Initial rejection:
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Feb 05, 2016

Dear Andrei,

Thanks for your patience with an extended review process. I've now heard from three referees and their comments are appended below. You'll see that the predominant recommendation is against publication in Cell. Both Reviewers 2 and 3 raised significant concerns. They share the view that the conclusions related to decoding are not supported by the data and that the insights into RelA are going to be of primary importance to a more focused readership. They note a number of more technical concerns as well. We've discussed the paper and the reviews editorially, and while we have considered Reviewer 1's more favorable assessment, we share the more conceptual concerns cited by the other reviewers and I'm afraid that we cannot recommend revision and resubmission in this instance.

Although we cannot offer to proceed further with this particular paper for Cell, the manuscript may be a stronger candidate for another journal within the Cell Press manuscript transfer system. If you are interested in learning more about transfer as an option, please contact your journal of interest directly, as each journal is editorially independent. Once you are ready to formally initiate transfer of your files and resubmission to the transfer journal, you can click on the link below.

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Declining to transfer will officially close out the manuscript in our system. If you do not accept or decline by May 05, 2016, the system will automatically decline on your behalf. If you prefer, you do have the choice of submitting this manuscript or a revised version of it to another Cell Press journal as a regular new submission, in which case you can decline the transfer.

I'm sorry that I cannot be delivering better news and hope that the reviewers' comments are helpful for you.

Best wishes,

Scientific Editor, Cell

Reviewers' Comments:

Reviewer #1: In this exciting paper, the authors have used state-of-the-art innovations in cryo-EM data collection and single particle analyses to determine 4 structures of E. coli 70S ribosomes in complex with RelA at near atomic resolution. RelA is a low abundance, stringent response factor that senses a cognate deacylated tRNA in the A-site during amino acid deprivation. Structure I has no A-site tRNA whereas Structures II-IV show an extended, and

somewhat bent RelA wrapped around the incoming cognate tRNA. To this point, only a few domains of RelA homologs have been solved at atomic resolution by X-ray crystallography and NMR. Hence, these new structures show RelA domain structure, positioning and rearrangements in the 70S ribosome, before and after arrival of the A-site tRNA. This is an important step towards deciphering the activation mechanism of the synthetase domain, which transfers a phosphoryl group from ATP to bound GDP/GTP to make small molecule alarmones ((p)ppGpp). The authors propose a novel mechanism by which structural rearrangements in the bound RelA in the presence of A-site tRNA may bring the various domains of this protein into proper alignment, to then bind nucleotide co-factors required for alarmone production.
Thus, the groundwork is now set to determine structures of RelA with bound nucleotide co-factors in order to further decipher the mechanism. This process activates transcription of genes in the stress response pathway and also inhibits various genes needed for growth, while modifying the expression of various metabolic genes. Upregulation of this pathway allows pathogenic bacteria to thrive under adverse conditions, whereas a loss of the pathway reduces pathogenicity. Hence, the stringent response provides yet another set of targets for antibiotic development. Strikingly, the RelA mechanism may require identification of the incoming deacylated tRNA in the A-site, so that the enzyme is only activated when cognate deacylated tRNAs are present in the A-site (as seen in Stucture IV). Thus, there appears to be an interaction between the terminal 3' base, A76, in the CCA motif of tRNA and RelA. Overall, the structural analysis has led the authors to identify a distant evolutionary kinship between RelA and metazoan innate immune sensors, which have almost no sequence identify but have some structural similarities including surfaces that may interact with nucleic acids.
Remarkably, careful classification followed by insightful analysis of Structures II-IV has revealed a road map of potential steps that may occur during the pre-accommodation of the EF-Tu-tRNA during the critical proofreading step of protein synthesis, to allow exquisite discrimination between non-cognate and cognate tRNAs. Since the resolution of RNA in the ribosome is quite good, the authors are able to propose an exciting 2 step model that relies on the sequential interaction of two critical flipped out bases of the ssu RNA (1492 and 530) with the nascent codon-anticodon helix during tRNA accommodation. Importantly, when an EF-Tu-tRNA structure is mapped onto Structure II, there is no clash with the ribosome and the GTPase site is about 10A away from the sarcin-ricin loop, so that the GTPase activity of the EF-Tu complex would not be prematurely activated during the proposed pre-accommodation steps. This model appears to confirm a number of other biophysical observations and can now be used as a basis for further experiments.

When taken together, this paper represents a significant and exciting step forward in our understanding of the stringent response and of critical first steps in protein synthesis.

What follows are a number points that if addressed should improve this well written paper.

Specific comments:

1. A linear cartoon of domains in RelA should be provided in the Figures, to help the reader quickly follow what is being described in the text.

2. Perhaps a bit of clarification is required concerning the point that is raised concerning RelA, which is far less abundant than 70S ribosomes. Since RelA is at the apex of a signaling cascade, and as an enzyme can create many alarmones to propagate the signal, every ribsosome in a stressed bacterium may not need to have a bound and activated RelA.

3. The authors note in the Discussion, and rightfully so, that the presence of RelA may bias conformations of the tRNA acceptor arm, such that they may differ a bit from those in the presence of EF-Tu. This is a point that needs raising, but given the requirement for base pairing between codon and anti-codon, and local constraints on the ribosomal RNAs, it seems likely that the basic RNA interactions at this interface will be similar. This point is driven home by the observation of Structure IV in a closed state for the ssu, with RelA and tRNA bound.

4. Perhaps Figures 2 and 3 might benefit from having an icon view to orient the reader when looking at these detailed interactions.

Reviewer #2: This paper describes a relatively high-resolution (3.8 - 4 Å resolution) cryo-EM structure of the bacterial 70S ribosome in complex with deacylated tRNA and the stringent-response factor RelA. The present study is a significant advance from previously

published cryo-EM study (10.8 Å resolution) by the Ramakrishnan and Valle groups of a very similar ribosome-RelA complex. In the present study, by extensive classification of the cryo-EM dataset, four unique conformational states of the complex have been identified, which allow authors to extract densities corresponding to almost complete RelA protein (744 amino acids). Available high-resolution structures of the 70S ribosome and homologs of RelA domains were used for molecular analysis the cryo-EM maps, except for the C-terminal domain, identified by authors as ribosome-intersubunit domain, which was modeled using a de-novo structure prediction software. The four conformational states of the 70S-deacylated-tRNA-RelA complex were used to interpret the mechanism of RelA-mediated (p)ppGpp synthesis, which is the main function of RelA a stringent response factor. In addition, authors make significant effort to interpret these conformational states with possible intermediates of the tRNA decoding process in the ribosomal A site. Overall, the paper is well written, but the functional interpretation of the map, especially the section relating to tRNA decoding, has gone far beyond the scope of the present study. Also, it appears that the resolution of the density corresponding to RelA protein itself is somewhat limited to draw a detailed mechanistic conclusion. Therefore, the paper cannot be recommended for publication in Cell but may be suitable for a more specialized journal after addressing the points outlined below:

Major Points:
1. One major problem with this paper is the interpretation of the ribosome-deacylated-tRNA-RelA complexes, where the deacylated-tRNA is partially wrapped around with elongated RelA molecule, for early stages of tRNA decoding process. While doing so authors argue that the various positions of deacylated tRNAs observed by them match with previously published single-molecule FRET data and open and close conformational states correlate with role of 30S subunit in the decoding process. But this whole section (page 18 to beginning of page 22) is bit of a stretch and somewhat dangerous, without experimentally ruling out that the observed RelA-wrapped tRNA positions are not solely and primarily related to different steps of RelA function. Also, there are some key structures of the complex with GTP and GDP are missing. Furthermore, there is no consideration of the impact that the binding of C-terminal domain of RelA alone might have on the aforementioned 30S conformation. Moreover, according to the schematic presented in Fig. 4, the binding of RelA precedes the tRNA binding. Isn't it?
2. From Fig. S1, it is obvious that the density corresponding to RelA in various structures are not as well resolved as the rest of the ribosome, and this should impact the molecular modeling and interpretation of the RelA. Fittings shown in Figs, S3 B-E and S4C-D are not convincing enough to judge the local quality of the map. It would help if interpretation of each RelA domain is supported by its local resolution map and domain-wise dockings.

Minor Points:
1. Abstract: It says 30S center, not clear which center.
2. Fig.1 will benefit from addition of a bar diagram identifying locations for various structural domains of RelA.
3. Page 4, 2nd para, line 4 from bottom: Citation of the Stark et al., 2001 paper here and elsewhere (e.g., pages 8 and 15) does not seem right.
4. Page 5, 2nd para, line 7: In conjunction with the Cate and Yusupov et al papers, cite the original paper describing the subunit rotation, without that non-rotated state of the ribosome sounds meaningless for the wide readership of Cell.
5. Page 7, 2nd para, line 5: mention of "954-960 loop": Fig. 2B says 950-954.
6. Page 8, line 5 from bottom: Should RIS be replaced by ACT?
7. Page 10, 2nd para: This comparative section is nice but somewhat distracts the flow of the manuscript. Authors may want to consider moving this section towards the end.
8. Page 12, last sentence: again rotated states may be better referred to an original subunit rotating paper.
9. Page 13, line 5: Note that the fact that EF-G occupies the A site of the 30S subunit was first observed in a cryo-EM study by the Frank group in late nineties.
10. Page 16, 2nd para: Description of ~2 Å movement from somewhat disordered NTD of RelA is difficult to assess. Moreover, the second last sentence of this paragraph sounds little presumptuous for reason described under major point 1 and may be modified.
11. Page 19, 1st para: An anology of RelA with EF-Tu does not work, since different sets of interactions take place between these two factors and the tRNA.
12. Fig. 7: An attractive model to propose, but it is like stretching the facts a bit too much to talk about decoding process using RelA complexes. Certainly, these RelA structures suggest a step-wise progression of tRNA. This progression may not have anything to do with actual decoding process, but to the RelA-dependent (p)ppGpp synthesis.
13. The reference list should be checked carefully. Couple of listings caught my eyes, e.g., the same Jenner et al., paper is cited twice as 2010a and 2010b while bibliography of Li et

al., 2015 is incomplete.

Reviewer #3: This manuscript describes four structures of RelA bound to the E. coli 70S ribosome (from one cryo-EM experiment) derived from a single initial complex formation containing 70S ribosome, a cognate mRNA-tRNA pair, and RelA. These structures are significantly better resolution than previous a previously published 70S-RelA structure and could inform on the mechanism of RelA action. However, over-interpretation of the presented structures and misinterpretation of the literature leading to models that don't make biological sense means there are fundamental flaws with this manuscript.
1. Model for RelA activation: Based upon these four structures, the authors propose a catalytic mechanism for RelA production of ppGpp. The structures presented here are an extension of low resolution cryo-EM structures published in 2013 (Agirrezabala et al. EMBO Reports 2013) in which a distorted A site tRNA was also seen upon RelA binding. The model presented in this manuscript for how RelA functions is hard to reconcile with what is known about protein synthesis. During stress conditions when nutrients are limiting, deacylated tRNA is brought to the ribosomal A site as a ternary complex with EF-Tu and GTP (in the A/T state). After GTP hydrolysis and accommodation, a deacylated tRNA, as a result of the lack of nutrients, will not fully occupy the A/A state but primarily samples the A/T state (this state is also seen structurally; Noller (Korostelev), Ramarkrishnan, Steitz, Yusupov structures). RelA recognizes this A/T state (which is termed A/R in this study) which is distinct from the aminoacylated A/A state. The mode by which RelA recognizes the A/T tRNA is based on a model presented in the 2013 low resolution EM 70S-RelA study (which used the same exact complex as in this study). Since RelA is much less abundant than ribosomes (as also mentioned by the authors), it seems highly unlikely that RelA is ribosome-bound "waiting" for a deacylated tRNA to be brought to the A site. If it were, the authors suggest that EF-Tu and RelA would need to simultaneously bind. The authors superimpose EF-Tu and RelA and show they would have minimal overlap but yet, it makes no sense to think of this model in terms of RelA "waiting": the vast concentration difference (RelA is remarkably less abundant than ribosomes) would mean few ribosomes would contain RelA and hence the activation of the stringent response would be very slow unlike what we know from in vivo studies. Recognition of this A/T tRNA state would be the only thing that RelA recognises and would mean that normal tRNA states seen during translation would not be recognised: For example, tRNAs aminoacylated (non stress conditions), once delivered to the A site would occur a A/A state (not recognised by RelA), spontaneous peptide bond formation would occur, with subsequent hybrid tRNA state forming (not recognised by RelA). As pointed out, all of these states would never be recognized by RelA and makes for a simply and elegant model as proposed by Agirrezabala et al. So in summary, the model in Figure 4 does not make sense. Previously it was seen that RelA binds to 70S ribosomes lacking A site tRNA AND that RelA can bind in the absence of nucleotide (Agirrezabala et al. EMBO Reports 2013). Clearly RelA needs nucleotide for its function (ATP and GTP) so these are likely nonfunctional states. The interpretation of the former state by these authors is based solely upon their structure and there is no evidence that RelA is always bound to the 70S waiting for deactylated tRNA to appear in vivo (in contrast to say trigger factor where there is almost a 1:1 ratio of trigger factor compared to ribosomes and it is likely always found bound at the protein exit tunnel). The authors likewise question whether RelA binding would overlap with EF-G but again, it is highly unlikely they recognize the same complex if one thinks about elongation (p12). EF-G binds to a hybrid tRNA state to move the tRNA-mRNA pairs on the 30S. RelA is unable to recognize hybrid state tRNAs because, as proposed by Agirrezabala et al. (EMBO Reports 2013), it recognizes an A/T tRNA state induced by the lack of aminoacyl group attached to the CCA end. So it seems obvious that RelA and EF-G would never simultaneously bind.

The authors state: "The structures reveal large-scale conformational rearrangements in RelA when it binds deacyl-tRNA entering the 30S A site, suggesting a mechanism of activation of the (p)ppGpp synthetase." Without a nucleotide present (as in the 2013 study), it is hard to reconcile this statement and the extrapolation to a detailed molecular mechanism is without basis in the data presented.
2. tRNA accommodation. The second major issue is that a significant section of the manuscript centers on how the authors have captured a new, previously unseen, pre-accommodation tRNA state. However, as the structures they solved do not contain EF-Tu and instead contain RelA, it is unclear and perplexing how one could come to this conclusion.

Additionally, to suggest that the "A/R" tRNA-RelA complexes containing a cognate tRNA-mRNA pair in the A site is informing us on the mechanism for how non-cognate tRNA selection occurs is simply without basis or merit. ("Visualizing this earliest step of tRNA accommodation could

provide mechanistic insight into how the ribosome prevents miscoding by non-cognate tRNAs.")
The authors have not locked a non or near cognate state but rather their complexes contain a cognate state (as has been observed numerous times) and therefore it has nothing to do with tRNA selection errors.

Also the "closed" conformation cited in Stark et al., 2001; Valle, 2002; Schmeing et al., 2009; Fischer et al., 2015 are discussed as if they were closed conformations because used antibiotics and non-hydrolysable GTP analogues caused the closure. This is not correct. The antibiotics in one 70S-EF-Tu structure was bound to EF-Tu (kirromycin) which does not cause a domain closure and neither do GTP analogues. These ribosome structures had closed conformations because the tRNA-mRNA interaction pair in the A site was cognate!

The statement regarding decoding as "The structural understanding of this mechanism is limited." (p14) seems either incredibly naïve or intentionally exaggerated. Decoding is the best studied aspect of translation and to purport that our understanding of this is limited is deliberately misleading. Likewise to suggest that a new kind of decoding event is being observed in these structures ignores the fact that these structures aren't representative of decoding state structures- they are deacylated tRNAs that because they adopt an A/T conformation, are sensed by RelA! Misinformation like this pervades the manuscript.

Another related aspect of these issues is that the model the authors propose for decoding does not fully take into account the recent work of Demeshkina (Nature 2012) or worse, lumps it together with the Ogle work. Demeshkina argues against the Ogle data.

3. A/R tRNA: Of the 4 structures solved, 3 contain a distorted tRNA-RelA bound at the A site. This interaction has been previously described and noted as such in 2013. The authors decide to make a new name for this tRNA positon and call it A/R tRNA. The A/T tRNA was previously seen in slightly different orientations in a number of published X-ray and EM structures when bound to EF-Tu and RelA. It seems the authors may be making unwarranted efforts to add novelty to their studies and by attempting to define a new tRNA orientation. This needs to be removed.
Later in the text, the authors attest to these similarities: p 21: "Fourth, the overall conformation of tRNA in each RelA-bound complex is similar to that in EF-Tu-bound ribosome complexes, suggesting that tRNA dynamics and interactions with the ribosome may be similar."
4. The section comparing RelA with nucleic acid sensors is odd and out of place. As noted by the authors, it seems there are more differences than similarities. Yes they all respond to stress but to completely different types of stress- RelA is in response to nutritional stress while OAS and cGAS recognize foreign RNA or DNA. It seems like a huge leap to suggest these are similar proteins. It would seem more worthwhile to compare RelA to other ppGpp synthases like the recently published Steinchen et al. PNAS 2015 structure.

5. There is a general lack of information regarding local resolution except when it is good, for example, when mentioned at the decoding center (3.0-3.5 Å). This makes it extremely difficult to judge how good the fit of RelA is when there is no information. To build side chains of a RelA homology model into low resolution (Fig 2B) is misleading. The maps shown in Fig S3B and C suggest this building may be premature.

Knowing what the local resolution is important in the context of discussing potential small changes (~2 Å; see below) that may be within experimental error. Therefore, the local resolutions of the regions that are being discussed should be directly stated (i.e. not just shown in Fig S2).
Where this could be an issue is in looking at changes such as: "The acceptor arm of A/R tRNA and the N-terminal domain of RelA shift toward the head of the 30S subunit by ~2 Å between Structures II and III, and by ~2 Å between Structure III to IV" (p16). What is the local resolution of these regions? If one compares the structures (which is not mentioned in the paper how they did this), what is associated error with these comparisons? If the local resolution is 6-8 Å (I'm guess from Fig S2), it is hard to distinguish true small movements such as ~2 Å from noise in the data.

After appealing:
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Dear Andrei,

Thanks for your patience. I'm very sorry for the time its taken me to respond to your request for reconsideration. We've now been back over the reviews and the manuscript in light of the points you included in your letters. Our initial decision on the manuscript was informed by both Reviewers 2 and 3 who felt the conclusions overreached the data and who each recommended publication in a different journal. In response to these criticisms, you've proposed to restructure the paper to back off on the conclusions centered on decoding to refocus the paper on RelA and the stringent response, and this was the primary focus of our editorial discussion. Although we appreciate the fundamental nature of this question and how this work extends from the earlier RelA structures, unfortunately, there is not sufficient enthusiasm from the team for the main message. Building the comparison between the stringent response and innate immune activation does offer an interesting perspective; however, we don't see it as sufficient to carry the paper for Cell.

We take author concerns about reviewer bias very seriously and looked back carefully at Reviewer 3's comments. In this instance, Reviewer 3 raises many of the same "big picture" concerns as Reviewer 2, which were what we focused on to help guide our thinking about the paper. It's unfortunate that there may have been errors in the detailed comments. While this is not the norm in reviews for the journal, we do see it happen and it becomes part of the dialog between author and reviewer and editor as a manuscript is assessed. In thinking about the specific points you included relative to this reviewer's criticisms, we don't see that your responses would change the overall conceptual message and as a consequence our view remains that the study would be best published elsewhere.

I'm sorry that I can't be delivering better news. If you're interested in having the paper considered at one of the other Cell Press journals, I would be happy to gauge the initial interest from the the relevant editor and help facilitate a transfer of the reviews and reviewer identities.

Once again, I'm very sorry for leaving you in limbo with regard to our editorial decision and I'm sorry for any inconvenience this delay has caused.

Best wishes,