Protein synthesis during cellular quiescence is inhibited by phosphorylation of a translational elongation factor

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In nature, most organisms experience conditions that are suboptimal for growth. To survive, cells must fine-tune energy-demanding metabolic processes in response to nutrient availability. Here, we describe a novel mechanism by which protein synthesis in starved cells is down-regulated by phosphorylation of the universally conserved elongation factor Tu (EF-Tu). Phosphorylation impairs the essential GTPase activity of EF-Tu, thereby preventing its release from the ribosome. As a consequence, phosphorylated EF-Tu has a dominant-negative effect in elongation, resulting in the overall inhibition of protein synthesis. Importantly, this mechanism allows a quick and robust regulation of one of the most abundant cellular proteins. Given that the threonine that serves as the primary site of phosphorylation is conserved in all translational GTPases from bacteria to humans, this mechanism may have important implications for growth-rate control in phylogenetically diverse organisms.

dormancy | sporulation | Ser/Thr kinase | GTPase | EF-Tu

Adaptation to nutrient availability is a fundamental cellular process. From unicellular prokaryotes to complex multicellular organisms, cells sense and adjust their metabolism to respond to variations in nutrient levels. Protein synthesis is one of the most energy-intensive cellular processes, and both the initiation and elongation stages of translation are down-regulated in response to nutrient limitation (1). Proteins that mediate translation, such as the essential and universally conserved GTPase Elongation factor Tu (EF-Tu), are observed in the phosphoproteomes of diverse organisms (2), suggesting that they are subject to regulatory phosphorylation. However, EF-Tu is the most abundant protein in bacteria, present at ∼100,000 copies per cell of growing *Escherichia coli* (3), but less than 10% of the EF-Tu molecules are phosphorylated (4). Thus, a key question is how this relatively small fraction of phosphorylated EF-Tu can cause a down-regulation of protein synthesis.

GTP-bound EF-Tu binds and delivers an aminoacyl-tRNA (aa-tRNA) molecule to the translating ribosome (3). Upon formation of a correctly base-paired mRNA codon–aa-tRNA anticodon interaction, the ribosome activates the GTPase activity of EF-Tu, followed by accommodation of the aa-tRNA into the ribosomal aa-tRNA binding (A) site, and release of the inactive GDP-bound EF-Tu from the ribosome. EF-Tu belongs to the GTPase superfamily which comprises molecular switches that share a core catalytic domain and mechanism but have evolved to perform diverse roles in many cellular processes (5). Central to their function is the hydrolysis of GTP, which controls the switch between the ON, GTP-bound, and the OFF, GDP-bound, states. Hydrolysis of GTP is followed by large conformational changes in two flexible regions known as "switch I" and "switch II." These regions are composed of highly conserved motifs that surround and contact the nucleotide and are involved in interactions with both exchange factors and effectors that regulate GTPase function.

GTPases also can be regulated by direct inhibition of their GTPase activity. For example, *Clostridium difficile* toxin B glycosylation of the small GTPases RhoA, Rac1, and Cdc42 reduces their intrinsic GTPase activity (6). In addition, during infection by the intracellular pathogen *Toxoplasma gondii*, a secreted serine/threonine (Ser/Thr) kinase phosphorylates the host immunity-related GTPases Irgb6 and Irga6 on conserved Thr residues located in the switch I region of the GTP-binding domain, abolishing GTP hydrolysis and inhibiting GTP-dependent oligomerization (7, 8). GTP hydrolysis also is necessary for the delivery of the aa-tRNA and the subsequent release of EF-Tu from the ribosome. Compounds that inhibit hydrolysis, such as nonhydrolysable GTP analogs, stabilize the association of EF-Tu with the ribosome (9). A likely consequence of this stabilization is that subsequent interaction of the ribosome with a new EF-Tu molecule is prevented, and elongation stalls. Thus, if the ability of phosphorylated EF-Tu to hydrolyze GTP were impaired, this phosphorylation could act as a regulatory switch to inhibit translation.

To investigate this question in a physiological context, we took advantage of the Gram-positive bacterium *Bacillus subtilis* that responds to nutrient limitation by forming a metabolically quiescent, highly resistant spore (10). In the onset of this process, called "sporulation," cells undergo an asymmetric division that generates two compartments with very distinct fates (Fig. 1*A*). The smaller compartment, called the "forespore," differentiates into a dormant cell (endospore), and the larger compartment, designated the "mother cell," remains metabolically active. Ultimately, the mother cell lyses and releases the mature dormant spore into the environment. Thus, *B. subtilis* sporulation allows the study of the differential regulation of protein synthesis in two adjacent cells that are genetically identical but experience different metabolic fates.

Here, we describe a novel posttranscriptional regulatory mechanism by which protein synthesis is down-regulated in dormancy. Underlying this mechanism is the reversible phosphorylation of EF-Tu on an absolutely conserved Thr that lies within the switch I

Significance

In nature, cells sense and fine-tune their metabolism in response to nutrient availability. Protein synthesis is one of the most energy-demanding metabolic processes and as such is subject to a tight regulation. A key open question, however, is how the components of the translation machinery, which are among the most abundant cellular proteins, can be regulated quickly and robustly in response to acute nutrient deprivation. We show that starved cells down-regulate protein synthesis by phosphorylation of essential and universally conserved translational GTPase Elongation factor tu (EF-Tu). Importantly, phosphorylated EF-Tu has a dominant-negative effect in elongation, resulting in the overall inhibition of protein synthesis. Thus, this novel regulatory mechanism allows for the quick and efficient regulation of protein synthesis.

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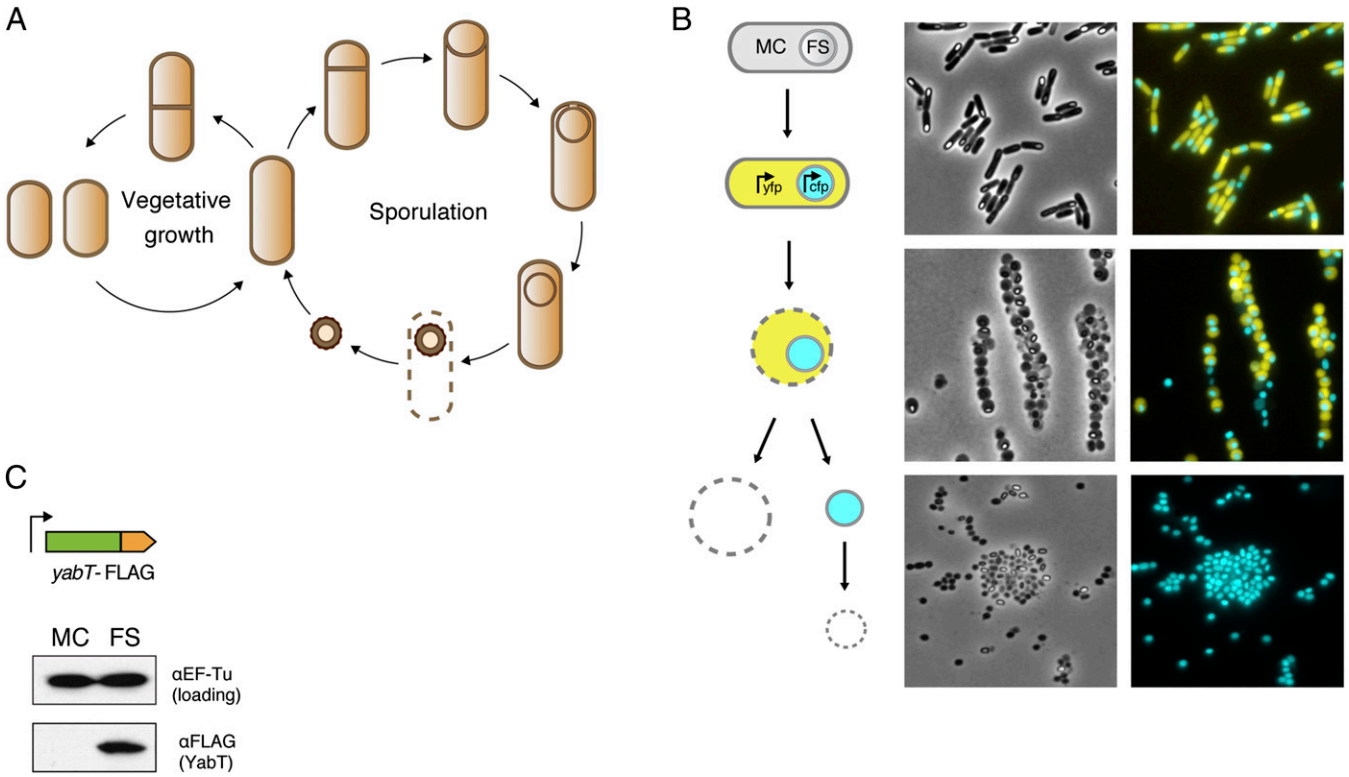

Fig. 1. Expression of the YabT kinase during sporulation. (*A*) The *B. subtilis* cell cycle. Upon nutrient limitation, *B. subtilis* ceases vegetative growth and initiates sporulation. This process starts with an asymmetric division at one pole and produces two cell compartments: the mother cell and the forespore. The mother cell engulfs the forespore and remains metabolically active throughout sporulation, synthesizing the protective layers that surround the forespore. During this process, the forespore becomes metabolically inactive, eventually becoming a fully dormant spore that is released into the environment following lysis of the mother cell. The mature spore can endure extreme stresses and will exit from dormancy and resume vegetative growth in favorable conditions. (*B*) Mother cell/forespore fractionation strategy. Sporulating cells were treated with lysozyme resulting in the formation of protoplasts, which then were lysed, releasing the forespore. Free forespores were separated from mother cell lysates and lysed separately. The fractionation process was monitored by fluorescent microscopy using fluorescent reporters specifically expressed in the mother cell (YFP) and forespore (CFP). (*C*) Expression of YabT in vivo. Mother cell (MC) and forespore (FS) fractions isolated from a sporulating *B. subtilis* strain expressing a FLAG-tagged YabT were analyzed by immunoblotting with a FLAG antibody.

region of the GTP-binding domain (11). This modification is accomplished by a eukaryotic-like Ser/Thr kinase that is expressed selectively during sporulation in the compartment that will become metabolically quiescent. We demonstrate that this phosphorylation stabilizes the interaction between the ribosome and EF-Tu and has a dominant-negative effect in elongation leading to the down-regulation of protein synthesis both in vitro and in vivo.

Results

YabT Ser/Thr Kinase Is Expressed in the Cell Compartment That Becomes Dormant. The *B. subtilis* Ser/Thr kinase YabT is expressed in sporulation (Fig. S1 and ref. 12) under the control of σ^F, a sigma factor that is active in the forespore but not in the mother cell (13). We confirmed that YabT is present only in the forespore by developing a technique to separate the two cells biochemically (Fig. 1*B*). This technique relies on the higher sensitivity to the cell wall hydrolase lysozyme of the mother cell compared with the forespore. Sporulating *B. subtilis* cells were protoplasted by lysozyme treatment and lysed to release the forespore. The mother cell fraction was collected, and free endospores were isolated and subsequently lysed. We monitored the fractionation process using fluorescent reporters specifically expressed in the mother cell (YFP) and forespore (CFP) (Fig. 1*B*), and, to detect YabT, we generated a C-terminal FLAG-tagged YabT fusion under its native promoter (Fig. 1*C*). Lysates prepared from both mother cells and forespores were analyzed by Western blotting with a FLAG antibody, and YabT expression was detected only in the forespore fraction (Fig. 1*C*). This observation is consistent with the involvement of YabT in spore development (14) and suggests a possible regulatory role for this kinase in the progressive metabolic quiescence of the forespore.

Phosphorylation of EF-Tu. *B. subtilis* EF-Tu is phosphorylated in vivo on multiple Ser/Thr residues by an as-yet-unidentified kinase (15, 16). We examined whether *B. subtilis* EF-Tu is an in vitro substrate of YabT by incubating the two proteins in the presence of [^{32}P]ATP and monitoring transphosphorylation using autoradiography. A band with mobility similar to that expected from YabT autophosphorylation (~33 kDa) and a closely spaced doublet with mobility similar to that expected from EF-Tu (~44 kDa) can be seen (Fig. 2*A*). Phosphorylation often causes the production of a doublet, presumably resulting from partial phosphorylation of a multiply-phosphorylated substrate. To confirm the identity of the doublet, the two bands were isolated and analyzed by mass spectrometry. This analysis indicated that both bands corresponded to phosphorylated EF-Tu and identified Thr-63, an absolutely conserved Thr residue within switch I of the GTP-binding domain (11), as the main phosphorylated residue (Fig. S2 *A*–*C*). We confirmed this observation by generating and purifying an EF-Tu–mutant protein in which Thr-63 was replaced by an alanine (Ala) residue (T63A). This protein was still transphosphorylated by YabT, but the upper band of the doublet was no longer observed (Fig. 2*A*). In addition, the mass spectrometry analysis identified three other phosphorylated residues: Ser-11, Thr-300, and Thr-385 (Fig. S2*D*). However, phosphorylation on these residues was identified only in the absence of Thr-63

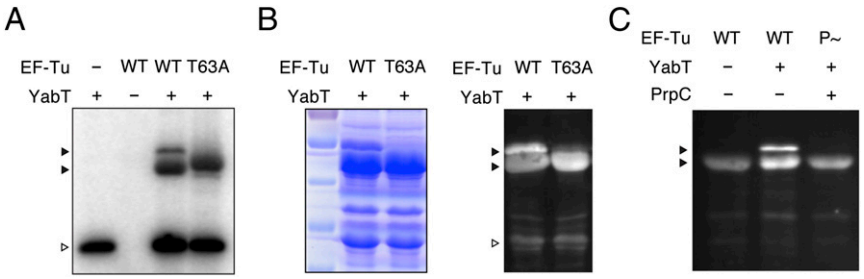

Fig. 2. Reversible phosphorylation in vitro and in vivo of EF-Tu Thr-63. (*A*) Purified wild-type *B. subtilis* EF-Tu or T63A point mutant (T63A) incubated in vitro with YabT kinase in the presence of [γ-^{32}P]ATP. Phosphorylation was assayed by phosphorimager analysis. (*B*, *Left*) SDS/PAGE and Coomassie blue staining of whole-cell lysates of *E. coli* coexpressing *B. subtilis* wild-type EF-Tu or the T63A point mutant (T63A) and YabT kinase. (*Right*) Phosphorylation of EF-Tu was assayed by transferring lysates to a PVDF membrane and staining with Pro-Q Diamond. (*C*) EF-Tu was phosphorylated in the presence of YabT in vitro, the reaction was split, and one half was incubated with the *B. subtilis* Ser/Thr phosphatase PrpC. Phosphorylation was assayed by Pro-Q Diamond staining. Positions of EF-Tu (closed arrowheads) and YabT (open arrowhead) in the gels are indicated.

(i.e., using the T63A EF-Tu mutant), as is consistent with Thr-63 being the primary phosphorylation site.

We further confirmed Thr-63 phosphorylation by constructing an *E. coli* strain that coexpressed *B. subtilis* YabT and EF-Tu. Lysates of this strain were subjected to SDS/PAGE, and phosphorylation was assayed by Pro-Q Diamond phosphoprotein staining. The double-band pattern was observed when YabT was coexpressed with wild-type EF-Tu, but the upper band was no longer present when YabT was coexpressed with the T63A mutant (Fig. 2*B*). Thus, EF-Tu is a bona fide YabT substrate, and Thr-63 is the primary site of phosphorylation.

EF-Tu is an in vitro substrate of Ser/Thr phosphatases in *Listeria monocytogenes* and in *B. subtilis* (17, 18). In the latter study, EF-Tu phosphorylated in vitro by the PrkC kinase was dephosphorylated by its cognate phosphatase PrpC. We determined that EF-Tu phosphorylated by YabT (P~EF-Tu) also was a substrate for PrpC by incubating P~EF-Tu with PrpC and assaying phosphorylation by Pro-Q Diamond staining (Fig. 2*C*). In the presence of PrpC, only the lower EF-Tu band was detected, and phosphorylation levels were close to background (EF-Tu in the absence of YabT). Thus, in vitro, YabT and PrpC can reversibly regulate the phosphorylation of EF-Tu Thr-63.

We investigated if phosphorylation of EF-Tu Thr-63 is restricted to spore-forming bacteria. The Gram-positive bacterium *Mycobacterium tuberculosis* also enters a state of metabolic dormancy, and in vitro phosphorylation of *M. tuberculosis* EF-Tu affects its interaction with GTP (19). Consistently, incubation of *M. tuberculosis* PknA, a homolog of YabT, with EF-Tu resulted in the phosphorylation of the homologous Thr residue (Thr64) (Figs. S2*B* and S3). The homologous Thr residue also is phosphorylated in vivo in *E. coli* (20), further indicating that this modification is phylogenetically conserved.

Phosphorylation of EF-Tu Inhibits GTP Hydrolysis and Translation Elongation. Crystal structures of *Thermus aquaticus* EF-Tu (21) and *Thermus thermophilus* EF-Tu (22) indicate that *B. subtilis* EF-Tu Thr-63 is located within the switch I region of the GTP-binding domain, in close proximity to the nucleotide (Fig. S2*C*). Consistently, substitution of the homologous Thr with an Ala residue results in a dramatic reduction in GTP hydrolysis activity of both *T. thermophilus* and *E. coli* EF-Tu (23, 24). Thus, phosphorylation could affect binding and/or nucleotide hydrolysis. To examine the effect of phosphorylation on GTP binding, we used the differential radial capillary action of ligand assay (DRaCALA), which is based on the ability of dry nitrocellulose to bind and sequester proteins at the application site (25). Protein–ligand complexes bind to nitrocellulose and are separated from unbound free ligand, which diffuses away. We incubated increasing concentrations of EF-Tu or P~EF-Tu with radiolabeled GTP and spotted the reactions on nitrocellulose (Fig. S4). As expected, EF-Tu significantly retarded ^{32}P-GTP diffusion compared with a GTP-binding–deficient (K138E) mutant of EF-Tu (26). Interestingly, incubation of ^{32}P-GTP with P~EF-Tu led to a slight, but not significant, decrease in ^{32}P-GTP migration, indicating that EF-Tu phosphorylation did not substantially affect GTP binding in this assay.

We then determined whether EF-Tu phosphorylation affects GTP hydrolysis using thin-layer chromatography (TLC) to separate radiolabeled GDP and GTP (Fig. 3*A*). Consistent with previous observations (27), ribosomes are required to stimulate EF-Tu–mediated GTP hydrolysis, and virtually no hydrolysis of GTP was detected in their absence. EF-Tu hydrolyzed GTP in the presence of ribosomes in a time-dependent manner, and by 30 min ~60% of the GTP was converted into GDP. The relatively modest levels of GTP hydrolysis observed likely are caused by the absence of mRNA and aa-tRNA in the reactions, which are required for full activation of EF-Tu GTPase activity (27). However, under the same conditions, GTP hydrolysis was reduced significantly in the presence of P~EF-Tu to levels similar to those observed in the absence of ribosomes. In addition, dephosphorylation of P~EF-Tu by PrpC restored GTP hydrolysis to wild-type levels. Taken together, these results indicate that phosphorylation of EF-Tu severely attenuates its GTPase activity.

To characterize further the effect of this modification on EF-Tu activity, we performed in vitro translation reactions using a fully purified, recombinant in vitro translation system (Fig. 3*B*, *Top*) (28). GTP hydrolysis is key to the catalytic cycle of EF-Tu, and when EF-Tu is incubated with 70S ribosomes in the presence of a nonhydrolysable GTP analog GDPNP, elongation is blocked (9). Similarly, the inhibition of GTP hydrolysis by phosphorylation also should block elongation. As expected, a polypeptide is synthesized in the presence of EF-Tu, and after 5-min incubation ~60% of the radiolabeled methionine was found as part of a tripeptide (Fig. 3*B*, *Bottom*). In contrast, P~EF-Tu exhibited a significant decrease in the rate of elongation, particularly noticeable in the levels of tripeptide, which corresponded to only 20% of the radiolabeled Met in the same incubation period (Fig. 3*B*, *Bottom*). We note that, in contrast to the GTP hydrolysis assay reported in Fig. 3*A*, technical challenges associated with phosphoenriching P~EF-Tu in the quantities required for this experiment prevented us from testing whether dephosphorylation of P~EF-Tu by PrpC could reverse the inhibitory effect that phosphorylation of EF-Tu has on the rate of elongation. Nonetheless, the observation that dephosphorylation of P~EF-Tu by PrpC reverses the inhibitory effect on GTP hydrolysis (Fig. 3*A*) strongly suggests that it likewise would reverse the inhibitory effect of phosphorylating EF-Tu on the rate of translation elongation.

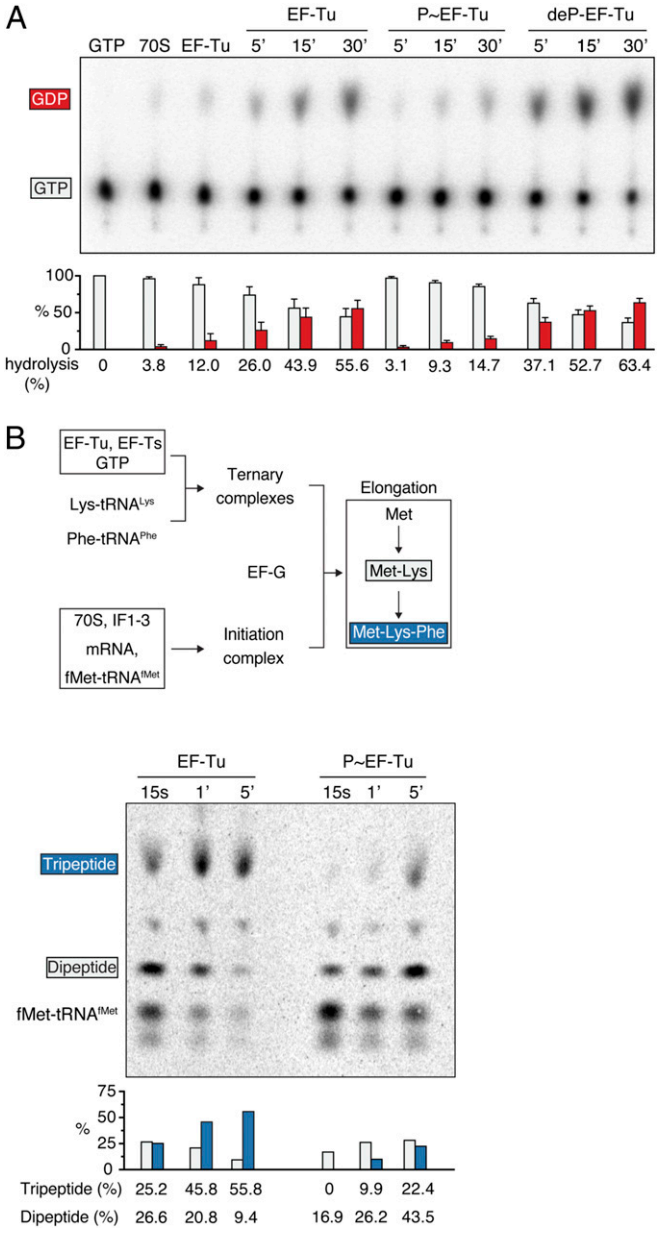

Fig. 3. Phosphorylation inhibits EF-Tu GTP hydrolysis activity and translation elongation in vitro. (*A*) EF-Tu GTP hydrolysis assay. Nonphosphorylated, phosphorylated, or dephosphorylated EF-Tu was incubated with [α-^{32}P]GTP in the presence of 70S ribosomes. Samples were collected at different time points, and GTP was separated from GDP by TLC. GTP hydrolysis was measured by phosphorimager analysis and is shown below a representative autoradiogram. Control reactions in which [α-^{32}P]GTP was incubated by itself or in the presence of either ribosomes or EF-Tu were carried out for 30 min. (*B*) Protein elongation assay. Nonphosphorylated or phosphorylated EF-Tu was incubated with EF-Ts and Lys-tRNA^{Lys} or Phe-tRNA^{Phe} in the presence of GTP. The resulting ternary complexes then were incubated with 70S ribosomes preloaded with a RNA message and f-[^{35}S]Met-tRNA^{fMet} in the presence of EF-G. Samples were collected at different time points, and TLC was used to separate the different peptide species. The percentage of radiolabeled Met incorporated into a dipeptide or a tripeptide was determined by phosphorimager analysis as above. Error bars indicate the SD for at least three independent experiments.

Phosphorylation Stabilizes the Interaction Between EF-Tu and the Ribosome. The antibiotic kirromycin prevents the conformational changes in EF-Tu that follow GTP hydrolysis, thereby blocking EF-Tu release from the ribosome (29). To determine if the inhibition of GTP hydrolysis by phosphorylation has a similar effect, we assayed the interaction between EF-Tu (nonphosphorylated, phosphorylated, or dephosphorylated) and 70S ribosomes in the presence of GTP. Free EF-Tu was separated from ribosome-bound EF-Tu by ultracentrifugation through a sucrose cushion, and both supernatant and pellet fractions were analyzed by Western blotting with antibodies against EF-Tu and the ribosomal protein S3. As expected, EF-Tu was found mostly in the supernatant fraction, independently of the presence of ribosomes (Fig. 4*A*). In the presence of P~EF-Tu, however, we observed a significant enrichment of EF-Tu in the pellet fraction with the ribosomes, indicating that phosphorylation of EF-Tu stabilizes its interaction with the ribosome, presumably by inhibiting GTP hydrolysis (Fig. 4*A*). Dephosphorylated EF-Tu was found almost exclusively in the supernatant, as is consistent with this modification having an inhibitory effect on EF-Tu release.

We next determined whether dephosphorylating P~EF-Tu that was preincubated with ribosomes reversed this effect. P~EF-Tu was incubated with ribosomes as before, but in this case the reaction was split, and one part of the reaction was incubated with PrpC (Fig. 4*B*). As in the previous assay, EF-Tu was significantly present in the pellet with ribosomes only when phosphorylated. In contrast, in the presence of the phosphatase, EF-Tu levels in the pellet fraction reverted to those observed in the nonphosphorylated EF-Tu. This observation is consistent with phosphatase being able to dephosphorylate P~EF-Tu that presumably is bound to the ribosome.

Phosphorylation of EF-Tu Occurs During *B. subtilis* Sporulation. The presence of YabT in the forespore (Fig. 1*C*) suggested that it could phosphorylate EF-Tu in this compartment. Because P~EF-Tu is stably associated with ribosomes (Fig. 4*A*), we fractionated sporulating *B. subtilis* into mother cell and forespore fractions (as in Fig. 1*B*), generated lysates, and isolated ribosomes by ultracentrifugation through sucrose cushions. The amount of ribosome-bound EF-Tu

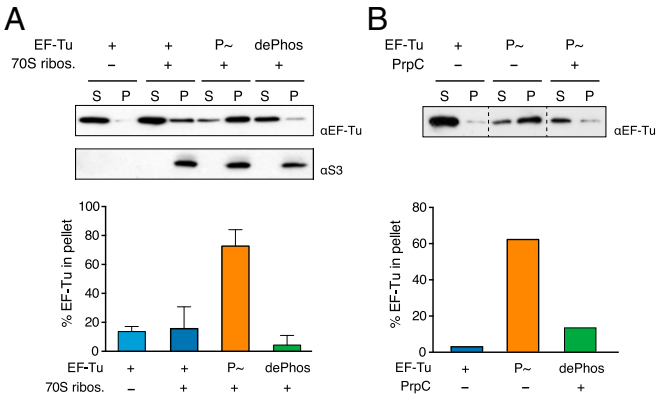

Fig. 4. EF-Tu–ribosome interaction is stabilized by phosphorylation in vitro. (*A*) EF-Tu/ribosome cosedimentation assay. Nonphosphorylated (WT), phosphorylated (P~), or dephosphorylated (dePhos) EF-Tu was incubated with 70S ribosomes in the presence of GTP. A reaction carried out in the absence of ribosomes was used as control. The binding reactions were layered into a sucrose cushion, and the ribosome-bound EF-Tu was separated from the free EF-Tu by ultracentrifugation. The supernatant (S) and pellet (P) fractions were probed with antibodies to EF-Tu and the ribosomal protein S3, and the relative amount of EF-Tu in the fractions was determined by quantitative densitometry and is shown below a representative Western blot. (*B*) Phosphorylated EF-Tu was incubated with 70S ribosomes in the presence of GTP. The reaction was split, and PrpC phosphatase was added to one half. Both reactions were incubated further for 30 min at 37 °C. Reactions then were pelleted in a sucrose cushion and analyzed as above. Dashed lines separate noncontiguous lanes. Error bars indicate the SD for at least three independent experiments.

then was determined by analyzing the pellets by Western blotting with an antibody against EF-Tu. There were equivalent amounts of EF-Tu in the mother cell fraction from both wild-type and Δ*yabT* strains (Fig. 5*A*, *Left*). In contrast, there was an approximately fourfold enrichment in the forespore fraction of the wild-type strain compared with a Δ*yabT* strain (Fig. 5*A*, *Center*). These results are consistent with the in vitro effect of EF-Tu phosphorylation and support the role of YabT as the kinase that phosphorylates EF-Tu in vivo in the developing spore. Next, we analyzed ribosomes isolated from mature spores. As in the forespore, we detected an enrichment of EF-Tu in the ribosome pellet only when the kinase was present (Fig. 5*A*, *Right*), confirming the role of this kinase during dormancy. Finally, we probed the ribosomal fraction isolated from forespores (Fig. 5*A*, *Center*) with a phospho-Thr antibody and observed that the antibody cross-reacted with EF-Tu from lysates derived from the wild-type strain but not from a strain lacking the YabT kinase (Fig. 5*B*). Thus, YabT is responsible for phosphorylating the EF-Tu molecules associated with ribosomes in the forespore.

Phosphorylation of EF-Tu Inhibits Protein Synthesis. EF-Tu in its active, GTP-bound state binds and delivers the aa-tRNA to the translating ribosome. Upon delivery, EF-Tu exits in its inactive, GDP-bound state, and the ribosome undergoes translocation, moving one codon forward and allowing a new elongation round to occur. Phosphorylated EF-Tu could have a dominant-negative effect on translation by halting ribosome progression. We tested this hypothesis using an in vitro transcription/translation system consisting of reconstituted *E. coli* components including EF-Tu. The yield of the reporter protein (CotE) is not affected significantly by the presence of nonphosphorylated *B. subtilis* EF-Tu (Fig. 6*A*). However, the addition of phosphorylated *B. subtilis* EF-Tu to the reaction reduced CotE levels threefold, suggesting that P∼EF-Tu inhibits translation even in the presence of the native *E. coli* EF-Tu. Unfortunately, technical challenges identical to those described for the translation elongation assay reported in Fig. 3*B* prevented us from testing directly whether dephosphorylation of P∼EF-Tu by PrpC could reverse the inhibitory effect that phosphorylation of EF-Tu has on the yield of CotE. Nonetheless, the observations that dephosphorylation of P∼EF-Tu reverses the inhibitory effect on GTP hydrolysis (Fig. 3*A*) and the stabilizing effect of EF-Tu phosphorylation on the interactions between EF-Tu and the ribosome (Fig. 4) strongly suggest that it likewise would reverse the inhibitory effects of phosphorylating EF-Tu on the yield of CotE.

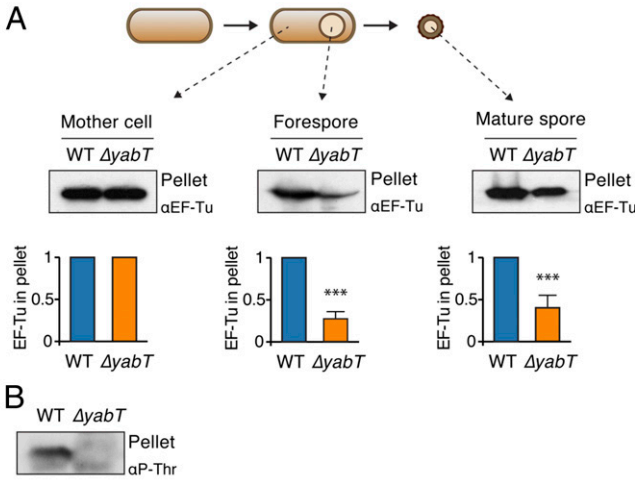

Fig. 5. Enrichment of EF-Tu in ribosomes isolated from spores. (*A*) Isolation of ribosomes from sporulating cells. Intact ribosomes from wild-type and Δ*yabT* strains were isolated from mother cells (*Left*), forespores (*Center*), and mature spores (*Right*) by ultracentrifugation through a sucrose cushion and were probed with an EF-Tu antibody. The relative amount of EF-Tu present in the ribosomal fraction (pellet) was determined by quantitative densitometry and is expressed as a fraction of WT EF-Tu. Quantification is shown below a representative Western blot. ***$P < 0.001$, unpaired t test with Welch's correction. Error bars indicate the SD for at least three independent experiments. (*B*) Forespore fractions shown in *A*, *Center* were probed with a phospho-Thr antibody.

The phosphorylation of EF-Tu in the forespore of sporulating cells (Fig. 5) suggests that this in vitro inhibition of translation (Fig. 6*A*) could occur in vivo as well. We investigated this possibility by constructing a strain expressing a *yfp* reporter gene under the control of an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter (Fig. 6*B*, *Left*). Thus, YFP levels following induction of the reporter should correspond to the ability of cells to translate YFP. Our protein expression data indicated that YabT is detected 2 h after sporulation initiation (T2; Fig. S1) and reaches maximum levels around 3–4 h (T3–T4) after sporulation initiation, so we induced YFP expression at T4 and 40 min later measured the fluorescence in the forespore. We observed higher fluorescent levels in the Δkinase cells than in wild-type cells (Fig. 6*B*, *Right*), but the *yfp* mRNA levels in the two strains were not significantly different (Fig. 6*C*), suggesting that the rate of protein synthesis is increased in the absence of the kinase. These results are consistent with an inhibitory effect of EF-Tu phosphorylation on translation in the cell that is becoming dormant.

Finally, we asked if the absence of this regulatory mechanism had phenotypic consequences for the spore. Dormant spores contain significant amounts of mRNA molecules (30–32), suggesting that, in principle, spores are capable of undergoing translation. We therefore reasoned that spores would have an increased predisposition to translate these mRNAs and perhaps initiate germination spontaneously if protein synthesis was not inhibited. That is, the absence of this metabolic "brake" would make spores less stable. Approximately half the spores from the Δkinase strain transitioned from phase-bright to phase-dark (a hallmark of germination) without being stimulated (Fig. 6*D*). Thus, consistent with the hypothesis, spores spontaneously initiate exit from a fully dormant state in the absence of the kinase.

Discussion

Here, we describe a novel mechanism for the regulation of protein synthesis involving phosphorylation of EF-Tu and demonstrate that it occurs during the process of sporulation when *B. subtilis* produces a metabolically dormant spore. EF-Tu supplies ribosomes with aa-tRNAs in each elongation cycle during growth (Fig. 7*A*), but EF-Tu is phosphorylated as the cell initiates dormancy (Fig. 7*B*). Because P∼EF-Tu is unable to hydrolyze GTP, it remains bound to translating ribosomes and stalls protein synthesis. When nutrients become available, the stalling could be reverted by the action of a phosphatase, which dephosphorylates EF-Tu, thereby releasing it from the ribosome and consequently allowing elongation to resume (Fig. 7*C*). This model predicts that a single phosphorylated EF-Tu would be sufficient to stall a ribosome on an mRNA. Thus, the ability of a single P∼EF-Tu to act as a dominant negative provides a mechanistic basis for the quick and robust regulation of the highly abundant components of the translation machinery observed in cells responding to nutrient limitation.

The low intrinsic GTPase activity of EF-Tu is stimulated by interaction with the ribosome. Specifically, a cognate codon–anticodon interaction in the ribosomal A site induces conformational changes in the ribosome and the aa-tRNA that are transmitted to the switch I and II regions of EF-Tu (3). The catalytic mechanism underlying the activation of the GTPase activity of EF-Tu is the subject of much recent debate (33–35). Nevertheless, it is clear that

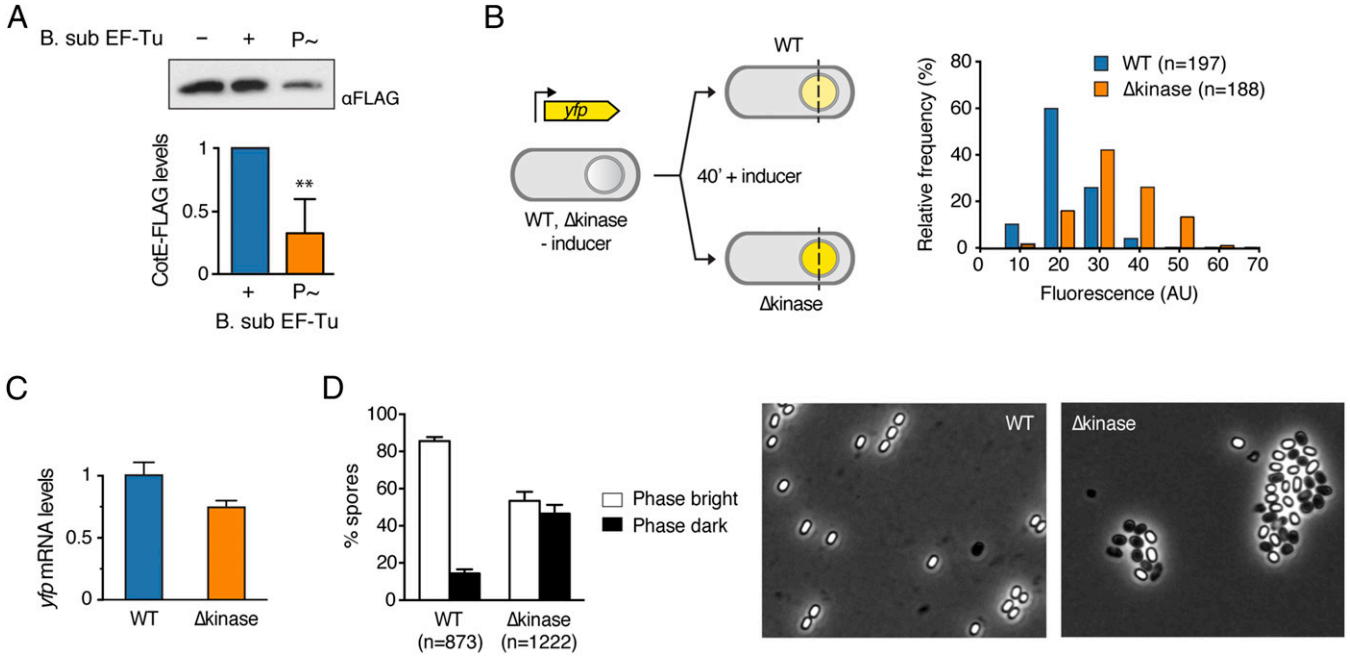

Fig. 6. EF-Tu phosphorylation inhibits protein translation. (*A*) In vitro synthesis of CotE-FLAG in the presence of *B. subtilis* EF-Tu. PURExpress reactions in the absence (–) and presence (+) of nonphosphorylated *B. subtilis* EF-Tu or phosphorylated *B. subtilis* EF-Tu (P~). The amount of CotE-FLAG was determined by quantitative densitometry and is expressed relative to CotE-FLAG synthesized in the absence of *B. subtilis* EF-Tu. Quantification is shown below a representative Western blot. $^{**}P < 0.005$, unpaired *t* test with Welch's correction. Error bars indicate the SD for at least three independent experiments. (*B*) The expression of the P_{spac}-*yfp* reporter was induced in wild-type and Δkinase (JDB3566) cells at hour 4 of sporulation. The magnitude of the YFP signal was measured across the spore 40 min following reporter induction. The distribution of fluorescence values observed in both strains is represented as the relative frequency of cells within each range of fluorescence values. Randomly selected fields were analyzed in at least three independent experiments. (*C*) *yfp* mRNA levels in forespores. Samples in *B* were processed as in Fig. 1*B* to isolate forespores, and total mRNA was extracted. *yfp* mRNA levels were determined by quantitative RT-PCR and are expressed relative to *tuf*. Error bars indicate the SD for at least three independent experiments. (*D*) Spores prepared from wild-type and Δkinase (JDB3566) were incubated in 10 mM Tris·HCl, pH 7.5, for 48 h at room temperature and visualized by phase-contrast microscopy. Randomly selected fields were analyzed in at least three independent experiments. Error bars indicate the SD. Quantification is shown next to representative fields.

the absolutely conserved Thr residue (Fig. S2*B* and ref. 11) within switch I of the GTP-binding domain of EF-Tu plays a key role by mediating the coordination of the Mg^{2+} ion, the catalytic water molecule, and the γ-phosphate of GTP and is directly involved in the activation of the GTPase activity of EF-Tu by the ribosome (36). Our in vitro kinase assays and mass spectrometry data indicate that this Thr residue, Thr-63 in *B. subtilis*, is the primary site of phosphorylation of EF-Tu by the YabT kinase (Fig. 2 and Fig. S2). However, because this residue is essential for EF-Tu function (23, 24), it is difficult or impossible to interpret experiments involving mutations at this site unambiguously. Therefore we cannot irrefutably ascribe the reduced GTPase activity of the phosphorylated protein to phosphorylation of this Thr residue. Nonetheless, the reduced GTPase activity that we observe for EF-Tu that has been primarily phosphorylated at Thr-63 (Fig. 3*A*) is consistent with previous reports that replacing the homologous Thr residue with an Ala residue is sufficient to reduce the GTPase activity very significantly in both *T. thermophilus* and *E. coli* EF-Tu (23, 24).

EF-Tu species from the nonsporulating Gram-positive bacterium *M. tuberculosis* (Fig. S3) and the Gram-negative bacterium *E. coli* (20) also are phosphorylated on this Thr residue. Thus, the mechanism that we describe here is very likely to be phylogenetically conserved. In addition, because this Thr residue is absolutely conserved among translational GTPases (Fig. S2*B* and ref. 11), our findings may have general implications for the mechanism of GTP hydrolysis in this family of proteins (37).

The inhibition of GTP hydrolysis by phosphorylation prevents EF-Tu from dissociating from the ribosome (Figs. 4 and 5). This effect is reminiscent of nonhydrolysable GTP analogs, which stabilize EF-Tu association with the ribosome (9). A similar inhibitory mechanism also has been observed previously for the GTPase eIF5B, the eukaryotic homolog of the translation initiation factor IF2. A mutation of a single Thr residue, which blocks GTP hydrolysis, prevents release of eIF5B from the ribosome and thereby inhibits translation (38). Thus, this mechanism of inhibition appears to be conserved in both eukaryotic and prokaryotic translational GTPases.

An unresolved aspect of the phosphorylation of the Thr residue located within the nucleotide-binding pocket is how the kinase gains access to this non–surface-exposed residue. In the case of isocitrate dehydrogenase (IDH), which is regulated by direct phosphorylation of an Ser residue in the active site, the flexible nature of the IDH catalytic center likely facilitates this modification (39). Interestingly, the switch I region of EF-Tu assumes an alpha-helical conformation in the closed form of EF-Tu but changes to a beta-hairpin conformation in the open form of EF-Tu (40, 41). Consistent with this flexibility, this region of EF-Tu is disordered in most EF-Tu structures, whether alone or in complex with the ribosome (36). Thus, the dynamics of switch I may facilitate access to Thr-63 by both the kinase and the phosphatase.

Translation elongation in eukaryotes also is subject to regulatory phosphorylation. For example, the eukaryotic EF-Tu homolog eEF1α is phosphorylated in glial cells in response to glutamate, and this modification was correlated with a reduction in the rate of polypeptide chain elongation (42). Also, phosphorylation of Thr-56 inhibits the activity of the eukaryotic EF-G homolog eEF2 by reducing its affinity for the ribosome (43). The eEF2K kinase responsible for eEF2 phosphorylation is itself subject to control by the AMP-activated kinase (44), suggesting a direct link between nutritional availability and the inhibition of protein synthesis. This mechanism protects tumor cells during acute nutrient deprivation, suggesting that it is a key switch in the fate of these cells (45).

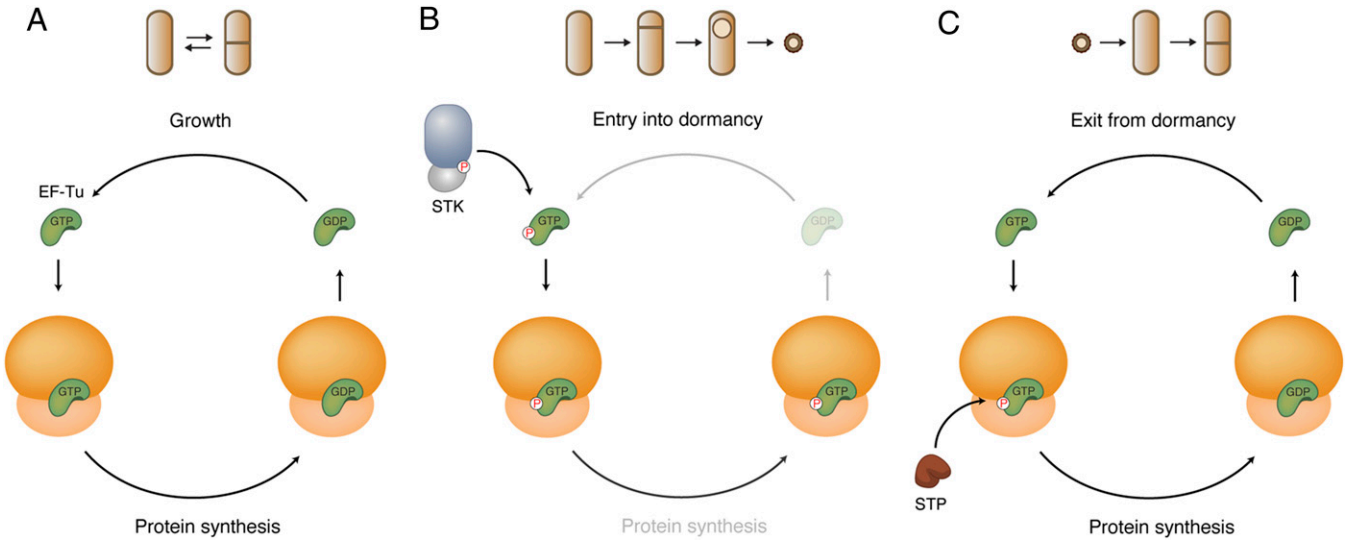

Fig. 7. Regulation of protein synthesis by reversible phosphorylation of EF-Tu. (*A*) During growth, the translation GTPase EF-Tu cycles rapidly between its active (GTP-bound) and inactive (GDP-bound) state. In the active state, EF-Tu binds and delivers the aa-tRNA to the ribosome. If there is a codon/anticodon match, GTP hydrolysis is activated, and EF-Tu releases the aa-tRNA and exits the ribosome. (*B*) In cells entering dormancy (e.g., in the forespore of sporulating bacteria), an Ser/Thr kinase (STK) is expressed and phosphorylates EF-Tu. This phosphorylation inhibits GTP hydrolysis; as a result, EF-Tu remains stably bound to the ribosome, stalling protein translation. Ribosomes are kept in this quiescent state throughout dormancy. (*C*) In cells exiting dormancy, EF-Tu can be dephosphorylated by an Ser/Thr phosphatase (STP). GTP hydrolysis can occur, and protein synthesis can resume.

Many organisms respond to nutrient limitation by entering a metabolically dormant or quiescent state. This response allows them to persist through unfavorable conditions until the environment becomes conducive for growth, whereupon they exit dormancy and reinitiate growth. Although dormancy traditionally has been associated with a relatively small number of bacterial species that generate specialized forms such as spores or cysts, the majority of the microbial biomass is quiescent (46). The ability to exist in such a state becomes advantageous when cells are faced with unfavorable environmental conditions. For example, metabolically quiescent pathogens show high tolerance to chemotherapy and host defenses and thus commonly are associated with recurrent and chronic infections. However, the advantage provided by this strategy relies on the ability of dormant cells to reverse this state under the appropriate conditions; if they cannot, they will be outcompeted by growing cells.

Our understanding of the mechanisms underlying entry into and exit from dormancy is still incomplete. An appealing hypothesis is that these two processes are mechanistically related. Hence, when cells reduce protein synthesis in response to nutrient limitation, they need to ensure that they can reverse this state efficiently to respond to changes in the environment. The observations presented in this paper suggest that reversible phosphorylation of the translation factor EF-Tu could function as part of this mechanism. Thus, the phosphorylation of EF-Tu that results in a stable association with the ribosome and thereby inhibits translation also enables the ribosome to resume translation quickly by ensuring that EF-Tu already is present as part of what can be termed the "ribosome holoenzyme."

Finally, the mechanism described here may have direct implications for the regulation of translation in eukaryotic organelles of bacterial origin. For example, mitochondrial protein translation is responsible for the synthesis of components of the respiratory chain complexes that are encoded in the mitochondrial genome and that are necessary for oxidative phosphorylation. The translation components of this organelle, including the elongation factor mtEF-Tu, are closely related to the bacteria homologs (47). Because the target of regulatory phosphorylation in *B. subtilis* EF-Tu (Thr-63) is conserved in both yeast and mammalian mtEF-Tu (Fig. S2*B*), a question to be addressed in future work is whether mtEF-Tu is subject to similar reversible regulation.

Materials and Methods

See *SI Materials and Methods* for descriptions of growth conditions and strain construction and detailed descriptions of experimental conditions and buffers. Strains, plasmids, and primers used in this study are listed in Tables S1–S3.

Fractionation of Mother Cells and Forespores. Cells sporulated for 5 h were protoplasted by incubation with 1 mg/mL of lysozyme for 10 min at 37 °C in protoplasting buffer. Protoplasts were lysed by vigorous vortexing in lysis buffer, and supernatant (lysed mother cells) and pellet (forespores) were separated by centrifugation at 13,000 × *g* for 3 min at 4 °C. Mother cell lysate was cleared at 20,000 × *g* for 20 min at 4 °C. Forespores were isolated from the pellet and lysed in the presence 4 mg/mL of lysozyme for 5 min at 37 °C, processed in a FastPrep homogenizer, and incubated with 1% Nonidet P-40. Forespore lysate then was cleared by centrifugation at 20,000 × *g* for 20 min at 4 °C. The strains used in this experiment were coat-deficient to facilitate lysis and contained fluorescent reporters specifically expressed in the mother cell (P_{spoIID}-*yfp*) or forespore (P_{spoIIQ}-*cfp*) compartments to monitor fractionation by fluorescence microscopy.

EF-Tu Phosphorylation. EF-Tu was phosphorylated in vitro by incubating kinases (0.5 μM) and substrates (2 μM) with 0.1 M unlabeled ATP and 1 μCi of [γ-32P]ATP in kinase buffer for 30 min at 37 °C. *B. subtilis* EF-Tu was phosphorylated in vivo by coexpression with a tagless YabT kinase domain from a pETDuet-derived plasmid in *E. coli* and was phosphoenriched using the Pro-Q Diamond phosphoprotein enrichment kit (Molecular Probes).

GTP Hydrolysis and Translation Elongation. GTP hydrolysis and translation elongation assays were adapted from ref. 28.

Cosedimentation Assay. The EF-Tu ribosome-binding assay was adapted from ref. 48.

In Vitro Translation of CotE-FLAG. The PURExpress system (New England Biolabs) was used to assay translation of CotE-FLAG in the presence of *B. subtilis* EF-Tu.

Isolation of Intact Ribosomes from *B. subtilis*. Ribosomes were isolated from lysed mother cells and forespores or mature spores by ultracentrifugation in sucrose cushions and were analyzed by immunoblotting.

Translation in Cells Entering Dormancy. Protein synthesis in endospores was measured by fluorescence microscopy by inducing the expression of an *yfp* reporter 4 h after initiation of sporulation. Quantitative RT-PCR was used to quantify expression of *yfp* under the same conditions.

Spontaneous Germination. Purified spores were incubated in 10 mM Tris·HCl, pH 7.5, for 48 h at room temperature.

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