molecular systems biology

Quantitative analysis of transient and sustained transforming growth factor-β signaling dynamics

Zhike Zi, Zipei Feng, Douglas A Chapnick, Markus Dahl, Difan Deng, Edda Klipp, Aristidis Moustakas and Xuedong Liu

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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 09 November 2010

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

The reviewers recognized that this work provides some potentially valuable information regarding the quantitative response of the TGF-beta pathway to different dose and stimulation patterns, however, they raise a variety of concerns that will require substantial additional clarification and in some cases new experimental work. Most importantly, the reviewers indicated that this work would greatly benefit from a direct experimental validation of the most novel modeling predictions (e.g. that ligand depletion affects the long-term Smad response), and the reviewers suggest testing the TGF-beta response in either cells grown in different media volumes or with inhibition of endocytosis. Other concerns may require additional experiments or controls to support the conclusiveness of your interpretations, in particular reviewer #1's concerns about the robustness of the Hill coefficients calculated in Fig. 4 seems important and may require the collection of additional data points.

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.

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Sincerely,

Editor
Molecular Systems Biology

REFEREE REPORTS

Reviewer #1 (Remarks to the Author):

The authors show that TGFb pulse stimulation results in transient pSMAD2 response whereas a train of pulses results in sustained pSMAD2 response, similar to constitutive stimulation. They also show that the transient response is graded or hyperbolic whereas the sustained response is ultra-sensitive. The authors provide a model that is consistent with these observations. Overall this is a study with some interesting aspects on TGFb signal processing that lacks experimental rigor. The qualitative features of the model are not well explained at the beginning of the article (some part of the discussion could be used in the introduction). For example, it could be stated at the start that ligand depletion is the key feature that distinguishes it from previous models. The reader remains wondering if alternative models as discussed by the authors at the end of the article can explain the same phenomena with equal probability. The quality of the manuscript would improve if competing models not including ligand depletion were quantitatively compared to the model of the authors.

Specific points:

The authors describe the dose of TGFb as X molecules per cell. What is the advantage of this as compared to a simple concentration of TGFb? I cannot believe the significance given to the numbers (6. 10^4 instead of 60000?).

Removal of TGFb after 30 sec of stimulus gives a transient SMAD2 response. What happens when cells are treated for longer times with TGFb? Does phospho-SMAD2 also decay to zero after TGFb washout, or is there an offset as in the constitutive stimulation?

How is the new and comprehensive model different to older published models? Could the authors describe the qualitative differences between their model and previous ones.

What is different in the refined model? Please describe in molecular mechanistic terms.

Why is there such a difference in the level of pSMAD2 between fig.2 A and D in the initial part of the time course (should this not be the same?).

More importantly, there is not much of a sustained pSMAD response after the initial 3h pulse of TGFb in fig.2. Compare this to fig.1A

Figure 3C should be quantified and plotted. This could then be compared to fig.3B. I am not sure that that the pulsed input gives much of the same response as the sustained input.

Fig. S5: again the blot data should be quantified with more time points to enable a better comparison to the model predictions. The general effect on pSMAD response is clear in this case. However the simulation experiment in Fig. S4 shows that concurrent inhibition of RLC with TGFb wash-out results in very low amplitude of pSMAD2. Can this be reproduced experimentally?

In Fig. 4A and B the fits are very poor. In the short-term exposure dose-response curve (A) the fit overshoots the low dose points whereas in the long exposure dose-response the fit undershoots the low dose points. I can easily imagine that there is a fit for both data sets that gives approximately the same Hill coefficient. Please provide better data with more convincing fits.

The authors should do experiments that confirm the qualitative predications of the model. For example, they could affect receptor trafficking by perturbing endocytosis and see how this affects the dose-response behavior.

Reviewer #2 (Remarks to the Author):

The manuscript by Zi et al addresses the sensitivity of the transforming growth factor beta (TGFb) response to varying the ligand dose and time of exposure. The authors take a combined approach of mathematical modeling and experimental testing. The data are interesting for a number of reasons. Specifically, they show that multiple very short treatments can result in a response similar to that seen with continuous stimulation, and that the dose response curve for Smad phosphorylation is quite different at a short time after stimulation from that at 24 hours. In general the work is clear and well presented (although I am not able to judge the mathematical models). However, I do have a number of concerns:

1. In Fig 3 they show that repeated 30 second pulses at 30 minute intervals are integrated into a similar response to that with continual stimulation. However, in Fig 1 they have already shown that the response to a 30 second pulse is still increasing between 15 and 45 minutes after the pulse. Wouldn't the more interesting experiment be to examine how the cell integrates a pulsatile stimulation where the duration between pulses was slightly longer - where the response to each pulse is beginning to decline?

2. The growth inhibitory response to TGFb is used in Fig 4, to examine whether a real response takes on the dose-response curve of a long or short term stimulation. I am not sure that growth inhibition is the best thing to look at, since it can only be measured after 24 hours of stimulation, not at 45 minutes. More interesting would be to compare gene expression responses at very short times and after sustained stimulation.

3. The authors suggest that the 24 hour stimulation generates an "ultrasensitive" switch-like response. While this is true in that the slope of the curve is much steeper than at a short time of stimulation, the word ultrasensitive is a bit misleading, since it can also be taken to mean that the cells are more sensitive to TGFb with longer stimulation. In fact they become less sensitive - 12000 molecules per cell gives no Smad2 phosphorylation at 24hrs, but robust phosphorylation at 45 minutes.

4. Presumably, this lack of response to the lower doses at 24hrs could be due to a number of adaptations of the cells - induction of negative regulatory mechanisms, such as dephosphorylation or ubiquitylation.

5. In Fig 1, I would have liked to see more time-points for the Smad2 phosphorylation by the 30 second pulse - what is happening between 15 and 45 minutes, and especially between 45 minutes and 4 hours?

Reviewer #3 (Remarks to the Author):

In this report, Zi et al combined quantitative modeling with experimental approach to determine the effect of TGFfl ligand doses and stimulation patterns on downstream signaling. They made several interesting discoveries: 1) Cells respond differently to continuous and pulsating TGFfl stimulation with transient activation response to a single pulse of ligand and sustained activation in response to repeated short pulses of ligand; 2) Cells can respond dynamically to repeated TGFfl stimulation 3 hours after the initial stimulation; 3) Short term TGFfl signaling activation (P-Smad2) is graded in response to ligand dosage while long term signaling activation)P-Smad2 and growth inhibition) is an ultra-sensitive response. This switch-like ultrasensitive long-term response is mainly caused by ligand depletion due to ligand-receptor interaction.

This study uncovered critical regulatory aspects of TGFfl signaling that have not been revealed using the conventional biochemical approaches. These are highly significant results that may have important implications in future experimental design and data interpretation and deserve to be published.

It would be of great interest to the field if the following questions can be addressed:

1. What are the percentages of receptor occupancy at various ligand concentrations? Does this factor play a role in the strength of signal activation and the threshold concentration at which graded response is converted to ultrasensitive response? For example, if a cell line with very different cell surface receptor numbers as HaCat is tested in the experiment shown in Fig. 4B, would this cell line show a different ligand concentration at the border of transient response and ultrasensitive response?

2. In term of ultrasensitivity of the long term signaling response, the authors have tested the cytostatic response. Do other TGFfl-induced responses show different threshold to the ligand dosage when graded early responses are converted to the ultrasensitive responses? For example, does transcription of difference TGFfl target genes show different dosage threshold (for converting to the ultrasensitive response)? Does genes like PAI-1 show a different ligand dosage threshold from genes like p21 in this model?

3. Does the model explain how TGFfl signaling specificity may be generated? i.e. will ligand dosage or stimulation duration determine whether the cells undergo growth arrest or EMT, given that HaCat cells can do both?

Reviewer #4 (Remarks to the Author):

In this manuscript, Zi et al. use both mathematical modeling and in vitro experiments to understand how the magnitude and duration of the extracellular signal (TGF-beta) is transduced into Smad2 phosphorylation levels in the TGF-beta signaling pathway. They find that while TGF-beta addition for 30s followed by ligand removal induces transient Smad2 phosphorylation, the repeated application of 30s ligand pulses spaced 30 minutes apart results in a more sustained response that is closer to that seen under continuous ligand stimulation. Smad2 phosphorylation levels at 45 minutes shows a sigmoidal relationship to TGF-beta dose (quantified as molecules/cell), while both 24 hour Smad2 activation levels and inhibition of cell proliferation show ultrasensitive dose responses with Hill coefficients > 4. Sensitivity analysis of the mathematical model reveals that the depletion of ligand molecules at low doses is a major cause of the ultrasensitivity in long-term responses.
The mathematical model (structure and governing equations) appears to be identical to the one previously published by the authors (Zi and Klipp, PLOS One, 2007). The observation that ligand depletion affects Smad activation kinetics has also been previously published by the authors (Clarke et al, Mol Cell Biol, 2009). Portions of the current manuscript lack clarity and some of the results are not discussed in depth. That said, the authors rigorously parameterize their model using existing datasets as well as some new datasets reported in the current manuscript. The manner in which the authors use modeling to reach the conclusion that ligand depletion affects the long-term dose response is also compelling. Overall, while the manuscript may be somewhat lacking in originality and depth, their approach is sound and the findings are likely to be of interest to the field of cell signaling.
Major concerns:
It is not clear which of the datasets used in model calibration are original datasets and which ones have been previously published. This needs to be clearly mentioned. Original data (datasets 1-3?) needs to be presented in the main manuscript. I don't understand why Fig. 1B is necessary. What did the authors learn from the "initial calibration of the model", what prompted them to change the model and what did this exercise reveal? Why not start with the "refined" model that is calibrated using all the data?
On a related note, the studies involving 2 successive additions of ligand with either Ton=Toff=1hr or Ton=Toff=3hr seem to have been designed in an ad hoc fashion and don't really appear to tie in with the rest of the manuscript. How do these results (Fig. 2) inform the rest of the manuscript? Do the model parameters change significantly when these results are included as part of the fit? Which parameters change, and what does this reveal about how the system operates? What are the implications of the inherent relaxation time of the pathway (between 1 and 3 hours) in the context of development? The manuscript in general and this section in particular would benefit from a more

detailed discussion of the results.
Experimental validation of the finding that ligand depletion affects long-term Smad phosphorylation would greatly strengthen the manuscript. Comparison of the Smad response to the same concentration of TGF-beta provided in different volumes of media can be used to demonstrate that ligand depletion is indeed important.

Minor comments:
1. Table S4: Rate expression for dx12/dt is incorrect - koff_Smads*x13 should be 2*koff_Smads*x13. If the model is coded this way, then simulations need to be re-run to verify the results.
2. The authors need to provide quantitative plots for all the western blots, particularly in cases where model simulations are being compared to experimental data (Fig. 1A, Fig. 3C, Fig. S5B).
3. Figure 4A,B: Legend needs to state that experimental data has been re-scaled to match the mathematical model so that p-Smad2 can be expressed as a fraction of total Smad2. Unless panels A and B were done as part of the same blot, it is not possible to compare the absolute P-Smad2 levels between them. This applies to Fig. S3A as well - were all measurements in the figure done as part of the same blot? How did the authors obtain p-Smad2 as a % of total Smad2?
4. Figure 4E: Y-axis label should be more descriptive (e.g.% of BrdU-labeled cells).
5. A discussion of why receptor depletion cannot be invoked to explain the dose response behavior (along the lines of the discussion in Clarke, 2009) would be useful.
6. Fig. 1B and associated text - replace "normal stimulation" with something more descriptive (e.g. sustained ligand stimulation).
7. Figure 2: It would help to have bars on the plots indicating the on and off phases of ligand addition.
8. Page 3, 1st paragraph - the Watanabe et al. reference is incomplete.
9. Page 4, 2nd paragraph: "The dynamic behavior... to alternating ligand exposure cannot be predicted by existing mathematical models..." is misleading. Any of the existing models (Vilar, Schmierer, Clarke) can be simulated under conditions where the ligand concentration varies arbitrarily with time.
10. Page 8, Line 1: "Keratinocytes" is misspelled.
11. Supp Mat, Page 6, 1st paragraph: "The estimated 9 parameter values have small variations..." is inaccurate. The on rate for non-specific ligand binding clearly has a large variability. This is not surprising since this occurs at a much faster time scale than the experimental sampling. Upper and lower bounds used for the various parameters during the optimization need to be included as part of Table S2.
12. Supp Mat, Page 6, Section 4: Provide the Smad4 level in HaCaT cells (only Smad2 level is given).
13. Table S2: Incorrect units for trimolecular rate constant ka_LRC.
14. Table S3: Highlight x2, x3, x4 and x5 and clearly mention that these initial concentrations change as a function of the receptor synthesis rates during parameter estimation.

There are several grammatical and typographical errors in the manuscript. Careful proofreading and editing is warranted.

1st Revision - authors' response 02 March 2011

Responses to Reviewer Comments
We are grateful to all the reviewers for their valuable and insightful comments on our work. Based on the remarks from the reviewers, we have performed additional experiments, model analyses and rewritten the manuscript so that the significance of the work is clearly presented and almost all the reviewer's concerns have been addressed. In addition, we have re-optimized the parameter sets in the final model with the latest experimental data sets that include more biological replicates. It is worth noting that the final model has the same network topology and reaction kinetics as the models presented in the first version of the manuscript. The estimated parameter values are slightly adjusted, and we have updated the model simulations and prediction results in the figures.

Point-by-Point Responses to Reviewer comments

Reviewer #1 (Remarks to the Author):

The authors show that TGFb pulse stimulation results in transient pSMAD2 response whereas a train of pulses results in sustained pSMAD2 response, similar to constitutive stimulation. They also show that the transient response is graded or hyperbolic, whereas the sustained response is ultra-sensitive. The authors provide a model that is consistent with these observations. Overall this is a study with some interesting aspects on TGFb signal processing that lacks experimental rigor. The qualitative features of the model are not well explained at the beginning of the article (some part of the discussion could be used in the introduction). For example, it could be stated at the start that ligand depletion is the key feature that distinguishes it from previous models. The reader remains wondering if alternative models as discussed by the authors at the end of the article can explain the same phenomena with equal probability. The quality of the manuscript would improve if competing models not including ligand depletion were quantitatively compared to the model of the authors.

We have performed additional experiments to strengthen our data with more biological replicates and added some new evidence. Some parts of the manuscript have been elaborated according to the reviewers' suggestions. We have also quantitatively compared our model to previous models not including ligand depletion with our model in the Supplementary Information (Figure S8). The models without ligand depletion fail to explain the experimental observations in this work (e.g., ultrasensitive P-Smad2 dose responses at 24 hour).

Specific points:
The authors describe the dose of TGFb as X molecules per cell. What is the advantage of this as compared to a simple concentration of TGFb? I cannot believe the significance given to the numbers (6. 10^4 instead of 60000?).

As briefly explained in the manuscript, our previous theoretical and experimental analyses indicate that cell density and medium volume will affect the signaling responses (Clarke et al, 2009, Mol Cell Biol 29: 2443, Zi et al, 2007, FEBS Lett 581:4589). We have demonstrated that the Smad signal is a function of the number of TGF- molecules per cell. The ligand number per cell is more precise than the final concentration of TGF- when quantifying the TGF- dose for determining Smad2 phosphorylation (P-Smad2) responses. For example, if one uses different medium volume or different cell number, the cell density if affected and P-Smad2 levels can in fact be quite different in varying experimental setups despite the fact that concentration of TGF- in the medium remains the same. However, if we look at the TGF- dose in terms of TGF- molecules per cell, P-Smad2 levels remain consistent in different experimental setups (Clarke et al, 2009). The reason for this is that the actual amount of available TGF- molecules for each cell can vary in different experimental setups even with a constant concentration of TGF- .
Of course, if one always uses the same cell number and medium volume, then the number of TGF- molecules per cell is just a scaled quantity of TGF- concentration. However, in reality different labs and experimental designs may use different cell seeding number or medium volume. In this case, the experimental results using a TGF- concentration scale would be less comparable than using the number of TGF- molecules per cell. In fact, this is what we find when we compare many published papers by different laboratories and even by the same laboratory. More in depth discussions about this issue are presented in response to major point 3 of the review #4 and in Clarke et al. MCB 2009.
Removal of TGFb after 30 sec of stimulus gives a transient SMAD2 response. What happens when cells are treated for longer times with TGFb? Does phospho-SMAD2 also decay to zero after TGFb washout, or is there an offset as in the constitutive stimulation?
We have repeated the experiments with TGF- stimulation of 30 seconds. We measured phospho-Smad2 levels with more time points (0 15' 30' 45' 2hr 3hr 4hr 5hr 6hr 8hr). The data indicate that phospho-Smad2 decays to the basal level (near to zero) after TGF- washout (Figure 1B). No offset is observed in the case of sustained TGF- stimulation.

How is the new and comprehensive model different to older published models? Could the authors describe the qualitative differences between their model and previous ones.

A major impetus for us to develop a new and comprehensive model is that almost all published mathematical kinetics models developed for TGF- /Smad signaling pathway fail to capture the observed qualitative trends of the phospho-Smad2 kinetics in response to different doses of TGF- stimulation and/or to 30 seconds single pulse of TGF- stimulation. One of the major qualitative differences is that previous models assume that loss of the T RI coupled with dephosphorylation is the underlying mechanism for the decrease in phospho-Smad2 that occurs during signaling. Our double stimulation experiment clearly suggests that loss of T RI cannot be invoked to explain the termination of TGF- /Smad signaling in response to different dose of ligand stimulation. Instead, we show that ligand depletion is a prevailing mechanism to account for variable duration of Smad signaling. Our new model takes into account TGF- depletion and makes no assumption of significant loss of T RI. This model is calibrated with more quantitative experimental data with different TGF- stimulation profiles and to published quantitative datasets. This results in a model that has more predictive power and is capable of non-intuitive insights into the quantitative nature of TGF- signaling. For example, long-term ultrasensitive Smad phosphorylation and cellular growth response are predicted by our new model, while this novel insight cannot be derived from published TGF- mathematical models.

What is different in the refined model? Please describe in molecular mechanistic terms.

The development of the refined model has been an iterative process. As discussed above, what set apart the model developed in this study from the previous ones are the introduction of ligand depletion and no assumption of significant reduction of T RI. Details about the features and differences of the new model to the older published models are discussed in the first paragraph of the section "1. Model development and major assumptions" in the supplementary information. The differences between our initial model and the refined model in this study are not based on molecular mechanism (network topology). The initial model was calibrated with a few experimental data sets and consequently lacks precision in estimating parameter values. As more experimental data was gathered, the model parameter set was refined. In the first submission, we intended to show the history of model development which might have caused some misunderstanding about the actual model we developed. In the revised manuscript, we removed the initial model analysis (previous Figure 1B) and focus our analyses on the final model.

Why is there such a difference in the level of pSMAD2 between Figure2 A and D in the initial part of the time course (should this not be the same?).

Perhaps the reviewer overlooked the fact that Figure 2A and Figure 2D are in different time scale and have different TGF- stimulation profiles. Previous Figure 2 is now renamed as Figure 3 in the revised manuscript. Hereafter, we refer it as Figure 3. Figure 3A has 1 hour of TGF- stimulation and 1 hour washout, while Figure 3D has 3 hours of TGF- stimulation and 3 hours washout. The comparable initial part of these two experiments is the first hour. They are consistent because both time courses peak at 1 hour. For the data points after 1 hour, they are different because they have different TGF- stimulation profiles. For example, from time 1 hour to 2 hour, TGF- is washed out for Figure 3A, but TGF- remains in the medium for Figure 3D.

More importantly, there is not much of a sustained pSMAD response after the initial 3h pulse of TGFb in Figure2. Compare this to Figure1A

Since the reviewer did not point out which panel of Figure 2 (Figure 3 in the revised manuscript. Hereafter, we refer Figure 2 as Figure 3) was compared to Figure 1A, we assume that the reviewer may refer to Figure 3F vs. Figure 1A. There is a fundamental difference in experiment design between Figure 3F and Figure 1A. Figure 1A right panel was the continuous stimulation with saturated TGF- without washout. This resulted in a more sustained response. In contrast, in Figure 3 the same amount of TGF- was applied but washed out at 3 hr and consequently P-Smad2 was reduced to a lower level at 6 hour. There should not be a sustained P-Smad2 response based on our model and related experimental data. Ligand washout causes a reduction in P-Smad2 signal. Figure 3C should be quantified and plotted. This could then be compared to Figure3B. I am not sure that that the pulsed input gives much of the same response as the sustained input.

We have repeated the experiment in Figure 3C of the previous manuscript. Now we have added the experimental data and the standard deviation in previous Figure 3B (Figure 4A in the revised manuscript) for comparison.

Figure S5: again the blot data should be quantified with more time points to enable a better comparison to the model predictions. The general effect on pSMAD response is clear in this case. However the simulation experiment in Figure S4 shows that concurrent inhibition of LRC with TGFb wash-out results in very low amplitude of pSMAD2. Can this be reproduced experimentally?

We have repeated the experiment in Figure S5 (Figure S4 in the revised manuscript) and monitored phospho-Smad2 level for a longer duration (up to 8 hours). The quantified experimental data has been shown in Figure S5 A (Figure S4A in the revised manuscript).
Concurrent inhibition of LRC with TGF- washout cannot be experimentally implemented due to the lack of tools that enable rapid reversible inhibition of LRC. This is the why we resorted to modeling analysis to predict possible behaviors of pSmad2 response in the first submission of this work. We concede the unverifiable and speculative nature of previous Figure S5B and have decided to remove this figure panel in the revised manuscript.

In Figure 4A and B the fits are very poor. In the short-term exposure dose-response curve (A) the fit overshoots the low dose points whereas in the long exposure dose-response the fit undershoots the low dose points. I can easily imagine that there is a fit for both data sets that gives approximately the same Hill coefficient. Please provide better data with more convincing fits.

The legend for Figure 4A, 4B in the first submission (Figure 5A, 5B in the revised manuscript) was not very clear. The blue curves in Figure 4A, 4B were not the data fitting curves with the Hill equation but were in fact the simulation curves predicted by our model. To clarify this, we have added new fitted curves with Hill equations and have created new, more clear legends.
The authors should do experiments that confirm the qualitative predications of the model. For example, they could affect receptor trafficking by perturbing endocytosis and see how this affects the dose-response behavior.

We agree with the reviewer that it is necessary to confirm the qualitative predictions of the model. Investigation of receptor trafficking regulation on the dose-response behavior is definitely important. However, we contend that one should be very cautious about over-interpreting the experimental results from perturbing endocytosis using conventional approaches. First, current reagents available to perturb endocytosis are either not very specific or cause pleiotropic changes in cells. Controversial reports on the effect of endocytosis on P-Smad2 responses (Lu et al., 2002, J Biol Chem; Di Guglielmo et al., 2003, Nat Cell Biol; Mitchell et al., 2004, Mol Biol Cell) underscore the challenge of correctly interpreting the results from these types of studies. Second, there are additional challenges to compare dose-responsive behavior in the long-term as most endocytosis inhibitors we tried become toxic to cells in 24 hr experiments. Even though we can introduce very specific perturbation through modeling simulations, the lack of specificity in available experimental perturbation tools makes it difficult to test model predictions with experimental outcomes.
We thought about an alternative way to design experiments to confirm the qualitative predications of the model based on the recommendations of the editor and reviewer #4. The goal is to demonstrate that alteration in ligand/receptor trafficking rate could affect the dose-response behavior. One way to prove this is by perturbing endocytosis directly. In the absence of reliable reagents or tools to specifically disrupt TGF- receptor trafficking while sparing the global endocytosis pathways, we consider this line of investigation is likely to yield more questions than answers.
An alternative way to address this issue is to alter the speed of ligand depletion. Our model predicts that ligand depletion speed should impact the shape of the dose response curve. Ligand depletion speed is a function of the concentration of ligand-receptor complex. The speed of ligand depletion can be adjusted by varying the volume of the media while keeping the same doses of TGF- molecules per cell. Our model simulation predicts that slowing down ligand depletion by increaseing medium volume should decrease the ultrasensitivity of long-term P-Smad2 dose

response at 24 hour (Figure 6A). This prediction is largely verified by the experimental results shown in the new Figure 6B,C,D,E,F,G. Thus the new data lends additional support for the validity of our new model.
Despite the above response, we are essentially in agreement with the reviewer's comment. When experimental means for specific molecular intervention on the endocytosis process become available, this important parameter needs to be revisited.

Reviewer #2 (Remarks to the Author):
The manuscript by Zi et al addresses the sensitivity of the transforming growth factor beta (TGFb) response to varying the ligand dose and time of exposure. The authors take a combined approach of mathematical modeling and experimental testing. The data are interesting for a number of reasons. Specifically, they show that multiple very short treatments can result in a response similar to that seen with continuous stimulation, and that the dose response curve for Smad phosphorylation is quite different at a short time after stimulation from that at 24 hours. In general the work is clear and well presented (although I am not able to judge the mathematical models). However, I do have a number of concerns:
1. In Fig 3 they show that repeated 30 second pulses at 30 minute intervals are integrated into a similar response to that with continual stimulation. However, in Fig 1 they have already shown that the response to a 30 second pulse is still increasing between 15 and 45 minutes after the pulse. Wouldn't the more interesting experiment be to examine how the cell integrates a pulsatile stimulation where the duration between pulses was slightly longer - where the response to each pulse is beginning to decline?

The suggested experiment is indeed very interesting to us. According to the comment from reviewer #1, we have monitored phospho-Smad2 (P-Smad2) response to a single pulse of 30 seconds TGF- stimulation at more time points (Figure 1B). The data shows that phospho-Smad2 level is decreased at 3 hours in this case. Therefore, we monitored P-Smad2 response to pulses of 30 seconds TGF- stimulation at 3 hours intervals. The experimental data shows that P-Smad2 undergoes periodic changes with response to the addition of 30 seconds TGF- stimulation and decreases to a lower level during 3 hours of TGF- washout interval (Figure 4F). This result is in agreement with our view that the cell is able to tell the difference in duration of ligand withdrawal. With a strong TGF- stimulation, the pre-bound receptors are capable of sustaining signaling for half an hour to bridge it to the next stimulus. When ligand is washed out for extended periods of time, the ability of pre-bound receptors to bridge signaling becomes weakened, and oscillations in P-Smad levels become more and more pronounced.

2. The growth inhibitory response to TGFb is used in Fig 4, to examine whether a real response takes on the dose-response curve of a long or short term stimulation. I am not sure that growth inhibition is the best thing to look at, since it can only be measured after 24 hours of stimulation, not at 45 minutes. More interesting would be to compare gene expression responses at very short times and after sustained stimulation.

In agreement with the reviewer, we have measured the expression of the early response gene Smad7 at 45 minutes to different doses of sustained TGF- stimulation by quantitative PCR. The experimental data shows a graded response with corresponding Hill coefficients of about 1.3 (Figure 5E), which is consistent with the graded TGF- response at 45 minutes (Figure 5A, 5C). For long-term translational responses we choose to measure translational induction of PAI-1 and p21, two known targets of TGF-beta as suggested by the reviewer. We show that PAI-1 translational induction is highly ultrasensitive with a Hill coefficient close to 6 while p21 induction is only modestly ultrasensitive with a Hill coefficient approximately 2 (Figure 5F, 5G).

3. The authors suggest that the 24 hour stimulation generates an "ultrasensitive" switch-like response. While this is true in that the slope of the curve is much steeper than at a short time of stimulation, the word ultrasensitive is a bit misleading, since it can also be taken to mean that the cells are more sensitive to TGFb with longer stimulation. In fact they become less sensitive - 12000 molecules per cell gives no Smad2 phosphorylation at 24hrs, but robust phosphorylation at 45 minutes.

We introduced the concept of "ultrasensitive" in the introduction part: "In an ultrasensitive response, a small change of stimulus within a certain range results in a large change in response". There is a certain range in which signal response is very sensitive to the change of stimulus. Ultrasensitive response doesn't mean the output is sensitive to the change of the input throughout the entire range. It shows how fast the output is switched from lower response to a higher response and sometimes is also referred as a "switch-like response." When the input is less than one threshold or it is higher than another threshold, the output doesn't change too much or become less sensitive. Therefore, Hill coefficient is normally used to quantify the ultrasensitive responses.
The point of the reviewer is also well taken. Therefore, we use switch-like along with ultrasensitive in many sentences to remind the reader what we mean by ultrasensitivity.

4. Presumably, this lack of response to the lower doses at 24hrs could be due to a number of adaptations of the cells - induction of negative regulatory mechanisms, such as dephosphorylation or ubiquitylation.

We believe the lack of response to the lower doses at 24hrs is primarily due to TGF- depletion. Our data (not shown) and previous studies demonstrated that Smad2 phosphatase PPM1A (dephosphorylation rate) does not change with different doses of TGF- stimulation. In addition, published works on negative regulatory mechanisms always use saturated TGF- doses. However, we cannot rule out that induction of negative feedback such as inhibitor production or ubiquitylation may also play a role in adaptation.

5. In Fig 1, I would have liked to see more time-points for the Smad2 phosphorylation by the 30 second pulse - what is happening between 15 and 45 minutes, and especially between 45 minutes and 4 hours?

To address this concern, we have added more time points (0 15' 30' 45' 2hr 3hr 4hr 5hr 6hr 8hr) to the p-Smad2 time course. The data indicates that phospho-Smad2 decays to the basal level (untreated) after TGF- washout (Figure 1B).

Reviewer #3 (Remarks to the Author):

In this report, Zi et al combined quantitative modeling with experimental approach to determine the effect of TGFfl ligand doses and stimulation patterns on downstream signaling. They made several interesting discoveries: 1) Cells respond differently to continuous and pulsating TGFfl stimulation with transient activation response to a single pulse of ligand and sustained activation in response to repeated short pulses of ligand; 2) Cells can respond dynamically to repeated TGFfl stimulation 3 hours after the initial stimulation; 3) Short term TGFfl signaling activation (P-Smad2) is graded in response to ligand dosage while long term signaling activation) P-Smad2 and growth inhibition) is an ultra-sensitive response. This switch-like ultrasensitive long-term response is mainly caused by ligand depletion due to ligand-receptor interaction.
This study uncovered critical regulatory aspects of TGFfl signaling that have not been revealed using the conventional biochemical approaches. These are highly significant results that may have important implications in future experimental design and data interpretation and deserve to be published.
It would be of great interest to the field if the following questions can be addressed:
1. What are the percentages of receptor occupancy at various ligand concentrations? Does this factor play a role in the strength of signal activation and the threshold concentration at which graded response is converted to ultrasensitive response? For example, if a cell line with very different cell surface receptor numbers as HaCat is tested in the experiment shown in Figure 4B, would this cell line show a different ligand concentration at the border of transient response and ultrasensitive response?

We appreciate the reviewer's positive assessment of this work. The reviewer raised a very interesting issue about the relationship between receptor occupancy and signaling responses. Directly measuring the exact fraction of receptor occupancy rate at various doses of TGF- is very challenging experimentally within the constraints of our system. The number of TGF- receptors on the cell surface is quite low (<10,000 per cell). While it is possible to measure the receptor occupancy with a saturated dose of TGF- , the signal from low doses, as tested in this study, would be too low to detect with the available reagents and technology.
Ligand-receptor interaction obeys the laws of chemical kinetics and physics. The percentage of receptor occupancy should be determined by the ligand concentration and the distribution of receptors at the cell surface. Since mathematical modeling can predict the levels of variables that are difficult to measure, we can indirectly deduce the relationship between receptor occupancy and signaling responses. This is really where we consider the modeling is most useful.
The ligand concentration can be accurately adjusted. Ligand depletion speed is a function of the occupancy of cell surface receptor. Our model simulation predicts that slowing down ligand depletion by increase medium volume should decrease the ultrasensitivity of long-term P-Smad2 dose response at 24 hour (Figure 6A). This prediction is largely verified by the experimental results shown in the new Figure 6B-6G. Thus, our data is consistent with the view that the percentages of receptor occupancy do play a role in converting the graded response to an ultrasensitive response.
Based on our modeling analysis, elevated T RII expression should increase the speed of ligand depletion analogous to increasing ligand concentration. Artificially elevating the number of T RII receptor on the surface may not be very straightforward. High levels of T RII will cause cell growth arrest and make it difficult to obtain homogenous clones. To make things even more complicated, a majority of overexpressed T RII receptor tends to get stuck in the ER. Increase in mRNA expression does not guarantee elevated cell surface expression of the receptors. While we will make more efforts in generating an array of cell lines with different levels of T RII which will undoubtedly take years to establish and carefully characterize, alternatively we hope the new experiment we offered in Figure 6 addressed this question satisfactorily.

2. In term of ultrasensitivity of the long term signaling response, the authors have tested the cytostatic response. Do other TGFβ-induced responses show different threshold to the ligand dosage when graded early responses are converted to the ultrasensitive responses? For example, does transcription of difference TGFβ target genes show different dosage threshold (for converting to the ultrasensitive response)? Does genes like PAI-1 show a different ligand dosage threshold from genes like p21 in this model?

TGF- induced gene transcription is often temporal. Different genes reach steady state at different times. Few TGF- inducible genes are known to have two steady states (early and late). Typically, early responsive genes peak early and return to basal state after prolonged exposure to TGF- . To investigate whether dose responses of early responsive gene induced by TGF- are graded or ultrasensitive, we measured Smad7 levels by quantitative PCR after stimulating cells with different doses of TGF- . The experimental data sets show a graded short-term Smad7 gene response with corresponding Hill coefficients of about 1.3 (Figure 5E). For long-term translational responses we chose to measure protein level of PAI-1 and p21, two known targets of TGF- that were suggested by the reviewer. We show that PAI-1 translational induction is highly ultrasensitive with a Hill coefficient close to 5 (Figure 5F), while p21 induction is only modest ultrasensitive with a Hill coefficient approximately 2 (Figure 5G). It appears that different TGF- targets do show different dosage thresholds.

3. Does the model explain how TGFβ signaling specificity may be generated? i.e. will ligand dosage or stimulation duration determine whether the cells undergo growth arrest or EMT, given that HaCat cells can do both?

Considering the correlation of P-Smad2 response duration and the cell proliferation assay at 24 hours, the model analysis indicates that the duration of Smad signaling response (e.g. sustained or transient P-Smad2) might determine whether cells undergo growth arrest. Therefore TGF- signaling specificity can be generated by how the cell ‘decodes’ the ligand dose which can be predicted by our new model, at least in HaCaT cells.
The reviewer is correct in that HaCaT has been shown to undergo EMT after prolonged TGF-beta

treatment (48 hr to 72 hr) (e.g. Zavadil et al., 2001; Lamouille and Rik Derynck, 2007). The typical assays for measuring EMT in HaCaT cells are cell morphology changes and disassembly of E-cadherin-mediated adherens junctions. The levels of E-cadherin do not exhibit dramatic changes in HaCaT during EMT. While one or two pictures can give qualitative assessment of EMT, it is not trivial to convert these assays into quantitative readouts. While blots or Brdu fluoresence images are relatively straightforward to quantify, automated quantification of EMT results is not yet feasible to engender confidence in quantitative claims. Moreover, the time scale of EMT (48 hr instead of 24 hr) also makes it difficult to compare growth arrest vs. EMT. Ultimately, we would like to develop robust quantitative assays to measure the dose responsive curves for a variety of TGF- processes. We hope the reviewer will agree that such endeavors are beyond the scope of this paper.

Reviewer #4 (Remarks to the Author):

In this manuscript, Zi et al. use both mathematical modeling and in vitro experiments to understand how the magnitude and duration of the extracellular signal (TGF-beta) is transduced into Smad2 phosphorylation levels in the TGF-beta signaling pathway. They find that while TGF-beta addition for 30s followed by ligand removal induces transient Smad2 phosphorylation, the repeated application of 30s ligand pulses spaced 30 minutes apart results in a more sustained response that is closer to that seen under continuous ligand stimulation. Smad2 phosphorylation levels at 45 minutes shows a sigmoidal relationship to TGF-beta dose (quantified as molecules/cell), while both 24 hour Smad2 activation levels and inhibition of cell proliferation show ultrasensitive dose responses with Hill coefficients > 4. Sensitivity analysis of the mathematical model reveals that the depletion of ligand molecules at low doses is a major cause of the ultrasensitivity in long-term responses.
The mathematical model (structure and governing equations) appears to be identical to the one previously published by the authors (Zi and Klipp, PLOS One, 2007). The observation that ligand depletion affects Smad activation kinetics has also been previously published by the authors (Clarke et al, Mol Cell Biol, 2009). Portions of the current manuscript lack clarity and some of the results are not discussed in depth. That said, the authors rigorously parameterize their model using existing datasets as well as some new datasets reported in the current manuscript. The manner in which the authors use modeling to reach the conclusion that ligand depletion affects the long-term dose response is also compelling. Overall, while the manuscript may be somewhat lacking in originality and depth, their approach is sound and the findings are likely to be of interest to the field of cell signaling.

The model is not identical to our previous model (Zi and Klipp, PLoS ONE, 2007) even though the network topology and equations are similar. We have made several important modifications to our old model. First, we have changed the scheme of Smad2/Smad2 and Smad2/Smad4 interactions that are based on the work of Schmierer et al (Schmierer et al, PNAS, 2008). Second, the receptor trafficking part is simplified and the ODE equation for TGF- is updated. Our previous model did not calibrate the ligand depletion due to the lack of experimental data sets at that time. Instead, our previous model assumed that loss of the T RI is the underlying mechanism for the decrease in phospho-Smad2 that occurs during signaling. More importantly, we have calibrated and validated the model with more data sets from different conditions. Such refinements of the prior model are important to achieve accurate model predictions, as illustrated in this work.

Major concerns:
1. It is not clear which of the datasets used in model calibration are original datasets and which ones have been previously published. This needs to be clearly mentioned. Original data (datasets 1-3?) needs to be presented in the main manuscript. I don't understand why Figure 1B is necessary. What did the authors learn from the "initial calibration of the model", what prompted them to change the model and what did this exercise reveal? Why not start with the "refined" model that is calibrated using all the data?

The experimental data sets are explicitly specified in the supplementary information (section 2.3 Experimental data sets for parameter estimation). Only experimental data set 7 and 8 were obtained

from previous publications. The experimental data sets 1-6 and other data sets for testing model predictions were measured in this work. This is now better explained in section 2.3 of the supplemental information.
We have followed the reviewer's suggestion and present only the final refined model in the revised manuscript. Figure 1B of the first version is now omitted. The initial model was calibrated with few data sets at the early stage of the work, which give us some preliminary predictions and inspired us to do additional experiments. Similar to the above response to the reviewer #1 presented above, we intended to show the history of work in the manuscript by mentioning the initial model analysis. Since this might cause some confusion, we have followed the suggestion of the reviewers by only presenting the final model.

2. On a related note, the studies involving 2 successive additions of ligand with either Ton=Toff=1hr or Ton=Toff=3hr seem to have been designed in an ad hoc fashion and don't really appear to tie in with the rest of the manuscript. How do these results (Figure 2) inform the rest of the manuscript? Do the model parameters change significantly when these results are included as part of the fit? Which parameters change, and what does this reveal about how the system operates? What are the implications of the inherent relaxation time of the pathway (between 1 and 3 hours) in the context of development? The manuscript in general and this section in particular would benefit from a more detailed discussion of the results.

The experimental results from Figure 2 (Figure 3 in the revised manuscript, Hereafter we refer it as Figure 3) are valuable for the manuscript. First, the result indicates that cells can periodically respond to pulses of TGF- stimulation with different doses and different periods. This is an interesting observation as remarked by reviewer #3. In addition, the availability of the data sets in Figure 3 allows us to validate the model. The data in Figure 3A-3E were not included during parameter estimation. We tested the model by checking whether the model can predict similar P-Smad2 response with different TGF- doses and different Ton and Toff (Figure 3). The estimated parameter values would not be significantly changed by including the data in Figure 3A-3E. It's also important to validate model performance by checking the model predictability for reproducing other experimental data sets from different conditions.

3. Experimental validation of the finding that ligand depletion affects long-term Smad phosphorylation would greatly strengthen the manuscript. Comparison of the Smad response to the same concentration of TGF-beta provided in different volumes of media can be used to demonstrate that ligand depletion is indeed important.

We appreciate the suggestion of the reviewer and performed two types of experiments for long-term P-Smad2 dose responses with 10 mL medium, which is as 5 times the normal amount of medium we used (2mL). In one assay, we use the same concentrations of TGF- with 10 mL medium as those with 2 mL volume. As shown in Figure 6G, P-Smad2 response with 10 mL medium was almost saturated with even at the lowest concentrations of TGF- . In contrast, the same low concentrations of TGF- gave almost no P-Smad2 response with 2 mL volume. This has confirmed that cells sense TGF- doses by molecules per cell, not by TGF- concentration.
In addition, we also performed the experiments to test the model prediction that decreasing TGF- depletion with larger volume of medium will affect P-Smad2 response at 24 hour to TGF- doses in terms of molecules per cell. We measured P-Smad2 level with 10 mL TGF- containing medium and stimulated cells with the same molecules per cell doses as those with 2 mL medium. The experimental data confirmed our model prediction that long-term P-Smad2 dose responses are changed from ultra-sensitive to more graded-like when cells are stimulated with 10 mL medium (Figure 6B-6E).

Minor comments:

We thank the reviewer #4 for carefully reading of the manuscript. We have also checked the grammar and spelling in the revised manuscript. All of the following specific minor comments such as typos, reference updates, figure and figure legend changes have been addressed. Below, we respond only to selected comments that require further clarification.

*1. Table S4: Rate expression for dx12/dt is incorrect - koff_Smads*x13 should be 2*koff_Smads*x13. If the model is coded this way, then simulations need to be re-run to verify the results.*

This was a typo of the equations in the preparation of the manuscript. The model was coded correctly and the simulations were verified. Once the manuscript is accepted, we will provide the SBML files or deposit them in public database (for example, BioModels database).

2. The authors need to provide quantitative plots for all the western blots, particularly in cases where model simulations are being compared to experimental data (Figure 1A, Figure 3C, Figure S5B).
Done as suggested.

3. Figure 4A,B: Legend needs to state that experimental data has been re-scaled to match the mathematical model so that p-Smad2 can be expressed as a fraction of total Smad2. Unless panels A and B were done as part of the same blot, it is not possible to compare the absolute P-Smad2 levels between them. This applies to Figure S3A as well - were all measurements in the figure done as part of the same blot? How did the authors obtain p-Smad2 as a % of total Smad2?

We have added a description in the figure legend as suggested by this reviewer. The P-Smad2 as percentage of total Smad2 was calculated by the number of P-Smad2 molecules/cell divided by the number of total Smad2 molecules/cell (1e5). In the revised manuscript, we plot P-Smad2 levels with "molecules/cell". We have added the corresponding assignment rule (totalPSmad2) in Table S4.

4. Figure 4E: Y-axis label should be more descriptive (e.g.% of BrdU-labeled cells).
Changes have been made.

5. A discussion of why receptor depletion cannot be invoked to explain the dose response behavior (along the lines of the discussion in Clarke, 2009) would be useful.
Done as suggested.

6. Figure 1B and associated text - replace "normal stimulation" with something more descriptive (e.g. sustained ligand stimulation).
Changes have been made as suggested.

7. Figure 2: It would help to have bars on the plots indicating the on and off phases of ligand addition.
Changes have been made as suggested.

8. Page 3, 1st paragraph - the Watanabe et al. reference is incomplete.
Changes have been made as suggested.

9. Page 4, 2nd paragraph: "The dynamic behavior... to alternating ligand exposure cannot be predicted by existing mathematical models..." is misleading. Any of the existing models (Vilar, Schmierer, Clarke) can be simulated under conditions where the ligand concentration varies arbitrarily with time.
To avoid any misunderstanding, we have changed the sentence to "The dynamic behavior of Smad2 phosphorylation in response to alternating ligand exposure cannot be predicted by the existing mathematical models of TGF- signaling pathway, which have not explicitly taken into account the ligand dynamics in the medium".

10. Page 8, Line 1: "Keratinocytes" is misspelled.
Corrected.

11. Supp Mat, Page 6, 1st paragraph: "The estimated 9 parameter values have small variations..." is inaccurate. The on rate for non-specific ligand binding clearly has a large variability. This is not surprising since this occurs at a much faster time scale than the experimental sampling. Upper and lower bounds used for the various parameters during the optimization need to be included as part of Table S2.
We have included the upper and lower bounds of the estimated parameter in optimization.

12. Supp Mat, Page 6, Section 4: Provide the Smad4 level in HaCaT cells (only Smad2 level is given).
The level of Smad4 in HaCaT is given.

13. Table S2: Incorrect units for trimolecular rate constant ka_LRC.
Correction has been made.

14. Table S3: Highlight x2, x3, x4 and x5 and clearly mention that these initial concentrations change as a function of the receptor synthesis rates during parameter estimation.
Correction has been made.

There are several grammatical and typographical errors in the manuscript. Careful proofreading and editing is warranted.

2nd Editorial Decision 31 March 2011

We have now heard back from the three referees who agreed to evaluate your revised work. As you will see, the referees felt that the key issues raised in the previous reviews had been sufficiently addressed, and they were generally supportive of publication. Some minor concerns remain, however, which we would ask you to carefully address in a final revision of the present work.

The first reviewer has some final concerns that s/he felt required some additional minor clarification in the text and abstract (see below).

In addition, the editor asks you to address the following data and format issues:

1. Please provide the mathematical model files in SBML (as noted in your supplemental information file). In addition, we strongly encourage you to submit these model files to a public repository such as BioModels or JWS Online. If possible, we ask that you also provide the source file used to generate Fig. 2 (i.e. the Cell Designer file). A single zip file containing all of these files can be provided as supplementary material.

2. We strongly encourage authors to submit the source numerical data for any experiments that were subsequently used to train mathematical models. To aid with this, 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>). Guidelines have been pasted at the end of this email.

3. The current synopsis is a bit short. In general, extended synopses are 500-1000 words, include a brief description of the experimental results (aimed at a broad audience), and may reference up to two figures from the main manuscript.

4. The Table of Contents at the beginning of the Supplementary Information pdf should list any additional resources provided as supplementary material (e.g. model files); figure source data is not listed here.

Thank you for submitting this paper to Molecular Systems Biology.

Yours sincerely,

Editor
Molecular Systems Biology

REFEREE REPORTS

Reviewer #2 (Remarks to the Author):

The revised manuscript by Zi et al is improved from the previous submission, and the authors have addressed most of my concerns. However, I still feel that their description of the results could cause some confusion to readers who are not experts in modeling.

For example, in response to my previous point 1 they have included pulsatile TGFb stimulation with longer intervals (3 hours) between pulses. This results not in a sustained phospho-Smad2 response (as stated in the abstract), put rather an oscillating one (see Fig 4E). So while repeated pulses at relatively short intervals are integrated, with a 3 hour gap they are not. This needs to be clarified in the abstract.

Similarly, I still feel that the use of the phrase "long-term ultrasensitive signaling", as in the final sentence of the abstract is also confusing. While they have included "switch-like" to explain this in some places, I feel that this should be very clearly explained up-front. The long term responses are not ultrasensitive to low doses of TGF , which it seems to me is an obvious interpretation of this statement. Rather they switch from off to on at a higher dose, in a manner that is "ultrasensitive" to a small change in TGF levels.

While this does not reflect a major criticism of the data or analysis, I do feel that these points of potential confusion should be cleared up in the text prior to publication.

Reviewer #3 (Remarks to the Author):

The authors have addressed my concerns in a satisfactory manner. I recommend acceptance.

Reviewer #4 (Remarks to the Author):

The authors have satisfactorily addressed my concerns, as well as - in my opinion - the concerns of the other reviewers in developing the revised manuscript. Additional experiments where the media volume is varied at a fixed TGF-beta concentration clearly demonstrate that ligand depletion plays an important role in the shape of the p-Smad2 dose response at 24 hours. The additional studies examining the expression of specific early and late-response genes as a function of TGF-beta dose also serve to strengthen the biological relevance of the manuscript.

2nd Revision - authors' response 07 April 2011

Enclosed please find the revised manuscript entitled " Quantitative Analysis of Transient and Sustained Transforming Growth Factor- Signaling Dynamics " by Zhike Zi, Zipei Feng, Douglas A. Chapnick, Markus Dahl, Difan Deng, Edda Klipp, Aristidis Moustakas and Xuedong Liu for consideration for publication in Molecular Systems Biology.
We thank the reviewers for their supports and insightful comments on our revised manuscript. Reviewer #2 pointed out that we need to clearly define the conditions that periodic short pulse

exposure of ligand can produce a sustained TGF- response. In addition, he/she also suggested that we should improve the definition of ultrasensitivity in the context of dose TGF- response behavior. We agree with these recommendations and we have rewritten the relevant part of the manuscript Abstract to clarify potential confusions. A typo in Figure 7 is corrected. In addition, we have provided the source Figure data, the SBML model and the CellDesigner file as recommended by you. Accordingly, the Supplementary Information is updated. We hope that you will find this version of the manuscript suitable for publication in MSB.

My colleagues and I appreciate your efforts on behalf of this manuscript. We look forward to hearing from you. Thank you very much for your consideration!

