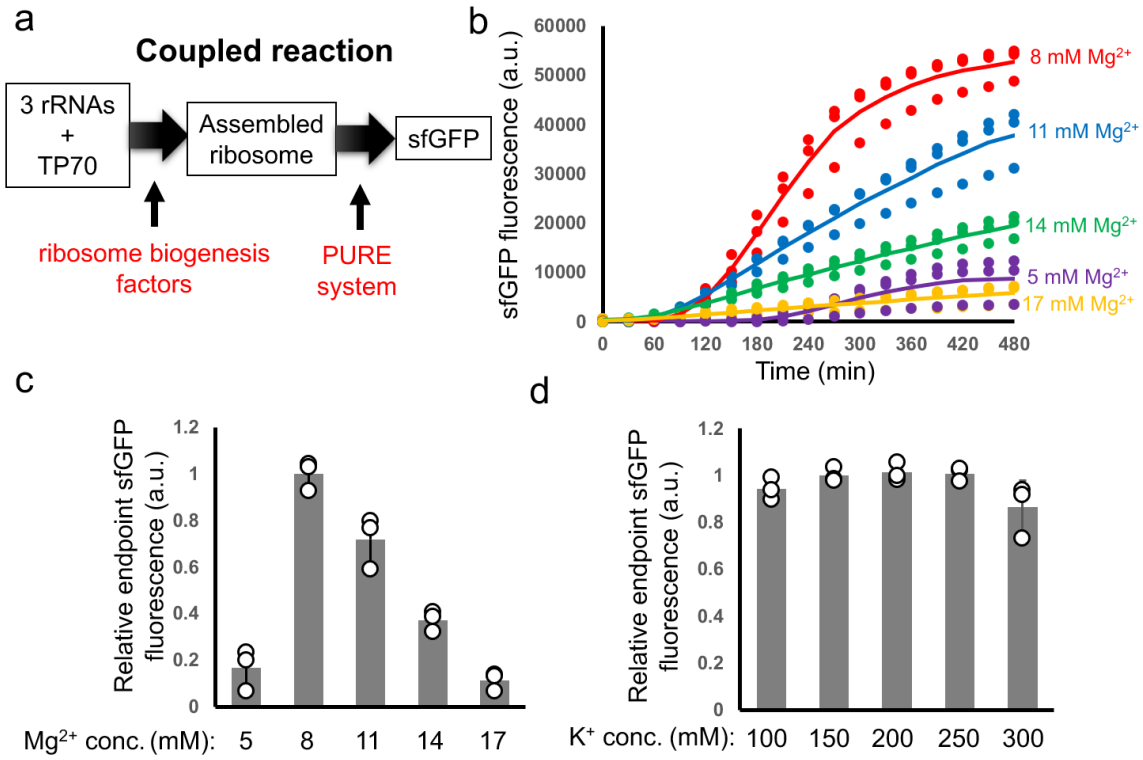


**Figure 4. Coupling of the assembly and translation reactions.** (a) Schematic of the coupled reaction. Three rRNAs (5S, 16S, and 23S) and TP70 are added into the ribosome-free PURE system. Ribosomes are assembled in the PURE system with a help of ribosome biogenesis factors and then, assembled ribosomes exhibit the translation activity, which can be detected as sfGFP fluorescence. (b) Time course of sfGFP fluorescence in the coupled reaction system. Effect of magnesium concentration is shown. (c) Effect of magnesium concentration on the assembled ribosome activity. (d) Effect of potassium concentration on the assembled ribosome activity. Relative endpoint sfGFP fluorescence at 8 h timepoint, compared to 8 mM magnesium or 150 mM potassium, respectively, is shown. Potassium concentration was fixed at 150 mM (b, c) and magnesium concentration was fixed at 8 mM (d).

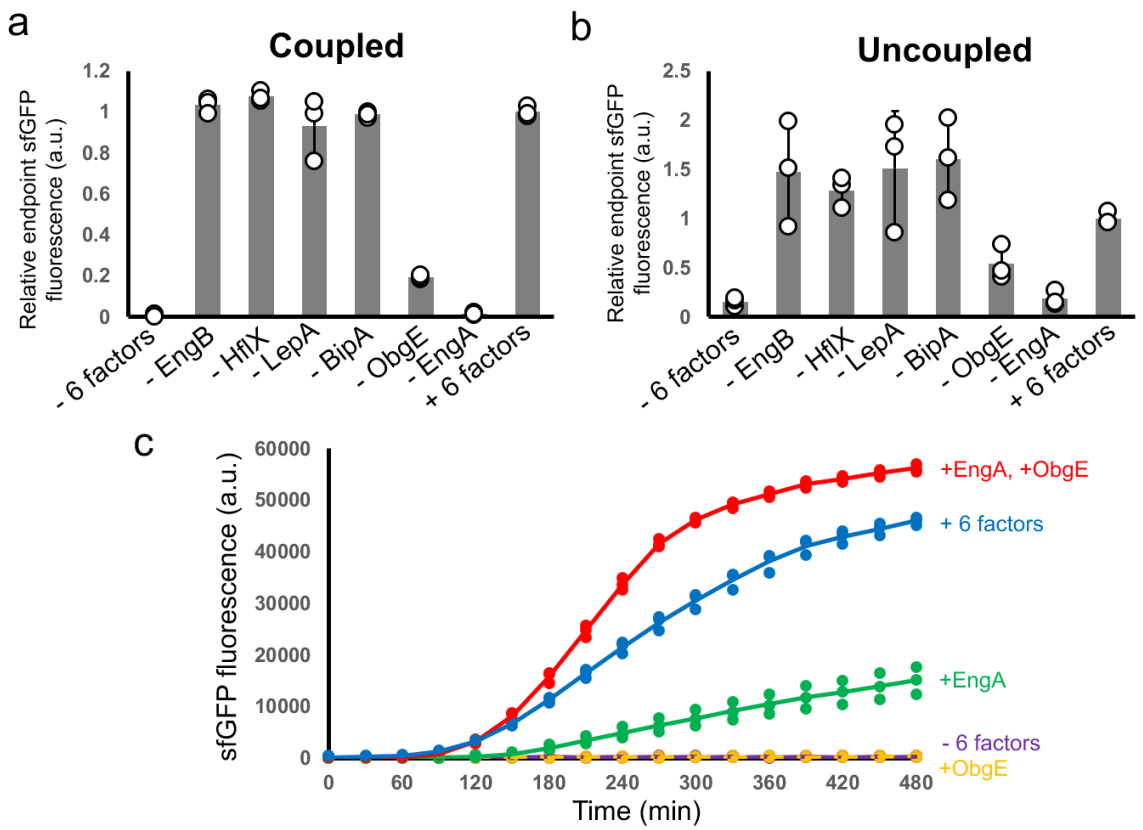


**Figure 5. Identification of the responsible factors.** (a) Ribosome activities assembled in the absence of each factor in the coupled system. (b) Ribosome activities assembled in the absence of each factor in the uncoupled system. (c) Ribosome activities assembled with only EngA and ObgE. When coupled system is applied, magnesium concentration was fixed at 8 mM and potassium concentration was fixed at 150 mM (a, c). When uncoupled system was used, magnesium concentration was fixed at 8 mM and potassium concentration was fixed at 250 mM for the ribosome assembly reaction (b). Relative endpoint sfGFP fluorescence at 8 h timepoint, compared to that using all six factors (a, b) or time course of sfGFP fluorescence (c) are shown.

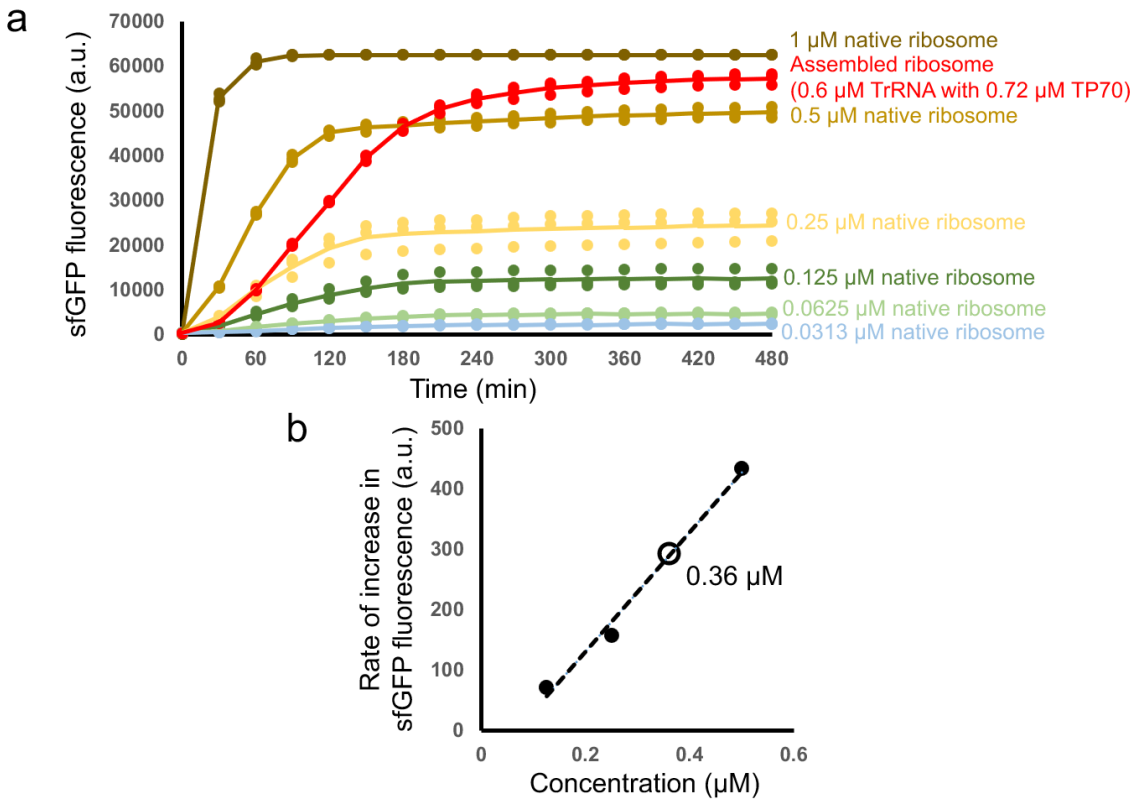


**Figure 6. Rate of increase in fluorescence of synthesized sfGFP.** (a) sfGFP synthesis was measured in the coupled system with varying concentrations of native ribosome. (b) The estimated concentration of assembled ribosomes was calculated from the native ribosome data. Black dots represent data of the native ribosome and a white dot represent the data of the assembled ribosome.

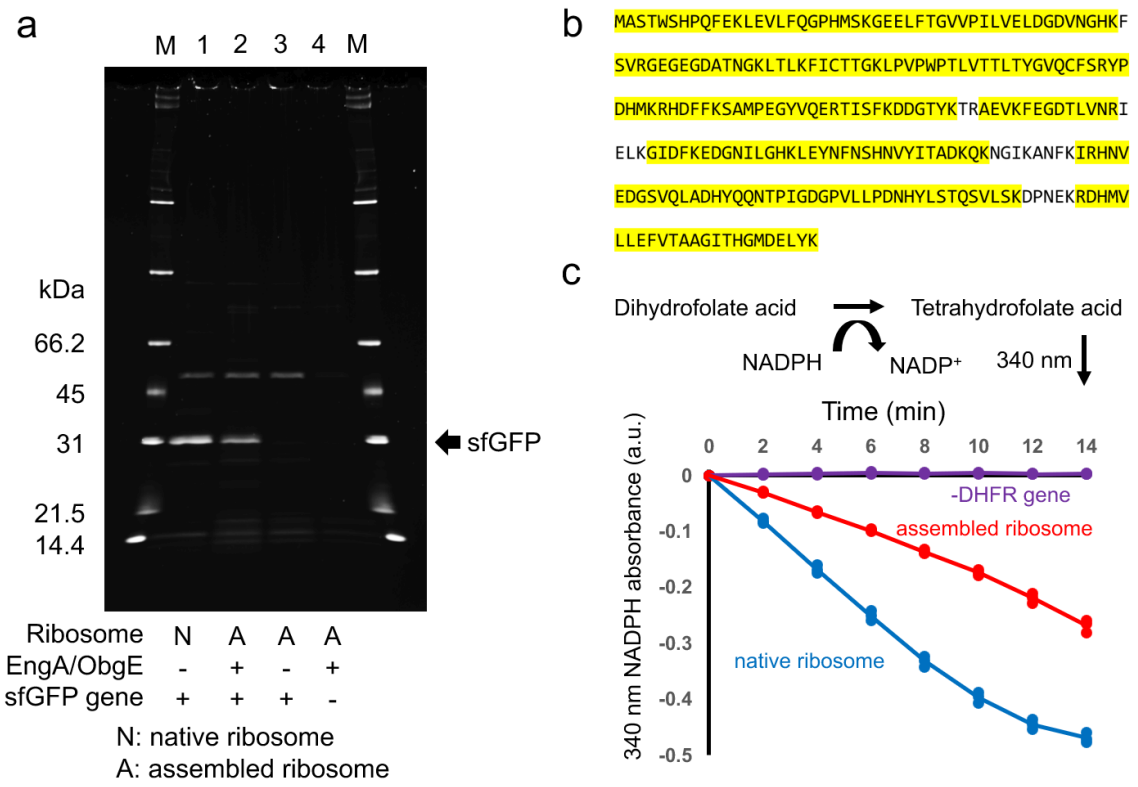


**Figure 7. Characterisitics of the synthesized proteins using the assembled ribosome.** (a) SDS-PAGE analysis of purified sfGFP. sfGFP synthesis was performed with native ribosome (lane 1) and assembled ribosome (lane 2-4). sfGFP was not synthesized in the absence of EngA and ObgE (lane 3) and in the absence of sfGFP gene (lane 4). M represents the protein size markers. (b) Mass spectrometry analysis of the purified sfGFP. Amino acid sequence of the synthesized sfGFP is shown and the MS-detected sequences are marked with yellow. (c) Activity measurement of the synthesized DHFR. DHFR catalyzes reduction of dihydrofolate acid into tetrahydrofolate acid, in which the reaction could be monitored through reduction of 340 nm NADPH absorbance. Activities of synthesized DHFR with native ribosome and assembled ribosome was measured.

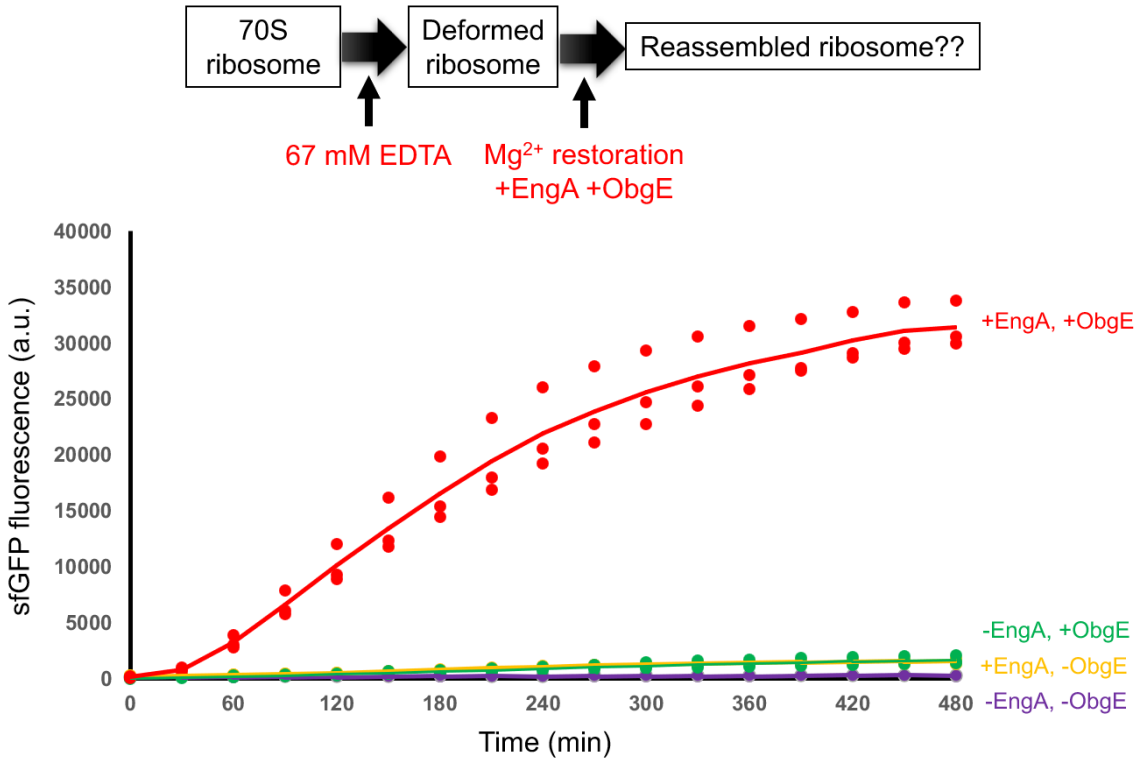


**Figure 8. Reassembly of unfolded ribosomes using EngA and ObgE.** Ribosomes were unfolded by the addition of high concentration of EDTA and then restored the environment by the addition of magnesium ions. Followingly, reassembly of the ribosome was addressed by using EngA and ObgE. Time course of sfGFP fluorescence are shown in the presence or absence of EngA and ObgE..