THE EMBO JOURNAL

Manuscript EMBO-2010-75485

Gcn4p-mediated transcriptional repression of ribosomal protein genes under amino acid starvation

Yoo Jin Joo, Jin-ha Kim, Un-Beom Kang, Myung Hee Yu and Joon Kim

Corresponding author: Joon Kim, Korea University

Review timeline:

Submission date:	12 February 2010
Editorial Decision:	25 March 2010
Resubmission:	26 July 2010
Editorial Decision:	01 September 2010
Revision received:	12 October 2010
Editorial Decision:	08 November 2010
Revision received:	13 November 2010
Accepted:	16 November 2010

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 25 March 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the exceptionally long delay in getting back to you with a decision. Unfortunately, one of the referees was not able to return his/her report as quickly as initially expected.

Your manuscript has now finally been seen by three referees whose comments to the authors are shown below. As you will see while all three referees consider the study as conceptually very interesting in principle, it becomes clear that the evidence put forward for the mechanistic aspect of your study is not sufficiently strong and convincing to justify publication at this point. I will not repeat all issues raised by the referees in detail, but essentially the referees feel that the functional as well as the physiological significance of the Rap1/Gcn4 interaction is not developed sufficiently. The issue is not necessarily the mechanistic depth of the study, but the referees (in particular referee 3) rather feel that the data presented are not conclusive enough to allow for your mechanistic model. Clearly, the referees point to major shortcomings of key aspects of the experimental evidence provided, and fair amount of further experimentation which includes crucial controls would be required to address these issues. Furthermore, the outcome of such experiments cannot be predicted at this point. I therefore see little choice but to come to the conclusion that we cannot offer to publish the manuscript at this stage.

We would, however, not exclude the possibility to consider a new submission on the same topic should future studies allow you to strengthen the study considerably along the lines suggested by the reviewers including the major concerns raised by referee 3. To be completely clear, however, I would like to stress that if you wish to send a new manuscript this will be treated as a new

submission rather than a revision and will be evaluated and reviewed afresh (involving our original referees again if available), also with respect to the literature and the novelty of your findings at the time of resubmission. At this stage of analysis, though, I am sorry to have to disappoint you.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This manuscript by Yoo Jin Joo et al. relates to the problem how ribosomal protein expression in yeast might be effected by amino acid starvation. Their main conclusion is that the transcriptional activator Gcn4 mediates in this respect transcriptional repression of the relevant promoters via its interaction with the general factor Rap1. As such the manuscript would not only be surprising but also of interest to a large audience even beyond those working on yeast.

The work provides a rather large set of data that principally would support the proposed model. Indeed, if one looks at the transcript levels of specific ribosomal protein genes it is undisputable that Gcn4 has a distinct effect on their regulation. Thus, my skepticism of this work only arises with respect to the mechanistic detail starting with some of the results in Figure 2. The authors perform a mass spectrometry analysis of Rap1 co-precipitating proteins claiming that one of the bands identified on an SDS gel is Gcn4. The arrow pointing towards this protein is either not very precise or the level is so low that it might make these data more or less un-interpretable. The following experiments depicted in this figure are based on pull downs with heavily expressed proteins. Although I tend to believe that the in vitro interactions defined by these assays are correct I am always worried about their in vivo relevance. Such an experiment seems to have been provided in panel D although the expression level of the two proteins seems also rather high to me. One question connected with the co-precipitation experiment in Fig. 2D is whether the proteins bind independently of or through a DNA bridge. DNase1 digestion may not quite be the appropriate approach because of the protection provided by the binding proteins. Sensitivity towards ethidium bromide might give here a more conclusive answer.

I would suggest that the real crucial experiments for the Rap1 Gcn4 interaction have to be found within the chromatin IPs. One of the problems with HA tagged proteins is the fact that this tag sometimes causes arte facts at regions of low nucleosomal densities. Therefore I am not completely convinced of the data provided in figure 3, especially if they are related to IP/input ratios instead of internal multiplex controls or real time PCR readouts. Finally I think a lot of my reservations could be shown as irrelevant if the authors could just provide a correlation of Rap1 binding and Gcn4 chromatin recruitment to the relevant RPG promoters using ChIP assays with a tiled primer system across the promoter region.

Referee #2 (Remarks to the Author):

Rap1 is a DNA binding factor found upstream of most ribosomal protein (RP) genes and glycolytic genes as well as many other places in the yeast genome. The authors show that Gcn4 can bind to Rap1, and suggest that this binding competes with the histone acetyl transferase Esa1, thus leading

to repression of RP gene expression. This is an attractive hypothesis because Gcn4 is (translationally) induced by amino acid starvation, a situation in which it would seem valuable for the cell to curtail resource-consuming ribosome synthesis.

There is a great amount of data, much of which seems sound and supports the authors' conclusion. There are some particularly nice experiments, such as the demonstration that the Gcn4S242L mutant affects transcriptional activation of the AA genes but does not affect their results with the RP genes. However, there are also a number of places where questions are raised. The key ones are #5 and #8 below, that require some further comment.

The following are not in order of importance:

1) p3l12 I wouldn't call the ribosome a "protein complex" since it is 2/3 RNA.

2) P8l6 RPS11 does not match number in Fig 1.

3) P8l9 "suggest" is too strong a word here. They could be just parallel.

4) P8l17 I am not sure that 0.1X the amount of amino acids is really limiting; it depends on the inoculum and the length of growing time. It would be useful to know if the cells are still doubling.

5) A key issue that the authors do not address is the generality of their observations. I hate to suggest more experiments for this data-packed paper, but it would be interesting to know if the effect they see on RP genes can be generalized to the RP genes such as RPL3 that do not appear to have Rap1 sites, as well as to the non-RP genes, e.g. the glycolytic enzymes that DO have Rap1 sites.

6) Many of the bar-graphs are VERY hard to see, even when blown up in my pdf. I suggest they be done with color, or somehow made more legible.

7) P11l16 "RPGs HIS3 and ADE3" they are not

8) Fig 4A One of the most distinctive results in the paper is the rapid decline in Fhl1 and Ifh1 in response to amino acid starvation. It seems to me that that observation should be a major part of their model, since this effect is far greater than the very marginal change of histone acetylation that they observe in Fig 5G.

9) P13l11 They bring in Hmo1, but they have NO experiments on it.

10) Fig 5G It is not clear what part of the RP genes they have used in this ChIP experiment. It would be useful to have the results for the RPG box as well as upstream and downstream locations, especially as it has been reported that the RPG boxes are usually histone-poor.

11) I do not understand Fig 6, nor do I think it an important part of the story .Amino acid deprivation should activate Gcn2 and thereby reduce translation AND change the polysomes profile toward smaller polysomes, in either +/- GCN4. But they do not see this. Also, Fig 6C tells us very little. After two hours what change were they expecting? How is this controlled, in terms of the amount of RNA put on the gel. How does it detect 'newly synthesized rRNA"?

Referee #3 (Remarks to the Author):

This paper addresses the molecular mechanism of the known down-regulation of ribosomal protein gene (RPG) mRNAs during amino acid starvation in a manner involving the transcriptional activator Gcn4. A previous study uncovered this phenomenon and suggested the likelihood that Gcn4 acted indirectly to repress RPGs in starved cells. In this paper, the authors wish to convince us that Gcn4 acts directly by physically impeding the ability of the Rap1 protein to recruit the KAT in NuA4, Esa1, to RPG promoters. The authors begin by confirming with Northern analysis the conclusions reached previously by the microarray analysis of Natarajan et al that there is indeed Gcn4-dependent

down-regulation of ribosomal protein gene (RPG) mRNAs during amino acid starvation, although one of the figures presented on this point (Fig. 1A) has innumerable technical problems. They present seriously flawed experiments designed to eliminate the possibility that Gcn4 reduces the stability of RPG mRNAs (Fig. S3), or that it functions indirectly to repress RPG transcription in the course of its established role in activating amino acid biosynthetic genes (Fig. 1G). They go on to present evidence, some of it convincing (in Fig. 2E), that Gcn4 interacts with Rap1 in vivo, and they claim to have mapped the interaction domains to specific segments of the two proteins, although the conclusion for the domain in Gcn4 is questionable. In addition, the physiological relevance of these binding domains are not tested by examining the effects of mutations in the critical domains on native Gcn4-Rap1 association using the co-IP assay of Fig. 2E. Indeed, a key experiment missing is to show that a mutation in Gcn4 that abrogates the Gcn4-Rap1 interaction in vivo would also eliminate the ability of Gcn4 to repress RPG expression. They go on to provide ChIP evidence, some convincing (eg. for RPS3 in Fig. 3A-B), that Gcn4 binds to RPG chromatin in vivo, but the occupancies at RPS11B and RPL19B do not appear to be statistically significant. Other ChIP evidence, which is mostly unconvincing, is presented that Esa1 occupancy at RPG genes is reduced by 3AT treatment, dependent on the presence of Gcn4. They provide compelling evidence by co-IP that native Esa1 and Rap1 interact in vivo. Other experiments, presented as evidence that Gcn4 can compete with Esa1 for binding to Rap1 are very hard to accept, because the proposed competition mechanism could work only if Gcn4 was present in amounts comparable to that of Rap1 and/or Esa1. This is very unlikely to be true for the in vivo experiment in Fig. 5D in which Rap1 and Esa1 are both highly overproduced but Gcn4 is not; and the condition is clearly not met for the in vitro experiments in Fig. 5E-F, where GST-Esa1 is in vast excess compared to Gcn4-His. It seems impossible that Gcn4 would be able to titrate Rap1 from all of the available GST-Esa1. In addition, these in vitro experiments lack the important control of a non-interacting Gcn4 mutant (like S242L) that could not compete with Esa1 for binding Rap1. They provide ChIP evidence indicating a moderate, Gcn4-dependent reduction in acetylated histone H4 on 3AT treatment. Based on these results, they wish to conclude that Gcn4 acts directly to repress RPG transcription by binding to Rap1 and competing for Esa1 association, lowering histone acetylation by Esa1 of RPG promoters.

As indicated above and detailed further below, there are many problems with the experiments and interpretations in this paper. The fact that there is a Gcn4-dependent reduction in RPG mRNAs was published long ago. It is indeed interesting that Gcn4 is found at the RPS3 promoter in vivo, that Gcn4 can be coimmunoprecipitated with Rap1, and that Rap1 can be coimmunoprecipitated with Esa1-all of which I find convincing. However, the evidence is not compelling that Gcn4 occupancy applies broadly to RPGs or that Esa1 occupancy significantly declines in parallel with an increase in Gcn4 occupancy at multiple RPGs. In addition, the evidence that Gcn4 is able to compete with Esa1 for binding to Rap1 appears to be seriously flawed. Finally, the study lacks a mutation in Gcn4 or Rap1 that would abrogate Gcn4-Rap1 association in vitro and in vivo, reduce Gcn4 occupancy at RPGs, and eliminate the putative reduction in Esa1 association with RPGs, in parallel with eliminating the Gcn4-dependent negative regulation of RPG transcription in 3AT-starved cells. They might also need to show that deleting the Rap1 binding site at an RPG promoter would eliminate Gcn4 occupancy of the promoter region. Thus, while there are some intriguing data here, and it is possible that the authors' model will ultimately be demonstrated convincingly, these results are much too preliminary and technically deficient for publication here.

Specific criticisms

1. There is a large number of problems with panels A-E of Fig. 1.

Panels A-B:

-There is no untagged GCN4 control strain included to confirm that the HA signals derive from Gcn4-HA.

-It is very unexpected that the amount of Gcn4-HA induced after 30 min of 3AT (lane 6) would be so low compared to 120 min 3AT signals (lane 6), especially considering the ChIP results in Fig.3A showing no significant differences in Gcn4 occupancy of HIS3 between these two time points

-It's impossible to determine exactly what condition applies to lane 5 as the description in panel B refers to two different conditions. Presumably, this represents a Leu starvation since their strain is

leu2-, but this was not explained

-The control blot for Pgk1 is too overexposed for accurate quantification.

-They never indicate what the repressed condition for Gcn4 would be, but presumably it is that in lane 3

-there are no error bars, suggesting no replicate experiments were done to confirm the results

-Importantly, the results do not support their conclusion that the RPS3 and RPL19B mRNA levels are inversely related to Gcn4 protein levels. The largest reduction in mRNA levels occurs with rapamycin treatment, which produces only a modest increase in Gcn4. In addition, the RPS3 mRNA levels are not significantly lower for conditions 5 and 7 versus the other conditions, and the same holds for RPL19B mRNA in condition 7

-the 3D graphing method employed makes it exceedingly difficult to analyze the results.

Panels C-E:

-panel E seems to be completely redundant with panel C

-it should be noted in the text that the results in panels C-F confirm the conclusions reached by Natarajan et al (2001) of a Gcn4-dependent reduction in levels of most RPS and RPL genes in response to histidine limitation by 3AT

2. p. 8: it makes no sense to refer to a pair of otherwise isogenic GCN4 and gcn4 strains as "GCN4 isogenic strains" when only one of them contains WT GCN4.

3. The results shown in Fig. S3 seem completely superfluous to the 0 min 3AT data points for GCN4 and gcn4 cells presented in Figs. 1C and E. Are the results/conclusions somehow different for these different experiments?

4. The data in Fig.S3 are not informative because the experiment was carried out in nonstarvation conditions when, in fact, the effect of deleting GCN4 on RP mRNAs is observed only in starvation conditions. The experiment would need to be repeated by including a 3-AT treatment.

5. p. 9 and Fig.1G: the claim on Fig. 9 that the S242L substitution would not reduce Gcn4 DNA binding activity is completely unsubstantiated, and in fact, it is shown later in Fig. 3C that it does seem to reduce Gcn4 occupancy at HIS4. Given this last result, it is very difficult to understand how the S242L variant can reduce activation of HIS4 in a dominant fashion in Fig. 1G, unless it is doing so by squelching the resident WT Gcn4 in this strain owing to its overexpression from the GAL promoter. Another big problem with the interpretation arises from the results presented later (Fig. S4) that the S242L mutation greatly weakens the Gcn4-Rap1 interaction. According to their model, this should weaken the ability of Gcn4 to down-regulate RPG transcription, but in Fig. 1G, it does not do so. There is really no excuse for designing the experiment in this way, and the vastly superior approach would be to examine a strain in which the gcn4-S242L allele is expressed at native levels as the only source of Gcn4, and to attempt to confirm that induction of HIS4/ADE in 3AT-treated cells is impaired, whereas repression of RP genes is unaffected, by the S242L mutation. Even if this result is obtained, however, I don't see how they would reconcile it with those in Fig. S4.

6. Fig. 2A: it is unacceptable to omit the results of the mass spec analysis, which is necessary to confirm that they identified a statistically significant number of peptides matching Gcn4 only from the TAP-tagged strain, and (presumably) only following induction of Gcn4 with 3AT (considering how little Gcn4 is present in uninduced cells). Merely showing the gel in Fig. 2A is meaningless. And where is the 2D analysis referred to in the text?

7. Fig. 2B. Many of the figures have type too tiny to see and the schematic here is a perfect example. Beyond that, it appears that each of the deletions in GST-Gcn4 reduces His-Rap1 binding by a certain amount (certainly true for delta 4), but they don't seem to notice this. An obvious experiment missing is to examine the effect of the S242L substitution on the GST-Gcn4-Rap1 interaction.

8. As noted above, a key experiment missing is to show that a mutation in Gcn4 that abrogates the Gcn4-Rap1 interaction in vivo will also eliminate the ability of Gcn4 to repress RPG expression. On the surface, the gcn4-S242L mutation should fit the bill, but as noted above, the results in Fig. 1G suggest that weakening Gcn4-Rap1 interaction has no effect on RPG expression.

9. Fig. 3A: no t-tests were conducted to establish the statistical significance of Gcn4 occupancies at RPS11B and RPL19B, which do not appear to be significantly higher than that given by the untagged control, except possibly for RPL19B at 120 min.

10. Fig. 3C: S242L seems to reduce occupancy at RPS3, consistent with the results in Fig. S4. Why is there no such effect at RPL19B, and as mentioned above, why doesn't this mutation affect RPS3 repression? Why does S242L reduce the signal for the POL1 CDS so dramatically? If corrected for this effect, S242L would not reduce Gcn4 binding at HIS4 or RPS3. Apparently, the ChIP results are inadequate to reach any firm conclusion about the effect of S242L on Gcn4 occupancy.

11. Fig. 4B, Esa1-HA and Fhl1-HA data: the differences in occupancies between the GCN4 and gcn4 strains in 3AT are unlikely to be statistically significant, except possibly for Esa1-HA at RPS11B. More replicate measurements are needed to reduce the standard errors of these mean values, and a t-test must be included.

11B. P. 13, line 11: what is the conclusion about Hmo1 binding based on?

12. Fig. 5D: This experiment would have to be repeated using native levels of Esa1 and Rap1.

13. Fig. 5E-F: As indicated above, the amount of Gcn4-His appears to be too small to titrate Rap1 from the vast amount of GST-Esa1 employed. In addition, at odds with their claim, the amount of His-Rap1 eluted in Fig. 5F does not increase with more Gcn4 being added. It seems possible that the Gcn4-His is non-specifically poisoning the GST-Esa1/Rap1 interaction, and to eliminate this, they need to show that a mutant Gcn4 defective for binding Rap1 would not be able to compete with Rap1 for binding GST-Esa1.

14. The experiment in Fig. 6 is misguided in design and incorrectly interpreted. It would be unlikely that 1 hr treatment with 3AT would significantly affect levels of ribosomes, which are very stable molecules. In addition, the decrease in translation observed in gcn4 cells treated with 3AT almost certainly results from the low levels of histidine in these cells and has to do with the defect in up-regulating HIS3 not in down-regulating RPGs.

15. The authors frequently don't indicate what strains were used or exactly how they were cultured in the legends to the figures.

Resubmission 26 July 2010

Response to referees' comments

Referee #1 (Remarks to the Author):

This manuscript by Yoo Jin Joo et al. relates to the problem how ribosomal protein expression in yeast might be effected by amino acid starvation. Their main conclusion is that the transcriptional activator Gcn4 mediates in this respect transcriptional repression of the relevant promoters via its interaction with the general factor Rap1. As such the manuscript would not only be surprising but also of interest to a large audience even beyond those working on yeast. The work provides a rather large set of data that principally would support the proposed model. Indeed, if one looks at the transcript levels of specific ribosomal protein genes it is undisputable that Gcn4 has a distinct effect on their regulation. Thus, my skepticism of this work only arises with respect to the mechanistic detail starting with some of the results in Figure 2. The authors perform a mass spectrometry analysis of Rap1 co-precipitating proteins claiming that one of the bands identified on an SDS gel is Gcn4. The arrow pointing towards this protein is either not

very precise or the level is so low that it might make these data more or less un-interpretable.

- Although the proteins from the significant bands of the silver-stained gel were identified by in-gel digestion, however, there could be other co-precipitated contaminating bands in the same fraction. Actually, other proteins were also identified at the same fraction for Gcn4p identification. Thus, in our experimental scale of protein identification, we could not depict the band as Gcn4p precisely, as you pointed out. Therefore, we re-wrote the mass data in Supplementary Figure S5.

The following experiments depicted in this figure are based on pull downs with heavily expressed proteins. Although I tend to believe that the in vitro interactions defined by these assays are correct I am always worried about their in vivo relevance. Such an experiment seems to have been provided in panel D although the expression level of the two proteins seems also rather high to me. One question connected with the co-precipitation experiment in Fig. 2D is whether the proteins bind independently of or through a DNA bridge. DNase1 digestion may not quite be the appropriate approach because of the protection provided by the binding proteins. Sensitivity towards ethidium bromide might give here a more conclusive answer.

- As shown in Figure 2D, CoIP had been performed with endogenous Rap1p and Gcn4p with EtBr. In the former manuscript, we missed the description of experimental procedure about EtBr usage. Gcn4p-Rap1p binding was confirmed *in vivo* convincingly.

I would suggest that the real crucial experiments for the Rap1 Gcn4 interaction have to be found within the chromatin IPs. One of the problems with HA tagged proteins is the fact that this tag sometimes causes arte facts at regions of low nucleosomal densities. Therefore I am not completely convinced of the data provided in figure 3, especially if they are related to IP/input ratios instead of internal multiplex controls or real time PCR readouts.

- Provided that HA tagged protein causes artifact signal of ChIP, other HA tagged proteins such as Ifh1-HA3, Sfp1-HA3 and Fhl1-HA3 would show the same patterns to Esa1-HA3. However, there are large discrepancies among the ChIP results of four different HA tagged proteins (Figure 5B~5E in the new manuscript). Therefore, we thought that arte facts at low nucleosomal region were not detected in the scale of our ChIP experiments.

Finally I think a lot of my reservations could be shown as irrelevant if the authors could just provide a correlation of Rap1 binding and Gcn4 chromatin recruitment to the relevant RPG promoters using ChIP assays with a tiled primer system across the promoter region.

- To evaluate functional relevance of this binding, we generated new strains which are harboring various mutants of Gcn4p (S242L and ΔC). These mutant strains have different binding affinity to Rap1p. From the set of experiments with these strains, we could get more convincing results supporting the hypothesis about correlation of Rap1p binding and Gcn4p chromatin recruitment (Figure 4).

Referee #2 (Remarks to the Author):

Joo et al EMBO

Rap1 is a DNA binding factor found upstream of most ribosomal protein (RP) genes and glycolytic genes as well as many other places in the yeast genome. The authors show that Gcn4 can bind to Rap1, and suggest that this binding competes with the histone acetyl transferase Esa1, thus leading to repression of RP gene expression. This is an attractive hypothesis because Gcn4 is (translationally) induced by amino acid starvation, a situation in which it would seem valuable for the cell to curtail resource-consuming ribosome synthesis.
There is a great amount of data, much of which seems sound and supports the authors' conclusion. There are some particularly nice experiments, such as the demonstration that the Gcn4S242L mutant affects transcriptional activation of the AA genes but does not affect their results with the RP genes. However, there are also a number of places where questions are raised. The key ones are #5 and #8 below, that require some further comment.

The following are not in order of importance:

1) p3l12 I wouldn't call the ribosome a "protein complex" since it is 2/3 RNA.

- It was corrected.

2) P8l6 RPS11 does not match number in Fig 1.

- Actually, Figure 1A and 1B were deleted in the new manuscript. New experiments were designed and performed to confirm the mutually exclusive expression pattern between RP gene and Gcn4p target genes.

3) P8l9 "suggest" is too strong a word here. They could be just parallel.

- It was re-written.

4) P8l17 I am not sure that 0.1X the amount of amino acids is really limiting; it depends on the inoculum and the length of growing time. It would be useful to know if the cells are still doubling.

- The yeast cell under 1/10X the amount of amino acid can grow. As shown in Figure 1A, the transcription of *TRP3*, which is one of the best-characterized Gcn4p target genes, was activated under this condition. Therefore, this is one of the amino acid starvation conditions to yeast cells.

5) A key issue that the authors do not address is the generality of their observations. I hate to suggest more experiments for this data-packed paper, but it would be interesting to know if the effect they see on RP genes can be generalized to the RP genes such as RPL3 that do not appear to have Rap1 sites, as well as to the non-RP genes, e.g. the glycolytic enzymes that DO have Rap1 sites.

- When we depicted the mutually exclusive transcription pattern between RP genes and Gcn4ptarget gene in Figure 1A, we also checked transcription levels of *RPL3*, *RPL18B* and *PGK1*. *RPL3* and *RPL18B* do not have Rap1-sites, and *PGK1* have Rap1p-site in its promoter region. As predicted, it was revealed that *RPL3* and *RPL18B* transcriptions were not regulated like other RP genes, but *PGK1* did. This result was presented in Figure 1A of the new manuscript.

6) Many of the bar-graphs are VERY hard to see, even when blown up in my pdf. I suggest they be done with color, or somehow made more legible.

- Resolutions of all graphs in manuscript were upgraded.

7) P11l16 "RPGs HIS3 and ADE3" they are not

- It was changed to"RP genes, *HIS3* and *ADE3*".

8) Fig 4A One of the most distinctive results in the paper is the rapid decline in Fhl1 and Ifh1 in response to amino acid starvation. It seems to me that that observation should be a major part of their model, since this effect is far greater than the very marginal change of histone acetylation that they observe in Fig 5G.

- Yes. The expression pattern and the regulation of promoter occupancy of these factors were more significant. However, it did occur in a Gcn4p-independent manner. We primarily focused on Gcn4p-dependent mechanism hear. Further experiment related with these observations were not performed any more.

9) P13l11 They bring in Hmo1, but they have NO experiments on it.

- It was corrected.

10) Fig 5G It is not clear what part of the RP genes they have used in this ChIP experiment. It would be useful to have the results for the RPG box as well as upstream and downstream locations, especially as it has been reported that the RPG boxes are usually histone-poor.

- All the primers used in this study were the same in each Figures. They amplified about 400 bp core promoter region including RPG box as well as upstream and downstream regions. Many previous studies revealed that promoters of heavily transcribed genes were histone-poor regions, however, some other reports demonstrated that histone-modification still occurred in this regions. From the results from others and ours, it was suggested that intensive histone modifications were occurred, so as to compensate less existence of histone in this region.

11) I do not understand Fig 6, nor do I think it an important part of the story. Amino acid deprivation should activate Gcn2 and thereby reduce translation AND change the polysomes profile toward smaller polysomes, in either +/- GCN4. But they do not see this. Also, Fig 6C tells us very little. After two hours what change were they expecting? How is this controlled, in terms of the amount of RNA put on the gel. How does it detect 'newly synthesized rRNA"?

- Figure 6 in old manuscript was deleted.

Referee #3 (Remarks to the Author):

This paper addresses the molecular mechanism of the known down-regulation of ribosomal protein gene (RPG) mRNAs during amino acid starvation in a manner involving the transcriptional activator Gcn4. A previous study uncovered this phenomenon and suggested the likelihood that Gcn4 acted indirectly to repress RPGs in starved cells. In this paper, the authors wish to convince us that Gcn4 acts directly by physically impeding the ability of the Rap1 protein to recruit the HAT in NuA4, Esa1, to RPG promoters. The authors begin by confirming with Northern analysis the conclusions reached previously by the microarray analysis of Natarajan et al that there is indeed Gcn4-dependent down-regulation of ribosomal protein gene (RPG) mRNAs during amino acid starvation, although one of the figures presented on this point (Fig. 1A) has innumerable technical problems. They present seriously flawed experiments designed to eliminate the possibility that Gcn4 reduces the stability of RPG mRNAs (Fig. S3), or that it functions indirectly to repress RPG transcription in the course of its established role in activating amino acid biosynthetic genes (Fig. 1G). They go on to present evidence, some of it convincing (in Fig. 2E), that Gcn4 interacts with Rap1 in vivo, and they claim to have mapped the interaction domains to specific segments of the two proteins, although the conclusion for the domain in Gcn4 is questionable. In addition, the physiological relevance of these binding domains are not tested by examining the effects of mutations in the critical domains on native Gcn4-Rap1 association using the co-IP assay of Fig. 2E. Indeed, a key experiment missing is to show that a mutation in Gcn4 that abrogates the Gcn4-Rap1 interaction in vivo would also eliminate the ability of Gcn4 to repress RPG expression. They go on to provide ChIP evidence, some convincing (eg. for RPS3 in Fig. 3A-B), that Gcn4 binds to RPG chromatin in vivo, but the occupancies at RPS11B and RPL19B do not appear to be statistically significant. Other ChIP evidence, which is mostly unconvincing, is presented that Esa1 occupancy at RPG genes is reduced by 3AT treatment, dependent on the presence of Gcn4. They provide compelling evidence by co-IP that native Esa1 and Rap1 interact in vivo. Other experiments, presented as evidence that Gcn4 can compete with Esa1 for binding to Rap1 are very hard to accept, because the proposed competition mechanism could work only if Gcn4 was present in amounts comparable to that of Rap1 and/or Esa1. This is very unlikely to be true for the in vivo experiment in Fig. 5D in which Rap1 and Esa1 are both highly overproduced but Gcn4 is not; and the condition is clearly not met for the in vitro experiments in Fig. 5E-F, where GST-Esa1 is in vast excess compared to Gcn4-His. It seems impossible that Gcn4 would be able to titrate Rap1 from all of the available GST-Esa1. In addition, these in vitro experiments lack the important control of a non-interacting Gcn4 mutant (like S242L) that could not compete with Esa1 for binding Rap1. They provide ChIP evidence indicating a moderate, Gcn4-dependent reduction in acetylated histone H4 on 3AT treatment. Based on these results, they wish to conclude that Gcn4 acts directly to repress RPG transcription by binding to Rap1 and competing for Esa1 association, lowering histone acetylation by Esa1 of RPG promoters.
As indicated above and detailed further below, there are many problems with the experiments and interpretations in this paper. The fact that there is a Gcn4-dependent reduction in RPG

mRNAs was published long ago. It is indeed interesting that Gcn4 is found at the RPS3 promoter in vivo, that Gcn4 can be coimmunoprecipitated with Rap1, and that Rap1 can be coimmunoprecipitated with Esa1-all of which I find convincing. However, the evidence is not compelling that Gcn4 occupancy applies broadly to RPGs or that Esa1 occupancy significantly declines in parallel with an increase in Gcn4 occupancy at multiple RPGs. In addition, the evidence that Gcn4 is able to compete with Esa1 for binding to Rap1 appears to be seriously flawed. Finally, the study lacks a mutation in Gcn4 or Rap1 that would abrogate Gcn4-Rap1 association in vitro and in vivo, reduce Gcn4 occupancy at RPGs, and eliminate the putative reduction in Esa1 association with RPGs, in parallel with eliminating the Gcn4-dependent negative regulation of RPG transcription in 3AT-starved cells. They might also need to show that deleting the Rap1 binding site at an RPG promoter would eliminate Gcn4 occupancy of the promoter region. Thus, while there are some intriguing data here, and it is possible that the authors' model will ultimately be demonstrated convincingly, these results are much too preliminary and technically deficient for publication here.

Specific criticisms

1. There is a large number of problems with panels A-E of Fig. 1.

Panels A-B:

-There is no untagged GCN4 control strain included to confirm that the HA signals derive from Gcn4-HA.

-It is very unexpected that the amount of Gcn4-HA induced after 30 min of 3AT (lane 6) would be so low compared to 120 min 3AT signals (lane 6), especially considering the ChIP results in Fig.3A showing no significant differences in Gcn4 occupancy of HIS3 between these two time points

-It's impossible to determine exactly what condition applies to lane 5 as the description in panel B refers to two different conditions. Presumably, this represents a Leu starvation since their strain is leu2-, but this was not explained

-The control blot for Pgk1 is too overexposed for accurate quantification.
-They never indicate what the repressed condition for Gcn4 would be, but presumably it is that in lane 3

-there are no error bars, suggesting no replicate experiments were done to confirm the results

-Importantly, the results do not support their conclusion that the RPS3 and RPL19B mRNA levels are inversely related to Gcn4 protein levels. The largest reduction in mRNA levels occurs with rapamycin treatment, which produces only a modest increase in Gcn4. In addition, the RPS3 mRNA levels are not significantly lower for conditions 5 and 7 versus the other conditions, and the same holds for RPL19B mRNA in condition 7

-the 3D graphing method employed makes it exceedingly difficult to analyze the results.

- The experiments in Figure 1A and 1B in old manuscript were performed in order to confirm the conclusions reached previously by the microarray analysis of Natarajan. Following your helpful comments, we totally redesigned an experiment for the same purpose (Figure 1A in new manuscript). Amino acid starvation conditions were simplified and many flaws were fixed. Moreover, we tried to examine other Rap1p-target genes to make it clear.

Panels C-E:

- panel E seems to be completely redundant with panel C

- The result of Figure 1E in former manuscript was eliminated.

- it should be noted in the text that the results in panels C-F confirm the conclusions reached by

Natarajan et al (2001) of a Gcn4-dependent reduction in levels of most RPS and RPL genes in response to histidine limitation by 3AT

- We clearly mentioned that in the new manuscript.

2. p. 8: it makes no sense to refer to a pair of otherwise isogenic GCN4 and gcn4 strains as "GCN4 isogenic strains" when only one of them contains WT GCN4.

- It was rewritten.

3. The results shown in Fig. S3 seem completely superfluous to the 0 min 3AT data points for GCN4 and gcn4 cells presented in Figs. 1C and E. Are the results/conclusions somehow different for these different experiments?

- No. Amounts of loaded total RNA were the same. However, in Figure S3, all RP mRNA decreased so fast that longer exposure should be technically required.

4. The data in Fig.S3 are not informative because the experiment was carried out in nonstarvation conditions when, in fact, the effect of deleting GCN4 on RP mRNAs is observed only in starvation conditions. The experiment would need to be repeated by including a 3-AT treatment.

- Investigation of RP mRNA stability under amino acid starvation was performed and presented in Figure S4.

5. p. 9 and Fig.1G: the claim on Fig. 9 that the S242L substitution would not reduce Gcn4 DNA binding activity is completely unsubstantiated, and in fact, it is shown later in Fig. 3C that it does seem to reduce Gcn4 occupancy at HIS4. Given this last result, it is very difficult to understand how the S242L variant can reduce activation of HIS4 in a dominant fashion in Fig. 1G, unless it is doing so by squelching the resident WT Gcn4 in this strain owing to its overexpression from the GAL promoter. Another big problem with the interpretation arises from the results presented later (Fig. S4) that the S242L mutation greatly weakens the Gcn4-Rap1 interaction. According to their model, this should weaken the ability of Gcn4 to downregulate RPG transcription, but in Fig. 1G, it does not do so. There is really no excuse for designing the experiment in this way, and the vastly superior approach would be to examine a strain in which the gcn4-S242L allele is expressed at native levels as the only source of Gcn4, and to attempt to confirm that induction of HIS4/ADE in 3AT-treated cells is impaired, whereas repression of RP genes is unaffected, by the S242L mutation. Even if this result is obtained, however, I don't see how they would reconcile it with those in Fig. S4.

- First of all, galactose induction for Gcn4WT-myc7 and Gcn4S242L-myc7 was performed in *gcn4* null strain (Figure 4B). Therefore, only source of Gcn4p came from the exogenous expression. Next, we thought that there were some misinterpretations about the definition of S242L mutant. We showed that S242L mutant can bind to Rap1p (Figure 4A) like wild type, but have no trans-activity (Figure 4B and 4D). To evaluate the functional relevance of Rpa1p binding *in vivo*, we generated new strains which is harboring various mutant version of Gcn4p (S242L and ΔC). These mutant strains have different binding affinity to Rap1p comparing to wild type Gcn4p. From set of these experiments in Figure 4, we showed that the binding affinity to Rap1p, not its trans-activity is important for a repressive role of Gcn4p in RP transcription. (see the details in main text)

6. Fig. 2A: it is unacceptable to omit the results of the mass spec analysis, which is necessary to confirm that they identified a statistically significant number of peptides matching Gcn4 only from the TAP-tagged strain, and (presumably) only following induction of Gcn4 with 3AT (considering how little Gcn4 is present in uninduced cells). Merely showing the gel in Fig. 2A is meaningless. And where is the 2D analysis referred to in the text?

- It was re-written. Mass spectrometry result presented in Figure S5. Moreover, the raw data of probability and coverage were also summarized.

7. Fig. 2B. Many of the figures have type too tiny to see and the schematic here is a perfect example. Beyond that, it appears that each of the deletions in GST-Gcn4 reduces His-Rap1 binding by a certain amount (certainly true for delta 4), but they don't seem to notice this. An obvious experiment missing is to examine the effect of the S242L substitution on the GST-Gcn4-Rap1 interaction.

- Resolution of all the Figures, especially the graphs, was increased in the new manuscript. Of course, we also notified that wild type and delta 1 mutant of GST-Gcn4 truncations bound to His6-Rap1 stronger. It might be caused by more adequate folding of these recombinant proteins, however, we decided that this difference was not enough to focus on because it was performed *in vitro*. Moreover, GST pull-down assay was performed to examine binding affinity of S242L mutant to Rap1 (Figure 4A).

8. As noted above, a key experiment missing is to show that a mutation in Gcn4 that abrogates the Gcn4-Rap1 interaction in vivo will also eliminate the ability of Gcn4 to repress RPG expression. On the surface, the gcn4-S242L mutation should fit the bill, but as noted above, the results in Fig. 1G suggest that weakening Gcn4-Rap1 interaction has no effect on RPG expression.

- As mentioned above, new experiments were performed with strains possessing endogenous level Gcn4p variants in order to identify the functional and mechanistic mechanism of Gcn4p-dependent regulation, majorly presented in Figure 4 of new manuscript. S242L mutant can bind to Rap1p like wild type, but does not have transcriptional activation activity. On the other hand Gcn4ΔC mutant does not have both activities such as Rap1p-binding and trans-activation. Using these mutants along with wild type and null strains appears to be sufficient for supporting our hypothesis.

9. Fig. 3A: no t-tests were conducted to establish the statistical significance of Gcn4 occupancies at RPS11B and RPL19B, which do not appear to be significantly higher than that given by the untagged control, except possibly for RPL19B at 120 min.

- We performed more ChIP assay repeatedly and t-tests of these raw data. The results of t-test were presented in Figure 3A.

10. Fig. 3C: S242L seems to reduce occupancy at RPS3, consistent with the results in Fig. S4. Why is there no such effect at RPL19B, and as mentioned above, why doesn't this mutation affect RPS3 repression? Why does S242L reduce the signal for the POL1 CDS so dramatically? If corrected for this effect, S242L would not reduce Gcn4 binding at HIS4 or RPS3. Apparently, the ChIP results are inadequate to reach any firm conclusion about the effect of S242L on Gcn4 occupancy.

- As noted above, there must be an explanation of S242L mutant in this manuscript. The reduction ratios of promoter occupancy by S242L to by wild type Gcn4p were much higher at *HIS4/ADE3* rather than RP genes no matter how Gcn4p was expressed (Figure 4C and 4E). Moreover, relatively low binding to RP promoter than to its target genes may be due to the indirect association through Rap1p.

11. Fig. 4B, Esa1-HA and Fhl1-HA data: the differences in occupancies between the GCN4 and gcn4 strains in 3AT are unlikely to be statistically significant, except possibly for Esa1-HA at RPS11B. More replicate measurements are needed to reduce the standard errors of these mean values, and a t-test must be included.

- More experiments were performed repeatedly only with Esa1-HA3. Subsequent t-test was also performed and presented in Figure 5D. We got a statistically more significant data for Esa1p binding at RP promoter region.

11B. P. 13, line 11: what is the conclusion about Hmo1 binding based on?

- It was revised.

12. Fig. 5D: This experiment would have to be repeated using native levels of Esa1 and Rap1.

- CoIP with native level Esa1p and Rap1p with or without 3-AT was performed (Figure 6E).

13. Fig. 5E-F: As indicated above, the amount of Gcn4-His appears to be too small to titrate Rap1 from the vast amount of GST-Esa1 employed. In addition, at odds with their claim, the amount of His-Rap1 eluted in Fig. 5F does not increase with more Gcn4 being added. It seems possible that the Gcn4-His is non-specifically poisoning the GST-Esa1/Rap1 interaction, and to eliminate this, they need to show that a mutant Gcn4 defective for binding Rap1 would not be able to compete with Rap1 for binding GST-Esa1.

- In the CoIP result in Figure 6D, the absolute expression moles among three proteins could not compared because the activity of antibodies used in this experiment were different from each other. To investigate molar ratio of Esa1p, Gcn4p and Rap1p in endogenous level, western blot was performed with strain harboring Esa1-HA3, Gcn4-HA3, and Rap1-HA3 (Figure S8). These three proteins, expressed native level, have the same tag (triple-HA) in the same architecture (C-terminus). Therefore, we were able to compare the expression level exactly. Gcn4p expression was not much lower than other proteins. Although, the expression level of Rap1p is about 4~5 fold higher than Esa1p and Gcn4p, our mechanistic model can be still possible because these proteins have lots of functions in various space in yeast cells. Some of derepressed Gcn4p bound to Rap1p at RP promoter. Not all Rap1p were masked by Gcn4p. To probe that another experiment should be designed to find specific circumstance for Rap1p-Gcn4p binding. It is important to note that Pina B. et al proposed that Rap1p alters its structure to bind to different versions of its DNA binding sequence. These structural changes may modulate the function of Rap1p domains, providing different interacting surfaces for binding to specific co-operating factors. Therefore, we performed a GSTpulldown with DNA bound His6-Rap1 and GST-Gcn4p. PCR products amplified from RP promoter region and point mutant of Rap1p-site on it were used as DNA to bind to recombinant Rap1p. However, no significant change was detected in Gcn4p-Rap1p binding. Of course could tell the suggestion of context dependent structural changes of Rap1p could be wrong. According to our observations, we also could suggest that epigenetic surroundings should be required for the mechanism proposed by Pina et al. Moreover, BSA control was enough to use as no binding protein control to Rap1p in Figure 6G. If Gcn4-His6 is nonspecifically poisoning the GST-Esa1/Rap1 interaction, BSA also can inhibit this association.
Finally, we thought that results in Figure 6F and 6G could be interpreted like this; “As amount of added Gcn4-His6 was increased, more His6-Rap1 could not bind to GST-Esa1”.

14. The experiment in Fig. 6 is misguided in design and incorrectly interpreted. It would be unlikely that 1 hr treatment with 3AT would significantly affect levels of ribosomes, which are very stable molecules. In addition, the decrease in translation observed in gcn4 cells treated with 3AT almost certainly results from the low levels of histidine in these cells and has to do with the defect in up-regulating HIS3 not in down-regulating RPGs.

- Figure 6 in former manuscript was removed.

15. The authors frequently don't indicate what strains were used or exactly how they were cultured in the legends to the figures.

- All the figure legends and section of ‘Materials and Methods’ were re-checked and revised.

2nd Editorial Decision 01 September 2010

Thank you for submitting a new version of your manuscript (original version EMBOJ-2010-73930) for consideration by The EMBO Journal. After some delay caused by problems with the availability of our original referees due to the current summer holiday season it has now been seen by our original referees 1 (now referee 2) and 3 (now referee 1) again. As you will see while, in principle, both referees are now more positive regarding publication of the paper here, referee 1 still raises concerns about the conclusiveness of part of the experimental evidence provided that, in his/her

view, need to be addressed by further experimentation. Given that he/she is in favour of giving you the chance to address these concerns in an additional round of revision I have come to the conclusion that we would be able to consider a re-revised manuscript. However, it will be indispensable to address all concerns of referee 1 to his/her full satisfaction, and I should stress that a final decision can only be made at that stage.

Furthermore, it will be highly advisable for you to take another careful look through the text and correct and improve the spelling and grammar throughout. I would therefore urge you to have a native English speaker read the final draft before returning it to our office.

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

Thank you again for the opportunity to consider your work for publication. I look forward to your re-revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This manuscript represents an improvement over the previous version; however, there are still a number of issues concerning the quality or completeness of the data, which impact certain key conclusions. The main improvement is that they included a Gcn4 mutant, variously named delta5 or deltaC, truncated at its C-terminus and thus missing the DNA binding domain, which they claim cannot interact with Rap1 in vitro and also cannot support the down-regulation of RP gene transcripts in response to amino acid starvation in vivo. This protein also is not detected at RP promoters by ChIP. By contrast, the gcn4-S242L mutant is not defective for interaction with Rap1 in vitro, or for the down-regulation of RP gene transcripts, and it is found at RP promoters by ChIP, even though the mutation impairs Gcn4 occupancy at a Gcn4 target gene (HIS4) and disrupts transcriptional activation of HIS4 and ADE3 by Gcn4 in starved cells. These findings provide good evidence that the ability to interact with Rap1, but not to bind canonical Gcn4 target gene promoters, is required for Gcn4 to down-regulate RP gene transcripts, thus providing evidence that Gcn4 acts directly to repress RP genes. Unfortunately, however, they did not complete the analysis and show that the gcn4-deltaC variant cannot be coimmunoprecipitated with Rap1 from cell extracts using the approaches employed in Fig. 2C-D to document interaction of the two wild-type proteins. This strikes me as a critical omission because the failure of deltaC to interact with Rap1 is at the crux of this important line of reasoning. Another reason for insisting about this experiment is that the data they presented to show that delta5/deltaC abrogates binding of Gcn4 to Rap1 in vitro in Fig. 2A is flawed by the fact that the Gst-Gcn4-delta5 fusion is present at lower levels compared to all of the other Gst-Gcn4 fusions being compared. (It's also not actually clear which band represents the full-length delta5 fusion.) Thus, it also seems necessary to titrate down the wild-type GST-Gcn4 to insure that Rap1 binding can be detected to the wild-type but not to delta5 at equal levels of the two fusion proteins.

-There are important flaws regarding the Northern data in Fig. 1A. Contrary to their claims, the levels of RPS11B, RPL10, and RPL19B mRNAs are not reduced by 3AT treatment, whereas RPL18B (says RPS18B in the text, but this must be a typo) mRNA is reduced by SM treatment for 2 hr. They make a comment in the text that the response to 3AT is faster than the other two methods used to induce amino acid starvation, but Fig. 1A indicates the contrary. Clearly, Fig. 1A needs to be replaced with a more careful analysis that includes quantification of results from multiple experiments after normalizing for the levels of ACT1 mRNA, and the text needs to be crafted more

carefully to make sure it accurately reflects the data in the figure.

-The experiment carried out to show that the rate of RP gene mRNA turnover is unaffected by deletion of GCN4 in Fig. S4 is flawed in several ways. First, they added thiolutin to inhibit transcription at the same time they shifted cells into amino acid limiting medium. As a result, they did not allow the cells to respond to starvation before inhibiting all cellular transcription. Clearly, the cells should be amino acid deprived first and only after they respond with a reduction in levels of RP gene transcripts (in wild-type) should thiolutin be added to measure decay rates. Second, they didn't quantify the data in Fig. S4 and calculate half-times for the RP gene transcripts. It seems possible that the mRNAs actually are stabilized somewhat in the gcn4 mutant.

-The authors state that the data in Fig. 5B reveal no change in Fhl1 occupancy of the RP gene promoters that is "related with Gcn4", but in fact the occupancies at both RPS3 and RPS11B appear to be higher in the gcn4 mutant versus the wild-type strain in the presence of 3AT. (The differences in ChIP signals are as great as other differences they judge to be significant.) This finding coincides with a higher level of total Fhl1 in gcn4 versus wild-type cells shown by Western analysis in panel A. Thus, it seems possible that the higher promoter occupancy of Fhl1 in the gcn4 cells could be an important contributing factor to the diminished repression of RP gene promoters in amino acid starved gcn4 cells, in addition to the higher level of Esa1 recruitment which they have shown convincingly.

-I still maintain that for the experimental results in Fig. 6F-G to make sense, it would be necessary that the authors have used stoichiometric amounts of Gcn4-His6 and His6-Rap1 in order to enable Gcn4-His6 to dissociate His6-Rap1 from GST-Esa1, but they have not provided information about the molar amounts, or even the relative amounts, of these purified proteins used in these assays. This is unacceptable.

-For the experiment in Fig. 6E, they observe that association of TAP-Esa1 with Rap1 in the gcn4 mutant is undetectable in the absence of 3-AT, whereas their model would predict constitutive Esa1-Rap1 association in this strain. No comment is made regarding this inexplicable finding.

-For the ChIP data in Fig. 7A, I find it rather difficult to accept that the difference in occupancy of acetylated H4 at RPS3 for the GCN4 strain in the presence and absence of 3-AT is significant with a P-value of less than 0.005 considering that the error bars nearly overlap and the results derive from only three replicate measurements. Similarly, a P-value of <0.05 for the corresponding data for RPL19B is surprising considering that these two error bars overlap completely.

In summary, the authors have provided considerable evidence in favor of their model, which is quite appealing intellectually on a number of levels. However, the experimental design or quality of data for some of the experiments do not meet the standards of this journal. In addition, a key experiment needs to be conducted to provide direct evidence that complex formation in vivo between Gcn4 and Rap1 is abolished by the deltaC gcn4 mutation.

Referee #2 (Remarks to the Author):

The authors have made a valiant attempt at countering the criticisms of all the reviewers. In my opinion, their experimental approach has been improved to a level that one might consider the main conclusion of their work as well supported. The point remaining as the most speculative one is perhaps the notion that the decrease of Esa1 recruitment by Rap1-Gcn4 is the primary cause for the down regulation of RP genes. Nevertheless, taking everything into consideration I could support publication of the work now on experimental merit. I have however some serious misgivings about the style of the manuscript. There are many typos or wrong use of words depending on one's interpretation such as derepressed, depressed and suppressed (too many to list in detail). Past and present tenses are not always used in a logical way. Some of the sentences are atrocious, especially in the later result chapters and the discussion section. Some of their speculation, for example on the physiological relevance of the down regulation of RP gene transcription, namely to provide more general transcription factors for stress related genes is naïve, not convincing or could easily be

tested. I therefore suggest to reword and to rewrite some of the more notorious parts of the manuscript, perhaps with some outside help.

1st Revision - authors' response 12 October 2010

Referee #1 (Remarks to the Author):

This manuscript represents an improvement over the previous version; however, there are still a number of issues concerning the quality or completeness of the data, which impact certain key conclusions. The main improvement is that they included a Gcn4 mutant, variously named delta5 or deltaC, truncated at its C-terminus and thus missing the DNA binding domain, which they claim cannot interact with Rap1 in vitro and also cannot support the down-regulation of RP gene transcripts in response to amino acid starvation in vivo. This protein also is not detected at RP promoters by ChIP. By contrast, the gcn4-S242L mutant is not defective for interaction with Rap1 in vitro, or for the down-regulation of RP gene transcripts, and it is found at RP promoters by ChIP, even though the mutation impairs Gcn4 occupancy at a Gcn4 target gene (HIS4) and disrupts transcriptional activation of HIS4 and ADE3 by Gcn4 in starved cells. These findings provide good evidence that the ability to interact with Rap1, but not to bind canonical Gcn4 target gene promoters, is required for Gcn4 to down-regulate RP gene transcripts, thus providing evidence that Gcn4 acts directly to repress RP genes. Unfortunately, however, they did not complete the analysis and show that the gcn4-deltaC variant cannot be coimmunoprecipitated with Rap1 from cell extracts using the approaches employed in Fig. 2C-D to document interaction of the two wild-type proteins. This strikes me as a critical omission because the failure of deltaC to interact with Rap1 is at the crux of this important line of reasoning. Another reason for insisting about this experiment is that the data they presented to show that delta5/deltaC abrogates binding of Gcn4 to Rap1 in vitro in Fig. 2A is flawed by the fact that the Gst-Gcn4-delta5 fusion is present at lower levels compared to all of the other Gst-Gcn4 fusions being compared. (It's also not actually clear which band represents the full-length delta5 fusion.) Thus, it also seems necessary to titrate down the wild-type GST-Gcn4 to insure that Rap1 binding can be detected to the wild-type but not to delta5 at equal levels of the two fusion proteins.

- We preformed coimmunoprecipitation with endogenous Rap1p and three mutants of Gcn4p including Gcn4ΔC mutant (Figure 4D). We found that Gcn4ΔC mutant did not bind to Rap1p in the endogenous level, and this result is consistent with in vitro binding data in Figure 2A.

There are important flaws regarding the Northern data in Fig. 1A. Contrary to their claims, the levels of RPS11B, RPL10, and RPL19B mRNAs are not reduced by 3AT treatment, whereas RPL18B (says RPS18B in the text, but this must be a typo) mRNA is reduced by SM treatment for 2 hr. They make a comment in the text that the response to 3AT is faster than the other two methods used to induce amino acid starvation, but Fig. 1A indicates the contrary. Clearly, Fig. 1A needs to be replaced with a more careful analysis that includes quantification of results from multiple

experiments after normalizing for the levels of ACT1 mRNA, and the text needs to be crafted more carefully to make sure it accurately reflects the data in the figure.

- We performed northern experiments more than three times with independent colonies. We substituted blotting data by graphs containing quantification results after normalizing for the levels of ACT1 mRNA. We also corrected the typo.

The experiment carried out to show that the rate of RP gene mRNA turnover is unaffected by deletion of GCN4 in Fig. S4 is flawed in several ways. First, they added thiolutin to inhibit transcription at the same time they shifted cells into amino acid limiting medium. As a result, they did not allow the cells to respond to starvation before inhibiting all cellular transcription. Clearly, the cells should be amino acid deprived first and only after they respond with a reduction in levels of RP gene transcripts (in wild-type) should thiolutin be added to measure decay rates. Second, they didn't quantify the data in Fig. S4 and calculate half-times for the RP gene transcripts. It seems possible that the mRNAs actually are stabilized somewhat in the gcn4 mutant.

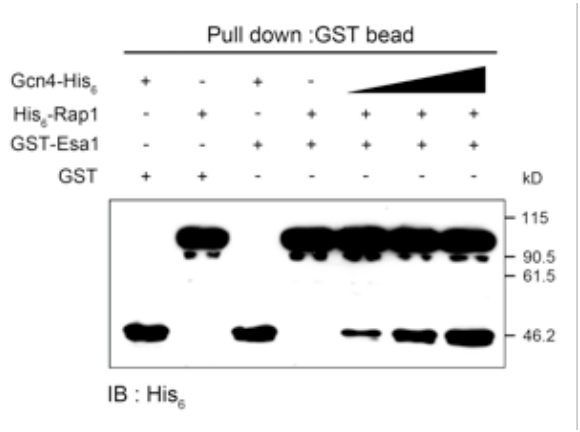
- We double-checked the half life of RP mRNA under amino acid starvation following your critical suggestion (Figure S5). However, we observed that initiation of TRP3 transcription still occurred 20~40 min after thilotin treatment. This result implies that transcription initiation still occurred even though thiolutin was treated. Therefore, we evaluated the half life of RP mRNA 20 min after thiolutin treatment and confirmed that no-changes of RP mRNA stability. Moreover, our previous data published in 2009 (Yoo Jin Joo, et al. 2009. BBA) clearly showed inhibition of transcription initiation of RP gene (RPS3) by 3-AT using reporter assay. According to these results, we can conclude that reduction of RP mRNA level under amino acid starvation is not due to the change of its mRNA stability, but is mainly due to the inhibition of transcriptional initiation process in the RP promoter region.

The authors state that the data in Fig. 5B reveal no change in Fhl1 occupancy of the RP gene promoters that is "related with Gcn4", but in fact the occupancies at both RPS3 and RPS11B appear to be higher in the gcn4 mutant versus the wild-type strain in the presence of 3AT. (The differences in ChIP signals are as great as other differences they judge to be significant.) This finding coincides with a higher level of total Fhl1 in gcn4 versus wild-type cells shown by Western analysis in panel A. Thus, it seems possible that the higher promoter occupancy of Fhl1 in the gcn4 cells could be an important contributing factor to the diminished repression of RP gene promoters in amino acid starved gcn4 cells, in addition to the higher level of Esa1 recruitment which they have shown convincingly.

- We had also recognized the observation which suggests that Gcn4p might have an effect on RP gene regulation indirectly through the modulation of Fhl1p expression level, finally resulting in the down-regulation the RP genes under amino acid starvation. Given that Gcn4p regulates the expression level of Fhl1p, it should occur outside of RP promoter region, and Gcn4p-dependent factor recruitment was less significant. Therefore, in present study, we only focused on Esa1p as a mechanistic target of Gcn4p. Other regulatory pathways for RP genes including this possibility were discussed in Discussion section of our revised manuscript.

I still maintain that for the experimental results in Fig. 6F-G to make sense, it would be necessary that the authors have used stoichiometric amounts of Gcn4-His6 and His6-Rap1 in order to enable Gcn4-His6 to dissociate His6-Rap1 from GST-Esa1, but they have not provided information about the molar amounts, or even the relative amounts, of these purified proteins used in these assays. This is unacceptable.

- GST pull down analysis in Figure 6F was performed with *E. coli* lysates containing each recombinant protein, and these lysates were mixed as indicated for the in vitro binding and competition. Therefore, we did not know the exact molar ratio of recombinant proteins used in this experiment. To compare relative molar ratio of tagged proteins, we performed immunoblotting with anti His antibody to detect mixed recombinant proteins, His_6-Rap1 and Gcn4-His_6.



As shown in the upper figure, relative amount of Gcn4-His_6 to His_6-Rap1 appears to be enough to compete the Rap1-Esa1 binding. In the case of GST pull down assay in Figure 6G, the molar ratio of both proteins added were presented in figure legend. However, actual ratio of Gcn4-His_6 to His_6-Rap1 should be much more than that indicated because not all His_6-Rap1 bound to GST-Esa1 and Gcn4-His_6 was added after washing the 1st GST-Esa1-His6-Rap1 binding complex. The observation that more competition in Figure 6G might be explainable based on this hypothesis. Therefore, we think that these in vitro analyses were enough to support our mechanistic model of Gcn4p.

For the experiment in Fig. 6E, they observe that association of TAP-Esa1 with Rap1 in the gcn4

mutant is undetectable in the absence of 3-AT, whereas their model would predict constitutive Esa1-Rap1 association in this strain. No comment is made regarding this inexplicable finding.

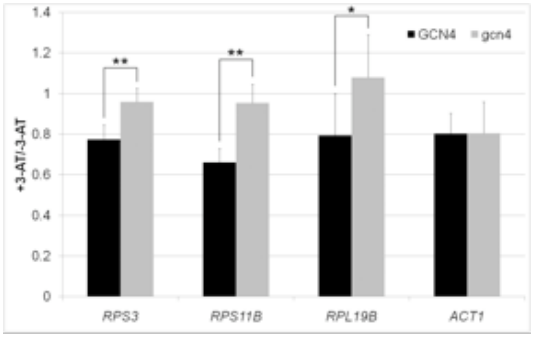
- Manuscript was re-written. This was mentioned and discussed in the result section of our revised manuscript.

For the ChIP data in Fig. 7A, I find it rather difficult to accept that the difference in occupancy of acetylated H4 at RPS3 for the GCN4 strain in the presence and absence of 3-AT is significant with a P-value of less than 0.005 considering that the error bars nearly overlap and the results derive from only three replicate measurements. Similarly, a P-value of <0.05 for the corresponding data for RPL19B is surprising considering that these two error bars overlap completely.

- The P-values were calculated based on a formula as followed:

mRNA of +3-AT sample/ mRNA of -3-AT sample

It was the same calculation used in Figure 5B~F. These ratios were summarized in a graph below.



We thought that ratios, not absolute values, were more conserved through all of independent experiments. This may explain the discrepancy between two graphs. However, for the consistency of data presentation between Figure 5D and Figure 7A, we decided to use the original version of graph in the revised manuscript.

In summary, the authors have provided considerable evidence in favor of their model, which is quite appealing intellectually on a number of levels. However, the experimental design or quality of data for some of the experiments do not meet the standards of this journal. In addition, a key experiment needs to be conducted to provide direct evidence that complex formation in vivo between Gcn4 and Rap1 is abolished by the deltaC gcn4 mutation.

- Co-immunoprecipitation experiment revealed that complex formation between Gcn4 and Rap1 is abolished by C-terminal deletion (Gcn4ΔC) in vivo.

Referee #2 (Remarks to the Author):

The authors have made a valiant attempt at countering the criticisms of all the reviewers. In my opinion, their experimental approach has been improved to a level that one might consider the main conclusion of their work as well supported. The point remaining as the most speculative one is perhaps the notion that the decrease of Esa1 recruitment by Rap1-Gcn4 is the primary cause for the down regulation of RP genes. Nevertheless, taking everything into consideration I could support publication of the work now on experimental merit. I have however some serious misgivings about the style of the manuscript. There are many typos or wrong use of words depending on one's interpretation such as derepressed, depressed and suppressed (too many to list in detail). Past and present tenses are not always used in a logical way. Some of the sentences are atrocious, especially in the later result chapters and the discussion section. Some of their speculation, for example on the physiological relevance of the down regulation of RP gene transcription, namely to provide more general transcription factors for stress related genes is naïve, not convincing or could easily be tested. I therefore suggest to reword and to rewrite some of the more notorious parts of the manuscript, perhaps with some outside help.

- We thoroughly checked and revised our manuscript with a professional proofreader whose mother language is English.

3rd Editorial Decision 08 November 2010

Thank you for sending us your (re)revised manuscript. Our original referee 1 has now seen it again. In general, he/she is now positive about publication of your paper. Still, he/she feels that there are a few minor issues that need to be addressed (see below) before we can ultimately accept your manuscript. I would therefore like to ask you to deal with the issues raised. Please let us have a suitably amended manuscript as soon as possible.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors have adequately satisfied all of my requests for additional results. However, one issue remains regarding the interpretation of the result in Fig. 6E indicating that Esa1-Rap1 interaction is reduced in nonstarvation conditions in the gcn4 mutant versus wild-type GCN4. It is surprising that GCN4 would promote the interaction between Esa1 and Rap1 under nonstarvation conditions but antagonize the same interaction under starvation conditions. In addition, the result in nonstarvation conditions seems incompatible with the fact that RP mRNA levels (Fig. 1) and Esa1 occupancies at RP genes (Fig. 5D) are not reduced by the absence of GCN4 under nonstarvation conditions (Fig. 1). Hence, it seems important to indicate that this puzzling result of Fig. 6E was reproduced in replicate experiments. In addition, the results seem to demand inclusion of a statement indicating that a tight interaction between Esa1 and Rap1, which enables co-IP of the two proteins, is not required under nonstarvation conditions in cells lacking GCN4 for efficient recruitment of Esa1 to RP promoters.

2nd Revision - authors' response 13 November 2010

The authors have adequately satisfied all of my requests for additional results. However, one issue remains regarding the interpretation of the result in Fig. 6E indicating that Esa1-Rap1 interaction is reduced in nonstarvation conditions in the gcn4 mutant versus wild-type GCN4. It is surprising that GCN4 would promote the interaction between Esa1 and Rap1 under nonstarvation conditions but antagonize the same interaction under starvation conditions. In addition, the result in nonstarvation conditions seems incompatible with the fact that RP mRNA levels (Fig. 1) and Esa1 occupancies at RP genes (Fig. 5D) are not reduced by the absence of GCN4 under nonstarvation conditions (Fig. 1). Hence, it seems important to indicate that this puzzling result of Fig. 6E was reproduced in replicate experiments. In addition, the results seem to demand inclusion of a statement indicating that a tight interaction between Esa1 and Rap1, which enables co-IP of the two proteins, is not required under nonstarvation conditions in cells lacking GCN4 for efficient recruitment of Esa1 to RP promoters.

- We already had performed the Co-IP as in Fig. 6E with three independent experiments. The results of triplicate experiments of the Co-IP were summarized in supplementary Figure S9 in the revised manuscript. Moreover, a statement indicating correct interpretation of these results was inserted in result section of the revised manuscript as you suggested.

