molecular systems biology

Network quantification of EGFR signaling unveils potential for targeted combination therapy

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1st Editorial Decision 04 April 2013

Thank you again for submitting your work to Molecular Systems Biology. First of all, I would like to greatly apologize for the delay in getting back to you. We have now finally heard back from the three referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. They raise, however, substantial concerns on your work, which, I am afraid to say, preclude its publication in its present form.

Without repeating all the points listed by all the referees, referee #2 raises two important concerns:
- the validity of the steady state assumption should be convincingly supported.
- the robustness of the results remains unclear in view of the use of "single best-fit parameter sets" instead a more complete description of the distribution and correlation structure of the parameters.

We would also kindly ask you to include the dataset corresponding to the systematic perturbations as 'dataset' in supplementary information as well as the 'source data files' for figure panels displaying quantitative data (see guidelines at <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.

REFEREE REPORTS

Reviewer #1 (Remarks to the Author):

The manuscript by Klinger et al reports an integrated study of colon cancer combining mathematical modeling, cell/molecular biology and in vivo mouse tumor work, the combination of which is quite novel. First, the authors collected a significant amount of signaling data from a panel of colon carcinoma cell lines, focusing on several known aberrant pathways in colon cancer. It is noteworthy that they looked at a panel as opposed to one cell line as has been common in similar studies. To comprehend this signaling data they use an interesting modeling approach based on modular response analysis. This seems to be a reasonable compromise between fully mechanistic models, which have direct physical interpretation but whose parameters can be difficult to precisely estimate, and logical models, which are easy to build and simulate but can lack quantitative rigor particularly in situations when there are feedforward and feedback loops and/or crosstalk. The methods they used to arrive at a final modular response analysis model also extend the current theory in a meaningful way, allowing one to find identifiable combinations of response coefficients from perturbation data sets that are common and quite straightforward to generate experimentally. There are however questions remaining as to how unique the final reported modular response models are: if one repeats the estimation procedure does one arrive at the same solution each time? Or can a family of such models describe the same data set? In moving forward, the author's model makes an interesting prediction, that Akt activity can be controlled by ERK, through a negative feedback to the EGF receptor. Extensive experimental data presented by the authors are consistent with this modeling-based prediction. Impressively, this prediction is shown to have in vivo therapeutic relevance, where dual blockade of ERK and EGFR lead to greater reduction in tumor mass than either alone, despite the fact that an activating Ras mutation is present in the xenograft model. This can have clinical ramifications because the status quo is that EGFR inhibitors do no good when Ras mutations are present, but this work suggests they can indeed do good if combined with inhibition of the ERK pathway. I therefore can recommend this manuscript for publication in Molecular Systems Biology, albeit with attention to a few minor points:

1. As touched upon above, it was unclear from the analysis presented whether the author's modeling procedure yields unique models, or if the estimation procedure is repeated several times, different models would arise (although each having "identifiable" parameters in the linear independence sense the authors refer to in the supplement). This is particularly unclear because there are stochastic elements in the modeling protocol.

2. On page 9 with the Fig. 5 experiments, the authors switch to EGF, when they were working with TGFa before to build the computational model. While these two ligands have some similar effects, EGF preferentially leads to receptor degradation, whereas TGFa preferentially leads to receptor recycling. This might have effects on the proposed negative feedback from ERK to EGFR, and it would be of interest to discuss why a model built with TGFa data can predict all the EGF responses. Is that to say receptor degradation doesn't play much of a role in the investigated phenomena?

3. In discussing the ERK feedback to the EGF receptor (pg. 13), the authors seem unaware of previous mechanistic modeling work that considers exactly this feedback in the context of the ErbB receptor signaling network (Birtwistle et al., Mol Sys Biol, 2007 and references therein). Model-based analysis suggests this feedback can have both positive and negative effects on ERK and Akt signaling, depending on the time after stimulation and on the expression levels of other ErbB receptors such as ErbB2. So one might need caution in extending the current results in colon cancer to other types of cancers. Moreover, ERK can directly phosphorylate the EGF receptor on T669, so it would be interesting to mutate this site and see if it mediates the reported feedback, or if the discovered feedback is a novel mechanism.

Reviewer #2 (Remarks to the Author):

This manuscript applies a previously published method for modeling signaling networks to studies of drug combinations. The problem posed is obviously of importance to the field of systems biology as one in which integrative systems analysis combining quantitative experiment with computational

modeling should be helpful. I hold concerns about the validity of the methodology, in two ways. First, if my understanding is correct it is based on perturbations from zero-gradient dynamics; this should be proper when the zero-gradient profiles are truly steady-states but not when they involve transient peaks. Since signaling networks are rarely at true steady-state, it is not clear how the fundamental premise of the methodology can be relevant. Second, it appears that the authors constrain their models with a goal of gaining a uniquely identifiable single best-fit parameter description. This is also artifactual and unlikely to provide robust results.

Reviewer #3 (Remarks to the Author):

This manuscript describes an approach to predict most suitable inhibitor combinations by modular response analysis. A panel of six colon cancer cell lines were genotyped by targeted sequencing. Then, these cells were stimulated with TGFalpha or IGF in combination with small molecule inhibitors against MEK, IKK, GSK3 and PI3K and a panel of eight phosphoproteins were measured using a Luminex-assay. Mathematical modeling was performed by modular response analysis. By a combination of identifiability analysis and parameter estimation, presence as well as strength of the connecting edges could be determined in a cell line-specific manner. Model analysis and further experiments revealed an unexpected side effect of the IKK inhibitor that was used. Furthermore, a negative feedback link from activated ERK to the EGFR was predicted by the model. As a consequence of this feedback, inhibition of MEK leads to increased phosphorylation of AKT. To circumvent this, the model predicted combined inhibition of RAF/MEK and the EGFR to inhibit cell proliferation. This combination was experimentally confirmed in cell culture and a xenograft model. Quantification of information flow in a network is highly needed, especially in cancer-related pathways such as the EGFR. Combinatorial therapy could be crucial to prevent escape mechanisms of cancer cells. The mathematical approach (modular response analysis) selected is appropriate for this specific question and has been carefully applied.

Major comments:

1) This manuscript nicely shows how an unwanted side effect of an inhibitor can be detected. Treatment of cells with the IKK inhibitor BMS345541 elevated phospho-ERK levels, while other, more specific IKK inhibitors did not. Furthermore, while this wrongly elevated phospho-ERK could increase phosphorylation of p70RSK, phospho-ERK target genes were even decreased. Thus, BMS345541 not only resulted in an artifact by requiring a connection between IKK and ERK, it also decoupled phospho-ERK from its biological function. As ERK is one of the central hubs in the network, this data is not suitable for model inference. There are two possibilities to correct for this artifact. The best way would be to repeat the IKK inhibitor experiments with one of the two specific inhibitors tested in Fig. 3C. If this is not possible, the data points corresponding to treatment with BMS345541 should be removed from the data set and model calibration should be repeated.

2) The authors used targeted sequencing of a panel of six colon cancer cell lines and could verify that these cell lines carry mutations found in tumor patients. Disappointingly, this very important data set could not be linked to the signaling wiring predicted by the mathematical model. For example, the negative feedback from ERK to the EGFR was reported in all cell lines tested, with no quantitative correlation to mutations in K-RAS or B-RAF. For targeted therapy, it would be crucial to be able to predict signaling wiring based on genomic data. Some discussion on this point would be helpful to guide future research.

3) The authors show that the model could predict a negative feedback from ERK to EGFR affecting AKT. We suggest considering also an alternative possibility of interaction between ERK and AKT since it has been published that a negative feedback occurs via Gab1 phosphorylation by ERK (Cheng Fang Yu, Zhen-Xiang Liu, and Lloyd G Cantley, J Biol Chem, 277(22):19382-8, May 2002).

4) The handling of experimental errors and data reproducibility throughout the manuscript requires clarification:
Fig. 2B: Luminex-based data are displayed as a heat map (log2 fold change). Are these single measurements or an average of biological replicates? Has this experiment been repeated? It is of course not possible to show error bars on a heat map, but the reproducibility of the data should be

demonstrated in the supplement (similar to Fig. 5A,C).
This is especially important because parameter estimation is based on a maximum likelihood approach resulting in an Chi-2 value (Fig. 3B and formula 18 in the supplement). For comparing model to experimental data, the error of the data has to be known.
Fig. 3C: Data is displayed as single dots without error bars, however, the legend reads "Luminex, n=3". Is this the mean value that is displayed? What does "n=3" stand for, biological or technical replicates?
Fig. 3D,E: Here, data points are displayed with error bars, but the legend reads "Western blot, n=2, qrt-PCR, n=2). What do the error bars indicate?

5) In the supplement, the modeling technique is nicely explained in principle. However, it is unclear what has exactly been done in this specific project. For example, how did the literature-derived starting network look like? Is it the network depicted in Fig. 2A? If yes, the elimination of RAS and the combination of C-RAF and B-RAF has not been discussed. Was this based on the automatic elimination of non-identifiable nodes? It would be helpful to re-display Fig. 3A in the supplement with the actual network topologies at each step (at least for on exemplary cell line).

Minor comments:

1) Fig. S2: In this experiment, cells were treated with EGF for 10 min (instead of TGFalpha for 20 min). The authors state that "the signal was adjusted to the new stimulus". How has this been done? Have all measurements been repeated, or just phospho-AKT as shown in Fig. 6B? If there are additional measurement, they should be displayed. If not, it should be clearly stated how the model was adjusted.

2) Fig. 4A: It would be helpful to explain filled (experiment) and open (model) triangles in a figure legend. Similarly, in Fig. 4B, it is slightly confusing that open and closed circles depict present and absent edges, respectively. Maybe it would be clearer to show symbols for present edges only.

1st Revision - authors' response 05 April 2013

Response to Reviewer #1 (Remarks to the Author):

The manuscript by Klinger et al reports an integrated study of colon cancer combining mathematical modeling, cell/molecular biology and in vivo mouse tumor work, the combination of which is quite novel. First, the authors collected a significant amount of signaling data from a panel of colon carcinoma cell lines, focusing on several known aberrant pathways in colon cancer. It is noteworthy that they looked at a panel as opposed to one cell line as has been common in similar studies. To comprehend this signaling data they use an interesting modeling approach based on modular response analysis. This seems to be a reasonable compromise between fully mechanistic models, which have direct physical interpretation but whose parameters can be difficult to precisely estimate, and logical models, which are easy to build and simulate but can lack quantitative rigor particularly in situations when there are feedforward and feedback loops and/or crosstalk. The methods they used to arrive at a
final modular response analysis model also extend the current theory in a meaningful way, allowing one to find identifiable combinations of response coefficients from perturbation data sets that are common and quite straightforward to generate experimentally. There are however questions remaining as to how unique the final reported modular response models are: if one repeats the estimation procedure does one arrive at the same solution each time? Or can a family of such models describe the same data set? In moving forward, the author's model makes an interesting prediction, that Akt activity can be controlled by ERK, through a negative feedback to the EGF receptor. Extensive experimental data presented by the authors are consistent with this modeling-based prediction. Impressively, this

prediction is shown to have in vivo therapeutic relevance, where dual blockade of ERK and EGFR lead to greater reduction in tumor mass than either alone, despite the fact that an activating Ras
mutation is present in the xenograft model. This can have clinical ramifications because the status quo is that EGFR inhibitors do no good when Ras mutations are present, but this work suggests they can indeed do good if combined with inhibition of the ERK pathway. I therefore can recommend this manuscript for publication in Molecular Systems Biology, albeit with attention to a few minor points.

1. *As touched upon above, it was unclear from the analysis presented whether the author's modeling procedure yields unique models, or if the estimation procedure is repeated several times, different models would arise (although each having "identifiable" parameters in the linear independence sense the authors refer to in the supplement). This is particularly unclear because there are stochastic elements in the modeling protocol.*

We agree that due to stochastic initial parameter sampling before optimization, each run could in principle end up with different parameters. We therefore tested our pipeline by running it 100 times (and each time initializing the random number generator with a random number from /dev/random, thus ensuring that they are not identical). For all five models, we never had a different parameter set. Thus, for a given data set, the model parameterization is unique.
To further investigate the robustness of the parameters, we applied our algorithm on data after random addition of gaussian noise. We observed that low confidence correlated with removed links and parameter distribution was mostly narrow. (see also Response to Referee 2).
We discuss both points in the manuscript and added the parameter distributions to the supplement .

Main additions to the manuscript in response to this comment:

Methods (page 18): “We verified that this initial parameter scan results in unique parameters since running the procedure 100 times on the same data, each time initializing the random number generator with a different number resulted in identical parameter sets.”

Results (Page 9): “By running the procedure on the data with simulated noise added, we confirm that the procedure robustly identifies the parameters (see Suppl. Fig. S4). In addition, we confirmed by 100 runs of our algorithm that for each data set the model structure and parameterization was identical, irrespective of initial parameterization.”

2. *On page 9 with the Fig. 5 experiments, the authors switch to EGF, when they were working with TGFa before to build the computational model. While these two ligands have some similar effects, EGF preferentially leads to receptor degradation, whereas TGFa preferentially leads to receptor recycling. This might have effects on the proposed negative feedback from ERK to EGFR, and it would be of interest to discuss why a model built with TGFa data can predict all the EGF responses. Is that to say receptor degradation doesn't play much of a role in the investigated phenomena?*

In the validation experiments, we switched to EGF, as EGF is a slightly more potent activator of EGFR and to show that the effect is ligand independent. Furthermore, we also used an earlier time point (10min) to have stronger fold-changes by the ligand (according to the time series data in S1). We think that the experiments cannot fully exclude that recycling/degradation is not important for the effect; therefore we rather would like to avoid this interpretation. We now repeated the validation experiments also with TGFa, and added these to Fig. 5 and 6. They show exactly the same behavior.

Main changes in the Manuscript:

Added data for TGFa in Figure 5A and 6A, removed EGF data from Figure 6A and changed the text accordingly.

3. *In discussing the ERK feedback to the EGF receptor (pg. 13), the authors seem unaware of previous mechanistic modeling work that considers exactly this feedback in the context of the ErbB receptor signaling network (Birtwistle et al., Mol Sys Biol, 2007 and references therein). Model-based analysis suggests this feedback can have both positive and negative effects on ERK and Akt signaling, depending on the time after stimulation and on the expression levels of other ErbB receptors such as ErbB2. So one might need caution in extending the current results in colon cancer to other types of cancers. Moreover, ERK can directly phosphorylate the EGF receptor on T669, so it would be interesting to mutate this site and see if it mediates the reported feedback, or if the discovered feedback is a novel mechanism.*

Thank you for pointing this out, we added this reference to the manuscript. We think that the initial, transient effects may not be important for drug treatment, and thus concentrate on the later time points (30 min like in Birtwistle et al), at which, in agreement with Birtwistle et al, the feedback is negative. In response to this and other comments we thus also used the 30min in Fig. 5A and 6A. We fully agree that the EGFR directed feedback may have different roles in other cell types, and that the cross-talk might be limited to colon cancer, whereas in other cell types with different levels of the other receptors may show even positive cross-talk. In colon cancer, we think that there is a contribution of multiple feedbacks, involving direct regulation, the regulation of CDC25, as suggested by the Bernards' lab for colon cancer and potentially also indirect effects on certain intermediates. To get a more comprehensive picture of which sides are important, we are currently screening for feedback phosphorylation sites, but that screen is not mature enough to say something definite in near future.

Main addition to the Manuscript:

Discussion (Page 15): "Phosphorylation of T669 by ERK affects EGFR turnover (Birtwistle *et al*, 2007)."
Discussion (Page 16): "It is likely that in other cell types different combinations of drugs may be more successful, as the role of specific feedbacks can be different in different cell types, and can even switch between positive and negative effects depending on receptor expression (Birtwistle *et al*, 2007)."

Response to Reviewer #2

This manuscript applies a previously published method for modeling signaling networks to studies of drug combinations. The problem posed is obviously of importance to the field of systems biology as one in which integrative systems analysis combining quantitative experiment with computational modeling should be helpful. I hold concerns about the validity of the methodology, in two ways. First, if my understanding is correct it is based on perturbations from zero-gradient dynamics; this should be proper when the zero-gradient profiles are truly steady-states but not when they involve transient peaks. Since signaling networks are rarely at true steady-state, it is not clear how the fundamental premise of the methodology can be relevant.

The referee is right that the method is derived by assuming steady state of an ODE and linearization around that steady state. Deviations from steady-state do however not necessarily invalidate the modeling approach as such, however it will be a more phenomenological model and will hamper primarily the interpretation: the parameters, the local response coefficients, would no longer be interpretable as normalized elements of the Jacobian matrix. For example, the same equations arise when using e.g. partial correlation or a maximum entropy approach to reduce transitivity in correlation between variables (see e.g. Hopf et al, Cell 2012, 149:1607-21). When lifting the interpretation of the response coefficients as entries of the Jacobian matrix, the main assumption that has to be fulfilled is that the effects between nodes are multiplicative along paths in the graph.

However, as we were clearly interested also in the interpretation of the coefficients, we investigated experimentally if our system is close to a steady state. To determine an optimal time point, we conducted time-series experiments before our screen and chose a time point after initial transient

dynamics (90 min after inhibitor treatment, and 30 min after stimulus). Thus we believe that the experimental system is close enough to steady state such that we can interpret the parameters as response coefficient.

In line with the comment of this referee we agree that the 10 min time point (figure 5A and 6A) that we chose in some of the validation experiments was clearly not a good choice, and thus we repeated these experiments for a 30 min time point, and changed the manuscript accordingly.

In the revised version of the manuscript, we discuss this issue and show the time series that we used to determine the time points in Fig S1.

Main changes to the manuscript:

Figure 5A,6A: Added data for 30min Timepoint for TGFa, removed EGF predictions (6A) and changed the results section and figure legends accordingly.

Results (Page 6):" As signaling typically displays a strong transient response followed by a long-term plateau, we performed time series experiments to determine optimal time points for the experiments (see supplemental Fig. S1). We find that peak transients are within the first 10 min after stimulation, and the response to TGFa as well as IGF has reached a plateau at 30 min after stimulation. Thus we chose the 30 min time point for further experiments, as the interpretation of modular response analysis requires the signaling network to be approximately in steady-state. "

Discussion (Page 14): Modular response analysis generally requires that the response of the system to perturbations can be modeled using linear equations, and the system is close to steady-state. Thus, the modeling procedure can be used to interpret perturbation screens, but parameters should be interpreted in a phenomenological rather than in a precise mechanistic way. Consequently, the procedure is helpful to interpret perturbation data and generate new hypotheses that can be subsequently tested, such as in a previous study of transient EGF/NGF signaling (Santos *et al*, 2007).

Supplementary Figure S1: Time series, showing that 30 min is an appropriate time point for IGF and TGFa.

> *Second, it appears that the authors constrain their models with a goal of gaining a uniquely identifiable single best-fit parameter description. This is also artifactual and unlikely to provide robust results.*

Maximum-likelihood approaches to estimate parameters such as the one applied in our manuscript are common in the field, and routinely done and accepted in systems biology. We agree with the referee that maximum likelihood approaches are often used when it is not suitable, particulary when there are non-identifiable parameters. To ensure that the parameters are identifiable, we intentionally reduced the parameters to identifiable parameter combinations.
In this revision we confirm systematically that the estimated parameters can be robustly identified. We tested this by simulating data sets by adding Gaussian noise to the original data, and then applied our methodology to each of those data sets. The majority of the parameters varies only slightly, indicating that the model is identifiable and the parameters can be well estimated. We then used these parameter distributions in the revision to generate confidence values for our predictions.

We added the confidence intervals to our prediction, the robustness analysis to the supplementary material, and discuss potential limitations in the discussion.

We would like to stress that our methodology is not intended to generate fully mechanistic models – we see the approach primarily as a pipeline that helps us to interpret screening data from cell line panels in terms of networks, and from there generate hypotheses for further testing. Therefore, our aim is to keep the procedure as simple as possible so that it scales to larger screens. Such models are helpful to generate hypotheses, but these will always require independent biological validation. In our manuscript, the model generated the hypothesis that the feedback generates cross-talk, and that successful therapy requires dual inhibition; our subsequent experiments validate the effect of

feedback and dual inhibitor treatment in cell lines, and in addition our prediction regarding the treatment of tumors is validated biologically in xenograft models.

Main changes to the manuscript:

Results (Page 9): "By running the procedure on the data with simulated noise added, we confirm that the procedure robustly identifies the parameters (see Suppl. Fig. S4). In addition, we confirmed by 100 runs of our algorithm that for each data set the model structure and parameterization was identical, irrespective of initial parameterization."

Discussion (Page 13): "In our study, the majority of parameters could be well estimated from the data. However, if there are strong uncertainties in the parameters, methods like MCMC (Hastings, 1970) or the profile likelihood (Raue *et al*, 2009) method can be readily applied to model parameters or structural uncertainties. "

Methods (page 18): "We verified that this initial parameter scan results in unique parameters since running the procedure 100 times on the same data, each time initializing the random number generator with a different number resulted in identical parameter sets."

Simulations and model fits shown in 6A have error bars corresponding to the parameter distributions obtained from "noised" data sets.

Supplement: Figure S4 (pages 8 and 9), containing the parameter distributions obtained from applying our framework 100 times to "noised" data.

Response to Reviewer #3

This manuscript describes an approach to predict most suitable inhibitor combinations by modular response analysis. A panel of six colon cancer cell lines were genotyped by targeted sequencing. Then, these cells were stimulated with TGFalpha or IGF in combination with small molecule inhibitors against MEK, IKK, GSK3 and PI3K and a panel of eight phosphoproteins were measured using a Luminex-assay. Mathematical modeling was performed by modular response analysis. By a combination of identifiability analysis and parameter estimation, presence as well as strength of the connecting edges could be determined in a cell line-specific manner. Model analysis and further experiments revealed an unexpected side effect of the IKK inhibitor that was used. Furthermore, a negative feedback link from activated ERK to the EGFR was predicted by the model. As a consequence of this feedback, inhibition of MEK leads to increased phosphorylation of AKT. To circumvent this, the model predicted combined inhibition of RAF/MEK and the EGFR to inhibit cell proliferation. This combination was experimentally confirmed in cell culture and a xenograft model.
Quantification of information flow in a network is highly needed, especially in cancer-related pathways such as the EGFR. Combinatorial therapy could be crucial to prevent escape mechanisms of cancer cells. The mathematical approach (modular response analysis) selected is appropriate for this specific question and has been carefully applied.

Major comments:

1) *This manuscript nicely shows how an unwanted side effect of an inhibitor can be detected. Treatment of cells with the IKK inhibitor BMS345541 elevated phospho-ERK levels, while other, more specific IKK inhibitors did not. Furthermore, while this wrongly elevated phospho-ERK could increase phosphorylation of p70RSK, phospho-ERK target genes were even decreased. Thus, BMS345541 not only resulted in an artifact by requiring a connection between IKK and ERK, it also decoupled phospho-ERK from its biological function. As ERK is one of the central hubs in the network, this data is not suitable for model inference. There are two possibilities to correct for this artifact. The best way would be to repeat the IKK inhibitor experiments with one of the two specific inhibitors tested in Fig. 3C. If this is not possible, the data points corresponding to treatment with BMS345541 should be removed from the data set and*

model calibration should be repeated.

We fully agree with the referee, removed BMS345541 from the analysis, and changed the manuscript, and figures 4-6 accordingly.

Main changes to the manuscript:
Changed Figures 4-6 with BMS345541 removed.
Results (Page 7): "…, and consequently we excluded the data of BMS345541 from further analysis."

2) *The authors used targeted sequencing of a panel of six colon cancer cell lines and could verify that these cell lines carry mutations found in tumor patients. Disappointingly, this very important data set could not be linked to the signaling wiring predicted by the mathematical model. For example, the negative feedback from ERK to the EGFR was reported in all cell lines tested, with no quantitative correlation to mutations in K-RAS or B-RAF. For targeted therapy, it would be crucial to be able to predict signaling wiring based on genomic data. Some discussion on this point would be helpful to guide future research.*

The referee is right the removal of the negative feedback to BRAF in HT29 cells is the only change in structure that could be linked to the genotype. In contrast the link from ERK to EGFR exists irrespective of genotype and hampers treatment under all mutation patterns that we investigated. For us this was rather surprising, as we thought that (in line with the paradigm) RAS-mutated cells would be insensitive to EGFR-directed therapy. In contrast, our predictions and experiments show that combinatorial treatment of MEK or RAF inhibitors with EGFR inhibitors should work irrespective of RAF or RAS mutations. This is particularly interesting as e.g. for RAS mutated colon tumors, no appropriate targeted therapy exists so far. Thus, it was essential to generate the genome data to show that such feedbacks exist irrespective of whether RAS or RAF is mutated. To further dissect the role of mutations on network wiring, we predict that we would require much larger cell line panels. We discuss this now in the manuscript.

Main changes to the manuscript:

Disussion (Page 14/15): "We also found qualitative differences between cell lines, such as the loss of the ERK-RAF-Feedback in HT29 cells which can be traced back to the BRAF V600E mutation (Friday *et al*, 2008). Similar studies on larger cell line collectives may unveil further differences in network wiring due to the underlying mutations.
Despite the diversity of mutations in the EGFR signaling network, we found in all five cell lines a conserved strong feedback from ERK to EGFR. ".
Discussion (Page 16): "For RAS-mutated tumors, so far no targeted therapy is avaible (Ward et al, 2012; Baines et al, 2011), and a mutation in RAS precludes EGFR-directed interventions. Our results suggest that RAS-mutated tumor cells can be successfully treated by EGFR inhibibitors if provided together with MEK or RAF inhibitors."

3) *The authors show that the model could predict a negative feedback from ERK to EGFR affecting AKT. We suggest considering also an alternative possibility of interaction between ERK and AKT since it has been published that a negative feedback occurs via Gab1 phosphorylation by ERK (Cheng Fang Yu, Zhen-Xiang Liu, and Lloyd G Cantley, J Biol Chem, 277(22):19382-8, May 2002).*

We thank you for the reference, and added it. As written in response to the first referee, we believe that there are multiple feedbacks targeting the EGFR or some adaptors involved, and we are currently screening for these as our data presented in this manuscript is not sufficient to disentangle the different mechanisms.

Main changes to the manuscript:
Discussion (Page 15): Adaptors shared between receptors, such as SOS (Douville & Downward, 1997; Shankaran & Wiley, 2010) or Gab1 (Yu *et al*, 2002), ….

4) *The handling of experimental errors and data reproducibility throughout the manuscript requires clarification:*
Fig. 2B: Luminex-based data are displayed as a heat map (log2 fold change). Are these single measurements or an average of biological replicates? Has this experiment been repeated? It is of course not possible to show error bars on a heat map, but the reproducibility of the data should be demonstrated in the supplement (similar to Fig. 5A,C).
This is especially important because parameter estimation is based on a maximum likelihood approach resulting in an Chi-2 value (Fig. 3B and formula 18 in the supplement). For comparing model to experimental data, the error of the data has to be known.

Indeed, we performed replicate measurements for several of the conditions, and from that trained an error model. This performs better than estimating the error for individual data points, since (similarly to microarray data), one systematically underestimates the error for half of the measurements. Thus we used an error model with additive and multiplicative noise terms. This is now in detail explained in the supplement. The raw data is also provided as spreadsheets linked to the figures. Depending on the assay, the coefficient of variation usually ranged between 10% and 30%.

Main changes to the Manuscript:
Added raw data
Supplementary text (Page 18): new section explaining the error model

> *Fig. 3C: Data is displayed as single dots without error bars, however, the legend reads "Luminex, n=3". Is this the mean value that is displayed?*
> *What does "n=3" stand for, biological or technical replicates?*

Thank you for pointing out our mistake. For Fig. 3C each time point is a single data points. We have corrected this in the figure legend.

> *Fig. 3D,E: Here, data points are displayed with error bars, but the legend reads "Western blot, n=2, qrt-PCR, n=2). What do the error bars indicate?*

We agree that this was confusing. The experiments were done using technical and biological replicates. The Westernblot has been conducted in two seperate experiments. In the first experiment each data point was measured twice (i.e. two identically treated wells of cells per condition were measured) and in the second experiment three replicates were measured. The qrt-PCR has been similarly conducted in 2 experiments, with 3 replicate wells, each. (See also corresponding raw data). Each displayed data point and standard deviation represented the mean and standard deviation within one experiment, respectively. The line then indicated the mean of the two experiments. To avoid any further confusion and statistical caveats, we decided to remove the error bars and only show the mean of the experiments and rewrote the corresponding figure legend.

5) *In the supplement, the modeling technique is nicely explained in principle. However, it is unclear what has exactly been done in this specific project. For example, how did the literature-derived starting network look like? Is it the network depicted in Fig. 2A? If yes, the elimination of RAS and the combination of C-RAF and B-RAF has not been discussed. Was this based on the automatic elimination of non-identifiable nodes? It would be helpful to re-display Fig. 3A in the supplement with the actual network topologies at each step (at least for on exemplary cell line).*

Since we had no means to directly perturb or measure RAS itself it has been removed - more precisely modularized together with EGFR (and IGFR). Likewise B-RAF and C-RAF have been merged. The modeling procedure would do that automatically, but we removed them manually in the beginning.
The Figure was therefore misleading and we replaced it. We followed your suggestion and added to the supplement a real example (cell line HT29), displaying how the model is reduced along with the identifiable parameter combinations.

Main changes to the Manuscript:
Figures 2 and 4 were changed

New supplementary figure S2 shows network reduction and parameterization.

Minor comments:

1) *Fig. S2: In this experiment, cells were treated with EGF for 10 min (instead of TGFalpha for 20 min). The authors state that "the signal was adjusted to the new stimulus". How has this been done? Have all measurements been repeated, or just phospho-AKT as shown in Fig. 6B? If there are additional measurement, they should be displayed. If not, it should be clearly stated how the model was adjusted.*

Reparameterisation has been done on the measurements on pAKT and pERK for single perturbations (shown in Figure 6A and Supplemental Figure S4 (formerly called S2)). Refitting was conducted as follows: as starting parameters we used the parameters derived from the intial screen model and the newly introduced inhibitors of RAF and EGFR were set to 1. Then we refitted the new data by only allowing the parameter for the inhibitors of RAF, EGFR and MEK, PI3K to be changed. A more detailed explanation has been added to the manuscript and pERK measurements used for the refit were added to the supplement. In addition, we removed the prediction for 10 minutes EGF (as pointed already out to Reviewer 2) and instead conducted the procedure for 30 min TGFa measurements.

Main changes to the manuscript:

Figure 6A: Added data for 30 min TGFa and removed EGF prediction.
Figure S5: display of all fitted data and parameter distribution of the new parameters
The refitting proceudure is now briefly described the methods section, and in detail in the legend of figure S5 which shows the re-fit.

2) *Fig. 4A: It would be helpful to explain filled (experiment) and open (model) triangles in a figure legend. Similarly, in Fig. 4B, it is slightly confusing that open and closed circles depict present and absent edges, respectively. Maybe it would be clearer to show symbols for present edges only.*

Main changes to the manuscript:

We changed Figure 4A and B accordingly.

Acceptance letter 07 May 2013

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.

Reviewer #1 (Remarks to the Author):

The authors have done an excellent job of addressing the original critiques and I recommend publication.

Reviewer #3 (Remarks to the Author):

The raised questions have been addressed and thereby the manuscript has significantly improved.

