molecular systems biology

Multi-layered stochasticity and paracrine signal propagation shape the type-I interferon response

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Transaction Report:

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

1st Editorial Decision 19 January 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from two of the three referees who agreed to evaluate your manuscript, and have decided to render a decision now to avoid further delay. As you will see from the reports below, the referees find the topic of your study of potential interest. They raise, however, some substantial concerns, which, I am afraid to say, preclude its publication in its present form.

In general, both reviewers felt that this work presented novel and potentially interesting results. They both indicated, though, that important experimental controls were needed to convincingly demonstrate that the IFN BAC reporter shows stochastic behavior that is comparable to the endogenous IFN-beta response. They also raised a series of more specific concerns, which in some cases may require further analyses or experimental work. In particular the last reviewer requested additional details and a deeper analysis of how the model accounts for cellular heterogeneity.

In addition, when preparing your revised work, we encourage you to provide numerical data supporting the figures in the main manuscript. We now provide a functionality that allows readers to directly download the 'source data' associated with selected figure panels (e.g. <http://tinyurl.com/365zpej>). This sort of figure-associated data may be particularly appropriate for many of the figure panels in this work (Fig 1 B,C; Fig. 2B; Fig. 3 A,C,E; Fig. 4; Fig. 6C, etc). Please see our Instructions of Authors for more details on preparation and formatting of figure source data (<http://www.nature.com/msb/authors/index.html#a3.4.3>).

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.

Sincerely,

Editor - Molecular Systems Biology
msb@embo.org

Referee reports:

Reviewer #2 (Remarks to the Author):

Rand et al report on a quantitative analysis of IFN signaling. This study is of broad interest. It is well designed, executed and described.

Some questions remain
Given that a major interest is the stochastic nature of signaling and gene control, it is important to establish that the integration site of the Bac IFN reporters does not introduce stochasticity. (One way to address this, is to show that several Bac clones behave equivalently.) Related: It is mentioned that IFN expression is stochastic, but no paper is cited. In fact showing that the BAC transgene behaves as the endogenous would be ideal.

It is curious that the variability in IFN expression is higher with polyIC than with virus (Figure 2). Is this the case no matter whether polyIC is added or transfected?

The fact that IRF and NF B pathways are so highly correlated is striking. I am concerned that this may be the result of over-expressing the reporters. A simply immunofluorescence experiment of nuclear localization of endogenous proeins would address this.

The degree to which the IFN promoter adds variability is not as clearly documented as it could be. The text says that only 9% of the cells do not make IFN when IRF7 is activated (Is there a figure for this? Is NF B activated also?) The time variability is shown in a scatter plot in 3E. But 3D is not well integrated in the text and may relate primarily to Fig.4.

Figure 4 is suboptimal in making an important point that the cell-to-cell variability is intrinsic rather than extrinsic as in e.g. apoptosis. It is not clear what the significance of the various measures are in E-H. Maybe indicate correlation coeefs in figure? When did sister cells divide?

Model and experimental study in Figure 6 ad 7 are nice. The model could be taken further, but for the scope of this study it seems ample.

Reviewer #3 (Remarks to the Author):

In this paper, Rand et al. study the cellular response to viral infection with single-cell resolution. The issue of heterogeneity of cellular response to ligands or to cell differentiation has been a hot topic in recent years. Here, Rand et al. track the response to viruses at the gene expression level and demonstrate convincingly that the heterogeneity of cellular response is cell-intrinsic rather than driven by fluctuations in viral infection.

There are few issues that would require more discussion for clarification-sake:

1) The authors built a BAC to monitor IFNb and IRF7 expression. Controls to demonstrate the

validity of this construct should be presented. In particular, one would need to check that the average cellular response for cells endowed with this additional BAC is comparable to the average endogenous response. This would settle the issue whether the stochasticity of cellular response is affected by the copy-number variation of IFNb and IRF7 introduced in these cells.

2) Details about the modeling results are missing in the main text (mostly by lack of space). The authors keep stating that the parameters were "appropriately calibrated". Supplementary information about the quality of the fit (e.g. with parameter sensitivity analysis and model reduction consideration) are needed. Moreover, it remains not clear to this reviewer how the model accounts for the cellular heterogeneity: were the cellular parameters drawn from a distribution (if so, details and experimental evidence about this are needed) or is the cellular heterogeneity related to the stochasticity in the chemical equations?

3) One critical conclusion of the paper is that cell-cell variability in response to viral infections is cell-intrinsic. This emerges from the bimodal cellular response (e.g. in terms of IRF7 expression - Fig 5) despite homogenous viral infection. The results from the modeling effort do not properly document this fact because it is not clear, at this stage, where the heterogeneity comes from in the model. Additional results would need to be presented: one would need to check how bimodal the distribution of IFNb and IRF7 is in the model.

4) The importance of noise quenching through paracrine communication with IFNb is interesting and consistent with a recent (but not as complete) publication by Hu et al. in PLoSOne. A direct discussion of this paracrine effect (by measuring the amount of secreted IFNb, by disrupting paracrine communication with blocking antibodies, and by modeling the effect of saturating cytokine) would make a more convincing case. For example, Supp. Figure 6 documents the natural disappearance of IFNb in the medium: does that play a role in the heterogeneity in IRF7 expression?

To conclude, this study by Rand et al. is original and will find a great audience at Molecular Systems Biology. Additional limited efforts in presentation would increase its readability and potential impact.

Minor issues:

1) instead of individual cells, one would need to see the distribution or the cumulative distribution of IFNb expression in Figure 3A.

2) When presenting the IFN response as a function of the viral load (Figure 7B), please plot the results in semilogx: there seems to be discrepancy in the low viral-titer regime. If so, please correct the model accordingly.

1st Revision - authors' response 26 March 2012

Response to Reviewer #2:

Comment 1a: "… , it is important to establish that the integration site of the Bac IFN reporters does not introduce stochasticity."

Response: We have carried out the experiment suggested by the reviewer. Comparing the frequency of reporter gene expression in different, independently created IFN-β-tGFP BAC reporter cell clones after NDV infection, we observed practically identical behavior of the time-dependent frequency of IFN-β-tGFP expression of the three clones. Before viral infection, IFN-β-tGFP was not expressed, and after 18 hours and 24 hours, expression was seen with very similar frequencies in all clones. Thus clone-specific integration sites do not affect stochastic expression of the reporter. These results further validate the utility of the BAC-based reporter for measuring authentic regulation of the *Ifnb* gene. They are added to the Supplement as Figure S2, which is referred to on page 5 of the manuscript.

Comment 1b: "Related: It is mentioned that IFN-beta; expression is stochastic, but no paper is cited."

Response: We apologize for this confusion arising from the fact that the relevant references were only cited in the paragraph following this introductory statement. We have now cited the two initial descriptions of heterogeneous type-IFN protein and mRNA expression (Zawatzky et al. 1985 and Hu et al. 2007, respectively) in the first sentence introducing this topic; the further citations follow at the relevant places in the discussion of potential underlying mechanisms (page 3).

Comment 2: "It is curious that the variability in IFN expression is higher with polyIC than with virus (Figure 2). Is this the case no matter whether polyIC is added or transfected?"

Response: We clarified in the main text (page 6) that only liposome-transfection of poly I:C but not extracellular poly I:C addition alone induced IFN-β expression. The behavior after NDV infection and poly I:C transfection is quantitatively similar, as all CVs have the same order of magnitude. This has been added for clarification on page 6. Slight quantitative differences are in our view most likely due to the somewhat different mode of action (transfected bolus of RNA analogue versus self-replicating virus).

Comment 3: "The fact that IRF and NF-κB pathways are so highly correlated is striking. …"

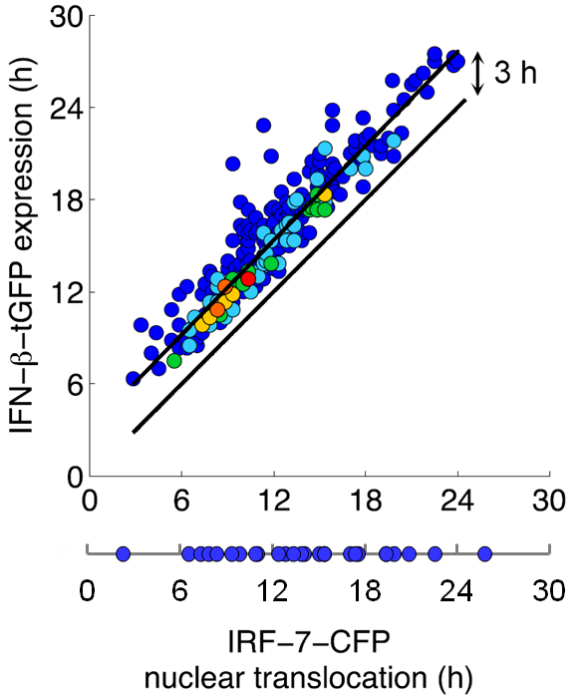
Response: We carried out the additional experiment suggested by the reviewer. To examine whether the correlation of IRF and NF-・B activation might be affected by overexpression of the fluorescently-tagged constructs, we stained the endogenous IRF-3 and p65 in NDV-infected or poly I:C-stimulated cells. In individual nuclei, both endogenous transcription factors together or none of them were found to be accumulated 8 hours after stimulation. These results corroborate our finding of synchronous activation of IRF-7 and NF-κB. The new data were added as Figure S6 to the Supplementary Information and highlighted in the main text on pages 6 and 7.

Comment 4: "The degree to which the IFN promoter adds variability is not as clearly documented as it could be. The text says that only 9% of the cells do not make IFN when IRF7 is activated (Is there a figure for this? Is NF-kB activated also?)"

Response: We find 9% of cells that show translocation of IRF-7 do not express IFN-β. Following the referee's suggestion, we have integrated this information graphically into the revised Figure 3E (reproduced for convenience below). This new figure shows that the small fraction of IFN-β non-expressers is distributed across the same range of IRF-7-CFP activation times as the majority of IFN-β expressers (new text on page 7).
This analysis was done with dual-reporter cells for IRF-7 translocation and IFN-β expression, as we have shown in separate experiments that IRFs and NF-κB colocalize to the nucleus in individual cells (Figure 3C; see also new Supplementary Figure S6).

The prominent novel finding in our manuscript is that stochastic transcription factor activation is a major source for heterogeneous IFN-β expression, as the vast majority (91%) of cells express IFN-β after activation of IRF-7. The failure to induce IFN-β in the remaining 9% fraction could be due, at least in part, to the stochastic mechanism of gene expression (Apostolou and Thanos, 2008). This is further emphasized in the Discussion (page 12).

Revised Figure 3E:



Comment 5: "Figure 4 is suboptimal in making an important point that the cell-to-cell variability is intrinsic rather than extrinsic as in e.g. apoptosis. It is not clear what the significance of the various measures are in E-H. Maybe indicate correlation coeefs in figure? When did sister cells divide?"

Response: We revised the corresponding paragraphs and Figure 4. First, we split the text into two separate paragraphs: (i) for the analysis of heterogeneity between sister cells after NDV (Fig. 4A,B) and poly I:C (Fig. 4E,F) stimulation and (ii) for the relation of heterogeneity and time elapsed after cell division (Fig. 4C,D and G,H for NDV and poly I:C, respectively). The text was revised for clarity. We added the appropriate coefficients of determination r^2 into Figure 4.

Response to Reviewer #3:

Comment 1: Validity of the IFN-β and IRF-7 reporter BACs.

Response: For the further validation of the IFN-β-tGFP reporter BAC see our response to comment 1 of reviewer #2. In addition we determined IRF-7-mCherry induction in different cell clones after treatment of cells with different IFN-β concentrations. The three tested IRF-7-mCherry clones behaved in a very similar manner, showing IFN-β

concentration-dependent bimodal IRF-7-mCherry expression This experiment adds further support to the validity of the BAC-based reporters. We added these results as Supplementary Figure S8 to the manuscript and referred to this control experiment in the main text on page 9.

Comment 2a: "… Supplementary information about the quality of the fit (e.g. with parameter sensitivity analysis and model reduction consideration) are needed."

Response: We would like to express our gratitude to the reviewer for suggesting us to revisit model parameterization and make it more rigorous.

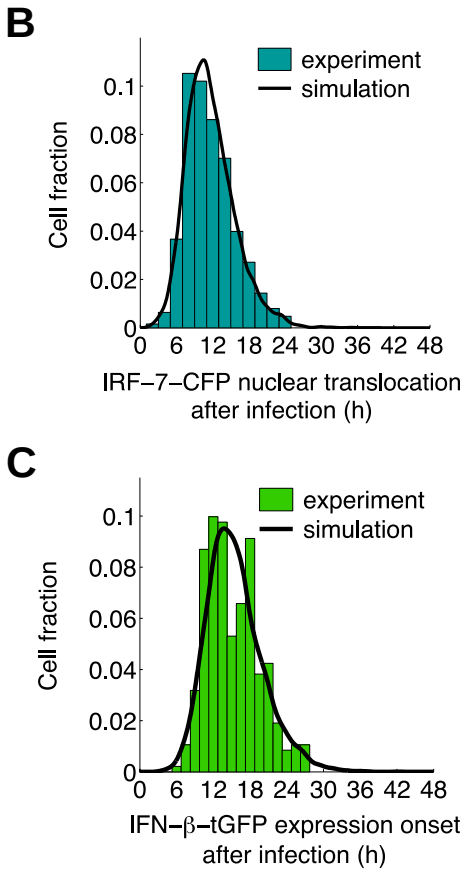
We have done additional work on our side to more fully exploit the available data for rigorous parameter estimation. This revision of the modeling part has substantiated our previous findings and, moreover, resulted in two biologically significant results. First, we have been able to make a new prediction based on parameter sensitivity analysis: Induction of RIG-I-mediated signalling by virus is predicted to be cooperative. Second, the qualitative discrepancy between theoretically predicted paracrine amplification and data that was present for the lowest viral infection dose tested (1 HAU/ml) has been resolved.

In the present case, long simulation times of the model arise from the fact that the cells are coupled by secreted IFN-β, and thus their internal stochastic dynamics cannot be iterated independently from one another. Hence the typical time step for creating a single realization of the model's dynamics with Gillespie's algorithm scales inversely with number of simulated cells. The resulting large simulation times (several hours for one stochastic trajectory) preclude a straightforward optimization approach with the full model for parameter estimation. Therefore we have now devised a computationally feasible "divide-and-conquer" strategy for parameter estimation that fully exploits the available, single-cell resolved data.

Briefly, we separately determine the parameters for (i) IRF/NF-κB activation, (ii) IFN-β expression as well as (iii) STAT1/2 activation and ISG expression. For the first two parts, coupling between the cells via secreted IFN-β can be neglected, as in our model there is no autocrine/paracrine feedback to IFN-β expression (in particular, the NIH3T3 fibroblasts already express IRF-7 – the prime mediator of positive feedback – prior to stimulation).

The absence of paracrine coupling means that IRF/NF-κB transcription factor activation and IFN-β induction can be simulated much faster. Thus we have been able to fit the model rigorously to the measured distributions of IRF-7 activation times and onset times of IFN-β expression (new Supplementary Figure S10 A-C). As a result, the stochastic simulations of the model fully reproduce these two distributions, which is now shown in the new Figures 3B and C.

Revised Figures 6B and C, taking into account improved parameter fit:



Using the profile-likelihood method for determining the quality of this fit, we have found that the induction of RIG-I-mediated signalling by virus must be cooperative (new Supplementary Figure S10B).

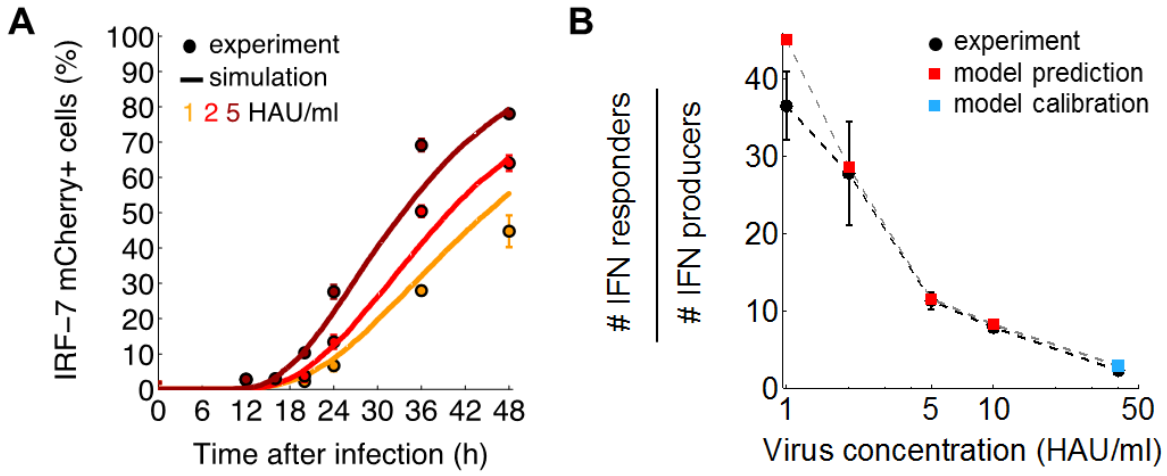
For the third part, STAT1/2 activation and ISG expression, paracrine coupling is relevant (implying impractically long simulation times for straightforward numerical parameter estimation). Despite this fact, we have been able to infer salient quantitative features of ISG expression from the experimental data by exploiting two experimentally measured relations: (1) the dose-response curve of IRF-7-expressing cells versus IFN-β concentration

(Figure 5A; new Supplementary Figure 10D) and the estimated IFN-β secretion rate per cells (determined from the data shown now in Figure 6D, green and blue traces). The latter ensures the correct prediction of extracellular IFN-β concentration. As a consequence, the former dose-response than yields the correct fraction of IRF-7(ISG)-expressing cells.

As a visible consequence of this improved parameterization of ISG induction, the predicted paracrine amplification (# of IFN responders / # IFN producers) has been improved for low-dose infection. Previously, the model showed a maximum of paracrine amplification at 2 HAU/ml NDV, with an increase from 1 to 2 HAU/ml. Now the model is in full accord with the data, showing a monotonic decrease of paracrine amplification (new Figure 7B).

The mathematical details are given in the expanded Supplementary Text S1 (new Section 2. Parameter estimation), alongside with the new Supplementary Figure S10.

Revised Figures 7A and B:



Comment 2b: "Moreover, it remains not clear to this reviewer how the model accounts for the cellular heterogeneity: were the cellular parameters drawn from a distribution (if so, details and experimental evidence about this are needed) or is the cellular heterogeneity related to the stochasticity in the chemical equations?"

Response: The reviewer correctly points out that there are intrinsic and extrinsic sources of cell-to-cell variability. As a novel finding of our work is the strong cell-intrinsic stochasticity of IFN-β and IRF-7 expression (and in particular the timing of these events), we focused the model on this property. Cellular heterogeneity in the model arises due to stochasticity in the reaction steps and is not due to variability of parameters. We have reorganized the paragraph on the model description to point this out clearly at the beginning (pages 9 and 10). Indeed, the measured distributions NF-κB/IRF activation as well as IFN-β and IRF-7 induction times can be fully accounted for within this framework. In a new paragraph in the Discussion, we have emphasized that distributed parameters could play an additional role in generating heterogeneity and cited relevant papers (page 13).

Comment 3: "One critical conclusion of the paper is that cell-cell variability in response to viral infections is cell-intrinsic. This emerges from the bimodal cellular response (e.g. in terms of IRF7 expression -Fig 5) despite homogenous viral infection. The results from the modeling effort do not properly document this fact because it is not clear, at this stage, where the heterogeneity comes from in the model. Additional results would need to be presented: one would need to check how bimodal the distribution of IFNb and IRF7 is in the model."

Response: We have now emphasized more clearly in the reorganized model description that both IFN-β and IRF-7 distributions are bimodal. Indeed, we exploited this experimentally established fact by modeling transitions between discrete (IFN-β or IRF-7) expressing and non-expressing cell states; hence the distributions are bimodal by model construction. In particular, this approach is supported by the observation that the waiting time to gene induction in an individual cell is much larger than the time needed to reach maximal expression after induction – in other words, induction is switch-like in time. These ideas are now explained more clearly on pages 9 and 10.

Comment 4: "The importance of noise quenching through paracrine communication with IFNb is interesting and consistent with a recent (but not as complete) publication by Hu et al. in PLoSOne. A direct discussion of this paracrine effect (by measuring the amount

of secreted IFNb, by disrupting paracrine communication with blocking antibodies, and by modeling the effect of saturating cytokine) would make a more convincing case. For example, Supp. Figure 6 documents the natural disappearance of IFNb in the medium: does that play a role in the heterogeneity in IRF7 expression?"

Response: We indeed measured quantitatively the time course of secreted IFN-β (Figure 6D, blue trace). From this measurement, we determined the production rate per cell. This parameter value is key to predicting the strong paracrine amplification (# of IFN responders / # IFN producers) at low infection doses (Figure 7B). Extracellular IFN-β degradation does not contribute to causing heterogeneity of IRF-7 expression in the model, as extracellular IFN-β levels remain elevated in the supernatant at least until 48 hours (Figure 6D, blue trace). These points are now emphasized more clearly in the text (page 10).

Minor issues:

1) "Instead of individual cells, one would need to see the distribution or the cumulative distribution of IFNb expression in Figure 3A."

Response: Distributions have been added in Supplementary Figure 5 for comparison.

2) "When presenting the IFN response as a function of the viral load (Figure 7B), please plot the results in semilogx: there seems to be discrepancy in the low viral-titer regime. If so, please correct the model accordingly."

Response: This has been done. The discrepancy at the lowest viral dose between model and experimental data has been resolved with the improved parameterization of the model (for details see response to Comment 2a).

Acceptance letter 24 April 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.

Before we can transmit this work to production, we have a few remaining format and content issues that we ask you to address:

1. In general Molecular Systems Biology requires that mathematical models are provided in a machine-readable form as supplementary material. Could you please supply the matlab files used for the model simulations (in a single zip file)? This additional file will also need to be listed in the Table of Contents at the beginning of the Supplementary Information pdf.

If you can provide the items above by tomorrow, April 25th (I know this is tight timing), there is a chance we might still be able to publish your article in May.

2. If possible, we also encourage you to supply numeric "source data" for some of the Figures in this work (e.g. <http://tinyurl.com/365zpej>). Please see our Instructions of Authors for more details on preparation and formatting of figure source data (<http://www.nature.com/msb/authors/index.html#a3.4.3>). These supporting files would need to be received by the end of this week if we are publish this work in May.

Revised files can be sent as attachments to a reply email.

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

Sincerely,

Editor - Molecular Systems Biology
msb@embo.org

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Reviewer #2 (Remarks to the Author):

The revised manuscript addresses my previous questions and concerns adequately. In some cases I feel that data in the supplement is of sufficient importance that it belongs into the main paper or vice versa, but generally presentation is improved also.

