molecular systems biology

Modelling reveals novel roles of two parallel signalling pathways and homeostatic feedbacks in yeast

Joerg Schaber, Rodrigo Baltanas, Alan Bush, Edda Klipp, Alejando Colman-Lerner

Corresponding author: Joerg Schaber, Otto von Guericke University

Review timeline:

Submission date:	26 March 2012
Editorial Decision:	09 May 2012
Revision received:	02 August 2012
Editorial Decision:	03 September 2012
Revision received:	12 September 2012
Accepted:	18 September 2012

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 09 May 2012

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 substantial concerns on your work, which, I am afraid to say, preclude its publication in its present form.

The points raised by the third reviewer appear to be the most fundamental. This reviewer felt that substantial additional work was needed both to support the robustness of your main conclusions to different initial modeling decisions (e.g. points #2 and #6), and to provide more convincing support for some of these conclusions. Addressing some of these issues may require additional experimental work. The reviewers also raised a series of more technical, but important, concerns regarding, for example, parameter identifiability and the justification for the use of the reduced model in some analyses.

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.

Please note, that in addition to our capacity to host datasets in our supplementary information section, we provide a new functionality that allows readers to directly download the 'source data' associated with selected figure panels (e.g. <http://tinyurl.com/365zpej>). This sort of figure-associated data may be particularly appropriate for this work. Please see our Instructions of Authors

for more details on preparation and formatting of figure source data (<http://www.nature.com/msb/authors/index.html#a3.4.3>).

Please note As part of EMBO Publications' Transparent Editorial Process, Molecular Systems Biology now publishes a Review Process File with each accepted manuscript. In the event of acceptance, your cover letter/point-by-point response will be included in this file, which will be available to the scientific community. Authors may opt out of the transparent process at any stage prior to publication (contact us at msb@embo.org). More information is available in our Instructions to Authors (http://tinyurl.com/79zy6ap).

Sincerely,

Editor - Molecular Systems Biology
msb@embo.org

Referee reports:

Reviewer #1 (Remarks to the Author):

The HOG pathway in yeast is one of the best-studied signaling pathways and serves as a prototype for MAP kinase signaling systems. This manuscript uses computational approaches to investigate different HOG regulatory and feedback mechanisms and their interaction in time. The authors also consider why yeast has maintained two redundant signaling pathways that converge on the MAPK Hog1. They systematically test different hypotheses against published data. By this approach they identify a parsimonious model that is best supported by the data. Among these, the models suggest that fast transient negative feedback loops serve to prevent oscillatory behavior, which may occur in systems with delayed negative feedback. The models suggest also that two branches render the system more robust to internal variations in cellular states and to molecular noise. They confirme experimentally that cells indeed recover significantly faster than the single branch mutants for low and intermediate osmotic shocks, and that the single branch mutants exhibit a higher variability than the wild type. The work appears to be well done and will be of broad interest to the readership of MSB.

Major point:

Much of the data used for modeling are obtained from Macia (2009) and Klipp (2005), but also others such as Hao (2007). There is an inherent danger to comparing data obtained by different investigators using different strains and even slightly different experimental conditions, which should be noted explicitly in the discussion. THis is not currently done, for example: "Our ensemble modelling approach also revealed a possible role of the suggested transient feedbacks on the signalling branches (Hao, et al., 2007; Macia, et al., 2009), besides the fact that their physical nature is still elusive, especially in the Sln1 branch." It is appropriate to cite both papers but they should not be lumped together in this manner since the 2007 Hao paper uses an ssk1- mutant derived from S288C and KCl as the stimulus while the 2009 Macia paper uses SSK1+ strain derived from W303a and NaCl as the stimulus. The authors should also cite Yamamoto K, Tatebayashi K, Tanaka K, Saito H. Mol Cell. 2010 Oct 8;40(1):87-98.).

Minor points:

p. 27, l. 35: "Data in F was derived by assuming that volume mirrors Hog1 profile and minimal volumes from (Schaber, et al., 2010)." Is this a valid assumption?

p. 27 l. 36: "Not all data points used for fitting are shown." Why not?

Reviewer #2 (Remarks to the Author):

The authors of the paper "Modelling reveals novel roles of two parallel signalling pathways and

homeostatic feedbacks in yeast" present a kinetic model detailing important mechanisms underlying osmo-adaptation in yeast. The model's focus is on the High Osmolarity Glycerol (HOG) pathway in S. cerevisiae, a prototypic signaling system in eukaryotes.

Glycerol accumulation plays a key role in short-term adaptation to osmotic shock in this system. This process is mediated by a primary integral feedback mechanism augmented by rapid transient negative feedbacks. The activation state of the protein Hog1 is central to the emergent behavior of these pathways.

The authors construct an ensemble of putative models, differing mechanistically and structurally, that attempt to capture system response to osmotic shock. Each model is scored based on quality of fit to training data and predictive capability against validation data, and a single best-fit model was chosen. A parsimonious modeling strategy was wisely chosen. This ensemble approach importantly curbs inherent bias of selecting a single model in the face of biological network uncertainty and represents an attractive technique for developing models of this size.

The model is convincingly and exhaustively validated and corrects previously misconstrued experimental explanations of the control systems underlying yeast osmo-adaptation. Of particular interest was analysis of the different mechanisms cooperating at different time scales in order to impart speed and robustness to the osmotic shock response. A fast post-translational modification signal is the initial driver of glycerol accumulation then a slow transcription-level mechanism takes over to restore homeostasis. Importantly, the model elucidates the role transient feedback mechanisms play in imparting system stability as well as the reason yeast has evolved two redundant pathways to perform similar osmo-adaptive functions.

I have the following minor concerns with the current state of this article:

1. In the stability analysis of the fast transient feedback mechanisms, a simplified generic model was constructed. The complete model was not used because it was apparently 'not possible with available standard software.' It's unclear to me why this is impossible. What specifically prevented the use of the complete model? I would rather have seen this analysis performed with the complete model.

2. In the discussion section, if possible, could you list specific examples of other eukaryotic homeostatic adaptive systems, specifically ones containing redundant signaling pathways like that of the osmo-adaptation response.

3. In the methods section concerning parameter identifiability, it is emphasized that models which contain a number of parameters exceeding the number of experimental data points renders many of the model parmeters unidentifiable. The language used here I think does not emphasize correctly the relationship between model parameter inferability and experimental data. Number of model parameters and shear number of experimental data points are only loosely related. What matters most is experimental design, so what you are measuring and at what times. More measurements of a particular species is not guaranteed to significantly improve identifiability.

4. Related to issue 3, given the one-dimensional likelihood profiles you've calculated would it be possible to suggest a set of measurements one could make in order to render the 7 unidentified parameters identifiable.

This study displays a creative and rigorous approach to dynamic network analysis of an important eukaryotic adaptative response system. I believe it to be a significant contribution toward advancing our understanding of the complex regulation underlying a eukaryotic cell subsystem.

Reviewer #3 (Remarks to the Author):

Review of Schaber et al for MSB. In this article the authors develop a set of ~200 models for the HOG pathway in budding yeast. The models are fit to a significant amount of data. The model most capable of fitting the data (based on AIC score) were identified and further analyzed. Additional models that scored well were also analyzed, albeit less completely. Upon analysis of the model, the authors conclude that non-transcriptional regulation of glycerol synthesis by Hog1 is the main mechanism behind adaptation to an osmotic stress, while transcriptional regulation increases

glycerol production on longer time scales. Finally, the authors' model supports the idea that fast negative feedbacks from Hog1 to upstream components of the signaling pathway stabilize the response to higher frequency oscillations. Some measurements on volume adaptation are also presented. As noted below, I have concerns regarding how solid these conclusions are. Additionally, the authors have already to published their model selection method and its 'proof-of-principle' application to the HOG pathway (Schaber et al. 2011; Analyzing Ensemble Modeling with modelMaGe: Analyzing Feedback Mechanisms in the Sho1 branch of the HOG pathway). This manuscript does reach some different conclusions, but the degree of novelty is certainly reduced.

Main concerns:

1. One of the main conclusions of the paper is that yeast osmoadaptation occurs mainly through Hog1-dependent post-translational glycerol production. This is described as being "in contrast to the commonly accepted opinion" that the main adaptation mechnanism is through transcriptional feedback regulation. However, over the last few years, several authors have concluded that the transcriptional activation of glycerol production by Hog1 is not responsible for immediate osmoadaptation (see, for instance, Mettetal 2008), and that transcriptional mechanisms slowly prepare the cell for further shocks (as the authors' model also demonstrates in Figure 5). Therefore, the authors' main conclusion is mostly against a 'straw man.' The authors should make it clear in the discussion that their model is supporting previously described results.

2. Moreover, the conclusion that post-transcriptional regulation in the primary mode of regulation may even depend on the particular form of glycerol production that the authors chose to use, without much explanation or support. Although all other reactions in table S4 are in the form of a mass reaction, the choice of the form of glycerol production, v13, is not obvious. It is possible that the change in its form will change the behavior of the model 22 as well as general rankings of different models. This form should be supported with more explanation/clarification, and the model must be shown to be robust to changes in the form of this term.

3. Another conclusion of the paper is that the presence of two branches in the wild-type results in less variability than the single branch mutants. Even though this result is corroborated by robustness to parameter and initial condition changes that the model displays, the authors' own experimental results are not significant (3 positive results out of 4 is not significant, especially because the less variable Sho1 branch result was obtained in the more reliable 0.2 M NaCl experiment).

4. I am also concerned about the claim that signaling output is stabilized by Hog1-feedback on signaling branches. For example, in Figure 7, the presence of 2 feedbacks only modestly decreases the fraction of models that oscillate (~71% to 66% for Gdp1+ models, ~38% to 25% in Gdp1+Hog1 models). Additionally, I have concerns regarding the analysis behind Figure 8 and Figure 9. In Figure 8, they do not show whether their reduced model in Figure 8 fits the data well, so it is not obvious whether this model is a good representation of the full network dynamics. As for Figure 9, the explanation is essentially incorrect. The real part of the eigenvalue determines the stability, while the imaginary part determines oscillations, so the authors' analysis of a real eigenvalue as having anything to do with oscillations is erroneous.

5. It is important to note which parameters are identifiable in a modeling paper. The number of unidentifiable parameters is misstated as 7 (there seem to be 8 in Figure S1: kHog1phos1, kHog1phos2, Ki1, k1, k2, k3, k9, and k15). Additionally the names of the parameters in Figure S1 do not correspond to the names given in Tables S7 and S11 making it impossible to understand this figure or ascertain which parameters were actually identifiable.

6. In the discussion, the authors mention that their model is more consistent with imperfect adaptation of Hog1 because the model was able to fit the Macia et al. (2009) data well. However, given that the model was selected based upon fitting to some of the Macia data and ranking based on how well it predicted other parts of the Macia data, it is unsurprising that it supports the conclusions regarding imperfect adaptation that were previously drawn from the Macia data. It would be helpful to see whether another model emerges as the best if data suggesting the opposite conclusion (such as

that from Muzzey et al. (2009)) was used in the model selection process. In particular, it is unclear to me why the data from the Mettetal et al Bode plot (amplitude vs. frequency plots) were not used to constrain the modeling.

7. Finally, the captions for Figures 4 through 11 are inadequate. This makes it difficult to understand many of these figures. For example: what is shaded in Figure 10 and 11? Standard error of the mean, standard deviation, 95% confidence interval, or entire range of all cell volumes they measured? Also: what are the extra unlabeled colors in Figure 6?

Other concerns/notes:
8. When mentioning the low ratio of parameters to data points, it might be helpful to note what the ratio is (20:515) and how that compares to the best other published HOG models.

9. It is not clear what is shown in Figure 2F. Why do the authors show simulated data even though the model does not fit these data very well? Why do they not fit their model to measured data (or ignore these simulated data completely)?

10. The text that explains the conclusions from Figure 4 must be rewritten to make the logic clear to the reader.

11. Figure 4 parts C and D should be switched.

12. Figure image quality in general was poor and the labels were difficult to read.

13. Figure S5 is missing

1st Revision - authors' response 02 August 2012

Please consider our revised manuscript entitled 'Modelling reveals novel roles of two parallel signalling pathways and homeostatic feedbacks in yeast' by Schaber et al. for publication in Mol. Syst. Biol.
We present a well-parameterized mathematical model for the HOG pathway in yeast, which is a prototypic eukaryotic mitogen activated protein kinase pathway. The model can excellently both recapitulate and predict an unprecedented amount of quantitative data. Importantly, the model exhibits several new features and proposes novel mechanisms and hypotheses about the functioning of this well-studied system. The model suggests that

i) the main mechanism for osmo-adaptation is a fast and transient non-transcriptional Hog1-mediated activation of glycerol production,

ii) the transcriptional response rather serves to maintain an increased steady state glycerol production with low steady state Hog1 activity after adaptation,

iii) a fast negative feedback of activated Hog1 on the upstream signalling branches serves to stabilize adaptation response by preventing oscillatory behaviour.

iv) two parallel redundant signalling branches elicit a more robust and swifter adaptation than a single branch alone, at least for low osmotic shock. This notion could be corroborated by dedicated measurements of single cell volume recovery for the wild type and single branch mutants, which were repeated for the revised version.

Our study also demonstrates that systematically testing an ensemble of models representing different biological hypotheses against available data has the potential to achieve a better and unbiased understanding of underlying molecular mechanisms.

Our new findings have implications beyond the HOG pathway in yeast. Because of the generality of the methodology and the prototypic biological system, our findings are of importance to a broad audience interested in modelling dynamic systems, signal pathways and their regulation as well as intracellular variability.

Below you will find a detailed point-by-point response to all concerns raised by the three reviewers. We are confident that we have satisfyingly addressed all issued by conducting additional experiments and more detailed computational and mathematical analyses where they seemed appropriate.

Reviewer #1 (Remarks to the Author):

Major point:

Much of the data used for modeling are obtained from Macia (2009) and Klipp (2005), but also others such as Hao (2007). There is an inherent danger to comparing data obtained by different investigators using different strains and even slightly different experimental conditions, which should be noted explicitly in the discussion. THis is not currently done, for example: "Our ensemble modelling approach also revealed a possible role of the suggested transient feedbacks on the signalling branches (Hao, et al., 2007; Macia, et al., 2009), besides the fact that their physical nature is still elusive, especially in the Sln1 branch." It is appropriate to cite both papers but they should not be lumped together in this manner since the 2007 Hao paper uses an ssk1- mutant derived from S288C and KCl as the stimulus while the 2009 Macia paper uses SSK1+ strain derived from W303a and NaCl as the stimulus. The authors should also cite Yamamoto K, Tatebayashi K, Tanaka K, Saito H. Mol Cell. 2010 Oct 8;40(1):87-98.).

Response:

Being aware of the problem of using data from different sources, we only used data from the same strain and same culture conditions (medium, temperature, etc.) to parameterize the models. Certainly, the predictions of the model cannot be transferred to other strains in a quantitative way, e.g. data from the Hao paper. That's why we do not show simulation and the Hao data in the same plot. However, we think it is instructive to show that the model is also able to predict data measured in other strains, at least qualitatively.

In the discussion, we try to address this problem by changing the above mentioned sentence to the following paragraph (p 17., l. 32ff):

"Several recent studies support the notion that there are post-translational Hog1-mediated rapid negative feedback mechanisms within the signalling branches of the HOG pathway. Two studies demonstrated experimentally, that phosphorylated Hog1 negatively regulates the Sho1 branch at different sites (Hao, et al., 2007; Yamamoto, et al., 2010) and Macia et al. (2009) proposed a rapid Hog1-mediated negative feedback within the Sln1 branch, which was indirectly supported by experimental data, but whose physical nature remains elusive. It should be noted that all three cited studies used different strains and Macia et al. (2009), whose data we used to parameterize our models, did not propose a transient negative feedback in the Sho1 branch. Thus, it still unclear whether there are Hog1-mediated post-translational feedbacks within both branches of the HOG pathway in the strain our data refers to (W303). Nevertheless, our model discrimination analysis shows that the data support the existence of such feedbacks. "

Indeed, the Yamamoto et al. paper must be cited, as it demonstrates yet another negative feedback in the Sho1-branch and also elucidates other potential roles of this feedback. We refer to this paper in the above mentioned paragraph and include the following sentence on p.4, 11ff:

"Recently, Yamamoto et al. (2010) showed that Hog1 phosphorylates Ste50 and thereby shortens the duration of Hog1 activation, which further supports the notion of a transient negative feedback within the Sho1 branch".

We also add this reference on p. 3, l. 1., p. 3, l.5, and and p. 10., l. 16.

Minor points:

p. 27, l. 35: "Data in F was derived by assuming that volume mirrors Hog1 profile and minimal volumes from (Schaber, et al., 2010)." Is this a valid assumption?

Response:

We think that yes. We explain this further in the method section (Data). Moreover, we actually measured volumes and show in Figure 1F that our approach is justified. The minimal volumes obtained in Schaber et al 2010 were measured using the same strain and similar culture conditions.

p. *27 l. 36: "Not all data points used for fitting are shown." Why not?*

Response:

For technical reasons, we repeated the first time point for each time series. These data points do not add any information about the dynamic behaviour and are therefore not shown. We make this more explicit by changing the second to last sentence of the caption of Figure 2: "For each time series we repeated the first data point 6 times for technical reasons, these are not shown."

Reviewer #2 (Remarks to the Author):

I have the following minor concerns with the current state of this article:

1. In the stability analysis of the fast transient feedback mechanisms, a simplified generic model was constructed. The complete model was not used because it was apparently 'not possible with available standard software.' It's unclear to me why this is impossible. What specifically prevented the use of the complete model? I would rather have seen this analysis performed with the complete model.

Response:

The full model includes conditional statements (see Supplementary Material, e.g. variable *Turgor* Table S5). Therefore, the complete model is not continuously differentiable, which impairs calculation of the Jacobian matrix and a stability analysis, at least with the available software.

In Figure 5 we display the behaviour of the complete best-approximating model upon deletion of various feedbacks. It can be seen that the best approximating model does not show oscillatory behaviour, when the transient feedbacks in the signalling branches are removed, but rather slightly damped oscillations (orange lines in Figure 5).

Using the simple model, we rather wanted to test in a more general sense, whether models without transient feedbacks are more prone to oscillations. To this end, we analyse a reduced but generic model, which is meant to qualitatively show the same behaviour as the complete model.

We try to make this idea more explicit by re-writing and/or modifying

a) the last part of the first paragraph of this sections (p. 12, l. 18ff):

"….This prompted the question as to why the best approximating model included these feedbacks at all and, the related question, as to what they are good for. First, we observed that the best approximating model without the branch feedbacks displayed slightly damped oscillations (orange lines in Figure 5). Secondly, as mentioned in the Methods section, we excluded models that showed oscillatory behaviour after adaptation from the model selection procedure (Supplementary Table S8), assuming that the real system does not exhibit this behaviour. Even careful single cell analyses did not reveal oscillatory behaviour (Mettetal, et al., 2008; Muzzey, et al., 2009). We analysed the percentage of models with oscillatory behaviour amongst models with or without transient

feedbacks on the signalling branches and with or without non-transcriptional Hog1-PP mediated glycerol production. We noticed that including either of these feedbacks reduced the percentage of oscillating models in the respective category (Figure 7). Thus, models including transient feedbacks had a higher chance of being part of the discrimination procedure, because they were less prone to oscillatory behaviour. "

b) The 2nd paragraph of this section (p13., l. 6ff):

"…For models including both transient feedback mechanisms only 25% showed oscillatory behaviour. Taken together, it seems that transient feedbacks stabilise the adaptation response in terms of avoiding or making it less prone to oscillatory behaviour, at least for our parameterized models.

We hypothesised that this might be a more general feature of delayed negative feedback systems including fast transient feedbacks. To further investigate this hypothesis, we developed a simple and more general model (Figure 8A)…."

2. In the discussion section, if possible, could you list specific examples of other eukaryotic homeostatic adaptive systems, specifically ones containing redundant signaling pathways like that of the osmo-adaptation response.

Response:

We include the following paragraph in the discussion (p. 19, l. 22ff):

"There are other reports on parallel signalling pathways that are activated by a single stimulus in eukaryotic systems. One example is the Erk MAP kinase cascade, which is activated at high doses of epidermal growth factor by both a phosphoinositide 3-kinase (PI3K) dependent and a PI3K independent pathway (Sampaio, et al., 2008). Another example is NF-kB activation upon genotoxic stress. In this case, at least two partially redundant parallel signalling pathways, one PIASy dependent and another ATM dependent, converge on NEMO/IKK activation (McCool and Miyamoto, 2012). In these examples stabilisation or acceleration of response time might be important, however, as in the HOG system, parallel signalling pathways probably serve additional purposes, e.g. crosstalk to other connected signalling pathways."

3. In the methods section concerning parameter identifiability, it is emphasized that models which contain a number of parameters exceeding the number of experimental data points renders many of the model parameters unidentifiable. The language used here I think does not emphasize correctly the relationship between model parameter inferability and experimental data. Number of model parameters and shear number of experimental data points are only loosely related. What matters most is experimental design, so what you are measuring and at what times. More measurements of a particular species is not guaranteed to significantly improve identifiability.

Response:

The reviewer probably refers to the first paragraph in the subsection "Model development". Here we state that if the number of parameters exceeds the number of data points used to fit those parameters, this leads to over-parameterised models with non-identifiable parameters. We think that this statement is correct. The reverse, however, is not correct, and this is what the reviewer refers to. More data does not automatically render parameters identifiable, it's indeed the experimental design that matters in this case. We clarify this by changing the last sentence of this paragraph (p 21., l. 10ff) to

"Therefore, we aimed at a model that had substantially less parameters than data points for parameterisation. Given an appropriate experimental design this increases the possibility of obtaining at least some identifiable parameters (Raue, et al., 2009; Schaber and Klipp, 2011)."

4. Related to issue 3, given the one-dimensional likelihood profiles you've calculated would it be possible to suggest a set of measurements one could make in order to render the 7 unidentified parameters identifiable.

Response:

It would certainly be possible to suggest a set of measurements which would increase the number of identifiable parameters. We suspect that more measurements especially shortly after osmotic shock, i.e. in the highly dynamic range, would render more parameter identifiable. However, we think that a proper analysis of the optimal experimental design to render more parameter identifiable is out of the scope of the paper and should be addressed in an extra paper.

Reviewer #3 (Remarks to the Author):

Main concerns:

1. One of the main conclusions of the paper is that yeast osmoadaptation occurs mainly through Hog1-dependent post-translational glycerol production. This is described as being "in contrast to the commonly accepted opinion" that the main adaptation mechanism is through transcriptional feedback regulation. However, over the last few years, several authors have concluded that the transcriptional activation of glycerol production by Hog1 is not responsible for immediate osmoadaptation (see, for instance, Mettetal 2008), and that transcriptional mechanisms slowly prepare the cell for further shocks (as the authors' model also demonstrates in Figure 5). Therefore, the authors' main conclusion is mostly against a 'straw man.' The authors should make it clear in the discussion that their model is supporting previously described results.

Response:

Indeed, there are previous reports that imply different negative feedbacks acting on different time scales, the first being Klipp et al (2005), which suggests the fast closure of the Fps1 channel leading to glycerol retention as a non-transcriptional negative feedback acting on a fast time scale and protein synthesis on the slow time scale. Mettetal et al. (2008) find basically the same and actually also suggest a Hog1-dependent fast negative feedback responsible for the recovery after mild osmotic shocks of 0.2 M NaCl, although they do not suggest a fast increase in glycerol production as a potential mechanism. These studies were the main reason why a large portion of our model family included different versions of Fps1 closure and opening. However, the model in Mettetal et al. was not instructed by knowledge of the underlying biology and, thus, the proposed mechanisms was speculative. The model of Klipp et al., yet biologically detailed, was fitted to few data and therefore not identifiable. Both facts inspired our study using a parsimonious yet biology-based model supported by a wealth of data. Our model supports a mechanism in which the fast feedback acts upon direct glycerol synthesis. This is in contrast to the mentioned studies, which are part of the "commonly accepted opinion".

We tried to clarify this notion by changing the paragraph in the discussion including the criticized phase "in contrast to the commonly accepted opinion" the following way (p. 17, l. 1ff):

"…First, the model suggested that the main adaptation mechanism is not via a feedback involving transcription of glycerol producing enzymes, but rather a fast, possibly post-translational, Hog1-mediated feedback on the glycerol production machinery. This is in contrast to previous studies, which also proposed a fast non-transcriptional Hog1-mediated feedback playing an important role for short term osmo-adaptation, but speculated that it is rather mediated via fast Fps1-channel closure and resulting glycerol retention (Klipp, et al., 2005; Mettetal, et al., 2008). Such a mechanism played a less pronounced role in our model. …."

Additionally, on p. 12, l. 9 we fully recognized that our model also supports Mettetal et al. conclusions in the sense that one consequence of the slow transcriptional feedback is that the cells can respond faster to subsequent shocks.

We also insert a reference to Mettetal et al. on p. 3, l. 15:

"…whereas others also see an important role in glycerol retention by closing the glyceroporin Fps1 (Klipp, et al., 2005; Luyten, et al., 1994; Mettetal, et al., 2008; Tamas, et al., 1999)."

In the Introduction we change p. 5, l. 1ff :

"…The model suggested that a) the main adaptation mechanism is through the increase in glycerol production by fast transient post-translational mechanisms, rather than translational mechanisms or

glycerol retention as previous studies had suggested; b) glycerol retention is the second mechanisms in importance, which is also fast and acts through closure of the glycerol channel;…"

2. Moreover, the conclusion that post-transcriptional regulation in the primary mode of regulation may even depend on the particular form of glycerol production that the authors chose to use, without much explanation or support. Although all other reactions in table S4 are in the form of a mass reaction, the choice of the form of glycerol production, v13, is not obvious. It is possible that the change in its form will change the behaviour of the model 22 as well as general rankings of different models. This form should be supported with more explanation/clarification, and the model must be shown to be robust to changes in the form of this term.

Response:

In the main text we introduced a new paragraph, where we provide a more detailed rationale of the kinetics we used in general and specifically for reaction v9 and v13 (Method Section p. 22, l. 3ff).:

"According to our guiding principle of parsimony, not only the structure of the model, but also the kinetic rate law formulations were kept as simple as possible, but as complex as necessary. In almost all rate laws the most simple rate law, i.e. mass action kinetics, was sufficient. However, in the course of model development we realised that assuming simple mass action kinetics for reactions v_9 and v_{13} could not explain the data well, making a more complex kinetic necessary. In these two reactions we used a simple saturation kinetic, which gave good results, implying that saturation seems to be an important feature of these reactions. In the Supplementary Material we describe a detailed derivation of such saturation kinetics (Alon, 2007). Due to the lack of knowledge about a mechanism of how Hog1 may potentially modify glycerol synthesis, we used a simple heuristic approach. Because this modifying influence of Hog1 turned out to be important for our results, we also tested several possible rate law formulations for reactions v_{13} for the three best approximating models (see Result Section and Supplementary Material Table S11 and S12). See Supplementary Tables S1-S8 for a detailed list of all reactions, their respective rationale and parameters. Figure 1 gives an overview of all reactions considered in the candidate models"

We provide more explanations and a detailed mechanistic derivation of the saturation kinetics used in reaction v9 and v13 in an additional section of the Supplementary Material (Section 2 Saturation and Inhibition Kinetics). Moreover, we test different kinetic rate laws for reaction v13 for the three best approximating model and show that our ranking is robust to a change in this kinetic.

In the result section we add a paragraph (p. 7, l. 19ff):

"In the course of model analysis reaction v_{13}, i.e. Hog1-modified glycerol production (Figure 1), turned out to be important for our conclusions (see below). As Hog1 modification of glycerol production was modelled by a simple heuristic approach due to the lack of a detailed mechanism, we also tested different possible kinetic rates laws for reaction v_{13} in the three best approximating models Nr. 22, Nr. 78 and Nr. 30 that had an $AICw > 0.05$ (see Method Section, Supplementary Tables S11 and S12). The kinetic rate law for reaction v_{13} used in the original model formulation was best supported by the data and, thus, the ranking, of at least the three best approximating models, was robust to changes in this kinetic rate law. The large difference in the performance between the original and the other tested kinetics suggests that this result is also valid for the other models (Supplementary Table S12). "

We like to remark that each kinetic rate law formulation assumes implicitly a certain model structure. Therefore, it can be expected that changing a kinetic rate law will have just as profound effects as changing model structure. It can be easily perceived that, if saturation is an important feature for reactions v9 and v13 (as it was noted in the course of model development), using mass action in these reactions will affect the results (see also ranking of kinetic K6 in Supplementary Table S12).

3. Another conclusion of the paper is that the presence of two branches in the wild-type results in less variability than the single branch mutants. Even though this result is corroborated by robustness to parameter and initial condition changes that the model displays, the authors' own experimental results are not significant (3 positive results out of 4 is not significant, especially

because the less variable Sho1 branch result was obtained in the more reliable 0.2 M NaCl experiment).

Response:

We agree that concerning robustness the data does not fully support our model predictions. We made additional experiments measuring single cell volume changes for 0.1M and 0.2M NaCl. These additional experiments confirmed that adaptation velocity and robustness is smallest for the wild type for 0.1M NaCl, whereas for 0.2 M NaCl this is true only for adaptation velocity (see updated Tables 1 and 2). Now, we also included statistical tests for the difference in scale in addition to the difference in location tests. Accordingly, we modify the manuscript in several places:

We change the abstract (p.2, l. 14ff)

"…We could corroborate this notion to a large extent by dedicated measurements of volume recovery in single cells…."

We change the last paragraph of the introduction (p.5, l. 17ff)

"The model also provided an explanation for why there are two redundant parallel signalling pathways. Simulation studies suggested that the mean adaptation time for the wild type yeast is faster and more robust to variations in initial state and parameters than for the single branch mutants, especially for weak stress. By dedicated experiments we could corroborate the prediction that wild type yeast adapts faster than single branch mutants. The notion that adaptation in wild type yeast is also more robust could only be corroborated for low osmotic stress, which might be more relevant in the natural environment."

In the results section we mentioned the statistical tests (p 15, l 5f):

"($P < 0.01$, using two robust tests for location, i.e. U-test and Kolmogoroff-Smirnov test)"

p 15, l 12f:

"($P < 0.01$, using two robust tests for scale, i.e. Siegel-Tukey test and Conover test)"

We change the last two paragraphs of the result section (p.16, l. 1ff):

"In terms of interquartile ranges, a robust measure of population variability, we observed that for an osmotic shock of 0.1M NaCl the wild type exhibits significantly less variability ($P < 0.01$) than the single branch mutants (Table 1). For an osmotic shock of 0.2 M NaCl, however, the Sho1 branch showed least variability, which was significant ($P < 0.01$), whereas the variability of the wild type and the Sln1 branch was not significantly different (Table 2).

Our Monte-Carlo analysis of adaptation times using the parameterised model support the hypothesis that a major consequence of maintaining two redundant signalling pathways is that they provide an advantage in recovery from hyper-osmotic shock, both in terms of speed and robustness. The advantage in speed was corroborated by our measurements, whereas the advantage in robustness was only supported for low osmotic shock (0.1M NaCl). "

We modify in the discussion the respective paragraph (p. 19, l. 1ff):

"…We could confirm by dedicated experiments that the wild type cells indeed recover significantly faster than the single branch mutants for low and intermediate osmotic shocks (0.1, 0.2M NaCl). In addition, as evidenced by the interquartile ranges of the adaptation times, we could show that the single branch mutants showed a significantly higher variability than the wild type for the low osmotic shock of 0.1M NaCl. For intermediate osmotic shock the Sho1 branch seems to be more robust than both wild type and Sln1 branch mutant, indicating other possible roles of Sho1 branch under this condition and the existence of additional processes, which are not considered by our model.

There are technical limitations to the precision with which the small changes in volume that occur in mild osmotic shocks can be measured. However, recovery of the wild type was up to five minutes faster than in the single branch mutants, which constitutes more than 5% of the cell cycle time under good growth conditions. This advantage might be sufficient to evolutionary conserve two parallel redundant signalling branches. Besides the hypothesis that parallel signalling pathways have evolved to improve noise suppression, which is partly supported by this study, there are, of course, other possible explanations, which we did not investigate here. It is known that, e.g., the Sho1 branch is also involved in the activation of the Fus3 and Kss1 MAP kinases, which regulate the

response to pheromone and starvation, respectively. Therefore, it seems clear that possible noise suppression is not the sole reason for the existence of the Sho1 branch."

4. I am also concerned about the claim that signaling output is stabilized by Hog1-feedback on signalling branches. For example, in Figure 7, the presence of 2 feedbacks only modestly decreases the fraction of models that oscillate (~71% to 66% for Gdp1+ models, ~38% to 25% in Gdp1+Hog1 models). Additionally, I have concerns regarding the analysis behind Figure 8 and Figure 9. In Figure 8, they do not show whether their reduced model in Figure 8 fits the data well, so it is not obvious whether this model is a good representation of the full network dynamics. As for Figure 9, the explanation is essentially incorrect. The real part of the eigenvalue determines the stability, while the imaginary part determines oscillations, so the authors' analysis of a real eigenvalue as having anything to do with oscillations is erroneous.

Response:

In the result section we state that (page 14., l. 12ff)

"… this provides support to the hypothesis that a potential biological role of those proposed transient feedbacks is the stabilisation of the adaptation response by avoiding oscillatory behaviour, which can occur in delayed negative feedback systems (Kholodenko, 2000)."

Thus, we rather argue for transient feedbacks in general. To make clear that in our model the effect of the Hog1-dependent feedback on glycerol production seems to be stronger, we added the following sentence to the end of the respective paragraph in the discussion (p. 18, l. 17ff);

"The stabilisation effect in our model, however, seems to be stronger for the transient Hog1-mediated feedback on the glycerol production rather than for the transient feedbacks on the signalling branches".

The reduced model is not supposed to fit the data quantitatively. It is rather supposed to illustrate the principle, and to check with a generic model, whether stabilization of adaptation response by fast transient feedback is a more general feature of delayed feedback systems (also see response to reviewer 1). Therefore, we refrained from fitting it to data. Figure 8 shows that the reduced model shows qualitatively the same behaviour as the full model and is therefore a valid simplification and generalisation.

Indeed, the maximum real part of the eigenvalue of the Jacobian only determines the stability of the steady state and not whether oscillations occur. The occurrence of oscillations in a non-linear system of a dimension higher than two can only be shown in very special cases (see, e.g., 'Guckenheimer and Holmes, Nonlinear oscillations, dynamical systems and bifurcation of vector fields'). One of these cases is, e.g., a Hopf-bifurcation. We can show by a computational analysis of the Eigenvalues that in our systems oscillations arise because of a supercriticial Hopf-bifurcation. Thus, in our case, change in stability of the steady state coincides with the emergence of stable oscillations.

We clarify this by modifying the respective paragraph in the result section (p. 13, l. 30ff)

"…In Figure 9 we plot the sign of the real part of the maximum eigenvalue of the linearised system at steady state. If negative, i.e. white squares in Figure 9, the steady state is stable and no sustained oscillations are possible. A computational analysis revealed that when the real part of the maximum eigenvalue changes from negative to positive, i.e. grey squares in Figure 9, there is a single pair of complex conjugated eigenvalues crossing the imaginary axis and the remaining two eigenvalues remain negative. This is the hallmark of a Hopf-bifurcation giving rise to, in our case, stable oscillations. We illustrate this by bifurcation diagrams of *Hog1PP* equilibria as a function of T_0 for selected values of *NaCl* (see Supplementary Figure S2). .. "

We also mention this in the caption of Figure 9.

5. It is important to note which parameters are identifiable in a modeling paper. The number of unidentifiable parameters is misstated as 7 (there seem to be 8 in Figure S1: kHog1phos1, kHog1phos2, Ki1, k1, k2, k3, k9, and k15). Additionally the names of the parameters in Figure S1 do not correspond to the names given in Tables S7 and S11 making it impossible to understand this figure or ascertain which parameters were actually identifiable.

Response:

Indeed, in the Figure S1, there were 8 parameters, which did not cross the confidence limit within the plotted interval. That was because the plotted interval was not chosen wide enough. We adjusted the interval for k3, such that it now crosses the confidence limit within the considered interval, too. We modified Figure S1 and corresponding text accordingly. Now, the number of unidentifiable parameters is 7 as stated. We also changed the naming of the parameters in Figure S1 as in table S7 and S11.

6. In the discussion, the authors mention that their model is more consistent with imperfect adaptation of Hog1 because the model was able to fit the Macia et al. (2009) data well. However, given that the model was selected based upon fitting to some of the Macia data and ranking based on how well it predicted other parts of the Macia data, it is unsurprising that it supports the conclusions regarding imperfect adaptation that were previously drawn from the Macia data. It would be helpful to see whether another model emerges as the best if data suggesting the opposite conclusion (such as that from Muzzey et al. (2009)) was used in the model selection process. In particular, it is unclear to me why the data from the Mettetal et al Bode plot (amplitude vs. frequency plots) were not used to constrain the modeling.

Response:

Indeed, it is unsurprising that the model supports the conclusions regarding imperfect adaptation that were drawn from the Macia data, because the model is fitted to this data. We try to clarify this by changing the sentence (p. 18, l. 22f)

"Since the model could fit the Macia et al. data well, it also implies a role for model components that were not observed during adaptation response."

To

"Since the model was fitted to the Macia et al. data and could explain this data well, it also implies a role for model components that were not observed during adaptation response."

In the respective paragraph we do not aim to analyse this result *per se*. We think that the model provides a more complete picture of this phenomenon, which is worth to take a short look at.

Yes, it would be interesting to see, whether the model could also be fitted to data showing perfect adaptation using data from the Muzzey paper. We actually thought about that, but refrained from this idea for several reasons:

1) The Muzzey paper basically provides data about Hog1 nuclear translocation and volume. Since there were no data on other components, like RNA, Protein, etc, we would have had to adjust the model to have at least some identifiable parameters. In this case, the resulting model would have been difficult to compare to our model. We concluded that this was out of the scope of this paper.

2) For fitting and constraining the model we only use data from comparable experiments, i.e. same strain, same culture conditions, same stress. We consider this a meaningful approach. Therefore, neither the data from the Muzzey nor from the Mettetal paper was used to complement our data sets.

Moreover, we find it critical to draw conclusions about the HOG pathway from repeated hypo-hyper-shock experiments as in the Mettetal paper. We have data showing that when hyper-shock is removed shortly after initiation, this induces a hypo-shock response (Mpk1 activation), which interferes with the HOG pathway. Therefore, we do not further embark on the multiple hyper-hypo-shock simulation in Figure 6. We make this more explicit by adding the following sentence to the last paragraph in the respective result section (p. 12, l. 10ff):

"However, this result should be taken with care, because the hypo-shock response, which activates the cell wall integrity pathway, might interfere with the hyper-shock response regulated by the HOG pathway."

7. Finally, the captions for Figures 4 through 11 are inadequate. This makes it difficult to understand many of these figures. For example: what is shaded in Figure 10 and 11? Standard

error of the mean, standard deviation, 95% confidence interval, or entire range of all cell volumes they measured? Also: what are the extra unlabeled colors in Figure 6?

Response:

We adjusted the captions of Figure 4 through 11 trying to make them more descriptive and clear. We also switched Figure 4C and 4D, and changed some labels for the sake of clarity.

Figure 4: Simulated Hog1 phosphorylation and corresponding intracellular glycerol concentrations. Shown are simulation scenarios, where all except one Hog1-mediated feedbacks (FBs) are blocked at time 0 by addition of 5mM SPP86 kinase inhibitor in the absence of osmotic shock. Blue line: all FBs are blocked in the respective branch (see also Figure 2D). Red line: only Hog1 feedback on upstream signalling is kept active. Grey line: Only Hog1 feedback on Fps1 closure is kept active. Green line: Only Hog1 mediated transcription is kept active. Orange line: Only Hog1-mediated glycerol production is kept active. **A**: Hog1 phosphorylation time series for the Sln1 branch. **B**: Hog1 phosphorylation time series for the Sho1 branch. **C**: Glycerol time series for the Sln1 branch. **D**: Glycerol time series for the Sho1 branch.

Figure 5: Simulated Hog1 phosphorylation and volume dynamics for 0.4M NaCl osmotic shock for cases where several feedback mechanisms are selectively shut-off. w/o denotes 'without'. FB denotes feedback. Blue line: wild type. Brown line: without Hog1-mediated feedback on both upstream signalling branches. Orange line: without Hog1-mediated feedback on the Sho1 branch. Light Orange line: without Hog1-mediated feedback on the Sln1 branch. Dark grey line: without Fps1 closure. Grey line: without Hog1-mediated Fps1 closure. Light grey line: without turgor mediated Fps1 closure. Green line: without transcription. Red line: without Hog1-mediated glycerol production.

Figure 6: Simulations for multiple consecutive osmotic shocks (A, left panels) and multiple hyper-hypo osmotic shocks (B, right panels). The upper panels show the respective input osmotic shocks [M NaCl]. The lower panels show the respective simulations. The full and light coloured lines show the corresponding model simulations with and without transcriptional feedback, respectively.

Figure 7: Percentage of oscillating models in four different model categories. **Gpd1 + <2BFBs**: transcriptional feedback (Gpd1) with less than two Hog1-mediated upstream signalling branch feedbacks (2BFBs). **Gpd1 + 2BFBs**: transcriptional feedback (Gpd1) with both Hog1-mediated upstream signalling branch feedbacks (2BFBs). **Gpd1 + Hog1 + <2BFBs**: transcriptional feedback (Gpd1) with Hog1-mediated glycerol production (Hog1) and less than two Hog1-mediated upstream signalling branch feedbacks (2BFBs). **Gpd1 + Hog1 + 2BFBs**: transcriptional feedback (Gpd1) with Hog1-mediated glycerol production (Hog1) and both Hog1-mediated upstream signalling branch feedbacks (2BFBs).

Figure 8: Generic HOG model. **A**: Wiring scheme. Optional feedbacks are dashed. For details on the implementation refer to the Supplementary Material **B**: Simulation with both transient feedbacks, i.e. dashed lines in A, **C**: simulation without transient feedbacks, i.e. dashed lines in A. Shown are simulations for NaCl (dotted line), activated Hog1 (grey line) and glycerol (black line). Simulation were done with T_0=0.02, $[Hog1]_0$=0.05, $[RNA]_0$=0.01, $[Protein]_0$=0.03, E_0=$[Glycerol]$=0.3, H_t=1, k=0.1, $[NaCl]$=0.5, K_i=0.1, n=2.

Figure 9: Real parts of the maximum eigenvalues of the Jacobian matrix of the simplified HOG model from Figure 8 at steady state including different feedback mechanisms. Grey squares indicates maximum eigenvalues > 0, i.e. unstable steady states, white squares indicate maximum eigenvalues < 0, i.e. stable steady states. Unstable steady states coincide with stable oscillations due to a Hopf-bifurcation, which was checked by a computational bifurcation analysis (see Supplementary Material and Supplementary Figure S2).

Figure 10: Volume adaptation simulations and measurements after 0.1M NaCl osmotic shock. For the time series shaded coloured regions indicate respective interquartile ranges. We show measured volume curves of one representative experiment, whereas adaptation times are pooled over four independent experiments (Table 1). In the box plot, solid lines are median, dashed lines means, boxes indicate the interquartile range, whiskers minimum and maximum and point are outliers beyond upper quartile+1.5*interquartile range.

Figure 11: Volume adaptation simulations and measurements after 0.2M NaCl osmotic shock. For the time series shaded coloured regions indicate respective interquartile ranges. We show measured volume curves of one representative experiment, whereas adaptation times are pooled over five

independent experiments (Table 2). In the box plot, solid lines are median, dashed lines means, boxes indicate the interquartile range, whiskers minimum and maximum and point are outliers beyond upper quartile+1.5*interquartile range.

Other concerns/notes:

8. When mentioning the low ratio of parameters to data points, it might be helpful to note what the ratio is (20:515) and how that compares to the best other published HOG models.

Response:

We changed the according sentence in the discussion (p. 16, l. 20ff):

"…To our knowledge, there was no model proposed yet with a lower ratio of number of parameters to number of fitted data points (20/390). Comparable models based on biological knowledge and fitted to data had ratios of, e.g., 70/33 (Klipp, et al., 2005) and 10/41 (Gennemark, et al., 2006). ..„

9. It is not clear what is shown in Figure 2F. Why do the authors show simulated data even though the model does not fit these data very well? Why do they not fit their model to measured data (or ignore these simulated data completely)?

Response: We consider it best scientific practice to show all fitted data and not only those that fitted best. Of course, this indicates a shortcoming of the model, but there is no perfect model. Figure 1F is indeed a little confusing, because we depicted here data which was used for fitting and data which was not used for fitting along with simulation results.

We also explain in the main text, why we did not use our measured volume data for fitting (p 20, l. 31ff):

"…As the model does not include growth, the artificial and simulated volumes level off below 100% of the initial volume, whereas the measured volume surpasses the initial volume. Therefore, and in the light of the excellent predictive properties, we refrained from refitting all models including the measured volumes."

10. The text that explains the conclusions from Figure 4 must be rewritten to make the logic clear to the reader.

Response:

We re-write the paragraph introducing Figure 4 (p. 9, l. 1ff):

"Thus, we decided to further investigate the importance of the individual mechanisms responsible for the measurements done by Macia et al. using our best approximating model (Nr. 22), which could reproduce and predict well all these data (Figure 2D, 3B, 3D). To this end, we again simulated Hog1 phosphorylation time series during a Hog1 kinase inhibition experiment as shown in Figures 2D, 3B and 3D. However, this time instead of removing (setting the appropriate parameters to zero) all modifying influences of activated Hog1, which would mimic complete inhibition of the kinase, we removed all but one of these modifying influences at a time. This way we could selectively test which of the proposed feedback mechanisms is responsible for the observed Hog1 phosphorylation upon kinase inhibition, because if the responsible feedback would still be active despite of other feedbacks being blocked, no Hog1 activation should be observed. Specifically, we tested situations where all Hog1-mediated feedbacks we deleted except one of the following four: a) upstream signalling branch feedback (branch FB only), b) Hog1-mediated induction of transcription (transcriptional FB only), c) direct activation of glycerol production (Hog1-glycerol FB only), or d) closure of Fps1 channel (Fps1 FB only). The results are shown in Figure 4."

We partly re-write the paragraph explaining Figure 4 (p. 9, l. 19ff):

"Sln1 branch mutant (disabled Sho1 branch)

When only the Hog1 feedback on upstream signalling remains functional (reactions v_1 and v_3 in Figure 1, "branch FB only"), after addition of Hog1 kinase inhibitor, Hog1 still becomes phosphorylated, though to a lesser extent than in the simulation with all feedbacks blocked ("all FBs

off") (Figure 4A, compare blue and red lines). A similar simulation output is observed when Hog1 activity remains functional only towards Fps1 closure (reaction v_{16} in Figure 2, Figure 4A, "Fps1 FB only", grey line). When only Hog1-dependent induction of gene expression remains enabled in the model, we see initially the same behaviour as if all feedbacks were blocked, but at later times Hog1 phosphorylation decreases again, reflecting increasing protein production and subsequent increased glycerol production (Figure 4A, "transcriptional FB only". green line). Thus, our model indicates that these feedbacks can only partly explain the observed behaviour. In contrast, there is almost no simulated Hog1 phosphorylation when the direct Hog1-mediated modification of glycerol production is the only remaining functional feedback upon kinase inhibition (Figure 4A, "Hog1 glycerol FB only", orange line). This demonstrates that in our model, and for the Sln1 branch, the main mechanism leading to Hog1 phosphorylation upon addition of inhibitor is inhibition of direct (non-transcriptional) Hog1-mediated glycerol production. Accordingly, simulations of the dynamics of intracellular glycerol concentration and corresponding branch activation (Figure 4C, Supplementary Figure S4) further support this mechanism, since inhibition of Hog1 activity leads to a rapid down-regulation of steady state glycerol production, leading to a decrease in the simulated internal glycerol. This reduction leads to an osmotic stress, which in turn leads to pathway activation."

11. Figure 4 parts C and D should be switched.

Response: Yes, we did (see response to point 7)

12. Figure image quality in general was poor and the labels were difficult to read.

Response: We improved the quality of all images.

13. Figure S5 is missing

Response: Figure S5 is the last image in supplementary section 7.3. We extended the captions of Figure S4 and S5, so they become clearer and also better distinguishable.

We further corrected the several typos and slightly changed some formulations which did not change the general meaning of the respective sentence.

The author list was also updated in the way that now AB is equally contributing second author.

2nd Editorial Decision 03 September 2012

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, the referees felt that the revisions had substantially improved this work. The last reviewer has one important concern, requiring some additional computational analysis, which we would ask you to carefully address in a final revision of the present work.

In addition please address the reviewers' more minor points, and the following format and content issues:

1. Molecular Systems Biology generally requires that new mathematical models are deposited in a public repository, such as BioModels or JWS Online. In this case, I encourage you to submit a SBML version of the best-fit model to one of these databases. The resulting accession should be

referenced in the Methods section of the main manuscript. This SBML model should also be included in the Dataset 1 zip file.

2. Molecular Systems Biology also, in principle, requires that numeric data is supplied supporting new experimental results, particularly when these data are used to train mathematical models. To make these datasets more accessible to readers, we provide a new functionality that allows authors to submit the 'source data' associated with selected figure panels (e.g. <http://tinyurl.com/365zpej>). This sort of figure-associated data may be particularly appropriate for this work. Please see our Instructions of Authors for more details on preparation and formatting of figure source data (<http://www.nature.com/msb/authors/index.html#a3.4.3>).

3. The resolution of the some of the smaller text in the figures is currently somewhat fuzzy/blocky. I would encourage you to provide the figures files as EPS files, retaining the line-art and model diagrams as vector graphics. This should substantially reduce the file sizes, and lead to a better final product.

4. The Table of Contents at the beginning of the Supplementary Information file should list all of the Supplementary Figures and Tables and their page numbers. The Supplementary Dataset should also be listed as "separate file".

Thank you for submitting this paper to Molecular Systems Biology.

Yours sincerely,

Editor - Molecular Systems Biology
msb@embo.org

Reviewer #1 (Remarks to the Author):

The authors have satisfied my relatively minor concerns.

The authors are correct to cite Yamamoto as another example of negative feedback, but they were actually the second group to show Hog1 phosphorylation of Ste50 (see PMID: 18854322) and both papers should be cited.

Reviewer #3 (Remarks to the Author):

The authors have resubmitted a much improved manuscript. I have only one major comment that I would like to see addressed:

I still think it would be interesting to see how the author's model performs when exposed to a series of osmotic shocks (previous point#6). I agree with the authors that since the Mettetal experiments are in a different strain background quantitatively fitting the data from the van Oudenaarden group would not necessarily be informative. However, one expects that the broad features of the amplitude response to different input frequencies would be preserved in both strains (the scaling relationship). It is important to see if the proposed model captures this relationship. Of course, it might not, which could be considered an argument that further considerations, such as Mpk1 activation that is proposed to be important for hypo-osmotic shocks, must be included. Performing this analysis should be relatively easy to do computationally.

Minor comment:

Double-check that all lines in figures are correctly labeled and that you refer to the correct figures in the text.

For example:

p7:11 Figure 3B?

p8:21,25 Macia's figures or yours?

Figure 3C green line missing?

Figure 4B,D blue line missing or covered?

2nd Revision - authors' response 12 September 2012

Please consider our final revised manuscript entitled 'Modelling reveals novel roles of two parallel signalling pathways and homeostatic feedbacks in yeast' by Schaber et al. for publication in Mol. Syst. Biol.
We present a well-parameterized mathematical model for the HOG pathway in yeast, which is a prototypic eukaryotic mitogen activated protein kinase pathway. The model can excellently both recapitulate and predict an unprecedented amount of quantitative data. Importantly, the model exhibits several new features and proposes novel mechanisms and hypotheses about the functioning of this well-studied system. The model suggests that

v) the main mechanism for osmo-adaptation is a fast and transient non-transcriptional Hog1-mediated activation of glycerol production,

vi) the transcriptional response rather serves to maintain an increased steady state glycerol production with low steady state Hog1 activity after adaptation,

vii) a fast negative feedback of activated Hog1 on the upstream signalling branches serves to stabilize adaptation response by preventing oscillatory behaviour.

viii) two parallel redundant signalling branches elicit a more robust and swifter adaptation than a single branch alone, at least for low osmotic shock. This notion could be corroborated by dedicated measurements of single cell volume recovery for the wild type and single branch mutants, which were repeated for the revised version.

Our study also demonstrates that systematically testing an ensemble of models representing different biological hypotheses against available data has the potential to achieve a better and unbiased understanding of underlying molecular mechanisms.

Our new findings have implications beyond the HOG pathway in yeast. Because of the generality of the methodology and the prototypic biological system, our findings are of importance to a broad audience interested in modelling dynamic systems, signal pathways and their regulation as well as intracellular variability.

Below you will find a detailed point-by-point response to all concerns raised by the three reviewers. We are confident that we have satisfyingly addressed all issued by conducting additional experiments and more detailed computational and mathematical analyses where they seemed appropriate.

Concerning the suggestions raised in the Decision letter we respond the following:

Ad 1) We submitted an SBML version of the best fit model to the Biomodels database (MODEL1209110001) and indicated this in the manuscript page 6, line 26f. We also included the SBML version in the Supplementary Material.

Ad 2) We submitted source data to all figures, where data was displayed.

Ad 3) We made quite some effort to improve figure quality, however, in some instances we did not seem to be very successful. Most of the figures are originally generated as high quality tif-

files from Mathematica software. Many figures are composite figures, which were generated the following: We pasted original high resolution tif-figures into Microsoft PowerPoint and additionally annotated them, partly together with original PowerPoint artwork. The resulting PowerPoint Figure was saved as high-quality-print pdf-file and subsequently converted to high resolution tif-file. We are happy to take any suggestions how to improve this. Please also indicate the figure which you feel need further improvement and we will do our best.

Ad 4) The Table of Contents at the beginning of the Supplementary Information file should now lists all of the Supplementary Figures and Tables and their page numbers. The Supplementary Dataset for Figure S2 is listed as "separate file" and also submitted.

Ad 5) Bullet points: see above points i-iv.

Ad 6) **Standfirst text:** We present a mathematical model for the HOG pathway in yeast, which can excellently both recapitulate and predict an unprecedented amount of quantitative data. The model exhibits several new features and proposes novel regulatory mechanisms that are corroborated by dedicated measurements.

Ad 7) We also submitted a thumbnail image. It is Figure 8A. However, I am afraid the quality is also poor. Suggestions for improvement are welcome.

Reviewer #1 (Remarks to the Author):

The authors have satisfied my relatively minor concerns.

The authors are correct to cite Yamamoto as another example of negative feedback, but they were actually the second group to show Hog1 phosphorylation of Ste50 (see PMID: 18854322) and both papers should be cited.

Response: We included the mentioned reference by changing p4, l. 10ff to:

"It was also shown that Hog1 phosphorylates Ste50 (Hao, et al., 2008; Yamamoto, et al., 2010) and thereby shortens the duration of Hog1 activation (Yamamoto, et al., 2010), which further supports the notion of a transient negative feedback within the Sho1 branch."

Reviewer #3 (Remarks to the Author):

The authors have resubmitted a much improved manuscript. I have only one major comment that I would like to see addressed:

I still think it would be interesting to see how the author's model performs when exposed to a series of osmotic shocks (previous point#6). I agree with the authors that since the Mettetal experiments are in a different strain background quantitatively fitting the data from the van Oudenaarden group would not necessarily be informative. However, one expects that the broad features of the amplitude response to different input frequencies would be preserved in both strains (the scaling relationship). It is important to see if the proposed model captures this relationship. Of course, it might not, which could be considered an argument that further considerations, such as Mpk1 activation that is proposed to be important for hypo-osmotic shocks, must be included. Performing this analysis should be relatively easy to do computationally.

Response: We included a frequency-response analysis as in Mettetal et al. (2008) and compared the result to re-analysed data from Mettetal et al. (2008) and Hersen et al. (2008).

In the main text (p. 7, l. 1ff) we include the following paragraph:

"The HOG pathway was shown to act as a low-pass filter regarding the frequency of salt shocks (Hersen, et al., 2008). We simulated the response of the best approximating model Nr. 22 to square-wave stimuli of 0.2M NaCl with periods ranging from P_0=2 min to P_0=64 min (Supplementary Figure S1). Using Fourier analysis, we approximated the simulations by sine wave oscillations with a period of P_0=2p/w and calculated frequency-dependent output amplitude A(w), which is represented in a Bode-plot (Supplementary Figure S3) as in Mettetal et al. (2008). We also

compared our simulated frequency-dependent amplitude $A(w)$ with re-analysed data from Mettetal et al. (2008) (Supplementary Figure S2). The model simulations show an increasing frequency-dependent amplitude $A(w)$ with decreasing frequency w, like both the results from Hersen et al. (2008) and the re-analysed data from Mettetal et al. (2008). Thus, the best approximating model can well reproduce the reported low-pass filter characteristics of the HOG pathway (for details refer to the Supplementary Material)."

In the Supplementary Material we add a section on the details of this analysis and show the details of the results (Figure S1 – S3).

Minor comment:

Double-check that all lines in figures are correctly labeled and that you refer to the correct figures in the text.

Response: We double checked all figure and legends (see below). Indeed, we noticed that Figure 3 was not correct. First, we missed one prediction that was referred to in the text (now Figure 3B) and, second, Figure 3D was from an older version, which was not longer referred to in the text. Figure 3D was now replaced by the former Figure 3B, as it was also referred to in the text. We changed the text and Figure legends accordingly.

For example:
p7:11 Figure 3B?

Response: That is correct.

p8:21,25 Macia's figures or yours?

Response: The data is from Macia, the simulation from our model. It escaped our attention, that the origin of the data in Figures 2D,2E and 3B, 3C and 3D was not explicitly mentioned. To clarify this, we add the following sentence in the method sections (p. 30, l31ff):

"The data in Figures 2D, 2E and 3B, 3C and 3D were also taken from Macia et *al*. (2009), but digitised from the original figures therein. "

In the respective figure legend we add explicit statements where the data come from. This is additionally mentioned in the corresponding source data files.

Figure 3C green line missing?

Response: No, but the line for 1uM inhibitor is the almost the same as for the wt, and therefore overlays the latter. We make this clear in the figure legend.

Figure 4B,D blue line missing or covered?

Response: Covered, we clarify this in the figure legend.

