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Structures and dynamics of hibernating ribosomes from *S. aureus* mediated by intermolecular interactions of HPF

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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 15 February 2017

Thank you for submitting your manuscript for consideration by The EMBO Journal and my apologies again for the extended duration of the review period. Your manuscript has now been seen by three referees and their comments are shown below. As you will see, while the referees express interest in the work and topic in principle, I am afraid they do not offer strong support for publication in The EMBO Journal.

I will not repeat all individual points of criticism here, but you will see that all three referees find that the resolution stated for the 100S complex is potentially misleading, since it reflects docking/modelling based on a lower-resolution structure. Combined with the flexibility seen in the dimers this leads the referees to question the overall conclusiveness of the presented interaction model. In addition, the referees find that additional work on the mechanism of inhibition and the implications for translational control would be needed for them to support publication here. Clearly, an extensive amount of further experimentation would be required to address these issues and to bring the study to the level of insight and significance required for publication here.

Given these consistent, negative opinions from the referees, I am afraid we are unable to offer

further steps towards publication in The EMBO Journal.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.

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REFEREE REPORTS

Referee #1:

Khusainov etal report on the structure of the hibernating 100S ribosome from Staphylococcus aureus using single particle cryo-EM. Under stress conditions, bacteria down regulate the protein synthesis, which is in some cases accompanied by dimerization of 70S ribosome to so-called 100S ribosomes by binding of a hibernation promoting factor (HPF). The mechanism of translation inhibition by protein factors such as HPF, YifA and pY is well-understood based on high resolution structures. However, the reasons for dimerization and the mechanistic implications of 100S dimer formation remain elusive.

Although the authors were able to obtain a reconstruction of the S. aureus 100S ribosomal dimers these insights may not be particularly useful for understanding the function since the relative arrangement of ribosomes in the 100S dimers is flexible and may be influenced by the cryo-EM grid preparation process. It is also unlikely that the features of the 100S dimer will be conserved across species raising questions about the mechanistic implications of the obtained structure.

Furthermore, there are numerous technical weaknesses of the manuscript related to the processing and analysis of the cryo-EM data, listed below.

Overall, this manuscript is not suitable for publication in EMBO J.

Major concerns:

1) According to the procedure described as strategy 2, cryo-EM reconstruction of the 70S ribosome was calculated using particle images of monomeric 70S ribosomes as well as of 70S ribosomes of 100S dimers. The resulting high resolution 70S map was twice fitted into a low resolution 100S map and the in silico reconstituted 100S map was used for the interpretation. Obviously, particle images of monomeric and dimeric 70S ribosomes have been merged to interpret the 100S dimer. Although the authors claim that HPF is bound to all 70S, there is no evidence that the conformation of the factor and of the ribosome is the same in the monomeric and the dimeric states. Furthermore, it is misleading to claim that the 100S particle was resolved at near-atomic resolution only by fitting the high resolution 70S map into the 100S map that was actually resolved at low resolution (11Å for the tight dimer). Strategy 2 is therefore not valid and the authors cannot claim to have resolved the 100S dimer at near-atomic resolution.

2) The C-terminal domain (CTD) of HPF is potentially the most interesting part of the structure, because it appears to mediate the 70S dimerization. However, the local resolution plots of the disomes indicate a local resolution of the 70S interaction sites of 15 Å. At this resolution it is not possible to unambiguously fit the model of the HPF-CTD. Furthermore, it is not clear from the text and the shown figures, whether the density for the HPF-CTD dimer was also resolved in the high-resolution 70S map. The density shown in Figure 3 assigned to HPF-CTD dimer seems to have a significant lower resolution compared to the surrounding density and it is not clear from which map ("density maps for unrotated ribosome-bound HPF") the carved-out density is derived from.

Minor concerns:

1) The 100S dimers used for structural analysis were in vitro assembled by incubating purified and high salt-washed 70S ribosomes with recombinantly expressed HPF. The sucrose gradient indicates that the majority of 70S ribosomes dimerize to form 100S disomes under the chosen conditions.

However, there are native 100S dimers present in the ribosome sample purified from S.aureus (see sucrose gradient in Fig. 1A). What are the reasons not to use these 100S dimers for structural analysis?

2) If HPF of S.aureus is present as dimer as predicted by the NMR analysis and binds the 70S ribosome as dimer, would it not also be more reasonable that the hibernating S.aureus ribosomes have a HPF dimer bound (see Fig. 6).

3) In contrast to features of the 2D class average of the tight and the loose dimer (see Fig. 2B) the 3D reconstruction of the loose dimer was resolved to "higher" resolution (9 Å) compared to the tight dimer (11 Å) using almost identical number of particle images. The authors should check and comment.

4) In the introduction, the hibernation mechanism of E.coli, which is the best studied so far, is compared with the mechanisms in other species. The authors state that the hibernation factors promote dimerization in different ways based on the species. How big are these differences between species expressing the long form of HPF? Also, if this is the case what is the purpose of studying dimers in one specific species?

5) The recombinantly expressed HPF was finally purified by SEC and the elution profile indicates two major peaks containing HPF - of which the one with the higher A260/A280 ratio was more efficient in formation of 100S dimers. This is suspicious and additional analysis of the sample by SDS-PAGE or RNA gel electrophoresis should be added to indicate the purity of the sample.

6) Expansion Figure 4 does not provide additional information to the reader and can be removed.

Referee #2:

The manuscript entitled "Structures and dynamics of hibernating ribosomes..." by Khusainov et al reports 11 A and 9 A cryo-EM structures of in vitro reconstituted hibernating 70S ribosome dimers (disomes) from Staphylococcus aureus. The authors modeled the structure of the N-terminal domain (NTD) of the S. aureus hibernation promoting factor (SaHPF) on the basis of its homology to the Escherichia coli- and plastid-specific homologs of SaHPF and, consistent with the E coli- and plastid-specific homologs of the SaHPF NTD, find that the NTD binds at the A site of the small ribosomal subunit. Because binding of the SaHPF NTD and its homologs at this position would sterically block the binding of initiation factors and tRNAs to the A site, the structure rationalizes the ability of these proteins to block translation during hibernation. The authors also find that the C-terminal domain (CTD) of SaHPF protrudes through the E site of the small ribosomal subunit, where it uses a CTD-CTD dimerization interaction to mediate the formation of the disome. The authors initially model the structure of the CTD using its sequence similarity to a protein of known structure, the Lmo2511/Y protein. They then validate this model using NMR structural studies of a construct consisting of only the isolated CTD. The NMR studies not only confirm the authors' modeling of the CTD structure, but they also provide strong evidence in support of the CTD-CTD dimerization interaction. In addition to the CTD-CTD dimerization interaction, disome formation is further strengthened through interactions between the 16S ribosomal RNA helix 26 of the two ribosomes in the disome. Finally, the authors find that disomes can form regardless of whether the ribosomal subunits occupy rotated or unrotated conformations.

In principle, the structures reported here represent a significant advance in the field and in our understanding of the mechanisms through which ribosomes hibernate during stress conditions. Nonetheless, I have several important questions and concerns regarding the methods and interpretation of the data that I think the authors would need to address before a determination could be made regarding publication in EMBO J. These are as follows:

1. It is not clear to me why the authors have chosen to in vitro constitute the hibernating ribosomes rather than simply purifying hibernating ribosomes from cells grown under stress conditions (e.g., purified from cells grown into stationary phase). This raises the question of whether or not structures of in vitro reconstituted hibernating ribosomes reflect structures of physiologically assembled hibernating ribosomes. Do the authors have any evidence that structures of in vitro reconstituted

hibernating ribosomes are physiologically relevant?

2. The description of the strategy for visualizing the disomes at near-atomic resolution is a bit convoluted and difficult to follow. If I understood it correctly, however, it seems that the authors have generated a near-atomic resolution model of the hibernating ribosomes by fitting a previously reported, cryo-EM-derived, 3.7 A structure of the S. aureus ribosome, a homology model of the SaHPF NTD, and an NMR-validated homology model of the SaHPF CTD into the 11 A resolution structure of the S. aureus "tight" 100S dimer. Given the uncertainties associated with an 11 A cryo-EM map and homology models, is it possible that the authors are over interpreting their models? For example, is it really justified to model the helix 26-helix 26 interactions, the bL31 movements, and the inter-small ribosomal subunit interactions in as high a level of detail as is shown in Fig 3A and 3B, Fig 4D, and Fig 5A and 5B, respectively?

3. The authors make spend a significant portion of the manuscript describing the fact that SaHPF can bind to ribosomes regardless of whether the ribosomes are rotated or unrotated. They interpret this as evidence that SaHPF can act to hibernate ribosomes during any step of translation. This raises several questions for me: (1) What ribosomal complex, or complexes, does SaHPF target? Is there any evidence that SaHPF targets and acts on a specific ribosomal complex or, more in line with what I think the authors are suggesting in their interpretation, that it can target and act on any ribosomal complex? (2) Could the authors observations and the answers to the questions I have raised here be influenced by the fact that the authors in vitro reconstituted the hibernating ribosomes rather than purified them out of cells grown under stress conditions? In other words, would the authors expect that physiologically assembled hibernating ribosomes purified out of cells grown under stressed conditions would exhibit the same subunit rotation properties or could this be an artifact of the in vitro reconstitution?

4. Given the structures that the authors have solved and the authors interpretations of these structures, can the authors explicitly propose a mechanism for SaHPF-mediated ribosome hibernation? The authors discuss some features of such a mechanism very briefly near the end of the Discussion section of the manuscript, but they stop short of proposing a mechanism. In any case, should it be possible for the authors to propose such a mechanism it would greatly increase the impact and significance of this manuscript.

5. This is more of a minor point, but I am not sure that the word "dynamics" is justified in the title of this manuscript. The manuscript describes cryo-EM structures of hibernating ribosomes, but it is not clear that these structures reveal anything about the dynamics of hibernating ribosomes.

Referee #3:

Hibernation promoting factor (HPF) causes Staphylococcus aureus ribosomes to dimerize. Khusainov et al present the structures of two different configurations of disome - solved at 9 Å and 11 Å. Using a masking approach they resolve the HPF-bound ribosome within the dimer to 3.7 Å. This higher-resolution map is used to model HPF. The N-terminal domain (NTD) binds at the intersubunit interface and prevents translation, as expected from homology with Escherichia coli HPF. The C-terminal domain (CTD) of S. aureus HPF is absent in E. coli and mediates dimerization of 70S ribosomes by forming a homo-dimer. The authors present an NMR structure of the CTD. Overall, their results demonstrate that the formation and morphology of disomes in S. aureus differs from in E. coli, which may have implications for the development of specific antibiotics.

Comments

1. The abstract needs to be revised as it currently suggests that the structure of the disome has been solved at near-atomic resolution. This is misleading as the best resolution for the disome is 9 Å. The wording needs to be carefully considered throughout the manuscript. For example, fitting the 3.7 Å structure into the lower resolution disome map does not "reconstruct a structure at near-atomic resolution of the 100S disome".

2. The abstract states "disomes form whether ribosomes adopt unrotated or rotated conformations, suggesting that translation can be paused by SaHPF at any stage". This sentence is misleading as

presumably 100S complexes can only form from empty 70S ribosomes - the bound tRNAs of actively translating ribosomes would prevent SaHPF binding. Similar statements appear throughout the manuscript. For example, why would accommodating rotated and unrotated states suggest "an efficient mechanism for stalling and resuming translation in a timely manner"?

3. The abstract and text elsewhere suggests that the dimerization mode of bacterial ribosomes is "species-specific". However, the presented structure only shows that S. aureus uses a different mechanism for E. coli. This may reflect a difference between γ-proteobacteria and all other ribosomes, rather than species-specific mechanisms.

4. In the introduction the CTD is described as being "marginally similar" to RMF. What does this mean? Are the authors implying a common evolutionary ancestor and is this in agreement with their structure?

5. The introduction says that the only available structural information is for the NTD of various HPFs. However, this ignores structures for the CTD (PDB IDs 3KA5 and 3K2T) that the authors themselves use to build a homology model.

6. The main text suggests that the NMR structure of the HPF CTD was used to model the EM density, however this doesn't agree with the material and methods, which says that a homology model was used and makes no mention of the NMR structure. This seems a wasted opportunity to use complementary information from cryo-EM, NMR and X-ray crystallography to build the best possible model for the CTD of the Staphylococcus aureus HPF.

7. "Prior to this work, no hibernation factors had been described that directly mediated dimerization" should be removed from the text.

8. The authors speculate that a single hibernation factor rather than two as in E. coli could "enhance bacteria's timeliness in blocking translation", but the flip side of this should also be mentioned - that the opportunities for regulation may be reduced.

9. Please expand upon the data acquisition settings. The statement "camera was set up to collect 7 frames (starting from the second one) out of 17 possible. Total exposure was 1 s" is unclear. Does this mean that 17 frames were collected over 1s, but only frames 2-8 were processed?

10. The "average resolution was...~9-12 Å for tight and loose disomes" doesn't agree with Table 1, which gives 11.0 Å for the tight disome and 9.0 Å for the loose disome.

11. Is there a reason that the tight disome is at a lower resolution than the loose disome despite similar numbers of particles? A tight disome might be expected to display less flexibility and therefore be resolved to a higher resolution. What is the difference in the interface between these two states?

12. Figure 2E - the title "In silico reconstituted 100S dimer at 3.7 Å" should be changed to remove the resolution estimate which is misleading.

13. The fit of the model of the CTD to the density in figure 3B is not convincing. Please provide additional views.

14. I would like to see some NMR figures (for example, EV6A and EV6D) moved to the main text.

15. The reference for Khusainov et al, 2016 should be updated now that the paper is published.

16. The authors present some AUC data, could AUC also be used to see if SaHPF is a dimer in solution? This would help answer one of the questions that the authors propose in the discussion.

17. "electron density" should be replaced with "map density" or equivalent.

18. I could not follow the significance of the bL31 movement. Is this a specific response to the binding of SaHPF or a signature of intersubunit rotation?

Additional Correspondence – Authors 16 February 2017

Thank you for sending us the comments from the three reviewers who accepted to evaluate our work. We are sorry about your editorial decision, although we understand it based on the tone of the reviewers comments.

However, as you can see from our draft of a point-by-point response below, we can address all of the reviewers' concerns through rewording of our manuscript. Hence, we basically find that the claims that our work shows "technical weaknesses" (reviewer #1) or that we do not have a high-resolution structure of hibernating ribosomes (reviewers #2 and #3) are not substantiated. Furthermore, reviewer #2 is overall positive and all comments from reviewer #3 are minor. The negative impression probably comes from our writing that we now see was in need of improvement.

Our data in themselves are neither ambiguous, nor lacking resolution or depth. We did obtain low resolution structures of the full dimer, but by focused refinement, we obtained a high resolution structure of each monomer within the dimer. In short, our strategy was a processing of the same data in two different ways, because both bring valuable insights. In this high resolution structure, we had density for the NTD of SaHPF, for L31, etc, as shown in several figures. Although the resolution of the CTD was too low to be able to build it, it was high enough to dock the structure we separately solved by NMR. We therefore have a structure at 3.7 angstroms of a hibernating ribosome from S. aureus, and not merely a 9 or 11 angstrom structure in which we docked a high resolution structure. We will explain this more clearly in the methods and the main text.

Our work therefore represents a breakthrough toward fully unraveling translation inhibition in a pathogen, as highlighted by reviewers #2 and #3 (we have reasons to believe that reviewer #1 may have conflicts of interest). Importantly, all our claims are supported by structural observations, without over-interpretation, in a manner similar to that of the paper by the Ban group of the chloroplast 70S ribosome that was published in yesterday's issue of the EMBO Journal (Feb 15), without any additional experimental data.

Intricate as it may seem -especially to colleagues outside of the structural biology and even cryo-EM fields- our strategy for obtaining high-resolution hibernating ribosomes is a standard way to process data of large assemblies. We indicate several references as examples in our response below. Again, the overall negativity of the reviewers probably stemmed from our lack of clarity in our writing, and their comments will now help us address them in full.

We hope that our arguments will encourage you to reconsider your decision regarding the suitability of our manuscript for the EMBO Journal, following text editing. As you already now know, there is a strong competition on this topic from what we have learned through the grapevine, and we were really counting on the EMBO Journal's reputation for prompt publishing.

Referee #1:

Khusainov etal report on the structure of the hibernating 100S ribosome from Staphylococcus aureus using single particle cryo-EM. Under stress conditions, bacteria down regulate the protein synthesis, which is in some cases accompanied by dimerization of 70S ribosome to so-called 100S ribosomes by binding of a hibernation promoting factor (HPF). The mechanism of translation inhibition by protein factors such as HPF, YifA and pY is well-understood based on high resolution structures. However, the reasons for dimerization and the mechanistic implications of 100S dimer formation remain elusive.

Although the authors were able to obtain a reconstruction of the S. aureus 100S ribosomal dimers these insights may not be particularly useful for understanding the function since the relative arrangement of ribosomes in the 100S dimers is flexible and may be influenced by the cryo-EM grid preparation process. It is also unlikely that the features of the 100S dimer will be conserved across species raising questions about the mechanistic implications of the obtained structure.
Furthermore, there are numerous technical weaknesses of the manuscript related to the processing and analysis of the cryo-EM data, listed below.

Overall, this manuscript is not suitable for publication in EMBO J.

Major concerns:
1) According to the procedure described as strategy 2, cryo-EM reconstruction of the 70S ribosome was calculated using particle images of monomeric 70S ribosomes as well as of 70S ribosomes of 100S dimers.

Yes, but in all cases ribosomes contained SaHPF, and they adopted rotated and unrotated conformations as revealed by the classification (figure EV2).

The resulting high resolution 70S map was twice fitted into a low resolution 100S map and the in silico reconstituted 100S map was used for the interpretation. Obviously, particle images of monomeric and dimeric 70S ribosomes have been merged to interpret the 100S dimer.

This sentence is the same as the first one in the paragraph. Particles from both 70S and 100S were included during processing, and they were divided into two categories (rotated and unrotated, but all of them contained SaHPF. It would not be possible to obtain 80,000 particles for each rotated and unrotated category of monomeric ribosomes bound to SaHPF with a 3.7 angstrom resolution (see figure EV5) if there was structural heterogeneity! Furthermore, we did remove >50% (185,000) of the particles in the process that did not fit in either of those two categories.

Although the authors claim that HPF is bound to all 70S, there is no evidence that the conformation of the factor and of the ribosome is the same in the monomeric and the dimeric states.

Perhaps we can reword our claims in that section, in order to make it more clear that we do not claim to visualize conformational changes of SaHPF. We merely show contacts between SaHPF and the ribosome in both the rotated and unrotated states, which are fully supported by our 3.7 angstrom map (see for example fig EV5).

Furthermore, it is misleading to claim that the 100S particle was resolved at near-atomic resolution only by fitting the high resolution 70S map into the 100S map that was actually resolved at low resolution (11Å for the tight dimer).

Yes, the reviewer is right that we did not solve the structure of 100S particles at 3.7 angstroms. As we say in our manuscript (for example, first sentence of the last paragraph of the introduction), "we obtained the structure at near-atomic resolution (3.7 Å) of hibernating SaHPF- bound ribosomes from S. aureus by cryo-electron microscopy". 'Hibernating' refers to inactive ribosomes bound to SaHPF. But we will edit our manuscript wherever we may have misleadingly written that 100S particles were solved at high resolution.

Our procedure is actually standard when working with complex architectures that comprise multiple subunits. A recent structure of an 80S ribosome was refined in a similar manner to ours, with the 40S and 60S subunits separately fitted in two maps, as follows (quote from the original article):"The Pf80S atomic model was refined as separate 60S and 40S subunits in the two maps that were obtained for either subunit in the focused refinements of the cryo-EM reconstructions." see model building and refinement section in https://elifesciences.org/content/3/e03080

Strategy 2 is therefore not valid and the authors cannot claim to have resolved the 100S dimer at near-atomic resolution.

Strategy 2 is valid in the sense that it did give a high resolution of individual ribosomes within dimers, and showed the sites of interaction between ribosomes.

2) The C-terminal domain (CTD) of HPF is potentially the most interesting part of the structure, because it appears to mediate the 70S dimerization. However, the local resolution plots of the disomes indicate a local resolution of the 70S interaction sites of 15 Å. At this resolution it is not possible to unambiguously fit the model of the HPF-CTD. Furthermore, it is not clear from the text and the shown figures, whether the density for the HPF-CTD dimer was also resolved in the high-resolution 70S map. The density shown in Figure 3 assigned to HPF-CTD dimer seems to have a

significant lower resolution compared to the surrounding density and it is not clear from which map ("density maps for unrotated ribosome-bound HPF") the carved-out density is derived from.

The low resolution at the site of interaction between the CTD (as one would expect from working with such a large assembly) motivated us to obtain a separate NMR structure of the CTD, in order to see more clearly the interface. Fitting this domain back into the low res map in order to be able to interpret it is a standard procedure in structural biology. Our resolution was sufficient to dock our NMR structure.

See for example:

in Cell: see figure 6 (C)
http://www.sciencedirect.com/science/article/pii/S0092867415011897

in Nature: "Rosetta electron-microscopy-guided symmetrical docking for the unresolved InvG N0 and N1 domains was carried as previously described2 using the InvG34–173 X-ray crystal structure (PDB accession number 4G08; truncated C terminus to residue 171) and the unaveraged basal body reconstruction as a restraint."
http://www.nature.com/nature/journal/v540/n7634/full/nature20576.html

in NSMB: the authors dock a structure solved at high resolution into a filtered map of a complex secretion system 2 of bacteria.
http://www.nature.com/nsmb/journal/v24/n2/full/nsmb.3350.html

Minor concerns:

1) The 100S dimers used for structural analysis were in vitro assembled by incubating purified and high salt-washed 70S ribosomes with recombinantly expressed HPF. The sucrose gradient indicates that the majority of 70S ribosomes dimerize to form 100S disomes under the chosen conditions. However, there are native 100S dimers present in the ribosome sample purified from S. aureus (see sucrose gradient in Fig. 1A). What are the reasons not to use these 100S dimers for structural analysis?

Please, see response to point #1 by reviewer #2 below

2) If HPF of S.aureus is present as dimer as predicted by the NMR analysis and binds the 70S ribosome as dimer, would it not also be more reasonable that the hibernating S.aureus ribosomes have a HPF dimer bound (see Fig. 6).

Only the CTD forms a dimer in solution, not the full-length protein and not the NTD (see reference to Ayupov et al 2016 in the manuscript). Additionally, please see response to minor point #5 reviewer 1.

3) In contrast to features of the 2D class average of the tight and the loose dimer (see Fig. 2B) the 3D reconstruction of the loose dimer was resolved to "higher" resolution (9 Å) compared to the tight dimer (11 Å) using almost identical number of particle images. The authors should check and comment.

Please, see response to point #11 by reviewer #2 below.

4) In the introduction, the hibernation mechanism of E.coli, which Is the best studied so far, is compared with the mechanisms in other species. The authors state that the hibernation factors promote dimerization in different ways based on the species. How big are these differences between species expressing the long form of HPF? Also, if this is the case what is the purpose of studying dimers in one specific species?

The work in our laboratories is focused on the translational aspects of Staphylococcus aureus. This bacterium is a severe human pathogen, which in addition is highly resistant to most of commonly used antibiotics. The translation machinery is a great target for development of new antimicrobials against S. aureus. And the finding of our research may be directly applied for medical reasons.

5) The recombinantly expressed HPF was finally purified by SEC and the elution profile indicates two major peaks containing HPF - of which the one with the higher A260/A280 ratio was more efficient in formation of 100S dimers. This is suspicious and additional analysis of the sample by SDS-PAGE or RNA gel electrophoresis should be added to indicate the purity of the sample.

We shared this unusual finding, which might be useful for the researchers working in the field. We also raised this question in the discussion section. The presence of an additional substance which made SaHPF protein active remains unclear. We proposed that this substance might prevent self dimerization of the protein in the cell. Additional SDS-PAGE analysis or RNA agarose gel electrophoresis didn't clarify this issue. Therefore, this will be a matter of further investigation.

6) Expansion Figure 4 does not provide additional information to the reader and can be removed.

We will remove this figure.

Referee #2:

The manuscript entitled "Structures and dynamics of hibernating ribosomes..." by Khusainov et al reports 11 A and 9 A cryo-EM structures of in vitro reconstituted hibernating 70S ribosome dimers (disomes) from Staphylococcus aureus. The authors modeled the structure of the N-terminal domain (NTD) of the S. aureus hibernation promoting factor (SaHPF) on the basis of its homology to the Escherichia coli- and plastid-specific homologs of SaHPF and, consistent with the E coli- and plastid-specific homologs of the SaHPF NTD, find that the NTD binds at the A site of the small ribosomal subunit. Because binding of the SaHPF NTD and its homologs at this position would sterically block the binding of initiation factors and tRNAs to the A site, the structure rationalizes the ability of these proteins to block translation during hibernation. The authors also find that the C-terminal domain (CTD) of SaHPF protrudes through the E site of the small ribosomal subunit, where it uses a CTD-CTD dimerization interaction to mediate the formation of the disome. The authors initially model the structure of the CTD using its sequence similarity to a protein of known structure, the Lmo2511/Y protein. They then validate this model using NMR structural studies of a construct consisting of only the isolated CTD. The NMR studies not only confirm the authors' modeling of the CTD structure, but they also provide strong evidence in support of the CTD-CTD dimerization interaction. In addition to the CTD-CTD dimerization interaction, disome formation is further strengthened through interactions between the 16S ribosomal RNA helix 26 of the two ribosomes in the disome. Finally, the authors find that disomes can form regardless of whether the ribosomal subunits occupy rotated or unrotated conformations.

In principle, the structures reported here represent a significant advance in the field and in our understanding of the mechanisms through which ribosomes hibernate during stress conditions. Nonetheless, I have several important questions and concerns regarding the methods and interpretation of the data that I think the authors would need to address before a determination could be made regarding publication in EMBO J. These are as follows:

1. It is not clear to me why the authors have chosen to in vitro constitute the hibernating ribosomes rather than simply purifying hibernating ribosomes from cells grown under stress conditions (e.g., purified from cells grown into stationary phase). This raises the question of whether or not structures of in vitro reconstituted hibernating ribosomes reflect structures of physiologically assembled hibernating ribosomes. Do the authors have any evidence that structures of in vitro reconstituted hibernating ribosomes are physiologically relevant?

We proceeded by reconstitution from purified partners in order to have a more homogenous material, for the sake of obtaining a sub 4 angstrom resolution. Additionally, we think that the purification of the stressed ribosomes could lead to non-specific aggregation of ribosomes, which would interfere with the analysis of unaltered and aggregated disomes.

2. The description of the strategy for visualizing the disomes at near-atomic resolution is a bit convoluted and difficult to follow. If I understood it correctly, however, it seems that the authors have generated a near-atomic resolution model of the hibernating ribosomes by fitting a previously

reported, cryo-EM-derived, 3.7 A structure of the S. aureus ribosome, a homology model of the SaHPF NTD, and an NMR-validated homology model of the SaHPF CTD into the 11 A resolution structure of the S. aureus "tight" 100S dimer.

From reviewer #2's analysis, it is clear we need to reformulate these aspects in our manuscript! We did not dock here a structure we proviously solved into a low-resolution map. We built a structure of the entire 70S ribosome at 3.7 angstroms, in a hibernating state, and therefore bound to SaHPF, fow which we see density in our complex (See figure EV5).

Given the uncertainties associated with an 11 A cryo-EM map and homology models, is it possible that the authors are over interpreting their models? For example, is it really justified to model the helix 26-helix 26 interactions, the bL31 movements, and the inter-small ribosomal subunit interactions in as high a level of detail as is shown in Fig 3A and 3B, Fig 4D, and Fig 5A and 5B, respectively?

All these structural aspects are observations we made from models in a 3.7 and not 11 angstrom map! The interpretation of the reviewer here is misled by the fact that s/he did not understand that all claims are based on a 3.7 angstrom map, which we show in figures 3B, 4D and EV5 in particular.

3. The authors make spend a significant portion of the manuscript describing the fact that SaHPF can bind to ribosomes regardless of whether the ribosomes are rotated or unrotated. They interpret this as evidence that SaHPF can act to hibernate ribosomes during any step of translation. This raises several questions for me: (1) What ribosomal complex, or complexes, does SaHPF target? Is there any evidence that SaHPF targets and acts on a specific ribosomal complex or, more in line with what I think the authors are suggesting in their interpretation, that it can target and act on any ribosomal complex? (2) Could the authors observations and the answers to the questions I have raised here be influenced by the fact that the authors in vitro reconstituted the hibernating ribosomes rather than purified them out of cells grown under stress conditions? In other words, would the authors expect that physiologically assembled hibernating ribosomes purified out of cells grown under stressed conditions would exhibit the same subunit rotation properties or could this be an artifact of the in vitro reconstitution?

Although this claim is legitimate, it narrows down to the classical criticism of in vitro work and its relevance to explain in vivo situations. We wish to point out that even physiologically assembled ribosomes would be susceptible to any alterations during the normal cryo-EM procedures, which could be similarly criticized by a reviewer! In vitro reconstitution and purification are the methods that allowed us to pinpoint only two major conformations at high resolution, which was unlikely to be obtainable from in vivo-extracted material in our experience. We don't exclude that there may be other conformational rearrangement and we propose to amend the manuscript to emphasize that point.

4. Given the structures that the authors have solved and the authors interpretations of these structures, can the authors explicitly propose a mechanism for SaHPF-mediated ribosome hibernation? The authors discuss some features of such a mechanism very briefly near the end of the Discussion section of the manuscript, but they stop short of proposing a mechanism. In any case, should it be possible for the authors to propose such a mechanism it would greatly increase the impact and significance of this manuscript.

We can expand our discussion in order to further highlight SaHPF-specific mechanistic aspects of ribosome hibernation, which we could derive from our structures and previous data. As always in this case, it is a fine line between formulating a useful hypothesis or simply speculations. Additional experiments that would help pinpoint the specific stages of the hibernation process are beyond the scope of a single manuscript…

5. This is more of a minor point, but I am not sure that the word "dynamics" is justified in the title of this manuscript. The manuscript describes cryo-EM structures of hibernating ribosomes, but it is not clear that these structures reveal anything about the dynamics of hibernating ribosomes.

From our point of view, even though ribosomes are not active in protein synthesis, they still demonstrate some dynamics in the relative orientations of small and large subunits in respect to each other when bound to the SaHPF protein. Therefore, we used the word 'dynamics' in the title to emphasize that aspect.

Referee #3:

Hibernation promoting factor (HPF) causes Staphylococcus aureus ribosomes to dimerize. Khusainov et al present the structures of two different configurations of disome - solved at 9 Å and 11 Å. Using a masking approach they resolve the HPF-bound ribosome within the dimer to 3.7 Å. This higher-resolution map is used to model HPF. The N-terminal domain (NTD) binds at the intersubunit interface and prevents translation, as expected from homology with Escherichia coli HPF. The C-terminal domain (CTD) of S. aureus HPF is absent in E. coli and mediates dimerization of 70S ribosomes by forming a homo-dimer. The authors present an NMR structure of the CTD. Overall, their results demonstrate that the formation and morphology of disomes in S. aureus differs from in E. coli, which may have implications for the development of specific antibiotics.

Comments

1. The abstract needs to be revised as it currently suggests that the structure of the disome has been solved at near-atomic resolution. This is misleading as the best resolution for the disome is 9 Å. The wording needs to be carefully considered throughout the manuscript. For example, fitting the 3.7 Å structure into the lower resolution disome map does not "reconstruct a structure at near-atomic resolution of the 100S disome".

We agree that the wording can be improved. By "reconstructed" we did not intend to mean "solved" although this could be confusing to the reader.

2. The abstract states "disomes form whether ribosomes adopt unrotated or rotated conformations, suggesting that translation can be paused by SaHPF at any stage". This sentence is misleading as presumably 100S complexes can only form from empty 70S ribosomes - the bound tRNAs of actively translating ribosomes would prevent SaHPF binding. Similar statements appear throughout the manuscript. For example, why would accommodating rotated and unrotated states suggest "an efficient mechanism for stalling and resuming translation in a timely manner"?

What is known in E. coli is that 100S dimers form from HPF-bound ribosomes. The situation may be different in S. aureus, as the reviewer points out. During translation in aminoacid starvation, the uncharged tRNA comes to the A site. RelA can sense this uncharged tRNA, and RelA induces synthesis of ppGpp, which in turn induces the synthesis of RMF protein. RMF binds to the ribosome at the E site to shut down translation. We don't have further information about the other stages that lead to dimer formation. Will mRNA dissociate only once RMF is bound? Does the ribosome become vacant before RMF can bind? In other words, we don't know whether 100S complexes can be formed only from empty 70S ribosomes. Most likely, allosteric changes are at play, which leads the 70S ribosome from an erroneous translational complex (i.e., with an uncharged tRNA) to a hibernating complex with bound RMF/HPF. As such, we can tone down in our manuscript our speculations about the actual mechanism of translation stalling mediated by SaHPF.

3. The abstract and text elsewhere suggests that the dimerization mode of bacterial ribosomes is "species-specific". However, the presented structure only shows that S. aureus uses a different mechanism for E. coli. This may reflect a difference between γ-proteobacteria and all other ribosomes, rather than species-specific mechanisms.

This is correct; the differences may be tied to various families of bacteria and not particular species. The text will be reworded accordingly.

4. In the introduction the CTD is described as being "marginally similar" to RMF. What does this mean? Are the authors implying a common evolutionary ancestor and is this in agreement with their structure?

We refer here to alignment shown in figure 6A in the paper by Ueta et al (2010). We only use this argument as indication that probably the CTD of SaHPF does not play the same role as RMF.

5. The introduction says that the only available structural information is for the NTD of various HPFs. However, this ignores structures for the CTD (PDB IDs 3KA5 and 3K2T) that the authors themselves use to build a homology model.

3KA5 and 3K2T are from a structural genomics study that does not seem to have been published. Here, only 3KA5 was assigned as 'ribosome-associated protein Y'. But we can amend the corresponding sentence in the manuscript to widen our net of what we consider to be legitimate 'structural information regarding ribosomal dimerization'.

6. The main text suggests that the NMR structure of the HPF CTD was used to model the EM density, however this doesn't agree with the material and methods, which says that a homology model was used and makes no mention of the NMR structure. This seems a wasted opportunity to use complementary information from cryo-EM, NMR and X-ray crystallography to build the best possible model for the CTD of the Staphylococcus aureus HPF.

Here, does the reviewer ask whether we used the NMR structure to interpret the EM density? Surely we did not use NMR data "to model" EM density. The misunderstanding comes from the fact that we did not update the corresponding section of the materials and methods, to indicate that the final and deposited structure has the NMR structure to model the CTD.

7. "Prior to this work, no hibernation factors had been described that directly mediated dimerization" should be removed from the text.

We can certainly remove that statement, but we would equally appreciate having the reference to prior work that did in fact show direct dimerization (and not indirect through ribosome-ribosome contacts).

8. The authors speculate that a single hibernation factor rather than two as in E. coli could "enhance bacteria's timeliness in blocking translation", but the flip side of this should also be mentioned - that the opportunities for regulation may be reduced.

Yes, this is a good point. The opportunities of the regulation of dimers formation in S. aureus might be reduced due to this fact, which makes for a strong argument in favor of developing more targeted strategies for combating S. aureus.

9. Please expand upon the data acquisition settings. The statement "camera was set up to collect 7 frames (starting from the second one) out of 17 possible. Total exposure was 1 s" is unclear. Does this mean that 17 frames were collected over 1s, but only frames 2-8 were processed?

The reviewer is absolutely right, we've collected 17 frames over 1 second, but only frames 2-8 were processed. We will clarify this in the text.

10. The "average resolution was...~9-12 Å for tight and loose disomes" doesn't agree with Table 1, which gives 11.0 Å for the tight disome and 9.0 Å for the loose disome.

We will correct the text to "The average resolution was ~ 3.7 Å for both rotated and unrotated monosomes and ~ 9-11 Å for 'loose' and 'tight' disomes respectively (Fig EV2).

11. Is there a reason that the tight disome is at a lower resolution than the loose disome despite similar numbers of particles? A tight disome might be expected to display less flexibility and therefore be resolved to a higher resolution. What is the difference in the interface between these two states?
As we discuss in the text and show in figure 5, the interface of these two state differs in the orientation of the two monosomes with respect to one other. The orientation in 'tight' disome is favorable for making an additional contact between 16S rRNA helixes 26 of both ribosomes, while this contact is distorted in 'loose' disomes. Despite of this, two half maps of the 'loose' disome correlate better in high frequences and, therefore contained more high resolution details then 'tight'

disome. This fact might be reflected to partially preferred orientation of the 'loose' disomes on the grid.

The further minor points below will be addressed by similarly altering the text according to the reviewer's comments.

12. Figure 2E - the title "In silico reconstituted 100S dimer at 3.7 Å" should be changed to remove the resolution estimate which is misleading.

Will be corrected

13. The fit of the model of the CTD to the density in figure 3B is not convincing. Please provide additional views.

We will include additional views

14. I would like to see some NMR figures (for example, EV6A and EV6D) moved to the main text.

We will move these NMR figures to the main text

15. The reference for Khusainov et al, 2016 should be updated now that the paper is published.

The reference will be updated

16. The authors present some AUC data, could AUC also be used to see if SaHPF is a dimer in solution? This would help answer one of the questions that the authors propose in the discussion.

Although AUC would be possible for a small protein (SaHPF is 22 kDa), it would most likely not lead to conclusive results, as in this case applying a centrifugal force over a long time may partially disrupt the dimer. The formation of full-length SaHPF dimer in solution will require a more thorough investigation in future projects.
17. "electron density" should be replaced with "map density" or equivalent.

Will be corrected

18. I could not follow the significance of the bL31 movement. Is this a specific response to the binding of SaHPF or a signature of intersubunit rotation?

It is more a signature of intersubunit rotation. As we know, the bL31 bridges the head of small subunit and the central protuberance of the large subunit. These parts of the ribosome are highly mobile during protein synthesis. Therefore, the correct position of bL31 on the ribosome will be crucial for the ribosome to ensure fast and correct restart of translation once bacterial cells will be transferred to favorable conditions and the SaHPF protein is dissociated.

Additional Correspondence - Editor 24 February 2017

Thank you for sending a point-by-point response to the concerns raised by our three referees. I have now consulted with an arbitrating technical advisor - specifically focusing on the resolution obtained and the docking in the disome structure - and this person finds that your response addresses the referee concerns in a satisfactory manner.

Based on this, I am therefore happy to invite you to submit a revised version of the manuscript, in which you address the concerns of our three referees as outlined in the preliminary response. 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.

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

1st Revision - authors' response 06 March 2017

Thank you for giving us the opportunity to submit a revised version of our manuscript describing the dimer architecture of the *S. aureus* 100S ribosome. We have now addressed the comments from all three reviewers, and we have adjusted our manuscript accordingly, including revised text and figures.

We hope you will find these revisions satisfactory and look forward to hearing from you.

Referee #1:

Khusainov etal report on the structure of the hibernating 100S ribosome from Staphylococcus aureus using single particle cryo-EM. Under stress conditions, bacteria down regulate the protein synthesis, which is in some cases accompanied by dimerization of 70S ribosome to so-called 100S ribosomes by binding of a hibernation promoting factor (HPF). The mechanism of translation inhibition by protein factors such as HPF, YifA and pY is well-understood based on high resolution structures. However, the reasons for dimerization and the mechanistic implications of 100S dimer formation remain elusive.

Although the authors were able to obtain a reconstruction of the S. aureus 100S ribosomal dimers these insights may not be particularly useful for understanding the function since the relative arrangement of ribosomes in the 100S dimers is flexible and may be influenced by the cryo-EM grid preparation process. It is also unlikely that the features of the 100S dimer will be conserved across species raising questions about the mechanistic implications of the obtained structure.

Furthermore, there are numerous technical weaknesses of the manuscript related to the processing and analysis of the cryo-EM data, listed below.

Overall, this manuscript is not suitable for publication in EMBO J.

Major concerns:

1) According to the procedure described as strategy 2, cryo-EM reconstruction of the 70S ribosome was calculated using particle images of monomeric 70S ribosomes as well as of 70S ribosomes of 100S dimers.

The resulting high resolution 70S map was twice fitted into a low resolution 100S map and the in silico reconstituted 100S map was used for the interpretation. Obviously, particle images of monomeric and dimeric 70S ribosomes have been merged to interpret the 100S dimer.

The particle picking step was performed including 70S particles of the dimers and individual monomeric 70S particles. As we can see from the cryo-EM micrograph (Fig 1A), most of the particles were picked from dimeric forms rather than monomeric forms. Additionally, not all the picked particles were included in the final reconstructions. Indeed, they were sorted out by 3D classifications (described in details in Materials and methods/Electron microscopy of disomes/Image processing) and resulted in two major classes: ~83,000 particles of 70S-SaHPF complex in the unrotated conformation and ~80,000 particles of 70S-SaHPF complex in the rotated conformation. The rest of the particles (~185,000) represented false positives (subunits only, small contaminants, damaged 70S, etc…), and were removed from further processing (figure EV2). Very importantly, we can see strong extra density near the platform of SSU (colored in grey on revised Figure 2D), which belongs to the neighboring 70S ribosome within a dimer. Therefore, we can conclude that our 3.7 angstrom 70S-SaHPF structures were derived from disomes. Regarding monomeric 70S, even if they were partly included into final reconstruction, they represented one of two conformations (Unrotated + SaHPF or Rotated +SaHPF), otherwise they were rejected from reconstruction after 3D classification. Finally, all of the particles picked as monomers are bound to SaHPF, which validates our approach and conclusions related to the structure of the dimers.

Although the authors claim that HPF is bound to all 70S, there is no evidence that the conformation of the factor and of the ribosome is the same in the monomeric and the dimeric states.

We do see SaHPF bound to all 70S used for final reconstructions. And, as mentioned in the paragraph above, additional density at the interface brings us an evidence that these 70S were derived from 100S. Therefore, the conformations of our 70S reconstructions correspond to the conformation within a disome. Regarding the HPF protein, we don't observe any conformational rearrangements based on rotation, however we could follow alterations of its contacts with SSU between two states. We see that some of the contacts with the body are distorted in the rotated state, when the head is buckled away from the body (Fig EV5). To summarize, the overall conformation of SaHPF is invariant, but some of its contacts depend mainly on the conformation of the 70S to which they belong, rotated or unrotated. It is interesting to highlight (as we now do in the Discussion) that in the absence of any other translation factors and tRNAs, due to the presence of SaHPF, only these two extreme conformations of the 70S can be observed, which in turn minimizes the conformational heterogeneity of SaHPF.

Furthermore, it is misleading to claim that the 100S particle was resolved at near-atomic resolution only by fitting the high resolution 70S map into the 100S map that was actually resolved at low resolution (11Å for the tight dimer).

We apologize for having misleadingly written the following phrase in the abstract: "Here, we present the structure of disomes…. that we solved at near-atomic resolution….". This sentence was corrected to: "Here, we present the structures of hibernating ribosomes from human pathogen Staphylococcus aureus containing a long variant of the hibernation promoting factor (SaHPF), that we solved using cryo-electron microscopy".

We wish to take this opportunity to clarify that 'hibernating' refers to inactive ribosomes bound to SaHPF, and therefore either in 70S or 100S form. We have modified the text throughout the manuscript to clarify that point.

Strategy 2 is therefore not valid and the authors cannot claim to have resolved the 100S dimer at near-atomic resolution.

We have modified the text where we have claimed that we solved 100S dimer at near-atomic resolution. Apart of the abstract, it was corrected in: Results/ Strategy for visualizing hibernating ribosomes at different resolution: "Further, in order to interpret the 11 Å resolution structure of 100S in greater details…"
Strategy 2 (resolving high resolution structures of 70S-SaHPF complex in context of dimers) revealed results that wouldn't be possible to obtain using only strategy 1 and not only regarding the nominal resolution. For example, the intersubunit rotations are hidden at the low resolution. The high-resolution reconstructions of 70S-SaHPF allowed us to interpret accurately the 30S-30S interface of dimerization and the structure of SaHPF.

2) The C-terminal domain (CTD) of HPF is potentially the most interesting part of the structure, because it appears to mediate the 70S dimerization. However, the local resolution plots of the disomes indicate a local resolution of the 70S interaction sites of 15 Å. At this resolution it is not possible to unambiguously fit the model of the HPF-CTD.

The referee is perfectly correct regarding the low-resolution but the 100S is only a fraction of our data, this is precisely why we endeavored to obtain a separate NMR structure of the CTD that was further rigid-body fitted into the interface region using both, low-resolution map and low-pass filtered high resolution map. Furthermore, the low-resolution extra density that was attributed to SaHPF-CTD is connected to SaHPF at its C-terminal domain. Taken together, our NMR structure, high-resolution 70S-SaHPF and low-resolution 100S drove our conclusions.
Furthermore, it is not clear from the text and the shown figures, whether the density for the HPF-CTD dimer was also resolved in the high-resolution 70S map.

We can see a clear density for CTD on our high-resolution maps after low-pass filtering to the appropriate local resolution of the map. Different views of this density and fitting are now shown on Figure 3D.

The density shown in Figure 3 assigned to HPF-CTD dimer seems to have a significant lower resolution compared to the surrounding density and it is not clear from which map ("density maps for unrotated ribosome-bound HPF") the carved-out density is derived from.

The density assigned for CTD dimer on Figure 3 is derived from low-pass filtered high resolution maps. We have changed the legend for Figure 3 to clarify this point.

Minor concerns:

1) The 100S dimers used for structural analysis were in vitro assembled by incubating purified and high salt-washed 70S ribosomes with recombinantly expressed HPF. The sucrose gradient indicates that the majority of 70S ribosomes dimerize to form 100S disomes under the chosen conditions. However, there are native 100S dimers present in the ribosome sample purified from S. aureus (see sucrose gradient in Fig. 1A). What are the reasons not to use these 100S dimers for structural analysis?

See response to point #1 of reviewer #2.

2) If HPF of S.aureus is present as dimer as predicted by the NMR analysis and binds the 70S ribosome as dimer, would it not also be more reasonable that the hibernating S.aureus ribosomes have a HPF dimer bound (see Fig. 6).

For NMR experiments, we purified only CTD and not the full-length HPF protein. This CTD forms a dimer in solution as was shown by NMR experiments. The referee indeed presents an interesting view point on the mechanism of dimerization in which ribosomes bind to preformed dimer of full SaHPF. In this case one would expect hibernating 70S to contain a dimer of SaHPF ready to accept a second 70S partner. However, in our cryo-EM 3D classification we didn't observe any monomeric 70S bound to an HPF dimer. Further future biochemical and structural experiments will have to be conducted to elucidate the step-by-step mechanism of dimerization, which is obviously beyond the scope of our paper.

3) In contrast to features of the 2D class average of the tight and the loose dimer (see Fig. 2B) the 3D reconstruction of the loose dimer was resolved to "higher" resolution (9 Å) compared to the tight dimer (11 Å) using almost identical number of particle images. The authors should check and comment.

See response to point #11 by reviewer #3 below.

4) In the introduction, the hibernation mechanism of E.coli, which Is the best studied so far, is compared with the mechanisms in other species. The authors state that the hibernation factors promote dimerization in different ways based on the species. How big are these differences between species expressing the long form of HPF? Also, if this is the case what is the purpose of studying dimers in one specific species?

The work in our laboratories is focused on the translational aspects of Staphylococcus aureus. This bacterium is a severe human pathogen, which in addition is resistant to most of commonly used antibiotics. The translation machinery is a potentially efficient target for development of new antimicrobials against S. aureus, as demonstrated by the fact that nearly half of the currently prescribed antibiotics target the translation machinery. According to our study, the differences in the hibernation process between gram negative and positive bacteria are substantial, and theses differences may be directly applied in medical research. For example, an antibiotic that prevents the SaHPF C-ter dimerization would inhibits the 100S formation, which would weaken the bacteria, rendering it more vulnerable to treatment.

5) The recombinantly expressed HPF was finally purified by SEC and the elution profile indicates two major peaks containing HPF - of which the one with the higher A260/A280 ratio was more efficient in formation of 100S dimers. This is suspicious and additional analysis of the sample by SDS-PAGE or RNA gel electrophoresis should be added to indicate the purity of the sample.

We shared this unusual finding, which might be useful for the researchers working in the field. We also raised this question in the discussion section. The presence of an additional substance which made SaHPF protein active remains unclear. We proposed that this substance might prevent self dimerization of the protein in the cell. Additional SDS-PAGE analysis or RNA agarose gel electrophoresis didn't clarify this issue. Therefore, this will be a matter of further investigation.

6) Expansion Figure 4 does not provide additional information to the reader and can be removed. We removed this figure.

Referee #2:

The manuscript entitled "Structures and dynamics of hibernating ribosomes..." by Khusainov et al reports 11 A and 9 A cryo-EM structures of in vitro reconstituted hibernating 70S ribosome dimers (disomes) from Staphylococcus aureus. The authors modeled the structure of the N-terminal domain (NTD) of the S. aureus hibernation promoting factor (SaHPF) on the basis of its homology to the Escherichia coli- and plastid-specific homologs of SaHPF and, consistent with the E coli- and plastid-specific homologs of the SaHPF NTD, find that the NTD binds at the A site of the small ribosomal subunit. Because binding of the SaHPF NTD and its homologs at this position would sterically block the binding of initiation factors and tRNAs to the A site, the structure rationalizes the ability of these proteins to block translation during hibernation. The authors also find that the C-terminal domain (CTD) of SaHPF protrudes through the E site of the small ribosomal subunit, where it uses a CTD-CTD dimerization interaction to mediate the formation of the disome. The authors initially model the structure of the CTD using its sequence similarity to a protein of known structure, the Lmo2511/Y protein. They then validate this model using NMR structural studies of a construct consisting of only the isolated CTD. The NMR studies not only confirm the authors' modeling of the CTD structure, but they also provide strong evidence in support of the CTD-CTD dimerization interaction. In addition to the CTD-CTD dimerization interaction, disome formation is further strengthened through interactions between the 16S ribosomal RNA helix 26 of the two ribosomes in the disome. Finally, the authors find that disomes can form regardless of whether the ribosomal subunits occupy rotated or unrotated conformations.

In principle, the structures reported here represent a significant advance in the field and in our understanding of the mechanisms through which ribosomes hibernate during stress conditions. Nonetheless, I have several important questions and concerns regarding the methods and interpretation of the data that I think the authors would need to address before a determination could be made regarding publication in EMBO J. These are as follows:

1. It is not clear to me why the authors have chosen to in vitro constitute the hibernating ribosomes rather than simply purifying hibernating ribosomes from cells grown under stress conditions (e.g., purified from cells grown into stationary phase). This raises the question of whether or not structures of in vitro reconstituted hibernating ribosomes reflect structures of physiologically assembled hibernating ribosomes. Do the authors have any evidence that structures of in vitro reconstituted hibernating ribosomes are physiologically relevant?

We proceeded by reconstitution from purified partners in order to have a more homogenous material, for the sake of obtaining a high-resolution structure (sub 4 angstrom resolution). Additionally, our experience of working with ribosomes told us that the purification of the stressed ribosomes could lead to non-specific aggregation of ribosomes, which would interfere with the analysis of unaltered and aggregated disomes.

2. The description of the strategy for visualizing the disomes at near-atomic resolution is a bit convoluted and difficult to follow. If I understood it correctly, however, it seems that the authors have generated a near-atomic resolution model of the hibernating ribosomes by fitting a previously reported, cryo-EM-derived, 3.7 A structure of the S. aureus ribosome, a homology model of the SaHPF NTD, and an NMR-validated homology model of the SaHPF CTD into the 11 A resolution structure of the S. aureus "tight" 100S dimer.

Based on the comments of all reviewers, it became clear that we needed to properly reformulate and clarify these aspects in our manuscript. We did not dock here a structure we previously solved into a

low-resolution map. In the current study, we independently solved 9-11 angstrom structures of 100S ribosome, and 3.7 angstrom structures of 70S ribosome in a hibernating state, and therefore bound to SaHPF, for which we see density in our complex (See Figure 2 C,D; Figure 3 A,B; Figure EV5 A,B). Necessary adjustments were applied to the manuscript accordingly.

Given the uncertainties associated with an 11 A cryo-EM map and homology models, is it possible that the authors are over interpreting their models? For example, is it really justified to model the helix 26-helix 26 interactions, the bL31 movements, and the inter-small ribosomal subunit interactions in as high a level of detail as is shown in Fig 3A and 3B, Fig 4D, and Fig 5A and 5B, respectively?

All these structural aspects (shown in particular in figures 3B, 4D and EV5) are observations we made from 3.7 angstrom structures of hibernating 70S-SaHPF complex solved in this study.

3. The authors make spend a significant portion of the manuscript describing the fact that SaHPF can bind to ribosomes regardless of whether the ribosomes are rotated or unrotated. They interpret this as evidence that SaHPF can act to hibernate ribosomes during any step of translation. This raises several questions for me: (1) What ribosomal complex, or complexes, does SaHPF target? Is there any evidence that SaHPF targets and acts on a specific ribosomal complex or, more in line with what I think the authors are suggesting in their interpretation, that it can target and act on any ribosomal complex?

The referee raises an interesting point. We have revised the manuscript and removed misleading sentences such as the following from the abstract: "Disomes form whether ribosomes adopt unrotated or rotated conformations, suggesting translation can be paused by SaHPF at any stage". We have now reworded the text in order to clarify that adopting rotated and unrotated conformations of subunits is more the feature of their re-involvement in protein synthesis rather than inhibiting particular complexes of translating ribosomes. This point is emphasized in the revised discussion.

(2) Could the authors observations and the answers to the questions I have raised here be influenced by the fact that the authors in vitro reconstituted the hibernating ribosomes rather than purified them out of cells grown under stress conditions? In other words, would the authors expect that physiologically assembled hibernating ribosomes purified out of cells grown under stressed conditions would exhibit the same subunit rotation properties or could this be an artifact of the in vitro reconstitution?

We don't exclude that there may be other conformational states in vivo. Based on our in vitro data, we now propose in the discussion that an equal distribution of rotated and unrotated ribosomes may represent a dynamic equilibrium between two most favorable conformations. Additionally, the ability to accept large scale rearrangements might serve as a signature that such ribosomes can be recruited for translation when required (more detailed explanations are provided in the revised discussion).

4. Given the structures that the authors have solved and the authors interpretations of these structures, can the authors explicitly propose a mechanism for SaHPF-mediated ribosome hibernation? The authors discuss some features of such a mechanism very briefly near the end of the Discussion section of the manuscript, but they stop short of proposing a mechanism. In any case, should it be possible for the authors to propose such a mechanism it would greatly increase the impact and significance of this manuscript.

We expanded our discussion in order to further highlight mechanistic aspects of ribosome hibernation, which we could derive from our structures and previous data, such as subunit rotation, features of SaHPF expression and purification, etc. The characterization of the specific stages of the hibernation process in details would require an independent study comprising additional experiments that are beyond the scope of a single manuscript… In our manuscript, the goal is to describe fro the first time the structure of the hibernation complex from a gram positive bacterium.

5. This is more of a minor point, but I am not sure that the word "dynamics" is justified in the title of this manuscript. The manuscript describes cryo-EM structures of hibernating ribosomes, but it is not clear that these structures reveal anything about the dynamics of hibernating ribosomes.

From our point of view, even though ribosomes are not active in protein synthesis when bound to SaHPF, they still demonstrate some dynamics in the relative orientations of small and large subunits with one another, as reflected by the various classes we obtained during data processing. We consider this finding important, as we explain in the revised discussion, therefore we think that it is important to keep the word 'dynamics' in the title to emphasize that aspect.

Referee #3:

Hibernation promoting factor (HPF) causes Staphylococcus aureus ribosomes to dimerize. Khusainov et al present the structures of two different configurations of disome - solved at 9 Å and 11 Å. Using a masking approach they resolve the HPF-bound ribosome within the dimer to 3.7 Å. This higher-resolution map is used to model HPF. The N-terminal domain (NTD) binds at the intersubunit interface and prevents translation, as expected from homology with Escherichia coli HPF. The C-terminal domain (CTD) of S. aureus HPF is absent in E. coli and mediates dimerization of 70S ribosomes by forming a homo-dimer. The authors present an NMR structure of the CTD. Overall, their results demonstrate that the formation and morphology of disomes in S. aureus differs from in E. coli, which may have implications for the development of specific antibiotics.

Comments

1. The abstract needs to be revised as it currently suggests that the structure of the disome has been solved at near-atomic resolution. This is misleading as the best resolution for the disome is 9 Å. The wording needs to be carefully considered throughout the manuscript. For example, fitting the 3.7 Å structure into the lower resolution disome map does not "reconstruct a structure at near-atomic resolution of the 100S disome".

We completely revised the abstract according to comments from all referees.

2. The abstract states "disomes form whether ribosomes adopt unrotated or rotated conformations, suggesting that translation can be paused by SaHPF at any stage". This sentence is misleading as presumably 100S complexes can only form from empty 70S ribosomes - the bound tRNAs of actively translating ribosomes would prevent SaHPF binding. Similar statements appear throughout the manuscript. For example, why would accommodating rotated and unrotated states suggest "an efficient mechanism for stalling and resuming translation in a timely manner"?

The referee raises an interesting point shared by referee #2. In the revised abstract, we have replaced that sentence with: "SaHPF is bound to ribosomes in both unrotated and rotated states, suggesting plasticity of 100S formation". Additionally, we expanded the view on the role or subunit rotations within the dimer in the discussion (paragraphs 3 and 4).

3. The abstract and text elsewhere suggests that the dimerization mode of bacterial ribosomes is "species-specific". However, the presented structure only shows that S. aureus uses a different mechanism for E. coli. This may reflect a difference between γ-proteobacteria and all other ribosomes, rather than species-specific mechanisms.

The referee is correct, the differences may be tied to various families of bacteria and not particular species. The text describing species-specific hibernation was reworded accordingly.

Abstract: " Overall, our work illustrates a specific mode of ribosome dimerization by long HPF, a finding that may help improve the selectivity of antimicrobials".
Introduction, paragraph 2: "The expression patterns of these factors differ between speceis bacteria"
Introduction, paragraph 3: "As their variations in structure, expression pattern, and functional role suggest, hibernation factors promote dimerization in different ways based on the species."

Introduction, paragraph 5: "Overall, our work paves the way for deciphering the species-specific diverse nature….."
Discussion, paragraph 2, last sentence: "mechanisms of dimer formation and the dimer architectures may be species-specific significantly vary across bacterial species.
Discussion, paragraph 8, last sentence: "….unraveling the mechanism for hibernation in a S. aureus species-specific manner, which should help guide the development of more selective treatments against S. aureus this pathogen."

However, we chose to keep the following sentence "These species-specific characteristics account for differential responses to stress…." in the paragraph 2 of introduction, since the stress response was shown to be indeed, species-specific.

4. In the introduction the CTD is described as being "marginally similar" to RMF. What does this mean? Are the authors implying a common evolutionary ancestor and is this in agreement with their structure?

We refer here to alignment shown in figure 6A in the paper by Ueta et al (2010). We use this argument as indication that probably the CTD of SaHPF does not play the same role as RMF.

5. The introduction says that the only available structural information is for the NTD of various HPFs. However, this ignores structures for the CTD (PDB IDs 3KA5 and 3K2T) that the authors themselves use to build a homology model.

3KA5 and 3K2T are from a structural genomics study that does not seem to have been published. Here, only 3KA5 was assigned as 'ribosome-associated protein Y'. We amended the text by adding the sentence in paragraph 4: "One structure for CTD assigned as ribosome-associated protein Y from Clostridium acetobutylicum was found in protein data bank (PDB ID 3KA5)".

6. The main text suggests that the NMR structure of the HPF CTD was used to model the EM density, however this doesn't agree with the material and methods, which says that a homology model was used and makes no mention of the NMR structure. This seems a wasted opportunity to use complementary information from cryo-EM, NMR and X-ray crystallography to build the best possible model for the CTD of the Staphylococcus aureus HPF.

Thank you for bringing up this point, and we apologize for the confusion. We have updated the corresponding section of the materials and methods, in order to indicate that the NMR structure was used for final interpretation of our data.

7. "Prior to this work, no hibernation factors had been described that directly mediated dimerization" should be removed from the text.

We can certainly remove that statement, but we would first appreciate having the reference to prior work that did in fact show dimerization through interactions of the hibernation promoting factor (and not indirect through ribosome-ribosome contacts).

8. The authors speculate that a single hibernation factor rather than two as in E. coli could "enhance bacteria's timeliness in blocking translation", but the flip side of this should also be mentioned - that the opportunities for regulation may be reduced.

We have rewritten paragraph 3 in discussion section "Shutting down translation by occupying… …antimicrobials against S. aureus" and included the point reised by the reviewer. Additionally we included the reference for recent YfiA-bound chloroplast ribosome: "….SaHPF binds to the small subunit similarly to its homologs EcHPF, EcYfiA, and a plastid-specific YfiA (Polikanov et al., 2012, Sharma, Donhofer et al., 2010, Vila-Sanjurjo et al., 2004, Bieri et al., 2017)..."

9. Please expand upon the data acquisition settings. The statement "camera was set up to collect 7 frames (starting from the second one) out of 17 possible. Total exposure was 1 s" is unclear. Does this mean that 17 frames were collected over 1s, but only frames 2-8 were processed?
"Frames 2–8 out of 17 possible frames were collected and used for image processing. Total exposure was 1 s with a dose of 60 $\bar{e}/Å^2$ (or 3.5 $\bar{e}/Å^2$ per frame)" – means that frames 2–8 were

collected and used for image processing, the other frames 1, 9–17 were not collected at all, although the exposure took place. We have corrected this phrase to: "Frames 2–8 out of 17 possible were collected and used for image processing. Total exposure was 1 s with a dose of 60 ē/Å^2 (or 3.5 ē/Å^2 per frame).

10. The "average resolution was...~9-12 Å for tight and loose disomes" doesn't agree with Table 1, which gives 11.0 Å for the tight disome and 9.0 Å for the loose disome.

We corrected the text to "The average resolution was ~ 3.7 Å for both rotated and unrotated monosomes and ~ 9-11 Å for 'loose' and 'tight' disomes respectively (Fig EV2)".

11. Is there a reason that the tight disome is at a lower resolution than the loose disome despite similar numbers of particles? A tight disome might be expected to display less flexibility and therefore be resolved to a higher resolution. What is the difference in the interface between these two states?

We think that this confusion comes from our nomenclature of "tight" and "loose". Indeed, this nomenclature doesn't reflect necessarily the rigidity of the 100S. As we discuss in the text and show in figure 5, the interface of these two states differs in the orientation of the two monosomes with respect to one other. The orientation in 'tight' disome is favorable for making an additional contact between 16S rRNA helixes 26 of both ribosomes, while this contact is distorted in 'loose' disomes. Despite this fact, two half maps of the 'loose' disome correlate better in high frequencies and, therefore contained more high-resolution details then 'tight' disome. This fact might be reflected to partially preferred orientation of the 'loose' disomes on the grid.

12. Figure 2E - the title "In silico reconstituted 100S dimer at 3.7 Å" should be changed to remove the resolution estimate which is misleading.

Corrected

13. The fit of the model of the CTD to the density in figure 3B is not convincing. Please provide additional views.

We attached more views. Now it became figure 3D.

14. I would like to see some NMR figures (for example, EV6A and EV6D) moved to the main text.

We moved figures EV6A and EV6D to main text as Figure 3C.

15. The reference for Khusainov et al, 2016 should be updated now that the paper is published.

Corrected

16. The authors present some AUC data, could AUC also be used to see if SaHPF is a dimer in solution? This would help answer one of the questions that the authors propose in the discussion.

Although AUC would be possible for a small protein (SaHPF is 22 kDa), it would most likely not lead to conclusive results, as in this case applying a centrifugal force over a long time may partially disrupt the dimer. The formation of full-length SaHPF dimer in solution will require a more thorough investigation in future projects.

17. "electron density" should be replaced with "map density" or equivalent.

Corrected to "Cryo-EM density map" or "Density map".

18. I could not follow the significance of the bL31 movement. Is this a specific response to the binding of SaHPF or a signature of intersubunit rotation?
As we know, the bL31 bridges the head of small subunit and the central protuberance of the large subunit. These parts of the ribosome are highly mobile during protein synthesis. Therefore, the correct position of bL31 on the ribosome will be crucial for the ribosome to ensure fast and correct

restart of translation once SaHPF protein is dissociated. We broadened our views on that subject in discussion section, paragraph 5: "Additionally, large rearrangements of the subunits interface are coupled with movements of bridging protein B-type bL31…."

2nd Editorial Decision 24 March 2017

Thank you for submitting a revised version of your manuscript. It has now been seen by one of the original referees whose comments are shown below. As you will see the referee finds that most of the original criticisms have been sufficiently addressed but still raises a few remaining points - both textual and experimental - that will have to be addressed before this person can recommend the manuscript for publication. I would therefore like to invite you to submit a final revision in which you address the following concerns:

-> The referee points to a number of instances where the data presented do not unambiguously support the conclusions made. Consequently, you should tone down and elaborate as requested by the referee.

-> In addition, the referee asks for additional experimental data on the dimerization status for the full-length SaHPF and for a more extensive discussion on the implications for ribosome binding. Furthermore, the referee asks for clarification of the elution profile with regard to nucleic acid binding. Given that these points were also raised in the original referee reports I would strongly encourage you to include this data in the final manuscript.

-> Please provide an ORCID identifier number for all corresponding authors (this needs to be updated directly in your user profile in our system and unfortunately we cannot do this linking for you)

-> Please provide figures as individual files for both main and EV figures. In addition, please make sure the legends for the EV figures are included in the main manuscript text file. We generally accommodate up to five typeset EV figures and noticed that your manuscript currently has seven. You can therefore either keep five of these as EV figures and move two to an Appendix file or turn the entire current 'Expanded View Content' into an Appendix. If you choose the latter option, please update the call-outs in the main manuscript text accordingly. Feel free to contact us with any questions regarding formatting.

-> Please fill out and include an author checklist as listed in our online guidelines (http://emboj.embopress.org/authorguide)

-> Please include database accession numbers for the structures generated (these can also be added at proof stage if not available at the moment)

-> We noticed that the reference style in the main text varies between listing one or two names before 'et al', could you please use the general journal style of 'one name +et al'?

-> Papers published in The EMBO Journal include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst - written by the handling editor - as well as 2-5 one sentence bullet points that summarise the paper and are provided by the authors. I would therefore ask you to include your suggestions for bullet points

-> In addition, I would encourage you to provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your revision.

REFEREE REPORT

Referee #3:

The paper by Khusainov and colleagues is much improved relative to the originally submitted manuscript. However, there are a few remaining concerns and a few new ones introduced by the revisions.

1) The new abstract includes the statement "SaHPF is bound to ribosomes in both unrotated and rotated states, suggesting plasticity of 100S formation." However, this ignores the possibility that subunit rotation only occurs after 100S particles have formed, as SaHPF does not bind the large subunit and so does not constrain the ribosome in a particular conformation. The authors are themselves more circumspect in the discussion "the question remains open as to whether the factor influences the transition between these two stages, or it binds to the preformed conformations". I would recommend modifying the sentence to a simple statement of fact, e.g. "ribosomes in the 100S particle adopt both rotated and unrotated conformations". I would also recommend adding a sentence about the NTD to the abstract.

2) The end of the statement "the C-terminal end in SaHPF (CTD) is only marginally similar to RMF" should also reference Ueta et al, 2010 which the authors mention in their reply to the first round of reviews.

3) The authors have included a new section in the discussion about the events after dissociation of SaHPF. It is highly speculative as the authors assume that dissociation of SaHPF leaves vacant 70S particles. Is there evidence that this is what happens in vivo? It is possible that 100S particles can only reenter the translation cycle after subunit dissociation.

4) I do not understand the authors' reticence to investigate whether full-length SaHPF forms a dimer in solution, which would strengthen their paper. Their argument that AUC would disrupt the dimer is inadequate as the 100S particle remains intact during centrifugation and the interface to this is mostly formed by the CTD dimer. Indeed they may already have the answer - in the figure legend to their size-exclusion chromatograms (Fig. EV1) they say "SaHPF eluted in the expected position" - is this the expected position for a monomer or a dimer? It should be possible to tell the difference between a 22 kDa monomer and a 44 kDa dimer with a calibrated size-exclusion column without the need to do additional AUC experiments.

5) SaHPF peak 1 is wrongly labeled in Figure EV1A - it currently points to the void volume peak.

6) The peaks with low and high 260:280 nm ratios appear to elute in exactly the same place (just one column volume apart). Doesn't this argue against the possibility that nucleic acids are coating the particle, as suggested in the revised discussion, as these would increase the mass and therefore change the elution profile? Could the presence of a small molecule (nucleotide) also explain the A260 peak? Furthermore, it is puzzling that they see another peak in the void volume in the second run - can the authors confirm that the trace is a continuation of the first run or whether more material has been loaded onto the column?

7) The authors also speculate whether SaHPF could be protected from self-dimerization by post-translational modifications. However they have not shown that full-length SaHPF is a dimer, that it needs to be protected from self-dimerization, or is post-translationally modified. Other possibilities are that SaHPF is a dimer in solution and simply doesn't inhibit ribosomes because of competition with translation factors/tRNAs during exponential growth, or that SaHPF is a monomer in solution in which the two domains bind one another in an autoinhibitory mechanism that is only released when it binds the ribosome.

8) The hypothesis that the "absence of nucleic acids in the first fraction could have led to an unproductive self-dimerization of free SaHPF" is incompatible with similar elution profiles between the two peaks. It is also not clear why self-dimerization would be unproductive. The authors either need to provide supporting evidence of the oligomeric state SaHPF in solution or remove unnecessary speculation.

9) The observation that SaHPF can bind 30S subunits (Basu and Yap, 2016) is explained as

"reflecting the ability of hibernation factors to trap 30S after recycling". However, an alternative explanation could be that SaHPF remains bound to 30S subunits after recycling of 100S particles.

2nd Revision - authors' response 12 April 2017

Thank you for giving us the opportunity to submit a final version of our manuscript describing the dimer architecture of the *S. aureus* 100S ribosome. We have now addressed the comments from the referee, and we have adjusted our manuscript accordingly, including revised text and figures. We also attach Synopsis with the figure and bullet points, author checklist and four pdb coordinates files of atomic reconstruction of 100S disomes in rotated and unrotated states.

We hope you will find these revisions satisfactory and look forward to hearing from you.

Referee #3:

The paper by Khusainov and colleagues is much improved relative to the originally submitted manuscript. However, there are a few remaining concerns and a few new ones introduced by the revisions.

1) The new abstract includes the statement "SaHPF is bound to ribosomes in both unrotated and rotated states, suggesting plasticity of 100S formation." However, this ignores the possibility that subunit rotation only occurs after 100S particles have formed, as SaHPF does not bind the large subunit and so does not constrain the ribosome in a particular conformation. The authors are themselves more circumspect in the discussion "the question remains open as to whether the factor influences the transition between these two stages, or it binds to the preformed conformations". I would recommend modifying the sentence to a simple statement of fact, e.g. "ribosomes in the 100S particle adopt both rotated and unrotated conformations". I would also recommend adding a sentence about the NTD to the abstract.

We modified the abstract as was suggested by the reviewer:

"….Our reconstructions reveal that the N-terminal domain (NTD) of SaHPF binds to the 30S subunit as observed for shorter variants of HPF in other species. The C-terminal domain (CTD) protrudes out of each ribosome in order to mediate dimerization. Using NMR, we characterized the interactions at the CTD-dimer interface. Secondary interactions are provided by helix 26 of the 16S ribosomal RNA. We also show that ribosomes in the 100S particle adopt both rotated and unrotated conformations. …."

2) The end of the statement "the C-terminal end in SaHPF (CTD) is only marginally similar to RMF" should also reference Ueta et al, 2010 which the authors mention in their reply to the first round of reviews.

Corrected.

3) The authors have included a new section in the discussion about the events after dissociation of SaHPF. It is highly speculative as the authors assume that dissociation of SaHPF leaves vacant 70S particles. Is there evidence that this is what happens in vivo? It is possible that 100S particles can only reenter the translation cycle after subunit dissociation.

We have modified this section and now discuss both possibilities, either restoring translation through subunits dissociation or as 70S particles as an alternative way.

See on page 10:
"This interplay could be crucial for the redistribution of ribosomes from hibernating to translationally competent states, upon release of SaHPF and dissociation of 100S ribosomes into free 30S an 50S subunits for canonical translation. Alternatively, vacant 70S particles could, for example, be involved in the translation of leaderless mRNAs… … Therefore, we hypothesize that

the presence of two extreme states of subunit rotation within hibernating ribosomes is a hallmark of their ability to be rapidly recruited for translation."

4) I do not understand the authors' reticence to investigate whether full-length SaHPF forms a dimer in solution, which would strengthen their paper. Their argument that AUC would disrupt the dimer is inadequate as the 100S particle remains intact during centrifugation and the interface to this is mostly formed by the CTD dimer. Indeed they may already have the answer - in the figure legend to their size-exclusion chromatograms (Fig. EV1) they say "SaHPF eluted in the expected position" - is this the expected position for a monomer or a dimer? It should be possible to tell the difference between a 22 kDa monomer and a 44 kDa dimer with a calibrated size-exclusion column without the need to do additional AUC experiments.

We carefully revised Figure EV1. First, we added the profiles of the Ni sepharose affinity chromatography, where we show how we separated fractions of the protein purification mostly absorbing at 280 nm (pool 1 on figure A) and those absorbing also at 260 nm (pool 2 on panel A). These profiles demonstrate that increased absorbance at 260 nm can lead to the increased yield on the following Superdex column (peak 2).

Second, to indicate that SaHPF is purified in the dimeric form (in the second Superdex column in both peaks), we included additional experimental data such as a native PAGE (panel C), we added the calibration curve for Superdex column (panel H) and we changed the figure legend where we mention that peak 1 corresponds to the position of SaHPF dimer (located at ~50 kDA position). We adjusted the main text accordingly (Methods/Isolation of full length SaHPF), so that it now reads as follows: "Two peaks containing SaHPF were eluted, the first one corresponding to pure SaHPF eluted at position of ~50 kDa, while the second contained extra absorbance at 260 nm and eluted after one column volume (Fig EV1 B,F), even though it also contains SaHPF in a dimeric form (Fig EV1 C)."

5) SaHPF peak 1 is wrongly labeled in Figure EV1A - it currently points to the void volume peak.

We included the calibration curve for the Superdex 75 column according to manufacturer's manual. According to it, position of peak 1 (10.5 mL) corresponds to ~50kDa, whereas the void volume of this column is 8 mL.

6) The peaks with low and high 260:280 nm ratios appear to elute in exactly the same place (just one column volume apart). Doesn't this argue against the possibility that nucleic acids are coating the particle, as suggested in the revised discussion, as these would increase the mass and therefore change the elution profile? Could the presence of a small molecule (nucleotide) also explain the A260 peak? Furthermore, it is puzzling that they see another peak in the void volume in the second run - can the authors confirm that the trace is a continuation of the first run or whether more material has been loaded onto the column?

We assume that the presence of nucleic acids in peak 2 might cause some interaction with the column resin (as we now mention on the figure legend), therefore, the material elutes close to the full column volume position in spite of the increased mass. We now demonstrate by native PAGE (Fig EV1, C) the protein from peak 2 and from peak 1 are detected at the same position, suggesting that the full SaHPF protein is present in solution in the dimeric form in both peaks. It would be of course interesting to investigate the nature of this nucleic acid molecule(s) in future studies. As mentioned by the reviewer, there is a possibility that it can be a single nucleotide or a short oligonucleotide that binds to the N-terminal domain of SaHPF (the domain which interacts with 16S rRNA) and, therefore, prevents non-regulated binding of SaHPF to the ribosome. We revised the text accordingly:

"This free SaHPF could be protected ~~from self-dimerization or~~ from unspecific binding to the ribosome by post-translational modifications or by coating with additional factors. The high A260/280 ratio we observed in the fraction of SaHPF that was able to promote dimer formation could be in support of coating by nucleic acids (oligonucleotide or even a single nucleotide). The absence of nucleic acids in the first fraction could have led to an inactive ~~self-dimerization of free~~ SaHPF".

7) The authors also speculate whether SaHPF could be protected from self-dimerization by post-translational modifications. However they have not shown that full-length SaHPF is a dimer, that it needs to be protected from self-dimerization, or is post-translationally modified. Other possibilities are that SaHPF is a dimer in solution and simply doesn't inhibit ribosomes because of competition with translation factors/tRNAs during exponential growth, or that SaHPF is a monomer in solution in which the two domains bind one another in an autoinhibitory mechanism that is only released when it binds the ribosome.
The dimeric conformation of full length SaHPF together with requirement of a nucleic acid component for the formation of 100S is now shown in revised figure EV1. The possibility of competition with translation factors/tRNAs is included in the discussion.

8) The hypothesis that the "absence of nucleic acids in the first fraction could have led to an unproductive self-dimerization of free SaHPF" is incompatible with similar elution profiles between the two peaks. It is also not clear why self-dimerization would be unproductive. The authors either need to provide supporting evidence of the oligomeric state SaHPF in solution or remove unnecessary speculation.

We observed a clear dependency on the amount of additional absorbance at 260 in the SaHPF sample and its ability to stimulate 100S formation. However, self-dimerization of SaHPF can be only one of the reasons behind the inactivation of this protein, therefore we replaced "self-dimerization of SaHPF" by "unproductive SaHPF". (See response to point #5 and #6)

9) The observation that SaHPF can bind 30S subunits (Basu and Yap, 2016) is explained as "reflecting the ability of hibernation factors to trap 30S after recycling". However, an alternative explanation could be that SaHPF remains bound to 30S subunits after recycling of 100S particles.

This part of the discussion is focused on the exponential growth phase, therefore, we did not include suggested alternative explanations that pertain to events taking place in the stationary phase. However, we revised the manuscript in order to include the possibility that the competition between tRNAs/factors and SaHPF may take place in the cell, which would shift the population of 30S complexes to 30S-SaHPF complexes when the production of SaHPF protein is elevated:
"Although these cells had artificially elevated expression of SaHPF, which would make it more competitive with tRNAs and translation factors, this fact reflects an ability of hibernation factors to trap 30S after recycling and before they enter initiation stages in vivo."

3rd Editorial Decision 08 May 2017

Thank you for submitting the final revision of your manuscript; I'm pleased to inform you that it has now been officially accepted for publication in The EMBO Journal.

I have one minor, remaining question: I noticed that you have provided two EV dataset files with additional information on the reconstruction of the 100S particles. These files are introduced in the author checklist but I couldn't find a specific reference to them in the manuscript file itself. Can you provide a brief mention or legend that would explain the content of these files to the reader? We can then add it to the manuscript in-house before transferring your files for production (and sorry if that information is already provided and I just failed to find it).

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here: http://emboj.embopress.org/about#Transparent_Process

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If you have any questions, feel free to contact me. Thank you for your contribution to The EMBO Journal and congratulations on this nicely executed work!

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Corresponding Author Name: Yaser Hashem
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-96105R1

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	NA
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	NA
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	EMDB ID: EMD-3638 (Tight 100S), EMD-3639 (Loose 100S); PDB ID: 5ND8 (Hibernating ribosome in unrotated state), 5ND9 (Hibernating ribosome in rotated state), 5NKO (CTD-SaHPF), BMRB ID 34120 (CTD-SaHPF). Additionally pdb files with reconstructions of 100S dimer are provided as supplementary data.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA