The glucose-deprivation network counteracts lapatinib-induced toxicity in resistant Erb B2-positive breast cancer cells

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Transaction Report:

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

1st Editorial Decision 14 October 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, I am afraid to say, preclude its publication in its present form.

Thus, additional analyses and experimentation are required to convincingly demonstrate the specificity (reviewer #1, points 1 & 2) and the robustness (reviewer #2 point 6 and reviewer #3 points 1 & 4) of the observed effects. The contribution of the NetWalk analysis as compared to a classical stat-of-the-art differential gene expression analysis (eg GSEA) should also be clarified. We would also like to refer to our instructions to authors with regard to our policy on data availability (http://www.nature.com/msb/authors). Please note that reference to "data not shown" is in principle not permitted in Molecular Systems Biology.

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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

http://www.nature.com/msb

Referee reports:

Reviewer #1 (Remarks to the Author):

In the manuscript by Komurav et al, the authors develop a lapatinib-resistant cell line by treating SKBR3 cells with sub-lethal doses of lapatinib for a year. Surprisingly they find that in the cell line with acquired resistance to lapatinib, treatment of this cell line with lapatinib abrogates ERK and AKT activation. This is contrast to established models of drug resistance whereby reaction of the AKT pathway is the mechanism of resistance. As a result, these findings could have a substantial biological and perhaps clinical impact. Pathways upregulated in the resistance cells are then identified and validated preliminarily through a network reconstruction algorithm the authors previously published. This is a very interesting paper on an important topic but a few additional controls are needed to more firmly substantiate the conclusions that have been drawn.

Major Comments

1.) To conclude that the SKBR3-R cells exhibit no differences in signaling through the EGFR/ErbB2 pathway (Fig 1D), the authors would need to assess more phosphorylation events. mTOR (e.g. pS6, PS6K, etc), pAKT T308 at a minimum are required. The activity status of downstream transcription factors (e.g. FOXO, JUN, MYC, HIF, etc) would help as well.

2.) Along the same lines, the authors will need to do more characterization in Figure 3 to conclude the resistant cells utilize the biological processes that they are claimed to do from their network analysis (UPR response, ER stress, AMPK activation, etc) Does p38 change? PERK signaling? The same can be said in Fig 4 for autophagy.

3.) As written, it's not clear what the network analysis provides that couldn't be obtained from established gene expression analysis methods. For examples, a comparison to the more commonly used GSEA would be helpful to demonstrate that this network-method gives results that couldn't be obtained from more standard practices.

4.) In figure 4, the authors associate individual nodes with clinical outcomes in HER2+ patients. The point the authors are making would be stronger if the authors correlate the presence of the network with outcome (see Taylor et al Nature Biotechnology 2009). Also a multivariate analysis that corrects for stage, grade, LVI, etc would be better if sufficient sample numbers are available.

Other comments:

1.) the title of the paper on the document is different from the title uploaded to the reviewer. Can the authors clarify this? I'd prefer the title uploaded.

2.) the authors need to present all data that are used the conclusions that are drawn or they need to remove statements that are not supported by data. For example, the authors mention that EGFR and ErbB3 were sequenced and no mutations were found but this is not shown. They also claim that they genotyped the SKBR3 cells and compared it to the Sanger and ATCC databases but these data are not presented.
In the paper titled "Network based analysis uncovers yin-yang of signaling and metabolic networks in acquired resistance" authors identified the pathways responsible for resistance to Lapatinib using network analysis. Methodology to identify is novel and validations are for this are also convincing and also paper is written and explained very well.

I have few concerns in the papers; most of them are related to computational aspects. There are

1) Authors identified genes using NetWalk analysis. If instead of using NetWalk, if genes that are highly differently expressed are selected then what result it will lead to. Will they get the same pathways and genes?
2) NetWalk method even though is published, authors should give a brief description if it in the paper, either in the main or supplementary material.
3) How the cutoff of 1000 and 500 genes are selected? What happens if this list is increased or decreased?
4) There should also be another class of genes, whose profiles are similar in both resistant and sensitive line before treatment but shows the differential expression only after treatment. These genes should be more informative to understand the mechanism of resistance.
5) Authors mentioned increased phosphorylation of TSC2 is found in both resistant and parental cell, but from figure it seems total TSC2 level is same and there seems some difference in p-TSC2 but that not much.
6) Authors did not perform replicates of microarray, so how robust is the result with just one replicate.
7) Is it possible, if the resistant line is grown in high glucose medium, will they become sensitive to Lapatinib again?
8) Why the genes with cox pvalue less than 0.05 are included. Idea behind EF score is that even if expression or cox value of each gene is not high its EF score could be high in the context of network and still shows some significance.
9) How the cox regression coefficients correlate with EF score. If relapse time and expression of gene correlates and if that in turn correlates with EF score then figure 4B is the figure that you should be expecting. Also what genes/pathways you will get if you use top genes from cox regression
10) Could dual treatment with Lapitinib and Pyrvinium will be more effective then treatment with Pyrvinium alone.

Minor Corrections:
1) In Figure 1D genes shown are different from what is mentioned in the text. There is no EGFR and MAPK1,2 in the figure.
2) References of subfigures for figure 3 are wrong.
3) No scale in figure S5
4) Authors should mention which independent dataset for breast cancer they used in figure S6 in the main text.
5) Typing mistake in caption for figure 5D. Same instead of Save

Reviewer #3 (Remarks to the Author):

The authors generated a polyclonal line from the SKBR3 cell line which is resistant to lapatinib. Upon validation of the new line the authors performed a transcriptional network analysis to identify key pathways upregulated in the resistant cells, including glucose deprivation and the unfolded protein response. Following these results and analysis of the connectivity map, the phenotypic response was determined upon glucose deprivation and drug treatment, and the authors observed increased susceptibility.

The network analysis is well done and the problem they aim to address is significant and exciting.
However, mechanistic detail in the phenotypic and metabolic analyses is lacking, which limits my enthusiasm for publication. More specific concerns are listed below.

Major comments.

1. The description of the generation of resistant cells is lacking. Were the control, parental cells maintained in parallel for the yearlong culture or were they cryopreserved. This must be clarified, as extensive passaging in vitro is very selective and can also cause metabolic and phenotypic changes in cells (Berdichevsky M et al. Biotech Prog 2008). The authors show clear modulation of the signaling response to lapatinib, so the cells are still responsive to the drug. Therefore, could their results arise independently of lapatinib treatment?

2. Given the extensive passaging described above, increased glucose consumption and lactate production is not surprising. What about other nutrients? What is the fate of the glucose that is taken up? This question could be addressed in part by calculating the ratio of lactate flux to glucose flux (in moles), but I cannot estimate this from their a.u. data.

3. In general, "no glucose" is not a particularly striking inducer of cell death. Many cancer cells are susceptible to glucose deprivation, and this is exacerbated by transforming events. The BT474 data is confusing, because 1 g/L glucose cannot be considered hypoglycemic - this is the normal concentration of glucose in plasma. Why is a response seen?

4. The drug identifications from the connectivity map are particularly interesting and the response to each drug is well done. However, small molecules often have pleiotropic effects. Given the lack of mechanistic detail in the described phenomena, testing in another "resistant" cell line would be helpful and warranted to strengthen their conclusions.

Alternatively, performing targeted knockdown of proteins along the UPR or autophagy pathway might provide more confirmation.

Minor comments

1. Some results are provided with no real explanation. For example, the authors demonstrate TSC2 phosphorylation is up basally in resistant cells and up in the lapatinib treated parental line. What sort of change would drive this signal being activated? Though very elegant, the transcriptional network analysis cannot explain this directly. Similarly, ROS can be elevated for many different reasons. While these results correlate with the authors' results, no clear mechanism is provided, detracting from the excitement of the manuscript.

Also, there is no description of the ROS measurements (DCFDA concentration, well plate versus flow cytometry), and the changes are very slight.

2. The authors claim that glucose deprivation induces "growth inhibition" of resistant cells. However, cell survival data is shown with no growth. The description of this data should be corrected since there is an important difference between growth, proliferation, and survival.

3. There were some typographical errors in the manuscript. In particular the second sentence of the abstract needs revising.

1st Revision - authors' response 13 January 2012

Response to reviewer comments

Reviewer #1 (Remarks to the Author):

Major Comments

1) To conclude that the SKBR3-R cells exhibit no differences in signaling through the EGFR/ErbB2 pathway (Fig 1D), the authors would need to assess more phosphorylation events. mTOR (e.g. pS6, pS6K, etc), pAKT T308 at a minimum are required. The activity
status of downstream transcription factors (e.g. FOXO, JUN, MYC, HIF, etc) would help as well.

**RESPONSE** - We have performed additional experiments as suggested and have included data from measuring the changes in phosphorylation in AKT, mTOR, S6 kinase, pS6 and pTSC2 on the AKT phosphorylation site and are included in revised Figure 1D. Text addition on page 4 paragraph 2. The resistance cells are very difficult to transfect and thus we were not able to perform the transcriptional reporter assays for FOXO, JUN etc.

2.) **Along the same lines, the authors will need to do more characterization in Figure 3 to conclude the resistant cells utilize the biological processes that they are claimed to do from their network analysis (UPR response, ER stress, AMPK activation, etc) Does p38 change? PERK signaling? The same can be said in Fig 4 for autophagy.**

**RESPONSE** - We have performed additional experiments as suggested and have included data from measuring the changes in p38 and PERK and AMPK activation and included them in Figure 3A. Text addition has been made on page 7 paragraph 1.

3.) **As written, it's not clear what the network analysis provides that couldn't be obtained from established gene expression analysis methods. For examples, a comparison to the more commonly used GSEA would be helpful to demonstrate that this network-method gives results that couldn't be obtained from more standard practices.**

**RESPONSE** – We have performed this analysis as suggested please see output file from GSEA on the lapatinib dataset with 8 conditions and 2 phenotypes. There is only ONE gene set with q < 0.05 showing that our network based method here is very useful in comparing and analyzing data sets. We have added the GSEA report as supplementary figure 5. Text changes have been on page 6 paragraph 1. A more detailed comparative analysis of our NetWalk method as compared to others is found in our PLoS Comp Biol paper 2010 (Komurov, K, White M, and Ram PT).

4.) **In figure 4, the authors associate individual nodes with clinical outcomes in HER2+ patients. The point the authors are making would be stronger if the authors correlate the presence of the network with outcome (see Taylor et al Nature Biotechnology 2009). Also a multivariate analysis that corrects for stage, grade, LVI, etc would be better if sufficient sample numbers are available.**

**RESPONSE** - These networks were constructed using as input into NetWalk the COX regression z-scores of association between gene expression and clinical outcome in the given dataset. So, these networks do represent those that are correlated with clinical outcome. Instead of including in our analyses different clinical parameters as variables, we limited our analysis to patients with high ERBB2 expression (highest 30th percentile) in line with the expected percentage of ERBB2 patients in breast cancers. We believe this analysis design is more relevant to our present study.

Other comments:

1.) **the title of the paper on the document is different from the title uploaded to the reviewer. Can the authors clarify this? I'd prefer the title uploaded.**

**RESPONSE** – The title was changed in consultation and recommendation of the senior editor.

2.) **the authors need to present all data that are used the conclusions that are drawn or they need to remove statements that are not supported by data. For example, the authors mention that EGFR and ErbB3 were sequenced and no mutations were found but this is not shown. They also claim that they genotyped the SKBR3 cells and compared it to the Sanger and ATCC databases but these data are not presented.**

**RESPONSE** - We apologize for not including these data in the initial submission since they were negative but we have included them in the supplementary data in this revised version.

3.) **there are no page numbers on the manuscript.**

**RESPONSE** – Added in the revised manuscript.
4.) the first page contains some typos when referring the figures.
RESPONSE – Corrected in the revised manuscript.

Reviewer #2 (Remarks to the Author):

1) Authors identified genes using NetWalk analysis. If instead of using NetWalk, if genes that are highly differently expressed are selected then what result it will lead to. Will they get the same pathways and genes?
RESPONSE- A clustering heatmap of most differentially expressed genes between the given conditions will generate a heatmap very similar to the one given in Fig.2. There is a large overlap between genes in the corresponding clusters in the EF heatmap in Fig.2 and this gene heatmap. However, interaction networks derived from the gene heatmap are not as clear as in the EF heatmap, due to low level of direct functional associations. We included a sentence in the text describing this and have added the gene heatmap to the supplementary material and our EF heatmap is behaving as expected from the gene heatmap, so the clustering in the EF heatmap is not artificial. This analysis is shown in Supplementary data 3. Edits in text on page 6 paragraph 1.

2) NetWalk method even though is published, authors should give a brief description if it in the paper, either in the main or supplementary material.
RESPONSE – We have added a brief description as requested in the methods section.

3) How the cutoff of 1000 and 500 genes are selected? What happens if this list is increased or decreased?
RESPONSE - EF heatmap using less EF values (480 instead of 1000) gave a very similar profile to that in Fig.2. This is added to the supplementary data. This data is shown in supplementary figure 2. Edits in text on page 5 paragraph 3.

4) There should also be another class of genes, whose profiles are similar in both resistant and sensitive line before treatment but shows the differential expression only after treatment. These genes should be more informative to understand the mechanism of resistance.
RESPONSE - Based on our analysis of most highly differentially expressed genes, major difference between resistant and parental cells in terms of response to lapatinib was in the magnitude of gene expression response, rather than the nature of genes per se. We believe that, since, according to our model, gene expression response to lapatinib in parental cells is primarily driven by the nutrient starvation after lapatinib treatment, EGFR/ERBB2-independent glucose uptake competency in resistant cells dampens this response, rather than alters it, and resistance is due to gene expression alterations prior to, rather than after, lapatinib treatment.

5) Authors mentioned increased phosphorylation of TSC2 is found in both resistant and parental cell, but from figure it seems total TSC2 level is same and there seems some difference in p-TSC2 but that not much.
RESPONSE - We have performed additional experiments and have include them as Figure 3B. We have probed for both the AKT and AMPK phosphorylation sites on TSC2 and have included these data. Edits in text page 7 paragraph 2.

6) Authors did not perform replicates of microarray, so how robust is the result with just one replicate.
RESPONSE - We apologize for not stating this in the methods section – the microarray was done using three independent sets of samples and was in fact repeated two different times with independent triplicate samples each time. In effect we have performed the microarray using 6 independent sample sets. The data shown are mean of three independent repeats. Page 15 paragraph 1.
7) Is it possible, if the resistant line is grown in high glucose medium, will they become sensitive to Lapatinib again?
RESPONSE – We have performed this experiment however we see no change in sensitivity to lapatinib.

8) Why the genes with cox pvalue less than 0.05 are included. Idea behind EF score is that even if expression or cox value of each gene is not high its EF score could be high in the context of network and still shows some significance.
RESPONSE - The network of highest-scoring z-scores are heavily dominated by cell cycle related processes. Due to high interaction density among these genes (cell cycle related complexes), the network of highest EF values from this analysis is also dominated by cell cycle-related genes of lesser significance, masking potentially non cell cycle-associated significant networks. Therefore, in order to reveal these non cell-cycle-related networks, we filtered this network to include only genes with p-values < 0.05.

9) How the cox regression coefficients correlate with EF score. If relapse time and expression of gene correlates and if that in turn correlates with EF score then figure 4B is the figure that you should be expecting. Also what genes/pathways you will get if you use top genes from cox regression
RESPONSE - EF scores are given to interactions, while COX z-scores are given to genes. So we cannot directly correlate the two. However, COX z-scores have a very high correlation with node visitation scores (which are used to calculate EF scores) (r2 = 0.88) Top genes from COX regression are primarily associated with cell cycle progression (cell division, DNA replication, M/G1 transition, S phase, etc…), as expected.

10) Could dual treatment with Lapatinib and Pyrvinium will be more effective then treatment with Pyrvinium alone.
RESPONSE – This is an excellent suggestion which we tried, the combination of lapatinib to pyrvinium did not have any additive or synergistic effect.

Minor Corrections:
1) In Figure 1D genes shown are different from what is mentioned in the text. There is no EGFR and MAPK1,2 in the figure.
RESPONSE – Corrected on page 22
2) References of subfigures for figure 3 are wrong.
RESPONSE – Corrected on page 23 in text
3) No scale in figure S5
RESPONSE - Corrected
4) Authors should mention which independent dataset for breast cancer they used in figure S6 in the main text.
RESPONSE- Corrected on page 18
5) Typing mistake in caption for figure 5D. Same instead of Save
RESPONSE – Corrected on page 24

Reviewer #3 (Remarks to the Author):

Major comments.
1. The description of the generation of resistant cells is lacking. Were the control, parental cells maintained in parallel for the yearlong culture or were they cryopreserved. This must be clarified, as extensive passaging in vitro is very selective and can also cause metabolic and phenotypic changes in cells (Berdichevsky M et al. Biotech Prog 2008). The authors show clear modulation of the signaling response to lapatinib, so the cells are still responsive to the drug. Therefore, could their results arise independently of lapatinib treatment?
RESPONSE – This is an excellent point and in fact the control cells were maintained in parallel for the yearlong culture, and we have clarified this in the text. Page 4 paragraph 1.
2. Given the extensive passaging described above, increased glucose consumption and lactate production is not surprising. What about other nutrients? What is the fate of the glucose that is taken up? This question could be addressed in part by calculating the ratio of lactate flux to glucose flux (in moles), but I cannot estimate this from their a.u. data.  
RESPONSE – The assays as performed do not allow us to determine the flux as pointed out by the reviewer. We are currently performing metabolomic analysis of these cells to determine other nutrients and metabolic pathways but do not yet have the data from this analysis. We have also initiated a collaboration to determine the fate of glucose taken in but these experiments are also ongoing.

3. In general, "no glucose" is not a particularly striking inducer of cell death. Many cancer cells are susceptible to glucose deprivation, and this is exacerbated by transforming events. The BT474 data is confusing, because 1 g/L glucose cannot be considered hypoglycemic - this is the normal concentration of glucose in plasma. Why is a response seen?  
RESPONSE – The normal levels of glucose in culture medium is 2 g/L, but in response to the comment we have repeated the experiment in 0.25 g/L and find that there is a reduction response to lapatinib. This new data is shown as figure 3G.

4. The drug identifications from the connectivity map are particularly interesting and the response to each drug is well done. However, small molecules often have pleiotropic effects. Given the lack of mechanistic detail in the described phenomena, testing in another "resistant" cell line would be helpful and warranted to strengthen their conclusions.  
RESPONSE – We have been unsuccessful in obtaining other lapatinib resistant cells from other investigators, however we have observed very similar results in herceptin resistant BT474 cells. In order to validate our findings beyond the cell line level we used breast cancer patient data and undertook the network analysis from the patient tumor data to determine the clinical implications of the potential resistance mechanisms which we have observed in the cell line model.

Alternatively, performing targeted knockdown of proteins along the UPR or autophagy pathway might provide more confirmation.  
RESPONSE – This is a very good point which we tried. Unfortunately, transfection of the resistant cells proved to be very challenging and we tried over 6 different transfection reagents all which proved to be toxic to the cells and we were not able to obtain any meaningful data. We have included data in the supplementary data with an additional drug Metformin that also targets the UPR transcription program. Supplementary figure 12. Text edits on page 11 paragraph 1.

Minor comments

1. Some results are provided with no real explanation. For example, the authors demonstrate TSC2 phosphorylation is up basally in resistant cells and up in the lapatinib treated parental line. What sort of change would drive this signal being activated? Though very elegant, the transcriptional network analysis cannot explain this directly.  
RESPONSE – We have performed additional experiments probing for both the AMPK and AKT phosphorylation sites on TSC2 and found that the AMPK site is the one that is upregulated in the resistant cells and increase in response to lapatinib, while the AKT site is blocked by lapatinib. These data suggests that one of the downstream effects of lapatinib may be alterations in the homeostasis of bioenergetics/glucose depravation and that the acquired resistant phenotype we have found could be an important target to overcome resistance. Figure 3B and text edits page 7 paragraph 3.

Similarly, ROS can be elevated for many different reasons. While these results correlate with the authors' results, no clear mechanism is provided, detracting from the excitement of the manuscript. Also, there is no description of the ROS measurements (DCFDA concentration, well plate versus flow cytometry), and the changes are very slight.  
RESPONSE - We have decided to remove the ROS data and agree with the reviewer that while it correlates with the results additional work will have to be performed to understand the role of ROS in lapatinib resistance.
2. The authors claim that glucose deprivation induces "growth inhibition" of resistant cells. However, cell survival data is shown with no growth. The description of this data should be corrected since there is an important difference between growth, proliferation, and survival.

RESPONSE – We have corrected the terminology in all the figures and now describe them as change in cell numbers. Figures 1, 3 and 5.

3. There were some typographical errors in the manuscript. In particular the second sentence of the abstract needs revising.

RESPONSE - Corrected

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate your revision. As you will see from the reports below, reviewer #1 and #2 are supportive. Reviewer #3 acknowledges that the manuscript has been significantly improved but still feels that key yet relatively straightforward metabolic measurements (lactate to glucose ratios, accounting for cell number and growth) are missing. Since these data were requested in the initial round already and the experiments required appear to be doable, we would ask you to revise the study accordingly in a second and exceptional round of revision.

*** PLEASE NOTE *** 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

http://www.nature.com/msb

Referee reports

Reviewer #1 (Remarks to the Author):

The authors have satisfactorily addressed most of my concerns. This is an important paper.

Reviewer #2 (Remarks to the Author):

Minor comment
Figure numbers are missing from supplementary figure.
Reviewer #3 (Remarks to the Author):

The revised manuscript has addressed some of the reviewer critiques, and again, I find their results timely, exciting, and important for the community. However, their conclusions are somewhat misleading and the lack of meaningful data on metabolism (after this was specifically requested) is disappointing.

Almost the entirety of their results implicate metabolic processes (e.g. glucose deprivation, gluconeogenesis, autophagy) and yet the only measurements provided are relative levels (arbitrary units) of glucose and lactate data with no accounting for cell growth/death. While detailed metabolomics studies are beyond the scope of this work, the authors can do better with respect to glucose and lactate measurement analysis. Does the ratio of lactate to glucose change in these cells (accounting for cell number and growth)? These measurements are not so difficult to do using the methods they describe - simply determining mol glucose taken up and mol lactate produced using standard curves is required.

At a bare minimum, discussing the metabolic implications of their work and the need for more extensive studies is required. How are these cells growing? They are more sensitive to inhibitors of autophagy and mitochondrial metabolism (see below) so something is going on.

Finally, no citation was included for their claim that metformin targets UPR. At most this results from indirect effects via AMPK. Only few citations are present connecting metformin to UPR. Metformin is thought to inhibit complex I and subsequently activate AMPK. From there many signaling and metabolic processes can be inhibited/activated - how this supports their UPR claim is not clear.

Response to reviewer comments

Reviewer #1 (Remarks to the Author):

The authors have satisfactorily addressed most of my concerns. This is an important paper.

Response – Thank you.

Reviewer #2 (Remarks to the Author):

Minor comment
Figure numbers are missing from supplementary figure.

Response – We have added the missing numbers.

Reviewer #3 (Remarks to the Author):

The revised manuscript has addressed some of the reviewer critiques, and again, I find their results timely, exciting, and important for the community.

Response – Thank you.
However, their conclusions are somewhat misleading and the lack of meaningful data on metabolism (after this was specifically requested) is disappointing. Almost the entirety of their results implicate metabolic processes (e.g. glucose deprivation, gluconeogenesis, autophagy) and yet the only measurements provided are relative levels (arbitrary units) of glucose and lactate data with no accounting for cell growth/death. While detailed metabolomics studies are beyond the scope of this work, the authors can do better with respect to glucose and lactate measurement analysis. Does the ratio of lactate to glucose change in these cells (accounting for cell number and growth)? These measurements are not so difficult to do using the methods they describe - simply determining mol glucose taken up and mol lac produced using standard curves is required. At a bare minimum, discussing the metabolic implications of their work and the need for more extensive studies is required. How are these cells growing? They are more sensitive to inhibitors of autophagy and mitochondrial metabolism (see below) so something is going on.

Response – We thank the reviewer for suggesting this experiment and we have performed the glucose and lactate flux analysis as requested and these data are included as Figures 3 C and 3D. We have also analyzed the lactate/glucose ratio accounting for cell number and growth and this is included in Figure 3 E. The results are described on page 8 paragraph 2. We have also included discussion of the changes in glucose and lactate flux and their ratios and how this relates to lapatinib response on pages 13 and 14. Briefly, the lapatinib resistant cells have a higher lactate/glucose ratio as compared to parental cells suggesting increased glucose metabolism in the resistant cells in line with the changes in gene expression and proteins in the cells. Interestingly in response to lapatinib there is a significant decrease in the lactate/glucose ratio only in the resistant cells suggesting a switch from glycolysis to the pentose phosphate shunt pathway and increased NADPH and ability to overcome reactive oxidative stress and decrease cell death as is functionally observed in the resistant cells but not in the parental cell.

Finally, no citation was included for their claim that metformin targets UPR. At most this results from indirect effects via AMPK. Only few citations are present connecting metformin to UPR. Metformin is thought to inhibit complex I and subsequently activate AMPK. From there many signaling and metabolic processes can be inhibited/activated - how this supports their UPR claim is not clear.

Response - We have added the citation, page 12 paragraph 1.

3rd Editorial Decision 15 May 2012

Thank you again for submitting your work to Molecular Systems Biology. We are now globally satisfied with the modifications made and we will be able to accept your manuscript for publication pending the following minor points:

- please prepare supplementary information according to our guidelines (single PDF, including a Table of Content) provided at http://www.nature.com/msb/authors/index.html#a3.4.6

- Functional genomics datasets need to be deposited in one of the major functional genomics public databases. Please include a sub-section entitle 'Data availability' in the Materials & Method section and list the relevant accession numbers.

- Finally, we would encourage you to upload the 'source data'--i. e. the tables of individual numerical values and measurements--that were used to generate figures 1A-B, 3C-H, 5C,D. These files are separate from the traditional supplementary information files and are submitted using the "figure source data" option in the tracking system. Source data are directly linked to specific figure panels so that interested readers can directly download the associated 'source data' (see, for example, <http://tinyurl.com/365zpej>), for the purpose of alternative visualization, re-analysis or integration with other data. Additional formatting guidelines for 'source data' are available at http://www.nature.com/msb/authors/source-data.pdf.

Thank you for submitting this paper to Molecular Systems Biology.
Yours sincerely,

Editor
Molecular Systems Biology

http://www.nature.com/msb

3rd Revision - authors' response 31 May 2012

Thank you for accepting our manuscript titled "The glucose-deprivation response network counteracts EGFR signaling in lapatinib resistant cells" for publication in Molecular Systems Biology. We have uploaded the required files and better quality figures as requested and have deposited the gene array data at GEO and have provided the accession number in the text.