molecular systems biology

Glutamine-driven oxidative phosphorylation is a major ATP source in transformed mammalian cells in both normoxia and hypoxia.

Jing Fan, Jurre J. Kamphorst, Robin Mathew, Michelle Keyi Chung, Eileen White, Tomer Shlomi, Joshua D. Rabinowitz

Corresponding author: Joshua D. Rabinowitz, Princeton University

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Transaction Report:

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

1st Editorial Decision 19 June 2013

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 manuscript. As you will see from the reports below, the referees raise substantial concerns on your work, which should be convincingly addressed in a revision of the manuscript.

Overall, reviewers #1 and #3 appreciate that the presented results are potentially interesting with regard to cancer metabolism. While some of the reviewers' comments refer to the need to clarify and better document a number of points throughout the manuscript, several major concerns have also been raised. Among the more fundamental issues are the following:
- Reviewer #3 is providing constructive comments and suggestions with respect to additional experimentation required to strengthen the validity of the presented conclusions. As suggested by this reviewer, it is required to examine the potential contribution of other NADH producing reactions associated with glycolysis as well as the differences in cytosolic versus mitochondrial NADH.
- The computational methods employed for the presented analyses need to be described in detail.
- The key novel findings of this work need to be presented/discussed more clearly, as requested by reviewer #2.
- A direct comparison of transformed iBMK cells grown in hypoxic versus normoxic conditions would strengthen the findings regarding the effect of hypoxia in cancer cells.

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.

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Referee reports:

Reviewer #1:

In the study entitled "Glutamine-driven oxidative phosphorylation is a major ATP....." Fan et al. take a systematic approach to model metabolic fluxes in immortalized and oncogene transformed cells to determine how ATP production is balanced by oxphos and aerobic glycolysis. Furthermore, they assess the relative contribution of glucose and glutamine to the metabolism of ibmk cells in normoxic and hypoxic conditions as well as those cells transformed by H-Ras and Akt. They show that the majority of ATP under all conditions, including hypoxia, is produced by oxphos. Additionally, they perform a quantitative analysis and develop redox balanced models of mammalian cellular respiration. This is a very interesting study that adds to our understanding of how normal and tumor cells utilize different carbon sources to fuel oxphos to generate ATP. While, there is limited functional studies in terms of what is critical for growth, this is not the central point of the paper and the metabolomic analyses and modeling are sufficient to stand on their own merits. It will be of interest to the scientific community. I have a few points to improve the paper.
-The authors should mention somewhere in the manuscript (possibly in the discussion) that overexpressing mutant H-ras (only rarely mutated in cancer) or the hyperactivated myr-AKT in mouse immortalized kidney cells are artificial and may not reflect the physiologic situation where Kras or PIK3CA are mutated.
- The finding that cells grown in hypoxia only have a modest reduction in oxphos is an interesting one. I would think that the authors would have compared the transformed IBMK cells (Ras and AKT) grown in hypoxia versus normoxia. This would be quite informative. I realize that they used the tumor cell lines to do such studies, but these are not as genetically defined as the IBMK cells, making direct comparisons more difficult.
-Applying the FBA to the NCI60 using publicly available data to confirm their findings certainly adds generality to the findings. However, some of this analysis should be shown (at least in the supplemental data) and not just mentioned in the text.
-The authors should perform some simple statistical analyses and show this in the figures to determine if many of the changes between samples rise to level of significance.
-Can the authors comment why, while glutamine withdrawal causes a significant decrease oxygen consumption in ASPC1 under normoxic condition while glucose withdrawal does not (fig 4C) but the major contribution to production of high energy electrons is from glucose (Fig 4B) under the same normoxic conditions?
-minor points:
-"In all cases, good agreement with the labeling data was obtained (Supp. Tables 1-4). As a further validation of the inferred fluxes, we measured the kinetic labeling of intracellular metabolites (for 72 hours) as well as their absolute concentrations, showing good agreement between kinetic labeling patterns and simulated patterns (Supp. Tables 1-4)". I believe the authors mean supplementary figures 1-4, not the tables.
-some data is mentioned in the text but there is no reference to specific figures/tables. For example: "The total ATP production rate in the parental iBMK cell-line was found to be 868 nmol/ul cells/h, with the contribution of oxidative phosphorylation and aerobic glycolysis being 80% and 20% respectively."
-fig 4c - the y-axis is unlabeled.
-fig 4b and 4c are not directly referred to in the text. The data is discussed but there is no reference to the figures.

Reviewer #2 (Remarks to the Author):

In the work by Fan et al. the authors carry out a flux analysis on a set of cell lines that differ in their expression of an oncogene (parental, KRAS, and AKT). They carry out flux measured using isotopically labeled nutrients (glucose and glutamine) and integrate these measurements with measurements of oxygen consumption. They use a model to estimate the best fit fluxes for each of the 3 cases and use the results to interpret some features of cancer metabolism. They find that all 3 lines derive substantial amounts of ATP from glutamine oxidation.

Comments

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-The main problem with this article I found is the lack of detail on computational methods. It is thus impossible to evaluate the validity of any of the results reported. There is nothing in this article I could find that points to the algorithms used, equations considered, etc. For example, FBA is mentioned but FBA requires a stoichiometry matrix, vmax constraints, and is very sensitive to boundary effects such as the "media" used. The authors have also previously published a report claiming the requirement of a "solvent capacity" constraint for reasonable FBA results in cancer metabolism (Plos Comp Bio 2011). It is not mentioned if this is used. The flux analysis calculation also requires a stoichiometry matrix and some way of estimating flux. There is no mention of any of this in the paper to my knowledge and not a single equation is mentioned throughout the text. As for the isotopomer modeling, it is not clear what was done. The ODEs used are not mentioned, no code is submitted or SBML file preferably. The error estimation is not clear as well.

- The next major concern is that it's not clear to me what the key results in this paper are. The authors estimate that glutamine has a major contribution to ATP generation but this is already known. Perhaps the key result is that the addition of oncogenes does not appear to change the resulting flux by much. However, this is perhaps to be expected since small changes in metabolism are usually observed when mammalian cells are cultured in artificial tissue culture conditions such as the presence of DMEM and 10% FBS.

Other points -

-The authors use the NCI-60 data from Jain, Science 2012 to match fluxes and compare to their case. As the authors are surely aware, those cells were grown in RPMI while their studies use DMEM. Cells exhibit substantial changes in metabolism depending on growth media used. It's not clear to me whether these results are transferrable.

-On page 4, the authors that NADH is not a major contributor to oxphos evidenced by 88% of it being consumed by pyruvate reduction. I was unclear on what this means. Could the authors elaborate?

- it's not clear in the methods whether dialyzed serum was used in the flux experiment. This will have a profound effect on the results on way or the other?

- from a rough estimate it appears that some of the fluxes in Figure 1 might not be balanced:
e.g.
reported O2 consumption, VO2=220 mmol/l/h. and the total NADH production flux is Vnadh=207, Vfadh2=76.

and:

If ATP production flux in cytosol Vatp(glycolytic)=232 mmol/l/h, Vatp(oxphos)=657.5 and Vatp(glycolytic) so the contribution would be 26%, not 20%
It's not clear if Ac-coA flux is balanced as well.

- I would expect reversibility to have big influences on the results but the reactions are all irreversible.

- Some of the internal references do not seem right. For example, it seems "Supp. Tables 1-4" in the third paragraph of the section "Quantifying ATP production routes via a redox-balanced metabolic flux model" should be "Supp. Figures 1-4"; "Supp. Figure 1" in the second paragraph of the section "Quantifying ATP production routes via a redox-balanced metabolic flux model" is not about "uptake and excretion rates of major nutrients"; "Supp. Table 3" in the first paragraph of the section "Redox-balanced metabolic flux analysis (MFA)" is not about the "metabolic network model of glycolysis and TCA cycle".

- no error bars are presented in supp table 3.

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Reviewer #3:

Understanding metabolic reprogramming in cancer is of particular interest and most metabolic studies to date have focused primarily on how the fate of carbons (or nitrogens) from nutrients into individual pathways changes in tumorigenesis. However, most metabolic reactions are coupled to inter-conversions of small co-factors, such as NAD(P)H, which have critical metabolic roles in their own accord by driving other metabolic reactions. Therefore, understanding how changes in metabolic fluxes support cancer cell proliferation requires studies that extend beyond carbon tracing, and, in particular, aim at elucidating how metabolic pathways maintain cellular redox potential.

In this paper, Fan et al. address this void by combining carbon flux analysis with metabolic modelling, nutrient exchange rates (between the cells and extracellular media) and oxygen consumption rates (as a surrogate of mitochondrial oxidative phosphorylation chain activity) to provide an integrated picture of the relative contribution of two major nutrients (glucose and glutamine) to cellular energy production during transformation by two of the most commonly mutated oncogenes, Ras and Akt, and under hypoxia.

Most importantly, the paper provides quantitative insights that help dispel one of the most commonly misunderstood concepts in cancer metabolism, namely the apparent paradox that, by enhancing glycolysis, cancer cells engage in a less efficient metabolic state, from an ATP-production standpoint, despite the high energetic demands of cell proliferation. The authors show a significant contribution of mitochondrial metabolism to ATP production both in hypoxia and during oncogene-driven transformation.

Because of the reasons above, this paper has the potential to be of significance to the field. Addressing the points below, would be important in completing an otherwise well-executed study.

1) It has been previously demonstrated that, in some cancer cells, a significant fraction of glucose carbons is diverted to serine and glycine biosynthesis [Locasale et al. Nat. Genet. 2011 43(9):869-74]. It has also been suggested that a significant fraction of a-ketoglutarate (a-KG) synthesis can be accounted for by the activity of this pathway by driving glutamate-dependent transamination [Posemato et al. Nature 2011 476:346-50].

As a-KG will not carry 13C in this case, this pathway may contribute to the decreased 13C incorporation from U-13C-glucose to a-KG relative to citrate (suppl. Fig. 1 A1) and therefore skew the predicted contribution of glucose to ATP synthesis. Furthermore, the involvement of the serine pathway is an important point to address because (a) PHGDH generates a molecule of NADH, (b) diversion of flux to the serine pathway is predicted to limit glucose carbon flux to pyruvate kinase and therefore will skew the predicted amount of ATP produced by glycolysis and (c) glucose carbon flux into the serine biosynthesis pathway is comparable to that for PEP even in cell lines not amplified for Phgdh, the rate-limiting enzyme in the serine pathway.

Do the authors observe any 13C incorporation from U-13C-glucose into serine in the cell lines used? If yes, is there a reason why this branch of the pathway was not taken into account in their model? If no, how would their model and conclusions hold in systems where a significant fraction of glucose is diverted to the serine pathway?

2) The necessity to account for all possible NADH-producing reactions associated with glycolysis is further underlined by the fact that there is no reference to the activity of the malate-aspartate or glycerol phosphate shuttles. Glucose and glutamine carbon metabolism contributes to ATP production in mitochondria through reactions that provide reducing power in the form of NADH or FADH to generate the proton gradient that drives ATP synthase. Depending on which shuttle is active in the cells used, and whether the NADH is derived from cytosolic versus mitochondrial reactions will affect the ATP yield per NADH. For their calculations, the authors infer the contribution of glucose and glutamine metabolism to reducing power for ATP production in mitochondria by using standard P/O ratios (p.3, par. 4). The authors' model doesn't seem to account for differences in cytosolic versus mitochondrial NADH and it would be desirable to highlight the rationale behind this choice by the authors. As in point 1: how sensitive would the model and conclusions be depending on the cell type (and therefore the relative contribution of the two shuttles

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to mitochondrial NADH).

3) An important experiment that would directly validate the paper's main claims is measuring NAD/NADH in cells following oncogene expression or glucose/glutamine deprivation. The LC-MS platform employed for other experiments would be useful in this respect but it would provide little information about metabolic compartmentalization in mitochondria. Fluorescent reporters [Zhao, Y. et al. 2011 Cell Metabolism, 14(4), 555-566; Hung, Y. P. et al., 2011Cell Metabolism, 14(4), 545-554] have been developed for this purpose. Does the sensitivity of mitochondrial NAD/NADH reflect the reported sensitivity of ATP production to mitochondrial inhibitors?

4) Does glucose deprivation affect glutamine uptake and vice versa?

Minor points:

p. 4 par. 2 "868 nmol/ul cells/h" and supplementary tables 2,3,4. The authors should provide a brief explanation why they choose to report ul cells as opposed to cell number.

Some typos, e.g.: p.6, lines 4, 5 and 11 respectively - "4T1" instead of "AT1", "phosphorylation" instead of "phosphorlytion" (twice).

1st Revision - authors' response 19 August 2013

Reviewer #1:

1. *The authors should mention somewhere in the manuscript (possibly in the discussion) that overexpressing mutant H-ras (only rarely mutated in cancer) or the hyperactivated myr-AKT in mouse immortalized kidney cells are artificial and may not reflect the physiologic situation where Kras or PIK3CA are mutated.*

   We now include this note in the first paragraph of the Results & Discussion section.

2. *The finding that cells grown in hypoxia only have a modest reduction in oxphos is an interesting one. I would think that the authors would have compared the transformed IBMK cells (Ras and AKT) grown in hypoxia versus normoxia. This would be quite informative. I realize that they used the tumor cell lines to do such studies, but these are not as genetically defined as the IBMK cells, making direct comparisons more difficult.*

   We measured the oxygen consumption rate of iBMK, Ras and Akt cells in hypoxia. Similar to what we have observed in the parental iBMK cell line, hypoxia moderately reduces oxphos in these cell lines (33% in Ras and 50% in Akt). Results are described and discussed in "Glutamine-supported oxidative phosphorylation is a major source of ATP also in hypoxia".

3. *Applying the FBA to the NCI60 using publicly available data to confirm their findings certainly adds generality to the findings. However, some of this analysis should be shown (at least in the supplemental data) and not just mentioned in the text.*

   We now show the distributions of predicted contribution of oxidative phosphorylation to total ATP making in Figure 4C. We further elaborate on the computational flux balance analysis method used to make these predictions in the Methods.

4. *The authors should perform some simple statistical analyses and show this in the figures to determine if many of the changes between samples rise to level of significance.*

   We performed student's T-test for selected key results (including differences in nutrient uptake following oncogene activation or hypoxia; differential response to electron transport chain

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inhibition or nutrient deprivation depending on oncogene activation), reporting the associated p-values in the text or figures.

5. *Can the authors comment why, while glutamine withdrawal causes a significant decrease oxygen consumption in ASPC1 under normoxic condition while glucose withdrawal does not (fig 4C) but the major contribution to production of high energy electrons is from glucose (Fig 4B) under the same normoxic conditions?*

   After careful consideration, we felt that the analysis of substrate contributions to oxidative phosphorylation was not robust in the absence of isotope tracer data. Accordingly, the associated analysis (which produced the inconsistency that the reviewer astutely noted) has been removed. This impacted only a single figure panel-- all of the other related figures are supported by isotope tracer data.

6. *"In all cases, good agreement with the labeling data was obtained (Supp. Tables 1-4). As a further validation of the inferred fluxes, we measured the kinetic labeling of intracellular metabolites (for 72 hours) as well as their absolute concentrations, showing good agreement between kinetic labeling patterns and simulated patterns (Supp. Tables 1-4)". I believe the authors mean supplementary figures 1-4, not the tables.*

   We thank the reviewer for pointing it out. We have fixed this in the revised version.

7. *Some data is mentioned in the text but there is no reference to specific figures/tables. For example: "The total ATP production rate in the parental iBMK cell-line was found to be 868 nmol/ul cells/h, with the contribution of oxidative phosphorylation and aerobic glycolysis being 80% and 20% respectively."*

   The references to the figures/ tables are added in revised paper.

8. *Fig 4c - the y-axis is unlabeled.*

   Fixed.

9. *Fig 4b and 4c are not directly referred to in the text. The data is discussed but there is no reference to the figures.*

   Fixed.

Reviewer #2:

1. *"The main problem with this article I found is the lack of detail on computational methods. It is thus impossible to evaluate the validity of any of the results reported. There is nothing in this article I could find that points to the algorithms used, equations considered, etc. "*

   Following the reviewer's comment, we now elaborate on both the steady-state and kinetic 13C flux analysis, and FBA analysis in the Methods.
   For steady-state 13C flux analysis, the complete model including reaction atom mapping is now given as Supp. Table 4. All flux constraints based on experimental measurements are included in Supp. Table 5. We now explicitly describe the non-convex optimization problem that is applied to search for a steady-state flux distribution that maximizes the log-likelihood of measured mass-isotopomer data, while also matching experimentally measured uptake and secretion rates (which utilizes the concept of Elementary Metabolite Units). We further elaborate on the method employed to compute flux confidence intervals using likelihood ratio test. We now further describe the set of ordinary differential equations used in computing metabolite labeling kinetics.
   For FBA analysis, we now describe the optimization problem and specific measurements of metabolite uptake and secretion rates taken from Jain et al that were used to predict metabolite fluxes in the NCI-60 cell lines.

2. *"For example, FBA is mentioned but FBA requires a stoichiometry matrix, vmax constraints, and is very sensitive to boundary effects such as the "media" used. "*

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As now explained, the stoichiometric matrix used in the FBA analysis is the same one used in the 13C flux analysis, and can be derived from Supp. Table 4. The specific optimization problem used is now given in the Methods. The optimization is formulated as to find a flux distribution in which the uptake of glucose and glutamine as well as lactate and glutamate secretion match measured rates.

3. *The authors have also previously published a report claiming the requirement of a "solvent capacity" constraint for reasonable FBA results in cancer metabolism (Plos Comp Bio 2011). It is not mentioned if this is used.*

   The usage of a "solvent capacity" constraint in Shlomi et al., PLoS CB (2011) (which was from Shlomi independent of the other authors of this paper) aimed to explain why cancer cells engage in inefficient ATP production via glycolysis instead of relying more heavily on oxidative phosphorylation (Warburg effect). That model was not constrained with measured metabolite uptake/secretion rates from any specific cell line.
   Here, our goal is to apply experimental data to determine the most likely flux distribution in specific cell lines, including in an exhaustive manner for the cell lines on which we focus, as well as more broadly using limited publicly available data for the NCI-60. To do that, we search for a flux distribution that optimally matches cell line specific measurements. We do not maximize ATP/biomass yield as done in some FBA applications. This optimization function did not account for a solvent capacity constraint. Following the reviewer's comment, we further examined whether the predicted mitochondrial respiration rate in consistent with the effect of the limited solvent capacity. Specifically, we considered the solvent capacity constraint from Vazquez et al, claiming that mitochondrial ATP production does not exceed a maximum value, with that maximum value in the range of 1.5-8.4 umole/uL-cells/h. We found that for all 60 cell lines analyzed here, mitochondrial ATP production is lower than the upper threshold of 8.4 umole/uL-cells/h, while in 41 cell lines it is also lower than 1.5 umole/uL-cells/h (now shown in Supp. Figure 7). Rerunning the analysis while constraining mitochondrial oxidative ATP production rate to be lower than 1.5 umole/uL-cells/h resulted in a significantly worse fit with the measured uptake and secretion rates; thus, we feel that the proper upper threshold is at the high end of the range proposed by Vazquez.

4. *As for the isotopomer modeling, it is not clear what was done. The ODEs used are not mentioned, no code is submitted or SBML file preferably.*

   The 13C metabolic flux analysis relies solely on steady-state metabolite labeling data, while kinetic labeling data is used strictly for validation of inferred fluxes (page 3). We now elaborate on both steady-state and kinetic 13C-flux analysis (Methods). Specifically, the ODEs used are now specified in the Methods and are similar to those used in previous papers (e.g. by Noack et al., J Biotechnol, 2011).

5. *"The error estimation is not clear as well. "*

   We now elaborate on the computation of flux confidence intervals: "To compute flux confidence intervals, we used the likelihood ratio test to compare the maximum log-likelihood estimation, computed by the above SQP optimization, to that obtained when constraining the flux to higher or lower values. Specifically, we iteratively run the SQP optimization to compute the maximum log-likelihood estimation while constraining the flux to increasing (and then decreasing) values (with a step size equal to 5% of the flux predicted in the initial maximum log-likelihood estimation). The confidence interval bounds were determined based on the 95% quantile of χ^2-distribution with one degree of freedom_ENREF_1".

6. *"The next major concern is that it's not clear to me what the key results in this paper are. The authors estimate that glutamine has a major contribution to ATP generation but this is already known. Perhaps the key result is that the addition of oncogenes does not appear to change the resulting flux by much. However, this is perhaps to be expected since small changes in metabolism are usually observed when mammalian cells are cultured in artificial tissue culture conditions such as the presence of DMEM and 10% FBS."*

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While the importance of glutamine catabolism to ATP generation has indeed been qualitatively investigated, this paper presents the first quantitative study of the contribution of glucose versus glutamine metabolism for ATP production and how it is affected by oncogene activation and hypoxia. The paper involves the substantial methodological innovation of redox-balanced (i.e., oxygen uptake-rate constrained) mammalian metabolic flux analysis. Moreover, it provides substantial evidence for the importance of oxidative metabolism even in cells expressing oncogenes that impair such metabolism (Ras) and also in hypoxia.

7. *"The authors use the NCI-60 data from Jain, Science 2012 to match fluxes and compare to their case. As the authors are surely aware, those cells were grown in RPMI while their studies use DMEM. Cells exhibit substantial changes in metabolism depending on growth media used. It's not clear to me whether these results are transferrable."*

   We thank the reviewer for pointing this out. We should note that fluxes are not constrained by uptake of any of the media components that differ between DMEM and RPMI. The fact that similar conclusions can be reached by quantitative reanalysis of data from Jain et al., despite the change in media composition, supports the generality of oxidative ATP production in transformed mammalian cells.

8. *"On page 4, the authors that NADH is not a major contributor to oxphos evidenced by 88% of it being consumed by pyruvate reduction. I was unclear on what this means. Could the authors elaborate? "*

   It is **glycolytic NADH** that is not a major contributor to oxidative phosphorylation due to the fact that 84% of it is being consumed by pyruvate reduction. The text was reworded to emphasize this point.

9. *"it's not clear in the methods whether dialyzed serum was used in the flux experiment. This will have a profound effect on the results on way or the other?"*

   All metabolomics experiments were done with dialyzed serum, which is metabolically well-defined. We now explicitly specify that in the Methods.

10. *"- from a rough estimate it appears that some of the fluxes in Figure 1 might not be balanced: e.g. reported O2 consumption, VO2=220 mmol/l/h. and the total NADH production flux is Vnadh=207, Vfadh2=76."*

    Figure 1 shows total O2 consumption rates, while the actual O2 consumption rates by oxidative phosphorylation (as determined by treating cells with an electron transport chain inhibitor) are lower (as now shown in Supp. Table 5). Specifically, in the parental iBMK cell line, the O2 consumption for oxidative phosphorylation is 170±16 nmol/μL/h. To maintain redox balance, we constrain the total NADH/FADH2 flux to be equal to be twice the O2 consumption by oxidative phosphorylation, though allowing a deviation of up to 2 standard deviations – hence total NADH/FADH2 production is within the feasible range.

    Notably, following the reviewers' comments, the computational analysis was redone to account for: (i) glycolytic flux divergence into serine biosynthesis and (ii) reversibility of reactions. These changes did not alter any of our major qualitative results. Here, we get that total NADH/FADH2 flux is 306 nmol/uL/h, which is in agreement with oxygen consumption rate of 170 nmol/uL/h (roughly being one standard deviation lower than the expected value of 340).

11. *"If ATP production flux in cytosol Vatp(glycolytic)=232 mmol/l/h, Vatp(oxphos)=657.5 and Vatp(glycolytic) so the contribution would be 26%, not 20% "*

    Glycolytic ATP production rate in previous version was found to be 182 nmol/uL-cells/h (as shown in Figure 1B), and not 232 nmol/uL-cells/h.

    In the updated analysis, we get glycolytic ATP production of 172.6 nmol/uL-cells/h and OxPhos ATP production of 688.3 nmol/uL-cells/h, which makes the glycolytic contribution 20% of total.

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12. *"It's not clear if Ac-coA flux is balanced as well. ""*

    Yes, it is enforced to be balanced by eqn. 6, which enforces flux balance for all internal metabolites. We have double checked that this is the case for Ac-CoA in both the prior version, and this updated version.

13. *"I would expect reversibility to have big influences on the results but the reactions are all irreversible. "*

    We thank the reviewer for bring this to our attention. Reexamining this, we note that reversibility of two reactions was also accounted for in the previous version of the paper: (i) citrate/isocitrate dehydrogenase and (ii) malic enzyme/pyruvate carboxylase, which in terms of 13C labeling, can be regarded as the same reaction working in opposite directions. Note that we observe identical labeling of malate and aspartate under all experimental conditions, implying that they both share the labeling pattern of oxaloacetate (which is too low abundance to directly measure).

    The lumped reaction converting alpha-ketoglutarate to malate was previously considered to be irreversible, due to the alpha-ketoglutarate dehydrogenase being highly exergonic. However, reexamining this following the reviewer's astute comment, we realized that backward flux may exist from malate to fumarate, which may affect the labeling pattern of malate (due to the symmetric structure of fumarate). The model was hence extended to account for forward/backward flux between fumarate and malate. As indicated above, the revised analysis resulted in qualitatively similar results.

    Other reactions in the model include: (i) citrate synthase which is highly exerogenic (and thus is commonly assumed to be irreversible) and (ii) a lumped reaction for entire glycolysis, as we do not detect glycolytic intermediates labeled from glutamine upstream of pyruvate.

14. *"Some of the internal references do not seem right. For example, it seems "Supp. Tables 1-4" in the third paragraph of the section "Quantifying ATP production routes via a redox-balanced metabolic flux model" should be "Supp. Figures 1-4"; "Supp. Figure 1" in the second paragraph of the section "Quantifying ATP production routes via a redox-balanced metabolic flux model" is not about "uptake and excretion rates of major nutrients"; "Supp. Table 3" in the first paragraph of the section "Redox-balanced metabolic flux analysis (MFA)" is not about the "metabolic network model of glycolysis and TCA cycle"."*

    They are corrected in the new version.

15. *No error bars are presented in supp table 3.*

    We now include standard deviation of these measurements in the revised version.

Reviewer #3:

1. *It has been previously demonstrated that, in some cancer cells, a significant fraction of glucose carbons is diverted to serine and glycine biosynthesis [Locasale et al. Nat. Genet. 2011 43(9):869-74]. It has also been suggested that a significant fraction of a-ketoglutarate (a-KG) synthesis can be accounted for by the activity of this pathway by driving glutamate-dependent transamination [Posemato et al. Nature 2011 476:346-50].*
   As a-KG will not carry 13C in this case, this pathway may contribute to the decreased 13C incorporation from U-13C-glucose to a-KG relative to citrate (suppl. Fig. 1 A1) and therefore skew the predicted contribution of glucose to ATP synthesis. Furthermore, the involvement of the serine pathway is an important point to address because (a) PHGDH generates a molecule of NADH, (b) diversion of flux to the serine pathway is predicted to limit glucose carbon flux to pyruvate kinase and therefore will skew the predicted amount of ATP produced by glycolysis and (c) glucose carbon flux into the serine biosynthesis pathway is comparable to that for PEP even in cell lines not amplified for Phgdh, the rate-limiting enzyme in the serine pathway.
   Do the authors observe any 13C incorporation from U-13C-glucose into serine in the cell lines

used? If yes, is there a reason why this branch of the pathway was not taken into account in their model? If no, how would their model and conclusions hold in systems where a significant fraction of glucose is diverted to the serine pathway?

The reviewer brings up an important point regarding serine synthesis pathway. In the revised version, this branch is added into the model. In addition to glucose and glutamine labeling, we conducted isotope tracer experiments with U-^{13}C-serine and also directly measured the serine uptake rate. Together these measurements enabled us to quantify *de novo* serine synthesis flux (see Methods). Consistent with prior literature, we find that a substantial fraction of serine is synthesized *de novo* from glucose. However, this biosynthetic flux is substantially less than glycolytic flux (3% or less) in the tested cell lines. Thus, the impact on carbon flow through glycolysis, NADH metabolism, and ATP production, is minimal. Note that the tested cell lines are not PHGDH amplified and have high serine uptake rates. All of the results in the new version of the paper include constraints based on the measured serine pathway fluxes, and, with this improvement, the conclusions regarding NADH balance and ATP metabolism remain the same.

2. *The necessity to account for all possible NADH-producing reactions associated with glycolysis is further underlined by the fact that there is no reference to the activity of the malate-aspartate or glycerol phosphate shuttles. Glucose and glutamine carbon metabolism contributes to ATP production in mitochondria through reactions that provide reducing power in the form of NADH or FADH to generate the proton gradient that drives ATP synthase. Depending on which shuttle is active in the cells used, and whether the NADH is derived from cytosolic versus mitochondrial reactions will affect the ATP yield per NADH. For their calculations, the authors infer the contribution of glucose and glutamine metabolism to reducing power for ATP production in mitochondria by using standard P/O ratios (p.3, par. 4). The authors' model doesn't seem to account for differences in cytosolic versus mitochondrial NADH and it would be desirable to highlight the rationale behind this choice by the authors.*
As in point 1: how sensitive would the model and conclusions be depending on the cell type (and therefore the relative contribution of the two shuttles to mitochondrial NADH).

We now discuss the above important issues in the section "Glutamine-supported oxidative phosphorylation is a major source of ATP in the parental iBMK cell line." Because most high-energy electrons produced in the cytosol are consumed by lactate excretion, and thus most high-energy electrons used for oxidative phosphorylation are generated by TCA metabolism, choice of the shuttle does not markedly impact our energy calculations. These results are now described quantitatively in the new version of the manuscript (page 4).

3. *An important experiment that would directly validate the paper's main claims is measuring NAD/NADH in cells following oncogene expression or glucose/glutamine deprivation. The LC-MS platform employed for other experiments would be useful in this respect but it would provide little information about metabolic compartmentalization in mitochondria. Fluorescent reporters [Zhao, Y. et al. 2011 _ENREF_2, 14(4), 555-566;, Hung Y. P. et al., 2011Cell Metabolism, 14(4), 545-554] have been developed for this purpose. Does the sensitivity of mitochondrial NAD/NADH reflect the reported sensitivity of ATP production to mitochondrial inhibitors?*

Following the reviewer's suggestion, we measured changes in NADH/NAD$^+$ ratio after treatment of mitochondria inhibitors or glucose/glutamine deprivation in cells with or without oncogene expression, in hypoxia and normoxia. Mitochondria inhibitors increase NADH/NAD$^+$. In case of glucose or glutamine deprivation, the lack of substrate decreases NADH/NAD$^+$. We agree that it would be valuable to do these measurements in a compartment specific manner, and the method the reviewer referred to is promising in this regard. However, this would require a great deal of effort, and it is not clear that it would be feasible under hypoxia, which is an important focus of our work. Thus, for the present manuscript, we elected to rely on whole cell measurements, which we believe are sufficient to support the claims of the paper

4. *Does glucose deprivation affect glutamine uptake and vice versa?*

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The profound perturbation of glucose or glutamine starvation will cause changes in growth rate and uptake of other nutrients, likely in a non-linear manner with respect to time. This is not a problem for oxygen whose consumption can be readily monitored on a minute-by-minute basis, but it is a problem for glucose and glutamine, where uptake measurements take many hours. Accordingly, we provide the oxygen uptake measurements but not the glucose and glutamine ones.

5. *p. 4 par. 2 "868 nmol/ul cells/h" and supplementary tables 2,3,4. The authors should provide a brief explanation why they choose to report µl cells as opposed to cell number.*

   We measured quantity of cells with both cell number and packed cell volume. Normalizing with either one gives the same result for relative flux. For iBMK cells, 1μl cell is ~ 10^6 cells. We have now clarified this in the main text – we appreciate the reviewer's helpful suggestion to do so. The reason we report all results in units of per ul cells is that reaction kinetics is influenced by metabolite concentration, and nmole per pack cell volume gives a more direct link to concentration than nmole per million cells.

6. *Some typos, e.g.: p.6, lines 4, 5 and 11 respectively - "4T1" instead of "AT1", "phosphorylation" instead of "phosphorlytion" (twice).*

   Thanks for pointing them out. They have been corrected in the new version.

2nd Editorial Decision 26 September 2013

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 manuscript. In the first round of review, reviewer #2 expressed a series of concerns regarding the computational methods. As unfortunately this referee was not available for reviewing the revised manuscript, we have asked an additional referee (#4) to specifically evaluate the employed methodology.

As you will see from the reports below, reviewers #1 and #3 feel that their main concerns have been satisfactorily addressed. However, reviewer #4 lists a few minor points, which we would like to ask you to address in a revision of the manuscript.

Thank you for submitting this paper to Molecular Systems Biology.

Referee reports:

Reviewer #1:

The authors have satisfactorily satisfied my concerns through experimental evidence as well through changes in the text and figures.

Reviewer #3:

The authors have adequately addressed all points raised in my first review.

Reviewer #4:

We have read "Glutamine-driven oxidative phosphorylation is a major ATP source in transformed mammalian cells in both normoxia and hypoxia" by Fan et. al. The authors use 13C tracing data to try to quantify the relative contribution of glucose and glutamine catabolism toward ATP production

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under normoxia and hypoxia. They tout the use of oxygen uptake rates in determining fluxes as a first in mammalian cells, though previously used in bacterial studies. The authors report that glutamine remains a major source of ATP under hypoxic conditions. The authors attempt to expand their results to additional cell lines, beyond those in their experiments, using flux balance analysis modeling with a subset of the consumption/release data recently published for the NCI-60 panel of cell lines. We were asked to evaluate the computational methods specifically, and thus will limit our discussion to these. In general, the 13C methods and minimal model used appear to be sound. The oxygen uptake rate is a key constraint for studying cancer cell energy metabolism and thus its measurement and use is to be applauded. We have only a few minor concerns, primarily with whether the FBA procedure used can truly determine the ATP production from glutamine in as high precision as reported for the NCI-60 cell lines.

1) The confidence intervals are quite small for certain reactions, per Figures 1, 2, and 3, for example glutamate flux to alpha-ketoglutarate being measured within 0.2% error. One wonders whether the probes used and experimental methods are genuinely sufficiently precise to determine the fluxes within this accuracy, or whether the choice of a minimal model and the particular approach for confidence interval calculation may artificially inflate the confidence. For example, do the authors account for proline production via glutamate, or the contribution of glutamate to biomass? There is an arrow in Figure 1B indicating biomass production from glutamate with no value, but glutamate -> biomass is not included in Supplementary Tables 4 or 5 when defining the MFA model so there appears to be a discrepancy. These fluxes may be small relative to the flux from glutamate entering the TCA cycle, and thus they may not affect the qualitative results, but they would at least affect the confidence intervals.

2) The flux balance analysis procedure is more questionable due to the lack of cell line-specific oxygen uptake rate data. The optimization procedure used will result in a unique solution, but no estimate of confidence in the face of unknown oxygen uptake was performed and the minimal model used may be overly constraining. I would recommend repeating the analysis with a global model such as human Recon 1 or human Recon 2, and use flux variability analysis with clearly defined ranges in cell line-specific oxygen uptake rates to identify whether the contribution of glutamine to ATP production can definitively be reported to be within some narrow range around 88% given the available constraints.

I wonder if this paper represents a lot of rediscovery. It has been known for over 20 years that glucose is mostly converted to lactate in transformed cells in culture, and that their high glutamine consumption rates is mostly related to the TCA cycle and oxidative phosphorylation. The Km value for oxygen uptake rate for most continuous cell lines is 0.5 to 1% of oxygen concentration relative to ambient. Thus normoxic and hypoxic conditions are hard to deal with experimentally.

2nd Revision - authors' response 15 October 2013

Reviewer #4:

1. *"The confidence intervals are quite small for certain reactions, per Figures 1, 2, and 3, for example glutamate flux to alpha-ketoglutarate being measured within 0.2% error. One wonders whether the probes used and experimental methods are genuinely sufficiently precise to determine the fluxes within this accuracy, or whether the choice of a minimal model and the particular approach for confidence interval calculation may artificially inflate the confidence. "*

We thank the reviewer for this insightful concern. The calculated standard deviations are obtained from the integration of numerous experimental measurements, including metabolite uptake and secretion, oxygen consumption, biomass composition, and steady-state labeling from both ^{13}C glucose and glutamine. Hence, some calculated standard deviations are indeed small (and are below the experimental standard deviations of single measurements). We note that the confidence intervals are calculated via a brute force approach, by repeating the flux analysis by constraining each flux to either increasing or decreasing values. This approach directly identifies the maximal flux deviations that are consistent with the entirety of the observed experimental data. It is thus actually more

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conservative than other literature approaches, at the expense of being more computationally intensive.
Despite the conservative nature of our calculations, we agree with the reviewer and that the minimal model may be inflating the confidence. It is not currently computationally feasible to integrate our diverse of experimental data within a genome-scale model; thus, we cannot say for sure what the error would be in a complete model of this sort. We note though, that using small-scale metabolic network models for ^{13}C metabolic flux analysis (due to computational limitations) is the common practice in this field. We concur, however, that flux from glutamate to alpha-ketoglutarate might well have an error greater than 0.2% in a genome-scale context. Given the validity of this concern, we added to the Results the statement: "note that reactions not included in the metabolic network model may introduce additional error beyond that reflected in the computed confidence intervals." in the third paragraph on page 3.

2. *"For example, do the authors account for proline production via glutamate, or the contribution of glutamate to biomass? There is an arrow in Figure 1B indicating biomass production from glutamate with no value, but glutamate -> biomass is not included in Supplementary Tables 4 or 5 when defining the MFA model so there appears to be a discrepancy. These fluxes may be small relative to the flux from glutamate entering the TCA cycle, and thus they may not affect the qualitative results, but they would at least affect the confidence intervals. "*

Yes, we account for glutamate and proline requirement for biomass when computing the glutamine flux into TCA cycle through alpha-ketoglutarate (F11) (see Supp. Table 5; "Demand flux: proteomic gln/glu/pro (nmol/uL cells/h)"). This demand flux amounts to ~5% of glutamine uptake. Thus, deviations in its value could potentially introduce error > 0.2% in flux from glutamate to ketoglutarate, but will not introduce errors large enough to impact our major conclusions. In calculating the quantitative flux confidence intervals we assumed a standard deviation of ±20% for biomass demand fluxes (see Supp. Table 5). The very tight error estimates for the flux from glutamate to ketoglutarate, in spite of this flexibility in the biomass demand fluxes, reflects the glutamate to ketoglutarate flux being constrained more directly by other experimental data (e.g., TCA labeling, O2 uptake).

3. *"The flux balance analysis procedure is more questionable due to the lack of cell line-specific oxygen uptake rate data. The optimization procedure used will result in a unique solution, but no estimate of confidence in the face of unknown oxygen uptake was performed and the minimal model used may be overly constraining. I would recommend repeating the analysis with a global model such as human Recon 1 or human Recon 2, and use flux variability analysis with clearly defined ranges in cell line-specific oxygen uptake rates to identify whether the contribution of glutamine to ATP production can definitively be reported to be within some narrow range around 88% given the available constraints."*

We thank the reviewer for this useful suggestion. We note that we do not quantify the contribution of glucose versus glutamine oxidation to making reducing power in the NCI 60 cell lines, as this would be unreliable without oxygen uptake and isotope tracer measurements. Following the reviewer's comment, we repeated the analysis of ATP production in the NCI-60 cell lines with a genome-scale human metabolic network model (Recon1). In addition to constraining influxes and effluxes based on experimental data from Jain et al., we constrained biomass production based on experimental growth rate data from NCI's DTP database. Then we employed Flux Balance Analysis (FBA) to find a flux distribution with maximal ATP production rate (with a second optimization minimizing the total sum of flux as in our previous analysis), finding an average ATP production of 84% through oxidative phosphorylation across NCI-60 cell lines (Supp. Figure 8A). An additional analysis was performed, without optimizing for ATP production, in which oxygen consumption by oxidative phosphorylation was constrained to be within between 75-225 nmol/uL-cells/h (where the lower bound is 50% lower and the upper bound is 50% higher than the average oxygen consumption by oxidative phosphorylation measured in the iBMK cell lines), and minimizing sum of flux. In this case, the mean ATP production by oxidative phosphorylation across the NCI60 cell lines is 70% (Supp. Table 8B). Notably, our results are robust with regard to biomass composition assumption; varying protein mass between 50%-90% as well as DNA/RNA and lipid mass between 3% and 20%

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changed the predicted contribution of oxidative phosphorylation by less than 1%. We now include these new results obtained with Recon1 in the main text (section "ATP production routes in cancer cell lines in both normoxia and hypoxia"), and a description of the associated calculations in the Methods.

We again thank the reviewer for encouraging us to make this important improvement.

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