



# Extended C-terminus and length of the linker connecting the G-domains are species-specific variations in the EngA family of GTPases

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## ABSTRACT

**EngA is an essential protein involved in ribosome biogenesis. It is a unique GTPase, possessing two consecutive G-domains. Using sequence and phylogenetic analysis, we found two intriguing variants among EngA homologues – one with a shorter linker joining the G-domains and another with a longer linker, which additionally possesses an extended C-terminus. Interestingly, while the former variant is mainly restricted to firmicutes, the latter is found in nonfirmicutes. Chimeric proteins with interchanged linkers and extensions were generated to gauge the importance of these elements. Ribosome interaction experiments employing the chimeric proteins suggest that a precise combination of the linker and C-terminal extension are important features regulating EngA ribosome interactions in a variant-specific manner.**

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## 1. Introduction

Ribosome biogenesis is one of the highly regulated and important cellular processes. Several nonribosomal factors play key roles in accomplishing this regulation [1]. Of these factors, GTPases like Era, Obg, YqeH, YlqF, YjeQ, EngA and YsxC were suggested to play important roles [2,3]. We had previously investigated the role of EngA from *Escherichia coli*, which is unique due to the regulation provided by two contiguous G-domains GD1 and GD2 [4]. It was shown to be essential for the maturation of the 50S ribosomal subunit [5]. Cells depleted of YphC, a homologue of EngA in *Bacillus subtilis*, were shown to accumulate 45S (a precursor of the mature 50S subunits) instead of the 50S subunits [6]. Crystal structures of EngA homologues Der from *Thermatoga maritima* and of YphC from *B. subtilis* reveal a common domain architecture, where the RNA binding KH domain which is C-terminal to the G-domains in the primary sequence, is sandwiched between the G domains, GD1 and GD2, in the three-dimensional structure [7]. However, a comparison of these structures reveals a strikingly large conformational change in the position of GD1: While GD1 depicts a movement of ~60 Å, the positions of GD2 and KH are almost unaltered (Supplementary material, Fig. S1) [8]. This difference is associated with the distinct nucleotide bound states of GD1 in the two homologues: In YphC, GD1 is bound to GDP, whereas in Der, it is believed to mimic the GTP bound conformation. Interestingly, the two G-domains of EngA share high sequence conservation, but show distinct nucleotide binding and hydrolyzing activities. GD1 has a higher

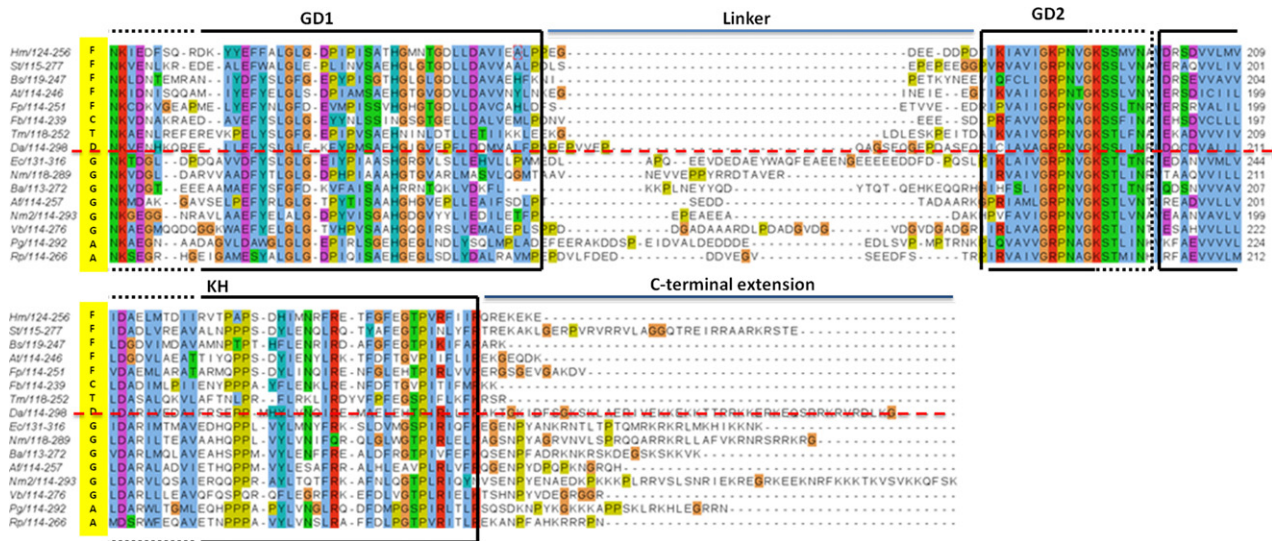
GTP hydrolysis rate but poor affinity for the nucleotide. On the contrary, GD2 possesses very high affinity for the nucleotide but exhibits poor GTP hydrolysis activity [7].

Most GTPases involved in ribosome biogenesis were shown to bind either the 30S or the 50S subunits [2,9,10]. In contrast, we showed two distinct ribosome-bound states for EngA by isolating the activities of the two G-domains. These two states, termed EngA[GD1<sup>GTP</sup>:GD2<sup>GTP</sup>] and EngA[GD1<sup>GDP</sup>:GD2<sup>GTP</sup>], are distinguished by the distinct nucleotides bound at the two G-domains [4]. Here, in the first state when both the G-domains are bound to GTP, EngA associates only with 50S [5,11]. However, in the second state when GD1, following GTP hydrolysis binds GDP (while GD2 continues to be GTP bound), it associates with 30S, 50S and 70S. The importance of GD1 in these, is also brought out by a construct of EngA devoid of GD1 ( $\Delta$ GD1-EngA), which shows a similar association with ribosomes as EngA[GD1<sup>GDP</sup>:GD2<sup>GTP</sup>] [4]. This suggests that the additional binding site for 30S results from an unmasking event triggered by the movement of GD1. This is in agreement with the conformational change in GD1, seen between the structures of Der and YphC [4,8].

In summary, the current studies suggest that nucleotide binding to the G-domains is clearly, a minimal requirement for ribosome association. However, the role of the two distinct ribosome-binding states of EngA in 50S maturation remains unexplored. In this work, based on a careful bioinformatics analysis, we report intriguing variations among the EngA homologues, which seem important in achieving the distinct ribosome-binding states. We find two variants of EngA homologues – one with a longer linker connecting GD1 and GD2, and possessing an extension at the C-terminus; the other lacks this extension and contains a shorter linker. Chimeric derivatives of EngA/YphC

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**Fig. 1.** Multiple sequence alignment of EngA homologues. A multiple sequence alignment of various EngA homologues was created. Here, only representative sequences (listed below) are shown to appreciate the variations in length of the linker connecting GD1 and GD2, and the C-terminal extension. To demarcate domain boundaries, only parts of the domains GD1, GD2 and KH are shown (see dotted lines). High conservation may be noticed within the domains, while significant variations are observed in the linker (see blue bar) and C-terminal regions (see purple bar). The length of the linker varies from 9 to 65 residues whereas an extension at the C-terminus is seen only in some of the homologues. However, irrespective of this variation, the charged character of both the regions is conserved. Among the representative sequences shown, *Escherichia coli* (Ec), *Nitrococcus mobilis* (Nm), *Baumanniac adellincola* (Ba), *Acidithiobacillus ferrooxidans* (Af), *Neisseria meningitidis* (Nm2) and *Verminephro bacterieiseniae* (Vb) belong to gamma-proteobacteria (G), *Phaebacter gallaeciensis* (Pg) and *Rhodopseudomonas palustris* (Rp) belong to alpha-proteobacteria (A). All of these represent homologues from nonfirmicutes, whereas *Helicobacillus mobilis* (Hm), *Symbiobacterium thermophilum* (St), *Bacillus subtilis* (Bs), *Anaerocellum thermophilum* (At) and *Faecalibacterium prausnitzii* (Fp) represents firmicutes (F). *Flavobacteriales bacterium* (Fb), *Thermatoga maritima* (Tm) and *Desulfobacterium autotrophicum* (Da) are from cyanobacteria (C), thermophilic (T) bacterial species and delta-proteobacterium (D), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proteins, with interchanged linkers and C-terminal extensions were generated to probe their significance. Ribosome binding experiments employing these, suggest a likely importance for these variations in enabling nucleotide specific ribosome association of EngA.

## 2. Materials and methods

### 2.1. Sequence alignment and phylogenetic analysis

A multiple sequence alignment of EngA protein sequences, obtained from diverse bacterial species using PSI-BLAST [12], was generated using ClustalX program [13]. Redundancy within the sequences was removed by employing 70% cut-off using CD-HIT program [14]. The sequence alignment shown in Fig. 1 was prepared using Jalview sequence editor [15], which presents 16 representative sequences from a complete dataset of 61 sequences. A phylogenetic tree of EngA homologues was generated using the Neighbor-Joining method [16] in MEGA4 program [17] with 1000 bootstrap replicates [18]. A condensed consensus tree is inferred where branches reproduced in less than 50% bootstrap replicates are collapsed. EngA homologues were clustered into firmicutes, gamma-proteobacteria, delta-proteobacteria, bacteroidetes, alpha-proteobacteria and cyanobacteria subgroups. All positions containing gaps and missing data were eliminated from the dataset using the option 'complete deletion' provided in the program.

### 2.2. Ribosome binding experiments

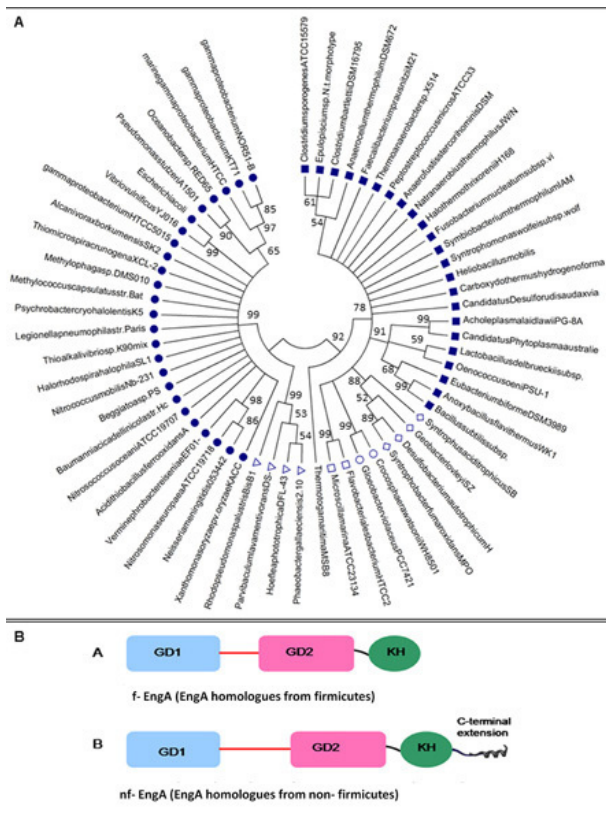
Ribosomes were purified and stored at  $-80^{\circ}\text{C}$ , employing similar protocol as described before [4]. For the binding studies, *E. coli* ribosomes were used with EngA proteins and their chimeric derivatives. Similarly for YphC proteins or its derivatives, *B. subtilis* ribosomes were used. Ribosome co-sedimentation experiments were performed according to protocols established earlier [4]; variations to these and methods for preparing the proteins employed here, are provided in Supplementary material.

## 3. Results

### 3.1. Multiple sequence alignment and phylogenetic analysis

A multiple sequence alignment of EngA proteins was generated as described in Section 2. The complete alignment has been provided (Supplementary material, Fig. S2). An alignment of 16 representative EngA homologues is shown in Fig. 1 for brevity. EngA homologues possess high sequence conservation in the regions spanning the G-domains and KH domain. The domain boundaries for GD1, GD2 and KH in *E. coli* EngA, correspond to residues 19–172, 218–390 and 392–503, respectively. Despite a high overall conservation, significant differences are found at the C-terminal end and the linker joining the G-domains. The length of the linker varies from 9 to 65 residues, whereas the C-terminus extension varies from 22 to 77 residues.

EngA is widely distributed in the bacterial kingdom. To distinguish species-specific features, if any, a phylogenetic tree was generated (Fig. 2A). In this we note that EngA homologues cluster into two distinct groups. Of these, one largely constitutes EngA from firmicutes while another consists of EngA from nonfirmicutes i.e. gamma, and alpha-proteobacteria. EngA from nonfirmicutes contain a long linker (15–65 residues) joining the two G-domains and simultaneously possess an extended C-terminus (22–77 residues). On the other hand, characteristic to EngA from firmicutes is a short linker (<15 residues) and the absence of a C-terminal extension (Fig. 2B). For instance, EngA from the nonfirmicute, *E. coli*, has a long linker of ~38 residues and a C-terminal extension of ~25 residues, while YphC from *B. subtilis* (firmicutes) has a shorter linker of ~20 amino acids and lacks an extended C-terminus (see Ec and Bs in Fig. 1). However, despite the fact that EngA from other bacterial species such as delta-proteobacteria, bacteroidetes and cyanobacteria, form a subgroup with firmicutes, they too display features similar to the nonfirmicute homologues. Given this conserved character, the clustering with firmicutes could be due to a higher similarity within the domains (and not the linker/C-terminal regions). Interestingly, we identify a correlation between the longer

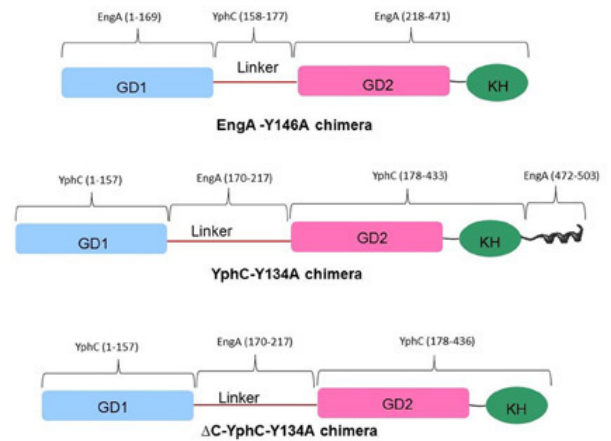


**Fig. 2.** Phylogenetic analysis of EngA proteins. (A) Phylogenetic analyses were conducted using the Neighbor-Joining method [16] in MEGA4 [17]. A bootstrap consensus tree is inferred from 1000 replicates [18] and branches which correspond to the partitions reproduced in less than 50% bootstrap replicates are collapsed. EngA sequences were clustered into firmicutes (■), gamma-proteobacteria (●), delta-proteobacteria (◇), bacteroidetes (○), alpha-proteobacteria (△) and cyanobacteria (□) subgroups. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 399 positions in the final dataset. (B) A schematic diagram representing EngA homologues from firmicutes and nonfirmicutes is shown. These can be classified into two groups, represented as (A) f-EngA and (B) nf-EngA. In nf-EngA, extension at the C-terminus is shown as an alpha helix, based on a secondary structure prediction suggested by JPred [19]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

linker and an extended C-terminus. While the C-terminus comprises basic residues like lysine and arginine, the linker is highly acidic and largely consists of aspartates and glutamates, irrespective of its length. The linker and the C-terminal extension thus possess a distinct charge that appears to be strictly maintained (Fig. 1). Therefore, we suggest that EngA homologues can be divided into two variants and termed f-EngA and nf-EngA signifying the firmicutes and nonfirmicute species that they are restricted to (Fig. 2B).

### 3.2. Ribosome binding assays to decipher the roles of C-terminal extension and linker length.

To address the significance of the aforesaid structural variations observed in f-EngA and nf-EngA, we employed nucleotide-dependent ribosome binding assays. For these, EngA and YphC proteins or their variants representing nf-EngA or f-EngA, respectively, were utilized. Like previously, we employed GST-EngA owing to a better solubility over His-tagged protein [4]. Also, we ensured the GST-tag does not significantly affect GTPase activity of EngA [4]. In contrast, for YphC, the His-tagged protein was found to have a better solubility. Previously, in *in vitro* ribosome binding experiments, where EngA and ribosome was supplied separately as purified components, we found

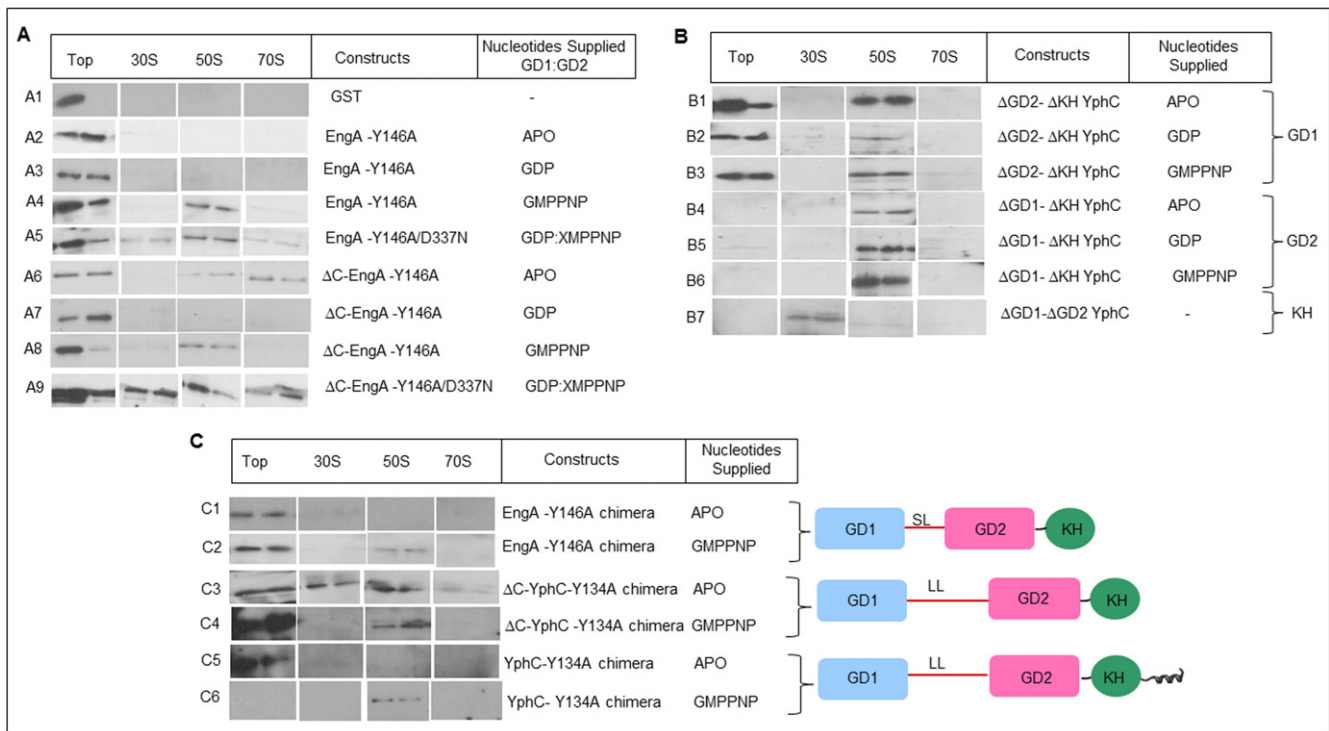


**Fig. 3.** Domain boundaries in the chimeric molecules. To generate the chimeric molecules, domain boundaries were determined by carefully analyzing the sequence alignment of EngA and YphC proteins. The precise domain boundaries with the residue numbers, corresponding to EngA and YphC is shown. An extension at C-terminus is depicted by a helix. EngA-chimera and YphC-chimera molecules mimic f-EngA and nf-EngA, respectively.

that purified EngA remains in a repressed state where it is unable to bind the ribosome [4]. We associated this inhibition to the tight hydrophobic interactions at GD1–KH interface. Further, a mutation Y146A in EngA or Y134A in YphC, at this interface compromises the inhibition and restores ribosome association [4]. The mutant proteins are therefore amicable for *in vitro* studies as they are considered to unfasten the hydrophobic interactions at GD1–KH interface (Supplementary material, Fig. S1) [4]. These mutations are not required if assays were performed using lysates of cells over-expressing EngA. Curiously, cell lysates do not seem to contain any additional factor as the addition of S100 extracts (lysates devoid of ribosomes) could not restore ribosome binding to EngA in *in vitro* assays [4]. It remains to be understood how unfastening the GD1–KH interface is achieved in the cellular environment. For these reasons, *in vitro* ribosome-binding assays reported here, employed mutants EngA–Y146A or YphC–Y134A.

#### 3.2.1. The role of C-terminal extension

We began by evaluating the effect of a C-terminal truncation on ribosome binding. Ribosome-binding assays were performed using full-length EngA or using  $\Delta$ C-EngA – a mutant where the C-terminal extension was truncated. Fig. 4A-A1 shows that a negative control, i.e. the GST tag alone, does not bind ribosomes. EngA–Y146A cofractionates with 50S alone, only when supplied with GMPPNP, the nonhydrolysable GTP analog (Fig. 4A-A4), but not with GDP (Fig. 4A-A3) or in absence of any nucleotides (Fig. 4A-A2). Similarly, the double mutant EngA–Y146A/D337N binds to 30S, 50S and 70S in presence of GDP and XMPPNP (Fig. 4A-A5). These concur with previous findings that D to N mutants of EngA exhibit an altered specificity and utilize xanthine as opposed to guanine nucleotides [4]. Here, the mutation, D337N allows selective binding of GDP to GD1 and XMPPNP to GD2 resulting in EngA [GD1<sup>GDP</sup>:GD2<sup>GTP</sup>] state. When ribosome-binding assays were carried out with the construct  $\Delta$ C-EngA–Y146A lacking the C-terminal 22 residue extension, it bound 50S in presence of GMPPNP (and not with GDP) (Fig. 4A-A8 and A7). Similarly,  $\Delta$ C-EngA–Y146A/D337N bound 30S, 50S and 70S in presence of GDP and XMPPNP (Fig. 4A-A9). While this behavior was similar to the full-length protein,  $\Delta$ C-EngA–Y146A bound ribosomes even in the Apo state, i.e. in the absence of nucleotides (Fig. 4A-A6). This is surprising, considering nucleotide binding by the G-domains is a minimal requirement to realize EngA–ribosome interactions [8,9]. Control nucleotide binding and hydrolysis assays employing the mutants, suggest that the mutation Y146A or truncation of the C-terminus do not affect nucleotide



**Fig. 4.** Appropriate combination of linker length and C-terminal extension is important for EngA-ribosome interactions. Proteins were subjected to ribosome co-fractionation experiments and detected by immunoblotting as described in Section 2. Peak fractions corresponding to 30S, 50S and 70S are shown. (A) A negative control, GST alone, does not bind the ribosome (A1). EngA-Y146A in presence of GMPPNP binds 50S (A4) but not in presence of GDP (A3) or in absence of nucleotides (A2). The double mutant (EngA-Y146A/D337N) binds 30S, 50S and 70S in presence of GDP and XMPPNP (A5). However,  $\Delta$ C-EngA-Y146A, binds ribosome even in the Apo state (A6) while it binds to 50S in presence of GMPPNP (A8), but not with GDP (A7) like the wild type protein. Similarly, it also binds to 30S, 50S and 70S in presence of GDP and XMPPNP (A9). (B) GD1, GD2 or KH domain alone were also subjected to ribosome co-fractionation experiments, in presence of the indicated nucleotides.  $\Delta$ GD2- $\Delta$ KH YphC (i.e. only GD1) or  $\Delta$ GD1- $\Delta$ KH YphC (i.e. only GD2) irrespective of their nucleotide bound states bind to 50S (B1-B6), whereas  $\Delta$ GD1- $\Delta$ GD2 YphC (i.e. only KH) binds to 30S (B7). (C) Importance of linker region and extended C-terminus was tested using chimeric molecules. EngA-Y146A chimera does not bind to ribosome in Apo state (C1) but retains specific ribosome binding to 50S in presence of GTP (C2).  $\Delta$ C-YphC-Y134A chimera loses specificity in binding the ribosome and binds 30S and 50S even in the absence of any nucleotide (C3); whereas, it retains specific binding to 50S in the GTP bound state (C4). When the 'C-terminal extension' of EngA is appended to it, YphC-Y134A chimera, specifically binds 50S only in presence of GTP (C6) and not in the Apo state (C5). A schematic to represent chimeric proteins is also shown where SL and LL denote short linker and long linker, respectively.

binding significantly (Supplementary material, Fig. S3).

### 3.2.2. Importance of the linker region

To comprehend loss of nucleotide-specific ribosome binding by  $\Delta$ C-EngA-Y146A, we examined the role of individual domains in rendering specificity for ribosome binding. Individual domains of YphC were therefore employed. When  $\Delta$ GD2- $\Delta$ KH-YphC (i.e. only GD1) or  $\Delta$ GD1- $\Delta$ KH-YphC (i.e. only GD2) was incubated with crude ribosomes from *B. subtilis*, they interact with the 50S subunit irrespective of their nucleotide bound states (Fig. 4B-B1-B6). On the other hand,  $\Delta$ GD1- $\Delta$ GD2-YphC (i.e. only KH) interacts with 30S (Fig. 4B-B7). These constitute control experiments, as the same constructs of EngA homologue were evaluated in an earlier work [4], which suggests that the specificities of the domains towards ribosomal subunits are not altered in the different homologues. Overall, these experiments suggest that the individual domains, in isolation, show an inherent ability to bind ribosomal subunits in a nucleotide-independent manner. This is unlike the full-length protein where these domains co-exist and ribosome binding is nucleotide dependent. This brings out an inter-domain regulation in the molecule. However, it is intriguing that deleting the C-terminal extension in nf-EngA ( $\Delta$ C-EngA-Y146A) results in the loss of nucleotide specific ribosome binding (Fig. 4A-A6). Based on this, we reasoned that the truncation of the C-terminal extension in nf-EngA might have inappropriately exposed ribosome binding sites and thereby realized ribosome binding even in the Apo state. Given the fact that nf-EngA and f-EngA differ only in the linker region and the C-terminal extension (Figs. 1 and 2B), it might be that

a regulation due to an interaction between these two regions is misplaced upon truncating the C-terminal extension. If this be the case, nonspecific ribosome binding in the Apo state should be abolished upon replacing the long linker in  $\Delta$ C-EngA with a shorter one: such a construct would mimic f-EngA and likely restore nucleotide-specific ribosome binding. To test this hypothesis, a chimeric construct of nf-EngA with a short linker was created. Therefore, the entire linker region of *E. coli* EngA (nf-EngA) was interchanged with that of *B. subtilis* YphC (f-EngA). This was preferred over deletions so that any perturbation to the natural charge distribution could be avoided. This construct was also created in Y146A background for reasons stated above and is referred as EngA-Y146A chimera (Fig. 3). In line with our reasoning, when EngA-Y146A chimera was employed in ribosome binding assays, nonspecific ribosome binding in the Apo state was abolished (Fig. 4C-C1), while GTP-specific binding to 50S was re-established (Fig. 4C-C2).

The importance of the linker and C-terminal extension in the EngA variants was further verified using converse chimeric molecules. In  $\Delta$ C-YphC-Y134A chimera construct, short linker of YphC was replaced with the longer linker of *E. coli* EngA. In terms of the linker, this construct mimics  $\Delta$ C-EngA (nf-EngA) and lacks the C-terminal extension. Here too, we anticipated nonspecific ribosome association in the Apo state. Indeed, a loss of nucleotide-specific ribosome binding was observed as  $\Delta$ C-YphC-Y134A chimera binds 30S and 50S in Apo state (Fig. 4C-C3). However, similar to  $\Delta$ C-EngA (Fig. 4A-A8),  $\Delta$ C-YphC-Y134A chimera also restores specific binding to 50S in the GTP bound state (Fig. 4C-C4). Importantly, when the C-terminal extension of *E. coli* EngA is appended to this construct to create YphC-Y134A

chimera (that mimics full-length EngA, Fig. 3), we observe that non-specific binding to ribosome could be abolished in the Apo state (Fig. 4C–C5). Also, similar to the other constructs, this too bound 50S in presence of GTP (Fig. 4C–C6). GTP hydrolysis assays show that all the chimeric proteins hydrolyze GTP (Supplementary Fig. S4). Overall, this study suggests that appropriate combination of the linker and C-terminal extension are important for determining specificity in EngA–ribosome interactions.

#### 4. Discussion

EngA homologues show high sequence conservation within the domains, GD1, GD2 and KH. Variations are observed only in the linker region or at the C-terminus (Fig. 1). EngA homologues from the non-firmicutes are longer (~500 residues) and have insertions in the linker (longer linker) together with an extension at the C-terminus, whereas EngA from firmicute species are smaller (~450 residues) and have a smaller linker without the C-terminal extension (Figs. 1 and 2B). The significance of these variations in EngA homologues was unknown. Our attempts to address their role lead us to recognize an intriguing correlation between the length of the linker and the extension at the C-terminus. These features appear unique to the two variants nf-EngA and f-EngA; and a correct combination of these appear critical for their function. Interestingly, secondary structure prediction using JPred suggests that the C-terminal extension in nf-EngA homologues would form an alpha helix, unlike in several proteins where such extensions are typically loops or unstructured regions (indicated in Fig. 2B). The C-terminal extension in nf-EngA indeed appears to render an important structural regulation, as inferred from nonspecific ribosome binding by  $\Delta$ C-EngA construct in the Apo state (Fig. 4A). Furthermore, the C-terminal extension in nf-EngA appears to stabilize the long linker, as suggested by ribosome binding assays employing chimeric proteins, in which the linker and C-terminal extension were swapped between EngA (nf-EngA) and YphC (f-EngA) proteins. It was possible to restore nucleotide-dependent ribosome binding, only when appropriate combinations of linker and C-terminal extension were provided (Fig. 4C). Taken together with the fact that isolated domains of EngA do not need nucleotides to bind ribosome (Fig. 4B), the following model for EngA–ribosome interaction may be proposed. Overall, it appears that ribosome binding sites in full length EngA are likely present at domain interfaces. Upon nucleotide binding at the G-domains, inter-domain interactions are unfastened, which then exposes these sites for ribosome binding [4]. Such a model would explain how truncating the C-terminus extension in nf-EngA results in 'misregulated' ribosome binding in the absence of nucleotides. It is possible to conceive an intricate regulation between the long linker and C-terminal extension, which is necessary to maintain a conformation that precludes ribosome binding in absence of nucleotides. A correct combination of the two is necessary to ascertain this regulation in distinct homologues of EngA. Since ribosome biogenesis is a critical, highly conserved and regulated process in all three kingdoms of life, perhaps such variations fine-tune ribosome assembly in a species-specific manner. It would not be surprising if such variations were found in other ribosome assembly factors too. Appreciating

such variations could provide a comprehensive understanding of this process.

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#### Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fob.2012.07.009.

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