molecular systems biology

Division of Labor by Dual Feedback Regulators Controls JAK2/STAT5 Signaling over Broad Ligand Range

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Review timeline:

Submission date:	21 December 2010
Editorial Decision:	10 February 2011
Revision received:	09 May 2011
Acceptance letter:	10 June 2011

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 10 February 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees whom we asked to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. However, they raise substantial concerns on your work, which should be addressed in a major revision of the present work.

The reviewers recognize the quality of the experimental work and the careful description of the model. However, they also express some important concerns with regard to the following points:

- the physiological relevance of the postulated concerted roles of CIS and SOCS in controlling STAT5 over a broad range of Epo concentrations remains unclear.
- the correlation between dynamical features of the system and apoptosis should be explored more systematically (reviewer #2 recommends PCA) rather than be restricted to the integral npSTAT response only.
- some important discrepancies between model and experimental measurements should be clarified.

NOTE: in addition to our capacity to host datasets in our supplementary in formation section, we provide a new functionality on our website, which allows readers to directly download the 'source data' associated with selected figure panels (eg <http://tinyurl.com/365zpej>), for the purpose of alternative visualization, re-analysis or integration with other data. These files are separate from the traditional supplementary information files and are directly linked to specific figure panels. In the case of this study, we would strongly encourage you to submit the relevant data files for the quantitative data displayed in the figures of this study. We provide below some general guidelines with regard to the format of such data tables.

As part of the EMBO Publications transparent editorial process initiative (see our Editorial at

http://www.nature.com/msb/journal/v6/n1/full/msb201072.html), Molecular Systems Biology will publish online a Review Process File to accompany accepted manuscripts. When preparing your letter of response, please be aware that in the event of acceptance, your cover letter/point-by-point document will be included as part of this File, which will be available to the scientific community. More information about this initiative is available in our Instructions to Authors. If you have any questions about this initiative, please contact the editorial office msb@embo.org.

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 favourable.

Yours sincerely,

Editor
Molecular Systems Biology

REFEREE REPORTS

Reviewer #1 (Remarks to the Author):

This manuscript offers a nicely constructed and communicated study of the quantitative dynamics of the JAK2/STAT5 pathway downstream of Epo treatment in hematopoietic cells, demonstrating how two different transcriptional feedback loops (CIS, SOCS3) operate at different Epo doses. In my view, this study ought to be more suitable for publication in mainstream biochemistry or cell biology journals, as opposed to seeming restricted to a systems biology journal; my reasoning is that the findings are more significant to an audience focused on the particular cell type and/or pathway rather than as a major general systems principle or methodological advance. It will be beneficial for the systems biology field to have this approach move into the mainstream of cell biology/biochemistry, remaining viewed as outside. However, I will leave this concern to the editors.

I have a two minor suggestions for the authors' consideration before final acceptance. First, it is not clear to me from the presentation of the model how particular phosphatase activities are included. Given the centrality of feedback loops to this study, it would be extremely helpful to have greater emphasis in the text on how the phosphatases are represented in affecting the various model species kinetics. Second, while I see the analysis of the quantitative dependence of cell survival on npSTAT5 as a wonderful facet of the work (instead of the more typical effort not endeavoring to connect signaling dynamics to phenotypic behavioral responses), it would be helpful for the authors to provide some comment on whether and why cell survival might appear to be depending solely on STAT5 in surprisingly uni-variate fashion at least under the treatment conditions employed here.

Reviewer #2 (Remarks to the Author):

In this study, Bachmann et al. presents a theoretical model for the STAT5 signaling response of primary erythroÔd progenitor cells to varied levels of EPO. The main insight relates to the non-redundant role of the JAK-STAT signaling regulators CIS and SOCS3. This is a very relevant issue as integration of signaling responses over "long" timescales (>1hr) is the best predictor of biological function (here anti-apoptosis response). This integration implies the accounting of transcriptional upregulation of JAK-STAT negative regulators, upon STAT5 phosphorylation. Bachmann et al. present such an experimentally-validated model of JAK-STAT signaling and transcriptional regulation to tease out the differential role of CIS and SOCS3: it is predicted that CIS impacts STAT5 signaling at low concentrations of EPO, while SOCS3 downregulates STAT5 signaling at high concentrations of EPO. This study is well designed and benefit from the interplay between experimental and theoretical insights. Few issues would benefit from further discussion before publication.

A) To model JAK-STAT signaling here, biochemical stochasticity has been neglected because of the high expression levels of signaling proteins and averaging for the signaling response measurements by Western Blot (cf Supplementary model - p9). However, Bachmann et al. present survival responses of individual cells (Tunnel assay) that are inconsistent with this model averaging: in Figure 5B, it is found that cells undergo apoptosis in an all-or-none manner, while the STAT5 phosphorylation response (integrated over 45min) varies smoothly with the EPO concentration (Figure 5C). The correlation between integrated npSTAT5 and survival percentage is impressive (Figure 5C) but somewhat spurious as it involves cell averaging. Hence there exists a critical shortcoming of the STAT5 signaling model that misses out the nonlinearity of this signaling response in individual cells. This discrepancy needs to be solved theoretically (essentially by tracing the source of nonlinearity and variability (Spencer, Gaudet et al. 2009)). Experimentally, this discrepancy could be resolved by correlating npSTAT5 accumulation with downstream signals (e.g. upregulation of anti-apoptotic molecules like Bcl-2 or Bcl-xL (Silva, Grillot et al. 1996)).

B) The most interesting predictions of the model is that CIS downregulation and SOCS3 downregulation impact STAT5 phosphorylation for different ranges of EPO concentrations (Figure 6). These predictions have not been validated experimentally. This is rather puzzling as the authors made the effort of over-expressing CIS and SOCS3 (Figure 3) and should attempt to downregulate CIS and SOCS3 by RNA interference (short of developing a conditional knock-out model for SOCS3 within the erythroÔd lineage, as it has been done for the lymphoid lineage (Alexander and Hilton 2004)).

C) There are discrepancies between the signaling measurements and their modeling output that need to be addressed:

a) According to the model in Figure 6, CIS mostly impacts EpoR phosphorylation (relevant at low EPO concentration) while SOCS3 mostly impacts JAK2 phosphorylation. In the model reported in Figure 3, CIS upregulation does not impact EpoR nor JAK2 phosphorylation.

b) More timepoints need to be taken to constrain better the theoretical model. At present time, only one timepoint is taken around the peak response (~5min timepoint) while it appears critical to understand the differential role of CIS and SOCS3 in the signaling response. Indeed, the measured pEpoR response seems to be greatly affected by CIS upregulation but not by SOCS3 upregulation, while the opposite is true for JAK2 phosphorylation. On the other hand, the model output (while dealing appropriately with the long-term responses > 30min) completely misses this differential effect for the peak phosphorylation response (cf Figure 3): it looks correct for pJAK2 but inconsistent for pEpoR.

c) According to the model in Figure 3, JAK2 and pEpoR phosphorylations are not affected by CIS upregulation but STAT5 phosphorylation is greatly reduced. The experimental results (with a concomitant downregulation of pEpoR and pSTAT5 upon CIS overexpression) do not match this theoretical prediction. Please justify this discrepancy.

d) CIS upregulation is inconsistent in the wild-type cells in the experimental sets top right panel and bottom right panel of Figure 3). Please clarify how reproducible these measurements are (e.g. how were error bars estimated and how many repeat experiments were performed).

The detailed description of the molecular model as well as its underlying assumptions and justifications, detailed in the supplementary materials -section S2-, are greatly appreciated.

D) In the section 2.3.3. of the supplementary materials, the time delay between STAT5 phosphorylation and SOCS/CIS mRNA expression is being tested. Additional information about the experimental measurements is necessary as the experimental method section about qRT-PCR presents a single set of primer for CIS and a single set for SOCS3. Specifically, how were the different maturation states of the mRNA resolved experimentally?

E) One interesting observation, reported in Figure 5, is that the integral of npSTAT5 correlates quantitatively with the anti-apoptosis response. The supporting reference for this insight (Asthagiri, Reinhart et al. 2000) applied to the ERK response and not to cytokine response. Hence, using the integral of npSTAT5 does not stem from any conceptual insight and appears somewhat arbitrary. To

generate a more objective signaling outuput parameter, we recommend applying a classical principal component analysis (Janes, Albeck et al. 2005) to correlate the anti-apoptosis response to the whole compendium of experimental observations (time kinetics of phosphorylation of EpoR, JAK, STAT5, as well as upregulation of signaling components): this statistical analysis would yield to an optimized and objective parameter to encompass the variability of apoptosis responses.

F) Minor points:

1) The time order of the gel loading in Figure S2 is puzzling: please justify this experimental idiosyncrasy.
2) The calibrated measurements for the expression levels of signaling components (as presented in Table S1) are of general relevance for the systems biology community. Please make sure to deposit these numbers in a repository (e.g. http://bionumbers.hms.harvard.edu).
3) On page 8, the authors state "27 parameters describing 30 reactions and 84 nuisance parameters, which is a reasonable number (...)". Please specify what "reasonable" means.

References:

Alexander, W. S. and D. J. Hilton (2004). "The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response." Annu Rev Immunol 22: 503-29.

Asthagiri, A. R., C. A. Reinhart, et al. (2000). "The role of transient ERK2 signals in fibronectin- and insulin-mediated DNA synthesis." J Cell Sci 113 Pt 24: 4499-510.

Janes, K. A., J. G. Albeck, et al. (2005). "A systems model of signaling identifies a molecular basis set for cytokine-induced apoptosis." Science 310(5754): 1646-53.

Silva, M., D. Grillot, et al. (1996). "Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-XL and Bcl-2." Blood 88(5): 1576-82.

Spencer, S. L., S. Gaudet, et al. (2009). "Non-genetic origins of cell-to-cell variability in TRAIL-induced apoptosis." Nature 459(7245): 428-32.

Reviewer #3 (Remarks to the Author):

Bachmann et al. examine the dynamics of Jak2/Stat5 signaling in erythroid progenitor cells in response to varying concentrations of the hormone erythropoietin (Epo), with particular focus on the roles of the transcriptional negative feedback mediators CIS and SOCS3 on pathway dynamics. The authors parameterize a mathematical model of the pathway using original datasets collected in wild-type cells and cells overexpressing CIS, SOCS3 and SHP1. Subsequent analysis with the model reveals that cell survival correlates with the level of nuclear pSTAT5 integrated over the first 45 mins. Further, negative feedback due to CIS and SOCS3 are shown to have prominent effects on the dynamics of STAT5 activation at low and high doses of Epo respectively, thereby pointing to the complementary nature of these proteins in controlling STAT5 dynamics over the wide range of hormone concentrations that would be encountered in vivo.

The authors demonstrate impressive rigor in collecting the appropriate quantitative datasets, designing a parsimonious mathematical model that can be informed by the data, and analyzing the uncertainty in their parameter estimates. As such, this is a well written manuscript that reports results obtained from a well designed study, and should be of interest to the readers of the journal.

My concern is that the impact of the main conclusion - that CIS and SOCS3 modulate STAT5 dynamics at different agonist concentrations - is somewhat unclear. This conclusion is a satisfying one from the perspective of a modeler who is interested in understanding the operating principles of a signaling circuit. However, it is unclear whether the subtle changes in signaling caused by CIS at low Epo concentrations, and the changes to steady-state signaling effected by SOCS3 at high Epo concentrations are physiologically relevant. The authors show that the nuclear pSTAT5 levels integrated over the first 45 mins correlate with an outcome (survival), but then choose to focus on the steady-state signaling level at 6 hours when they analyze the potential roles of CIS- and SOCS3-mediated feedback. A more thorough discussion of the effects observed in Fig. 6A in the context of

erythroid biology would strengthen the manuscript.

Minor comments:

It is difficult to compare the results across different figures due to differences in the units for agonist dose. The authors should provide the Epo concentration in units/cell for all figures to enable comparison. On a related note why does 0.05 U/ml in Fig. 5A elicit a response (peak nuclear pSTAT5 of ~30nM) comparable to the 4 U/ml dose in Fig. 6A? Is SOCS3 the primary regulator of STAT5 dynamics in both these scenarios?

Pg. 8 - Replace "84 nuisance parameters" with something more descriptive explaining that these parameters relate to scaling the model predictions to enable comparison with the data.

Fig. 3B - The quantitative mass spectrometry results are plotted in an odd fashion with sampling apparently occurring at negative times. What do the individual points represent? Are these biological replicates?

Figure 4 - Is there an explanation for why the model cannot accurately capture the effects of SOCS3 and CIS overexpression on the pEpoR levels?

1st Revision - authors' response 09 May 2011

Please find enclosed our revised manuscript "Division of Labor by Dual Feedback Regulators Controls JAK2/STAT5 Signaling over Broad Ligand Range" that we would like to re-submit to Molecular Systems Biology.
We addressed in depth all the reviewersí comments point-by-point as outlined below. We performed additional experiments suggested by the reviewers and re-fitted our experimental data to improve the model performance explaining all data points.

Briefly, the most important additions are:
o The physiological relevance of CIS and SOCS3 in controlling STAT5 over a broad range of Epo concentrations was clarified in a completely new paragraph in the discussion.
o We applied standard regression statistics for linear models and investigated the impact of stochasticity on the npSTAT5 dynamics to explore the correlation between the different pathway components and apoptosis more systematically.
o Additionally, we performed new experiments analyzing STAT5-induced anti-apoptotic target genes and revealed that the pro-survival factor Pim-1 correlates with the integral of STAT5 activity.
o We modified the structure of our mathematical model and completely re-fitted the model parameters to improve model performance explaining all data points.

Furthermore, we provide you with the following data sets to enable alternative visualization orintegration of data and models as suggested:

o SBML file of our model: jak2_stat5_feedbacks_sbml
o Source data for Figure 1B
o Source data for Figure 1C
o Source data for Figure 3A
o Source data for Figure 3B
o Source data for Figure 3C
o Source data for Figure 4
o Source data for Figure 5C

We believe that the manuscript gained substantially by your and the reviewer's suggestions and hope that you will find the revised manuscript acceptable for publication in Molecular Systems Biology.

Reviewer #1 (Remarks to the Author):

This manuscript offers a nicely constructed and communicated study of the quantitative dynamics of the JAK2/STAT5 pathway downstream of Epo treatment in hematopoietic cells, demonstrating how two different transcriptional feedback loops (CIS, SOCS3) operate at different Epo doses. In my view, this study ought to be more suitable for publication in mainstream biochemistry or cell biology journals, as opposed to seeming restricted to a systems biology journal; my reasoning is that the findings are more significant to an audience focused on the particular cell type and/or pathway rather than as a major general systems principle or methodological advance. It will be beneficial for the systems biology field to have this approach move into the mainstream of cell biology/biochemistry, remaining viewed as outside. However, I will leave this concern to the editors.

We have established a comprehensive modeling framework in combination with quantitative data employing different experimental techniques. By linking quantitative signaling information with phenotypic cellular decisions using mathematical modeling, we contributed to the advanced understanding of how signaling is correlated to cellular decisions, which is of interest to the entire systems biology community.

I have a two minor suggestions for the authors' consideration before final acceptance.
First, it is not clear to me from the presentation of the model how particular phosphatase activities are included. Given the centrality of feedback loops to this study, it would be extremely helpful to have greater emphasis in the text on how the phosphatases are represented in affecting the various model species kinetics.

We apologize for the insufficient explanation of how phosphatases were included in the model and how they affect the model species kinetics. To clarify this issue, we moved the following text from the Supplementary Material into the Results section, page 7:
Additional negative regulators of Epo-induced JAK-STAT signaling such as (...) hematopoietic protein tyrosine phosphatases (PTPs) usually do not show stimulationdependent induction but are constitutively expressed. To confirm this in primary erythroid progenitor cells, we compiled expression profiles of the (...) hematopoietic phosphatases SHP1, SHP2, PTP1B and PTPRC (CD45) (Supplementary Figure S1). In line with previous reports, stimulation-dependent induction of these genes was not detected.
And in the Results section " Dynamic pathway model (…)", page 7:
Briefly, the tyrosine phosphatase SHP1, which is constitutively expressed (Supplementary Fig S2B) has previously been shown to require binding to the specific phosphorylated tyrosine residue Tyr429 on the EpoR to activate phosphatase activity (Klingm¸ller et al, 1995; Pei et al, 1994). Thus, we included an activated form of SHP1 in our model that in turn catalyzes dephosphorylation of JAK2 and EpoR.
For an extended explanation, we modified the following paragraph in the Supplemental Material Section 2.2, page 17:
In general, phosphatase activities were included in the model as dephosphorylation reactions that are proportional to the substrate with a time-independent kinetics. Ligand-dependent attenuation, for instance by receptor internalization and dephosphorylation was summarized by including SHP1-mediated deactivation of JAK2 and EpoR as described in a previous model of CFU-E cells (Schilling et al., 2009). The tyrosine phosphatase SHP1 has previously been shown to require binding to the specific phosphorylated tyrosine residue Tyr429 on the EpoR to activate phosphatase activity (Pei et al., 1996; Klingm¸ller et al., 1995). Therefore, SHP1 was considered to exist in two states in the model: one active and one inactive form. The transition of inactive to catalytically active SHP1 is mediated by phosphorylated EpoR and JAK2 that reflects the recruitment to the specific phosphotyrosine residue. The activated form of SHP1 then catalyzes dephosphorylation of JAK2 and EpoR.
(pEpoR -> EpoR, catalyzed by actSHP1).
To clarify dephosphorylation of STAT5 in the model by phosphatases we added the following paragraph to the Results section, page 8:
To enter a new cycle of activation, STAT5 is dephosphorylated and exported from the nucleus into the cytoplasm in our model by a single reaction. Since various phosphatases are known to faciltate dephosphorylation of STAT5 in the nucleus and in the cytoplasm (Aoki et al, 2000; Hoyt et al,

2007; Yu et al, 2000), we assumed constitutive inactivation of STAT5 during traffic from nucleus to cytoplasm (npSTAT5 -> STAT5).

Second, while I see the analysis of the quantitative dependence of cell survival on npSTAT5 as a wonderful facet of the work (instead of the more typical effort not endeavoring to connect signaling dynamics to phenotypic behavioral responses), it would be helpful for the authors to provide some comment on whether and why cell survival might appear to be depending solely on STAT5 in surprisingly uni-variate fashion at least under the treatment conditions employed here.

We thank the reviewer for this critical comment. To clarify the contribution of STAT5 to survival we rephrased and added the following paragraph in the Results section, page 11, of our manuscript:
To systematically test if npSTAT5 is the major factor that contributes to survival decisions we additionally parameterized the contributions to the survival signal of the different pathway species (pEpoR, pJAK2, npSTAT5, CIS and SOCS3) in an additive way (see new Supplementary Material Section 1.1). By comparing the contributions of various signaling components to the anti-apoptosis response, we detect that the npSTAT5 signal contributed more than 99%.
To discuss the contribution of npSTAT5 compared to additional pathways, we rephrased the following paragraph in the Discussion section of our manuscript, page 15:
Our finding that the integral of npSTAT5 is a good predictor for survival does not imply that survival is exclusively depending on STAT5. The integral of STAT5 activity in the nucleus transfers quantitative information about extracellular ligand concentrations to downstream signals, i.e. expression of anti-apoptotic target genes such as Pim-1 that contribute to the ultimate cellular response. Additional pro-survival factors such as the PI3K/AKT pathway that have been shown previously to be involved in prevention of apoptosis in CFU-E cells (Bouscary et al, 2003) may also contribute. However, it was previously shown that overexpression of constitutively active AKT could not substitute for the apoptosissuppressing function of the EpoR-STAT5 pathway in JAK2-/- erythroid cells (Ghaffari et al, 2006). Hence, though we cannot rule out the involvement of other pathways, our study underlines the direct relationship of the integral STAT5 response and survival decisions of primary erythroid progenitor cells.

Reviewer #2 (Remarks to the Author):
In this study, Bachmann et al. presents a theoretical model for the STAT5 signaling response of primary erythroïd progenitor cells to varied levels of EPO. The main insight relates to the non-redundant role of the JAK-STAT signaling regulators CIS and SOCS3. This is a very relevant issue as integration of signaling responses over "long" timescales (>1hr) is the best predictor of biological function (here anti-apoptosis response). This integration implies the accounting of transcriptional upregulation of JAK-STAT negative regulators, upon STAT5 phosphorylation. Bachmann et al. present such an experimentally-validated model of JAK-STAT signaling and transcriptional regulation to tease out the differential role of CIS and SOCS3: it is predicted that CIS impacts STAT5 signaling at low concentrations of EPO, while SOCS3 downregulates STAT5 signaling at high concentrations of EPO. This study is well designed and benefit from the interplay between experimental and theoreticalinsights. Few issues would benefit from further discussion before publication.
A) To model JAK-STAT signaling here, biochemical stochasticity has been neglected because of the high expression levels of signaling proteins and averaging for the signaling response measurements by Western Blot (cf Supplementary model - p9). However, Bachmann et al. present survival responses of individual cells (Tunnel assay) that are inconsistent with this model averaging: in Figure 5B, it is found that cells undergo apoptosis in an all-or-none manner, while the STAT5 phosphorylation response (integrated over 45min) varies smoothly with the EPO concentration (Figure 5C). The correlation between integrated npSTAT5 and survival percentage is impressive (Figure 5C) but somewhat spurious as it involves cell averaging. Hence there exists a critical shortcoming of the STAT5 signaling model that misses out the nonlinearity of this signaling response in individual cells.
This discrepancy needs to be solved theoretically (essentially by tracing the source of nonlinearity and variability (Spencer, Gaudet et al. 2009)).

We thank the reviewer for raising the interesting point of biochemical stochasticity. Following the reviewers suggestion, we investigated the impact of intrinsic and extrinsic stochasticity on

the model dynamics, with special focus on npSTAT5, the important factor for apoptosis in our system.
We explained our new results in details in the new Supplementary Material section, page 11:
1.4 Impact of stochasticity on nuclear phosphorylated STAT5 Intrinsic stochasticity was simulated using the Stochastic Simulation Algorithm (Gillespie, 1977), see Fig. S8. To mimic the effect of extrinsic stochasticity of a cell population, we simulated the ODE model for multiple initial concentrations of proteins drawn from a normal distributed centered around the estimated value with 10% variation, see Fig. S9 and Spencer et al, (2009). If the variability in the STAT5 response in single cells simulated by intrinsic or extrinsic stochasticity is the main source to explain the all-or-none survival decision, a bistable behavior would be expected. However, in both cases, the trajectory of npSTAT5 is rather negligibly affected by noise and does not show bi-stability. We therefore suggest that other individual cell-to-cell differences cause the all-or-none response of cell death or survival.

To summarize these new results, we added the following paragraph to the Results section, page 11:
As apoptosis is an all-or-none decision, we performed simulations describing the effect of intrinsic (Fig. S8) and extrinsic stochasticity (Fig. S9) on npSTAT5. In both cases, the trajectory of npSTAT5 is rather negligibly affected by noise and does not show bi-stability.

Experimentally, this discrepancy could be resolved by correlating npSTAT5 accumulation with downstream signals (e.g. upregulation of anti-apoptotic molecules like Bcl-2 or Bcl-xL (Silva, Grillot et al. 1996)).

To investigate this experimentally, we determined, as suggested by the Reviewer, the induction of an important anti-apoptotic target gene at the mRNA level in primary erythroid progenitor cells (see also new Supplementary Material Section 1.3). We selected Pim-1 since this kinase was previously associated with STAT5-mediated anti-apoptotic responses. Our results demonstrate that the dose-dependent behavior of Pim-1 mirrors the behavior of the integral pSTAT5 response in the nucleus.
These new results are described in the following paragraph of the Results section, page 11/12:
To further analyze the direct correlation between the integral response of phosphorylated STAT5 in the nucleus and the survival decision of erythroid progenitor cells (Fig. 6C), we investigated the induction of anti-apoptotic target genes. First, global gene expression data was used to identify a panel of Epo-regulated anti-apoptotic genes. Among the preselected candidates (Bcl-xL, BIM, Bcl-2, Pim-1) that were previously associated with Epo-dependent regulation, exclusively Pim-1 showed rapid and significant induction compared to untreated control cells (Figure S7A). The dose-dependent behavior of Pim-1 (Fig. S7) mirrored the behavior of the integral npSTAT5 response (Fig. 6C) and continuously increased over different Epo doses in an averaged cell population. Thus, the integral of nuclear phosphorylated STAT5 correlates with Pim-1 induction, while the individual cell fate may be influenced by cell-to-cell variability (Spencer et al., 2009).
The selection of the anti-apoptotic gene Pim-1 and the qRT-PCR results are described in details in the following new paragraph in the Supplementary material, page 9:
1.3 Expression of anti-apoptotic STAT5 target gene Pim-1
To further analyze the direct correlation between the integral response of phosphorylated STAT5 in the nucleus and the survival decision of erythroid progenitor cells (Fig. 6C), we investigated the induction of anti-apoptotic target genes.
Global gene expression demonstrated that exclusively Pim-1 is rapidly induced compared to untreated control cells (Figure S7A). Because it was also previously identified as STAT5-modulated anti-apoptotic effector (Sathyanarayana et al., 2008, Menon et al., 2006), we selected Pim-1 as representative candidate for further experiments. In contrast, Bcl-xL, which was discussed earlier to be important for survival in erythroid cell lines (Solokovsky, 1999; Silva, Grillot et al. 1996), showed upregulation only very late after 15-20 h and Bcl-2 showed no significant modulation. BIM as pro-apoptotic factor was repressed upon Epo stimulation.
These results are in line with the expression pattern of pro-survival factors observed by Sathyanarayana et al. (2008) that investigated Epo-dependent regulation of pro-survival factors in primary murine Kit(+)CD71(high)Ter119(-) erythroblasts.
To examine if Pim-1 follows the integrated nuclear STAT5 response over the entire range of Epo concentrations, the upregulation of Pim-1 was determined after 2h for various Epo concentrations (Fig. S7B). The dose-dependent expression of Pim-1 mirrors the behavior of

the integral pSTAT5 response (Figure6C), implying that the integral STAT5 response is directly proportional to transcriptional responses of direct target genes.

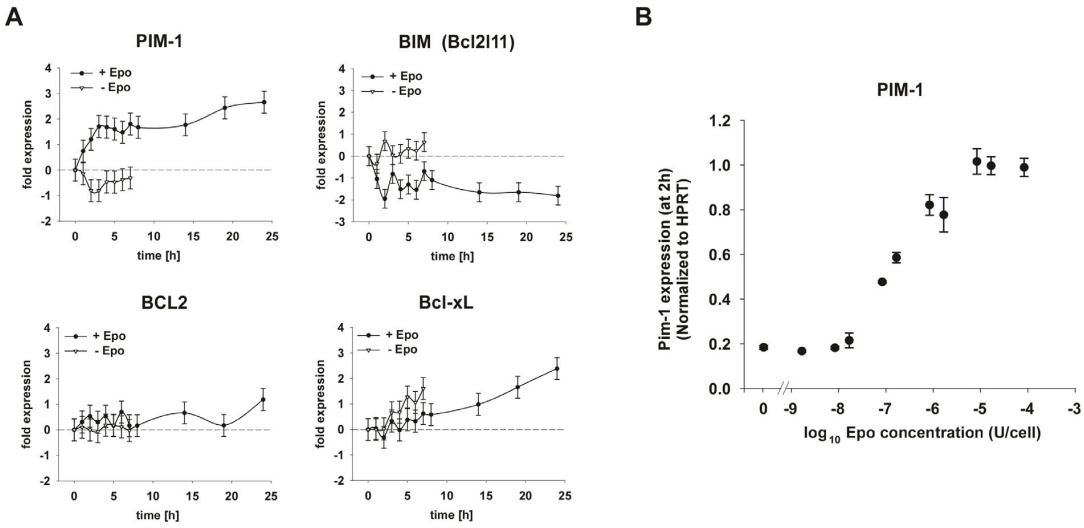

Figure S7. Epo-dependent expression of anti-apoptotic factors in primary erythroid progenitor cells at the CFU-E stage. (A) Time series of Pim-1, BIM, BCL-2 and Bcl-xL with 0.5 U/ml and without Epo stimulation as control. Freshly isolated and MACS sorted primary CFU-E cells from murine fetal livers were starved, stimulated or left untreated over a period of 8 h or 24 h. After 8 h the untreated time course was stopped due to increase of CFU-E cell death without Epo stimulation. RNA was extracted at different time-points and subjected to microarray analysis. Log2-fold change of mRNA levels were calculated relative to the time-point t = 0. (B) Quantitative RT-PCR analysis of Pim-1 expression stimulated with various Epo concentrations at 2 h. Freshly isolated CFU-E cells were starved and stimulated with the indicated Epo concentrations, prepared RNA was reverse transcribed and used in qRT-PCR analysis. Values represent means with SD for n = 3 independent reverse transcription samples. Relative concentrations were normalized using HPRT as a reference gene.

B) The most interesting predictions of the model is that CIS downregulation and SOCS3 downregulation impact STAT5 phosphorylation for different ranges of EPO concentrations (Figure 6). These predictions have not been validated experimentally. This is rather puzzling as the authors made the effort of over-expressing CIS and SOCS3 (Figure 3) and should attempt to downregulate CIS and SOCS3 by RNA interference (short of developing a conditional knock-out model for SOCS3 within the erythroïd lineage, as it has been done for the lymphoid lineage (Alexander and Hilton 2004)).

We agree with the reviewer that knockdown of CIS and SOCS3 would be interesting; however, due to technical limitations this could not be tested experimentally but rather addressed in silico. To explain this in detail we included a new paragraph in the Supplementary Material, page 9:
1.2 Insufficient siRNA knockdown efficiency of SOCS members in primary murine CFU-E cells
We tested different techniques to generate siRNA knockdowns of the SOCS family members in primary erythroid progenitor cells. Retro-, lenti- and adenoviral vectors were used expressing different shRNA constructs targeting the mRNA of one of the SOCS family members in primary erythroid cells at the CFU-E stage. However, the knockdown efficiencies achieved with these experimental techniques were insufficient. A potential explanation for these results is the time period available for experiments of 12-20 h after transduction that may be too short to observe siRNA-mediated knockdown in these cells. The time frame of the experiments, however, cannot be extended since erythroid progenitor cells start to terminally differentiate after 12 h. The study of Yu

et al (2003) (reviewed in Alexander and Hilton, 2004) generated stable Th2 cell lines overexpressing SOCS3 antisense constructs in the lymphoid lineage, a method that cannot be applied to primary cells.
Another possible explanation of the insufficient knockdown in the primary cells is the rapid turnover (Larsson et al, 2010) of SOCS mRNA that could limit siRNA efficacy. Larsson et al (2010) have shown that high pre-existing mRNA turnover rate is associated with reduced susceptibility to silencing by siRNAs.
Furthermore, we added the following sentence into the Results section of the Main Manuscript, page 9:
We focused on overexpression since RNAi- mediated knockdown was not addressable with the currently available techniques in these cells.

C) There are discrepancies between the signaling measurements and their modeling output that need to be addressed:
a) According to the model in Figure 6, CIS mostly impacts EpoR phosphorylation (relevant at low EPO concentration) while SOCS3 mostly impacts JAK2 phosphorylation. In the model reported in Figure 3, CIS upregulation does not impact EpoR nor JAK2 phosphorylation.

We apologize that the cartoon in Fig. 6C was confusing. Our intention was to show that CIS mostly impacts pEpoR-dependent STAT5 activation (at low Epo concentrations), while SOCS3 mostly impacts pJAK2-dependent STAT5 phosphorylation (at high Epo concentrations). We improved the cartoon to clarify this point.
To better explain the different inhibitory mechanism of the two negative regulators we included the following text to the figure legend in Figure 6C:
At low Epo levels, CIS mostly impacts pEpoR-dependent STAT5 activation by preventing binding of STAT5 via its SH2 domain to the specific pTyr sites on the EpoR receptor. At high Epo concentrations SOCS3 mostly impacts pJAK2-dependent STAT5 phosphorylation by inhibiting the activation of the JAK2 via its kinase inhibitory region (KIR).

b) More timepoints need to be taken to constrain better the theoretical model. At present time, only one timepoint is taken around the peak response (~5min timepoint) while it appears critical to understand the differential role of CIS and SOCS3 in the signaling response. Indeed, the measured pEpoR response seems to be greatly affected by CIS upregulation but not by SOCS3 upregulation, while the opposite is true for JAK2 phosphorylation. On the other hand, the model output (while dealing appropriately with the long-term responses > 30min) completely misses this differential effect for the peak phosphorylation response (cf Figure 3): it looks correct for pJAK2 but inconsistent for pEpoR.

As suggested by the reviewer, we repeated the experiment for the CIS overexpression and measured two time points in the peak response for the EpoR phosphorylation. This result was consistent with our previous experiment, demonstrating a reduction of pEpoR in case of CIS overexpression for early time points.
As our model was not able to capture this effect, we adapted the model by now taking into account the different phosphotyrosine residues at the EpoR. Please see our answer to comment c) for the details of the adapted model.
The adapted model was superior in describing the effects of CIS overexpression and also slightly improved the fit in the case of SOCS3 overexpression.
We completely re-calibrated our improved model including this new data set. The new data set and the corresponding model trajectory is depicted in Figure S17 and the new data points of pEpoR in case of CIS overexpression have been added to Figure 4.
c) According to the model in Figure 3, JAK2 and pEpoR phosphorylations are not affected by CIS upregulation but STAT5 phosphorylation is greatly reduced. The experimental results (with a concomitant downregulation of pEpoR and pSTAT5 upon CIS overexpression) do not match this theoretical prediction. Please justify this discrepancy.

We thank the reviewer for pointing out this discrepancy between model and experimental data. Mathematical modeling of biological systems always comprises a trade-off between accuracy in

describing the experimental data and model complexity that may prevent identifiability of parameters and thereby the predictive power of the model. Based on the reviewerís comments we have therefore explored if we can increase the complexity of our model in a way that would improve the goodness-of-fit while still keeping the relevant model parameters identifiable. To this aim, we have now revisited the model assumptions to analyze why the model could not explain the reduction of EpoR phosphorylation in the CIS overexpression experiment. We concluded that our model structure was not sufficiently detailed to explain the behavior of the pEpoR data in this particular experiment. Therefore, we refined our modeling of the EpoR level by considering that the EpoR harbors eight different tyrosine sites that can be phosphorylated. In the previous version of the manuscript, we summarized these sites with the variable pEpoR. However, as the reviewer correctly pointed out, this simplification renders the model unable to describe the receptor phosphorylation in the case of CIS overexpression.
It has been previously shown that CIS-GFP can bind unspecifically to the EpoR lacking the STAT5 recruitment site Y401 if expressed in high amounts (Ketteler et al, 2003). To include this information in our new model, different subclasses of phosphorylated tyrosines at the receptor had to be considered: (i) p1_EpoR_pJAK2 represents the phosphorylated tyrosine sites Y343 and Y401 that serve as recruitment sites for STAT5 and thereby facilitate STAT5 phosphorylation. CIS inhibits recruitment of STAT5 to these sites. (ii) p2_EpoR_pJAK2 represents the remaining phosphorylated tyrosines sites of the EpoR that do not recruit STAT5. These residues are represented in our new model by the variable p2_EpoR and phosphorylation of these residues is inhibited by high levels of CIS.
After introducing this adaptation of our mathematical model, we re-estimated all model parameters and repeated subsequent analyses. We observed a significantly better fit of the pEpoR data in the CIS overexpression time course, whereas all other fits were not affected significantly. Importantly, the major conclusions of our manuscript remained unchanged. Due to the additional data set even the certainty in the prediction could be increased and all the relevant parameters remained identifiable.
The details of the modified model are now included in the Supplementary Model results, page 15-17 (Section 2.2 Definition of dual negative feedback model of JAK2/STAT5 signaling).
It has been previously shown that CIS-GFP can bind unspecifically to the EpoR lacking the STAT5 recruitment site Y401 if expressed in high amounts (Ketteler et al, 2003). To include this information in the model, two subclasses of phosphorylated tyrosines at the receptor were incorporated into the model: (i) p1_EpoR_pJAK2 represents the phosphorylated tyrosine sites Y343 and Y401 that serve as recruitment sites for STAT5 and thereby facilitate STAT5 phosphorylation. CIS inhibits phosphorylation of STAT5 induced by these sites. (ii) p2_EpoR_pJAK2 represents the remaining phosphorylated tyrosines sites of the EpoR that do not recruit STAT5. Phosphorylation of these sites can be inhibited by high levels of CIS due to unspecific binding of CIS to the EpoR (EpoRJAK2_CIS). p12_EpoR_JAK2 represents the completely phosphorylated complex.
We also modified Figure 2 to indicate that the EpoR is phosphorylated on different tyrosine residues. We added to Figure 2 legend:
The pEpoRpJAK2 complex was modeled by considering different subclasses of phosphorylated tyrosines at the receptor (see Supplementary section 2.2).

d) CIS upregulation is inconsistent in the wild-type cells in the experimental sets top right panel and bottom right panel of Figure 3). Please clarify how reproducible these measurements are (e.g. how were error bars estimated and how many repeat experiments were performed).

Please note that when comparing the CIS upregulation in Figure 4 right panel the different time scales on the x-axes have to be considered. If the different scaling is taken into account CIS upregulation is consistent in all plots. We now mention the different time scales in the corresponding figure legend for clarification. The estimation of error bars and all available repetitions are summarized in the Supplementary Material.
Figure 4 legend:
Positively transduced cells were starved and stimulated with 5 U/ml Epo for 60 min or 120 min, respectively.

The detailed description of the molecular model as well as its underlying assumptions and justifications, detailed in the supplementary materials -section S2-, are greatly appreciated.
D) In the section 2.3.3. of the supplementary materials, the time delay between STAT5 phosphorylation and SOCS/CIS mRNA expression is being tested. Additional information about the experimental measurements is necessary as the experimental method section about qRT-PCR presents a single set of primer for CIS and a single set for SOCS3. Specifically, how were the different maturation states of the mRNA resolved experimentally?

We apologize for the unclear numbering of the mRNA species. We do not discriminate experimentally between the different maturation states of the mRNA. The plots in Figure S13 show replicate experiments of the fully spliced mRNA of CIS and SOCS3. We modified the the following text in the Materials and Methods (page 21) to better explain this procedure:
UPL Probes and primer sequences were selected with the Universal ProbeLibrary Assay Design Center (RocheDiagnostics) (...) According to the manufacturesí instruction the selected probes are intron-spanning, thereby ensuring the amplification of fully matured mRNA.
And we added to the legend of Figure S13:
The time course experiments of mature CIS and SOCS3 mRNA expression was performed independently three time and the different replicates are shown.

E) One interesting observation, reported in Figure 5, is that the integral of npSTAT5 correlates quantitatively with the anti-apoptosis response. The supporting reference for this insight (Asthagiri, Reinhart et al. 2000) applied to the ERK response and not to cytokine response. Hence, using the integral of npSTAT5 does not stem from any conceptual insight and appears somewhat arbitrary. To generate a more objective signaling output parameter, we recommend applying a classical principal component analysis (Janes, Albeck et al. 2005) to correlate the anti-apoptosis response to the whole compendium of experimental observations (time kinetics of phosphorylation of EpoR, JAK, STAT5, as well as upregulation of signaling components): this statistical analysis would yield to an optimized and objective parameter to encompass the variability of apoptosis responses. The supporting reference for this insight (Asthagiri, Reinhart et al. 2000) applied to the ERK response and not to cytokine response."]

We thank the reviewer for this comment that encouraged us to improve our manuscript. Janes et al (2005) applied the principal component analysis (PCA) to a multi-input system, in which the human colon adenocarcinoma cell line HT-29 treated with combinations of the prodeath cytokine tumor necrosis factor- (TNF) and the pro-survival growth factors epidermal growth factor (EGF) and insulin. In contrast to this approach, we studied a single input, Epo, which is the essential factor at this stage of erythropoiesis to activate pro-survival factors in primary cells. To exclude the possibility that other signaling components contribute stronger to survival decisions than STAT5, we performed regression statistics for linear models that allows for similar conclusions like PCA in the present situation (Janes et al, 2005), as the referee suggested. Therefore, we considered the contributions of various signaling components (activated EpoR, JAK2, STAT5, CIS and SOCS3) to the anti-apoptosis response, as the referee suggested. This yields a more objective parameter that allows for alternative and mixed contributions to the survival signal.
To explain our results in detail we added the following new section to the Supplementary Material, page 9:
1.1 Contributions to survival signal
In order to infer the contributions to the survival signal shown in Fig. 5 of the main text the following function was considered:
[survival] = p0 + p1 * [pEpoRauc] + p2 * [pJAK2auc] + p3 * [npSTAT5auc] + p4 * [CISauc] + p5 * [SOCS3auc]; (1) where p0 is an offset parameter and ëaucí denotes the integrated signal of the respective species until 60 minutes, see in Fig. S5. We apply standard regression statistics for linear models, see in Table S2 and in Backhaus et al. (1996). The p-value of the one-sided t-test indicated that only the parameters p3 and p5 do have statistically significant non-zero contributions. Via the standardized regression coefficients we can translate the values of the parameters to percentage contributions of explained variance. p3 and its corresponding variable, the integrated npSTAT5 response, contribute more than 99%.
Please note that our formulation allows for mixing of the signals like PCA, as the referee

suggested.
Furthermore, we included the following text in the Results section of the Main Manuscript, page 11:
To systematically test if npSTAT5 is the major factor that contributes to survival decisions we additionally parameterized the contributions to the survival signal of the different pathway species (pEpoR, pJAK2, npSTAT5, CIS and SOCS3) in an additive way (see new Supplementary Material Section 1.1). By comparing the contributions of various signaling components to the anti-apoptosis response, we detect that the npSTAT5 signal contributed more than 99%.
Following the reviewers comments we have clarified the use of the supporting reference Asthagiri, Reinhard et al (2000) in the Results section of the Main Manuscript, page 10:
We focused on the integral response, which is the area under the curve, because this entity captures both the kinetics and magnitude of the signal. It has been demonstrated that processes with slow kinetics (i.e. genetic networks and cell fate decisions) downstream of processes with fast kinetics (i.e. phosphorylation-based signaling networks) act as integrators capable of measuring how long the upstream signal has been on (Behar et al, 2007). Moreover, besides cytokine responses, in MAPK signaling the integral response of a transcription factor was used previously to link ERK activity and DNA synthesis (Asthagiri et al, 2000).

F) Minor points:
1) The time order of the gel loading in Figure S2 is puzzling: please justify this experimental idiosyncrasy.

We apologize for not sufficiently explaining our immunoblotting procedures.
We have previously demonstrated (Schilling et al. (2005) FEBS J 272, 6400-6411; Schilling et al. (2005) IEE Proc Syst Biol 152, 193-200) that in immunoblotting, neighboring lanes show correlated errors due to inhomogenities in the polyacrylamide gel and the transfer to the membrane. This can be corrected by loading the samples not in chronological, but in a randomized order on the gel. These strategies, including spline-based data normalization using normalizers (housekeeping proteins) and calibrators (recombinant proteins to correct for errors in immunoprecipitation) have successfully been applied recently (c.f. Schilling et al. (2009) Mol Syst Biol 5, 334; Becker et al. (2010). Science 328, 1404-1408; Raia et al. (2011) Cancer Res 71, 693-704).
We added an additional explanation to the legend of Figure S2, page 4, Supplementary Material:
To prevent correlated errors in neighboring lanes due to inhomogenities in the polyacrylamide gel and the transfer to the membrane, samples were loaded in a randomized order on the gel (Schilling et al., 2005).

2) The calibrated measurements for the expression levels of signaling components (as presented in Table S1) are of general relevance for the systems biology community. Please make sure to deposit these numbers in a repository (e.g. http://bionumbers.hms.harvard.edu).

We are aware of this database and we will deposit our numbers in a repository as soon as the manuscript is being published.

3) On page 8, the authors state "27 parameters describing 30 reactions and 84 nuisance parameters,
which is a reasonable number (...)". Please specify what "reasonable" means.

We apologize for the imprecise formulation. The mentioned sentence was changed to: Finally, the model comprised 29 parameters describing 30 reactions and 86 nuisance parameters, such as scaling and offset parameters of the experimental data. Considering the total of 541 experimental data points the estimation uncertainties of the model parameters are small enough to allow for accurate model predictions (see Supplementary Material Section2.5).

References:
Alexander, W. S. and D. J. Hilton (2004). "The role of suppressors of cytokine signaling (SOCS)

proteins in regulation of the immune response." Annu Rev Immunol 22: 503-29.
Asthagiri, A. R., C. A. Reinhart, et al. (2000). "The role of transient ERK2 signals in fibronectin- and insulin-mediated DNA synthesis." J Cell Sci 113 Pt 24: 4499-510.
Janes, K. A., J. G. Albeck, et al. (2005). "A systems model of signaling identifies a molecular basis set for cytokine-induced apoptosis." Science 310(5754): 1646-53.
Silva, M., D. Grillot, et al. (1996). "Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-XL and Bcl-2." Blood 88(5): 1576-82.
Spencer, S. L., S. Gaudet, et al. (2009). "Non-genetic origins of cell-to-cell variability in TRAIL-induced apoptosis." Nature 459(7245): 428-32.

Reviewer #3 (Remarks to the Author):
Bachmann et al. examine the dynamics of Jak2/Stat5 signaling in erythroid progenitor cells in response to varying concentrations of the hormone erythropoietin (Epo), with particular focus on the roles of the transcriptional negative feedback mediators CIS and SOCS3 on pathway dynamics. The authors parameterize a mathematical model of the pathway using original datasets collected in wildtype cells and cells overexpressing CIS, SOCS3 and SHP1. Subsequent analysis with the model reveals that cell survival correlates with the level of nuclear pSTAT5 integrated over the first 45 mins.
Further, negative feedback due to CIS and SOCS3 are shown to have prominent effects on the dynamics of STAT5 activation at low and high doses of Epo respectively, thereby pointing to the complementary nature of these proteins in controlling STAT5 dynamics over the wide range of hormone concentrations that would be encountered in vivo.
The authors demonstrate impressive rigor in collecting the appropriate quantitative datasets, designing a parsimonious mathematical model that can be informed by the data, and analyzing the uncertainty in their parameter estimates. As such, this is a well written manuscript that reports results obtained from a well designed study, and should be of interest to the readers of the journal.
My concern is that the impact of the main conclusion - that CIS and SOCS3 modulate STAT5 dynamics at different agonist concentrations - is somewhat unclear. This conclusion is a satisfying one from the perspective of a modeler who is interested in understanding the operating principles of a signaling circuit. However, it is unclear whether the subtle changes in signaling caused by CIS at low Epo concentrations, and the changes to steady-state signaling effected by SOCS3 at high Epo concentrations are physiologically relevant. The authors show that the nuclear pSTAT5 levels integrated over the first 45 mins correlate with an outcome (survival), but then choose to focus on the steady-state signaling level at 6 hours when they analyze the potential roles of CIS- and SOCS3-mediated feedback. A more thorough discussion of the effects observed in Fig. 6A in the context of erythroid biology would strengthen the manuscript.

We thank the reviewer for this critical comment. We have now rephrased the discussion concerning this issue to better explain the role of CIS and SOCS3 in erythroid biology. We included the following section, page 17/18:
Our findings raise the question why it is important for the cell to tightly control the long-term steady-state signaling level of STAT5 by transcriptional feedback regulators over the entire range of high and low Epo doses although the first hour of STAT5 activation is predictive for the survival decision. We hypothesize that when the decision for survival has occurred, it is essential to constrain signaling to a residual steady state level in order to prevent aberrant events that could lead to uncontrolled erythroid progenitor growth. Constitutive phosphorylation of the JAK2/STAT5 pathway caused by activating JAK2 mutations plays a crucial role in the onset of polycythemia vera (PV), a disease that is characterized by the formation of endogenous colonies with Epo-independent differentiation (Kota et al, 2008; Prchal et al, 1974; Weinberg et al, 1989). Moreover, human erythroid progenitor cells transduced with a constitutive phosphorylated form of STAT5 were reported to survive, proliferate and differentiate in the absence of Epo and in this way mimic the PV phenotype.
The essential requirement for progenitor cells to tightly constrict the Epo input signal after 1 hour of stimulation over the broad range of physiological Epo concentrations that can vary over 1000-fold is already apparent at the upstream receptor level. Different studies have shown that activation of EpoR and JAK2 is rapidly terminated within the first hour by dephosphorylation as well as internalization and degradation of Epo (Becker et al, 2010; Gross et al, 2006).
We propose that the transcriptional feedback proteins CIS and SOCS3 are required to tightly

adjust the phosphorylation level of STAT5 after 1 hour of stimulation. This hypothesis is supported by reports demonstrating the crucial role of SOCS3 in embryonic development. Mice lacking the SOCS3 gene exhibit embryonic lethality at days E12-16. Marine et al (1999) showed that these mice display erythrocytosis with dramatic expansion of erythropoiesis within the fetal liver as well as throughout the embryo. Roberts et al (2001) demonstrated that the death is associated with abnormalities in the placenta. Vice versa, enforced expression of SOCS3 in vivo specifically suppressed fetal liver erythropoiesis (Marine et al, 1999). Moreover, a loss-of-function mutation of SOCS3 has been proposed to contribute to the onset of myeloproliferative disease in polycythemia vera patients (Suessmuth et al, 2009). In cancer, high input doses are mimicked by aberrant activation of JAKs that are frequently mutated in many tumor cells. Interestingly, there are numerous recent studies showing that in several malignant tumors JAK activating mutations are complemented by gene silencing of SOCS3 and SOCS1, the two SOCS members which contain a KIR domain (Chim et al, 2004; He et al, 2003; Johan et al, 2005; Jost et al, 2007). Thus, the absence of SOCS3 severely impacts the growth and survival of erythroid progenitor cells and is essential to abrogate signaling downstream of the EpoR at long-term steady-state phosphorylation levels upon high input conditions.
In contrast to SOCS3, our model predicts that CIS acts most efficiently at a single point of the network at low ligand concentrations. This is line with other reports that demonstrated the major role of CIS as a specific competitive binding inhibitor of STAT5 at the pY401 position of the EpoR (Ketteler et al, 2002; Matsumoto et al, 1997; Verdier et al, 1998). The specific inhibition of STAT5-mediated responses by CIS is also supported by transgenic mice that overexpress CIS. These animals display diminished expression of STAT5-mediated responses in growth hormone (GH) and prolactin (PRL) signaling, similar to STAT5 N/ N knockout mice (Matsumoto et al, 1999). In contrast to SOCS3 knockout mice, CIS knockout mice are viable, but show an increase in hematopoietic progenitor cells (Kubo et al, 2003), which is in line with our model prediction of CIS as modulator of fine-tuned pSTAT5 responses at basal level Epo input.

Minor comments:
It is difficult to compare the results across different figures due to differences in the units for agonist dose. The authors should provide the Epo concentration in units/cell for all figures to enable comparison. On a related note why does 0.05 U/ml in Fig. 5A elicit a response (peak nuclear pSTAT5 of ~30nM) comparable to the 4 U/ml dose in Fig. 6A? Is SOCS3 the primary regulator of STAT5 dynamics in both these scenarios?

We apologize for the different notations of the Epo units. We followed the suggestions of the reviewer and provided all the Epo units in units/cell. Please see Figure 5 and Figure 6 and the corresponding legends.

In Figure 5A, SOCS3 overexpression is simulated and in Figure 6A, SOCS3 knockdown is predicted, which both has significant impact on the STAT5 phosphorylation, demonstrating that SOCS is the primary regulator at these concentrations (10-6.78 and 10-6 U/cell, respectively).
Figure 5 legend:
(…) A representative example at Epo = 10-6.78 U/cell is depicted.(…)
Histograms show the representative result of a TUNEL assay with Epo = 10-6.78 U/cell.
Figure 6 legend:
For two exemplary Epo concentrations, (I) Epo = 10-9 U/cell and and (II) Epol= 10-6 U/cell, the time profiles of npSTAT5 are shown.
Main manuscript, Results section, page 11:
A representative example at Epo = 10-6.78 U/cell is displayed (Figure5B), which demonstrates that SOCS3 overexpression affects survival to a larger extent than CIS considering similar overexpression levels
Pg. 8 - Replace "84 nuisance parameters" with something more descriptive explaining that these parameters relate to scaling the model predictions to enable comparison with the data.

We followed the suggestion of the referee and replaced the paragraph with:
"nuisance parameter, such as scaling and offset parameters of the experimental data."

Fig. 3B - The quantitative mass spectrometry results are plotted in an odd fashion with sampling apparently occurring at negative times. What do the individual points represent? Are these biological replicates?

We apologize for confusing labels of the x-axes of this plot. The mass spectrometry data represent indeed four biological replicates of the time points t = 0 min and t = 10 min. We modified the labels in the figure and added an explanation to the legend of Figure 3B:
"Mass spectrometry data represent replicates of 4 independent experiments."

Figure 4 - Is there an explanation for why the model cannot accurately capture the effects of SOCS3 and CIS overexpression on the pEpoR levels?

We thank the reviewer for pointing out this discrepancy between model and experimental data. Mathematical modeling of biological systems always comprises a trade-off between accuracy in describing the experimental data and model complexity that may prevent identifiability of parameters and thereby the predictive power of the model. Based on the reviewerís comments we have therefore explored if we can increase the complexity of our model in a way that would improve the goodness-of-fit while still keeping the relevant model parameters identifiable. To this aim, we have now revisited the model assumptions to analyze why the model could not explain the reduction of EpoR phosphorylation in the CIS overexpression experiment. We concluded that our model structure was not sufficiently detailed to explain the behavior of the pEpoR data in this particular experiment. Therefore, we refined our modeling of the EpoR level by considering that the EpoR harbors eight different tyrosine sites that can be phosphorylated. In the previous version of the manuscript, we summarized these sites with the variable pEpoR. However, as the reviewer correctly pointed out, this simplification renders the model unable to describe the receptor phosphorylation in the case of CIS overexpression.
It has been previously shown that CIS-GFP can bind unspecifically to the EpoR lacking the STAT5 recruitment site Y401 if expressed in high amounts (Ketteler et al, 2003). To include this information in our new model, different subclasses of phosphorylated tyrosines at the receptor had to be considered: (i) The phosphorylated tyrosines sites Y343 and Y401 that facilitate recruitment of STAT5. These residues are represented in our new model by the variable p1_EpoR. CIS inhibits recruitment of STAT5 to these sites. (ii) The remaining phosphorylated tyrosines sites of the EpoR that do not recruit STAT5. These residues are represented in our new model by the variable p2_EpoR and phosphorylation of these residues is inhibited by high levels of CIS.
Additionally, we repeated the experiment for CIS overexpression and measured two time points in the peak response for the EpoR phosphorylation. The result was consistent with our previous experiment, demonstrating a reduction of pEpoR in case of CIS overexpression for early time points. The new data set and the corresponding model trajectory is depicted in Figure S17 and the new data points of pEpoR in case of CIS overexpression have been added to Figure 4.
After adapting our mathematical model, we re-estimated the model parameters including the new data set and repeated all subsequent analyses. We observed a significantly better fit of the pEpoR data in the CIS overexpression time course and also a slightly improved fit of the SOCS3 overexpression experiment. All other fits were not affected significantly. Importantly, the major conclusions of our manuscript remained unchanged. Due to the additional data set even the certainty in the prediction could be increased and all the relevant parameters remained identifiable.
The details of the modified model are now included in the Supplementary Model results, page 15-17 (Section 2.2 Definition of dual negative feedback model of JAK2/STAT5 signaling).
It has been previously shown that CIS-GFP can bind unspecifically to the EpoR lacking the STAT5 recruitment site Y401 if expressed in high amounts (Ketteler et al, 2003). To include this information in the model, two subclasses of phosphorylated tyrosines at the receptor were incorporated into the model: (i) p1_EpoR_pJAK2. The phosphorylated tyrosine sites Y343 and Y401 that serve as recruitment sites for STAT5 and thereby facilitate STAT5 phosphorylation. CIS inhibits phosphorylation of STAT5 induced by these sites. (ii) p2_EpoR_pJAK2. The remaining phosphorylated tyrosines sites of the EpoR that do not recruit STAT5. Phosphorylation of these sites can be inhibited by high levels of CIS due to unspecific binding of CIS to the EpoR (EpoRJAK2_CIS). p12_EpoR_JAK2 represents the completely phosphorylated complex.
We also modified Figure 2 to indicate that the EpoR is phosphorylated on different tyrosine

residues. We added to Figure legend 2:
The pEpoRpJAK2 complex was modeled by considering different subclasses of phosphorylated tyrosines at the receptor (see Supplementary section 2.2).

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Acceptance letter 10 June 2011

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.

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

Sincerely,

Editor
Molecular Systems Biology

REFEREE REPORTS:

Reviewer #2 (Remarks to the Author):

The authors made a significant effort to address the concerns raised in the first round of review. Corrections to the model (to match the effect of CIS upregulation on EPOr phosphorylation) are satisfactory. Correlations to more functional readout (e.g. Pim-1) appear significant and should be documented quantitatively in the final version.

Although some issues remain unresolved (namely the origin of bi-stability in STAT5 phosphorylation and digitalness in apoptosis response), they should be subject to future studies.

This paper is significant in its quantitative breath and functional insight. It will raise the bar for

future Systems Biology studies of signal transduction. I strongly recommend that this paper get published in MSB in its current form.

Reviewer #3 (Remarks to the Author):

In response to the reviews the authors have: 1) refined their mathematical model to address previous discrepancies between model predictions and the experimental data, 2) performed statistical analysis to objectively demonstrate that npStat5 levels are the best predictor for cell survival, 3) clarified the biological relevance of their findings and 4) identified a potential intermediate factor between Stat5 activation and the phenotypic response. In so doing they have satisfactorily addressed my concerns, as well as - in my opinion - the concerns of the other reviewers. I believe that the manuscript is acceptable for publication in its current form.

