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GTP hydrolysis by EF-G synchronizes tRNA movement on small and large ribosomal subunits

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

23 December 2013

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, ref#1 and ref#3 are positive about your findings and support publication in The EMBO Journal, pending extensive revision of the text to more clearly acknowledge the recent literature and to make the data and conclusions more accessible for the non-specialist reader. Ref#2 on the other hand is rather critical and points out that while the presented data are of high quality, multiple individual observations had already been reported in previous papers. A main caveat for ref#2 appears to be the recently published structural work in PNAS that presents the suggested A/P2 state on the ribosome; however, as you probably know The EMBO Journal offers full scooping protection for all manuscripts submitted for consideration here and as such these points do not count against the novelty of your findings in our assessment since the two PNAS studies only appeared while your study was under review.

In light of our scooping policy and the overall positive recommendations by the referees, I would therefore like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. Since the revisions required by all three refs mainly relate to textual changes, I would ask you to revise and return the manuscript to us as soon as possible to ensure a timely appearance with the PNAS studies.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For

more details on our Transparent Editorial Process, please visit our website:
<http://www.nature.com/emboj/about/process.html>

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

In the manuscript by Holtkamp et al., the authors probe the mechanism by which elongation factor G accelerates mRNA and tRNA translocation on the ribosome due to GTP hydrolysis. The authors first introduce a new fluorescent labeling scheme that allows them to track the short nascent peptide attached to the 3'-end of peptidyl-tRNA. This BODIPY (Bpy) probe can be tracked entirely independently of the probe used to track movement of the mRNA (Alx). Using these probes, the authors employ a suite of biochemical perturbations to dissect the step-wise translocation of mRNA and tRNA on the ribosome. These include non-hydrolyzable GTP analogs, antibiotics, the concentration of magnesium, and well-characterized mutations in EF-G that interfere with certain aspects of the translocation reaction.

Although the data presented in this manuscript are interesting, and of a quality in general suitable for The EMBO Journal, it is presently not very accessible to the non-specialist. I therefore think the authors need to work on key aspects of the presentation, as well as interpretation of one set of experiments, before the paper can be published. My comments are given below.

1. The abstract is not written with a broad enough audience in mind. For example, the authors use antibiotics quite effectively in their experiments, but fail to mention that the translocation reaction is a key step inhibited by many classes of antibiotics.
2. Sentences 4-5 of the abstract will be very hard for the non-specialist (and even some specialists) to follow, especially given the naming convention. It would be worthwhile finding a way to present the results without resorting to the naming conventions at this point in the manuscript.
3. In the Introduction, the authors dive into the known details of the translocation reaction far too quickly. Again, 1-2 sentences early on bringing in the antibiotic connection would be helpful here.
4. A second modification to the Introduction would be to move the last sentence of the 2nd paragraph (bottom of p. 4) to half-way through the first paragraph, for example right after the sentence that ends with "...L1 stalk." Then, the authors could start a new paragraph with the sentence, "One controversial issue concerns the mechanism..."
5. As the authors proceed through their experiments, the varied naming conventions used by different groups for different substeps are introduced as needed. I think it would be a great service to the ribosome community if the authors were to provide a Table at some point, in which they align the various naming conventions to the steps they think these correspond to, based on the present and previous experiments.
6. There is one significant issue with interpretation of the data presented in the manuscript that the authors need to address, namely the use of K_M values to conclude that "EF-G binds more efficiently to the classical state". This is not supported by such an experiment. The fundamental flaw with this analysis is that the "apparent affinity of EF-G" for the ribosome is by definition undefined. Regardless of how many microstates are involved, it is obvious that EF-G is bound to the ribosome in three different nucleotide states (GTP, GDP-Pi, and GDP), each of which may have a different

affinity for the ribosome, and more importantly different affinities for the different conformational states. So, the statement that "EF-G binds more efficiently to the classical state" is not meaningful.

7. A second problem with the experiment in Figure 2 relates to results from Chen et al. (2013). Although the present authors criticize the single-molecule work as 100-fold slower than the bulk experiments; beyond arrival times, this is in fact not true at all. Chen et al. achieve time resolutions of tens of milliseconds (i.e. see last paragraph on p. 720 and Fig. 3c of that paper). Chen et al. observe that EF-G binding to the classical state is unproductive for translocation (Fig. 5 and 6 in Chen et al.). Furthermore, Chen et al. provide strong evidence that EF-G/GTP binds to rotated state of the ribosome more tightly (see last paragraph, p. 722). These are serious discrepancies with the present results that the authors need to address more clearly.

8. Chen et al. proposed that three substeps are kinetically resolvable that contribute to translocation (Figure 6), and use antibiotics to probe these substeps (Figure 7). The present authors need to do more to connect the present results to those in Chen et al.

9. The authors do not do a good job of labeling the substeps of translocation in Figure 5. This figure will be hard for the non-specialist to follow.

Referee #2:

The article "GTP hydrolysis by EF-G synchronizes..." by Holtkamp, et al. reports a comprehensive, rapid kinetic study of the movements of the CCA end of the A-site peptidyl-tRNA on the 50S subunit and the anticodon of the A-site peptidyl-tRNA/mRNA on the 30S subunit during EF-G-catalyzed translocation. The authors find that EF-G-GTP facilitates peptidyl-tRNA movement into an early post-translocation state synchronously on the two subunits. Binding of EF-G-GTP to the pre-translocation ribosome brings about translocation on the 50S subunit, resulting in an intermediate pre-translocation state (A/P2). GTP hydrolysis subsequently completes translocation on the 50S subunit and catalyzes rapid translocation on the 30S subunit. The experiments that are presented have been carefully designed and executed and the appropriate controls have been performed. The data that are presented are of the highest quality and have been properly analyzed. The interpretation of the data and the conclusions that are drawn are appropriate, but there are a few gaps, as addressed in further detail below, that have not been addressed. Regardless, the results and findings presented in this manuscript are somewhat incremental, as much of the data and the findings have been previously reported. In addition, two articles reporting the cryo-electron microscopy structure of what seems to be the A/P2 state described in this manuscript have just been published in PNAS; a development that, unfortunately, further limits the impact of the current manuscript. Finally, there are a number of misstatements and referencing errors that should be corrected. A more detailed description of the above listed shortcomings are provided below:

1. Similar rapid kinetic studies of the movements of the A-site peptidyl-tRNA body, using a proflavin-labeled tRNA (Rodnina et al (1997) Nature; Savelsbergh et al (2003) Mol Cell), and the anticodon of the A-site peptidyl-tRNA/mRNA, using a fluorescein-labeled mRNA (Peske et al (2004) J Mol Biol), have been published by the same group and have arrived at conclusions, at least in regards to movements of the A-site peptidyl-tRNA body and the anticodon of the A-site peptidyl-tRNA/mRNA, that are similar to those reported in the current manuscript. The novelty of the current manuscript, therefore, is largely limited to the rapid kinetic study of the movements of the CCA end of the peptidyl-tRNA on the 50S subunit using a BODIPY-labeled, A-site peptidyl-tRNA. However, it should be noted that several rapid kinetic studies of the movements of the CCA end of the peptidyl-tRNA on the 50S subunit using puromycin reactivity have been previously published by various groups (Semenkov et al (2004) FEBS Letters; Sharma et al (2004) RNA; Pan et al (2007) Mol Cell) and that these studies arrived at conclusions regarding the movements of the CCA end of the peptidyl-tRNA on the 50S subunit that overlap with the conclusions that are presented in the current manuscript. Similarly, rapid kinetic studies of translocation using labeled tRNAs, labeled mRNA, and/or puromycin reactivity have been used to study the effects of viomycin (Peske et al (2004) J Mol Biol), spectinomycin (Peske et al (2004) J Mol Biol), streptomycin (Peske et al (2004) J Mol Biol), hygromycin B (Peske et al (2004) J Mol Biol), EF-G(H91A) (Cunha et al (2013) Translation), EF-G(Δ 4/5) (Savelsbergh et al (2000) J Mol Biol), and EF-G(H583K) (Savelsbergh et al (2000) J Mol Biol) on translocation, again arriving at conclusions that overlap with the

conclusions presented in the current manuscript. In light of these previous studies, therefore, the novelty, significance, and impact of the current manuscript seem somewhat incremental.

2. Two cryo-electron microscopy studies of EF-G-bound pre-translocation complexes carrying an A-site peptidyl-tRNA have been recently published in PNAS (Brilot et al (2013) PNAS; Ramrath et al (2013) PNAS). The conformation of the A-site peptidyl-tRNA in these structures is unique relative to the conformation of the A-site peptidyl-tRNA that is observed in pre-translocation complexes imaged in the absence of EF-G (e.g., Agirrezabala et al (2008) Cell; Julian (2008) PNAS) and is apparently only observed in the presence of bound EF-G. Based on the fact that it is observed exclusively in the presence of bound EF-G, it is exceedingly likely that the conformation of the A-site peptidyl-tRNA that is observed in these cryo-electron microscopy studies is equivalent to the A/P2 conformation of the A-site peptidyl-tRNA that is also observed exclusively in the presence of bound EF-G in the current work. Unfortunately, this further limits the novelty, significance, and impact of the current manuscript.

3. On pg3, par1 of the manuscript, the authors note that "the hybrid/rotated state is not a single intermediate" and that "a variety of distinct hybrid states have been identified." Nonetheless, the data analysis, interpretations, conclusions, and mechanistic model that are presented in the current manuscript treat the hybrid/rotated state as a single intermediate. No data or arguments are presented to justify this simplified treatment and, although it certainly makes the data analysis, interpretations, conclusions, and mechanistic model simpler, it is not clear that this simplified treatment is justified.

4. There are several misstatements and referencing errors, as listed below, that should be corrected:

(a) pg3, par1

"This pre-translocation (PRE) complex is dynamic and fluctuates between the classical state, where the tRNAs are located in the A and P sites on both 30S and 50S subunits (A/A and P/P states), and hybrid states, where the acceptor domains of the tRNAs are moved towards the P and E sites, while the anticodon domains remain bound in the A and P sites (A/P and P/E states) (Moazed & Noller, 1989)."

This statement is lacking a reference. The finding that the pre-translocation complex is dynamic, such that the tRNAs fluctuate between classical and hybrid states was first reported by Blanchard et al (2004) PNAS; this article should be referenced here.

(b) p3 par1

"Structurally, the hybrid/rotated state is not a single intermediate, because a variety of distinct hybrid states have been identified. These sub-states (denoted as states H1 and H2 (Munro et al, 2010a), MSI and MSII (Fu et al, 2011), classes 2, 4A, 4B, 5 and 6 (Agirrezabala et al, 2012), or PRE1-5 states (Fischer et al, 2010)) differ in the orientation of the tRNAs, the degree of subunit rotation, the conformation of the 30S subunit, and the position of the L1 stalk."

There are several misstatements and a few missing references in this statement. Fu et al (2011) PNAS makes no reference to structures labeled "MSI" or "MSII" and, generally speaking, structures from the Frank group labeled "MSI" typically refer to a classical/unrotated state, not a hybrid/rotated state. Similarly, classes 2 and 4A in Agirrezabala et al (2012) PNAS refer to classical/unrotated states, not hybrid/rotated states. In addition, classes 5 and 6 in Agirrezabala et al (2012) PNAS are very similar to each other and to the MSII hybrid/rotated structure that has been previously characterized by the Frank group (Agirrezabala et al (2008) Cell). Strictly speaking, Fischer et al (2010) Nature refers to structures relevant to the process of tRNA-binding-induced retro-translocation; it is unclear how these structures relate to the process of EF-G-catalyzed translocation; indeed, this is not clearly stated or discussed anywhere in the current manuscript despite the fact that findings reported in Fisher et al (2010) Nature are cited as relevant for EF-G-catalyzed translocation throughout the current manuscript. Regardless, classes PRE1 and, possibly, PRE2 in Fischer et al (2010) Nature refer to classical/unrotated states, not hybrid/rotated states. Finally, the GS2 state described in Fei et al (2008) Mol Cell and the R2 state described in Zhang et al (2009) Science should also be included and cited in this list.

(c) p5, par5

"The ribosome is intrinsically dynamic and functions as a Brownian machine that undergoes spontaneous conformational fluctuations driven by thermal energy, which can be coupled to directed motion (Fischer et al, 2010; Munro et al, 2010b)."

There are some missing references here: Spirin (2009) J Biol Chem and Frank et al (2010) Ann Rev Biochem.

(d) p8, par2

"Previous cryo-EM studies have shown that the ribosomes in classical and rotated/hybrid conformations are in equilibrium with each other under experimental conditions similar to those used here (Agirrezabala et al, 2008; Fischer et al, 2010; Julian et al, 2008)."

The fact that pretranslocation ribosomes in classical/unrotated and hybrid/rotated conformations are in equilibrium with each other under experimental conditions similar to those used here was first shown by Blanchard et al (2004) PNAS, Munro et al (2007) Mol Cell, Fei et al (2008) Mol Cell, and Cornish et al (2008) Mol Cell; these papers should be referenced here.

(e) p8, par2

"EF-G binding strongly shifts the equilibrium towards the hybrid/rotated state by reducing the rate of the conversion from the hybrid to classical state (Cornish et al, 2008; Munro et al, 2010b);"

The first article to report that EF-G binding strongly shifts the equilibrium towards the hybrid/rotated state by reducing the rate of the conversion from the hybrid to classical state was Fei et al (2008) Mol Cell; this article should be referenced here.

(f) p15, par1

"Thus, while the hybrid/rotated states are authentic intermediates of translocation reflecting the intrinsic dynamics of the ribosome that are crucial for translocation, the kinetics of these fluctuations are unlikely to be rate-limiting for EF-G recruitment or tRNA translocation."

This statement seems like a broad generalization and an oversimplification. While the kinetics of fluctuations between classical/unrotated and hybrid/rotated states seem to not be rate-limiting for EF-G recruitment or tRNA translocation under the conditions investigated in the present work, one can easily imagine conditions under which these fluctuations could easily become rate limiting. Indeed, any obligatory step in a complex, multi-step biochemical reaction such as translocation could easily become rate-limiting under the appropriate conditions.

Referee #3:

Review of Koltkamp et al., "GTP Hydrolysis by EF-G synchronizes tRNA movement...."

This paper describes kinetic experiments using fluorescent labels attached to the mRNA and to the peptidyl moiety of tRNA to measure movement of mRNA and of the acceptor end of tRNA during EF-G-dependent translocation. The reaction is followed at different temperatures, as a function of Mg ion and EF-G concentrations, and in the presence of a series of antibiotics and with mutant versions of EF-G that affect different aspects of the mechanism. In the absence of GTP hydrolysis, neither movement of mRNA on the 30S subunit nor complete movement of the acceptor end of the peptidyl-tRNA into the 50S P site take place. The authors conclude that the final steps of translocation on both the 30S and 50S subunits depend on hydrolysis of GTP by EF-G.

I believe that this is an excellent paper that clarifies several critical aspects of the translocation mechanism, and that it should be published in the EMBO J. The following points need to be addressed:

1. p.8, paragraph 1: "...movement of the 3' end of deacylated tRNA on the 50S subunit..." As the experiment mentioned uses the Prf20 label, doesn't the experiment show movement of the tRNA elbow, rather than its 3' end, strictly speaking?
2. Fig. 2A: What is meant by "P" in the diagram? I am guessing that it is the post-translocation state, but it could also mean "products".
3. p.10, bottom line: How can binding of streptomycin trap the 30S subunit in a conformation that is inherently more prone to rapid translocation and at the same time inhibit translocation?
4. p.15, 2nd paragraph, line 10: "...all three reactions..." It is not obvious which three reactions are

meant.

1st Revision - authors' response

28 January 2014

Referee #1:

In the manuscript by Holtkamp et al., the authors probe the mechanism by which elongation factor G accelerates mRNA and tRNA translocation on the ribosome due to GTP hydrolysis. The authors first introduce a new fluorescent labeling scheme that allows them to track the short nascent peptide attached to the 3'-end of peptidyl-tRNA. This BODIPY (Bpy) probe can be tracked entirely independently of the probe used to track movement of the mRNA (Aix). Using these probes, the authors employ a suite of biochemical perturbations to dissect the step-wise translocation of mRNA and tRNA on the ribosome. These include non-hydrolyzable GTP analogs, antibiotics, the concentration of magnesium, and well-characterized mutations in EF-G that interfere with certain aspects of the translocation reaction.

Although the data presented in this manuscript are interesting, and of a quality in general suitable for The EMBO Journal, it is presently not very accessible to the non-specialist. I therefore think the authors need to work on key aspects of the presentation, as well as interpretation of one set of experiments, before the paper can be published. My comments are given below.

Abstract

1. The abstract is not written with a broad enough audience in mind. For example, the authors use antibiotics quite effectively in their experiments, but fail to mention that the translocation reaction is a key step inhibited by many classes of antibiotics.

2. Sentences 4-5 of the abstract will be very hard for the non-specialist (and even some specialists) to follow, especially given the naming convention. It would be worthwhile finding a way to present the results without resorting to the naming conventions at this point in the manuscript.

We have changed the abstract in a way suggested by the referee, avoiding the complex naming conventions and mentioning the antibiotics.

Intro

3. In the Introduction, the authors dive into the known details of the translocation reaction far too quickly. Again, 1-2 sentences early on bringing in the antibiotic connection would be helpful here.

Done as suggested by the referee.

4. A second modification to the Introduction would be to move the last sentence of the 2nd paragraph (bottom of p. 4) to half-way through the first paragraph, for example right after the sentence that ends with "...L1 stalk." Then, the authors could start a new paragraph with the sentence, "One controversial issue concerns the mechanism..."

Done as suggested by the referee.

5. As the authors proceed through their experiments, the varied naming conventions used by different groups for different substeps are introduced as needed. I think it would be a great service to the ribosome community if the authors were to provide a Table at some point, in which they align the various naming conventions to the steps they think these correspond to, based on the present and previous experiments.

It would be very difficult to provide such a Table, because the identity of the intermediates from various naming conventions is often uncertain. We are rather confident in including the intermediate described by Cooperman et al (INT), which we can reproduce and assign in our kinetic scheme (p. 8-9). The position on the time axis of translocation of various intermediates identified by single molecule techniques is less certain, because the rates often cannot be compared with those measured

in bulk experiments. The intermediates identified by cryo-EM and X-ray are even more difficult as they lack the time axis for comparison and we do not feel in the position to assign them; it would be rather a task for structure specialists. In the discussion, we make some suggestions as to their appearance during translocation (p. 18-19), mostly based on the effect of antibiotics.

6. There is one significant issue with interpretation of the data presented in the manuscript that the authors need to address, namely the use of K_M values to conclude that "EF-G binds more efficiently to the classical state". This is not supported by such an experiment. The fundamental flaw with this analysis is that the "apparent affinity of EF-G" for the ribosome is by definition undefined. Regardless of how many microstates are involved, it is obvious that EF-G is bound to the ribosome in three different nucleotide states (GTP, GDP-Pi, and GDP), each of which may have a different affinity for the ribosome, and more importantly different affinities for the different conformational states. So, the statement that "EF-G binds more efficiently to the classical state" is not meaningful.

We simplified and corrected the text on p. 10. Most importantly, the rate of translocation was decreased by two-fold at most when most of ribosomes were starting at the classical state, suggesting that the conversion from C to H state is either not crucial or not rate limiting for translocation. Having a K_M value is an advantage, rather than a "fundamental flaw", because it reports on the overall affinity in the system before the reaction happens, regardless of the microstates and including the different nucleotide states, and accounts for all binding intermediates, also rare and transient ones which may escape the single molecule detection.

7. A second problem with the experiment in Figure 2 relates to results from Chen et al. (2013). Although the present authors criticize the single-molecule work as 100-fold slower than the bulk experiments; beyond arrival times, this is in fact not true at all. Chen et al. achieve time resolutions of tens of milliseconds (i.e. see last paragraph on p. 720 and Fig. 3c of that paper).

Chen et al. collect the data at 30 frames per second (p. 721, Figure legend for Fig. 3c and Materials and methods), which means that their integration time is 33 ms. This is nicely shown in Fig. 3c, where the integration time is shaded. This means that the transition between rotated and non-rotated state occurs within one frame, implying that this experiment does not provide the time resolution for translocation. For comparison, our Supplemental Figure 1 – just as an example, because the data there are shown on a logarithmic scale – shows that after 33 ms the reaction measured by Bpy is essentially over, implying that the kinetics of this reaction ($20\text{-}30\text{ s}^{-1}$) cannot be properly measured with the time resolution of the single-molecule TIRF setup described in Chen et al. However, after considering the respective paragraph, we decided to remove the comparison with the single-molecule rates on p. 15, as it may appear overly aggressive and is not necessary for the argument.

Chen et al. observe that EF-G binding to the classical state is unproductive for translocation (Fig. 5 and 6 in Chen et al.).

We are actually grateful to the reviewer for drawing our attention to these Figures, because there is an interesting observation which may explain the apparent inconsistency in a relatively simple way. Fig. 5 a and b in Chen et al. addresses the binding properties of EF-G-GDPNP and EF-G-GDP to the rotated/ non-rotated states and show that the arrival times are similar for the rotated and non-rotated states, while the dwell times for EF-G-GDPNP are different. In contrast, the dwell times for EF-G-GTP are quite similar for the two states (Fig. 6a,b). This may indicate that the GTP-bound form of EF-G (and, after GTP hydrolysis, GDP-Pi and GDP forms) appear to have a different preference for the rotated/non-rotated state than the GDPNP-bound form. This discussion is now included on p. 15.

Concerning the preference to the classical state, also Cooperman et al. and Gonzalez et al. show that translocation can occur from the classical state and that, at some conditions, the rotated state may be formed only transiently (Chen et al., 2011a, Fei et al., 2011), as described on p. 5 and 14.

Furthermore, Chen et al. (2013) provide strong evidence that EF-G/GTP binds to rotated state of the ribosome more tightly (see last paragraph, p. 722). These are serious discrepancies with the present results that the authors need to address more clearly.

It is EF-G/GDPNP, not EF-G/GTP, which binds more tightly to the rotated than to non-rotated state (Fig. 5b of Chen et al.). For EF-G/GTP, the distribution of sampling dwell times is quite similar (with $\tau = 64$ ms and 76 ms, respectively (compare Fig. 6a and b); there is no standard deviation given, but the measured points in panels a and b practically coincide if you overlay the two panels). Also the arrival rates are comparable: On p 722, the average value is $1.9 \mu\text{M}^{-1}\text{s}^{-1}$ for the rotated state compared to $0.84 \pm 0.5 \mu\text{M}^{-1}\text{s}^{-1}$ for the non-rotated state (average calculated from the data of Fig. 3b). So the initial interaction of EF-G with the rotated and non-rotated state seems very similar not only in this work, but also in Chen et al. 2013.

8. Chen et al. proposed that three substeps are kinetically resolvable that contribute to translocation (Figure 6), and use antibiotics to probe these substeps (Figure 7). The present authors need to do more to connect the present results to those in Chen et al.

The existence of multiple steps prior to back rotation, such as GTP hydrolysis, Pi release, movements of the 30S head, etc. is known from the work of several groups, including ours (Rodnina et al., 1997, Savelsbergh 2003, 2005, Pan et al., 2007, recent Noller's work, etc.). Also the complex Poisson distributions have been described earlier (Wen et al., 2008). Nevertheless, we added the reference to Chen to acknowledge that they also see multiple states of EF-G dynamics (p. 20).

Concerning the antibiotics, Chen et al. used viomycin and spectinomycin to probe the EF-G sampling. The conclusion of these experiments, i.e. that the antibiotics inhibit translocation steps after GTP hydrolysis, is not novel, as we have shown this a long time ago (Rodnina et al., 1997; Peske et al., 2004); notably, the authors (Chen et al.) do not cite this earlier work. We have added the citation to Chen et al 2013 to indicate that the antibiotics perturb EF-G dynamics on p. 11.

9. The authors do not do a good job of labeling the substeps of translocation in Figure 5. This figure will be hard for the non-specialist to follow.

We introduced additional labels and more information in Figure 5 and rearranged it to better fit on page.

Referee #2:

The article "GTP hydrolysis by EF-G synchronizes..." by Holtkamp, et al. reports a comprehensive, rapid kinetic study of the movements of the CCA end of the A-site peptidyl-tRNA on the 50S subunit and the anticodon of the A-site peptidyl-tRNA/mRNA on the 30S subunit during EF-G-catalyzed translocation. The authors find that EF-G-GTP facilitates peptidyl-tRNA movement into an early post-translocation state synchronously on the two subunits. Binding of EF-G-GTP to the pre-translocation ribosome brings about translocation on the 50S subunit, resulting in an intermediate pre-translocation state (A/P2). GTP hydrolysis subsequently completes translocation on the 50S subunit and catalyzes rapid translocation on the 30S subunit. The experiments that are presented have been carefully designed and executed and the appropriate controls have been performed. The data that are presented are of the highest quality and have been properly analyzed. The interpretation of the data and the conclusions that are drawn are appropriate, but there are a few gaps, as addressed in further detail below, that have not been addressed. Regardless, the results and findings presented in this manuscript are somewhat incremental, as much of the data and the findings have been previously reported. In addition, two articles reporting the cryo-electron microscopy structure of what seems to be the A/P2 state described in this manuscript have just been published in PNAS; a development that, unfortunately, further limits the impact of the current manuscript. Finally, there are a number of misstatements and referencing errors that should be corrected. A more detailed description of the above listed shortcomings are provided below:

1. Similar rapid kinetic studies of the movements of the A-site peptidyl-tRNA body, using a proflavin-labeled tRNA (Rodnina et al (1997) Nature; Savelsbergh et al (2003) Mol Cell), and the anticodon of the A-site peptidyl-tRNA/mRNA, using a fluorescein-labeled mRNA (Peske et al (2004) J Mol Biol), have been published by the same group and have arrived at conclusions, at least in regards to movements of the A-site peptidyl-tRNA body and the anticodon of the A-site peptidyl-tRNA/mRNA, that are similar to those reported in the current manuscript. The novelty of the current

manuscript, therefore, is largely limited to the rapid kinetic study of the movements of the CCA end of the peptidyl-tRNA on the 50S subunit using a BODIPY-labeled, A-site peptidyl-tRNA.

The main focus of this paper is the timing of tRNA movement on the 50S subunit, compared to the movement of mRNA and tRNA on the 30S subunit. This is stated on p. 6: “In contrast [to the movement on the 30S subunit], due to the lack of suitable reporters, the movement on the tRNA acceptor end of the 50S subunit -... has not been examined kinetically”. We for the first time use a fluorescence reporter group which monitors the movement on the 50S subunit and the kinetic information about this step was not available before. Measurements with labels in the mRNA or the tRNA body are necessary to compare the rates of 50S and 30S translocation, as explained on p. 6-7. We note that the outcome of the present experiments is rather surprising, because the current models propose that 50S translocation is complete before the 30S translocation takes place. Furthermore, parallel analysis of translocation of the 30S and 50S subunit allowed us to elucidate the coupling between EF-G domain 4 and GTP hydrolysis (p. 12-13). These conclusions are novel and were not possible without following translocation separately on the 30S and 50S subunit.

However, it should be noted that several rapid kinetic studies of the movements of the CCA end of the peptidyl-tRNA on the 50S subunit using puromycin reactivity have been previously published by various groups (Semenkov et al (2004) FEBS Letters; Sharma et al (2004) RNA; Pan et al (2007) Mol Cell) and that these studies arrived at conclusions regarding the movements of the CCA end of the peptidyl-tRNA on the 50S subunit that overlap with the conclusions that are presented in the current manuscript.

In this paper, we for the first time use a time-resolve coupled translocation-puromycin assay, which allowed us to deduce the rate of tRNA movement into the authentic puromycin-reactive P site on the 50S subunit. This provides an additional validation for the measurements with the BODIPY label on the peptidyl-tRNA. In all previous publications, puromycin was used either as a test to follow very slow translocation (such as observed in the presence of antibiotics), or to measure the rate of puromycin reaction itself in a particular state, such as in A/P state (Semenkov, Sharma) or an antibiotic-stabilized state (Pan); however, the rate of rapid, authentic translocation was not monitored. Nobody has so far made a deconvolution of coupled time-resolved translocation and puromycin reaction, which we apply here for the first time. The new assay was developed specifically to provide an additional independent observable for the rate of translocation on the 50S subunit. We added a few words on p. 7 to state this fact more clearly

Similarly, rapid kinetic studies of translocation using labeled tRNAs, labeled mRNA, and/or puromycin reactivity have been used to study the effects of viomycin (Peske et al (2004) J Mol Biol), spectinomycin (Peske et al (2004) J Mol Biol), streptomycin (Peske et al (2004) J Mol Biol), hygromycin B (Peske et al (2004) J Mol Biol), EF-G(H91A) (Cunha et al (2013) Translation), EF-G(delta4/5) (Savelsbergh et al (2000) J Mol Biol), and EF-G(H583K) (Savelsbergh et al (2000) J Mol Biol) on translocation, again arriving at conclusions that overlap with the conclusions presented in the current manuscript. In light of these previous studies, therefore, the novelty, significance, and impact of the current manuscript seem somewhat incremental.

Previously, we and others could only use fluorescence reporters monitoring translocation on the 30S subunit. Puromycin assays, which were all carried out in a way which did not allow to resolve rapid translocation kinetics (see explanations above), provided information about slow reactions only. In contrast to these previous publications, we now show intermediate states of translocation on the 50S subunits and assign the role of GTP hydrolysis and domain 4 of EF-G by comparing the timing of translocation on the two subunits. Antibiotics and EF-G mutants (which both are well-characterized and validated) are exploited as tools to challenge the system and to elucidate different contributions e.g. of GTP hydrolysis or domain 4-promoted reactions. This is explained in the text, starting from p.5 on.

2. Two cryo-electron microscopy studies of EF-G-bound pre-translocation complexes carrying an A-site peptidyl-tRNA have been recently published in PNAS (Brilot et al (2013) PNAS; Ramrath et al (2013) PNAS). The conformation of the A-site peptidyl-tRNA in these structures is unique relative to the conformation of the A-site peptidyl-tRNA that is observed in pre-translocation complexes imaged in the absence of EF-G (e.g., Agirrezabala et al (2008) Cell; Julian (2008)

PNAS) and is apparently only observed in the presence of bound EF-G. Based on the fact that it is observed exclusively in the presence of bound EF-G, it is exceedingly likely that the conformation of the A-site peptidyl-tRNA that is observed in these cryo-electron microscopy studies is equivalent to the A/P2 conformation of the A-site peptidyl-tRNA that is also observed exclusively in the presence of bound EF-G in the current work. Unfortunately, this further limits the novelty, significance, and impact of the current manuscript.

We included the discussion about the states observed by Brillot et al. and Ramrath et al. on p. 4 and 18. Brillot et al. report a structure of a viomycin-stalled intermediate, which – as our data would imply – is distinct from the A/P2 state. Also the intermediate state described by Ramrath et al. is probably a much later event, because the 50S translocation and the movement of the mRNA are had occur in that state stalled by fusidic acid and GDP. Notably, both papers appeared after we submitted the manuscript. Furthermore, cryo-EM (or any other structural method) cannot determine the timing of the appearance of a particular intermediate. Kinetic methods provide the time axis for the structural studies and are thus by definition provide information that is orthogonal to the structural work. Thus, we do not consider the above cited structural papers as limiting the impact of our work, as the results nicely complement each other.

3. On pg3, par1 of the manuscript, the authors note that "the hybrid/rotated state is not a single intermediate" and that "a variety of distinct hybrid states have been identified." Nonetheless, the data analysis, interpretations, conclusions, and mechanistic model that are presented in the current manuscript treat the hybrid/rotated state as a single intermediate. No data or arguments are presented to justify this simplified treatment and, although it certainly makes the data analysis, interpretations, conclusions, and mechanistic model simpler, it is not clear that this simplified treatment is justified.

The simplified treatment is justified by the fact that all these presumed distinct hybrid states are not puromycin-reactive to any significant extent (Semenkov, Sharma, and our measurements shown in Fig. 1B). Furthermore, we show that conformation of the ribosome from the predominantly classical to predominantly hybrid state does not change the fluorescence of our Bodipy reporter (p. 9). Thus, all reactions that we study do not pertain to the hybrid state(s), but rather to later intermediates of translocation which are specifically induced by EF-G. Furthermore, 50S translocation follows predominantly single-exponential kinetics (p. 7), which implies that there is no heterogeneity with respect to the different pathways from different hybrid/rotated states).

4. There are several misstatements and referencing errors, as listed below, that should be corrected:

(a) pg3, par1

"This pre-translocation (PRE) complex is dynamic and fluctuates between the classical state, where the tRNAs are located in the A and P sites on both 30S and 50S subunits (A/A and P/P states), and hybrid states, where the acceptor domains of the tRNAs are moved towards the P and E sites, while the anticodon domains remain bound in the A and P sites (A/P and P/E states) (Moazed & Noller, 1989)."

This statement is lacking a reference. The finding that the pre-translocation complex is dynamic, such that the tRNAs fluctuate between classical and hybrid states was first reported by Blanchard et al (2004) PNAS; this article should be referenced here.

We have introduced that reference as requested.

(b) p3 par1

"Structurally, the hybrid/rotated state is not a single intermediate, because a variety of distinct hybrid states have been identified. These sub-states (denoted as states H1 and H2 (Munro et al, 2010a), MSI and MSII (Fu et al, 2011), classes 2, 4A, 4B, 5 and 6 (Agirrezabala et al, 2012), or PRE1-5 states (Fischer et al, 2010)) differ in the orientation of the tRNAs, the degree of subunit rotation, the conformation of the 30S subunit, and the position of the L1 stalk."

There are several misstatements and a few missing references in this statement. Fu et al (2011) PNAS makes no reference to structures labeled "MSI" or "MSII" and, generally speaking, structures from the Frank group labeled "MSI" typically refer to a classical/unrotated state, not a hybrid/rotated state. Similarly, classes 2 and 4A in Agirrezabala et al (2012) PNAS refer to classical/unrotated states, not hybrid/rotated states. In addition, classes 5 and 6 in Agirrezabala et al (2012) PNAS are very similar to each other and to the MSII hybrid/rotated structure that has been previously

characterized by the Frank group (Agirrezabala et al (2008) Cell). Strictly speaking, Fischer et al (2010) Nature refers to structures relevant to the process of tRNA-binding-induced retro-translocation; it is unclear how these structures relate to the process of EF-G-catalyzed translocation; indeed, this is not clearly stated or discussed anywhere in the current manuscript despite the fact that findings reported in Fisher et al (2010) Nature are cited as relevant for EF-G-catalyzed translocation throughout the current manuscript. Regardless, classes PRE1 and, possibly, PRE2 in Fischer et al (2010) Nature refer to classical/unrotated states, not hybrid/rotated states. Finally, the GS2 state described in Fei et al (2008) Mol Cell and the R2 state described in Zhang et al (2009) Science should also be included and cited in this list.

The sentence was actually meant to refer to the classical AND rotated/hybrid state. The respective change was introduced in the sentence "the classical and rotated/hybrid states (denotes as...). The reference to Fu et al. was replaced by the correct reference (Frank and Gonzalez). The other two references were also included.

Concerning the detailed comparison between different states reported by Frank et al. and other groups, we are not in the position to perform such detailed analysis, as this should be done by specialists in cryo-EM. We just summarize the states which have been reported in the literature. Further structural analysis and comparisons is beyond the scope of the present paper, which deals with kinetics of EF-G-promoted translocation, rather than with cryo-EM structures.

(c) p5, par5

"The ribosome is intrinsically dynamic and functions as a Brownian machine that undergoes spontaneous conformational fluctuations driven by thermal energy, which can be coupled to directed motion (Fischer et al, 2010; Munro et al, 2010b)."

There are some missing references here: Spirin (2009) J Biol Chem and Frank et al (2010) Ann Rev Biochem.

The references were added.

(d) p8, par2

"Previous cryo-EM studies have shown that the ribosomes in classical and rotated/hybrid conformations are in equilibrium with each other under experimental conditions similar to those used here (Agirrezabala et al, 2008; Fischer et al, 2010; Julian et al, 2008)."

The fact that pretranslocation ribosomes in classical/unrotated and hybrid/rotated conformations are in equilibrium with each other under experimental conditions similar to those used here was first shown by Blanchard et al (2004) PNAS, Munro et al (2007) Mol Cell, Fei et al (2008) Mol Cell, and Cornish et al (2008) Mol Cell; these papers should be referenced here.

The references were included, although we note that the conditions of the single molecule experiments are quite different from those used in cryo-EM or bulk kinetic studies.

(e) p8, par2

"EF-G binding strongly shifts the equilibrium towards the hybrid/rotated state by reducing the rate of the conversion from the hybrid to classical state (Cornish et al, 2008; Munro et al, 2010b);"

The first article to report that EF-G binding strongly shifts the equilibrium towards the hybrid/rotated state by reducing the rate of the conversion from the hybrid to classical state was Fei et al (2008) Mol Cell; this article should be referenced here.

Done. The sentence has been shifted to a later part of the text.

(f) p15, par1

"Thus, while the hybrid/rotated states are authentic intermediates of translocation reflecting the intrinsic dynamics of the ribosome that are crucial for translocation, the kinetics of these fluctuations are unlikely to be rate-limiting for EF-G recruitment or tRNA translocation."

This statement seems like a broad generalization and an oversimplification. While the kinetics of fluctuations between classical/unrotated and hybrid/rotated states seem to not be rate-limiting for EF-G recruitment or tRNA translocation under the conditions investigated in the present work, one can easily imagine conditions under which these fluctuations could easily become rate limiting.

Indeed, any obligatory step in a complex, multi-step biochemical reaction such as translocation could easily become rate-limiting under the appropriate conditions.

We changed the sentence to avoid over-generalization.

Referee #3:

Review of Holtkamp et al., "GTP Hydrolysis by EF-G synchronizes tRNA movement...."

This paper describes kinetic experiments using fluorescent labels attached to the mRNA and to the peptidyl moiety of tRNA to measure movement of mRNA and of the acceptor end of tRNA during EF-G-dependent translocation. The reaction is followed at different temperatures, as a function of Mg ion and EF-G concentrations, and in the presence of a series of antibiotics and with mutant versions of EF-G that affect different aspects of the mechanism. In the absence of GTP hydrolysis, neither movement of mRNA on the 30S subunit nor complete movement of the acceptor end of the peptidyl-tRNA into the 50S P site take place. The authors conclude that the final steps of translocation on both the 30S and 50S subunits depend on hydrolysis of GTP by EF-G.

I believe that this is an excellent paper that clarifies several critical aspects of the translocation mechanism, and that it should be published in the EMBO J. The following points need to be addressed:

1. p.8, paragraph 1: "...movement of the 3' end of deacylated tRNA on the 50S subunit..." As the experiment mentioned uses the Prf20 label, doesn't the experiment show movement of the tRNA elbow, rather than its 3' end, strictly speaking?

Absolutely. The sentence was corrected.

2. Fig. 2A: What is meant by "P" in the diagram? I am guessing that it is the post-translocation state, but it could also mean "products".

Yes, it is POST; we changed the scheme respectively.

3. p.10, bottom line: How can binding of streptomycin trap the 30S subunit in a conformation that is inherently more prone to rapid translocation and at the same time inhibit translocation?

This has been deduced by comparing the effects of streptomycin on the ground state and transition state of translocation in Peske et al., 2004. We changed the text on p. 11 to explain this.

4. p.15, 2nd paragraph, line 10: "...all three reactions..." It is not obvious which three reactions are meant.

Changed to make clear which reactions are meant.

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Thank you very much for submitting the revised version of your manuscript. It has now been seen by one of the original referees who finds that all original criticisms have been sufficiently addressed (comment included below). I am therefore happy to inform you that your manuscript has

been accepted for publication in *The EMBO Journal*

REFEREE REPORT

Referee #3:

The authors have responded to all my points. I am satisfied with the revised manuscript.