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A multifaceted small RNA modulates gene expression upon glucose limitation in *Staphylococcus aureus*

Delphine Bronesky, Emma Desgranges, Anna Corvaglia, Patrice François, Carlos J. Caballero, Laura Prado, Alejandro Toledo-Arana, Inigo Lasa, Karen Moreau, François Vandenesch, Stefano Marzi, Pascale Romby and Isabelle Caldelari

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Transaction Report:

(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 20th Apr 2018

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

As you will see from the reports, the three referees all express interest in the findings reported in your manuscript but also raise a number of points that will have to be addressed before they can support publication here. In particular, the referees ask for more insight on the functional context for RsaI-dependent regulation of both mRNAs and sRNAs as well as a better understanding on its mechanism of action. Furthermore, they list a number of constructive control experiments that should help strengthen the conclusiveness of the work and ask that you integrate the findings better with the existing literature. Finally, ref #3 points out that there is currently little data to show that RsaI is required for bacterial survival upon a shift in glucose availability. I have discussed this point with the other referees and while there may be good technical reasons why a RsaI deletion may not give a strong growth phenotype, we would encourage to include such loss of function experiments or at least comment on their outcome.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://emboj.embopress.org/about#Transparent_Process

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

REFEREE REPORTS:

Referee #1:

The authors have investigated the nature and function of a non-coding sRNA, RsaI, of Staphylococcus aureus. Major results/findings are: 1): the expression of RsaI is under the control of the carbon catabolite protein A (CcpA) and therefore inhibited by glucose; 2) RsaI interacts with a number of mRNAs and several sRNAs through base-pairing by two distinct regions; 3) RsaI inhibits translation of several target mRNAs such as glcU2, fn3K, and treB by interacting with their ribosome binding sites; 4) RsaI interacts with the 3'UTR of icaR mRNA encoding the repressor of the ica operon affecting the synthesis of the exopolysaccharides required for biofilm formation; 5) RsaI affects either negatively or positively the levels of many other mRNAs.

Based on these results, the authors conclude that RsaI is a multifunctional base-pairing sRNA to act as a metabolic switch responding to nutrient availability by regulating numerous mRNA and sRNA targets.

This work/paper reports unique interesting characteristics of RsaI of Staphylococcus aureus, a bacterial regulatory sRNA. While the data are overall clear and convincing to support the conclusion, the manuscript contains less convincing data/arguments.
1) The authors argue that RsaI serves as an RNA sponge to control the metabolic balance a by modulating the activities of three sRNAs (RsaG, RsaD, and RsaE) based on the observations that RsaI is able to base-pairs with three sRNAs and form ternary complexes with RsaG along with RsaI target mRNAs. Unfortunately, however, there is no experimental evidence to support this important proposal. Thus, the physiological consequence of the interaction between RsaI and three sRNAs is not clear at this stage.
2) The data and argument regarding the effect of RsaI on icaR mRNA and biofilm formation are confusing and ambiguous. Firstly, the ability of plasmid derived RsaI is extremely weak compared to the endogenous RsaI concerning the synthesis of PIA-PNAG exopolysaccharide (Fig. 4B). Second, there is no direct evidence for the inhibitory effect of RsaI on the icaR translation. I am wondering whether PIA-PNAG exopolysaccharide simply reflects the repression of icaR mRNA.

Referee #2:

The study by Bronesky et al. reports on the function of one of the most conserved staphylcoccal sRNAs, RsaI (a.k.a. RsaOG) both as a modulator of S. aureus carbon and energy metabolism and as an sRNA sponge in this organism. The authors show that RsaI expression is under control of the glucose-dependent global carbon catabolite repression control protein CcpA. By targeting a number of mRNAs involved in sugar uptake and utilization, RsaI is embedded into the complex regulatory network controlling central carbon flow in S. aureus. This function seems to be further supported by the interaction of RsaI with other sRNAs supposed to play their own roles in the control of metabolic functions. Finally, by targeting icaR (the repressor of polysaccharide intercellular adhesin (PIA)-mediated biofilm expression) RsaI supports biofilm formation in S. aureus.

The study is of high interest for both noncoding RNA biology and general microbiology. In addition, this study represents the first application of the MAPS technique to a gram-positive bacterium, which will be of interest to many other labs that are getting into finding targets of sRNAs in their organism of interest. The data generally is of high quality but we have a number of

comments, questions and suggestions to improve the manuscript prior to publication.

Major comments:

1: RsaI effects on global gene transcription: The authors state in line 245 that 'Significant differences were mostly observed between the mutant rsaI versus the same strain expressing RsaI from a plasmid.' A Northern blot should be included to assess the difference in RsaI expression between the wildtype situation and the overexpression of RsaI from the plasmid. Further along this line, when differences on global gene expression only occur upon overexpression of RsaI, I find it a bit daring to consider RsaI as a 'key regulator' (Discussion Ln. 396). Maybe it rather represents a factor that fine tunes metabolism to distinct requirements?

2. Page 10: If RsaI binding does not involve the anti-SD-motif, do the authors know which part of the the icaR-3'UTR is mediating the interaction instead? This would be of particular interest for understandig the overall function of RsaI in the process. Also, does the interaction result in enhanced RNaseIII-driven decay of icaR mRNA? And, finally, does RsaI binding influence the interaction between the icaR-5' and 3'UTRs? Performing gel retardation assays of icaR-5'/ 3'UTR binding in presence and absence of RsaI would help to answer this question.

3. RsaI connects sugar metabolism and NO stress through sRNA binding, Figure 5: Here, binding controls of RsaG with the respective mRNA targets are lacking.

4. The authors should do a better job putting their work into context with what is known about the regulatory logic of other well-characterized sRNAs with functions in carbon metabolism, foremost Spot42 and SgrS. For example, the G6P and other phosphosugar detoxification function of SgrS which involves both mRNA repression and mRNA activation. We felt that these aspects and how RsaI compares to those models sRNAs deserved more discussion.

Minor comments:

5. Lines 79-82: 'Indeed a S. aureus ccpA deletion mutant....' The meaning of this sentence is not clear. Please restate.

6. Lines 112: Instead of naming the different mRNAs, clearly state the general nature of the RsaI targets: sRNAs and mRNAs involved in various cellular processes (such as carbon/energy metabolism and biofilm formation).

7. Line 180: 'best mRNA candidate' On what criterion is this ranking based on?

8. Figure 1 (and also Fig. 5B) loading controls were run with similar RNA aliquots on separate gels. An explanation for doing so (similar size of RsaI and 5S rRNA) is only provided in Fig. S1. This information should also be given in the main text. In the strict sense, this actually does not represent genuine loading controls. Re-probing of the blots with a 5S rRNA probe would certainly have been the better approach.

9. Figure 1C: Is there a specific reason why a codY mutant was used as a control in the experiments?

10. Figure 1D: Please indicate the medium used (i.e. BHI) in the panel.

11. Figure 1E: Please indicate which strain was used in this experiment (incl. in the legend). How do the authors explain that fructose has a more striking effect than glucose on RsaI expression? Further regarding glucose, the blot of Fig. 1A does not match very well that of panel E.

12. Lines 156-160: Design and outcome of this experiment is interesting and should be added to the manuscript.

13. Line 180: Again, provide the criterion for the 'best candidate' selection.

14. Figures 2 B-C and S2: Please indicate the nM range of RsaI used in all assays. Otherwise it is

difficult to estimate the molar ratios and binding activities between RsaI and its targets.

15. Figure 2B: Generally, these gel retardation assays are difficult to judge. Thus, full-length mRNAs (app. 1kb in size) were employed in the experiments. Binding of the rather small RsaI is expected to cause only a very minor size difference and shift upon interaction. Therefore, at least controls of the mRNA targets without RsaI and, likewise, of RsaI without target mRNAs should be shown.

16. Figure 2A: Deleting the entire unpaired stretch in mut4 appears to be a rather harsh approach which is likely to hamper the overall structure of RsaI that might (unspecifically?) influence its binding activity. Or did the authors exclude that possibility? Why not exchanging the nucleotides or, even better, exchange the supposed interaction sites in the target mRNAs.

17. Lines 219-221: 'The data showed that RsaI mut3 bind to all three mRNAs similarly to the WT RsaI...' Is this really true? It appears in Fig. 2B that at least the interaction with icaR and fnk3 mRNAs are weaker than with wildtype RsaI.

18. Line 238: The effects described in this section would not be on gene TRANSCRIPTION but more generally on gene expression (steady-state levels).

19. Figure 3A: Again, please indicate glcU and fn3K mRNA concentrations used, and mark RBS and start codons (as indicated in the figure legend).

20. Line 313: Wrong figure reference: It should read Fig. 4A instead of Figure 2.

21. Figure 4A: In this figure, again, controls and RsaI concentrations are lacking. What does lane C in the right-hand panel stand for?

22. PIA/PNAG detection in WT, rsaI mutant and complemented strain: Although this experiment clearly shows that both RsaI and an intact icaR-3'UTR are required to trigger PIA expression, the overall design of the experiment is critical. Thus, another wildtype strain is used in this experiment which produces high amounts of PIA upon NaCl addition to standard TSB medium (which contains glucose). NaCl is known as an efficient trigger of biofilm production in S. aureus and as such NaCl represents a novel player and stimulus whose role for RsaI expression is not defined yet. This issue should at least be actively addressed in the discussion section.

23. Line 348: Could the authors please explain why 'formation of a ternary complex is a good indication that RsaG binding does not interfere with the regulatory functions of RsaI.'

24. With respect to biofilm formation, Figure 6 is confusing. Here, we strongly recommend the key features of icaR-dependent autoregulation and the role of RsaI in the process be shown in a separate scheme. As it stands now, the reader gets the impression that RsaI inhibits icaR under glucose-rich conditions. However, as the authors have convincingly shown, RsaI comes only into play when glucose becomes scarce. Also, in the discussion section the authors should elaborate a bit more on this interesting issue. Particularly, they should put their findings into perspective with previous data (obtained by numerous groups) showing that glucose is generally an efficient inducer of PIA biofilm formation both in S. aureus and S. epidermidis.

25. Line 512: The Görke and Vogel 2009 review is sort of outdated. Consider Papenfort K, Vogel J 2014 Front Cell Infect Microbiol. (PMID:25077072).

Referee #3:

The manuscript by Bronesky, et al., attempts to characterize a small RNA of unknown function in Staphylococcus aureus, RsaI. The authors use two global approaches to characterize RsaI interactions with other RNAs, and RsaI-dependent changes in gene expression. Additional experiments looked in vitro at RsaI interactions with putative targets, and the in vivo PIA-PNAG production phenotype of strains with and without RsaI. The results are intriguing, but the authors'

interpretations overstate the results. I hope my comments will be helpful to the authors as they prepare a revised version of the manuscript.

Major Results and Critiques
1. Levels of RsaI are sensitive to glucose- rsaI is repressed by glucose in a CcpA-dependent manner (Fig. 1).
a. The authors assert that RsaI is involved in a metabolic switch from glucose-rich to glucose-poor conditions. The expression would be consistent with that model, but there are no phenotypic data to support this claim.
b. The growth curve of the wild-type strain in the two different media Fig. 1B is not very informative and does not support this model.
2. The authors identify a set of RNAs, both mRNAs and sRNAs that are enriched in pull-downs with RsaI.
a. There is no organization or deep analysis of the data from the pull-downs. Lots of targets were enriched in the pulldown with RsaI. The authors focus on only a few putative targets, but it's not clear why those were chosen over others. It would be very helpful to organize Table S3 in some way to highlight genes in functional groups, and to provide additional explanation of why certain potential targets were further characterized and others were not. Some of the RNAs were enriched 1000s of fold, and were not characterized while RNAs that were only 3 or 4-fold enriched were characterized. It would also be useful to correlate the information from MAPS and RNA-seq. Which targets were enriched in MAPS and strongly differentially regulated in RNA-seq? These would seem like the most obvious ones for further validation.
b. Table 1 is hard to understand at first glance. In particular the 2 different interactions shown for RsaI and icaR, and HG001_02520 are confusing.
3. The authors demonstrate in vitro sRNA-mRNA and sRNA-sRNA interactions for a subset of putative targets (Fig. 2).
a. For Fig. 2, it would be VERY helpful to have models illustrating the putative interactions for each sRNA-mRNA or sRNA-sRNA complex. This would help the reader better understand the predicted effects of different mutations, and evaluate the conclusions.
b. For icaR binding, mut3 looks impaired. Please clarify this point.
4. The authors identify differentially expressed genes in strains with variations in RsaI via RNA-seq and find a broad spectrum of changes, though it is not possible to tell direct from indirect effects.
a. Lines 244-260, it is hard to tell which strains are being used for comparisons. I think lines 249-256 are comparisons between rsaI mutant and complemented mutant, but then line 258 describes levels of two sRNAs that were enhanced in the WT strain (compared to what?). Please clarify.
b. The long description in the text of genes that were differentially regulated in RNA-seq would be more clearly presented as a table in the main body of the manuscript, with genes grouped by putative functional categories. Then the text description could be much shorter and more concise.
c. The transition to the next section (line 277) should be made more clear. After the explanation of the RNA-seq results, I was expecting the next step to be validation of some of the putative targets identified in RNA-seq. Instead, the authors return to two targets that were relatively poorly enriched in MAPS and not differentially regulated in RNA-seq.
5. Direct inhibition of ribosome-mRNA complex formation by RsaI is shown by toeprinting assays for two targets (glcU and fn3K). Translational repression by RsaI is shown for these two targets plus three more (Fig. 3).
a. It would be helpful to have diagrams with this figure, showing the putative base pairing interactions and the positions of these interactions relative to the ribosome binding sites. Even though it would be redundant with the table, it helps the reader assimilate the information more easily.
b. Lines 296-298, interpretation of these experiments goes too far. RsaI regulation of translation initiation of glcU and fn3K is supported by the data. The reporters for the other mRNAs show only translational regulation by RsaI, but do not provide information on the mechanism of that regulation.
6. Interaction of RsaI with the icaR 3' UTR is demonstrated, along with the impact of RsaI and mutations in the icaR 3' UTR on PIA-PNAG production phenotypes (Fig. 4).
a. This section on icaR was really hard to follow. A cartoon demonstrating the intramolecular icaR interaction, and the putative RsaI-icaR interaction, would be very helpful. It is also important to explicitly state the proposed mechanism: RsaI represses production of a repressor, which should enhance PIA-PNAG production. The authors didn't fully explain the model or provide predicted outcomes, which made it hard to evaluate and interpret the data.
b. What is the icaR SUBST mutation, and how is that predicted to impact the intramolecular and

intermolecular (with RsaI) interactions?
c. Is RsaI's impact on PIA-PNAG levels solely through regulation of icaR? This may be hard to get at, but the data imply this might be true because mut5 still increases PIA-PNAG.
7. It was demonstrated in vitro that RsaI-RsaG and RsaI-mRNA complexes are not mutually exclusive. In vivo, RsaI/G do not impact one another's stability. (Fig. 5)
a. The data are overinterpreted- e.g., lines 348-349: "The formation of a ternary complex is a good indication that RsaG binding does not interfere with the regulatory functions of RsaI." The in vitro data do not address this point. In vivo experiments to demonstrate this are necessary if this claim is to be made.
b. The experiment with RsaG and RsaD at the end of this section don't logically flow.
c. Lines 380-381 "These data suggested that through the binding of sRNAs, RsaI would link sugar metabolism pathways, carbon source utilization, energy production and stress responses." There is no direct evidence that sRNA-sRNA interactions are physiologically relevant, or that these interactions alter the activities of the sRNAs. In fact, the in vitro data suggest that sRNA-sRNA and sRNA-mRNA interactions can occur simultaneously, which could suggest that RsaI regulation of mRNA targets is not impaired when RsaG pairs.
8. Discussion:
a. There is no evidence of sponge-like activity. In fact (as mentioned above), the data suggest that the sRNA-sRNA interactions do not impair the ability of RsaI to regulate (at least some) targets. This would not be consistent with a sponge-like activity.
b. There is a lot of very interesting speculation regarding the role of RsaI in coordinating a metabolic switch. This would be very exciting if true. But there are no data to directly support this model in this manuscript.

Minor comments
1. The abstract could be more clear on the regulation of rsaI, i.e., that when glucose is high, RsaI is low and vice versa.
2. The abstract overstates the findings, e.g., lines 40-41. There is no direct evidence that RsaI regulates the activities of other sRNAs.
3. Tables and figures should be numbered and introduced in the order that they are discussed in the text (e.g., Fig. 1 comes before Table 1 in the text).
4. In general, the sections were disconnected from one another. It was not clear that results from one set of experiments were flowing logically to guide the next experiments.

1st Revision - authors' response 6th Nov 2018

Authors' response to Reviewers:

Referee's 1 comments

« The authors have investigated the nature and function of a non-coding sRNA, RsaI, of Staphylococcus aureus. Major results/findings are: 1): the expression of RsaI is under the control of the carbon catabolite protein A (CcpA) and therefore inhibited by glucose; 2) RsaI interacts with a number of mRNAs and several sRNAs through base-pairing by two distinct regions; 3) RsaI inhibits translation of several target mRNAs such as glcU2, fn3K, and treB by interacting with their ribosome binding sites; 4) RsaI interacts with the 3'UTR of icaR mRNA encoding the repressor of the ica operon affecting the synthesis of the exopolysaccharides required for biofilm formation; 5) RsaI affects either negatively or positively the levels of many other mRNAs. Based on these results, the authors conclude that RsaI is a multifunctional base-pairing sRNA to act as a metabolic switch responding to nutrient availability by regulating numerous mRNA and sRNA targets. This work/paper reports unique interesting characteristics of RsaI of Staphylococcus aureus, a bacterial regulatory sRNA. While the data are overall clear and convincing to support the conclusion, the manuscript contains less convincing data/arguments. »

We thank the referee for the positive comments and we hope that the revised version of our manuscript fulfill the reviewer' expectations.

"1) The authors argue that RsaI serves as an RNA sponge to control the metabolic balance a by modulating the activities of three sRNAs (RsaG, RsaD, and RsaE) based on the observations that RsaI is able to base-pairs with three sRNAs and form ternary complexes with RsaG along with RsaI

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target mRNAs. Unfortunately, however, there is no experimental evidence to support this important proposal. Thus, the physiological consequence of the interaction between RsaI and three sRNAs is not clear at this stage."

> *Response. We have shown in Figure 5 that RsaG did not prevent the formation of complexes involving RsaI and at least three of its targets and that RsaG was not able to bind efficiently to these mRNAs. In addition, in vivo we showed that the specific RsaI-dependent repression of fn3K is not affected in the WT and mutant ΔrsaG strains. We also have performed the MAPS approach on several RsaI mutants, which support the fact that RsaI uses independent regions to bind either its mRNA targets or RsaG. We also would like to mention that in the presence of G6-P, RsaG is highly transcribed whereas the steady state yield of RsaI is strongly reduced. Taken together these data strongly suggested that RsaG does not affect the regulatory activities of RsaI, although RsaG slightly enhances the half-life of RsaI, in agreement with the MAPS data that the two RNAs interact in vivo.*

"2) The data and argument regarding the effect of RsaI on icaR mRNA and biofilm formation are confusing and ambiguous. Firstly, the ability of plasmid derived RsaI is extremely weak compared to the endogenous RsaI concerning the synthesis of PIA-PNAG exopolysaccharide (Fig. 4B). Second, there is no direct evidence for the inhibitory effect of RsaI on the icaR translation. I am wondering whether PIA-PNAG exopolysaccharide simply reflects the repression of icaR mRNA."

> *Response. The comparative transcriptomic analysis (Tables 1, S6) showed that the steady state levels of icaR mRNA do not significantly vary in the WT strain, in the ΔrsaI mutant strain, and in the same strain complemented with a plasmid expressing RsaI. This data suggested that the transcriptional control is not affected in the ΔrsaI mutant strain. We have reproduced the dot blot experiment to visualize the PIA-PNAG synthesis. The data showed that RsaI deletion led to a significant and reproducible decrease in PIA-PNAG production (Figure 4C), which is restored by complementation with a plasmid expressing RsaI WT. The complementation is much less efficient with RsaI mut5 suggesting that the mutation has altered the regulatory activity of RsaI. Finally, we have also performed additional gel retardation assays in order to analyze the binding of RsaI to the 5' UTR and the 3'UTR of icaR. We showed that RsaI preferentially binds to the 3'UTR and does not prevent the pairings between the two UTRs of icaR mRNA (Figure S3). The mutation mut5 however strongly altered the binding of RsaI mut5 to the 3'UTR. Taking into account our data, it is tempting to propose that RsaI enhances the synthesis of PIA-PNAG through the repression of icaR at the translational level. Although the mechanism is not yet defined, we postulate that binding of RsaI to the 3'UTR of icaR mRNA might compete with a trans-acting activator of icaR translation, or contribute to the stabilization of the interaction between the SD sequence and the anti-SD located in the 3'UTR (Figure 6).*

Referee's 2 comments

"The study by Bronesky et al. reports on the function of one of the most conserved staphylcoccal sRNAs, RsaI (a.k.a. RsaOG) both as a modulator of S. aureus carbon and energy metabolism and as an sRNA sponge in this organism. The authors show that RsaI expression is under control of the glucose-dependent global carbon catabolite repression control protein CcpA. By targeting a number of mRNAs involved in sugar uptake and utilization, RsaI is embedded into the complex regulatory network controlling central carbon flow in S. aureus. This function seems to be further supported by the interaction of RsaI with other sRNAs supposed to play their own roles in the control of metabolic functions. Finally, by targeting icaR (the repressor of polysaccharide intercelular adhesin (PIA)-mediated biofilm expression) RsaI supports biofilm formation in S. aureus. The study is of high interest for both noncoding RNA biology and general microbiology. In addition, this study represents the first application of the MAPS technique to a gram-positive bacterium, which will be of interest to many other labs that are getting into finding targets of sRNAs in their organism of interest. The data generally is a of high quality but we have a number of comments, questions and suggestions to improve the manuscript prior to publication."

> *We thank the referee for the positive and thoughtful comments.*

1: "RsaI effects on global gene transcription: The authors state in line 245 that 'Significant differences were mostly observed between the mutant rsaI versus the same strain expressing RsaI from a plasmid.' A Northern blot should be included to assess the difference in RsaI expression between the wildtype situation and the overexpression of RsaI from the plasmid. Further along this

line, when differences on global gene expression only occur upon overexpression of RsaI, I find it a bit daring to consider RsaI as a 'key regulator' (Discussion Ln. 396). Maybe it rather represents a factor that fine tunes metabolism to distinct requirements?"

Response. In the Table S6, the RNAseq data revealed that RsaI is 17-fold overexpressed in the complemented strain than in the wild-type strain due to the plasmid copy number. We have also added a figure representing the northern blot with the samples used for the transcriptomic analyses (Figure S1E). This shows clearly that RsaI is indeed overexpressed in the complemented strain as compared to the WT strain. Although the comparative transcriptomic data has revealed more pronounced effects between the mutant strain and the complemented mutant isolate, we nevertheless observed significant and reproducible effects between the mutant and WT strains including the repression of enzymes involved in the pentose phosphate pathway and in the thiamine synthesis, and the activation of genes involved in fermentation. We nevertheless agree with the referee that the RNA most likely fine-tunes metabolism in response to glucose consumption. We have modified accordingly the discussion in the revised version of the manuscript.

2. "Page 10: If RsaI binding does not involve the anti-SD-motif, do the authors know which part of the icaR-3'UTR is mediating the interaction instead? This would be of particular interest for understanding the overall function of RsaI in the process. Also, does the interaction result in enhanced RNaseIII-driven decay of icaR mRNA? And, finally, does RsaI binding influence the interaction between the icaR-5' and 3'UTRs? Performing gel retardation assays of icaR-5'/ 3'UTR binding in presence and absence of RsaI would help to answer this question".

Response. We obtained preliminary data showing that using a MS2-tagged icaR either full length or its 3'-UTR, RsaI was recovered as one of the first targets in vivo. Using algorithm prediction, we identified a region of base-pairing complementarity (Table 1) located in the 3'UTR of icaR, which does not overlap with the anti-SD sequence. In addition, the gel retardation assays as shown in Figure 4B demonstrates in vitro that the 5'UTR of icaR is only weakly recognized by RsaI. A model of the icaR-RsaI complex has been added in Figure 4A. As proposed by the reviewer, we have also performed a new gel retardation assay to monitor the effect of RsaI on the formation of pairings between the 5' and 3'UTRs of icaR (Figure S3A). In this experiment, radiolabelled 3'UTR was mixed with 500 nM of 5'UTR (which leads to ca. 50% of 3'UTR bound to 5'UTR (Figure S3A, lane 2)) and with increasing concentrations of RsaI (Figure S3A, lanes 4 to 8). In addition to the binary 3'UTR-RsaI complex, a signal was detected corresponding to the formation of a ternary complex between the three RNA species. We conclude that in vitro RsaI does not displace the 3'UTR from the 5'UTR. Because the PIA-PNAG synthesis is reduced in the mutant strain, we hypothesized that binding of RsaI to the 3'UTR of icaR mRNA would contribute to the inhibition of icaR translation in an indirect manner either through the stabilization of the mRNA circularization or by preventing the binding of a translational activator. We have introduced new figures and have modified the discussion accordingly.

3. "Figure 5: Here, binding controls of RsaG with the respective mRNA targets are lacking."

Response. As the referee suggested, we have now added a gel retardation experiment demonstrating that RsaG does not bind to several mRNA targets of RsaI, i.e glcU_2 and HG001_1242 mRNAs (Figure 5B). Therefore, we conclude that the formation of ternary complexes do not result from a binding of RsaG to the mRNA but rather to RsaI.

4. "The authors should do a better job putting their work into context with what is known about the regulatory logic of other well characterized sRNAs with functions in carbon metabolism, foremost Spot42 and SgrS. For example, the G6P and other phosphosugar detoxification function of SgrS, which involves both mRNA repression and mRNA activation. We felt that these aspects and how RsaI compares to those models sRNAs deserved more discussion."

Response. Sugars are indeed major sources of energy for the bacterial growth but conversely accumulation of glucose phosphate intermediates can also be detrimental to the cell and cause growth inhibition. Indeed the work of several teams such as K. Vanderpool made important contributions showing how a sRNA is able to control networks regulating glucose uptake and efflux, and metabolic enzymes to restore metabolic homeostasis and to help the bacteria to overcome the stress. We have taken into account the referee's suggestion and have modified the discussion accordingly. RsaI has a different role than SgrS but also modulate glucose uptake, and enzymes involved in essential metabolic pathways such as the

pentose-phosphate pathway. In our study, we think that RsaI contributes to adapt the metabolism when glucose is metabolized but also represses genes that are no more necessary. When RsaI is produced from a constitutive promoter, a phenotype on cell growth was observed suggesting that the CcpA-mediated repression of RsaI is essential. We do not know what are the regulated genes responsible for this phenotype although the metabolic enzymes or FN3K might be obvious candidates. Therefore, our data suggested that RsaI represents a signature of a metabolic change as the result of glucose consumption, and contribute to fine-tune the regulation of metabolic pathways of the bacterial population to enter the stationary phase of growth.

Minor comments:
"5. Lines 79-82: 'Indeed a S. aureus ccpA deletion mutant....' The meaning of this sentence is not clear. Please restate."

Response. We have modified the sentence.

"6. Lines 112: Instead of naming the different mRNAs, clearly state the general nature of the RsaI targets: sRNAs and mRNAs involved in various cellular processes (such as carbon/energy metabolism and biofilm formation)."

Response. The genes, for which the function has been attributed, have been now clearly mentioned in the text.

"7. Line 180: 'best mRNA candidate' On what criterion is this ranking based on?"

Response. We have estimated the enrichment values for the MAPS using DEseq2 including statistical analysis process as described in the "material and methods". We have used a stringent value in order to take into account only the candidate mRNAs with a p-value < 0.05 and with a fold change of at least 4.

"8. Figure 1 (and also Fig. 5B) loading controls were run with similar RNA aliquots on separate gels. An explanation for doing so (similar size of RsaI and 5S rRNA) is only provided in Fig. S1. This information should also be given in the main text. In the strict sense, this actually does not represent genuine loading controls. Re-probing of the blots with a 5S rRNA probe would certainly have been the better approach."

Response. The information was added in the legend of Figure 1 too. We agree with the reviewer that this does not represent genuine loading controls. However, because sizes of 5S and RsaI are indeed very close, de-hybridization of DIG probes are not very efficient as seen in figure S1C. This is why we usually tend to run two different gels with the same samples.

"9. Figure 1C: Is there a specific reason why a codY mutant was used as a control in the experiments?"

Response. We also used CodY which is a global regulator activated in vitro by branched amino-acids. CodY controls expression of numerous genes involved in metabolism and in virulence, as did CcpA. Because we were not sure that CcpA would be the regulator of RsaI transcription, we also have probed the effect of several known regulators triggering alteration of the metabolism. This information was emphasized in the text.

"10. Figure 1D: Please indicate the medium used (i.e. BHI) in the panel."

Response. We have indicated the medium above the panel.

"11. Figure 1E: Please indicate which strain was used in this experiment (incl. in the legend). How do the authors explain that fructose has a more striking effect than glucose on RsaI expression? Further regarding glucose, the blot of Fig. 1A does not match very well that of panel E."

Response. The wild type strain HG001 was used in the experiment as indicated in the figure. The experiment was made at least three times with high reproducibility. In the first submission of the manuscript, we included a rather low exposition of the autoradiography of the northern blot in Figure 1A. Now we have modified the figure showing the same experiment but with a higher exposition time similar to the experiment performed with glucose. Although the metabolism of fructose and glucose share similar intermediate structures, they also have different metabolic fates explaining why the repression of RsaI seems to be longer with fructose than with glucose.

"12. Lines 156-160: Design and outcome of this experiment is interesting and should be added to the manuscript."

> *Response. The result of the experiment was added as Figure S1E. We have indeed observed that RsaI expressed from the plasmid under its own promoter is repressed by glucose in the MHB medium.*

"13. Line 180: Again, provide the criterion for the 'best candidate' selection."

> *Response. See comment above responding to point 7*

"14. Figures 2 B-C and S2: Please indicate the nM range of RsaI used in all assays. Otherwise it is difficult to estimate the molar ratios and binding activities between RsaI and its targets. "

> *Response. In all the gel retardation assays presented in Figure 2B and S2A, RsaI (144 nucleotides) has been radiolabelled. The concentration of RsaI is considered as negligible (< 1 pM). This is why we can estimate the Kd value as the concentration of the mRNA causing 50% of shift. We added this aspect in the material and methods section.*

"15. Figure 2B: Generally, these gel retardation assays are difficult to judge. Thus, full-length mRNAs (app. 1kb in size) were employed in the experiments. Binding of the rather small RsaI is expected to cause only a very minor size difference and shift upon interaction. Therefore, at least controls of the mRNA targets without RsaI and, likewise, of RsaI without target mRNAs should be shown.

> *Response. The control of RsaI alone is always shown in the first lane of the gels. Increasing concentrations of cold mRNA (ca. 700-1000 nucleotides in size) is mixed with radiolabelled RsaI. Hence under these conditions, a large delay of migration is expected to occur for the RsaI-mRNA complex. Cold mRNA alone will not be seen on the autoradiography. We checked the legends of figures to be sure that this information has been provided.*

"16. Figure 2A: Deleting the entire unpaired stretch in mut4 appears to be a rather harsh approach which is likely to hamper the overall structure of RsaI that might (unspecifically?) influence its binding activity. Or did the authors exclude that possibility? Why not exchanging the nucleotides or, even better, exchange the supposed interaction sites in the target mRNAs."

> *Response. We have decided to delete the region of RsaI (27 bases), which would include the predicted nucleotides involved in the binding with its major mRNA targets. Using the Mfold software, the general structure of the RNA was predicted to be preserved. Moreover, the RNA carrying the same deletion was tagged with MS2, and the RNA-seq data performed after the affinity chromatography revealed that MS2-RsaI mut4 was still able to bind efficiently RsaG, which suggests that at least the second track of G is available (see Table S4).*

"17. Lines 219-221: 'The data showed that RsaI mut3 bind to all three mRNAs similarly to the WT RsaI...' Is this really true? It appears in Fig. 2B that at least the interaction with icaR and fnk3 mRNAs are weaker than with wildtype RsaI."

> *Response. For icaR, we show another set of experiments, which better showed that the binding affinity for RsaI mut3 is very similar to that for the RsaI wild type. We have also quantified signals on gel retardation experiments by using ImageQuant (see Figure 2B) supporting that the mutation in RsaI mut3 did not impair interactions with mRNAs, but strongly affect the recognition of RsaG (Figure 2C).*

"18. Line 238: The effects described in this section would not be on gene TRANSCRIPTION but more generally on gene expression (steady-state levels)."

> *Response. We have changed "transcription" by "expression" as indeed RNAseq data variation would result not only from impaired/enhanced transcription but also from stabilization/destabilization of the RNA by ribonucleases.*

"19. Figure 3A: Again, please indicate glcU and fn3K mRNA concentrations used, and mark RBS and start codons (as indicated in the figure legend)."

> *Response. We have completed the legend of figure 3A with the concentration of mRNAs used in the experiment (50 nM) and added the RBS and start codons.*

"20. Line 313: Wrong figure reference: It should read Fig. 4A instead of Figure 2."

> *Response. Actually both figures show the binding between RsaI and icaR. We have added the*

reference to figure 4B in the text.

"21. Figure 4A: In this figure, again, controls and RsaI concentrations are lacking. What does lane C in the right-hand panel stand for?"

Response. We apologize for the lack of important information concerning the gel retardation assays. We have now completed the description of the method in the legend. Lane C represents the control of binding between radiolabelled RsaI and full-length icaR mRNA (50 nM).

22. PIA/PNAG detection in WT, rsaI mutant and complemented strain: Although this experiment clearly shows that both RsaI and an intact icaR-3'UTR are required to trigger PIA expression, the overall design of the experiment is critical. Thus, another wildtype strain is used in this experiment, which produces high amounts of PIA upon NaCl addition to standard TSB medium (which contains glucose). NaCl is known as an efficient trigger of biofilm production in S. aureus and as such NaCl represents a novel player and stimulus whose role for RsaI expression is not defined yet. This issue should at least be actively addressed in the discussion section.

Response. Because RsaI is conserved in Staphylococcaceae and the strain HG001 did not show detectable PIA-PNAG production, we switched to the strain 132. This has been mentioned in the text of the results section. The NaCl concentration used in the experiment is 3% and was included in all strain cultures. The addition of NaCl is required for PIA-PNAG production in 132 strain (Vergara-Irigaray et al, 2009, Infect Immun 77(9): 3978). As seen in the northern blot of the figure 4C, under these conditions of growth, RsaI is expressed at high level. Moreover in these experiments, RsaI has been expressed under the control of the constitutive and strong promoter PblaZ (Charpentier et al, 2004, Appl Environ Microbiol).

"23. Line 348: Could the authors please explain why 'formation of a ternary complex is a good indication that RsaG binding does not interfere with the regulatory functions of RsaI."

Response. We have shown in Figure 2 and Table S4 that RsaI carries two distinct domains by using MAPS approach combined to gel retardation assays, i.e. the G-track motif recognizing RsaG and the CU-rich interhelical region recognizing several mRNA targets. We also have shown that RsaG does not bind efficiently to the mRNA targets of RsaI. Therefore, the formation of a ternary RsaG-RsaI-mRNA shows that RsaG does not prevent the mRNA binding to RsaI. We have performed an additional experiment showing that the RsaI-dependent repression of fn3k-lacZ fusion is not altered in the WT and ΔrsaG mutant strains. Hence, it is tempting to propose that RsaG does not significantly alter the regulatory activities of RsaI on its target mRNAs, although RsaG slightly enhanced the half-life of RsaI. We have modified the discussion accordingly to give a more complete message.

24. With respect to biofilm formation, Figure 6 is confusing. Here, we strongly recommend the key features of icaR-dependent autoregulation and the role of RsaI in the process be shown in a separate scheme. As it stands now, the reader gets the impression that RsaI inhibits icaR under glucose-rich conditions. However, as the authors have convincingly shown, RsaI comes only into play when glucose becomes scarce. Also, in the discussion section the authors should elaborate a bit more on this interesting issue. Particularly, they should put their findings into perspective with previous data (obtained by numerous groups) showing that glucose is generally an efficient inducer of PIA biofilm formation both in *S. aureus* and *S. epidermidis*.

Response. As the referee suggested, we have modified figure 6, which now contains two panels, when RsaI expression is repressed (with glucose/G6P) or alleviated (glucose consumed). We also added an insert summarizing the potential mechanisms by which RsaI would repress the synthesis of IcaR. We hope that the new figure is a better illustration of our data.

The role of glucose in inducing PIA biofilm formation, and the fact that CcpA enhanced icaA and PIA formation are indeed well documented (Seidl et al., 2008). Nevertheless, in the PIA/PNAG dot blot experiment shown in Figure 4C, RsaI was not expressed under its native promoter, and therefore the effect of RsaI on PIA repression is occurring independently of the presence of glucose. Nevertheless we have modified the discussion according to the referee's suggestions.

"25. Line 512: The Görke and Vogel 2009 review is sort of outdated. Consider Papenfort K, Vogel J 2014 Front Cell Infect Microbiol. (PMID:25077072)."

Response. We have introduced the reference in the manuscript.

Referee's 3 comments

"The manuscript by Bronesky, et al., attempts to characterize a small RNA of unknown function in Staphylococcus aureus, RsaI. The authors use two global approaches to characterize RsaI interactions with other RNAs, and RsaI-dependent changes in gene expression. Additional experiments looked in vitro at RsaI interactions with putative targets, and the in vivo PIA-PNAG production phenotype of strains with and without RsaI. The results are intriguing, but the authors' interpretations overstate the results. I hope my comments will be helpful to the authors as they prepare a revised version of the manuscript.
Major Results and Critiques
1. Levels of RsaI are sensitive to glucose- rsaI is repressed by glucose in a CcpA-dependent manner (Fig. 1).
a. The authors assert that RsaI is involved in a metabolic switch from glucose-rich to glucose-poor conditions. The expression would be consistent with that model, but there are no phenotypic data to support this claim."

Response. Growth curves of the WT strain 132 and its ΔrsaI deleted mutant strain complemented with a vector expressing rsaI under a strong constitutive promoter were analyzed (figure S5). In parallel we have monitored the steady-state levels of RsaI during cell growth. We showed that the yields of RsaI are the highest for the WT strain complemented with the plasmid overexpressing RsaI. This is only under these conditions that we have observed a reproducible effect on cell growth. Therefore, as mentioned above (point 4 referee 2), we think that the CcpA-dependent repression of RsaI is essential to prevent the repression of essential genes when glucose is present. Although we do not known the nature of the essential genes responsible for this phenotype, the metabolic enzymes and FN3K might be obvious candidates. The data have been presented in the results section and were added in the discussion of the manuscript.

b. The growth curve of the wild-type strain in the two different media Fig. 1B is not very informative and does not support this model. "

Response. Because the panel B, which represented the comparison of the growth curve of the wild-type strain HG001 in BHI or MHB poor in glucose, was not very informative, we decided to remove the figure from the manuscript.

2. The authors identify a set of RNAs, both mRNAs and sRNAs that are enriched in pull-downs with RsaI.
a. There is no organization or deep analysis of the data from the pull-downs. Lots of targets were enriched in the pulldown with RsaI. The authors focus on only a few putative targets, but it's not clear why those were chosen over others. It would be very helpful to organize Table S3 in some way to highlight genes in functional groups, and to provide additional explanation of why certain potential targets were further characterized and others were not. Some of the RNAs were enriched 1000s of fold, and were not characterized while RNAs that were only 3 or 4-fold enriched were characterized. It would also be useful to correlate the information from MAPS and RNA-seq. Which targets were enriched in MAPS and strongly differentially regulated in RNA-seq? These would seem like the most obvious ones for further validation."

Response. We apologize for the confusion in the description of the MAPS data and we have modified the text accordingly. Table 1 is a shortened version of Table S3 with only the best 16 candidates having a fold change higher than 4 that we have analyzed more deeply. Table S3 represents all RNAs, which were pulled out specifically with MS2-RsaI listed following a decreasing fold change. This ratio corresponds to the number of reads obtained from the MS2-RsaI purification versus the number of reads obtained from the MS2 alone as control. Since the MS2 tag alone is expressed in the wild-type background, the untagged RsaI is also present. The highest enrichment value that we obtained is 33 for icaR (not 1000 as mentioned by the reviewer). In the last column of the table 1, we indicated the fold change obtained in transcriptomic analysis. As stated in the manuscript, most of RsaI targets identified by MAPS did not show any significant mRNA level variations when RsaI was deleted or overexpressed. We postulated that MAPS preferentially selected mRNAs that are translationally regulated by RsaI. The MAPS approach has also been carried out on two

other mutants RsaI (mut2 and mut4), and the data presented in Table S4 showed that the deletion of the G-track motif has a major decrease of the enrichment of RsaG whereas most of the mRNAs were still enriched at a very similar level. Conversely, the deletion of the C/U rich unpaired region (MS2-RsaI mut4) has altered considerably the RNA patterns since a strong decrease of many mRNAs was observed while RsaG was still detected at a reasonable enrichment fold. Therefore, we are confident that the most enriched RNAs, which were detected by the MAPS represented the main targets of RsaI. Such an observation was also made for RsaA (Romilly et al., 2014; Tomasini et al., 2017), and also in E. coli (work of Eric Massé's lab).

b. Table 1 is hard to understand at first glance. In particular the 2 different interactions shown for RsaI and icaR, and HG001_02520 are confusing."

Response. The title of Table 1 was modified for clarity. We have re-run the update version of the program IntaRNA2.0 (Mann et al, Nucleic acid Research, 2017) with the full-length mRNAs of icaR and HG001_02520. We obtained only one site of interaction per mRNA (see Table 1), which is consistent with the gel retardation assays presented in the manuscript.

3. The authors demonstrate in vitro sRNA-mRNA and sRNA-sRNA interactions for a subset of putative targets (Fig. 2).
a. For Fig. 2, it would be VERY helpful to have models illustrating the putative interactions for each sRNA-mRNA or sRNA-sRNA complex. This would help the reader better understand the predicted effects of different mutations, and evaluate the conclusions."

Response. The base-pairings between RsaI and RsaG was present in the original figure; we added the predicted interactions for RsaI and icaR, glcU_2 and fn3K below the autoradiographies of the gels.

"b. For icaR binding, mut3 looks impaired. Please clarify this point."

Response. As mentioned for the point 17 of the reviewer 2, we have done again the experiments with RsaI mut3 and quantify the experiments. Our data showed that the mutation in RsaI mut3 did not impair interactions with mRNAs (Figure 2B).

4. The authors identify differentially expressed genes in strains with variations in RsaI via RNA-seq and find a broad spectrum of changes, though it is not possible to tell direct from indirect effects.
a. Lines 244-260, it is hard to tell which strains are being used for comparisons. I think lines 249-256 are comparisons between rsaI mutant and complemented mutant, but then line 258 describes levels of two sRNAs that were enhanced in the WT strain (compared to what?). Please clarify."

Response. Indeed these sentences were not clear and we have corrected them in the manuscript. Most of the comparisons that were statistically different arose between the ΔrsaI mutant and the complemented strains. Nevertheless the same pathways were also found slightly deregulated in the ΔrsaI mutant versus the WT strains.

b. The long description in the text of genes that were differentially regulated in RNA-seq would be more clearly presented as a table in the main body of the manuscript, with genes grouped by putative functional categories. Then the text description could be much shorter and more concise."

Response. We have introduced a Table S5 (the previous Table S5 becoming S6), which describes the up and downregulated RNAs with a ratio of twofold and their biological functions by comparing the ΔrsaI mutant strain and the complemented strain. The text was modified accordingly and hopefully clarified. We nevertheless decided to keep this Table as a supplemental material.

c. The transition to the next section (line 277) should be made more clear. After the explanation of the RNA-seq results, I was expecting the next step to be validation of some of the putative targets identified in RNA-seq. Instead, the authors return to two targets that were relatively poorly enriched in MAPS and not differentially regulated in RNA-seq."

Response. We agree that the order of the presentation of the data following the transcriptomic analysis was not logical. We decided to move the transcriptomic analysis at the end of the result section. Although the data were obtained from triplicate biological samples with high reproducibility (p values<0.05), surprisingly we did not observe changes of the levels of the mRNAs, which were enriched in the MAPS approach. As described above, we think that the MAPS approach has primarily enriched the mRNAs that are regulated at

the translational level.
We have chosen the glcU_2 and fn3K mRNAs, which were significantly enriched (4-times) in the MAPS, based on their functions. Indeed, these two proteins are functionally related and involved in glucose metabolism (i.e., glucose entry, and quality control of protein). Although the enrichment looks rather low, we are confident that these mRNAs are direct RsaI targets. We indeed have shown that RsaI binds directly to these mRNAs, competes with ribosome binding and reporter gene fusion analysis showed that RsaI strongly represses the translation of these two mRNAs.

5. Direct inhibition of ribosome-mRNA complex formation by RsaI is shown by toeprinting assays for two targets (glcU and fn3K). Translational repression by RsaI is shown for these two targets plus three more (Fig. 3).
a. It would be helpful to have diagrams with this figure, showing the putative base pairing interactions and the positions of these interactions relative to the ribosome binding sites. Even though it would be redundant with the table, it helps the reader assimilate the information more easily."
Response. We have included the predicted base-pairing interactions between RsaI and glcU_2/fn3K mRNAs below the autoradiographies showing the toeprinting data but not for the targets assessed only in ß-galactosidase experiment, the figure would become overloaded.

b. Lines 296-298, interpretation of these experiments goes too far. RsaI regulation of translation initiation of glcU and fn3K is supported by the data. The reporters for the other mRNAs show only translational regulation by RsaI, but do not provide information on the mechanism of that regulation."
Response. We agree with the comments and have adapted the text accordingly. Nevertheless, we showed in vivo that glcU and fn3K mRNAs were no more enriched with MS2-RsaI mut4 carrying a deletion of the mRNA binding site supporting the in vitro binding assays. Because the mRNA yields do not vary in the ΔrsaI mutant strain and in the same strain complemented with the expression of RsaI, we are thus confident that the primary effect of RsaI binding is to prevent the initiation of translation in agreement with the toeprinting experiments.

6. Interaction of RsaI with the icaR 3' UTR is demonstrated, along with the impact of RsaI and mutations in the icaR 3' UTR on PIA-PNAG production phenotypes (Fig. 4).
a. This section on icaR was really hard to follow. A cartoon demonstrating the intramolecular icaR interaction, and the putative RsaI-icaR interaction, would be very helpful. It is also important to explicitly state the proposed mechanism: RsaI represses production of a repressor, which should enhance PIA-PNAG production. The authors didn't fully explain the model or provide predicted outcomes, which made it hard to evaluate and interpret the data."
Response. We have significantly modified the presentation of the data and the discussion as well. As suggested by the reviewer, we have improved the design of Figure 4, which shows now in panel A the intramolecular base-pairing interactions between the 5' and 3'-UTR in icaR as well as the RsaI binding site. In addition, Figure 6 has been changed so that we can better appreciate the action of RsaI when glucose is consumed, and as an insert we introduced a schematic drawing summarizing the possible RsaI-dependent regulatory mechanisms.

b. What is the icaR SUBST mutation, and how is that predicted to impact the intramolecular and intermolecular (with RsaI) interactions?"
Response. The SUBST mutation stands for the substitution of UCCCCUG sequence by AGGGGAC (see legend of Figure 4). The mutation was described previously by Ruiz de los Mozos et al. (2013) to inhibit the interaction between the 5' and 3'-UTRs allowing an enhanced translation of IcaR. As shown in figure 4B, the substitution did not impact the binding of RsaI in vitro supporting our prediction that RsaI binds to another region of icaR mRNA (illustrated in Figure 4A).

c. Is RsaI's impact on PIA-PNAG levels solely through regulation of icaR? This may be hard to get at, but the data imply this might be true because mut5 still increases PIA-PNAG."

Response. We have reproduced the dot blot experiment several times with rather good reproducibility, and we decided to replace panel C. The data showed that RsaI deletion led to a decrease in PIA-PNAG production (Figure 4C), which is restored by complementation with a plasmid expressing RsaI WT while the complementation is less efficient with RsaI mut5 meaning that the substituted nucleotides are important. Interestingly, the deletion of the 3'UTR strongly decreased the synthesis of PIA-PNAG in contrast to the WT strain, and the PIA-PNAG production cannot be restore by overexpression of RsaI indicating that the effect of RsaI on PIA-PNAG synthesis requires icaR 3'UTR. All these data suggested that the synthesis of PIA-PNAG is highly sensitive to the concentration of RsaI (compare WT and ΔrsaI mutant strains). New gel retardation assays also showed that the mutation mut5 in RsaI strongly affected binding to the 3'UTR of icaR. The fact that the WT strain in which RsaI mut5 is overexpressed still produce PIA-PNAG could be due to the fact that its binding to icaR is not totally abolished but strongly reduced. Alternatively, we cannot exclude that RsaI might play additional roles in PIA-PNAG synthesis.

7. It was demonstrated in vitro that RsaI-RsaG and RsaI-mRNA complexes are not mutually exclusive. In vivo, RsaI/G do not impact one another's stability. (Fig. 5)
a. The data are overinterpreted- e.g., lines 348-349: "The formation of a ternary complex is a good indication that RsaG binding does not interfere with the regulatory functions of RsaI." The in vitro data do not address this point. In vivo experiments to demonstrate this are necessary if this claim is to be made."

Response. We agree with the referee. We did not show that RsaG does not interfere with the regulatory activity of RsaI in vivo. However, the fact that RsaG does not bind to the mRNA targets of RsaI and is able to form a ternary complex strongly suggested that RsaG does not compete with the mRNAs for RsaI binding.

We have performed a new experiment where the fn3k-lacZ fusion has been expressed in the WT strain and the same strain overexpressing RsaI. Under these conditions, we detected a reproducible inhibition of the ß-galactosidase synthesis. This effect is specific since the overexpression of RsaI mut4 alleviated the inhibition. Identical data were obtained with the ΔrsaG mutant strain. These data indicate that under conditions where RsaG is expressed (in the WT context), no major effect on the RsaI-dependent repression of fn3k-lacZ fusion was observed.

b. The experiment with RsaG and RsaD at the end of this section don't logically flow."

Response. We have re-organized Figure S4 and changed the text accordingly. Now we first describe the expression pattern of RsaG followed by the functional consequences of the interaction between RsaI and RsaG, and we introduced a specific paragraph for RsaD.

"c. Lines 380-381 "These data suggested that through the binding of sRNAs, RsaI would link sugar metabolism pathways, carbon source utilization, energy production and stress responses." There is no direct evidence that sRNA-sRNA interactions are physiologically relevant, or that these interactions alter the activities of the sRNAs. In fact, the in vitro data suggest that sRNA-sRNA and sRNA-mRNA interactions can occur simultaneously, which could suggest that RsaI regulation of mRNA targets is not impaired when RsaG pairs."

Response. This comment is very similar to the point "a" just above. We agree that our data strongly suggested that RsaI-RsaG and RsaI-mRNA might occur simultaneously. Our new set of data strongly suggested that RsaG has no major impact on RsaI function.

8. Discussion:
a. There is no evidence of sponge-like activity. In fact (as mentioned above), the data suggest that the sRNA-sRNA interactions do not impair the ability of RsaI to regulate (at least some) targets. This would not be consistent with a sponge-like activity.
b. There is a lot of very interesting speculation regarding the role of RsaI in coordinating a metabolic switch. This would be very exciting if true. But there are no data to directly support this model in this manuscript.

Response. We performed new experiments showing that the CcpA-dependent repression of RsaI transcription is an important factor for optimal growth although we do not know what are the regulated mRNAs that are responsible for the growth phenotype. Other experiment supports the idea that RsaI is not regulated by RsaG. Following these observations, we have slightly modified the message of the manuscript. We think that RsaI expression is a signature

of a metabolic change due to glucose consumption, and that its CcpA-mediated repression is essential for the bacterial growth.

"Minor comments
1. The abstract could be more clear on the regulation of rsaI, i.e., that when glucose is high, RsaI is low and vice versa. "

Response. We have modified the abstract with respect to the reviewer's comment.

"2. The abstract overstates the findings, e.g., lines 40-41. There is no direct evidence that RsaI regulates the activities of other sRNAs."

Response. We have modified the sentence. The sponge hypothesis has been removed from the manuscript.

"3. Tables and figures should be numbered and introduced in the order that they are discussed in the text (e.g., Fig. 1 comes before Table 1 in the text)."

Response. We paid attention to figures and tables numbering.

"4. In general, the sections were disconnected from one another. It was not clear that results from one set of experiments were flowing logically to guide the next experiments."

Response. We have changed the order in the results section and we think that the message will be more logical.

2nd Editorial Decision 26th Nov 2018

Thank you for submitting a revised version of your manuscript. It has now been seen by two of the original referees whose comments are shown below.

As you will see, ref #2 is satisfied with the revised version while ref #3 raises a number of points that require additional clarification before we can go on to officially accept your study for publication here. In my view, most of the points can be addressed via additional discussion and by acknowledging the limitations in the system (points 8, 10, 11 and 12). Given the discrepancy between the reports I consulted with ref #2 on the comments by ref #3 and the outcome is included below along with the referee reports.
In conclusion, I would like to invite you to submit a final revision in which you address the remaining referee concerns and incorporate the following editorial points concerning text and figures:

REFEREE REPORTS:

Referee #2:

The authors have done a great job revising their manuscript. We have no further comments and recommend the manuscript now be published.

Referee #3:

In this revised manuscript by Bronesky, et al., the authors explore the regulation and activities of an sRNA in Staph aureus, RsaI. The results show that RsaI synthesis is responsive to glucose (and fructose) in a CcpA-dependent manner, where RsaI levels remain low in the presence of glucose, and rise as glucose is depleted. Pull-down experiments and further in vitro validation experiments demonstrate convincingly that RsaI base pairs with several mRNAs to repress their translation via inhibition of ribosome binding at the translation initiation region. Evidence that RsaI interacts with other sRNAs through sequences distinct from those involved in pairing with mRNAs is also presented. Many of my previous concerns have been addressed - the molecular aspects of regulation by RsaI are well supported by the data presented in the paper and the paper is now much clearer and

easier to follow. The story falls short in linking the molecular aspects of regulation to clear physiological impacts, and the authors still oversell this aspect of the work. In my view, the overinterpretation and speculation regarding the physiological role of this sRNA detracts from the strengths of the manuscript, and make me less enthusiastic about the whole package.

Specific comments:
1. I appreciate that the title has been modified so that it's more in line with the actual results. However, I wonder if there's a way to more clearly convey what is meant by "multifaceted?" My initial thought was that the authors might mean "multifunctional," or dual-function, which is a term used for sRNAs that also code for proteins. I think it would be reasonable to say something about the sRNA pairing with both mRNAs and other sRNAs in the title, and that would intrigue a lot of readers.
2. Line 35 - "metabolism," not "metabolization."
3. Line 80 - define "TSST"
4. Line 138 - experiment does not show that transcription of RsaI was stopped, rather RsaI levels dropped, which could be due to reduced transcription or increased degradation.
5. Lines 155-156 - "...data suggest that the inhibition of RsaI transcription is only dependent on hexose availability." This is misleading - since the authors have demonstrated that CcpA is involved, the response to glucose and fructose - two PTS sugars - is expected because of the way that CcpA activity is controlled. It isn't related to pentose vs. hexose, per se?
6. Lines 199-202 - These lines should be deleted. This is an overinterpretation of the data that were just presented. This speculation is more appropriate for the discussion.
7. Line 265 - Section heading is unclear - "their RBS" is not defined.
8. The results in Fig 4 are intriguing and a highlight of the paper. It seems like such a strong regulatory phenotype and is obvious in the mutant vs the wild-type (not requiring ectopic overexpression). Perhaps the authors should consider looking at physiological conditions where PIA-PNAG production is important for Staph.
9. The interaction between RsaI-RsaG is very interesting, and the data support the idea that this interaction does not impact the ability of RsaI to interact with mRNA targets. However, based on what is known about the conditions under which RsaI, RsaG and RsaI target mRNAs are expressed, do you expect tri-partite complexes to exist in vivo? Again, this is a comment regarding linking the molecular results to physiology.
10. The section on RsaD seemed out of place. The authors haven't clearly demonstrated an interaction between RsaI-RsaD, so, this section does not add to the overall story.
11. I appreciate the clearer explanation of the RNA-seq results. However, the experiments were done under conditions where RsaI had been expressed for a long time, and not by a pulse-expression. This makes it impossible to discern the immediate, direct impact of RsaI production verses indirect effects that happen over the long timescale of the experiment. So, the fact that many genes change expression in the RsaI expression strain compared to the mutant is not that informative. It certainly hints at RsaI's involvement in metabolic regulation, but that much was obvious from the MAPS experiments and subsequent validation.
12. The growth experiment does not add to the study. First, why was it done in a different strain background than most of the other experiments? The authors mention that they could not generate the constitutive expression construct in the HG001 strain. Is this physiologically meaningful? Or a technical issue? The growth difference does not look like a delay, rather it looks like a very mild reduction in the doubling time. This is hard to determine because the growth curve is not plotted on a log scale. I am also buzzled why the very mild phenotype is only seen in the wild-type + RsaI background, and not in the delta rsaI +RsaI background. The Northern blots show very similar levels of RsaI in both strains, so the phenotype doesn't seem to correlate well with measured RsaI levels. Finally, constitutive overexpression is not a very meaningful condition to use, especially when the authors already know how rsaI expression is regulated.

Editor-referee exchange in remaining ref concerns:

Editor: Point #8 is a good suggestion but in my view more of a further-reaching point for a separate study

Ref #2: I also think that this should be investigated in a follow-up study. Although I agree with that other referee that these phenotypes without sRNA overexpression are intriguing and a highlight, the

key aspect of this paper is the application of MAPS in a gram-positive species, combined with proper validation experiments for the predicted targets.

Editor: Point #10: would it harm to leave the data in, even if it doesn't add too much?

Ref #2: It's true that the RsaD data is a bit out of place or rather a bit of a loose end, but at the end of the day, if the authors feel strongly about including them, fine--it's their paper. And these results for RsaD would otherwise probably be put in the drawer and be not accessible to anyone. So, why not leave them in.

Editor: Point #11: I would think it's sufficient to acknowledge the potential for indirect effects in the discussion.

Ref #2: I agree with you, the authors should make this not being a true pulse-expression and consequences thereof clearer in in the discussion.

Editor: Point #12: Do you think additional experimental data is needed to make the growth experiment conclusive?

Ref #2: I went back to the Fig. S5 and looking at it again, I thought that the other referee had a very good point: the difference in RsaI overexpression effects in the wt vs knockout strains is puzzling. By contrast, the lack of a strong growth phenotype in the ko strain is less unexpected, loss of bacterial sRNA functions rarely translate into measurable growth phenotypes. I would be more concerned that the RsaI overexpression does not really give a stronger phenotype, given the number and importance of the targets of this sRNA. In any case, my recommendation is to leave this data out here, there is obviously more work to do. Instead, the authors should just clearly state in the discussion that they are yet to identify growth conditions under which a contribution of RsaI can be studied robustly when using growth rate as a readout.

2nd Revision - authors' response 17th Dec 2018

Authors' response to Reviewers 2 and 3:

<u>Referee's 3 comments</u> "In this revised manuscript by Bronesky, et al., the authors explore the regulation and activities of an sRNA in Staph aureus, RsaI. The results show that RsaI synthesis is responsive to glucose (and fructose) in a CcpA-dependent manner, where RsaI levels remain low in the presence of glucose, and rise as glucose is depleted. Pull-down experiments and further in vitro validation experiments demonstrate convincingly that RsaI base pairs with several mRNAs to repress their translation via inhibition of ribosome binding at the translation initiation region. Evidence that RsaI interacts with other sRNAs through sequences distinct from those involved in pairing with mRNAs is also presented. Many of my previous concerns have been addressed - the molecular aspects of regulation by RsaI are well supported by the data presented in the paper and the paper is now much clearer and easier to follow. The story falls short in linking the molecular aspects of regulation to clear physiological impacts, and the authors still oversell this aspect of the work. In my view, the overinterpretation and speculation regarding the physiological role of this sRNA detracts from the strengths of the manuscript, and make me less enthusiastic about the whole package."

> *Response. We thank the referee for his comments. We have amended the discussion regarding the physiological role of the sRNA in a less speculative manner.*

"1. **Ref #3 :** I appreciate that the title has been modified so that it's more in line with the actual results. However, I wonder if there's a way to more clearly convey what is meant by "multifaceted?" My initial thought was that the authors might mean "multifunctional," or dual-function, which is a term used for sRNAs that also code for proteins. I think it would be reasonable to say something about the sRNA pairing with both mRNAs and other sRNAs in the title, and that would intrigue a lot of readers. "

> *Response. We thank the reviewer for this suggestion; unfortunately the constraint of length limitation did not allow us to find a more informative title. We therefore kept the title, which implies that the sRNA might regulate various targets using different mechanisms.*

"2. **Ref #3 :** Line 35 - "metabolism," not "metabolization." ; 3. Line 80 - define "TSST"

Response. We have corrected this mistake.

"4. **Ref #3 :** Line 138 - experiment does not show that transcription of RsaI was stopped, rather RsaI levels dropped, which could be due to reduced transcription or increased degradation."

Response. We agree with the reviewer suggestions and we have changed the sentence accordingly.

"5. **Ref #3 :** Lines 155-156 - "...data suggest that the inhibition of RsaI transcription is only dependent on hexose availability." This is misleading - since the authors have demonstrated that CcpA is involved, the response to glucose and fructose - two PTS sugars - is expected because of the way that CcpA activity is controlled. It isn't related to pentose vs. hexose, per se?"

Response. We apologize for this misunderstanding and we have clarified the text.

"6. **Ref #3 :** Lines 199-202 - These lines should be deleted. This is an overinterpretation of the data that were just presented. This speculation is more appropriate for the discussion."

Response. We have shortened the end of the paragraph to avoid speculation.

"7. **Ref #3 :** Line 265 - Section heading is unclear - "their RBS" is not defined. "

Response. RBS is for ribosome binding site used for initiation of translation. It includes the Shine and Dalgarno sequence and the AUG initiation codon. We have modified the title of the section heading.

"8. **Ref #3:** The results in Fig 4 are intriguing and a highlight of the paper. It seems like such a strong regulatory phenotype and is obvious in the mutant vs the wild-type (not requiring ectopic overexpression). Perhaps the authors should consider looking at physiological conditions where PIA-PNAG production is important for Staph." "**Editor**: Point #8 is a good suggestion but in my view more of a further-reaching point for a separate study". "**Ref #2:** I also think that this should be investigated in a follow-up study. Although I agree with that other referee that these phenotypes without sRNA overexpression are intriguing and a highlight, the key aspect of this paper is the application of MAPS in a gram-positive species, combined with proper validation experiments for the predicted targets."

Response. Understanding the mechanism by which RsaI induces the synthesis of PIA-PNAG production is presently under study. We indeed do not exclude that RsaI might also regulate PIA/PNAG synthesis in an indirect manner through the regulation of another regulator of ica operon. In fact, S. aureus ica operon expression is extremely complex and multifactorial, and can be considered as a hub gene that is regulated by both cis-acting regulatory elements (circularization of the 3'-5'UTRs) and by a variety of regulators including sRNAs and proteins to control biofilm formation. Recent MAPS experiment performed with icaR mRNA fused to MS2, revealed significant enrichment of numerous sRNAs including RsaE and RsaI, in agreement with our present study. In addition, SarA and SrrAB are known to be transcriptional activators of icaADBC operon to induce the synthesis of PIA/PNAG. Moreover, many S. aureus isolates do not produce PIA/PNAG although they all encode the ica operon and the relative importance of the regulators vary between different strains. Finally, during the infection, multiple regulators are co-expressed raising the question how PIA/PNAG is regulated. We have slightly modified the discussion and introduced a review (Cue et al., 2012) describing the complexity of the regulation of ica operon and of the synthesis of PIA/PNAG.

"9. **Ref #3 :** The interaction between RsaI-RsaG is very interesting, and the data support the idea that this interaction does not impact the ability of RsaI to interact with mRNA targets. However, based on what is known about the conditions under which RsaI, RsaG and RsaI target mRNAs are expressed, do you expect tri-partite complexes to exist in vivo? Again, this is a comment regarding linking the molecular results to physiology."

Response. At the stationary phase of growth in BHI medium, the steady state levels of RsaI are high while RsaG is present but at a rather low yield. Under these conditions, we do not exclude that RsaI can simultaneously bind to some of its target mRNAs and to RsaG. Under G6P stress, the steady state levels of RsaI strongly decreased while those of RsaG are

strongly enhanced, suggesting that RsaG would exert its regulatory functions in response to G6P entry. These aspects have been mentioned in the discussion.

“10. **Ref #3:** The section on RsaD seemed out of place. The authors haven't clearly demonstrated an interaction between RsaI-RsaD, so, this section does not add to the overall story.” “**Editor**: Point #10: would it harm to leave the data in, even if it doesn't add too much?” “**Ref #2:** It's true that the RsaD data is a bit out of place or rather a bit of a loose end, but at the end of the day, if the authors feel strongly about including them, fine--it's their paper. And these results for RsaD would otherwise probably be put in the drawer and be not accessible to anyone. So, why not leave them in”.

Response. Although we agree that our present data on RsaD do not add so much to the study on RsaI, we however would like to include these experiments in the manuscript for the following reasons: (1) Gel retardation assays showed that RsaI is able to form basepairing interactions with RsaD albeit with a lower binding affinity than with RsaG (Fig EV2). (2) RsaD but also RsaE (enriched with the MS2-RsaI) are both part of the SrrAB regulon, which responds to hypoxia and nitric oxide (NO) resistance. As it was previously shown for RsaE, the steady state levels of RsaD enhanced in response to NO stress. (3) Transcriptomics analysis reveals that the expression of RsaI induces the synthesis of ldh1, hmp and qoxABCD operon (Table EV6), most likely through an indirect mechanism. Interestingly, S. aureus employed specific strategies to resist the NO effects by inducing ldh1, which has been shown by the team of J. Richardson, to permit the redox homeostasis when bacteria shift to a fermentative metabolism to fight against NO. The induction of ldh1 is most probably the results of the weak repression of rex as observed in the mutant strain expressing high concentrations of RsaI in the complemented strain. In addition, the flavohemoprotein Hmp is able to detoxify NO to nitrate when respiration is limited, and is induced by SrrAB. Although we could not provide a specific explanation for all these effects, our data strongly suggested that RsaD and RsaE are part of complex regulatory networks mediated by SrrAB in maintaining redox homeostasis, which appears to be induced under the conditions of growth where RsaI is expressed.

“11. **Ref #3**: I appreciate the clearer explanation of the RNA-seq results. However, the experiments were done under conditions where RsaI had been expressed for a long time, and not by a pulse-expression. This makes it impossible to discern the immediate, direct impact of RsaI production verses indirect effects that happen over the long timescale of the experiment. So, the fact that many genes change expression in the RsaI expression strain compared to the mutant is not that informative. It certainly hints at RsaI's involvement in metabolic regulation, but that much was obvious from the MAPS experiments and subsequent validation.” “**Editor**: Point #11: I would think it's sufficient to acknowledge the potential for indirect effects in the discussion.” “**Ref #2**: I agree with you, the authors should make this not being a true pulse-expression and consequences thereof clearer in in the discussion.”

Response. We certainly agree that we cannot distinguish primary from secondary events under the conditions of the experiments used for the comparative transcriptomic analysis. As we discussed in the manuscript, the fact that we did not observe overlap between the MAPS and the transcriptomic data suggested that the MAPS has primarily enriched the mRNAs for which the stability was not affected. Unfortunately for S. aureus, no appropriate expression system is yet available to perform pulse-expression of sRNA, and this is why we have complemented the mutant strain with plasmid expressing RsaI under its own promoter to avoid growth effects linked to a strong overexpression of RsaI. Nevertheless the pathways that are deregulated are of interest since the most repressed genes encode enzymes involved in the pentose phosphate pathway, which is an alternative route for glucose metabolism, and in the thiamine pathway, which provided co-factor for the transkelotase (Table EV6). Concomitantly, RsaI expression enhances the synthesis of proteins involved in NO response and resistance (part of SrrAB regulon), and in fermentation. Among these genes, the qoxABCD operon required for NO resistance (Kinkel et al., 2014) was found slightly enriched in the MAPS, and was enhanced when RsaI was expressed from a plasmid. Although it is difficult presently to understand the relationships between glucose metabolism and SrrAB regulon, we nevertheless think that RsaI expression is occurring at a specific metabolic transition of the bacteria. However, we have taken into account the advices of the referees and we were more careful in the presentation of the data and in the discussion.

"12. **Ref #3:** The growth experiment does not add to the study. First, why was it done in a different strain background than most of the other experiments? The authors mention that they could not generate the constitutive expression construct in the HG001 strain. Is this physiologically meaningful? Or a technical issue? The growth difference does not look like a delay, rather it looks like a very mild reduction in the doubling time. This is hard to determine because the growth curve is not plotted on a log scale. I am also buzzled why the very mild phenotype is only seen in the wild-type + RsaI background, and not in the delta rsaI +RsaI background. The Northern blots show very similar levels of RsaI in both strains, so the phenotype doesn't seem to correlate well with measured RsaI levels. Finally, constitutive overexpression is not a very meaningful condition to use, especially when the authors already know how rsaI expression is regulated.
Editor-referee exchange in remaining ref concerns." "**Editor**: Point #12: Do you think additional experimental data is needed to make the growth experiment conclusive?" "**Ref #2**: I went back to the Fig. S5 and looking at it again, I thought that the other referee had a very good point: the difference in RsaI overexpression effects in the wt vs knockout strains is puzzling. By contrast, the lack of a strong growth phenotype in the ko strain is less unexpected, loss of bacterial sRNA functions rarely translate into measurable growth phenotypes. I would be more concerned that the RsaI overexpression does not really give a stronger phenotype, given the number and importance of the targets of this sRNA. In any case, my recommendation is to leave this data out here, there is obviously more work to do. Instead, the authors should just clearly state in the discussion that they are yet to identify growth conditions under which a contribution of RsaI can be studied robustly when using growth rate as a readout."

> *Response. The growth experiment was performed in parallel on various strains (HG001, 132) and we never managed to transform the strain HG001 with the plasmid overexpressing RsaI constitutively. This was not a technical issue as successful transformation of the same strain was obtained with various plasmids expressing RsaI under its own promoter. This is why we decided to look more precisely the effect of RsaI overexpression in strain 132, which we used in the PIA-PNAG experiments (Fig 4). We have represented the curves in semi-log and and we observed a weak delay in the growth of the WT strain transformed with the plasmid expressing RsaI from a constitutive promoter. Even if the growth phenotype is very weak, it has been reproducibly observed in four distinct experiments. We have in parallel analysed the steady state levels of RsaI in the various strains and quantification of the gels taking into account the loading control performed with 5S RNA showed that the expression of RsaI was significantly enhanced at 2h of growth in the WT strain expressing RsaI from the plasmid as compared to the WT strain, and this level was much higher than in the mutant strain (see the graph next to the autoradiography in Fig EV5B). This is why we think that the CcpA-dependent repression of RsaI when glucose is present in the medium is critical for optimal growth. A very similar effect was observed with a ΔccpA mutant strain at the exponential level, which displays a slower growth with a mild effect on the doubling time as compared to the WT strain, when cultures were done in the presence of glucose. Although we would appreciate to keep the figure as the supplementary material, we have nevertheless reduced considerably the discussion to avoid additional confusion. We agree with the referees that we need to better identify the stress conditions under which RsaI can be studied robustly. This work suggests that RsaI might be associated with the regulation of redox homeostasis, either through SrrAB or Rex proteins. This is certainly one of the option, we would like to continue in the nearest future.*

All requested editorial changes were made.

3rd Editorial Decision 21st Jan 2019

Thank you for submitting a revised version of your manuscript. I have taken over handling of your manuscript from my colleague Anne Nielsen, who has meanwhile left our office. The revision has now been seen by one of the referees, who finds it suitable for publication. I am now pleased to inform you that your manuscript has been accepted for publication in The EMBO Journal. Congratulations on nice work!

REFEREE REPORTS :

Referee #2:

This is the re-re-review of the manuscript in question. The authors have adequately addressed the remaining issues of referee 3 and myself (referee 2) and have also responded constructively to the editorial input by Anne Nielsen. I think that this paper should now be published.

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Corresponding Author Name: Caldelari Isabelle, Romby Pascale
Journal Submitted to: EMBO Journal
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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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.

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

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- 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.).
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  - 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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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