molecular systems biology

A Mechanism for Robust Circadian Timekeeping via Stoichiometric Balance

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Review timeline:

Submission:	16 July 2012
Editorial Decision:	20 August 2012
Revision received:	18 September 2012
Accepted:	19 October 2012

Transaction Report:

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

Editorial Decision 20 August 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the four referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. They raise, however, substantial concerns on your study, which should be convincingly addressed in a revision of this work. Addressing the major points raised by reviewer #2 appears particularly important in this regard.

IMPORTANT: in view of our policy on material, data and software sharing (www.nature.com/msb/authors), we would also kindly ask you to include in supplementary information a machine-readable version of your model, in principle in SBML format, and to deposit it in one of the public resources (eg Biomodels) and include the accession number in the Material & Methods section.

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

http://www.nature.com/msb

Referee reports:

Reviewer #1 (Remarks to the Author):

This paper provides an incremental advance on previous models of the circadian clockwork. The authors principally use the mammalian system as their basis for modelling, and show that similar logic could be applicable to the Drosophila clockwork.

The main novelty of the work lies in the finding that stoichiometry is highly influential in determining the properties of the circadian oscillation. In particular, stoichiometric balance, likely afforded by an additional negative feedback loop (supported by recent in vivo evidence from Reverb a/b mouse studies).

A weakness of this largely theoretical paper is the lack of any clear ways in which the model may actually be tested in a wet lab. The authors purport that the findings could inform experimental work, but it might be useful to give some examples of how this might be done. For example, they state that manipulating stoichiometry should significantly effect the clockwork, and may be an opportunity for clinical translation. However, previous attempts to do this in all but very simplified systems has been fraught with difficulties. RNAi approaches, such as the Baggs / Hogenesch study, which did do this for a limited number of clock factors showed that stoichiometry may well be important already. Tuning the levels of different components simultaneously is just not possible with current molecular biology / cell biology, in any model system.

So, while the study is no doubt of interest, potentially guiding wet lab experiments, it is hard to see how it significantly advances our knowledge of how the clock works or general organizing principles. For example, we already know that the Reverba/b axis is more important than we thought previously because of 'wet' experiments. That the authors' model confirms this is great, but does it suggest anything more than what we know from experimental data already?

Reviewer #2 (Remarks to the Author):

Kim and Forger, Molecular Systems Biology MSB-12-3904

The authors improve their previous negative feedback loop model of the mammalian molecular circadian clock to predict more accurately the circadian phenotypes of clock gene knockouts, time courses of expression of clock genes, and relative expression levels of mRNAs and proteins. In their model, the authors show how stable activators, tight binding of repressors to activators (rather than directly to promoters), a balance of activators and repressors, and a double negative feedback loop architecture (which helps to keep this balance) are important for stable circadian oscillations, whereas adding a positive feedback loop allows greater tuning of period.

Major Concerns:

A) As experimental evidence that a balanced repressor:activator ratio is important for stable rhythmicity, the authors cite Lee (2011) as showing that damped rhythms in WT fibroblasts (which have a low ratio) can be rescued by PER overexpression (pp. 5, 8). However, Lee (2011) shows only that cell population rhythms damp, and other studies show that this is due to dephasing among cells, not damping of single fibroblasts (Welsh, Curr Biol 14:2289, 2004; Leise, PLoS One 7:e33334, 2012). Therefore, PER overexpression might reduce damping of cell populations by improving synchrony among cells rather than altering rhythms in single cells.

B) In Table 1, the authors compare clock gene knockout phenotype experimental data to predictions of their single cell model (and others). However, SCN slice and behavioral phenotypes often differ from single cell phenotypes due to SCN network interactions (Liu, 2007; Ko, 2010). Therefore,

single cell model performance should be compared not with SCN or behavioral phenotypes, but with single cell phenotypes. Also, adding Per2 and CKI mutant phenotypes would be of interest.

C) In cases where their model accurately predicts knockout phenotypes, the authors do not determine what features of the model account for its success, missing an opportunity to gain insight into biological mechanisms. Perhaps a sensitivity analysis of the new model might be a whole paper in itself.

D) Explain more clearly how a balance of activators and repressors enhances rhythm stability, e.g. as in Buchler, J Mol Biol 384:1106, 2008.

E) The simple model seems rigged to give optimal results at 1:1 stoichiometry of activators and repressors. Acknowledge this or refute.

F) In Fig. 3A, exactly how is repressor:activator ratio calculated for various mutants? The authors seem to ignore the complexities of multimeric complexes and variations in biochemical activity. In particular, amplitude of Cry1-/- and Cry2-/- are shown as similar in Fig. 3A, but single cell experimental data show that the amplitudes are actually very different (Liu, 2007) despite similar stoichiometry. This can be explained by CRY1's stronger repressor activity (Khan, J Biol Chem 287:25917, 2012).

G) Explain how the NNF model includes inhibition as by RevErba, whereas the PNF model includes activation by as by Rors, and the evidence that inhibition is more important (Liu, PLoS Genet 4(2), 2008).

Minor Comments:

1) Omit, qualify, or justify such broad, sweeping claims as "the most accurate and detailed mathematical model... to date" (p. 2).

2) In Fig. 1, use consistent colors for molecules in parts A and B. Fig. 1B should indicate the inhibition by PER/CRY.

3) In Fig. 2A and 2B, label vertical axes as relative mRNA and protein abundance, respectively (not amplitude).

4) In Fig. 3C, the placement of "Kd" near the upper arrow is misleading.

5) Fig. 4C indicates that the average level of repressors (0.25) and activators (0.75) in WT, their stoichiometry is about 0.33 and not close to 1. Is this consistent with your theory? In the legend, explain more clearly the sentence "The average of relative sensitivity...."

6) In Fig. 5, show NNF, SNF, and PNF areas in 3 separate panels, instead of overlaying them. Consider using color coding to provide period information, as in Fig. 6. Label A = mammalian, B = detailed mammalian, C = fly.

7) Change ", however, " to "; however, " (p. 8), "the rhythms whose period is robust to gene dosages" to "rhythms with periods robust to gene dosage" (p. 15), "too much repressors" to "overabundance of repressors" (p. 15), "data also supports" to "data also support" (p. 16), "more pivotal role" to "a more pivotal role" (p. 16), "model based on the protein... who focuses" to "model based on sequestration... which focuses" (p. 17), "the rhythm generation (e.g. High-hill" to "rhythm generation (e.g., high Hill" (p. 18), "data of mammalian" to "data on the mammalian" (p. 18), "Bmals" to "Bmal" (p. 31), "data form" to "data from" (p. 32), "degrade quickly" to "degrades quickly" (p. 33), "degrade slowly" to "degrades slowly" (p. 33).

Reviewer #3 (Remarks to the Author):

In this paper, Kim and Forger put forward in intriguing hypothesis for the regulation of mammalian circadian timekeeping. Circadian rhythms in mammals have been shown to be robust to a variety of perturbations: knockdown of important clock genes, general transcriptional repression, etc. While this resilience is important for the function of the system, it has made it difficult for researchers to pin down exactly which features of the system are truly essential. Kim and Forger suggest that the resilience of the mammalian clock lies in maintaining a roughly 1:1 stoichiometry between the activators (CLOCK, BMAL1) and the repressors (PER, CRY) acting on the E-box. They suggest that the major role of the secondary feedback loop involving the REV-ERB genes is to ensure that this stoichiometry is maintained. To support this argument, they present both a highly detailed model of the mammalian clock that incorporates a broad range of mechanistic data and a very simplified Goodwin-type model that is more amenable to analytical analysis, most of which is described in the supplementary information. While the detailed model is a (much-needed) update of the 2003 model by Forger and Peskin and will undoubtedly be used by other researchers in the future much as the F&P model has been, the detailed model does not occupy center stage in this manuscript -- the focus is on transcription factor stoichiometry and its biological consequences.

My concerns with this paper are fairly minor:

Pg. 7: The authors define stoichiometry as the "average ratio between the concentrations of repressors ... to that of activators." It would help if this definition were made more explicit -- if I'm not mistaken, the average in question is a time average over the circadian period. Also, the authors seem to have an unfortunate habit of using "activator" and "repressor" as shorthand for "E/E'-box activator" and "E/E'-box repressor" -- this could be very confusing for readers who might be tempted to extend the conclusions to other types of circadian activators and repressors. For the record, I believe that the standard English usage would be "average ratio between the concentration of repressors ... and that of activators."

Pg. 10: Are you defining <S> as A/P or P/A?

Pg. 12: Your prediction that REV-ERB knockouts will maintain rhythmicity in cell types with a nearly-balanced stoichiometry but lose it in poorly-balanced cells appears to be experimentally tractable. This is an interesting prediction, since it may have relevance for the differences between SCN and peripheral clocks, and it should be highlighted in the summary of experimental predictions on page 17.

Pg. 15: "Both our detailed and simplified NNF models predict these results" -- this isn't really a prediction, since the experimental results were already published when you made the model. It would be more correct to say "reproduce" or "confirm." (FWIW, this distinction is made quite well elsewhere in the manuscript.)

Pg. 16: "The elimination of additional positive feedback ... based on the PNF structure" -- I'm not quite sure what you're talking about here; all of the discussion so far has focused on the role of an additional *negative* feedback loop and an *NNF* structure. Is this a typo, or did I miss something really important?

Pg. 20: "Modifications and extensions of the detailed model" -- somewhere in this paragraph you should mention the reference for the previous version of the detailed model. I know you have it elsewhere in the manuscript, but readers not familiar with your previous work will appreciate having it here.

Pg. 21: "this large number of parameters, which is still less than other models," -- this makes it sound like you're apologizing for having lots of parameters. More importantly, what do you mean when you say you have "better" estimates of the parameters? You certainly demonstrate that your model does a good job at reproducing system-level behaviors, but there is evidence (James Sethna's work on "sloppy models", for example) that correct system-level behavior does not imply accurate estimations of model parameters, and that these two goals might often be incompatible. In other words, you restrict your parameters to a biologically reasonable range, but it might be entirely possible to get equally-good fits to your data with physically unreasonable parameter values. If you

just mean that you're using experimental data to put inequality constraints on your parameter estimates (which seems to be implied by your points 2-5), then say that instead.

Pg. 21-22: It would be nice to see a little more description of the simulated annealing protocol. Also, describing your fitting protocol without saying anything about your cost function doesn't really help anyone understand what you were doing; please say more about your cost function.

The quality of the English in this manuscript is quite high up until the Discussion section (page 14), but I felt like the latter portion of the document contained far more mistakes and rough spots. It would probably be worth it to look over the final sections with this in mind. A few specific issues:

Pg. 15: "more stable activators than repressors" makes it sound like the number of stable activators is larger than the number of repressors. You might want to try "higher stability for activators than for repressors" or something similar. Also "too much repressors" a few sentences down could be "elevated repressor concentrations."

Pg. 15: "yield opposite effects" should be "yields"

Pg. 16: "Our study also proposes the underlying mechanism ... of a previous experimental study" -- try "Our study also suggests an underlying mechanism ... for a previous experimental observation..." or something similar.

Pg. 16: "...studies have shown more pivotal role of the additional negative feedback loop" -- try "...studies have confirmed a pivotal role for the additional negative feedback loop"

Pg. 17: "Our model also makes important predictions, which can be tested..." -- comma is unnecessary.

Pg. 17: "...a previous model based on the protein sequestration ... who focuses on other mechanisms ..." -- try "a previous model based on protein sequestration ... which focuses on other mechanisms ..."

Pg. 18: "mechanisms of the rhythm generation" -- try "mechanisms for rhythm generation"

Pg. 18: "High-hill coefficients" should be "high Hill coefficients"

Pg. 18: "roles of the additional negative feedback loop" -- try "roles for the additional negative feedback loop"

Pg. 18: "experimental data of mammalian circadian clock" -- try "experimental data on the mammalian circadian clock"

Pg. 22: "which should a good fit" -- did you mean "which showed a good fit" ?

Reviewer #4 (Remarks to the Author):

The authors develop here a fairly comprehensive mathematical model of the mammalian circadian oscillator, which includes most of the recent experimental observations. The predictions of this model match with observed phenotypes of circadian knock-out strains better than previous models. In the center of this model is the new and original concept of 'stoichiometric balance' of activators and repressors. Then, they add to the core model two alternative extensions, one negative feedback loop, or a positive feedback loop. Interestingly, only the slow negative feedback loop model is capable to predict the behavior of the mammalian circadian feedback loop with its stoichiometric balance, while the other feedback loop model is more suitable for other (circadian) oscillators, e.g. the Neurospora circadian oscillator without obvious stoichiometry occurring.
Over all, the paper is well written, well documented and suitable in its length and displayed items. The Material and Methods part is well structured and can be followed even by non-specialists. However, one question remains:
Suppl. Information, page 4 (5): why is it necessary to include light - inducibility of Per1-2 into the

model? The model was intended to provide stable periodicity of the oscillator (page 5 'maintaining periodicity is crucial'). Light - inducibility of Per1-2 causes a phase shift of the circadian oscillator, which is probably based on a (transient) period change. In addition, the effect is completely dependent on the time, when the light pulse is given (phase-advance vs. phase-delay). How prominent is the light - inducibility parameter on the over all system? Does light - induction change the stoichiometry and would this explain e.g. the difference of type I and type 0 phase resetting?

I would also probably change the title to highlight the new concept of stoichiometric balance in their model.

1st Revision - authors' response 18 September 2012

Thank you for considering our manuscript for publication in Molecular Systems Biology and providing an opportunity to respond to the reviews.

We also thank all reviewers for carefully considering the manuscript. We now provide more testable predictions based on the comments of reviewer 1, and highlight the differences between single cell, network and whole organism data for reviewer 2. Additional analysis is preformed (e.g. sensitivity analysis or further simulations of mutations prompted by reviewer 2). More details about model generation are provided for reviewer 3 and further discussion of the role of light is provided for reviewer 4. Many other changes have now been provided.

We have also provided the additional information that you requested. We have deposited the models in ModelDB. Our simple model is available in 5 formats including SBML. Our more complex model is available in 3 formats including Matlab, Mathematica and XPP. The accession numbers are provided in the materials and methods.

The manuscript is much improved, to the point, we hope, that it is acceptable for publication at Molecular Systems Biology. Thank you again for your further consideration.

Sincerely,

Daniel Forger and Jae Kyoung Kim

Note: In the following document, we quote all reviews in black and provide responses to the points they raise in blue.

Reviewer #1 (Remarks to the Author):

This paper provides an incremental advance on previous models of the circadian clockwork. The authors principally use the mammalian system as their basis for modelling, and show that similar logic could be applicable to the Drosophila clockwork. The main novelty of the work lies in the finding that stoichiometry is highly influential in determining the properties of the circadian oscillation. In particular, stoichiometric balance, likely afforded by an additional negative feedback loop (supported by recent in vivo evidence from Reverb a/b mouse studies).

- We agree with the reviewer that the "main novelty" of our work is in the role of stoichiometry and the double negative feedback loop structure. That being said, we feel that the improvements to the model are substantial rather than incremental. For example, quoting reviewer 3, "the detailed model is a (much-needed) update of the 2003 model by Forger and Peskin and will undoubtedly be used by other researchers in the future much as the F&P model has been." Perhaps the most fair comparison is shown in Table 1, which indicates that our current model indeed matches much more experimental data than previous models. Additionally, the model is based on a novel mechanism of oscillation generation in circadian models. Thus, we hope the readers will be impressed, not only with our findings regarding our mechanism for oscillation generation, but also with the availability of a new detailed and a new simplified model.

A weakness of this largely theoretical paper is the lack of any clear ways in which the model may actually be tested in a wet lab. The authors purport that the findings could inform experimental work, but it might be useful to give some examples of how this might be done. For example, they state that manipulating stoichiometry should significantly effect the clockwork, and may be an opportunity for clinical translation. However, previous attempts to do this in all but very simplified systems has been fraught with difficulties. RNAi approaches, such as the Baggs / Hogenesch study, which did do this for a limited number of clock factors showed that stoichiometry may well be important already. Tuning the levels of different components simultaneously is just not possible with current molecular biology / cell biology, in any model system. So, while the study is no doubt of interest, potentially guiding wet lab experiments, it is hard to see how it significantly advances our knowledge of how the clock works or general organizing principles. For example, we already know that the Reverba/b axis is more important than we thought previously because of 'wet' experiments. That the authors' model confirms this is great, but does it suggest anything more than what we know from experimental data already?

- The reviewer points out an important limitation of our previous manuscript. In particular, our discussion section focused almost exclusively on putting our work within the context of other studies. While this is important, the readers of Molecular Systems Biology may be most interested in specific predictions, of reasonable difficulty, which could be tested in the lab.
We now have added general approaches to test our model (listed below).

- The reviewer is also correct that "Tuning the levels of different components simultaneously" is beyond current techniques. Therefore, we focus on predictions where this is not needed. This is possible since changes to only one component are sufficient to change the stoichiometry. Also at the reviewer's suggestion, we have removed some of the discussion about future translational applications.

- The following text has been added to a new section of the discussion: Proposed experiments based on model predictions (page 20-22). Each of the following six paragraphs highlights at least one way in which our model's predictions could be experimentally tested in the lab.

"**Proposed experiments based on model predictions**
Our most important prediction may be the following: when the stoichiometry between activators and repressors is within a fixed range, oscillations are sustained, and outside this range oscillations are damped (Figure 3). This can be tested by measuring the relative concentration of activators and repressors in many tissues and in the presence of several possible mutations which lead to damped or sustained rhythms. This has been done in WT fibroblasts and liver (Lee et al, 2001; Lee et al, 2011), but has not been done in other tissues or mutants. Moreover, we note that these previous experiments were done in population cell assays, whereas single cell measurements may be needed to determine whether damped oscillations are the result of damped rhythms in single cell, or greater population desynchrony (Leise et al, 2012; Welsh et al, 2004).

The behavior of isolated SCN neurons is similar to fibroblasts in that mutations of circadian genes can easily lead to arrhythmicity (Liu et al, 2007). We note that intercellular coupling in the SCN not only synchronizes SCN neurons, but also increases transcription of *per1* and *per2* (Yamaguchi et al, 2003), which may balance stoichiometry and help sustain rhythms when repressors are effectively removed (Table 1 and 2). Thus, we predict that increasing transcription of *per1* and/or *per2* could enhance rhythmicity in isolated SCN neurons similar to what is seen in fibroblasts (Lee et al, 2001). Moreover, our model predicts that cells with low stoichiometry (e.g. isolated SCN neurons) shows larger phase-shifts in response to light than cells with 1-1

stoichiometry (e.g. SCN slices) (data not shown). It would be interesting future work to see whether different cell types have different PRCs depending on their stoichiometry.

We also predict that tight binding between activators and repressors is required for rhythmicity (Figure 3D). Several studies have identified binding sites for PER and CRY on BMAL1 and CLOCK (Langmesser et al, 2008; Sato et al, 2006; Ye et al, 2011). Point mutations in binding sites can generate different binding affinities between PER-CRY and BMAL1-CLOCK. Comparing the experimentally measured binding affinities of these mutants, with the resultant rhythms, or lack thereof, would directly test this prediction.

Loss of the additional negative feedback loop (e.g. in the Rev-erbs -/-, constitutive expression of Rev-erbs or constitutive expression of BMAL) is predicted to cause the intracellular circadian clock to oscillate over a much narrower range of conditions (Figure 5). It would be interesting to test whether these cells would have less temperature compensation or would lose rhythms more easily when other genes are knocked out (e.g. *Cry2*$^{-/-}$, *Per1*$^{-/-}$). Moreover, we predict that in the Rev-erbs$^{-/-}$, rhythms persist in cell types with a balanced stoichiometry, but not in poorly balanced cells (Figure 5). It would be interesting future work to investigate whether SCN and peripheral clocks have different phenotypes of Rev-erbs$^{-/-}$ depending on their stoichiometry. We also predict that Rev-erbs$^{-/-}$ cells show a wider period distribution than WT (Figure 6).

Our modeling and analysis also predicts that relatively stable activators (e.g. BMAL1 and CLOCK) in the additional negative feedback loop allow rhythmicity over a wide range of conditions (Supplementary Figure 5D). These activators can be destabilized with point mutations (Sahar et al, 2010). Simply destabilizing the activators might lead to lower activator concentrations and unbalance stoichiometry, which is also predicted to reduce rhythmicity. However, we predict a loss of rhythmicity when these activators are destabilized, even when the overall activator concentrations are controlled for.

Perhaps the most direct way to test our model is to build the clock described in our simple NNF model using the tools of synthetic biology. Other synthetic clocks have been built, and the design we propose is not more complex than what has been previously built (Stricker et al, 2008; Tigges et al, 2009; Tsai et al, 2008). Validation could first be done in an analog electric circuit, even though this might be much less convincing. Building a synthetic clock would be of particular importance since it would be the first synthetic clock predicted to have a tightly regulated period."

- We also now make the model much more accessible by providing in several formats: SBML, Matlab, Mathematica and XPP. These have been deposited them in a public resource (Model DB). Our experience, at least based on the original Forger and Peskin model, is that the community will use models in very unanticipated ways. For example, we would have never anticipated that our model be used as was done in Wilkins el al, 2007 validating the importance of PER2, or the study by Dibner et al. 2009 testing overall changes in transcription rates. Thus, we hope that the model be tested in even more ways than the predictions now presented in the manuscript.

Reviewer #2 (Remarks to the Author):
Kim and Forger, Molecular Systems Biology MSB-12-3904

The authors improve their previous negative feedback loop model of the mammalian molecular circadian clock to predict more accurately the circadian phenotypes of clock gene knockouts, time courses of expression of clock genes, and relative expression levels of mRNAs and proteins. In

their model, the authors show how stable activators, tight binding of repressors to activators (rather than directly to promoters), a balance of activators and repressors, and a double negative feedback loop architecture (which helps to keep this balance) are important for stable circadian oscillations, whereas adding a positive feedback loop allows greater tuning of period.

- We thank the reviewer for this accurate summary of our work.

Major Concerns:

A) As experimental evidence that a balanced repressor:activator ratio is important for stable rhythmicity, the authors cite Lee (2011) as showing that damped rhythms in WT fibroblasts (which have a low ratio) can be rescued by PER overexpression (pp. 5, 8). However, Lee (2011) shows only that cell population rhythms damp, and other studies show that this is due to dephasing among cells, not damping of single fibroblasts (Welsh, Curr Biol 14:2289, 2004; (Leise et al, 2012; Welsh et al, 2004), PLoS One 7:e33334, 2012). Therefore, PER overexpression might reduce damping of cell populations by improving synchrony among cells rather than altering rhythms in single cells.

- The reviewer makes an important point that Lee et al (2011) only looked at population rhythms, and that damped population rhythms could arise either because of damped individual rhythms, or greater desynchrony. We now indicate this as a limitation of the Lee et al (2011) study and our interpretations of it. To address this, we now mention that the Lee et al (2011) study measures rhythmicity in a population of cells. The implications of this are also noted:

"Population rhythmicity in mouse embryonic fibroblasts shows much lower amplitude than in liver (Lee et al, 2011). The ratio of repressors to activators is significantly lower in fibroblasts than that found in liver (Lee et al, 2001; Lee et al, 2011)." (Introduction, page 4, bottom)

"This matches a recent experimental study showing that the amplitude and sustainability of population rhythms increase when the level of PER-CRY is increased closer to that of BMAL1-CLOCK in mouse fibroblasts (Lee et al, 2011)." (Results, page 8, bottom)

"Our most important prediction may be the following: when the stoichiometry between activators and repressors is within a fixed range, oscillations are sustained, and outside this range oscillations are damped (Figure 3). This can be tested by measuring the relative concentration of activators and repressors in many tissues and in the presence of several possible mutations which lead to damped or sustained rhythms. This has been done in WT fibroblasts and liver (Lee et al, 2001; Lee et al, 2011), but has not been done in other tissues or mutants. Moreover, we note that these previous experiments were done in population cell assays, whereas single cell measurements may be needed to determine whether damped oscillations are the result of damped rhythms in single cell, or greater population desynchrony (Leise et al, 2012; Welsh et al, 2004)." (Discussion, page 20, 2nd paragraph)

B) In Table 1, the authors compare clock gene knockout phenotype experimental data to predictions of their single cell model (and others). However, SCN slice and behavioral phenotypes often differ from single cell phenotypes due to SCN network interactions (Liu, 2007; Ko, 2010). Therefore, single cell model performance should be compared not with SCN or behavioral phenotypes, but with single cell phenotypes.

- The reviewer is correct to point out that SCN slice and behavioral phenotypes can be different than single cell phenotypes. In particular, rhythms can be seen in slice or whole animal behavior even when isolated neurons are incapable of generating autonomous rhythmicity (Liu and Welsh

et al, 2007; Ko 2010). Our model accurately matched slice or behavioral phenotypes. We agree with the reviewer that our model should be compared with single cell phenotypes, and this is now done (see below). However, since not all mutant phenotypes have been studied using single cell measurement, and also since we feel more data comparison is better than less, we have also kept the slice or behavioral data in our manuscript. In fact, this has lead to an interesting prediction of our model (see below).

- Coupling of SCN neurons in slice is mediated through the CREB/CRE pathway, which turns on the expression of repressor genes *per1* and *per2*. Thus coupling increases the concentration of *per1* and *per2*. For this reason, it is important that when the comparison with single cell data, *per1 and per2* expression should be reduced. (e.g. see Yamaguchi et al, Science (2003) from which it can be estimated by about 60%). When we reduced *per1* and *per2* expression in our model, our model is able to accurately reproduce the single cell phenotypes (Liu and Welsh et al, 2007). We have added the following text, and included a new table to indicate this (page 7, top):

"Our model accurately predicts the phenotype of known mutations of genes in the central circadian clock (SCN) (Baggs et al, 2009; Ko et al, 2010; Yoo et al, 2005), which other models do not predict (See Table 1) (Forger & Peskin, 2003; Leloup & Goldbeter, 2003; Mirsky et al, 2009; Relógio et al, 2011). Interestingly, our model shows opposite phenotypes for *Cry1*$^{-/-}$ and *Cry2*$^{-/-}$ matching experimental data (Liu et al, 2007). The opposite phenotypes of *Clock*$^{-/-}$ (null mutation) and *Clock*$^{\Delta 19/+}$ (dominant negative mutation) are also correctly simulated in a model for the first time (Debruyne et al, 2006; Herzog et al, 1998; Vitaterna et al, 1994). Moreover, our model also predicts the mutant phenotypes of isolated SCN neurons, which are different from the SCN slices (Liu et al, 2007). We note that SCN slices have significantly higher gene expression of *per1* and *per2* through CREB/CRE pathway than isolated SCN neurons (Yamaguchi et al, 2003). Interestingly, when we reduced *per1* and *per2* expression about 60% in our model, our model was able to accurately reproduce the phenotypes of isolated SCN neurons (Table 2)."

"Table 2. Comparison of modified model predictions with experimental data of single SCN neurons on the phenotypes of circadian mutations

Gene	dSCN	Model
Cry1$^{-/-}$	AR	AR
Cry2$^{-/-}$	Long	+2.3
Per1$^{-/-}$		AR
Per1^{ldc}	AR	
Bmal1$^{-/-}$	AR*	AR

Here we indicate whether the phenotype predicted by our model, or seen in experimental data is arrhythmic (AR) or shows a change in period in hours. Experimental data can be found in (Liu and Welsh et al, 2007), except those marked with * which can be found in (Ko et al., 2010). See the Materials and methods for details."

- We also suggest an experiment to compare the stoichiometry of single SCN neurons and SCN slices in discussion (page 20, bottom).

"The behavior of isolated SCN neurons is similar to fibroblasts in that mutations of circadian genes can easily lead to arrhythmicity (Liu et al, 2007). We note that intercellular coupling in the SCN not only synchronizes SCN neurons, but also increases transcription of *per1* and *per2* (Yamaguchi et al, 2003), which may balance stoichiometry and help sustain rhythms when repressors are effectively removed (Table 1 and 2). Thus, we predict that increasing transcription of *per1* and/or *per2* could enhance rhythmicity in isolated SCN neurons similar to what is seen in fibroblasts (Lee et al, 2001). Moreover, our model predicts that cells with low stoichiometry (e.g. isolated SCN neurons) shows larger phase-shifts in response to light than cells with 1-1 stoichiometry (e.g. SCN slices) (data not shown). It would be interesting future work to see whether different cell types have different PRCs depending on their stoichiometry."

- This is a very significant, and perhaps provocative prediction, which we hope will spark much future discussion. However, while making this prediction, we also want to make sure that it does not overshadow our other results, nor refocus our paper on the differences between single cell and network behavior. This has been studied in many other manuscripts (e.g. Liu and Welsh et al. 2007 and Ko et al. 2010).

Also, adding Per2 and CKI mutant phenotypes would be of interest.

- We now include the CK1 tau mutation in Table 1, which our model can accurately predict.

"

Gene	SCN	Animal	New Model	Relogio (2011)	Mirsky (2009)	Leloup (2003)	Forger (2003)
***Cry1*$^{-/-}$**	Short	Short	-1	Long	AR	Short	WT
***Cry2*$^{-/-}$**	Long	Long	+1.6	Long	Long	Short	Long
***Per1*$^{-/-}$**			WT	AR	AR	Short	Long
***Per1*^{ldc}**	WT	Short/AR					
***Per2*$^{-/-}$**			AR	AR	AR	Short	Short
***Per2*^{ldc}**		Short/AR					
***Bmal1*$^{-/-}$**	SR		AR	AR	AR	AR	AR
***Bmal1*$^{-/+}$**	WT*		+0.1	AR	Na	AR	Long
***Clock*$^{-/-}$**	WT	Short	-0.2	Long	AR	AR	AR
***Clock*$^{\Delta 19/\Delta 19}$**	AR*	Long	AR	Long	Na	Na	na
***Clock*$^{\Delta 19/+}$**	Long*		+1.1	Long	Na	Na	na
***Npas2*$^{-/-}$**	WT	Short	WT	na	Na	Na	na
***Rev-erbα*$^{-/-}$**		Short	-0.2	AR	Short	Na	WT
***CK1ε*$^{tau/tau}$**	Short	Short	-3	na	Na	Short	Short

"

- The PER2 mutations are more complex. While several PER2 "knockouts" exist, what specifically has been mutated remains somewhat debated. Our model can simulate a true knockout. The PER2 brdm1 actually is a mutation of the PAS domain of PER2. However, the role of the PAS domain remains debated. While it is known that Per2 brdm1 inhibits the nucleus entry of PER-CRY, the inhibition of PER-CRY entry cannot explain shortening of Per2 brdm1 since deletion of the NLD lengthens the period. We contacted the senior author (David Weaver) of one of the PER2 mutant studies (Per2 ldc) who also confirmed that not enough is known about this mutant to distinguish it.

C) In cases where their model accurately predicts knockout phenotypes, the authors do not determine what features of the model account for its success, missing an opportunity to gain insight into biological mechanisms. Perhaps a sensitivity analysis of the new model might be a whole paper in itself.

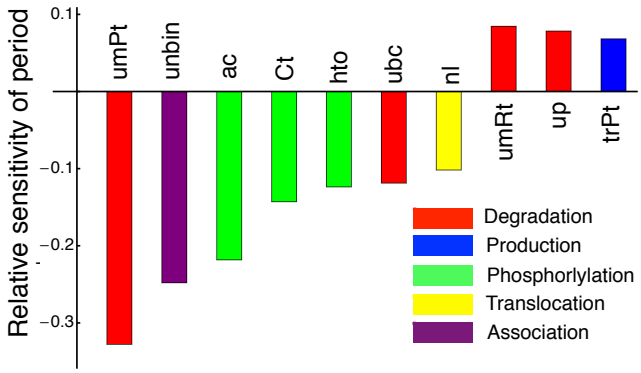
- We agree with the reviewer that it is important to explain what features of the model allow accurate prediction of knockout phenotypes. As we explained in the previous manuscript, the stoichiometry seems the key to determining the mutant phenotypes in our model:

"Since our mathematical model can accurately predict the phenotype of known mutations of the mammalian circadian clock, we next looked for a mechanism that could explain why some phenotypes were rhythmic, while others were not. We found that stoichiometry plays a key role in determining which mutations showed rhythmic phenotypes." (page 8, top)

- We believe that this provides an explanation and insight into the behavior of knockout phenotypes. Moreover, we think that our models predict the mutant phenotypes more correctly than previous models since our model is based on a novel mechanism of oscillation generation in circadian models including protein sequestration and stoichiometric balance.

- The reviewer is correct that much future work could be done studying sensitivity in detail. In the previous versions of the manuscript, we included a sensitivity analysis to stoichiometry in our simple model (See Figure 4, Figure S3 and mathematical analysis in the supplement). Furthermore, to fully satisfy the reviewer's comments, we also have conducted a numerical sensitivity analysis of the detailed model. Some of the results are similar to previous sensitivity analysis. When we searched for the most important parameters in period determination, as was done by Wilkinson et al, 2007 for the original F&P 2003 model, we find that four of the five top parameters remain, which matches their story about PER2 being important for period determination. While we are glad our model matches their study, we also note that this sensitivity analysis, nor its implications for PER2 are not at the core of the findings of our model. Thus the following has been added to the main text:

"We also conducted a sensitivity analysis to look at what parameters determine the period of our model. Four of the top five high parameters, in our sensitivity analysis, were also in the top five found in a previous sensitivity analysis with the original Forger and Peskin model and which was used to conclude that PER2 plays a dominant role in period determination (Wilkins et al, 2007) (See Supplementary Figure 1)." (page 7, bottom)



"Supplementary Figure 1. Top 10 ranked sensitivities for period. The changes of period are measured in the presence of 1% perturbation of each parameter. Relative sensitivity equation used is

$$\text{Re}\,lative\ Senstivity = \frac{d(Period)}{d(Parameter)}\frac{Parameter}{Period} = \frac{d\ln(Period)}{d\ln(Parameter)}$$

The model shows large sensitivities to perturbation of parameters related with degradation rate or phosphorylation of PER2."

D) Explain more clearly how a balance of activators and repressors enhances rhythm stability, e.g. as in Buchler, J Mol Biol 384:1106, 2008.

- Thank you for pointing out how we improve the discussion of our mechanism. We now provide a more detailed and clear explanation how 1-1 stoichiometry can generate rhythms and cite Buchler et al, 2008. (page 10, bottom):

"Many previous studies have argued that ultrasensitive responses (e.g. a large change in transcription rate for a small change in repressor or activator concentration) can cause oscillations in feedback loops (Buchler & Louis, 2008; Forger, 2011; Kim & Ferrell, 2007; Novak & Tyson, 2008). A previous study showed that an ultrasensitive response can be generated by tight binding of activators and repressors in a synthetic system (Buchler & Cross, 2009). Taken together, this provides a potential mechanism of rhythm generation. That is, when the total concentration of repressor is higher than that of activators, the repressor sequesters and buffers activator and inhibits transcription completely (Buchler & Louis, 2008). As the repressor is depleted, the excess free activators are no longer sequestered by repressors and are free to turn on the transcription. At this threshold, transcription of repressor shows an ultrasensitive response to the concentration of repressor or activator. Ultrasensitive responses amplify rhythms and prevent rhythms from dampening (Forger, 2011). In both our simple and our detailed model, we found ultrasensitive responses around a 1-1 stoichiometry (Supplementary Figure 3A). When the stoichiometry was not around 1-1, an ultrasensitive response was not seen, and both models did not show sustained rhythms.

Over the course of a day, as levels of repressor and activator change, the stoichiometry and also sensitivity change as well. We found that the 1-1 average stoichiometry is required to generate the ultrasensitive response which causes rhythms through mathematical analysis, confirming our simulation results (Figure 3D). That is, via both local and global stability analysis, we derived an approximate range of the stoichiometries ($\langle S \rangle$) that permit oscillations

$$\frac{8}{9} < \langle S \rangle < \frac{2}{7\sqrt{7\sqrt{K_d/2}}}$$

(See supplemental information). In agreement with our simulations shown in Figure 3D, this mathematical analysis also suggests that: 1) Oscillations are seen around a 1-1 stoichiometry; 2) the stoichiometry needs to be greater than 8/9 for sustained rhythmicity; 3) as the binding between activators and repressors becomes tighter, the upper bound on stoichiometry increases; 4) if the binding is too weak (e.g. $K_d=10^{-3}$), sustained oscillations do not occur."

E) The simple model seems rigged to give optimal results at 1:1 stoichiometry of activators and repressors. Acknowledge this or refute.

- While the model does show oscillations with the largest amplitude at a 1:1 stoichiometry, this comes about naturally from the mechanism we describe to produce a high sensitivity. We did not choose or rig the model to do this. A 1:1 stoichiometry gives the greatest sensitivity, which leads to the largest amplitude oscillation. We also show that this result does not depend on the choice of other parameters, except when our proposed mechanism is violated (e.g. when the activator degrades fast or the Kd is high).

F) In Fig. 3A, exactly how is repressor:activator ratio calculated for various mutants? The authors seem to ignore the complexities of multimeric complexes and variations in biochemical activity. In particular, amplitude of Cry1-/- and Cry2-/- are shown as similar in Fig. 3A, but single cell experimental data show that the amplitudes are actually very different (Liu, 2007) despite similar stoichiometry. This can be explained by CRY1's stronger repressor activity (Khan, J Biol Chem 287:25917, 2012).

- We used as a common definition of stoichiometry, the average ratio between the concentrations of repressors (all forms of PER and CRY in the nucleus) to that of activators (all forms of BMAL-CLOCK/NPAS2 in the nucleus) for every mutants. However, depending on the underlying mechanisms for the difference between repressor activity of CRY1 and CRY2, one may need a more sophisticated definition of stoichiometry. That is, since CRY1 and CRY2 bind to the CLOCK-BMAL1-E-box complex with the same binding affinity (Ye et al, 2011), Khan et al, 2012 proposed that the difference of their repressor activity might be due to differential post-translational mechanisms or different intrinsic repression activities. If the difference is due to a different post-translational mechanism (e.g. binding between PER and CRY, which could affect the repressor concentration in the nucleus), the current definition of stoichiometry can be used since the current definition of stoichiometry only uses concentrations in the nucleus. However, if the difference is due to different intrinsic repression activities, the concentration of CRY1 should be more weighted than that of CRY2 when the stoichiometry is calculated. Now, we describe this in the results (page 9, top):

"We defined the stoichiometry as the average ratio between the total concentrations of repressors to that of activators over a period. However, recent work has shown that CRY1 has stronger repressor activity than CRY2. The underlying biochemical mechanisms for this result have not been fully identified (Khan et al, 2012). If the difference is due to a different post-translational mechanism (e.g. binding between PER and CRY, which could affect the repressor concentration in the nucleus), the current definition of stoichiometry can be kept. Otherwise, a more sophisticated definition of stoichiometry may be needed (e.g. one that gives more weight to concentration of CRY1 than that of CRY2)."

- We agree to the reviewer that the different repressor activity of CRY1 and CRY2 may be the reason for the difference between amplitude of Cry1-/- and Cry2-/-. However, as we mentioned above, the underlying mechanisms of the different repressor activities of CRY1 and CRY2, which are critical for the simulation, have not been found. Furthermore, we now show that if we reduce the Per1 and Per2 transcription rates to match the model to the single SCN cells, CRY1-/- cells becomes arrhythmic (see above for details)

G) Explain how the NNF model includes inhibition as by RevErba, whereas the PNF model includes activation by as by Rors, and the evidence that inhibition is more important (Liu, PLoS Genet 4(2), 2008).

- We now clarify this in the text when we introduce the NNF and the PNF model and discuss the role of additional negative feedback loops:

"We tested these structures by including an additional protein R (Rev-ERBs or RORs in the mammalian circadian clocks) that is transcribed in a similar way to P. R then represses (as in the Rev-erbs) or promotes (as in the Rors) the production of A in the negative-negative feedback loop (NNF) or the positive-negative feedback loop (PNF) structure, respectively (Figure 4A)." (page 12, top)

"… However, when we changed the transcription rate of the repressor (*Per*) and activator (*Bmal*, *Clock* and *Npas2*), the original model (with a NNF structure) had the widest range of parameters where oscillations occur while the PNF structure had the narrowest range of parameters (Figure 5B). Interestingly, experiments have shown that REV-ERBs play a more dominant role than the RORs indicating that our proposed mechanism may play an important role in *in vivo* timekeeping (Liu et al, 2008). Thus, the choice of the additional feedback greatly affected the range of parameters where oscillations are seen." (page 15, top)

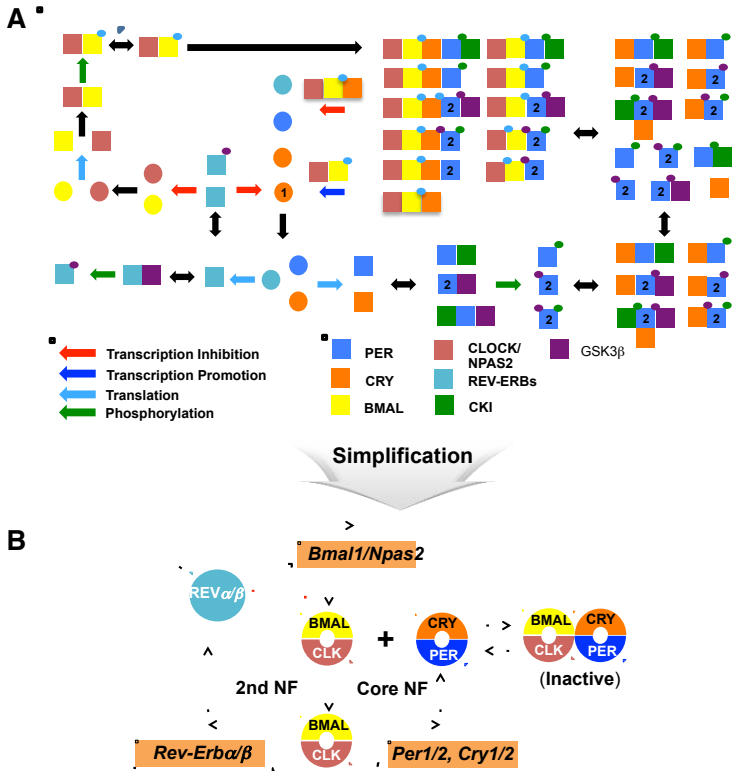
Minor Comments:

1) Omit, qualify, or justify such broad, sweeping claims as "the most accurate and detailed mathematical model... to date" (p. 2).

- We have changed this to "a detailed mathematical model of the mammalian circadian clock" (page 2)

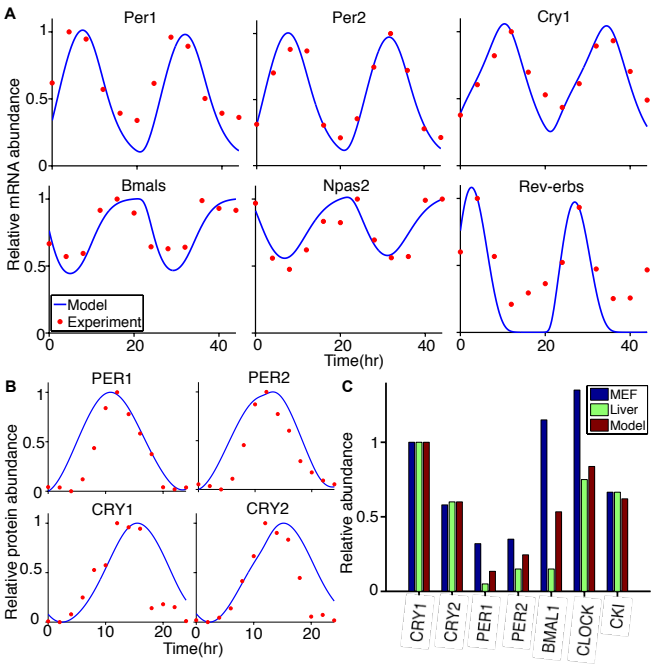
2) In Fig. 1, use consistent colors for molecules in parts A and B. Fig. 1B should indicate the inhibition by PER/CRY.

- Thank you for pointing this out. Now, we use consistent colors which shows a clearer relationship between two diagrams.



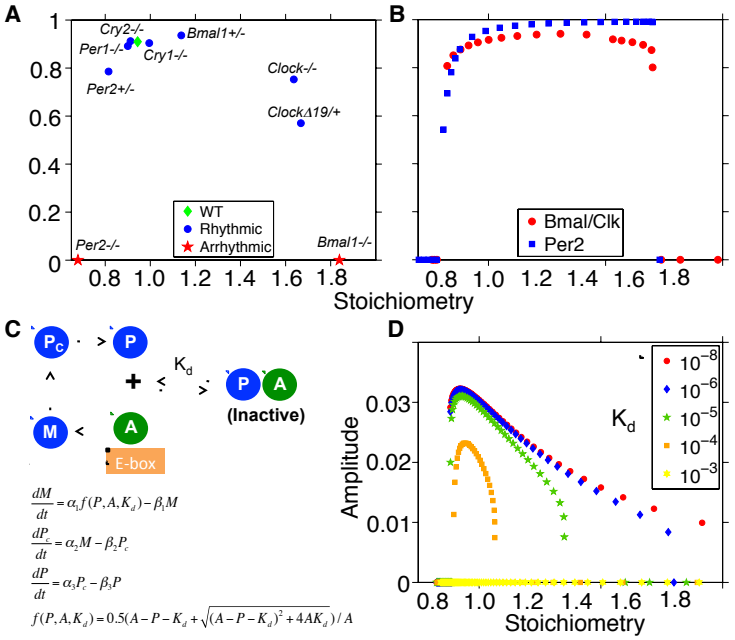
3) In Fig. 2A and 2B, label vertical axes as relative mRNA and protein abundance, respectively (not amplitude).

- Thank you the reviewer for these comment. Now, the labels have been corrected.



4) In Fig. 3C, the placement of "Kd" near the upper arrow is misleading.

- Thank you for this comment. Now, we have edited the Fig. 3C to clarify the meaning of "Kd".



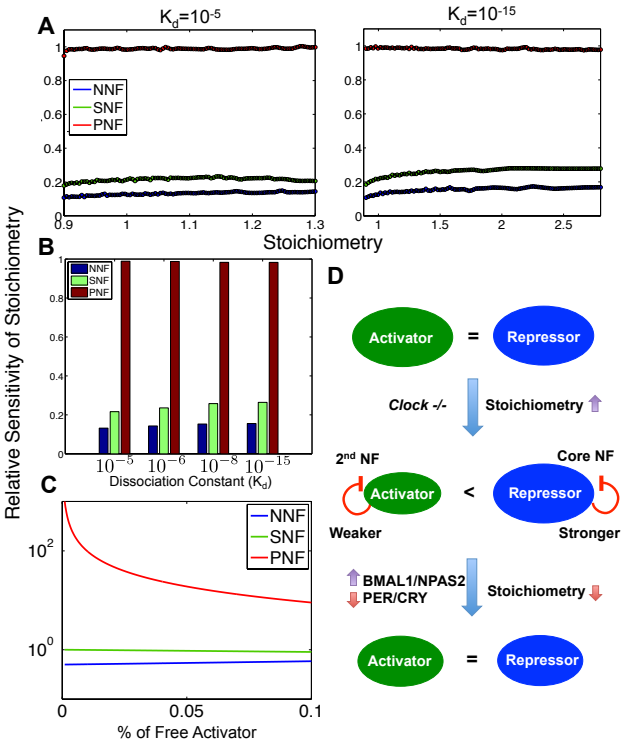
5) Fig. 4C indicates that the average level of repressors (0.25) and activators (0.75) in WT, their stoichiometry is about 0.33 and not close to 1. Is this consistent with your theory?

- Figure 4C indicates the activities of promoters of repressor or activator genes. This does not directly indicate the concentration of the repressors and activators. Therefore, the level in Figure 4C cannot be used to compare the concentration of repressor and activators.

In the legend, explain more clearly the sentence "The average of relative sensitivity...."

- Now, we have explained more detail in the legend and provided a new supplementary figure (Supplementary Figure 4A) to clarify the meaning of "the average of relative sensitivity":

"The relative sensitivity (% change in mean level of stoichiometry per % change in transcription rate of repressor) in the simple models with SNF, NNF, and PNF structure were measured over a range of the transcription rates of repressor (see Supplementary Figure 4A). Then, we calculated the average of relative sensitivity over the range of parameters. On average, the relative sensitivity of the NNF model is about 2 fold less sensitive that of the SNF model, but that of the PNF model is about 4 fold more sensitive than that of the SNF model. (See Supplementary information and Supplementary Figures 4A-C for details)." (page 38, top)



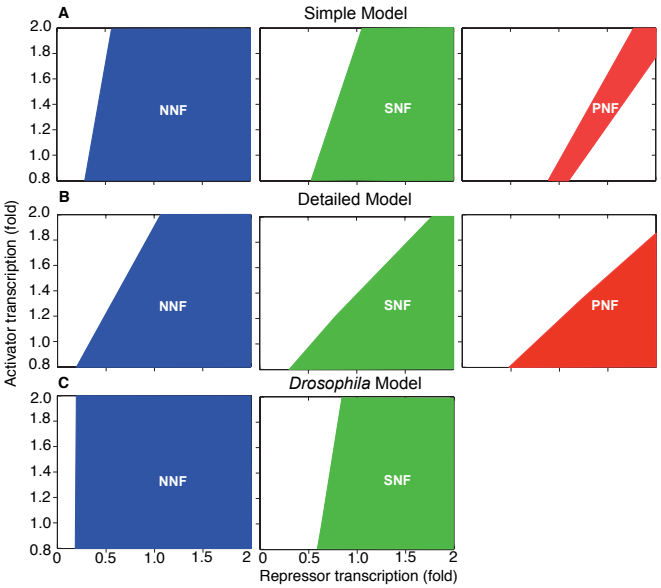
"Supplementary Figure 4. Controlling the activator concentration with an additional negative feedback loop maintains stoichiometry in balance. (A) The relative sensitivity of the stoichiometry with respect to transcription rate change in the SNF, NNF, and PNF was measured over the range of transcription rates with which the stoichiometry are in the appropriate range for rhythm generation seen Figure 3D. The relative sensitivity equation used is

$$\text{Re}lative\ Senstivity = \frac{d(Stoichiometry)}{d(Transcription\ Rate)}\frac{Transcription\ Rate}{Stoichiometry}$$

This equation is the % change in the stoichiometry (repressor/activator) per % change in transcription rate of repressor. (B) We calculated the average of relative sensitivity over the range of parameters. On average, the relative sensitivity of the NNF model is about 2 fold less sensitive that of the SNF model, but that of the PNF model is about 4 fold more sensitive than that of the SNF model regardless of the binding affinity. This implies that the NNF is best to maintain the stoichiometry balance when the transcription rate is perturbed. (Here we assumed δ=0.2. When this assumption was relaxed, the result is similar. See Supplementary information and Supplementary Figure 4C)….."

6) In Fig. 5, show NNF, SNF, and PNF areas in 3 separate panels, instead of overlaying them. Consider using color coding to provide period information, as in Fig. 6. Label A = mammalian, B = detailed mammalian, C = fly.

- We now include the labels indicating the type of models and separate the panels. However, we decide not to discuss the period at the Figure 5 since we would like to focus on how the NNF structure has the widest range of parameters where the rhythms occur at the Figure 5 and to postpone the discussion about the period to later in the manuscript (Figure 6).



7) Change ", however, " to "; however, " (p. 8), "the rhythms whose period is robust to gene dosages" to "rhythms with periods robust to gene dosage" (p. 15), "too much repressors" to "overabundance of repressors" (p. 15), "data also supports" to "data also support" (p. 16), "more pivotal role" to "a more pivotal role" (p. 16), "model based on the protein... who focuses" to "model based on sequestration... which focuses" (p. 17), "the rhythm generation (e.g. High-hill" to "rhythm generation (e.g., high Hill" (p. 18), "data of mammalian" to "data on the mammalian" (p. 18), "Bmals" to "Bmal" (p. 31), "data form" to "data from" (p. 32), "degrade quickly" to "degrades quickly" (p. 33), "degrade slowly" to "degrades slowly" (p. 33).

- We appreciate for pointing these out. Now, all the grammatical mistakes and misspell have been corrected.

Reviewer #3 (Remarks to the Author):

In this paper, Kim and Forger put forward in intriguing hypothesis for the regulation of mammalian circadian timekeeping. Circadian rhythms in mammals have been shown to be robust to a variety of perturbations: knockdown of important clock genes, general transcriptional repression, etc. While this resilience is important for the function of the system, it has made it difficult for researchers to pin down exactly which features of the system are truly essential. Kim and Forger suggest that the resilience of the mammalian clock lies in maintaining a roughly 1:1 stoichiometry between the activators (CLOCK, BMAL1) and the repressors (PER, CRY) acting on the E-box. They suggest that the major role of the secondary feedback loop involving the REV-ERB genes is to ensure that this stoichiometry is maintained. To support this argument, they present both a highly detailed model of the mammalian clock that incorporates a broad range of mechanistic data and a very simplified Goodwin-type model that is more amenable to analytical analysis, most of which is described in the supplementary information. While the detailed model is a (much-needed) update of the 2003 model by Forger and Peskin and will undoubtedly be used by other researchers in the future much as the F&P model has been, the detailed model does not occupy center stage in this manuscript -- the focus is on transcription factor stoichiometry and its biological consequences.

- We thank the reviewer for this accurate summary and potential of our work.

My concerns with this paper are fairly minor:

Pg. 7: The authors define stoichiometry as the "average ratio between the concentrations of repressors ... to that of activators." It would help if this definition were made more explicit -- if I'm not mistaken, the average in question is a time average over the circadian period. Also, the authors seem to have an unfortunate habit of using "activator" and "repressor" as shorthand for "E/E'-box activator" and "E/E'-box repressor" -- this could be very confusing for readers who might be tempted to extend the conclusions to other types of circadian activators and repressors. For the record, I believe that the standard English usage would be "average ratio between the concentration of repressors ... and that of activators."

- We thank to the reviewer for pointing this out. Now, we clarify the definition of stoichiometry, repressor and activator in the beginning of the manuscript:

"Here we define stoichiometry as the average ratio between the concentration of repressors (all forms of PER and CRY in the nucleus) to that of activators (all forms of BMAL-CLOCK/NPAS2 in the nucleus) over a period. Moreover, we specifically refer to repressors and activators of E/E'-boxes when discussing stoichiometry." (page 8, top)

Pg. 10: Are you defining as A/P or P/A?

- We apologized for this confusion. It is P/A. We now define the stoichiometry of the simple model at bottom of page 9:

"When we changed the activator concentration, which changed the stoichiometry (average ratio between the level of repressor (P) to the level of activator (A)), sustained oscillations were only seen at around a 1-1 stoichiometry similar to our detailed model (Figure 3D)."

Pg. 12: Your prediction that REV-ERB knockouts will maintain rhythmicity in cell types with a nearly-balanced stoichiometry but lose it in poorly-balanced cells appears to be experimentally tractable. This is an interesting prediction, since it may have relevance for the differences between SCN and peripheral clocks, and it should be highlighted in the summary of experimental predictions on page 17.

- We are grateful to the reviewer for suggesting this. Indeed, it will be interesting experiment. Now we highlighted it in the discussion (page 21, bottom):

"Loss of the additional negative feedback loop (e.g. in the Rev-erbs -/-, constitutive expression of Rev-erbs or constitutive expression of BMAL) is predicted to cause the intracellular circadian clock to oscillate over a much narrower range of conditions (Figure 5). It would be interesting to test whether these cells would have less temperature compensation or would lose rhythms more easily when other genes are knocked out (e.g. *Cry2*$^{-/-}$, *Per1*$^{-/-}$). Moreover, we predict that in the Rev-erbs$^{-/-}$, rhythms persist in cell types with a balanced stoichiometry, but not in poorly balanced cells (Figure 5). It would be interesting future work to investigate whether SCN and peripheral clocks have different phenotypes of Rev-erbs$^{-/-}$ depending on their stoichiometry. We also predict that Rev-erbs$^{-/-}$ cells show a wider period distribution than WT (Figure 6)."

Pg. 15: "Both our detailed and simplified NNF models predict these results" -- this isn't really a prediction, since the experimental results were already published when you made the model. It would be more correct to say "reproduce" or "confirm." (FWIW, this distinction is made quite well elsewhere in the manuscript.)

- Now, we changed the words "predict" to "confirm":

"Both our detailed and simplified NNF models confirm these results (Figure 4B and 4C and Supplementary Figure 3C)." (page 17, middle)

Pg. 16: "The elimination of additional positive feedback ... based on the PNF structure" -- I'm not quite sure what you're talking about here; all of the discussion so far has focused on the role of an additional *negative* feedback loop and an *NNF* structure. Is this a typo, or did I miss something really important?

- We apologize for the confusion. We wanted to emphasize that circadian clocks have a NNF structure by mentioning that the elimination of the existing additional positive feedback loop has little effect on circadian clocks unlike additional negative feedback loop.

Pg. 20: "Modifications and extensions of the detailed model" -- somewhere in this paragraph you should mention the reference for the previous version of the detailed model. I know you have it elsewhere in the manuscript, but readers not familiar with your previous work will appreciate having it here.

- Now, we add the reference of the original model (page 23, bottom):

"Modifications and extensions of the detailed model
The modification and extension of the detailed model from the original model (Forger and Peskin, 2003) are listed. See Supplementary information and Supplementary Tables 1-2 for details."

Pg. 21: "this large number of parameters, which is still less than other models," -- this makes it sound like you're apologizing for having lots of parameters. More importantly, what do you mean when you say you have "better" estimates of the parameters? You certainly demonstrate that your model does a good job at reproducing system-level behaviors, but there is evidence (James Sethna's work on "sloppy models", for example) that correct system-level behavior does not imply accurate estimations of model parameters, and that these two goals might often be incompatible. In other words, you restrict your parameters to a biologically reasonable range, but it might be entirely possible to get equally-good fits to your data with physically unreasonable parameter values. If you just mean that you're using experimental data to put inequality constraints on your parameter estimates (which seems to be implied by your points 2-5), then say that instead.

- We wholeheartedly agree with the reviewer. Indeed, the fitting to the system-level behavior does not guarantee accurate estimation of parameters. It would be interesting future work to find the sloppy and stiff parameters of our model. Now, we have removed the ambiguous word "better estimates of parameters" and provided more detail explanation about the parameter estimation compared to the original model (page 25, top):

"While the original model used 36 parameters, the new model has the 75 parameters due to the extensions and modifications of the model. Despite the increased number of parameters, we could get tighter restriction on the range of parameters with newly published data (listed below). Over these ranges, parameters are estimated by fitting to more various types of data: timecourses of gene expressions and proteins, abundance of proteins, and mutation phenotypes."

Pg. 21-22: It would be nice to see a little more description of the simulated annealing protocol. Also, describing your fitting protocol without saying anything about your cost function doesn't really help anyone understand what you were doing; please say more about your cost function.

- We have now described more detail of simulated annealing protocol and cost functions (pages 26-27):

"Within these restrictions, a simulated annealing method (SA, a global stochastic parameter searcher) (Gonzalez et al, 2007) was used to estimate the parameters in two steps. First, we found parameters that provides a good fit with mRNA and protein time profiles measured in mouse suprachiasmatic nuclei (SCN) (Reppert & Weaver, 2001; Ueda et al, 2005) and relative abundance of clock proteins measured in mouse liver (Lee et al, 2001) and fibroblast (Lee et al, 2009; Lee et al, 2011) (Figures 2A-C). In this fitting, we used a similar cost function to that used in estimating the parameters of the original model (Forger & Peskin, 2003)

$$\sqrt{\sum_{j=1}^{10}\sum_{i=1}^{n_j} w_{ij}\frac{(s_{ij}-e_{ij})^2}{n_j}+\sum_k (pm_k-p_k)^2}$$

Here, j runs through 6 mRNAs and 4 proteins. n_j is the number of data points (12 for mRNA and 13 for protein). s_{ij} and e_{ij} are simulated timecourses and experimentally measured timecourses, respectively. s_{ij} are normalized, matching experimental data (see Fig. 2 for details). w_{ij} = 5 when e_{ij} = 1 and w_{ij} = 1 otherwise, so that the cost function has more weight at the peak time than other times. pm_k and p_k are maximum value of protein abundance, respectively. pm_k and p_k are normalized, so that the maximum abundance of the CRY1 protein is 1.

After the first round of SA, we found several parameter sets qualitatively matching with experimental data on phenotypes of mutations of mice (WT, short, long and AR) (Table 1). Then we used these parameter sets as initial parameter sets for another round of SA to get the final parameter set, which shows a quantitatively good fit with knockout mutation phenotype as well as time profiles (Supplementary Table 3). The cost function used for the second round is followed.

$$\sqrt{\sum_{j=1}^{10}\sum_{i=1}^{n_j} w_{ij}\frac{(s_{ij}-e_{ij})^2}{n_j}+\sum_k (pm_k-p_k)^2}+\sqrt{\sum_l (mp_l/m_l-1)^2+\sum_n (ma_n)^2}$$

mp_l and m_l are simulated period and experimentally measured period of rhythmic phenotypes of mutations, respectively. ma_n are simulated relative amplitude of arrhythmic phenotypes of mutation (e.g. $Per2^{-/-}$ or $Bmal1^{-/-}$)."

The quality of the English in this manuscript is quite high up until the Discussion section (page 14), but I felt like the latter portion of the document contained far more mistakes and rough spots. It would probably be worth it to look over the final sections with this in mind. A few specific issues:

- We appreciate the reviewer for pointing out following misspell and grammatical errors. All these have been corrected.

Pg. 15: "yield opposite effects" should be "yields"
Pg. 17: "Our model also makes important predictions, which can be tested..." -- comma is unnecessary.
Pg. 17: "...a previous model based on the protein sequestration ... who focuses on other mechanisms ..." -- try "a previous model based on protein sequestration ... which focuses on other mechanisms ..."
Pg. 18: "High-hill coefficients" should be "high Hill coefficients"
Pg. 22: "which should a good fit" -- did you mean "which showed a good fit" ?

- We are grateful to the reviewer for the following suggestions. Now, we have clarified these statements according to reviewer's suggestions.

Pg. 15: "more stable activators than repressors" makes it sound like the number of stable activators is larger than the number of repressors. You might want to try "higher stability for

activators than for repressors" or something similar. Also "too much repressors" a few sentences down could be "elevated repressor concentrations."

"… longer half-life of activators than repressors" (page 16, bottom)

"For instance, the increased stoichiometry (elevated repressor concentrations) strengthens the repression…" (page 16, bottom)

Pg. 16: "Our study also proposes the underlying mechanism ... of a previous experimental study" -- try "Our study also suggests an underlying mechanism ... for a previous experimental observation..." or something similar.

"Our study also suggests an underlying mechanism (ultrasensitive response) for a previous experimental observation …" (page 17, bottom)

Pg. 16: "...studies have shown more pivotal role of the additional negative feedback loop" -- try "...studies have confirmed a pivotal role for the additional negative feedback loop"

"Moreover, recent studies have confirmed a pivotal role for the additional negative feedback loop …" (page 18, middle)

Pg. 18: "mechanisms of the rhythm generation" -- try "mechanisms for rhythm generation"

"Previous models have used different mechanisms for rhythm generation (e.g. high-Hill coefficients)…" (page 19, middle)

Pg. 18: "roles of the additional negative feedback loop" -- try "roles for the additional negative feedback loop"

"…have proposed different roles for the additional negative feedback loop." (page 19, middle)

Pg. 18: "experimental data of mammalian circadian clock" -- try "experimental data on the mammalian circadian clock"

"…which does not match with recent experimental data on the mammalian circadian clock…" (page 19, bottom)

Reviewer #4 (Remarks to the Author):

The authors develop here a fairly comprehensive mathematical model of the mammalian circadian oscillator, which includes most of the recent experimental observations. The predictions of this model match with observed phenotypes of circadian knock-out strains better than previous models. In the center of this model is the new and original concept of 'stoichiometric balance' of activators and repressors. Then, they add to the core model two alternative extensions, one negative feedback loop, or a positive feedback loop. Interestingly, only the slow negative feedback loop model is capable to predict the behavior of the mammalian circadian feedback loop with its stoichiometric balance, while the other feedback loop model is more suitable for other (circadian) oscillators, e.g. the Neurospora circadian oscillator without obvious stoichiometry occurring.

Over all, the paper is well written, well documented and suitable in its length and displayed items. The Material and Methods part is well structured and can be followed even by non-specialists.

- We appreciate the reviewer for this accurate summary of our work and the positive comment about our manuscript.

However, one question remains:
Suppl. Information, page 4 (5): why is it necessary to include light - inducibility of Per1-2 into the model?

- We thank the reviewer for asking for clarification. We included light into the model for several reasons. When the timecourses of clock gene expression or proteins are measured, mice are entrained to the Light-Dark cycle for several weeks. Then, during the following day, the timecourses are measured in darkness. To match this experimental protocol, we included light in the model as that has been done in the previous model (Forger and Peskin, 2003). Moreover, including light will be beneficial for the circadian clock research communities since many studies of circadian clocks consider the effects of light.

The model was intended to provide stable periodicity of the oscillator (page 5 'maintaining periodicity is crucial'). Light - inducibility of Per1-2 causes a phase shift of the circadian oscillator, which is probably based on a (transient) period change. In addition, the effect is completely dependent on the time, when the light pulse is given (phase-advance vs. phase-delay). How prominent is the light - inducibility parameter on the over all system? Does light - induction change the stoichiometry and would this explain e.g. the difference of type I and type 0 phase resetting?

- It is very interesting idea to explore the effect of light on the stoichiometry. Since light induces the expression of *per1* and *per2* genes, it will increase the stoichiometry. The increased stoichiometry can change the amplitude and period of rhythms as we shown in Figure 3 and 6. To see this, we simulated PRCs to light with model with different stoichiometry as the reviewer suggested. Surprisingly, the models show different type of PRCs depending on their stoichiometry. That is, a model with low stoichiometry or low amplitude shows more dramatic phase change to the light than a model with 1-1 stoichiometry. Now, we describe this in the text (page 20, bottom):

"The behavior of isolated SCN neurons is similar to fibroblasts in that mutations of circadian genes can easily lead to arrhythmicity (Liu et al, 2007). We note that intercellular coupling in the SCN not only synchronizes SCN neurons, but also increases transcription of *per1* and *per2* (Yamaguchi et al, 2003), which may balance stoichiometry and help sustain rhythms when repressors are effectively removed (Table 1 and 2). Thus, we predict that increasing transcription of *per1* and/or *per2* could enhance rhythmicity in isolated SCN neurons similar to what is seen in fibroblasts (Lee et al, 2001). Moreover, our model predicts that cells with low stoichiometry (e.g. isolated SCN neurons) shows larger phase-shifts in response to light than cells with 1-1 stoichiometry (e.g. SCN slices) (data not shown). It would be interesting future work to see whether different cell types have different PRCs depending on their stoichiometry."

- For the question of how prominent light is in the model, the 12:12 LD cycle with 100 lux light increases the amplitude of *per1* and *per2* gene expression about 16% and 30%, respectively, matching experimental data (Challet et al, 2003). Now, detail is described in the supplementary information (page 5, top):

"(5) *Precise description of the effect of light on the circadian clocks*: We included a previously model of the effect of light on the circadian clocks (Kronauer et al, 1999), which quantified the human circadian pacemaker response to the light successfully. In this model, the light increase on the transcription rate of Per1-2 decreases as more light is presented. This matches experiment

data (Wilsbacher et al, 2002). In addition, we include a higher increase in Per2 transcription by light than the increase in Per1 transcription also matching experimental data (Challet et al, 2003). That is, the amplitude of *per1* and *per2* gene expression are higher about 16% and 30%, respectively, under 12:12 LD cycle with 100 lux than 12:12 DD cycle. Because light is known to increase the transcription rate of Per1/2 regardless of the E-box state (Okamura et al, 1999; Reppert & Weaver, 2002), we assumed that the light effect was independent of the E-box state as occurred in the original Forger and Peskin model."

I would also probably change the title to highlight the new concept of stoichiometric balance in their model.

- We have changed the title to include 'stoichiometric balance', which we agree better reflects the contents of the manuscript:

"A mechanism for robust circadian timekeeping via stoichiometric balance"

Acceptance letter 19 October 2012

Thank you again for sending us your revised manuscript. We are now satisfied with the modifications made and I am pleased to inform you that your paper has been accepted for publication.

NOTES
-Reviewer #2 is asking for some additional explanations. We would be grateful if you could forward us as soon as possible an amended text.
-Please remove password protection in ModelDB.
-We would be grateful if you could also prepare two zip archives (including a README file) for the detailed and simple models, so that we can add them to the supplementary information section, for permanent archival.

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

Best wishes,

Editor
Molecular Systems Biology

http://www.nature.com/msb

Reviewer #2 (Remarks to the Author):

I urge the authors to consider including an even more detailed explanation of why the model gives the correct phenotypes for various knockouts, esp. Cry1 vs. Cry2. In all other respects, the authors have done an excellent job in addressing my concerns.

