molecular systems biology

Asymmetric Positive Feedback Loops Reliably Control Biological Responses

Alexander V. Ratushny, Ramsey A. Saleem, Katherine Sitko, Stephen A. Ramsey, John D. Aitchison

Corresponding author: John D. Aitchison, Institute for Systems Biology

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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 30 December 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 raise substantial concerns on your work, which, I am afraid to say, preclude its publication.

While the reviewers did find the description of the ASSURE motif potentially interesting, they remained substantially unconvinced regarding key claims about the properties and evolutionary advantages of this motif. Most importantly, the reviewers were not convinced that the experimental work with the Oaf1p/Pip2p system provided conclusive evidence that the ASSURE motif provides a clear fitness advantage (reviewer #2, point 2, reviewer #3, point 3). Their doubts seem to center on concerns that the engineered strain could be disadvantaged for a variety of reasons that may not be directly related to the ASSURE motif itself (e.g. greater protein expression burden). This concern was clearly shared by the first reviewer, who was otherwise more positive. In addition, the last two reviewers had concerns regarding the model-based analysis, which they felt were sufficient to cast some doubt on some of the most important predicted properties of the ASSURE motif, in particular the physiological relevance of the increased speed compared to symmetric feedback systems.

Given these important concerns, and since two reviewers clearly indicated that they felt that the main claims in this work remained insufficiently supported, we feel we have no choice but to return this work with the message that we cannot offer to publish it.

Nevertheless, the reviewers did express interest in this topic, and their comments suggest that additional experiments and analysis could potentially provide clearer support for the claims made in

this work. As such, we would like to suggest that we may be willing to reconsider a new submission based on this work. Any resubmitted work would need to provide new evidence that directly and conclusively addresses the reviewers' main concerns, including new experimental evidence that better demonstrates the direct fitness advantages of the ASSURE motif. We recognize that this would involve substantial additional work, and we would therefore understand if you decided to submit this work, instead, to another journal.

A resubmitted manuscript would receive a new number and receipt date, and we can give no guarantee about its eventual acceptability. If you do decide to follow this course, it would be helpful to enclose with your re-submission an account of how the work has been altered in response to the points raised in the present review.

I am sorry that the review of your work did not result in a more favorable outcome on this occasion, but I hope that you will not be discouraged from sending your work to Molecular Systems Biology in the future.

Thank you for the opportunity to examine this work.

Sincerely,
Editor - Molecular Systems Biology

Reviewer #1 (Remarks to the Author):

This paper studies a new network motifs:
Positive feedback carried out by a heterodimer, such that only one of the monomers has positive autrogerulation- named ASSURE by the authors.
It lists numerous systems where this motif appears, analyzes its dynamics and robustness mathematically, and compares it to a simpler circuit desing experimentally in yeast.
This is an important paper due to the large number of appearances of this motif across organisms.
The combination of math and experiment make this an excellent paradigm for studies of network motifs. I like the competition experiments in varying environments.

I warmly recommend publication and suggest the following suggestions for improvement (i stress that no additional experiments are essential).

Mathematical modeling:
The authors nicely compare ASSURE to all reasonable simpler circuits- those with no auto-regulation and symmetric regulation. It would help if they clarify better what effort was made to make a controlled comparison. I recommend putting this in the context of 'mathematically controlled comparison' (a term coined by Savageau, described in Alon's book An Into to Systems Biology, CRC press): keeping the largest number of internal and external parameters equal between the two circuits compared (eg protein production/decay rates, Kds are internal parameters, dynamic range is an external parameter).

Can an intuitive reason for increased robustness and faster response be better explained?

Since natural selection is at play, one may assume that optimal (or near optimal) parameters can arise: in optimal parameters (that is, given controlled comparison of all possible parameters, the parameters that optimize a feature such as response time)- is the response time and robustness of symmetric and asymmetric circuits different? By what amount? Can this be found analytically? Data in the SI seem to show that the optimal parameters give similar behavior to the two circuits, but I may have misunderstood.

p-values such as E^-61 are too small to be measured numerically- and so were estimated by a statistical model- it would be good to be conservative with such numbers (eg $p<1/n$ where n is number of numerical samples).

Experiment:
Again, evidence that this is a controlled comparison would strengthen the paper. For example, data

that the maximal and baseline expression, as well as cell-cell variation, in the two constructs is as close as possible (except of course for the protein whose regulation was shifted in a way central to the different circuit designs).

Can you comment on the potential reason for the reduced fitness of the symmetric construct? Is it protein cost of the target, or changes in response time, or some other effect?

Reviewer #2 (Remarks to the Author):

Ratushny et al. describe a positive feedback topology in which a heterodimeric transcription factor upregulates the expression of only one of its subunits. The authors use computational modeling to compare this asymmetric feedback topology (ASSURE) to a symmetric positive feedback (SPF) analog and they suggest that the ASSURE motif confers a competitive advantage in response tuning and robustness, which may have evolutionary implications. I found the topic to be interesting, but I do not feel that the analyses and experiments rigorously support the authors' conclusions.

Major points

1) The authors do not discuss differences between homodimerization and heterodimerization reactions on model sensitivity or robustness, independent of the feedback topology. One would naturally expect these two reactions to have fundamentally different sensitivities to protein concentrations, since the underlying reaction rates are different (with homodimer formation depending on the square of the monomer concentration). Thus, the study of symmetry/asymmetry in the feedback topologies is confounded by differences in the molecular reactions that lead to dimer formation. Furthermore, I do not think that the SPF topology can be recapitulated experimentally by introducing introducing two feedback loops into a heterodimer system because there is still no homodimer reaction step. Thus, the experimental system used by the authors does not appear to capture an important difference between SPF and ASSURE motifs. The authors also do not model the motif in Figure 3B to computationally show how it compares to SPF and ASSURE motifs.

2) The authors suggest that the wild-type single-feedback ASSURE motif in the yeast Oaf1p/Pip2p system confers a competitive advantage over yeast engineered with a system containing two positive feedback loops due to something intrinsic to the ASSURE motif. I am not convinced that the data support this claim. An alternative (perhaps even likelier) explanation is that cells with two positive feedback loops have an increased burden of protein synthesis and therefore grow more slowly compared to cells with just one feedback, so the two-feedback cells will be diluted over time.

3) Some of the analysis is a bit muddled by focusing on the effects of protein concentration (P) and Kd separately. The key parameter would appear to be the ratio, P/Kd, so the presentation would be clearer and more succinct by focusing on response relative to this ratio. I am not sure that I would consider Kd values ~10^(-7) M to constitute 'high' affinities (p.5), but again the important parameter is P/Kd. If it has been shown that P is much greater than Kd in these examples, then the absolute Kd value does not really matter. Similarly, in Figure 2H, it would be informative to use P/Kd instead of 'low Kd' and 'high Kd'.

4) When parameters in the SPF and ASSURE models are varied, the authors suggest that the ASSURE response is more robust because it has a significantly faster mean response time (p.6). Why is the mean speed of the response considered as the measure of robustness? Why isn't the variance of the distribution of response times a more appropriate metric?

Minor points

5) The information in Figure 1 is redundant with information given Table 1 and Figure 2, so these three display items could be streamlined into two more concise ones. For example, I think it would be helpful to the reader if the authors could add a column to Table 1 and state whether each motif is ASSURE I or ASSURE II. These general schematics would then only be needed in Figure 2.

6) Figure 2C,D are cited before Figure 2A,B in the main text.
7) Figure 2E could be removed.
8) Figure 2E,F need x-axis labels.

9) In Figure S2, why do the steady-state levels exceed the level of the limiting protein?

Reviewer #3 (Remarks to the Author):

The authors perform a numerical in silico analysis of an "asymmetric" positive feedback loop (PFL), defined as a PFL where a protein needs to dimerize with another partner which is found "out of the loop" in order for the PFL to be active. The in-silico analysis is then partially corroborated by experimental evidences obtained by modifying a naturally occurring asymmetric PFL in yeast.

The topic of this work is interesting, but there are some parts of this work which in my opinion need to be further clarified, as I will detail below. However my othermajor concern regards both the model and the numerical analysis.

Regarding the model, the Eq.s at page 14 and 15 are not intuitive at all and the functional form of d(.) is very unusual and the authors do not offer any explanation of its derivation. I guess it comes from a dimerisation reaction, but the authors should say that explicitely. Moreover is it not clear to me why there is a nested function, that is f(d(d(.))). I am sure there is a good reason but a derivation of the equations should be given in the suppl. material.

Regarding the numerical analysis of the model, in my opinion it would add more support to the conclusion if the authors had performed also a well known analysis to investigate the dynamics of the system, such as bifurcation analysis. For example one of the main feature of a PFL is its bistability, and it would interesting to know how, and if, bistability is different between a standard PFL and the ASSURE motif. This kind of analysis is standard and the authors should perform it. Moreover, it would be useful to add a parameter sensitivity analysis, to corroborate the authors'claim about the robustness of the ASSURE motif compared to the PFL. Again this is a standard analysis implemented in well known tools such as PottersWheel.

Some other claims about the ASSURE motif are not convincing yet; for example, the authors claim that:

(1) The ASSURE motif is faster than the PFL. This conclusion is drawn by random parametrisation of the model as shown in Figure 2I. However, the two distributions of tau_0.5 seem to be centered aroung the same peak (i.e. mean tau_0.5 is the same) but the PFL has a longer tail than the ASSURE motif. This causes the significance (low p value) but it does not mean that the difference in tau_0.5 is biologically significant, since the effects even in simulation are so small. A more convincing evidence of the difference in tau_0.5 are in Figure 2H in the case of high Kd, however the slow down in the PFL as compared to ASSURE in this case, is probably due to the differences in the bistability region between the two motifs (ASSURE and PFL), hence the importance of this analysis.

(2)In the experimental results Fig. 3D the authors claim that the response is slower in the symmetrical PFL compared to the WT ASSURE motif, however it would be interesting to quantify the rise time and the rate of increase of fluorescence, which can be easily done from their experimental data, since to me they appear very similar.

(3) The authors claim that the ASSURE motif gives a fitness advantage. In my opinion this claim cannot be done since the fitness disadvantage of the engineered strain is to be expected when compared to a WT strain.

Minor points:

A recent paper in the same journal (PLOS Comp Biol) has been published describing a theoretical and experimental analysis of a PFL in mammalian cells (Siciliano V et al, PLOS COMP BIOL). This work should be cited by the authors.

Reviewer #1 (Remarks to the Author):

This paper studies a new network motifs:
Positive feedback carried out by a heterodimer, such that only one of the monomers has positive autrogerulation- named ASSURE by the authors.
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This is an important paper due to the large number of appearances of this motif across organisms. The combination of math and experiment make this an excellent paradigm for studies of network motifs. I like the competition experiments in varying environments.

I warmly recommend publication and suggest the following suggestions for improvement (i stress that no additional experiments are essential).

Mathematical modeling:
The authors nicely compare ASSURE to all reasonable simpler circuits- those with no auto-regulation and symmetric regulation. It would help if they clarify better what effort was made to make a controlled comparison. I recommend putting this in the context of 'mathematically controlled comparison' (a term coined by Savageau, described in Alon's book An Into to Systems Biology, CRC press): keeping the largest number of internal and external parameters equal between the two circuits compared (eg protein production/decay rates, Kds are internal parameters, dynamic range is an external parameter).
The Results section has been revised to clarify that model comparisons were mathematically controlled in the sense defined by Savageau.

Can an intuitive reason for increased robustness and faster response be better explained?
To improve clarity, we have revised the explanation for the faster response and improved robustness of the ASSURE vs. SPF networks, in the Results section. To better contextualize the robustness property, we have added a figure showing the sensitivity of the response time with respect to the dimer affinity (Supplementary Figure 4 in revised manuscript). Intuitively, the faster response and the robustness of the ASSURE motif to changes in K_d is likely due to the imbalance in the abundance of P_1 and P_2, as we explain in more detail in the revised manuscript.

Since natural selection is at play, one may assume that optimal (or near optimal) parameters can arise: in optimal parameters (that is, given controlled comparison of all possible parameters, the parameters that optimize a feature such as response time)- is the response time and robustness of symmetric and asymmetric circuits different? By what amount? Can this be found analytically? Data in the SI seem to show that the optimal parameters give similar behavior to the two circuits, but I may have misunderstood.
We agree with the reviewer that one can possibly find such parameter sets for both (symmetric and asymmetric) systems when the system responses are very similar and call these “optimal

parameters". However, we believe an important point of this work is that, when one investigates the possible system evolution, one must understand how certain characteristics of the system are sensitive or tolerant to parameter changes. Therefore, we quantitatively explored how K_d and other critical parameters would affect the response time of the system and its sensitivity/robustness to the parameter perturbations. A key point of the MS is that the potential "optimal parameter" range for the asymmetric positive feedback system is wider than for the symmetric one facilitating evolvability of the ASSURE system.

p-values such as E^-61 are too small to be measured numerically- and so were estimated by a statistical model- it would be good to be conservative with such numbers (eg p<1/n where n is number of numerical samples).

We agree with the reviewer's suggestion and now provide a more conservative estimate of the p-value in Figure 2, as $P < 10^{-4}$.

Experiment:

Again, evidence that this is a controlled comparison would strengthen the paper. For example, data that the maximal and baseline expression, as well as cell-cell variation, in the two constructs is as close as possible (except of course for the protein whose regulation was shifted in a way central to the different circuit designs).

We have now added this statement in the Materials and Methods – Strain construction:

> "The wild type and engineered strains are genetically identical, with the exception of the insertion of the *PIP2* promoter at the 5' end of the OAF1 ORF (and an HPH drug resistance gene upstream of the ectopic *PIP2* promoter) in the engineered strain, which generates the SPF I topology (Supplementary Figure 12) with respect to the oleate responsive transcription factors, Oaf1 and Pip2."

We also performed a semi-quantitative western blot analysis of the levels of Oaf1 in the engineered strain and Pip2 in the wild type strain (Reviewer Figure 1). We find levels of these transcription factors are comparable, with Oaf1 under the control of the *PIP2* promoter in the engineered strain mimicking the expression profile of Pip2 in the wild type strain.

The FACS analysis we performed does give readouts of individual cells (10,000 cells per time point) and we have a matrix of experimental conditions that we tested for expression of the Pot1-GFP protein. We now reference this, as well as adding an additional Supplementary Figure. In the results (page 9):

> "The oleate response of the WT and engineered strains was measured at the protein level using flow cytometry, replicated 6 times over a matrix of varying conditions (Figure 3D and Supplementary Figures 13 and 14)."

And in the Materials and Methods (page 16):

> "Six replicates of each strain were done over a matrix of experimental conditions, varying pre-culture conditions (2% glucose or 0.15% glucose), cell densities (OD_{600} of 0.4, 0.8, or 1.6) and oleate concentrations (0.2% or 1%)."

We also performed multiple competitive growth analyses of the wild type and SPF I strains and found that the engineered strain showed no defect in the presence of glucose. This has been included as Supplementary Figure 15 (see our response to Reviewer 3 below). We also now refer to this experiment in the text (page 10):

> "Under glucose growth conditions no fitness disadvantage for the engineered strain was observed (Supplementary Figure 15)."

Can you comment on the potential reason for the reduced fitness of the symmetric construct? Is it protein cost of the target, or changes in response time, or some other effect?

We thank the reviewer for this suggestion. We have added a paragraph on the potential reason for the reduced fitness of the symmetric construct in the Discussion section on page 11. See also response to Reviewer 2 – below.

Reviewer #2 (Remarks to the Author):

Ratushny et al. describe a positive feedback topology in which a heterodimeric transcription factor upregulates the expression of only one of its subunits. The authors use computational modeling to compare this asymmetric feedback topology (ASSURE) to a symmetric positive feedback (SPF) analog and they suggest that the ASSURE motif confers a competitive advantage in response tuning and robustness, which may have evolutionary implications. I found the topic to be interesting, but I do not feel that the analyses and experiments rigorously support the authors' conclusions.

Major points

1) The authors do not discuss differences between homodimerization and heterodimerization reactions on model sensitivity or robustness, independent of the feedback topology. One would naturally expect these two reactions to have fundamentally different sensitivities to protein concentrations, since the underlying reaction rates are different (with homodimer formation depending on the square of the monomer concentration). Thus, the study of symmetry/asymmetry in the feedback topologies is confounded by differences in the molecular reactions that lead to dimer formation. Furthermore, I do not think that the SPF topology can be recapitulated experimentally by introducing two feedback loops into a heterodimer system because there is still no homodimer reaction step. Thus, the experimental system used by the authors does not appear to capture an important difference between SPF and ASSURE motifs. The authors also do not model the motif in Figure 3B to computationally show how it compares to SPF and ASSURE motifs.

We had previously addressed the nature of homo versus heterodimer formation in the Supplementary Materials; however, as suggested, we have expanded on this original analysis. In Supplementary Figure 3 we model the responses of both homo and heterodimerization for both low and high affinity interactions (panels A and B respectively). With low affinity, the system would have higher heterodimer than homodimer levels within the "ASSURE rescue zone" (Supplementary Figure 3A). With increasing affinity between interacting partners (homo or hetero) the differences in dimer levels decreases to equivalent levels within the ASSURE rescue zone. We further model the level of transcriptional responses of homo and heterodimers at high and low K_d (Figure 2H and Supplementary Figure 3C and D).

This analysis has now been expanded upon in order to address any concerns regarding (1) the mathematical equivalency of homo and heterodimers and (2) the ability to experimentally test the SPF system. Supplemental Figure S12 examines the SPF I system, mimicking the engineered strain presented in this manuscript. This analysis importantly demonstrates that, if the levels of each heterodimer partner degrade at equivalent rates, the response profiles of the SPF I motif follows that of the SPF motif precisely. Alterations to the levels of interacting proteins P_1 versus P_2 do generate some shifts in the response but retain a very similar profile compared to that seen for SPF. These responses were modeled at both high (Supplementary Figure 12B, C and D) and low (Supplementary Figure 12E and F) K_d values. The synthesis of both Oaf1 and Pip2 would be expected to occur at the same rate in the engineered (SPF I strain), as they are both under the control of the same promoter. Literature values for the half-life of Oaf1 and Pip2 are quite similar. Oaf1p protein half-life (55 min; Beyer *et al.*, MCP, 2004) is slightly longer than Pip2p protein half-life (40 min; Belle *et al.*, PNAS, 2007). This difference in protein half-life may be compensated for by the opposite differences in the half-life of the corresponding mRNAs (15 min for OAF1 mRNA and 22 min for PIP2 mRNA; Holstege *et al.*, Cell, 1998), increasing our confidence that the engineered system closely mimics the symmetric feedback network. We have now described this explicitly in the text on pages 8 and 9 along with the additional analysis of the SPF I model presented in Supplemental Figure S12.

2) The authors suggest that the wild-type single-feedback ASSURE motif in the yeast Oaf1p/Pip2p system confers a competitive advantage over yeast engineered with a system containing two positive feedback loops due to something intrinsic to the ASSURE motif. I am not convinced that the data support this claim. An alternative (perhaps even likelier) explanation is that cells with two positive feedback loops have an increased burden of protein synthesis and therefore grow more slowly compared to cells with just one feedback, so the two-feedback cells will be diluted over time.

To experimentally address this concern we used a competitive growth assay in which the cells were incubated in oleate for only short periods of time (3-6 hours) and then allowed to recover in 0.15% glucose. Based on our FACS data (Figure 3D) at these shorter time periods, the wild type strain has an increased protein synthesis burden (of oleate-responsive targets of the Oaf1/Pip2 dimer) relative to the engineered strain, yet as seen in Reviewer Figure 2, the engineered strain is

still competitively disadvantaged relative to the wild type strain. This defect is specific to growth in oleate as seen in Supplementary Figure 15, in which no competitive growth defect is seen in growth in glucose. This suggests that an increased protein synthesis burden is not the reason for the disadvantage.

A key argument presented in this manuscript is that the transcriptional response profile of the ASSURE motif confers a specific, advantageous response strategy to the cell, which we believe with the benefit of modeling is evident and leads to downstream effects that disadvantage cells with SPF I. One such downstream effect speculated on by this reviewer is protein synthesis burden. While the new experiments were designed to mitigate this possibility, we do note this this possibility in the Discussion:

> "Another key difference between these motifs is the ability to control the level of the response. More of the reporter protein Pot1-GFP was observed in the engineered strain at later stages of induction, which may also underlie the oleate specific competitive disadvantage of this strain."

Additional downstream effects of transcriptional dynamics are (perhaps more) likely. For example, altered dynamics of peroxisome biogenesis, which is required to metabolize oleic acid, would be expected to cause a significant disadvantage and we also now present this possibility in the Discussion:

> "These altered transcriptional rates likely result in a fitness disadvantage through a multitude of downstream effects such as delayed organelle biogenesis, potential increases in reactive oxygen species at early time points or a potential increase in the translational burden at later time points. Accordingly, delayed expression of targets of Oaf1p and Pip2p, conferred by, for example deletion of the histone variant Htz1 (Saleem et al, 2010; Wan et al, 2009) lead to delay in biogenesis and reduced viability in oleate conditions (Lockshon et al, 2007; Smith et al, 2006)."

A critical question is the origin of the oleate specific decrease in viability seen in the engineered strain. We have predicted, through model analyses, and demonstrated, through experimentation, that the SPF I motif alters transcription. Given the genetic identity of the two strains, the predictive power of our model and the experimental evidence presented herein, the results and interpretation in the context of the current literature indicate that the origin of the competitive disadvantage lies in the altered transcription (predicted and validated), and this altered transcription is paramount to downstream effects that contribute to the different resulting phenotypes.

3) Some of the analysis is a bit muddled by focusing on the effects of protein concentration (P) and Kd separately. The key parameter would appear to be the ratio, P/Kd, so the presentation would be clearer and more succinct by focusing on response relative to this ratio. I am not sure that I would consider Kd values ~10^(-7) M to constitute 'high' affinities (p.5), but again the important parameter is P/Kd. If it has been shown that P is much greater than Kd in these examples, then the absolute Kd value does not really matter. Similarly, in Figure 2H, it would be informative to use P/Kd instead of 'low Kd' and 'high Kd'.

While we appreciate the utility of the reviewer's suggestion in similar experimental systems, in particular a homodimer at steady state, the modeling results of Figure 2H clearly show that the SPF model is not in the K_d-insensitive regime. We have chosen to present the data with protein concentrations and K_d values separately for the above reason as well as the following additional reasons:

1. We are focused on the Oaf1-Pip2 heterodimer. We would have to introduce two ratios, P_1/K_d and P_2/K_d. For this reason alone we feel that that data are best presented as we chose to present it.
2. We are dealing with dynamic protein concentrations, so again we would need further ratios to define different points in the induction, which we believe readers would find unnecessarily confusing.
3. We explicitly provide all the necessary quantities (e.g. protein levels and K_d values we used in the model) in the text, which can be used to calculate (homo-)heterodimer levels. The formula is also provided.

In the interest of providing complete information to enable determination of P/K_d, we explicitly calculated the (homo-)heterodimer level for two representative sets of these quantities (Supplementary Figure 3).

4) When parameters in the SPF and ASSURE models are varied, the authors suggest that the ASSURE response is more robust because it has a significantly faster mean response time (p.6). Why is the mean speed of the response considered as the measure of robustness? Why isn't the variance of the distribution of response times a more appropriate metric?

We apologize for the confusion. We do not consider the absolute response time as a measure of robustness *per se*. When we refer to the robustness of the ASSURE network's response time with respect to K_d changes, we are referring to the parametric sensitivity of $\tau_{0.5}$ with respect to K_d, which can be calculated as $d\log(\tau_{0.5})/d\log(K_d)$ or as $\Delta\tau_{0.5}$. We have edited the text on page 6 to clarify this point. Regarding the distribution of $\tau_{0.5}$ across an ensemble of random perturbations of model parameters, we have also added a sentence in the text stating that "the coefficient of variation of the ASSURE $\tau_{0.5}$ distribution is 21.4% lower than the coefficient of variation of the SPF $\tau_{0.5}$ distribution".

Minor points

5) The information in Figure 1 is redundant with information given Table 1 and Figure 2, so these three display items could be streamlined into two more concise ones. For example, I think it would be helpful to the reader if the authors could add a column to Table 1 and state whether each motif is ASSURE I or ASSURE II. These general schematics would then only be needed in Figure 2.

We have added the proposed additional column to Table 1.

6) Figure 2C,D are cited before Figure 2A,B in the main text.

We removed this citation.

7) Figure 2E could be removed.
We feel that this figure increases clarity and propose to leave it in.

8) Figure 2E,F need x-axis labels.
This has been fixed.

9) In Figure S2, why do the steady-state levels exceed the level of the limiting protein?
In the model, the steady-state level of the target is defined by the balance between the synthesis and degradation rates (the synthesis rate being affected by the limiting protein) and can be more or less than the level of the limiting protein.

Reviewer #3 (Remarks to the Author):

The authors perform a numerical in silico analysis of an "asymmetric" positive feedback loop (PFL), defined as a PFL where a protein needs to dimerize with another partner which is found "out of the loop" in order for the PFL to be active. The in-silico analysis is then partially corroborated by experimental evidences obtained by modifying a naturally occurring asymmetric PFL in yeast.

The topic of this work is interesting, but there are some parts of this work which in my opinion need to be further clarified, as I will detail below. However my other major concern regards both the model and the numerical analysis.

Regarding the model, the Eq.s at page 14 and 15 are not intuitive at all and the functional form of d(.) is very unusual and the authors do not offer any explanation of its derivation. I guess it comes from a dimerisation reaction, but the authors should say that explicitely. Moreover is it not clear to me why there is a nested function, that is f(d(d(.))). I am sure there is a good reason but a derivation of the equations should be given in the suppl. material.
It is correct. $d(x, y, K)$ is a solution of the quadratic equation $(x-d)(y-d) = Kd$ that is derived from the steady-state assumption for the reversible biomolecular reaction $x + y \overset{K}{\Leftrightarrow} d$. In the case of a homodimer, $x = y$. We use the nested function *f(d(d(.)))* to compactly formulate the mathematical models. *f(z)* represents a fractional activity of the target gene which is a function of the activator *z*. *f(z)* is calculated using the following equation:

$$f(z) = \frac{k_0 + \left(\frac{z}{k_a}\right)^h}{1 + \left(\frac{z}{k_a}\right)^h}.$$

For example, $f(d(d(s,P_1,K_{sp}),P_2,K_d))$ represents the fractional activity of the ASSURE I target gene that is a function of the concentration of the heterodimer between P_2 and another heterodimer between s and P_1 in the model with K_d and K_{sp} dissociation constants, respectively. We have added more detailed explanation of the model equations and how they are derived into Mathematical modeling section of Materials and Methods.

Regarding the numerical analysis of the model, in my opinion it would add more support to the conclusion if the authors had performed also a well known analysis to investigate the dynamics of the system, such as bifurcation analysis. For example one of the main feature of a PFL is its bistability, and it would interesting to know how, and if, bistability is different between a standard PFL and the ASSURE motif. This kind of analysis is standard and the authors should perform it. Moreover, it would be useful to add a parameter sensitivity analysis, to corroborate the authors'claim about the robustness of the ASSURE motif compared to the PFL. Again this is a standard analysis implemented in well known tools such as PottersWheel.

We thank the reviewer for the suggestions. Bifurcation analysis of the SPF and ASSURE models revealed that both models have bistable responses for a particular range of parameters. Importantly, and in support of our contention that the ASSURE motif is advantageous, the ASSURE system shows bistability over a significantly smaller range of k_a and K_d parameter values compared to the SPF model (Supplementary Figure 11). Advantages conferred by this this property of the ASSURE system may include, preserving, the system's monostability and controllability over a wide range of heterodimer affinities (K_d) and activities (k_a) that are subject to change during evolution. To illustrate this interesting result we have added an additional Supplementary Figure 11 and a paragraph in the Results section of the manuscript.

The global parameter sensitivity analysis with respect to the response time model quantity is presented in Supplementary Figure 9 and discussed in the manuscript in the context of system evolvability. We have also performed a local parameter sensitivity analysis for the SPF and ASSURE model response time dependence on K_d values (Supplementary Figure 4). This analysis demonstrates that the effect of varying K_d on the sensitivity of the $\tau_{0.5}$ with respect to K_d ($d\log(\tau_{0.5})/d\log(K_d)$) is significantly larger for the SPF model compared to the ASSURE model.

Some other claims about the ASSURE motif are not convincing yet; for example, the authors claim that:

(1) The ASSURE motif is faster than the PFL. This conclusion is drawn by random parametrisation of the model as shown in Figure 2I. However, the two distributions of tau_0.5 seem to be centered aroung the same peak (i.e. mean tau_0.5 is the same) but the PFL has a longer tail than the ASSURE motif. This causes the significance (low p value) but it does not mean that the difference in tau_0.5 is biologically significant, since the effects even in simulation are so small. A more convincing evidence of the difference in tau_0.5 are in Figure 2H in the case of high Kd, however the slow down in the PFL as compared to ASSURE in this case, is

probably due to the differences in the bistability region between the two motifs (ASSURE and PFL), hence the importance of this analysis.

The analysis that we present in the manuscript allows us to understand and predict quantitative characteristics of the SPF and ASSURE systems. We have shown that there are parameter set ranges when the SPF and ASSURE responses differ by the level of response and $\tau_{0.5}$. We have also shown that there are parameter set ranges where the SPF and ASSURE responses are almost identical. To show in an unbiased manner that the ASSURE system has a more rapid response compared to the SPF system, we chose extended ranges of the random parameter sets (Supplementary Table 1) that include all scenarios that were discovered in the manuscript. Please note that Figure 2I should be viewed with Supplementary Figure 9B that represents the response time difference between the SPF and ASSURE models ($\tau_{0.5}^{SPF}$ - $\tau_{0.5}^{ASSURE}$) and shows that the majority of $\Delta\tau_{0.5}$ is positive. Additionally, it is a key point of the manuscript that we now quantitatively understand what makes $\Delta\tau_{0.5}$ close to zero or drastically high.

(2)In the experimental results Fig. 3D the authors claim that the response is slower in the symmetrical PFL compared to the WT ASSURE motif, however it would be interesting to quantify the rise time and the rate of increase of fluorescence, which can be easily done from their experimental data, since to me they appear very similar.

We thank the reviewer for the suggestion, and have added the estimated response time for WT ($\tau_{0.2}$ ~ 3 h) and the engineered strain ($\tau_{0.2}$ ~ 8 h) to the manuscript.

(3) The authors claim that the ASSURE motif gives a fitness advantage. In my opinion this claim cannot be done since the fitness disadvantage of the engineered strain is to be expected when compared to a WT strain.

This is an excellent point by the reviewer, and is addressed above in Response to Reviewer 2. To reiterate somewhat, these strains are identical with the exception of the insertion of the *PIP2* promoter at the 5' of the *OAF1* gene and an HPH cassette upstream from the ectopically positioned *PIP2* promoter, which we used to select for the insertion of the promoter. In order to address concerns regarding the nonspecific viability of the engineered strain, we ran an extensive competitive growth analysis in a constant glucose condition (0.15% glucose). In our oleate competitive growth defect we see complete elimination of the engineered strain by 72 h of incubation in oleate. In the absence of oleate, however, we see no fitness disadvantage for the engineered strain compared to wild type. We have included these data as Supplementary Figure 15. We also refer to this experiment in the text (page 9):

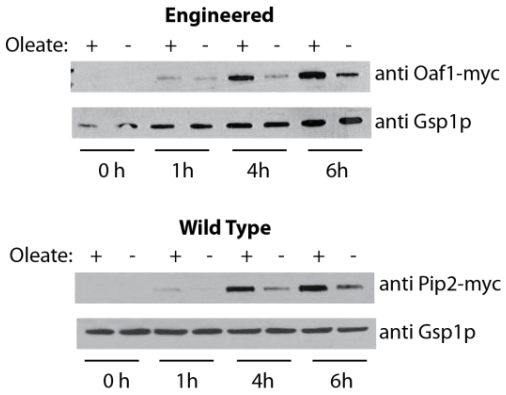
> "Under glucose growth conditions no fitness disadvantage for the engineered strain was observed (Supplementary Figure 15)."

Minor points:

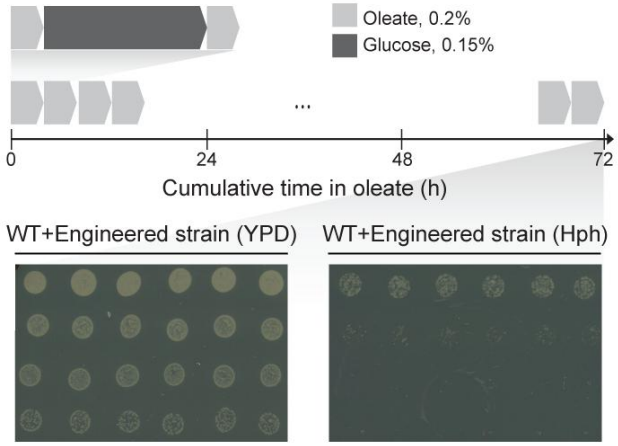
A recent paper in the same journal (PLOS Comp Biol) has been published describing a theoretical and experimental analysis of a PFL in mammalian cells (Siciliano V et al, PLOS COMP BIOL). This work should be cited by the authors.

Thank you for alerting us to this paper; it is now cited appropriately in the Introduction.

Reviewer Figures



Reviewer Figure 1. Expression of Oaf1 under the control of the *PIP2* promoter in the engineered strain mimics the expression of Pip2 in the wild type strain over a 6 h period of oleate incubation.



Reviewer Figure 2. The ASSURE network confers a competitive fitness advantage when exposed to short oleate pulses. Cells from wild type and engineered strains were mixed 1:1 and grown under time varying oleate and glucose conditions. Cultures were incubated in oleate for short periods of time (3-6 hours) and then allowed to recover in 0.15% glucose – conditions that do not place a burden of protein synthesis from oleate-responsive genes on the engineered strain. Multiple replicates of equal volumes of dilutions were plated onto YPD and YPD with 400 ug/ml Hygromycin B (Sigma) medium, to select for the engineered strain (see Material and Methods). Based on the FACS data (Figure 3D). Under these conditions the wild type strain is expected to have an increased protein synthesis burden relative to the engineered strain, yet the engineered strain is still competitively disadvantaged relative to the wild type strain.

2nd Editorial Decision 27 February 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 your revised manuscript. As you will see from the reports below, the referees felt that the revisions made had substantially improved this work. Both referees have, however, important remaining concerns, which, I am afraid to say, preclude publication of this work in its present form.

In general, Molecular Systems Biology only allows a single round of major revision. But given that these remaining concerns may be addressable with additional clarification and some new analyses, we would like to offer you a final chance to submit a revised work. The editor would like to highlight, in particular, points #1 and #2 by the second reviewer, which this reviewer seemed to feel were important enough to cast some doubt on the physiological relevance of the conclusions derived from the model-based comparisons of the ASSURE and SPF motifs. These points, and the others raised by the reviewers, will need to be convincingly addressed before this work would be appropriate for publication at Molecular Systems Biology.

When preparing your revised work, we also ask that you address the following content and format issues:

1. Molecular Systems Biology generally requires that authors provide biological models in a common machine readable format as supplementary material, and encourages the use of SBML and deposition at a public repository (e.g. BioModels), whenever possible.

2. Please provide a 'standfirst text' summarizing your study in one or two sentences (approx. 250 characters), as well as, three to four 'bullet points' highlighting the main findings.

3. Please include an author contributions statement after the Acknowledgements section (see http://www.nature.com/msb/authors/index.html#Submission)

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. We reserve the right to send any revised manuscript back to one or both of these reviewers, and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

*** 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).

Sincerely,

Editor - Molecular Systems Biology
msb@embo.org

Referee reports:

Reviewer #1 (Remarks to the Author):

This is a revised version of the manuscript thatI had previously reviewed. The authors have addressed all the points I raised in my previous review. However, I am still not completely convinced by the fitness advantage of the ASSURE motif, also because a mechanistic explanation of why the ASSURE motif should be better in the case of the oleate response is not given.

Reviewer #2 (Remarks to the Author):

The authors have done a good job in addressing my previous comments and the inclusion of new simulations and experiments has substantially improved the manuscript. However, there are still a few points that should be addressed.

1) The simulations of the SPF topology are still not fully clear to me. In particular, I do not know what concentrations of P are being used (and how this lends itself to appropriate comparison to the analogous ASSURE simulations). For example, in Supplemental Figures 3A and 3B, the homodimer level almost always exceeds P/2, which suggests that either the x-axis does not represent total P or there is something wrong with the mass balance of P in the model.

2) An important feature of the ASSURE motif seems to be that one component of the heterodimer is constant and limiting, which constrains response times and steady-state levels, whereas such a constraint does not exist in the SPF model. However, it seems that constraints on the cellular machinery involved in feedback may serve the "constant and limiting" role in the SPF motif, which may make the computationally observed differences in robustness more modest in an actual cell.

3) Based on Supplemental Figure 11 for ka=40, it appears that the steady-state SPF response should be bistable with respect to Kd. The shape of the SPF curve in Supplemental Figure 8 suggests that this response is also bistable, so the full bistable response should be shown in this figure.

4) The results in Supplemental Figures 13 and 14 should be more clearly explained. What does the OD600 value in each plot represent (starting value, constant OD value, etc.)? What is the interpretation of these results (e.g., why does OD600=0.4 give similar responses at short times)? Also, it appears that the data in Supplemental Figure 13 is entirely contained within Supplemental Figure 14, so the presentation could be condensed into one figure (perhaps using broken axes to show the final time point).

Reviewer #1 (Remarks to the Author):

This is a revised version of the manuscript that I had previously reviewed. The authors have addressed all the points I raised in my previous review. However, I am still not completely convinced by the fitness advantage of the ASSURE motif, also because a mechanistic explanation of why the ASSURE motif should be better in the case of the oleate response is not given.

We propose that the mechanism by which the wild-type (ASSURE network) confers a growth advantage over the engineered strain on alternating oleate/glucose media is as follows:

1. Peroxisome biogenesis is required for the yeast *Saccharomyces cerevisiae* to grow when oleic acid is presented as the sole carbon source (Lockshon et al., 2007; Saleem et al., 2008; Saleem et al., 2010; Smith et al., 2006).
2. Induction of the oleate-sensing gene regulatory network is required to produce peroxisomes (Saleem et al., 2008; Saleem et al., 2010; Smith et al., 2007).
3. Wild type yeast, whose oleate-sensing network has the ASSURE motif, induce downstream genes in oleate conditions faster than the engineered (SPF) strain (Figure 3D).
4. In an environment which is switching back and forth between oleic acid as the sole carbon source and glucose as sole carbon source, the organism has to repeatedly activate the peroxisome biogenesis gene regulatory network and induce peroxisomes.
5. Over multiple cycles, the response of the engineered strain is slower, the cells are less efficient at utilizing oleic acid and therefore, do not grow as well as wild type cells when oleic acid is the sole carbon source (Figure 3E).

In addition to our explanation in the Discussion section we have also expanded our interpretation of the mechanism in the Results section (pages 10-11).

Reviewer #2 (Remarks to the Author):

The authors have done a good job in addressing my previous comments and the inclusion of new simulations and experiments has substantially improved the manuscript. However, there are still a few points that should be addressed.

1) The simulations of the SPF topology are still not fully clear to me. In particular, I do not know what concentrations of P are being used (and how this lends itself to appropriate comparison to the analogous ASSURE simulations). For example, in Supplemental Figures 3A and 3B, the homodimer level almost always exceeds P/2, which suggests that either the x-axis does not represent total P or there is something wrong with the mass balance of P in the model.

We thank the reviewer for bringing this to our attention. The *x*-axis of Supplementary Figures 3A and B represents half of total *P*. The figure is appropriately corrected now.

2) An important feature of the ASSURE motif seems to be that one component of the heterodimer is constant and limiting, which constrains response times and steady-state levels, whereas such a constraint does not exist in the SPF model. However, it seems that constraints on the cellular machinery involved in feedback may serve the "constant and limiting" role in the SPF motif, which may make the computationally observed differences in robustness more modest in an actual cell.

Constraints associated with the general machinery of protein expression (translation, folding, translocation to the nucleus, activation, and binding to DNA) are accounted for in the models by representing the fractional activity of genes using a saturation function. This point is addressed now in the results section of the manuscript (page 4). It is possible that aspects of the transcriptional activation machinery (such as, an obligate cofactor that is constitutively expressed) may exert a net constraint on positive feedback in the context of the SPF model. However, within the context of our model, if such a constraint were significant and limiting, the SPF network would have a temporal response that is similar to the ASSURE network. In contrast we observed a substantial difference in the temporal responses of the *POT1* reporter in the wild-type and engineered strains in a glucose to oleate transition (Fig. 3D); the wild-type strain clearly induces more rapidly than the engineered strain.

3) Based on Supplemental Figure 11 for ka=40, it appears that the steady-state SPF response should be bistable with respect to Kd. The shape of the SPF curve in Supplemental Figure 8 suggests that this response is also bistable, so the full bistable response should be shown in this figure.

We thank the reviewer for pointing this out. Indeed the steady-state SPF response is bistable with respect to K_d for this parameter set. We have corrected the figure.

4) The results in Supplemental Figures 13 and 14 should be more clearly explained. What does the OD600 value in each plot represent (starting value, constant OD value, etc.)? What is the

interpretation of these results (e.g., why does OD600=0.4 give similar responses at short times)? Also, it appears that the data in Supplemental Figure 13 is entirely contained within Supplemental Figure 14, so the presentation could be condensed into one figure (perhaps using broken axes to show the final time point).

The OD_{600} of the culture is at the start of oleate incubation. We have revised the caption to Supplementary Figures 13 and 14 (which are now combined) to further clarify the experiment. We sought to test the effect of the ASSURE motif over a matrix of conditions. To this end we were able to manipulate three parameters; the concentration of cells, the concentration of oleate used in the induction process, and the concentration of glucose used in the pre-culture conditions.

Clearly there is some effect of the conditions in the response and importantly, all the responses observed over the matrix of conditions were within the range of response profiles predicted by the mathematical model. The differences in responses over the matrix of experimental conditions gives us further confidence of the ability of the model to predict cellular outputs in the ASSURE motif. With respect to the reviewers concerns regarding the response observed for OD_{600} 0.4, the OD_{600} of the culture is not a parameter in the model and we cannot directly determine what parameters are inherent to a particular OD_{600}. However there are parameter sets (e.g. particular binding affinities or degradation rates) which yield model responses like that observed for OD_{600} 0.4 (see Figures 3F, G and H and Supplementary Figures 12E and F). This particular condition possibly changes one or several of those parameters that are critical for the observed type of response.

Summary of Changes to Manuscript

We have updated the manuscript to address the reviewers' concerns, as described below.

•Supplementary Figures 13 and 14 have been combined. The legend of the combined figure now reads:

> **Supplementary Figure 13.** Response of the wild-type (blue) and engineered (red) budding yeast cells to oleate induction over a matrix of indicated experimental conditions. Shown are line plots of the early time points of induction and bar plots for the 21 h time point. The percentage glucose used for preculture, the percentage of oleate used during induction, and the optical density (OD_{600}) of the culture at the start of oleate incubation are indicated in the panel. OD_{600} values of 0.4, 0.8 and 1.6 represent early, mid and late log phase growth of the cultures. The time course of Pot1p protein level was measured in individual cells using flow cytometry in the wild-type strain with a Pot1p-GFP reporter and in the engineered strain with the same reporter under oleate growth conditions. The *y*-axis is the $\log_{10}$ level of fluorescence. Error bars represent standard error of means of Pot1p-GFP protein level from six independent replicates.

•In the results section of the manuscript (pages 9-10) we have now added:

> The oleate response of the WT and engineered strains was measured at the protein level using flow cytometry, replicated 6 times over a matrix of varying conditions (Figure 3D and Supplementary Figures 13). Despite the potential for additional control mechanisms operating in the context of the larger cellular network, for example the differences in response times between the early log phase and mid/late log phase cultures (Supplementary Figure 13), the *in vivo* responses measured here mirrored the predictions from the model simulations discussed above.

•In the discussion (page 13) we have now added:

> From an evolutionary standpoint there are many factors that can operate to control levels of the constitutive protein (synthesis, turnover, localization etc.). We expect that these factors contribute in combination to control and buffer systems that contain the ASSURE network motif. Indeed in our experimental exploration of induction parameters by flow cytometry (Supplementary Figure 13), we identified conditions that change the response types within the predicted range of profiles (Figure 3 and Supplementary Figures 12).

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