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Crosstalk between ABC transporter mRNAs via a target mRNA-derived sponge of the GcvB small RNA

Masatoshi Miyakoshi, Yanjie Chao and Jörg Vogel

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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 10 December 2014

Thank you again 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, all referees express high interest in the findings reported in your manuscript and support publication following a limited number of rather minor textual changes. Given these 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.

In addition, I would also ask you to address the following editorial points in the revised manuscript:

As you know papers published in The EMBO Journal include a 'Synopsis' to further enhance its discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis will include a short standfirst - written by the handling editor - as well as 2-5 one sentence bullet points that summarise the paper and are provided by the authors.
-> I would therefore ask you to include your suggestions for bullet points.
-> In addition, I would encourage you to provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

We also encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in

the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

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.

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

REFEREE REPORTS

Referee #1:

This nicely written manuscript provides a large number of high quality experiments that explain the unusual weak stability of the non coding RNA GcvB, which regulates at the post-transcriptional level the synthesis of many transporters of amino acids and short peptides, and of major transcription factors. Unexpectedly the authors found that the turn over of GcvB is controlled by one of its target mRNA encoding an aminoacid ABC transporter. Interestingly, perturbing this crosstalk impairs bacterial growth under conditions when peptides are the sole carbon and nitrogen sources. Although it was already known that mRNAs could act as sponge RNAs to influence the decay of small non coding RNAs, there are at least two reasons why the manuscript is original and appropriate for publication in EMBO J. First, this is the first time that a stable fragment of a GcvB-dependent mRNA target, is demonstrated as the key regulator that induces the RNase E-dependent degradation of GcvB, revealing a novel function of the 3'UTR of mRNAs. Second, the authors provide the basis for the molecular mechanism of a particular RNA sponge and the functional importance of these complexed mRNA-SroC sRNA-GcvB-mRNA crosstalks. This paper is also a nice illustration that small RNA-dependent regulation in bacteria and eukaryotes shares common rules although the machineries are highly different. Overall this manuscript is certainly of general interest and I have no doubt that it will be well cited.
Only some minor changes are suggested below:
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regulated by these sRNAs have been found deregulated in the mutant ΔsroC strain.

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SroC is one of Hfq-binding small RNAs (sRNAs) identified in enteric bacteria. Previous studies suggested that SroC derives from an internal region of the gltIJKL operon presumably by processing of gltI mRNA. This study was initiated to understand the function of SroC in Salmonella. The authors first found that SroC overexpression activates a number of mRNAs, and represses several mRNAs and sRNAs including GcvB. They noticed that many of SroC-activated mRNAs are known targets of GcvB which is an Hfq-binding sRNA involved in regulation of numerous mRNAs encoding short peptides transporters through base-pairing mechanism. This led the authors to test the idea that SroC down-regulates GcvB which in turn up-regulates GcvB targets such as dpp and opp mRNAs. They demonstrated, based on a series of genetic and biochemical experiments, that 1) the activation of mRNAs by SroC indeed results from depletion of GcvB by SroC and the SroC-mediated regulation requires Hfq; 2) SroC destabilizes GcvB through base-pairing mechanism and RNase E responsible for the destabilization of GcvB; 3) SroC itself is generated from the gltI-sroC transcript or pre-SroC in an RNase E-dependent manner both in vivo and in vitro; 4) SroC regulation of GcvB-target mRNAs including the gltI-sroC mRNA (parental mRNA of SroC) occurs in a chromosomal context under physiological conditions and SroC inactivation impairs cell growth when peptides are used as carbon and nitrogen sources.

Taken together, the authors conclude that the gltI-sroC mRNA provides both, the template for synthesis an ABC transporter, and the precursor of an Hfq-binding sRNA (SroC) which acts to destabilize GcvB, another Hfq-dependent sRNA, through base-pairing resulting in activation of GcvB-target mRNAs including the gltI-sroC.

This is a fascinating study that revealed novel aspects regarding both action and biogenesis of bacterial regulatory sRNAs. The experiments are well designed, and executed thoroughly and carefully. The data and arguments are mostly clear and convincing to support the conclusion. I only have a few comments.

1) SroC overexpression affects the expression of RNAs other than known targets of GcvB. It would be better to touch briefly the possible mechanism of this GcvB-independent SroC function. In particular, it is intriguing that several sRNAs in addition to GcvB are down-regulated by SroC.
2) While GcvB is dramatically destabilized by SroC, SroC itself is apparently very stable (Fig. 3B). This simply suggests that the SroC-GcvB complex is not subjected to the coupled degradation by RNaseE. It is also better to mention about this in the text.

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This study describes the novel finding that a "sponge" small RNA titrates GcvB sRNA and inhibits its activity on other target mRNAs. The sponge sRNA, SroC, is processed from a larger operon mRNA that itself is a target of GcvB-mediated post-transcriptional regulation. The authors provide excellent evidence supporting a direct interaction and show that SroC antagonizes GcvB activity by promoting its turnover through specific base pairing interactions that lead to RNase E-dependent GcvB degradation. Overall, this is a great story and the experimental data are very strong. This study expands our understanding of the ways in which small RNA activities are regulated, and has interesting and important implications for understanding "crosstalk" (or competition) among the mRNAs that are regulated by a given sRNA. I have some suggestions that I hope will help clarify certain issues.

Major comments:
• p8: The authors note that in the rneR169K mutant, longer processing intermediates of SroC accumulate , yet GcvB is still regulated by SroC in this background. This is maybe not so surprising, since there appears to be some small amount of mature SroC in the R169K background, and in Fig. 4A (e.g., lanes 2, 6) we see that not much mature SroC Is needed to effectively regulate GcvB.
• The growth experiment is the weakest link in the study. The sroC mutation has a very modest effect on doubling time, and the data are hidden in the supplementary material. It's tough to assert that the effect of the sroC mutation on growth on peptides is due to GcvB regulation of Opp/Dpp/Tpp, which the authors imply but do not test directly. The physiological significance of SroC regulation of GcvB in this case will likely take some time to work out. I think the mechanistic work stands well on it's own and this growth experiment is very interesting and worth including, but should not be oversold.

Minor comments:
• Abstract: use of the term "RNA sponges" in the second sentence is ambiguous. The term hasn't yet been defined for the reader and it's meaning here won't be obvious for non-experts.
• Introduction: It would be useful to state early, and very clearly what is meant by the term "RNA sponge."
• Introduction: Define "competitive endogenous RNAs."
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• p9, last paragraph. It would be useful to explicitly state whether the regions of GcvB that interact with SroC overlap the regions that interact with mRNA targets. This is mentioned in the discussion, but it is pertinent in this section of the results.
• I think the aspect of crosstalk among mRNA targets of GcvB is a little bit overemphasized. Fig 7 does provide evidence supporting the idea that the sroC function (derived from the mRNA of one GcvB target mRNA) does impact the activity of GcvB on another target (oppA). However, there are no data to directly address the aspect of crosstalk in a quantitative or physiological way.
• Crosstalk is really competition among targets for a limited pool of sRNA, right? It seems like this might be a more clear way to discuss this issue. The outcomes of competition will depend of how good a competitor SroC is compared to other targets. This will be influenced by gltI operon synthesis/SroC levels and the affinity/kinetics of SroC-GcvB association compared with the same properties of GcvB association with other targets. Again, this doesn't change the punchline of this paper, it might just provide a bit more clarity for the non-expert reader.
• p13, second paragraph ("This novel mRNA-sRNA-sRNA-mRNA scheme...") is very ambiguous in meaning and speculative. I don't think it adds much to the discussion.

1st Revision - authors' response 12 December 2014

Rebuttal, manuscript EMBOJ-2014-90546 by Miyakoshi, Chao and Vogel

Referee #1:

This nicely written manuscript provides a large number of high quality experiments that explain the unusual weak stability of the non coding RNA GcvB, which regulates at the post-transcriptional level the synthesis of many transporters of amino acids and short peptides, and of major transcription factors. Unexpectedly the authors found that the turn over of GcvB is controlled by one of its target mRNA encoding an aminoacid ABC transporter. Interestingly, perturbing this crosstalk impairs bacterial growth under conditions when peptides are the sole carbon and nitrogen sources. Although it was already known that mRNAs could act as sponge RNAs to influence the decay of small non coding RNAs, there are at least two reasons why the manuscript is original and appropriate for publication in EMBO J. First, this is the first time that a stable fragment of a GcvB-dependent mRNA target, is demonstrated as the key regulator that induces the RNase E-dependent degradation of GcvB, revealing a
novel function of the 3'UTR of mRNAs. Second, the authors provide the basis for the molecular mechanism of a particular RNA sponge and the functional importance of these complexed mRNA-SroC sRNA-GcvB-mRNA crosstalks. This paper is also a nice illustration that small RNA-dependent regulation in bacteria and eukaryotes shares common rules although the machineries are highly different. Overall this manuscript is certainly of general interest and I have no doubt that it will be well cited.

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Reply: We agree that the Seitz review discussing mRNA crosstalk via eukaryotic microRNAs should be referred to in the introduction and have added the reference to the text (page 3).

2- It is not clearly mentioned whether the sponge RNA SroC is cleaved (or not) by RNase E when it binds to GcvB. It is indeed intriguing that the 5' A-rich tail of SroC is not accessible to RNase E. Could it be that this 5' tail contained a strong Hfq binding site that would be the reason for its high stability? Did the authors map the in vitro cleavage sites of RNase E in GcvB bound to Hfq and SroC and vice versa?

Reply: Both reviewers #1 and #2 point out that the sponge RNA SroC is very stable and may not decay along with GcvB through coupled degradation, as seen with many Hfq-dependent sRNA-mRNA pairs. This has been clarified in the text (page 11).

The detailed molecular mechanism of SroC-GcvB degradation is of great interest to us, and we have already performed a series of experiments to address it. Our preliminary results indeed show that, as predicted by this reviewer, the 5'A-rich tail of SroC is an Hfq binding site that may protect the 5' end of this RNA fragment from further degradation. Moreover, we have data to suggest further RNase E *in vivo* cleavage sites in GcvB which are located in the vicinity of

the two SroC binding sites. These data and other data on RNase E, SroC and GcvB are currently being prepared for publication in a follow-up paper.

3- Table S1, Figure 1: Deletion of SroC alters the yield of at least 7 sRNAs, among them OxyS GlmZ, and spot42. These sRNAs are also known to regulate complex networks of genes, for instance spot42 fine tune carbon metabolism. Moreover, the expression of Spot42 is slightly affected in the ΔgcvB strain while GlmZ is not affected. The authors should discuss briefly the possible functional links between SroC, GcvB and these sRNAs. It is surprising that none of the genes regulated by these sRNAs have been found deregulated in the mutant ΔsroC strain.

Reply: The reviewer refers to the microarray experiments with SroC overexpression (Figure 1B and Table S1). Indeed, the pulse overexpression of SroC alters the levels of several other sRNAs. However, compared to 5.4-fold decrease of the GcvB level, other sRNAs were only mildly affected (~2-fold reduction). We consider the changes of these other sRNAs nonspecific, resulting from Hfq titration when the Hfq-binding SroC RNA is overexpressed; indeed, all these additional sRNAs are associated with Hfq *in vivo* under the growth condition assayed here (Chao Y et al. 2012 EMBO J). We predict that when SroC binds much of the available Hfq *in vivo*, upon which other sRNAs become susceptible to RNase E and their levels decrease. It is not surprising, though, that the levels of the target mRNAs of these other sRNAs do not measurably change in this pulse expression experiment since 10 minute-pulse would be too short to affect these downstream mRNAs.

Hfq titration effects have been reported (Hussein and Lim, 2011; Moon and Gottesman, 2011; Papenfort et al., 2009). We have added these examples and the above explanation to the revised manuscript (page 5).

4- In Figure 5, several nucleotides in GcvB within the hybrid are missing around positions 50-56. It seems that binding of SroC induces reactivity changes that extend nts 14-18. It would be more informative to draw on the free RNA the cleavages induced by T1 and Pb ions and to highlight the changes induced by the formation of the complex.

Reply: We thank this reviewer for pointing out this mistake. We have corrected the nucleotide sequence of GcvB in the hybrid structure in Figure 5. As per this reviewer's suggestion, T1 cleavage sites in GcvB and SroC were also marked in the improved figure.

Referee #2:

SroC is one of Hfq-binding small RNAs (sRNAs) identified in enteric bacteria. Previous studies suggested that SroC derives from an internal region of the gltIJKL operon presumably by processing of gltI mRNA. This study was initiated to understand the function of SroC in Salmonella. The authors first found that SroC overexpression activates a number of mRNAs, and represses several mRNAs and sRNAs including GcvB. They noticed that many of SroC-activated mRNAs are known targets of GcvB which is an Hfq-binding sRNA involved in regulation of

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Reply: For affected sRNAs, see our reply to reviewer #1, comment 3. We also agree that there are some mRNAs that may be regulated by SroC in a GcvB-independent manner, foremost the mRNA of the *Salmonella*-specific STM2728 gene encoding a protein of unknown function. As mentioned in the Discussion part, these would be interesting candidates for future studies. One possible mechanism is that SroC regulates these mRNAs directly, for example, by Hfq-dependent base pairing. However, as we have not been able to predict convincing target sites of SroC in these mRNAs thus far, we would like to refrain from any speculation at the moment.

2) While GcvB is dramatically destabilized by SroC, SroC itself is apparently very stable (Fig. 3B). This simply suggests that the SroC-GcvB complex is not subjected to the coupled degradation by RNaseE. It is also better to mention about this in the text.

Reply: We agree and have added such as statement to the text; see our reply to reviewer #1, comment 2.

Referee #3:

This study describes the novel finding that a "sponge" small RNA titrates GcvB sRNA and inhibits its activity on other target mRNAs. The sponge sRNA, SroC, is processed from a larger operon mRNA that itself is a target of GcvB-mediated post-transcriptional regulation. The authors provide excellent evidence supporting a direct interaction and show that SroC antagonizes GcvB activity by promoting its turnover through specific base pairing interactions that lead to RNase E-dependent GcvB degradation. Overall, this is a great story and the experimental data are very strong. This study expands our understanding of the ways in which small RNA activities are regulated, and has interesting and important implications for understanding "crosstalk" (or competition) among the mRNAs that are regulated by a given sRNA. I have some suggestions that I hope will help clarify certain issues.

Major comments:

• p8: The authors note that in the rneR169K mutant, longer processing intermediates of SroC accumulate , yet GcvB is still regulated by SroC in this background. This is maybe not so surprising, since there appears to be some small amount of mature SroC in the R169K background, and in Fig. 4A (e.g., lanes 2, 6) we see that not much mature SroC Is needed to effectively regulate GcvB.

Reply: We fully agree with this reviewer on this point; as evident from Figure 4A, even a small amount of SroC can induce GcvB depletion. Nevertheless, the result in Fig. 4C provides an additional interesting point that degradation of GcvB still occurs in the R169K mutant, suggesting that the 5'-sensor pocket of RNase E is not essential for the decay of this sRNA. In addition, we have preliminary data showing that SroC cannot trigger GcvB degradation in *rne701* mutant (lacking the RNase E scaffold region for degradosome assembly). These results highlight that RNase E mediates SroC processing and GcvB degradation in two distinct pathways, which will be described in more detail in the above mentioned follow-up paper. For clarity, we have also added more explanation to the revised version of this manuscript (page 9).

• The growth experiment is the weakest link in the study. The sroC mutation has a very modest effect on doubling time, and the data are hidden in the supplementary material. It's tough to assert that the effect of the sroC mutation on growth on peptides is due to GcvB regulation of Opp/Dpp/Tpp, which the authors imply but do not test directly. The physiological significance of SroC regulation of GcvB in this case will likely take some time to work out. I think the mechanistic work stands well on it's own and this growth experiment is very interesting and worth including, but should not be oversold.

Reply: The affect of the SroC mutation on the doubling time of *Salmonella* may seem moderate, but we still think it is intriguing and worth emphasizing as very few growth phenotypes have been reported for sRNA mutants. Ironically, this includes the global amino acid regulator GcvB itself. The discovery of a growth phenotype for SroC provides a departure point for genetic screens to look for other contributing factors in the GcvB/SroC regulatory network. In addition, it further illustrates that profiling sRNAs under different growth conditions is necessary to identify measurable phenotypes.

For clarification, we have moved the growth data to a small table (Table 1) in the main manuscript. As the reviewer also suggested, we also rephrased the abstract and body text to describe the physiological significance more precisely.

Minor comments:
• Abstract: use of the term "RNA sponges" in the second sentence is ambiguous. The term hasn't yet been defined for the reader and it's meaning here won't be obvious for non-experts.

Reply: We have rephrased the second sentence of the abstract to briefly explain what a RNA sponges does.

• Introduction: It would be useful to state early, and very clearly what is meant by the term "RNA sponge."
• Introduction: Define "competitive endogenous RNAs."
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• Introduction: The description of the ChiX effect on chitobiose mRNAs is not clearly described. Essentially, the authors should just explicitly state that a piece of the chitobiose operon mRNA titrates ChiX away from another target (chiP mRNA), leaving more chiP accessible for translation

Reply: We have rephrased these sentences in the introduction.

• p9, last paragraph. It would be useful to explicitly state whether the regions of GcvB that interact with SroC overlap the regions that interact with mRNA targets. This is mentioned in the discussion, but it is pertinent in this section of the results.

Reply: We added the following sentence on page 10: “While the BS1 region of GcvB has not been implicated in base pairing with other targets before, the BS2 site partially overlaps with the GcvB target region for the *phoP* mRNA in *E. coli* (Coornaert et al. 2013 PLoS Genetics)”.

• I think the aspect of crosstalk among mRNA targets of GcvB is a little bit overemphasized. Fig 7 does provide evidence supporting the idea that the sroC function (derived from the mRNA of one GcvB target mRNA) does impact the activity of GcvB on another target (oppA). However, there are no data to directly address the aspect of crosstalk in a quantitative or physiological way.

Reply: We agree that it will be important to study the quantitative aspects of the discovered crosstalk in the future (see the next comment). Regarding its scope, however, we think that pulse-expression experiment in Figure 1 clearly shows a truly global cross-regulation of ABC transporter mRNAs via GcvB.

• Crosstalk is really competition among targets for a limited pool of sRNA, right? It seems like this might be a more clear way to discuss this issue. The outcomes of competition will depend of how good a competitor SroC is compared to other targets. This will be influenced by gltI operon

synthesis/SroC levels and the affinity/kinetics of SroC-GcvB association compared with the same properties of GcvB association with other targets. Again, this doesn't change the punchline of this paper, it might just provide a bit more clarity for the non-expert reader.

Reply: Excellent points. We have expanded on these and other potential factor in the Discussion (page 13).

• p13, second paragraph ("This novel mRNA-sRNA-sRNA-mRNA scheme...") is very ambiguous in meaning and speculative. I don't think it adds much to the discussion.

Reply: We have clarified that this sentence is a speculation as to the potential benefits of releasing SroC from the *glt* mRNA for it to act on GcvB.