molecular systems biology

Synthetic conversion of a graded receptor signal into a tunable, reversible switch

Santhosh Palani and Casim A. Sarkar

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Rebuttal:	22 November 2010
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Transaction Report:

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

1st Editorial Decision 12 November 2010

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from two of the three referees whom we asked to evaluate your manuscript, and have decided to render a decision now to avoid further delay. As you will see from the reports below, the first reviewer raised a series of substantial concerns on your work, which, I am afraid to say, preclude its publication.

Most importantly, the first reviewer was not convinced that this work provides a generic strategy for reversible switch design. This reviewer indicates that this design is likely to require specific molecular requirements -- in particular, enzymatic reactions showing zero-order ultrasensitivity -- which could significantly hinder the adaption of this design to other receptor systems. This reviewer also felt that the conceptual connections between the results presented in this work, and the theoretical framework described in Palani & Sarkar 2008 remained insufficiently explored.

Because these concerns raise important doubts about the broad applicability of this design strategy, a key claim of this work, we feel we have no choice but to return this work with the message that we cannot offer to publish it.

Nevertheless, the reviewers expressed interest in the topic. In the event that you can provide conclusive additional evidence demonstrating the generality of this design strategy, including its ability to provide reliable bistability in the absence of saturated enzyme kinetics, then we may be

willing to consider a new submission based on this work. This would have a new number and receipt date. We recognize that this would involve further experimentation and analysis, and we can give no guarantee about its eventual acceptability. However, if you do decide to follow this course then 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 Molecular Systems Biology in the future.

Thank you for the opportunity to examine this work.

Sincerely,

Editor

Molecular Systems Biology

_____

Reviewer #2 (Remarks to the Author):

Synopsis: The authors modify a synthetic two-component signaling pathway (Cre1p from Arabidopsis) in yeast, such that IP-activated Cre1p phosphorylates endogenous response regulator (Skn7p). Activated Skn7p binds to SSRE cis-regulatory motif in a modified MEL1 promoter to express EGFP, a fluorescent reporter of Skn7p activity. The authors modified the two-component signaling pathway by adding two positive feedback loops: (1) SSRE binding sites in SKN7 promoter (transcription factor-mediated feedback), such that activated Skn7p produces more Skn7p and (2) SSRE binding sites in AtCRE1 promoter (receptor-mediated feedback), such that activated Skn7p produces more receptor that activates Skn7p.

Their central result is highlighted in Fig. 2E-F, where they show that TF-mediated feedback can generate bistability. Receptor-mediated feedback by itself cannot generate bistability (simple, reversible switch with nH=2), but it can increase the hysteresis of the TF-mediated feedback. At the bifurcation point, the authors could get very sharp transitions, nH ~ 20.

I have several serious concerns about the manuscript.

1. The authors claim that the dual feedback switch provides rational tuning of ultrasensitivity, amplitude, and bistability: see Abstract, Introduction, Discussion, Figure 1, and Table II. This tunability is highlighted as the significant and novel feature of their work. Yet, the claims of tunability stem from theoretical work that is not appropriately described in the manuscript or Supporting material. The results of Figure 1 and Table II appear magically. This is unacceptable. Furthermore, the experimental data in Fig. 2 do not show any tunability that can be interpreted or understood from first principles. I found it very odd that the authors have a simple, published model that analyzes dual feedback switch (Palani & Sarkar, 2008), yet there was no attempt to relate this model with the measured kinetic and steady state data. Please justify this omission.

2. The abstract claims to describe a "robust, easily constructed network topology (two positive feedback loops) to create highly ultrasensitive responses to a synthetic ligand/receptor complex." I would have to strongly disagree, as neither theory nor experiments support this claim. The authors

never experimentally test the source of ultrasensitivity (e.g. cooperativity, zero-order ultrasensitivity, phosphorylation, etc..) and/or bistability.

If I take the simplified model from Palani & Sarkar, 2008 and set F1=0, F2=0 (no feedback anywhere), then the active TF still exhibits an ultrasensitive response as a function of ligand! Despite the claims in their 2008 abstract "network topology (i.e. two positive feedback loops) suggests a novel mechanism for achieving robust bistability ... without molecular cooperativity", the ultrasensitivity has nothing to do with the presence of two positive feedback loops or the topology of the network. Rather, it has everything to do with the molecular mechanism of the network. Namely, there is zero-order ultrasensitivity (Goldbeter & Koshland) between signaling kinase and phosphatase competing for the same substrate (TF). NOTE: Figure 1a of the current manuscript is misleading: there should also be an arrow from active TF to inactive TF (i.e. lose the phosphate). Indeed, if you look at the parameters in Palani & Sarkar, 2008, the kinase and phosphatase are saturated and exhibit zeroth-order kinetics. Please clarify.

I agree that if zero-order ultrasensitivity is combined with positive feedback loops, then bistability can result as shown in Palani & Sarkar, 2008. However, on pg. 8 of the current manuscript, it would be more accurate to say: "if the basic pathway response were completely Michaelian and the TF feedback exhibited no cooperativity, then zero-order ultrasensitivity would be absolutely necessary to achieve any level of bistability through positive feedback".

3. The authors interpret their experimental results too easily. For example, they state that the P_SSRE-EGFP measurements for different strains are transcription-factor limited or receptor-limited regimes, without demonstrating or testing this claim by measuring abundances or performing ligand / receptor / TF dosage experiments. On pg. 6, the authors state "Increasing the receptor feedback strength (TR-SSRE > SSRE > CYC1) did not change ...". Where is the data indicating that TR-SSRE had higher expression than SSRE than CYC1? Assumption or fact?

4. One observation of Palani & Sarkar, 2008 was that receptor-mediated feedback was more critical than TF-mediated feedback for bistability and ultrasensitivity (at the bifurcation point). Yet, the experimental results in this manuscript appear to contradict this -- the TF-mediated feedback is responsible for bistability whereas receptor-mediated feedback has no effect. Please clarify.

Specific comments:

5. The authors' assertion that their method is more applicable and tunable when compared to other methods (refs on pg. 3) is an exaggeration. Their method relies on specific ligand/receptor-kinase interaction, coupled to saturated enzymatic reactions (zero-order ultrasensitivity) and positive feedback loops to get bistability. These are very specific molecular requirements, and it is unclear that this translates *easily* to other ligand/receptor pairs. Matter of fact, it's unclear to me whether saturated enzyme kinetics is even occurring in their Cre1/Skn7p system (as envisioned in their double feedback model of ligand/receptor).

6. On pg. 7, the authors state "The double feedback strains had the slowest degradation rate (~8.5 hr), which could be due to the higher accumulation of phopho-SKN7 at steady-state or a higher level of residual bound IP due to the higher receptor expression level".

The binding affinity of IP for Cre1 receptor is not in the regime of being effectively irreversible. Romanov et al, J. Exp. Botany 57: 4051 (2006) have measured the Kd of IP for Cre1 receptor, Kd ~20 nM.

7. The last paragraph and Table II are not supported by anything in the manuscript. They should be removed.

8. In Materials and Methods, I was confused why strains were grown in SC galactose. After significant background reading, it became apparent that TM182 strain is sln1-del GAL1-PTP2, which is necessary to abrogate the lethality that results from sln1-del. The authors should write several sentences describing their strain background, their reason for using it, and describing why PTP2 expression is needed.

9. Figure 1 with no accompanying equations and parameters is not useful or reproducible.

10. In Figure S1, I found it strange that the low peak ("OFF" state) of tRtTF (of the bimodal distribution) is lower than the original histogram at the top (i.e. 24 hours, 0.06 uM IP). Supposedly, the top histogram is uninduced by IP (or "OFF"). The flow cytometry data would be more interpretable if the authors included a negative control (TM182) and reporter-only control.

Reviewer #3 (Remarks to the Author):

This is a particularly elegant example of biochemical circuit design for creation of ultrasensitive response to a graded input signal. The authors clearly show, with no fudging that they can tune the behavior of the circuit in a synthetically generated circuit in yeast, while overall characteristics of the circuit response are robust to variation of kinetic parameters.

I'm rather at a loss to find anything specific to criticize about this manuscript; the presentation is elegant and results are crystal-clear. One minor thing the authors may address in he manuscript is to explain explicitly how the exogenous AtCRE1

happens to specifically phosphorylate endogenous SKN7 via the phospho-transfer protein YPD1. Even though published by Lu, et al. it would be good to briefly explain so that readers may not think that some reengineering of components allowed the circuit to work as designed, rather than the explicit network topology as the authors suggest. I believe that this manuscript should be published in MSB.

Additional correspondence 14 November 2010

Thank you for your evaluation of our manuscript. We have carefully gone through all of the comments and we believe that the concerns of Reviewer #2 do not apply to our system, which was intentionally selected to not have these enzymatic reactions such as cascading or

zero-order effects that engender ultrasensitivity. This point was, in fact, appreciated by Reviewer #3. The other points from Reviewer #2, including conceptual links to the model, can also be readily clarified.

In light of this, I am writing to ask if you would be willing to reconsider your decision and either wait for feedback from Reviewer #1 or allow us the opportunity to rebut the current critiques.

I thank you again for your consideration and look forward to hearing from you.

Additional correspondence 19 November 2010

Thank you for your response to our decision letter regarding your work entitled "Synthetic conversion of a graded receptor signal into a tunable, reversible switch." I have now had time to look again at your manuscript and the comments raised by the two reviewers.

I acknowledge that this work provides a potentially interesting case study of a synthetically created receptor-based switch, however, given the concerns raised by Reviewer #2, it seems that the generality of this design strategy remains unclear.

Importantly, Reviewer #2 was not convinced that this design would provide predictable switch-like behavior across different receptor systems. Indeed, your manuscript acknowledges that the CRE1-SKN7 pathway exhibits some basal ultrasensitivity, even in the absence of both transcriptional feedback loops. For these reasons, demonstrating the generality of this approach appears to require additional experimental evidence that this design can generate switch-like behavior in a receptor system with different molecular properties (ideally a system that lacks basal bistability).

For this reason, the presence or absence of zero-order ultrasensitivity in your system does not appear to be the main source of concern. Indeed, Reviewer #2 writes that "it's unclear to me whether saturated enzyme kinetics is even occurring in their Cre1/Skn7p system." Rather this reviewer's main concern appears to be that, since the model presented in Palani & Sarkar 2008 appeared to require zero-order ultrasensitivity, it remains unclear whether the double feedback mechanism presented in this work is sufficient to produce reliable switch behavior in the absence of very specific molecular properties.

As such, we feel that, in the absence of additional convincing evidence supporting the generality of this design approach, this work would be better suited for a more specialized journal.

I hope this helps to clarified our decision, and I apologize if my previous letter was confusing in any way.

Best Regards,

Editor

Molecular Systems Biology

Rebuttal 22 November 2010

Thank you very much for your response to my inquiry. If you will allow it, I would still like to make the case for reconsidering our manuscript because I believe there has been some misunderstanding of our work.

As you note below, Reviewer #2's main concern appears to hinge on the perceived need for a basal molecular requirement -- namely zero-order ultrasensitivity -- in order for our earlier model (Palani and Sarkar, Biophys J, 2008) to generate a switch-like output via the two feedback loops. This is, in fact, not true. Our two-feedback topology can convert a purely hyperbolic signaling pathway (nH = 1) into a switch-like response. The system does require a basal inactivation step in the pathway, as noted by Reviewer #2, but this reaction is present in virtually all signaling pathways and, importantly, there are no stringent parametric requirements for this inactivation (in contrast to the specific parametric constraints that are necessary to generate zero-order ultrasensitivity). Thus, we strongly believe that our dual-feedback topology is indeed generally useful in generating switch-like responses, even for basal signaling systems that are completely non-ultrasensitive.

As you also note, our linear AtCRE1/SKN7 signaling pathway exhibits a slight degree of ultrasensitivity. I would argue that one would be hard pressed to actually find a linear signaling pathway in a cellular context that is not at least slightly ultrasensitive (nH > 1), given the plethora of specific molecular mechanisms (including zero-order ultrasensitivity, multi-step hosphorylation, substrate competition, and cascading) that exist for generating this systems-level property between the receptor level and the transcription factor level. We specifically chose the AtCRE1/SKN7 pathway because it is not known to rely on any of the above molecular mechanisms (it is a phospho-transfer pathway, as noted by Reviewer #3). Nevertheless, slight ultrasensitivity could still arise in our system through well-known generic non-idealities in the cell such as molecular crowding, stochastic focusing, or dimensionally restricted reactions.

Again, it is important to emphasize that our model, which is the basis for our switch designs, does not depend on any such basal ultrasensitivity. Furthermore, our model assumes non-cooperative feedback loops, which is a stringent assumption; feedback is generally appreciated to be cooperative due to the nature of the transcriptional and translational steps and this cooperativity would only further enhance the switch-like character. Thus, although our two-feedback design may be overly stringent (i.e., not fully *necessary* in all cases, particularly when basal ultrasensitivity is high or feedback is strongly cooperative), it is indeed *sufficient* for generating switch-like responses across a very large region of parameter space (as further detailed in the Shah and Sarkar manuscript that we submitted as a supplementary document). This sufficiency speaks to the generality of our method. It is also worth mentioning that our topology-based approach, which involves incorporation of two feedback loops, should be relatively easy to implement in any receptor/transcription factor system of interest; by contrast, other mechanisms such as multi-step phosphorylation and molecular cooperativity, which rely on specific molecular components/interactions to generate ultrasensitivity, may be very difficult to generalize.

I hope that this clarifies any misunderstanding about our work. I would be more than happy to discuss this with you in more detail on the phone if that would be helpful. We are quite excited about this study and sincerely hope that we will have the opportunity to revise our manuscript for Molecular Systems Biology. Thank you once again for your time and consideration. I look forward to hearing from you.

2nd Editorial Decision 02 December 2010

Thank you for sending us your appeal letter regarding our decision on your work entitled "Synthetic conversion of a graded receptor signal into a tunable, reversible switch." We have now had time to fully consider the points raised in your letter. In addition, we have also received an evaluation of this manuscript from a third reviewer. As you will see below, this additional reviewer was generally quite positive.

Previously, we indicated that we may be willing to consider a substantially revised version of this work. Considering that two reviewers were clearly encouraged by the performance of this system, we would like to reiterate this offer. Nonetheless, Reviewer #2 does seem to raise substantial concerns regarding the actual molecular mechanisms underlying the function of this circuit, and we feel that these concerns would need to be convincingly addressed. Given the points in your appeal letter, we do agree that it may be possible for you to address these concerns without investigating a separate receptor system. For a revised work to be considered we feel that two important issues would need to be addressed:

1. Zero-order ultrasensitivity. At the theoretical level, your model indicates indeed that the two feedback topology is sufficient to create switch-like output without basal ultrasensitivity. Nonetheless, we note that the related Shah and Sarkar manuscript clearly acknowledges the potential importance of zero-order ultrasensitivity within the Cre1/Skn7p circuit (p.19). It is thus not completely clear to what extent the theoretical model applies to the actual in vivo implementation presented, a point that was also raised by Reviewer #2. Therefore, we feel that a revision of this work would need to convincingly analyze the role of basal ultrasensitivity within the actual in vivo circuit presented in this work, and explicitly discuss how these effects influence the behavior of this circuit design. This may require new analyses and possibly additional experimentation.

2. Generality. Given Reviewer #2 concern's, and in the absence of additional experimental evidence showing that this design operates in receptor systems with different molecular characteristics, the claims with regard to the broad generality of this design should be toned down appropriately.

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

Yours sincerely,

Editor

Molecular Systems Biology

REFEREE REPORTS:

Additional Reviewer (comments to the Author):

This paper builds on a previous theoretical paper by the same authors (Biophys J 2008) showing that a combination of two positive feedback loops can, in principle, generate a robust, tunable bistable response. The present paper implements the design through a synthetic biology approach and shows that, indeed, it does yield a bistable response that depends upon the presence of both loops. Although others have implemented synthetic bistable switches, the Biophys J paper argues that this design is a particularly good one, and the successful implementation of the switch underscores the point nicely. This is a strong paper that merits publication in MSB.

I have two extremely minor suggestions, plus a question the authors might consider addressing:

1. p 3 If I remember correctly, the Buchler and Cross (2009) paper does not actually construct a bistable switch-their switch was a monostable one with a threshold.

2. p 9. "...this two-feedback topology is highly parametrically robust with respect to ultrasensitivity...." I would like to suggest a re-wording: "...the ultrasensitivity of this two-feedback topology is highly robust with respect to parameter variation..."

3. Ferrell (Curr Biol 2008) pointed out that a two-loop system with mirror image positive feedback and double-negative feedback loops (inspired by the Cdk1/Wee1/Cdc25 system) yields robust bistability. How does the authors' nested positive feedback topology compare with Ferrell's system in terms of robustness, tunability, etc.?

1st Revision - authors' response 14 January 2011

Reviewer #1

This paper builds on a previous theoretical paper by the same authors (Biophys J 2008) showing that a combination of two positive feedback loops can, in principle, generate a robust, tunable bistable response. The present paper implements the design through a synthetic biology approach and shows that, indeed, it does yield a bistable response that depends upon the presence of both loops. Although others have implemented synthetic bistable switches, the Biophys J paper argues that this design is a particularly good one, and the successful implementation of the switch underscores the point nicely. This is a strong paper that merits publication in MSB.

We thank the reviewer for this positive assessment of our manuscript.

I have two extremely minor suggestions, plus a question the authors might consider addressing:

1. p 3 If I remember correctly, the Buchler and Cross (2009) paper does not actually construct a bistable switch-their switch was a monostable one with a threshold.

We thank the reviewer for catching this. We have now removed the Buchler and Cross (2009) paper from the list of bistable switch references on p.3.

2. p 9. "...this two-feedback topology is highly parametrically robust with respect to ultrasensitivity...." I would like to suggest a re-wording: "...the ultrasensitivity of this two-feedback topology is highly robust with respect to parameter variation..."

This rewording does indeed read more clearly and we have changed the sentence accordingly (p.10).

3. Ferrell (Curr Biol 2008) pointed out that a two-loop system with mirror image positive feedback and double- negative feedback loops (inspired by the Cdk1/Wee1/Cdc25 system) yields robust bistability. How does the authors' nested positive feedback topology compare with Ferrell's system in terms of robustness, tunability, etc.?

An important difference between the Cdk1/Wee1/Cdc25 topology and the one presented here is the functionality of the components. The topology presented in the Ferrell (Curr Biol 2008) paper consists only of enzymes; in contrast, our topology is a hybrid of enzymatic activation and transcriptional feedback. For synthetic biology applications, we believe that our hybrid enzyme/transcription factor topology may have advantages over a pure enzyme topology in terms of tunability and robustness. First, tuning of an enzyme-only system might require protein engineering of the enzymes themselves to modulate the system response; by contrast, our approach involves relatively simple promoter engineering at the DNA level. We have added a phrase to highlight this point (p.10, second paragraph). Second, we have another manuscript under review in which we show that hybrid enzyme/transcription factor networks are generally more robust with respect to parameter variation than pure enzyme networks (since this point is somewhat tangential to the present work, we do not address it here directly, but it is a main focus of the other manuscript). However, nature may choose to use the Cdk1/Wee1/Cdc25 topology for other reasons, including: 1) a pure enzyme network enables faster switching, which may be important during cell cycle progression and 2) tunability of the switch properties may not be particularly desirable in this native context.

Reviewer #2

Synopsis: The authors modify a synthetic two-component signaling pathway (Cre1p from Arabidopsis) in yeast, such that IP-activated Cre1p phosphorylates endogenous response regulator (Skn7p). Activated Skn7p binds to SSRE cis-regulatory motif in a modified MEL1 promoter to express EGFP, a fluorescent reporter of Skn7p activity. The authors modified the two-component signaling pathway by adding two positive feedback loops: (1) SSRE binding sites in SKN7 promoter (transcription factor-mediated feedback), such that activated Skn7p produces more Skn7p and (2) SSRE binding sites in AtCRE1 promoter (receptor-mediated feedback), such that activated Skn7p produces more receptor that activates Skn7p.

Their central result is highlighted in Fig. 2E-F, where they show that TF-mediated feedback can generate bistability. Receptor-mediated feedback by itself cannot generate bistability (simple, reversible switch with nH=2), but it can increase the hysteresis of the TF-mediated feedback. At the bifurcation point, the authors could get very sharp transitions, nH ~ 20.

I have several serious concerns about the manuscript.

1. The authors claim that the dual feedback switch provides rational tuning of ultrasensitivity, amplitude, and bistability: see Abstract, Introduction, Discussion, Figure 1, and Table II. This tunability is highlighted as the significant and novel feature of their work. Yet, the claims of tunability stem from theoretical work that is not appropriately described in the manuscript or Supporting material. The results of Figure 1 and Table II appear magically. This is unacceptable.

We have now provided a complete description of the model that was used to simulate the topology shown in Figure 1A and to generate Figures 1B-E (Tables S1 (p.S10) and S2 (p.S11)). We have also added a new column to Table II (p.20) to provide direct experimental and theoretical examples of the tunability suggested in this table.

Furthermore, the experimental data in Fig. 2 do not show any tunability that can be interpreted or understood from first principles. I found it very odd that the authors have a simple, published model that analyzes dual feedback switch (Palani & Sarkar, 2008), yet there was no attempt to relate this model with the measured kinetic and steady state data. Please justify this omission.

We have now provided more explicit connections between our experimental observations in Figure 2 and our computational results in Figure 1 (please see p.7-8).

The steady-state level of active SKN7P (and consequently GFP) can be limited by either insufficient signaling through AtCRE1 or insufficient inactive SKN7 substrate; the former can be modulated by the receptor feedback strength and the latter by the transcription factor feedback strength. Figures

2A and 2B reveal that the network can operate in either receptor-limited or transcription factor-limited regimes, as shown by our computational model (Figure 1C).

It is well appreciated that feedback loops involving new protein synthesis can slow the time to reach steady state (e.g., Xiong and Ferrell, Nature 426:460-465.(2003)). As predicted by our model (Figure 1D), varying the number and strength of the feedback loops can tune the system kinetics, with slower dynamics being observed for stronger overall feedback (Figures 2C and 2D).

Figure 1E computationally shows how different feedback combinations can tune steady-state properties in our topology. Analogous experimental tuning via different feedback strengths is shown in Figures 2E and 2F.

We have now also linked the results in Figure 2 to Table II, which summarizes the switch designs.

2. The abstract claims to describe a "robust, easily constructed network topology (two positive feedback loops) to create highly ultrasensitive responses to a synthetic ligand/receptor complex." I would have to strongly disagree, as neither theory nor experiments support this claim. The authors never experimentally test the source of ultrasensitivity (e.g. cooperativity, zero-order ultrasensitivity, phosphorylation, etc..) and/or bistability.

If I take the simplified model from Palani & Sarkar, 2008 and set F1=0, F2=0 (no feedback anywhere), then the active TF still exhibits an ultrasensitive response as a function of ligand! Despite the claims in their 2008 abstract "network topology (i.e. two positive feedback loops) suggests a novel mechanism for achieving robust bistability ... without molecular cooperativity", the ultrasensitivity has nothing to do with the presence of two positive feedback loops or the topology of the network. Rather, it has everything to do with the molecular mechanism of the network. Namely, there is zero-order ultrasensitivity (Goldbeter & Koshland) between signaling kinase and phosphatase competing for the same substrate (TF). NOTE: Figure 1a of the current manuscript is misleading: there should also be an arrow from active TF to inactive TF (i.e. lose the phosphate). Indeed, if you look at the parameters in Palani & Sarkar, 2008, the kinase and phosphatase are saturated and exhibit zeroth-order kinetics. Please clarify.

I agree that if zero-order ultrasensitivity is combined with positive feedback loops, then bistability can result as shown in Palani & Sarkar, 2008. However, on pg. 8 of the current manuscript, it would be more accurate to say: "if the basic pathway response were completely Michaelian and the TF feedback exhibited no cooperativity, then zero- order ultrasensitivity would be absolutely necessary to achieve any level of bistability through positive feedback".

The reviewer brings up an important point about the potentially confounding effect of zero-order ultrasensitivity. We have now completely reworked our computational model to demonstrate that zero-order ultrasensitivity is not required to achieve bistability in this topology; rather, bistability is an emergent property of the two interconnected, but linear, positive feedback loops.

We have now further reduced our previous model so that it only consists of the reactions shown in Figure 1A, as well as cellular processes of basal protein synthesis and first-order protein degradation. The differential equations, initial conditions, and parameter values for this model are given in Tables S1 (p.S10) and S2 (p.S11). Of note, there are no inactivating enzymes (e.g., phosphatases) in this system, so it cannot exhibit zero-order ultrasensitivity in any parameter regime. We have also eliminated the Michaelis-Menten approximation in modeling enzymatic activation of the transcription factor, instead using the more general mass-action kinetics framework to describe this reaction. This new model does not contain any canonical mechanisms for generating ultrasensitivity, such as zero-order ultrasensitivity, cascading, multistep activation, or molecular cooperativity. Nevertheless, the network can still generate bistable responses (Figure 1B), can operate under receptor- or transcription-factor limited regimes (Figure 1C), and can exhibit tunability in kinetic (Figure 1D) and steady-state (Figure 1E) properties.

We have also simulated topological perturbations of this basic receptor/transcription factor network (Figure S1 (p.S7)). Without either feedback loop, the system response is truly hyperbolic (Hill coefficient (nH) = 1.00); with only one of the linear feedback loops, the response is only mildly ultrasensitive (nH < 2) and does not give rise to a switch-like response. (The presence of any other sources of ultrasensitivity in the actual in vivo setting cannot hurt in achieving the desired bistable

response, but it is important to note that they are not fundamentally necessary to achieve this switch-like behavior as long as both feedback loops are present.)

The mechanism leading to bistability is therefore novel and appears to be an emergent property of the two interconnected, but linear, feedback loops. To demonstrate the necessity and sufficiency of these two feedback loops in a truly minimal context, we examined several topological variations of a one-component system (p.S2-S6). The convergence of the two linear feedbacks at the step of transcription factor activation mimics cooperative feedback, resulting in a bistable response.

3. The authors interpret their experimental results too easily. For example, they state that the P_SSRE-EGFP measurements for different strains are transcription-factor limited or receptor-limited regimes, without demonstrating or testing this claim by measuring abundances or performing ligand / receptor / TF dosage experiments. On pg. 6, the authors state "Increasing the receptor feedback strength (TR-SSRE > SSRE > CYC1) did not change ...". Where is the data indicating that TR-SSRE had higher expression than SSRE than CYC1? Assumption or fact?

CYC1 is a constitutive promoter, so its feedback strength is zero. The relative strengths of the synthetic SKN7- inducible promoters (TR-SSRE > SSRE) have been previously established by Chen and Weiss (2005)*. We have now included this reference in this sentence on p.6. * Chen MT, Weiss R (2005) Artificial cell-cell communication in yeast Saccharomyces cerevisiae using signaling elements from Arabidopsis thaliana. Nat Biotechnol 23: 1551-1555

4. One observation of Palani & Sarkar, 2008 was that receptor-mediated feedback was more critical than TF- mediated feedback for bistability and ultrasensitivity (at the bifurcation point). Yet, the experimental results in this manuscript appear to contradict this -- the TF-mediated feedback is responsible for bistability whereas receptor- mediated feedback has no effect. Please clarify.

The Palani & Sarkar 2008 model had other possible confounding reactions (e.g., inactivation by phosphatase leading to zero-order ultrasensitivity) which would make it difficult to make direct comparisons between this earlier model and the current experiments. For the present work, we have now reconstructed the entire model to match the network topology in Figure 1A; in this updated version of the model, neither linear feedback alone is capable of generating bistability when the basal signaling pathway is hyperbolic (nH = 1) (Figure S1 (p.S7)).

In our experiments, the weak bistability observed in the cRtTF strain (Figure 2F) is likely due to the fact that the basal signaling pathway in the cRcTF strain is weakly ultrasensitive (nH ~ 2) (Figure 2E) and transcription factor- limited (see discussion on Figures 2A and 2B). The presence of any basal ultrasensitivity in the actual in vivo setting can relax the requirement for both feedback loops, but it is important to note that basal ultrasensitivity is not fundamentally necessary to achieve this switch-like behavior as long as both feedback loops are present.

Specific comments:

*5. The authors' assertion that their method is more applicable and tunable when compared to other methods (refs on pg. 3) is an exaggeration. Their method relies on specific ligand/receptor-kinase interaction, coupled to saturated enzymatic reactions (zero-order ultrasensitivity) and positive feedback loops to get bistability. These are very specific molecular requirements, and it is unclear that this translates *easily* to other ligand/receptor pairs. Matter of fact, it's unclear to me whether saturated enzyme kinetics is even occurring in their Cre1/Skn7p system (as envisioned in their double feedback model of ligand/receptor).*

As noted above, there is no specific requirement for saturated enzymatic reactions or zero-order ultrasensitivity in our method. Our approach does depend on direct or indirect enzymatic activation of a transcription factor via a receptor complex; such a linkage is relatively common in natural

signaling pathways and it may be engineered to achieve synthetic signaling (as in the AtCRE1/SKN7 system). An advantage of using feedback strengths to tune the system dynamics is that promoter engineering is generally easier than protein engineering. Nevertheless, we agree that in this nascent stage of synthetic biology, engineering cellular function remains challenging and we have tempered some of the language in our manuscript.

6. On pg. 7, the authors state "The double feedback strains had the slowest degradation rate (~8.5 hr), which could be due to the higher accumulation of phopho-SKN7 at steady-state or a higher level of residual bound IP due to the higher receptor expression level".

The binding affinity of IP for Cre1 receptor is not in the regime of being effectively irreversible. Romanov et al, J. Exp. Botany 57: 4051 (2006) have measured the Kd of IP for Cre1 receptor, Kd ~20 nM.

We fully agree that the time scale for dissociation of residually bound IP is much faster than the half-life for GFP degradation, so we have changed the sentence accordingly on p.8.

7. The last paragraph and Table II are not supported by anything in the manuscript. They should be removed.

We have now inserted a new column in Table II (p.20) to link these designs to relevant figures in the manuscript.

8. In Materials and Methods, I was confused why strains were grown in SC galactose. After significant background reading, it became apparent that TM182 strain is sln1-del GAL1-PTP2, which is necessary to abrogate the lethality that results from sln1-del. The authors should write several sentences describing their strain background, their reason for using it, and describing why PTP2 expression is needed.

We have added a description of the background strain on p.11 and provide its genotype in Table S4 (p.S12).

9. Figure 1 with no accompanying equations and parameters is not useful or reproducible.

We have now included the complete description of the model with differential equations, initial conditions, and parameter values (Tables S1 (p.S10) and S2 (p.S11)).

10. In Figure S1, I found it strange that the low peak ("OFF" state) of tRtTF (of the bimodal distribution) is lower than the original histogram at the top (i.e. 24 hours, 0.06 uM IP). Supposedly, the top histogram is uninduced by IP (or "OFF"). The flow cytometry data would be more interpretable if the authors included a negative control (TM182) and reporter-only control.

The small peak observed at 12 hours (0.05 M IP) in the tRtTF strain is not an ‘off’ population but cell debris, as very high levels of SKN7 can be toxic. When the cells were spun down and washed after 12 hours, this debris was removed and was therefore not observed at 24 hours. We have clarified this point in Figure S2 (p.S8).

Reviewer #3

This is a particularly elegant example of biochemical circuit design for creation of ultrasensitive response to a graded input signal. The authors clearly show, with no fudging that they can tune the

behavior of the circuit in a synthetically generated circuit in yeast, while overall characteristics of the circuit response are robust to variation of kinetic parameters.

I'm rather at a loss to find anything specific to criticize about this manuscript; the presentation is elegant and results are crystal-clear. One minor thing the authors may address in he manuscript is to explain explicitly how the exogenous AtCRE1 happens to specifically phosphorylate endogenous SKN7 via the phospho-transfer protein YPD1. Even though published by Lu, et al. it would be good to briefly explain so that readers may not think that some reengineering of components allowed the circuit to work as designed, rather than the explicit network topology as the authors suggest. I believe that this manuscript should be published in MSB.

We appreciate the reviewer's positive assessment of our work.

We also thank the reviewer for suggesting that we detail how the signal is relayed, since this basic phospho-transfer pathway should not contain any inherent mechanisms for engendering ultrasensitivity. On p.5, we have now explicitly mentioned how endogenous SKN7 is activated by AtCRE1.

Acceptance 10 February 2011

Thank you again for sending us your revised manuscript. We have now heard back from Reviewer #2, the reviewer who raised the most substantial concerns during the first round of review. This reviewer indicated that the revisions made to this work have addressed his/her concerns, and as such I am pleased to inform you that your paper has been accepted for publication.

Before we can send this work to production, we would like to ask you to address the following format and content issues:

1. Molecular Systems Biology generally requires that authors provide machine readable versions of mathematical models as supplementary material in a common format, and we feel this will be important for the simulations in Fig. 1. When appropriate we strongly encourage authors to supply models in the SBML format, and to submit models to a public database like BioModels or JWS Online. Additional supplementary model files should be referenced in the Methods section of the manuscript, and listed in the Table of Contents at the beginning of the Supplementary Information pdf file.

2. 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 the data in Fig. 2. Guidelines have been pasted at the end of this email.

3. The resolution of the supplied Figure images is a bit low, please provide final high-resolution figure images in TIFF, EPS, or PDF formats.

Thank you very much for submitting your work to Molecular Systems Biology.

Sincerely,

Editor

Molecular Systems Biology

