molecular systems biology

Overexpression limits of fission yeast cell-cycle regulators *in vivo* and *in silico*

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 16 March 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. They raise, however, substantial concerns on your work, which, I am afraid to say, preclude its publication in its present form.

The referees recognized that the genetic tug-of-war (gTOW) experiments were well-conducted, and provided some interesting new details regarding robustness within the fission yeast cell-cycle network, as well as a potentially useful comparison to your previous budding yeast results. Nonetheless, the reviewers had significant concerns that the experiments regarding cyclin/cdc2 competition remain somewhat inconclusive, and that the advance provided by the new mathematical model was currently unclear. Given these concerns, two reviewers were not convinced that this work currently provides a sufficient level of broad and conclusive biological insight. Addressing these concerns will likely require additional analysis and experimentation:

- The reviewers indicated that the observed reduction in cyclin copy number limits, upon cdc2 overexpression, could result from increased deleterious effects of the overexpressed cyclin-cdc2 complex, and in this case the evidence could be interpreted as supporting the existence of competition. Additional experiments seem required to rigorously distinguish between these alternative explanations.

- The reviewers were not entirely convinced of the practical value of the new fission yeast cell-cycle mathematical model, and they indicate that it would be important to validate this new model with an independent data set that was not used in the model building/optimizing process, or, ideally, to specifically test novel biological predictions that arise from this new model.

On a more editorial note, Molecular Systems Biology strongly encourages authors, whenever possible, to provide models in the SBML format, and to deposit them in a public repository such as BioModels or JWS Online. Also, I note that some network diagrams are represented in SBGN (e.g. Fig. 6A and 7C), while others are not. You may wish to consider using a uniform SBGN representation across all diagrams, to help improve the clarity and consistency of the Figures.

*** PLEASE NOTE *** As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://www.nature.com/msb/journal/v6/n1/full/msb201072.html), Molecular Systems Biology will publish online a Review Process File to accompany accepted manuscripts. When preparing your letter of response, please be aware that in the event of acceptance, your cover letter/point-by-point document will be included as part of this File, which will be available to the scientific community. More information about this initiative is available in our Instructions to Authors. If you have any questions about this initiative, please contact the editorial office msb@embo.org.

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

Yours sincerely,

Editor
Molecular Systems Biology

Referee reports:

Reviewer #1 (Remarks to the Author):

The gTOW method balances the need of the cell for up to 50 copies of a given plasmid with the potentially deleterious effects of having as many copies of a particular cell cycle gene. This is an exciting new method and has produced some of the best experimental work to date on robustness. This paper follows the seminal work on the budding yeast cell cycle using the same method and the comparison of the two works yields some very interesting results, e.g., that there is a fragile core of the cell cycle that regulates mitotic entry. Also, that fission yeast is sensitive to imbalanced expression of spg1 and byr4 is also quite interesting. Taken together, this is an insightful new paper that well deserves publication in MSB. I have only a few minor quibbles.

1. Fig2 (and parts of other figures) aren't that professionally put together. Labels & legends are missing, fonts are minuscule etc... It looks like a matlab screeendump rather than a figure in a high impact journal.
2. Figure2 row1 col2 Why is the distribution for the L- and M- bimodal? If this indicates loss of plasmid, how can the cells grow? Or are these dead/dying cells that have lost their plasmids?
3. Regarding figure 5: It is interesting to note that the 'fragile core' isn't all that fragile as these cells are getting several fold over-expression. I agree that they are more fragile relative to the 50x over-expression though. I'll leave it to the authors discretion as to whether they want to rephrase this language.
4.Figure6c: This is really interesting and deserves quantification. If possible, it would be interesting to monitor these cells using time-lapse microscopy and score their color as a function of time and correlate with proliferation rate.
5. The conclusion of the 'cyclins are not in competition to form complexes with Cdc2' section needs to be revisited. It don't think the conclusion is that there is no competition between cyclins for the Cdk can be drawn from this data. A more likely reason that increased Cdc2 levels leads to lower limits of cyclin over-expression is that there is indeed competition for the Cdk. This competition is alleviated by increased Cdc2 leading to higher CDK activity leading to cell cycle arrest (one mechanism would be failure to exit mitosis). The recent paper by Coudreuse and Nurse (among others) demonstrates significant functional overlap between cyclins which would also argue against the authors interpretation that their data implies that there is no competition.

Reviewer #2 (Remarks to the Author):

The G-TOW strategy was previously applied to budding yeast by these authors to measure copy number limits for many cell cycle regulators. The overall conclusion was that copy number limits are extremely high for most genes, with a small subset identified as a 'fragile' core that had to be maintained at closer levels. This paper applies this methodology to fission yeast.

The G-TOW method is ingenious, and it clearly required some clever experimentation to work out the method in fission yeast, again looking at cell cycle control. The G-TOW experiments are done in a very carefully controlled and quantitative way that provide good assurance that the results are reliable. As in budding yeast, many genes can be present at extremely high copy numbers without evident defects; an interesting subset shows restricted permissible copy number. This information provides a novel angle on the system, indicating in a broad sense where in the network the sensitive points are located. Interesting similarities and differences from the budding yeast results are pointed out.

In this fission yeast system, methods have been worked out using a GFP marker for copy number estimation, in which the fate of high-copy cells can be followed. This is useful although the phenotypic information is limited so far to a rough analysis of cell morphology.

The copy number limits are compared with a previously developed ODE model for fission yeast cell cycle control. Reasonable agreement is noted for the limited number of components in the model (many fewer than the number of genes tested by G-TOW). Unfortunately, some of the most dosage-sensitive components from G-TOW are not yet in the model. If dosage sensitivity identifies important control points for the network, this is somewhat unsettling. But at the least, these findings imply that the computational model is reasonably well titrated in terms of balance of components.

It is stated that the basic ODE model was improved in some way to give better fitting to G-TOW results. The way this was done is not described; one supposes by-hand parameter adjustments, since no algorithmic approach is specified. I don't see that this contributes much, since we lack an independent set of data to test whether the apparent improvements are genuine, or are due to overfitting, and it is not stated whether these changes yield some additional insight into the system.

A 2-D G-TOW was carried out to test the idea that cdc2 might be limiting, at least under G-TOW conditions. It should be noted (although it is not discussed in the text) that cdc2 and cyclin abundances have been measured with some accuracy in several systems, including Xenopus and budding yeast, and it is known that cyclins are not in competition. With a gene copy increase up to 100-fold, though, this is clearly no longer a safe assumption. The proposal is tested that some cyclin (such as cig2) might have a relatively low tolerable copy number because it titrates cdc2, eliminating the ability of the essential cdc13 cyclin to find a cdc2 partner. To do this, cig2 copy number limits were determined in the presence of high cdc2 levels. The result is that high cdc2 lowers cig2 copy number limits; it is then concluded that cdc2 is not limiting. This is a possible conclusion but not the only one. Perhaps high cig2 is lethal both because of high cig2-cdc2 activity and because of depletion of cdc13-cdc2 complexes; increasing cdc2 in the presence of high cig2 will then enhance the first even while remedying the second. It is critical to know the phenotypes to distinguish these possibilities. A computational conclusion is reached that cdc2 is in considerable excess and binds tightly to B-type cyclins; there is already evidence that both of these things are true in other systems, and this should be referenced and discussed.

So I think the paper develops a useful methodology and employs it to get a rough-cut idea of overall fragility/robustness to copy number in fission yeast cell cycle control. These are substantive positives. The paper provides rather limited biological insight beyond this.

Reviewer #3 (Remarks to the Author):

This paper explores the role of genes in the control of cell cycle progression in S. pombe. They used a genetic "tug-of-war" (gTOW) method which they have created (but used before in a previous publication) to assess the over-expression limit of certain genes. The presented data suggested the importance and conservation of certain of these genes.

Though I believe the experiments have been done quite well and are presented quite well, and I am comfortable that there are not any glaring flaws, I have a more philosophic problem with this paper: I do not see where the novelty lies, or what it tell use new about cell cycle controls in S. pombe. All of the genes have been published before and in all cases their functions characterized. No striking new functions for any are proposed in this work. Instead the experiments simply describe how tolerant cell are to (artificial) copy numbers of the genes. In some cases cells are more sensitive, in other cases less sensitive. And in some cases the "fragile core" genes are conserved in the other yeast model organism S. cerevisiae. Almost all of this information is not new.

Finally the authors take their observations and integrate them into a new model of fission yeast cell cycle regulation. They claim the "presented "gTOW" model is the most detailed model of fission yeast cell cycle regulation so far". But to me what is crucial about any new model is the predictions that can be experimentally tested from it. This is not done from the presented model in any meaningful way. Some predictions are tested, but they are not that surprising, and others are not included in this paper.

1st Revision - authors' response 30 September 2011

Response to the editor's comment:

(1) *The reviewers indicated that the observed reduction in cyclin copy number limits, upon cdc2 overexpression, could result from increased deleterious effects of the overexpressed cyclin-cdc2 complex, and in this case the evidence could be interpreted as supporting the existence of competition. Additional experiments seem required to rigorously distinguish between these alternative explanations.*

We have replied to this comment with the General reply 1.

(2) *The reviewers were not entirely convinced of the practical value of the new fission yeast cell-cycle mathematical model, and they indicate that it would be important to validate this new model with an independent data set that was not used in the model building/optimizing process, or, ideally, to specifically test novel biological predictions that arise from this new model.*

We have replied to this comment with the General reply 2.

(3) *On a more editorial note, Molecular Systems Biology strongly encourages authors, whenever possible, to provide models in the SBML format, and to deposit them in a public repository such as BioModels or JWS Online. Also, I note that some network diagrams are represented in SBGN (e.g. Fig. 6A and 7C), while others are not. You may wish to consider using a uniform SBGN representation across all diagrams, to help improve the clarity and consistency of the Figures.*

We re-constituted the gTOW model using CellDesigner4.1, software compatible with SBML and SBGN. And we put the model diagram in Figure 7A. The CellDesigner4.1 file (a xml file), and the SBML file (a sbml file) are attached to the Supplementary Information. We will deposit our model to BioMdels.net by following the acceptance of our manuscript. In some cases, description of diagrams in SBGN seems not suitable to present what we want to show, and we gave up to use SGBN for those diagrams.

Response to the reviewer's comment:

We sincerely appreciate the reviewer's fair and suggestive comments. We added various descriptions in order to respond the comments of the reviewers. We also did some experiments and added the new results into our manuscript. We think these processes made our manuscript much improved.

General reply

At first, we will describe about major changes in our manuscript below, as a general reply to the editor and reviewers.

(G-1) About "Cyclins are not in competition to form complexes with Cdc2" section (in response to the reviewer's comment 1-5 and 2-3);

We thought that our aim of this part seemed not correctly introduced because our description was not clear. Probably the section title and the conclusion were inaccurate. We thus changed the description (Page 9-) of this part and the figure (Figure 8A), to clearly show the aim and the interpretation of the results in Figure 8B. In addition to this change, we added another experimental evidence supporting our conclusion (Figure S7). Detail of the changes and explanations are described below.

The aim of this part of the study is (as reviewer #2 clearly summarized) to know either of two potential mechanisms causes the cell cycle dysfunction upon high copy of each cyclin. The mechanisms are, in the case of *cig2* for example, "increase of Cdc2/Cig2 activity itself causes the dysfunction (Case1)", and "depletion of Cdc2/Cdc13 activity due to the competition of cyclins for Cdc2 causes the dysfunction (Case2)" (Figure 8A-2).

Increasing *cdc2* in the presence of high *cdc2* enhance the first even while remedying the second. If the Case1 mechanism determines the upper limit *cig2* (in a native *cdc2* level), above condition will lead to the decrease of the limit of *cig2*, due to the principle of gTOW. And if the Case2 mechanism determines the limit, above condition will lead to the increase of the limit of *cig2*. Our experiment showed that in high *cdc2*, the limits of cyclins (*cig1*, *cig2*, and *puc1*) were all decreased. We thus concluded that the Case1 was true, namely, that upper limits of cyclin genes are determined because of the high

Cdc2/cyclin (Cig1, Cig2, and Puc1) activity itself, but not the depletion of Cdc2/Cdc13.

In addition to the result, we added another experimental evidence that support this conclusion. We observed the phenotypes (morphologies) of the cells in gTOW-*cig1*+, -*cig2*+, and -*puc1*+ (Figure S7). If the Case2 above is true, increased these cyclin gens cause the depletion of Cdc2/Cdc13, which will lead to "*cdc*" phenotypes (elongated cellular morphology). However, non of cells showed elongated cellular morphology in the gTOW-cyclin experiment (Figure S14). This is another supportive evidence of our conclusion.

(G-2) About the practical value of the new fission yeast cell-cycle mathematical model (in response to the reviewer's comment 2-2 and 3-2);

To show the practical value of the model, we have tested the model using a data set (upper limits of the cell cycle regulators in 4 deletion mutant strains), which is not used in the development of the model (Figure 9A). This analysis indicated that there were additional (unknown) mechanisms conferring the robustness of the fission yeast cell cycle, and suggested that this model is a practically useful reference to find unknown regulations. We added this result in Figure 9, and the description to the Result section "Comparison of 2D-gTOW data *in silico* and *in vivo* revealed uncovered regulations in the cell cycle. " (page 11), and to Discussion (page 13, 3rd paragraph). Our comments on this issue and detail of the explanations are described below.

The mathematical model of the fission yeast cell cycle developed in this study can reproduce 42 mutant phenotypes experimentally tested. Remaining this constraint, we successfully reproduced the limits of overexpression of 14 genes measured in this study using gTOW. We think this fact itself is a significant outcome of this study.

However, we think that this model is not complete at al, although this model is the most detailed model that integrates the knowledge obtained in the fission yeast cell cycle research so far. We thus think that we need to further refine the model by evaluating the predictions, so that the model can reproduce every property of the cellular activity, which model is one of the "ultimate goal" of the systems biology.

On the other hand, as reviewers #2 and #3 pointed, we agree that it is very important to show that what are the predictions of the current model, and how we can test the prediction. This will show the degree of completion of the model, and how we can

extract novel knowledge from the predictions, which is the practical value of the model.

We thus evaluated the model prediction (limits of cell cycle regulators in 4 deletion mutant strains) with gTOW experiment (Figure 9A). We found some discrepancies between the model predictions and the experimental results, indicating that the model is not perfect, as we expected. However, this comparison provided us a hint to find additional (unknown) regulatory mechanisms that was not implemented into the model.

We thus could show the evidence that the model was a practically useful reference to find unknown regulation, when combined with the gTOW experiment. Of course, we need to further refine the model suggested in the comparison between prediction and experiment. And we need to further evaluate the model predictions with the gTOW experiment in the deletion mutants. However, this will require additional large experimental efforts, and we think, will be beyond the scope of this study.

Followings are the reply for each reviewer.

About the reviewer#1's comment:

(1-1) *Fig2 (and parts of other figures) aren't that professionally put together. Labels & legends are missing, fonts are minuscule etc... It looks like a matlab screeendump rather than a figure in a high impact journal.*

We put an enlarged figure in Figure S5. Actually, this data was a part of database that contains experimental data shown in Figure 2 for all genes. However, the database is too large to attach to Supplementary Information. We thus decided to provide the database upon request, and added the description in the legend of Figure 2.

(1-2) *Figure 2 row1 col2 Why is the distribution for the L- and M- bimodal? If this indicates loss of plasmid, how can the cells grow? Or are these dead/dying cells that have lost their plasmids?*

pTOWsp plasmid containing *ars3002x2* origin seems to be unstable (shown in Figure 1C). The bimodality is thus probably due to the plasmid loss. We added the description in the legend of Figure 2.

(1-3) *Regarding figure 5: It is interesting to note that the 'fragile core' isn't all that fragile as these cells are getting several fold over-expression. I agree that they are more fragile relative to the 50x over-expression though. I'll leave it to the authors discretion as to whether they want to rephrase this language.*

We consider that robustness and fragility are **relative** characteristics. The genes involved in the "fragile core" have **relatively** low limits compared to the whole cell cycle genes. We thus want to keep this description.

(1-4) *Figure6c: This is really interesting and deserves quantification. If possible, it would be interesting to monitor these cells using time-lapse microscopy and score their color as a function of time and correlate with proliferation rate.*

As described in Discussion, we agree that we should do time-lapse microscopic observations of gTOW. However, it requires some experimental set-up to perform the experiments, and we think it will be a future experiment.

(1-5) *The conclusion of the 'cyclins are not in competition to form complexes with Cdc2' section needs to be revisited. It don't think the conclusion is that there is no competition between cyclins for the Cdk can be drawn from this data. A more likely reason that increased Cdc2 levels leads to lower limits of cyclin over-expression is that there is indeed competition for the Cdk. This competition is alleviated by increased Cdc2 leading to higher CDK activity leading to cell cycle arrest (one mechanism would be failure to exit mitosis). The recent paper by Coudreuse and Nurse (among others) demonstrates significant functional overlap between cyclins which would also argue against the authors interpretation that their data implies that there is no competition.*

We have replied to this comment with General reply 1 above.

About the reviewer#2's comment:

(2-1) *It is stated that the basic ODE model was improved in some way to give better fitting to G-TOW results. The way this was done is not described; one supposes by-hand parameter adjustments, since no algorithmic approach is specified.*

We did hand parameters adjustments. We added the description in the result section (page 9), and the modeling part of the Supplementary Information (page 28).

(2-2) *I don't see that this contributes much, since we lack an independent set of data to test whether the apparent improvements are genuine, or are due to overfitting, and it is not stated whether these changes yield some additional insight into the system.*

We have replied to this comment with General reply 2 above.

(2-3) *A 2-D G-TOW was carried out to test the idea that cdc2 might be limiting, at least under G-TOW conditions. It should be noted (although it is not discussed in the text) that cdc2 and cyclin abundances have been measured with some accuracy in several systems, including Xenopus and budding yeast, and it is known that cyclins are not in competition. With a gene copy increase up to 100-fold, though, this is clearly no longer a safe assumption. The proposal is tested that some cyclin (such as cig2) might have a relatively low tolerable copy number because it titrates cdc2, eliminating the ability of the essential cdc13 cyclin to find a cdc2 partner. To do this, cig2 copy number limits were determined in the presence of high cdc2 levels. The result is that high cdc2 lowers cig2 copy number limits; it is then concluded that cdc2 is not limiting. This is a possible conclusion but not the only one. Perhaps high cig2 is lethal both because of high cig2-cdc2 activity and because of depletion of cdc13-cdc2 complexes; increasing cdc2 in the presence of high cig2 will then enhance the first even while remedying the second.*

It is critical to know the phenotypes to distinguish these possibilities. A computational conclusion is reached that cdc2 is in considerable excess and binds tightly to B-type cyclins; there is already evidence that both of these things are true in other systems, and this should be referenced and discussed.

We have replied to this comment with General reply 1 above. We referred a reference stating the abundance of CDK and cyclins in the budding yeast (Cross et al., 2002)(Page 10, 2nd sentence).

About the reviewer #3's comment:

(3-1) *I have a more philosophic problem with this paper: I do not see where the novelty lies, or what it tell use new about cell cycle controls in S. pombe. All of the genes have been published before and in all cases their functions characterized. No striking new functions for any are proposed in this work. Instead the experiments simply describe how tolerant cell are to (artificial) copy numbers of the genes. In some cases cells are more sensitive, in other cases less sensitive. And in some cases the "fragile core" genes are conserved in the other yeast model organism S. cerevisiae. Almost all of this information is not new.*

Biological robustness is one of the central issues of the systems biology. By gTOW, we can obtain unique quantitative data set reflecting the robustness against gene overexpression of cellular systems. Although gTOW was already developed in the budding yeast, we have developed this method in the fission yeast in this study. We think it is a significant breakthrough for the understanding of the design principle of eukaryotic system from the perspective of robustness, because both yeasts are highly distantly related.

(3-2) *Finally the authors take their observations and integrate them into a new model of fission yeast cell cycle regulation. They claim the "presented "gTOW" model is the most detailed model of fission yeast cell cycle regulation so far". But to me what is crucial about any new model is the predictions that can be experimentally tested from it. This is not done from the presented model in any meaningful way. Some predictions are tested, but they are not that surprising, and others are not included in this paper.*

We have replied to this comment with General reply 2 above.

2nd Editorial Decision 02 November 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the two referees who agreed to evaluate this revised study. As you will see below, the referees felt that the revisions made to this work had addressed their concerns and they are now largely supportive of publication. The second reviewer has some suggestions for additional discussion, and we have some remaining content and format issues, that we ask to address in a final minor revision of this work.

1. Please submit your SMBL model to BioModels, and incorporate the accession number into the Methods section of the main manuscript.

2. The resolution of the existing figures is a bit low; the text and line-art is noticeably blurry when zooming in. Please provide higher resolution figure files (EPS, TIFF, or PDF), and feel free to contact us if you would like additional advice on figure file preparation. In general, you will get the best results if the figures are created in a high-quality vector graphics program like Illustrator or the free, opensource alternative Inkscape, and then saved directly in EPS or PDF formats.

Please resubmit your revised manuscript online, with a covering letter listing amendments and responses to each point raised by the referees. Please resubmit the paper **within one month** and ideally as soon as possible. If we do not receive the revised manuscript within this time period, the file might be closed and any subsequent resubmission would be treated as a new manuscript. Please use the Manuscript Number (above) in all correspondence.

Thank you for submitting this paper to Molecular Systems Biology.

Yours sincerely,

Editor - Molecular Systems Biology

Referee reports

Reviewer #1 (Remarks to the Author):

The authors have sufficiently addressed my concerns.

Reviewer #2 (Remarks to the Author):

I thought the paper was quite interesting and well executed even in the first submission, especially the empirical work. In revision, the work has been extended with additional useful experiments. An issue with the previous version was the ad hoc nature of the model, and the lack of an attempt to determine if the model is predictive, overfitted, etc. In revision, it is attempted to validate the model with additional data not included in the dataset used for model development. This is most certainly the right thing to do; the finding that the model fails to capture some significant proportion of the new results is, in fact, just what one would expect, not a bad thing for the modeling or for the experiments. One thing that I missed here: the modelers clearly have a lot of insight into what makes their model tick, and they presumably have a lot of experience fitting new data into an old model. Not to ask them to do that again here with the new data, because it's really not the point, but don't the authors have some insight into why their model might fail to some extent, with the new data?

2nd Revision - authors' response 07 November 2011

Format and Content issues:

1. *Please submit your SMBL model to BioModels, and incorporate the accession number into the Methods section of the main manuscript.*

We submitted our SBML model to BioModels, and acquired ID MODEL1111040000. We incorporated the ID into the Methods section.

2. Please provide a 'standfirst text' summarizing the study in one or two sentences (approx. 250 characters).

We created a method to measure robustness of the fission cellular system, and revealed conserved fragility in the cell cycle among eukaryotes. Using the data, we developed a mathematical model, which reproduces the robustness of the cell cycle.

3. The resolution of the existing figures is a bit low; the text and line-art is noticeably blurry when zooming in…

We have improved the resolution of the figures, according to the editor's suggestion.

Below is the response to the reviewer#2's comment.

One thing that I missed here: the modelers clearly have a lot of insight into what makes their model tick, and they presumably have a lot of experience fitting new data into an old model. Not to ask them to do that again here with the new data, because it's really not the point, but don't the authors have some insight into why their model might fail to some extent, with the new data?

We actually argued about what is missing in the model, and how we can improve the model. We actually found some ideas to fit the model with the new data (some of them are described in the Discussion section of the current version of our ms.), but we decided to wait to refine the model until we get whole 2D-gTOW data and additional physiological data, which will be obtained in future experiments.