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Oncogenic K-Ras decouples glucose and glutamine metabolism to support cancer cell growth

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(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   06 December 2010

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.

The major concerns raised by the reviewers refer to the need to discriminate between the causes and consequences of the reported metabolic alterations. Additional investigations are therefore required to convincingly demonstrate that the metabolic alterations depend on K-Ras and do not merely reflect indirect effects and to provide more functional evidence that the observed decoupling "supports cancer cell growth". The reviewers also raise additional important methodological concerns with regard to the flux analysis and transcriptome analysis.

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.

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Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favourable.

Yours sincerely,

Editor
Molecular Systems Biology

Referee reports:

Reviewer #1 (Remarks to the Author):

The executive summary of the Paper presented by Gaglio et al is that in cells with oncogenic k-ras, glutamine is (increasingly) used for anabolism rather than oxidation. To this respect, both mouse and breast cancer cell lines are subject to carbon and nitrogen labeling experiments to characterize the breakdown of glutamine. Further, the analysis of transcripts for the involved enzymes suggests that the difference is mainly cause by a change in protein expression. The fundamental question is whether the mutation in k-ras is directly responsible for the observed shift towards reductive metabolism of glutamine. In my view, neither causality nor sufficiency have been demonstrated.

Major concerns:
-I was looking for proliferation of growth rates to rule out that the metabolic changes observed are linked to growth, rather than ras directly. The best I could find is the flux of metabolites to biomass formation (Table I) which is supposedly proportional to specific growth. To my surprise, the normal cells seem to grow faster than the transformed. Moreover, the ratio seems almost identical to the change in flux observed downstream of pyruvate in mitochondria. In the absence of proper controls, I therefore challenge the view that the effect seen here is ascribable to a regulatory response governed by k-ras rather than secondary effects.
-The study of transcriptional levels specific of enzymes linked to glutamine degradation is puzzling. I can't really understand why Mdh2 (aka the mitochondrial malate dehydrogenase) is so important for synthesis of aspartate from glutamine (Page 8)? It's only used if the glutamine goes through the whole TCA cycle, but then it is just one enzyme among ten. Reading further on Page 9, it is even stated that aspartate can be synthesized from glutamine through Mdh2 and Acly. Please clarify because I don't see how this could be the case (cfr. Figure S1). Because of this problem, I can't understand why the AKG is argued to be important (Page 9).
-Among the panel of breast cancer cell lines typically used (cfr Neve 2006), the MDA-MB-231 belongs to those with the lowest oxygen consumption. Since this line seems to generally lack respiratory/oxidative capacity, it is not surprising to observe that glutamine is to a large extent reduced. However, there are other "triple-negative" breast cancer cell lines that show the same physiology but don't have a mutation in k-ras, eg BT549. This indicates that glutamine reduction is likely independent of k-ras.
-The occurrence of the labeling pattern in aspartate M3 specific for reductive carboxylation from glutamine is di per se NOT demonstrating that transformed cell DEPEND on glutamine for biosynthesis (Page 6). Hence, the view that the observed metabolic change is necessary for tumorigenesis and therefore induced by k-ras remains arguable. It is also puzzling that the aspartate levels are much higher in the transformed cells, which points to a potential overcapacity compared the demand.

On another note, I was very much intrigued by the combination of 13C-methods and transcript information. The impression is that the transcriptional response of enzymes upon k-ras activation has been described earlier, even by detailed work previously published by the same Authors. Although some new targets are presented here (e.g. Pdk, Pfkfb), I could not scavenge new aspects emerging from the transcriptional analysis. In contrast, the flux analysis seems to provide novel elements on metabolic activity. Unfortunately, the model used for flux analysis is insufficiently described. Key information was not provided in the Supplement. A few examples:

-The assumption that CO2 is 100% 12CO2 clearly conflicts with common sense. Why do the Authors think that this is not biasing the fluxes?

-What is the basis to assume that alanine is primarily synthesized in the mitochondria? To my knowledge, alanine aminotransferases in the cytosol are the main source to fuel protein biosynthesis and therefore alanine could be

-Why was the mitochondrial malic enzyme preferred over the cytosolic?

-How were the MS measurements propagated in the model for the flux fitting? For example, the MID measured for pyruvic acid can neither be used to constrain the mitochondrial nor the cytosolic fraction (in theory only the sum weighted by the relative amounts). The same applies to aspartic acid.

-It is not clear why some measurements were included/omitted for the calculations, and why the set differs for the two cell lines (cfr Supplement)

-What is the difference between OAA and Oac? (cfr Supplement) It is also not clear what happens to the Oac overflow. Experts would claim it flows back to PEP via carboxykinase (eg Maier 2008 in Metabolic Engineering).

-What about the ca. 1 g/L of amino acids in the medium? Please add a short note why these are assumed to not influence the labeling and therefore omitted.

-There is an apparent discrepancy between the model listed in the Supplement and the reactions in Table I: reversibilities change (eg PPP), reactions not listed.

Reviewer #2 (Remarks to the Author):

Gaglio et al. perform an anaylsis of the metabolic fluxes of glutamine and glucose through normal and Kras transformed cell lines. In addition to these studies, they also associate the metabolic finding with transcriptional profiling data of their cell lines of study. While the authors confirm previous findings that k-ras transformation increases glycolysis, they also interestingly find the glutamine metabolism fuels a branched pathway of the TCA cycle that is able to drive citrate synthesis. This paper would be the first to examine carbon flux through the TCA cycle in Kras driven tumor cells. While this finding is very important for cancer biology, this paper must be classified as entirely 'descriptive' in nature. Actually testing the proposed mechanisms for tumor cell metabolism was not done. The title of the paper should read-"Oncogenic K-ras with p53 mutation decouples glucose and glutamine metabolism" as this function for cell growth is not confirmed. However, due to the growing interesting in tumor cell metabolism I recommend the paper for publication if they address my major concern.

Major concern:
The paper overstates the conclusion. The breast cancer cells they have utilized are p53 mutant along with Kras mutation. Loss of p53 diminishes mitochondrial activity.
I strongly suggest they should examine Kras driven HCT116 p53+/+ vs HCT116 p53-/- cells. These cells are widely available. It could be that p53 decides the degree to which Kras driven tumor cells perform reductive carboxylation.

Minor Comments:

1. One the interesting observations of this paper is that glutamine is important for biosynthetic reactions. As flux through the reductive caboxylation cycle could drive the creation of citrate for lipid synthesis, it may be useful to look more downstream for glutamine incorporation into lipids as a more functional readout for your studies.

2. This results of this paper are entirely descriptive, it could be made much stronger by incorporating experiments pharmacologically inhibiting the decoupled TCA-cycle to test if the pathway is required for k-ras dependent tumor cell growth. Previous paper by Weinberg et al in PNAS 2010 manipulated the glutamine pathways to demonstrate the relevance of this pathway.

Reviewer #3 (Remarks to the Author):

In the present paper Gaglio et al. investigate the metabolic flux consequences of expression of activated k-Ras in NIH3T3 mouse fibroblasts. The paper shows that k-Ras expression impairs PDH activity and citrate synthesis from glucose, while leaving metabolism of glutamine largely unchanged. In this respect, k-Ras alters the balance of TCA cycle inputs. The paper is the first to my knowledge to use metabolic flux analysis is a substantial way (covering meaningful fraction of

central carbon metabolism) to profile flux changes in response to an oncogene. Accordingly, I see this as a potentially important contribution, even though most of the flux changes reported by the authors would have been anticipated based on prior literature.

Major concerns:
1) Methodological gaps (or at least lack of clarity).
A) Why are data reported as time series, when the perturbation involves (to the best of my reading) stable expression of k-Ras? What defines time zero?
B) How were the transcripts normalized? What defines "1"? This is not a detail: basically I could not effectively understand any of the transcriptional data for lack of clarity on this point. Given this caveat, my impression is that the transcriptional responses are much more varied and complex than the authors claim in the abstract and do not support their conclusions.
C) Were the metabolic experiments performed in dialyzed serum? If not, how did small molecules in serum impact the analysis?

2) Validity of flux analysis
A) The flux analysis is based on a relatively small number of compounds measured only at steady-state. There is limited data (not all isotopic forms) provided for citrate; these other forms are quite useful for understanding TCA fluxes. Without more extensive data, it is hard to know whether flux model is valid, i.e., would additional data (on other compounds or labeling dynamics) match the model? Additional data is probably needed.
B) It appears in reading the paper that the quantitative TCA fluxes are derived solely using 13C-glutamine labeling data. Hopefully this is just a misunderstanding, but it is essential to see supplementary data showing fit of glucose labeling between model and data, as is shown now for glutamine.

3) Minor comments:
A) Table 1 is not readily readable due to small size. It is also hard to digest due to information on many unconstrained exchange fluxes which add no useful information to a general reader.
B) In Figure 5, how is decreased gln uptake compatible with unchanged flux from gln to glu from FBA perspective?
C) The word robust is misused in the text.
D) The authors claim in the abstract that they "surprisingly" see non-canonical labeling of aspartate, although actually there are a spate of careful recent papers (starting from seminal work of Kelleher and Stephanopoulos) that have made this pathway (and more recently its significance in cancer) quite clear. Thus, the results regarding IDH2 flux are not surprising at all.

1st Revision - authors' response   11 April 2011

Please find enclosed the revision of the manuscript "Oncogenic K-ras decouples glucose and glutamine metabolism to support cancer cell growth" by Daniela Gaglio, Christian M. Metallo, Paulo A. Gameiro, Karsten Hiller, Lara Sala Danna, Chiara Balestrieri, Lilia Alberghina, Gregory Stephanopoulos and Ferdinando Chiaradonna to be re-submitted as a Research Article to Molecular Systems Biology. We have addressed the reviewersí critiques by adding new experimental data and clarifying the text where necessary.
Here you will find our reply to regarding the Remarks for the Author and the specific issues addressed by you and by the reviewers.

Remarks for the Author by Editor and by Reviewers
Main concern
Based on comments from the Editor and referees, we now demonstrate that the metabolic alterations depend on oncogenic K-Ras by using a cell line expressing a dominant negative form of oncogenic K-ras. This cell line, NIH-GEF-DN (revertant), expresses a dominant negative Guanine Exchange Factor (CDC25) that attenuates the activity of oncogenic K- Ras. Cells expressing this molecule have been described in numerous publications: *The Journal of Biological Chemistry, 1999: Vol.274*

pp. 36656-36662. Characterization and properties of dominant-negative mutants of the ras-specific guanine nucleotide exchange factor CDC25(Mm); Oncogene, 2000: Vol.19 pp.2147-2154. A dominant negative RAS- specific guanine nucleotide exchange factor reverses neoplastic phenotype in K-ras transformed mouse fibroblasts; Biochimica et Biophysica Acta, 2006a: Vol.1757 pp.1338-1356. Expression of transforming K-Ras oncogene affects mitochondrial function and morphology in mouse fibroblasts; Oncogene, 2006b: Vol.25 pp. 5391-5404, Ras-dependent carbon metabolism and transformation in mouse fibroblasts; PlosONE, 2009: Vol.4 no.3 pp.   e4715   Glutamine   deprivation   induces abortive s-phase rescued by deoxyribonucleotides in k-ras transformed fibroblasts.

As reported in Figure 5 of the revised manuscript, by using enzymatic assays, we measured the endogenous levels of four metabolites, citrate, glutamate, malate and aspartate, shown in Figure 1 of our manuscript to change in transformed cells as compared to normal ones. Of note, the enzymatic measurements were highly consistent with GC/MS metabolic analysis (e.g. see the increased levels of aspartate and citrate in transformed cells) and demonstrate the similarity of metabolite abundances between NIH-3T3 cells and NIH- GEF-DN. The analysis was conducted, as in the other assays, at 54 hours time at which all three cell lines have the same proliferation rate, as shown by cell count (data not shown). In addition, we have used specific inhibitors of glutamine metabolism, such as AOA and EGCG, to analyze their effect on cell proliferation and survival of the three cell lines above described. As shown in Figures 5E, 5F and 6, the behavior of NIH-GEF-DN (revertant) closely resembles that of normal cells and is strikingly different from that observed in transformed cells, especially in terms of proliferation upon AOA treatment (see Figure 5F) and in terms of cell death upon EGCG treatment (Figure 6). Furthermore, addition of metabolites that arise downstream of glutamine (i.e., dimethyl aspartate (DMD, 1mM) and dimethyl AKG (DMK, 2mM)) in low glutamine growth condition (Figure S9) or in presence of AOA or EGCG (Figure S9), is to a large extent able to restore the proliferation or survival of transformed cells. Taken together these findings support the important role of glutamine and hence of its metabolizing enzymes for transformed cells growth and further underline the metabolic dissimilarities between NIH-GEF-DN cells (revertant) and NIH-K-Ras cells (transformed) and similarities between NIH-GEF-DN cells and NIH3T3 cells (normal).

Hence we trust that you will be satisfied by these findings which support the notion that attenuation of oncogenic K-Ras activity is able to restore a metabolism almost similar to that of normal NIH3T3 cells and that the metabolic alterations observed in transformed cells depend on K-Ras and do not merely reflect indirect effects of transformation. In addition these set of experiments as well as the experiments showed in Figure S9, provide more functional evidences that the observed decoupling in the metabolism of glucose and glutamine "supports cancer cell growth".

Remarks for the Author by Reviewers Reviewer #1

Point 1
"I was looking for proliferation of growth rates to rule out that the metabolic changes observed are linked to growth, rather than ras directly. The best I could find is the flux of metabolites to biomass formation (Table I) which is supposedly proportional to specific
growth. To my surprise, the normal cells seem to grow faster than the transformed. Moreover, the ratio seems almost identical to the change in flux observed downstream of pyruvate in mitochondria. In the absence of proper controls, I therefore challenge the view that the effect seen here is ascribable to a regulatory response governed by k-ras rather than secondary effects".

We did not adequately describe the MFA estimation and confidence interval calculations in the initial submission, so in this resubmission we added more pertinent details to the Supplemental Methods. Please also see Metabolic engineering, 2006: Vol. 8 pp. 324-337. Determination of confidence intervals of metabolic fluxes estimated from stable isotope measurements for a detailed description of flux confidence interval calculations. In short, we add the following statement: while the flux values provide some description of flow through the network, the confidence intervals generate a more precise quantification of how similar or different a given flux is when comparing two experimental conditions. The confidence intervals for flux to biomass listed in Table I and in the Supplement overlap. Therefore, there is no statistically significant change in flux of metabolites to biomass, which is consistent with cell proliferation data. On the other hand, the model results highlight a significant change in pyruvate dehydrogenase (PDH) flux and downstream reactions in the TCA cycle. Hope that this clarification suits the Reviewer.

Point 2
"I can't really understand why Mdh2, Acly and AKG are so important for synthesis of aspartate from glutamine. Please clarify because I don't see how this could be the case".

Aspartate is synthesized from oxaloacetate (OAA). OAA is obtained from glutamine through oxidative TCA metabolism, of which the final reaction is catalyzed by Mdh2. Glutamine-derived citrate (through oxidative and reductive pathways) is converted to OAA and acetyl coenzyme A by Acly. Finally, AKG is the entry point in which glutamine carbon enters the TCA cycle through anaplerosis. We have more clearly explained the role of Mdh2, Acly and AKG by adding the scheme in Figure 1F and explaining the issue throughout the paper.

Point 3
"Among the panel of breast cancer cell lines typically used (cfr Neve 2006), the MDA-MB- 231 belongs to those with the lowest oxygen consumption. Since this line seems to generally lack respiratory/oxidative capacity, it is not surprising to observe that glutamine is to a large extent reduced. However, there are other "triple-negative" breast cancer cell lines that show the same physiology but don't have a mutation in k-ras, eg BT549. This indicates that glutamine reduction is likely independent of k-ras".

Also for us it is not surprising that glutamine consumption is reduced in MDA-MB-231 cells as compared to mouse transformed and normal cells, because the human cell line has more deranged mitochondria as compared to mouse transformed cells. On the other hand proliferation analysis conducted in 25mM glucose plus 0.5mM glutamine indicate that MDA-MB-231 cells as well as mouse transformed cells (the results for mouse cells have been published in PlosONE Gaglio et al., 2009) rely on glutamine to sustain their proliferation (Figure 3A). On the contrary, no effect of glutamine reduction has been observed in normal cells. The experiments described in Figure 5 and 6 address this issue, clearly showing that glutamine utilization is dependent on oncogenic K-Ras expression.
We acknowledge that additional oncogenic alterations in cells/tumors may elicit similar metabolic changes. However, this fact does not mean the metabolic alterations we describe are independent of K-ras. For example, mutations in PTEN (as in BT549 cells) cause activation of the PI3K pathway, which itself is known to affect glycolytic metabolism. PTEN inactivation and Ras activation impinge with the same effect on the PI3K pathway as reported in references: Science 4 September 2009: Vol. 325 no. 5945 pp. 1261-1265. Activation of the PI3K Pathway in Cancer Through Inhibition of PTEN by Exchange Factor P-REX2a; Nature: 2006 May 25;441(7092):424-30. Ras, PI(3)K and mTOR signalling controls tumour cell growth. Cancer Res: December 1, 2000 60; 6750. K-ras Codon 12 Mutation Induces Higher Level of Resistance to Apoptosis and Predisposition to Anchorage-independent Growth Than Codon 13 Mutation or Proto-Oncogene Overexpression. Cell: 2007 June Volume 129, Issue 5, 1, Pages 957-968. Binding of Ras to Phosphoinositide 3-Kinase p110 Is Required for Ras- Driven Tumorigenesis in Mice.

Point 4
"The occurrence of the labeling pattern in aspartate M3 specific for reductive carboxylation from glutamine is di per se NOT demonstrating that transformed cell DEPEND on glutamine for biosynthesis (Page 6). Hence, the view that the observed metabolic change is necessary for tumorigenesis and therefore induced by k-ras remains arguable. It is also puzzling that the aspartate levels are much higher in the transformed cells, which points to a potential overcapacity compared the demand".

We agree with the referee on the issue that M3 aspartate labeling through reductive carboxylation cannot be the only indication that transformed cells depend on glutamine for biosyntheses. We do not observe a specific increase in reductive carboxylation, which appears to have a constant flux both in normal and transformed mouse fibroblasts. In transformed cells PDH and oxidative TCA fluxes decrease. On the other hand, we now demonstrate that transformed cells require glutamine to sustain their anabolic processes by using specific inhibitors of enzymes involved in glutamine metabolism (see Figure 5 and 6). These results show that glutamine carbon is necessary for biosynthesis and that the metabolic changes described in transformed cells support cancer cell

growth.

Support to this contention has also been given by the results presented by the paper:

Targeting Mitochondrial Glutaminase Activity Inhibits Oncogenic Transformation. Jian- Bin Wang et al. Cancer Cell 18, 207ñ219, September 14, 2010.

Taken together these observations suggest reductive carboxylation, though we acknowledge that conclusive evidence of the use of this pathway is pending. This point will be addressed in future investigations.

"It is also puzzling that the aspartate levels are much higher in the transformed cells, which points to a potential overcapacity compared the demand".

Indeed, the reviewer makes an excellent point regarding the increased aspartate abundance detected in transformed cells, as we did not adequately demonstrate an increased demand for this metabolite in K-ras transformed cells. The new data which utilizes the Got1 and Got2 inhibitor, AOA, demonstrates that transformed cells are more dependent upon aspartate synthesis relative to N and R cells. Importantly, all metabolite abundance measurements were conducted on proliferating cells under conditions where the demand is easily met, potentially leading to an overcapacity. Such an overabundance does not preclude the fact that aspartate is in greater demand upon K-ras transformation.

Point 5

"On another note, I was very much intrigued by the combination of 13C-methods and transcript information. The impression is that the transcriptional response of enzymes upon k-ras activation has been described earlier, even by detailed work previously published by the same Authors. Although some new targets are presented here (e.g. Pdk, Pfkfb), I could not scavenge new aspects emerging from the transcriptional analysis.

In our previous works we did not describe genes related to glutamine metabolism or genes involved in purine synthesis, and we did not take into consideration a comparative analysis between normal breast tissue and MDA-MB-231 cells.

The reason for doing the integrated analysis reported in this paper is to ascertain whether the metabolic response is due primarily to changes in transcription or not.

The integration of both metabolic analysis and transcriptional data, as shown in Figure 4 of the revised manuscript is very informative and may help to shed light on mechanisms leading to cancer cell metabolic alterations.

Point 6

"In contrast, the flux analysis seems to provide novel elements on metabolic activity. Unfortunately, the model used for flux analysis is insufficiently described. Key information was not provided in the Supplement. A few examples:

-The assumption that CO2 is 100% 12CO2 clearly conflicts with common sense. Why do the Authors think that this is not biasing the fluxes"?

We agree with the Referee, the CO2 story was poorly worded in our initial submission. We do not assume that CO2 is 100% 12CO2. Rather, we do not balance CO2 as a metabolite, and naturally labeled CO2 (accounting for natural abundance of 13C and 18O) freely exchanges from the environment with that generated by the cells.

The paragraph has been rewritten accordingly.

Point 7

"What is the basis to assume that alanine is primarily synthesized in the mitochondria? To my knowledge, alanine aminotransferases in the cytosol are the main source to fuel protein biosynthesis and therefore alanine could be".

The Reviewer is correct in saying cytosolic alanine aminotransferases should be active. The assumption in question as well as pyruvate compartmentalization was made to reconcile differences

in labeling observed between pyruvate, lactate, and alanine. Alanine acquires label to a much larger extent from glutamine carbon as compared to pyruvate and lactate (4% versus 1% in NIH3T3 cells). In order to obtain an acceptable fit of the labeling data (all metabolites used for analyses are extensively validated) we made this assumption. A possible explanation for it may be given by the flux to protein biosynthesis itself. The cytosolic pool of alanine may actually be quite small as compared to the contribution of the mitochondrial pool, and our measurements may reflect the mitochondrial pool more closely. We have strived to minimize the amount of compartmentalized measurements included in the model, given that separate measurements cannot be reliably made and therefore excessive compartmentalization will increase the degrees of freedom of the model and cause many fluxes to remain unresolved. That is, fits can be obtained but the confidence intervals will increase significantly.

Point 8
"Why was the mitochondrial malic enzyme preferred over the cytosolic"?

As above, this assumption was required to reconcile the increased labeling of alanine (4%) from glutamine carbon that was not observed in pyruvate or lactate. Importantly, glutamine carbon must be converted to pyruvate to generate label in alanine, and enzymes participating in pyruvate cycling (e.g. malic enzyme, pyruvate carboxylase) catalyze these reactions. Inclusion of the mitochondrial isozyme resolved this labeling issue and enabled a fit of the data. We certainly can include additional reactions, but it should be noted that such fluxes would be unresolvable without compartmentalized measurements. Even by limiting the number of reactions as we did, fluxes involved in pyruvate cycling are difficult to resolve accurately, as evidenced by the relatively large confidence intervals estimated in the pathway.

Point 9
"How were the MS measurements propagated in the model for the flux fitting? For example, the MID measured for pyruvic acid can neither be used to constrain the mitochondrial nor the cytosolic fraction (in theory only the sum weighted by the relative amounts). The same applies to aspartic acid".

Pyruvate is the only measured metabolite assumed in our model to be compartmentalized between the cytosol and mitochondria, and the MS measurements are made up from a weighted sum of both. The fractionation itself is a fitted "mixing flux," and this treatment recapitulates the mixing that occurs upon extraction of metabolites.
Other metabolites, such as aspartate and components of the TCA cycle, are not compartmentalized within the model. These compounds are assumed to be either at isotopic steady state across compartments or predominantly exist in one compartment or another. The only other exceptions are succinate and citrate, and for these metabolites it was necessary to include dilution pools to achieve a statistically acceptable fit. While a full compartmentalized model can be generated to more easily fit the data, the added degrees of freedom will decrease the utility of the fit due to potential for multiple solutions and poor confidence intervals. In order to consider the refereeís concern, we have expanded the discussion of compartmentalization and mixing fluxes in the supplement to better describe these issues.

Point 10
"What is the difference between OAA and Oac? (cfr Supplement) It is also not clear what happens to the Oac overflow. Experts would claim it flows back to PEP via carboxykinase (eg Maier 2008 in Metabolic Engineering)".

OAA and Oac are the same, we have corrected this mistake in the writing. In our model OAA is a balanced metabolite and is converted to citrate, aspartate, or malate. The results of Maier et al were obtained in a hepatoma cell line with stronger gluconeogenic potential, while NIH3T3 cells are reported to not express high levels of PEPCK enzyme (J Biol Chem. 1991 May 5;266(13):8416-25. Hormonal Control of Interacting Promoters Introduced into Cells by Retroviruses). In any event, simplifications were made within the model amongst reactions involved in pyruvate cycling, analogous to those made by Sriram et al., Mol. Gen & Metabolism 2007. Significant label was not

detected in pyruvate or lactate from glutamine carbon, indicative of the fact that minimal flux occurs from OAA to PEP or Mal to Pyr relative to glycolytic fluxes.

Point 11
"What about the ca. 1 g/L of amino acids in the medium? Please add a short note why these are assumed to not influence the labeling and therefore omitted".

The culture medium used (DMEM) does not include any of the amino acids directly measured (asparate, glutamate, alanine) nor any amino acids immediately downstream of those amino acids (asparagine, proline). In our experience we have not observed significant oxidation of other amino acids (e.g. isoleucine) into the TCA cycle as AcCoA. Since low concentrations of some amino acids might be present in the culture medium from serum, we performed a quick rinse before extracting samples to minimize media contamination. More importantly, we were able to achieve a good fit in metabolites such as citrate, where the unlabeled AcCoA arising from amino acid oxidation would be detected. We have modified the test to add some explanation for the exclusion of amino acid incorporation and oxidation. In the case of newly added 13C-glucose MFA results, the labeled tracer pool was diluted within our model to account for measured dilution from serum.

Point 12
"There is an apparent discrepancy between the model listed in the Supplement and the reactions in Table I: reversibilities change (eg PPP), reactions not listed".

In an effort to simplify the table we omitted some reactions, as we focus primarily on reactions that are well resolved in the model (i.e. have reasonable confidence intervals). PPP reactions are now included, although exchange fluxes present in the model but undetectable by our MFA model (i.e., confidence intervals ranging to infinity) are now omitted (as requested by Reviewer 3 below). A complete table including all results is now included in the supplement for each MFA fit and data set. Remarks for the Author by Reviewers

Reviewer #2
Point 1 (major concern)
"The paper overstates the conclusion. The breast cancer cells they have utilized are p53 mutant along with Kras mutation. Loss of p53 diminishes mitochondrial activity.
I strongly suggest they should examine Kras driven HCT116 p53+/+ vs HCT116 p53-/- cells. These cells are widely available. It could be that p53 decides the degree to which Kras driven tumor cells perform reductive carboxylation".

The reviewer makes an interesting point, and to address this question we now include experiments describing metabolite abundances with HCT116 p53+/+ and HCT116 p53-/- cells, reported in Figure S7. As shown in Figure S7, based upon enzymatic measurements of intracellular glutamate, aspartate, citrate and malate (Figure S7C through F) as well as proliferation analysis under nutrients deprivation (Figure S7A and B), no significant difference between the two HCT116 cell lines was detected, indicating that the metabolic reprograming observed in this paper does not rely upon p53. We also point out that NIH3T3 cells are p53 wild type (Cell research, 1999: Vol. 9 pp. 261-269. NIH 3T3 cells malignantly transformed by mot-2 show inactivation and cytoplasmic sequestration of the p53 protein.), while MDA-MB-231 cells carry an inactive mutant of p53 (Human mutation, 2002: Vol. 19 pp. 607. The IARC TP53 database: new online mutation analysis and recommendations to users.). They show similar metabolic changes, as demonstrated throughout the revised manuscript, so we could argue that these changes are p53-independent. In addition, we recently published that mouse K-Ras transformed mouse cells show a strong reduction of respiratory activity that, since they carry a p53 wild type, it should be p53-independent (Biochimica et Biophysica Acta, 2010: Vol, 1797 pp. 314-323. Mitochondrial Complex I decrease is responsible for bioenergetic dysfunction in K-ras transformed cells.). We hope that taken together these findings satisfy the refereeís concern.

Minor Comments:
1) "One the interesting observations of this paper is that glutamine is important for biosynthetic reactions. As flux through the reductive caboxylation cycle could drive the creation of citrate for lipid synthesis, it may be useful to look more downstream for glutamine incorporation into lipids as a more functional readout for your studies".

We agree that a demonstration of the use of reductive carboxylation for lipid synthesis would be interesting. However, such data are beyond the scope of the current study, whose aim is to show that oxidative TCA cycle flux decreases in transformed cells due to lower PDH flux, and more importantly that glutamine is utilized by transformed cells following the pathways summarized in Fig. 7 of the revised manuscript.

2 "This results of this paper are entirely descriptive, it could be made much stronger by incorporating experiments pharmacologically inhibiting the decoupled TCA-cycle to test if the pathway is required for k-ras dependent tumor cell growth. Previous paper by Weinberg et al in PNAS 2010 manipulated the glutamine pathways to demonstrate the relevance of this pathway".

This point has been addressed by a new series of experiments using specific inhibitors of glutamine metabolism, such as aminooxyacetate (AOA), an inhibitor of aminotransferase activity, and epigallocatechin gallate (EGCG), an inhibitor of glutamate dehydrogenase activity. Both inhibitors have been used to analyze their effects on cell proliferation and survival of the three mouse cell lines described in the re-submitted paper. As shown in Figures 5E, 5F and 6, the behavior of NIH-GEF-DN, in which the oncogenic activity of K- ras is strongly attenuated (Vanoni et al 1999, Bossˇ et al 2000, Chiaradonna et al 2006a, Chiaradonna et al 2006b, Gaglio et al 2009), closely resemble that of normal cells and is strikingly different from that observed in transformed cells, especially in terms of proliferation upon AOA treatment (see Figure 5F) and in terms of cell death upon EGCG treatment (Figure 6). Furthermore, addition of metabolites that arise downstream of glutamine (i.e., dimethyl aspartate -DMD, 1mM- and dimethyl AKG -DMK, 2mM-) in low glutamine growth condition (Figure S9) or in presence of AOA or EGCG, is to a large extent able to restore the proliferation or survival of transformed cells. These findings clearly support the important role of glutamine and hence of its metabolizing enzymes for transformed cells growth and further underline the metabolic dissimilarities between NIH- GEF-DN cells and NIH-K-Ras cells and similarities between NIH-GEF-DN cells and NIH3T3 cells.

Referee #3

Major concerns: 1) Methodological gaps (or at least lack of clarity).
A) "Why are data reported as time series, when the perturbation involves (to the best of my reading) stable expression of k-Ras? What defines time zero"?

Time zero refers to a change of medium. It was necessary to designate such a time point for flux calculations as well as for proliferation analyses and transcriptional data generation. At this time the medium is replaced with a new medium containing 25 mM glucose plus 4 mM glutamine. In this manner we start our analysis when the cells are again actively proliferating and we can measure the glucose consumed and lactate produced.

B) "How were the transcripts normalized? What defines "1"?

This is not a detail: basically I could not effectively understand any of the transcriptional data for lack of clarity on this point. Given this caveat, my impression is that the transcriptional responses are much more varied and complex than the authors claim in the abstract and do not support their conclusions".

The normalization procedure has been added in the material and methods section of the revised paper. However, for Figure 1F, we have preferred to use a linear scale in order to display the absolute expression intensity, where the value "1" represents the normal expression intensity for the probe set of the genes indicated.

C) Were the metabolic experiments performed in dialyzed serum? If not, how did small molecules in serum impact the analysis?

The serum used in these studies was not dialyzed. While some additional small molecules are therefore added to the culture medium, dilution of tracer molecules (e.g. glutamine or glucose) is accounted for within the model by assuming incomplete enrichment of the tracer pool. While free fatty acids and some amino acids might also be present in the medium, the levels at which these cells are taken up are not appreciable or can be accounted for by reactions within the model to accommodate a statistically acceptable fit.
We note that a dilution flux of citrate was included to account for an unlabeled fraction of this metabolite, but otherwise we were able to fit the measured data to the model very well.

2) Validity of flux analysis
A) "The flux analysis is based on a relatively small number of compounds measured only at steady-state. There is limited data (not all isotopic forms) provided for citrate; these other forms are quite useful for understanding TCA fluxes. Without more extensive data, it is hard to know whether flux model is valid, i.e., would additional data (on other compounds or labeling dynamics) match the model? Additional data is probably needed".

As fluxes within the TCA cycle are the focus of this study, we feel that there is adequate coverage of metabolites along this pathway and that a significant number of measurements were included for steady-state MFA. While non-stationary flux analyses using dynamic labeling can also be employed for such experiments, this method requires measurement and/or estimation of metabolite pool sizes and more complicated and therefore less reliable mathematical solutions. Importantly, our system is highly overdetermined, meaning there are many more measurements (MIDs and extracellular fluxes) as compared to degrees of freedom (i.e., fluxes). Furthermore, our fitted data are statistically significant based upon confidence interval calculations and are coherent with the biological findings reported in the manuscript.
It is unclear what is meant by isotopic forms of citrate. We include measurements of 2 citrate fragments for all analyses (more than most other studies, including Biotechnol Bioeng, 2008: Vol. 100 pp. 355ñ370. Identification of metabolic fluxes in hepatic cells from transient C-13-labeling experiments: Part II. Flux estimation.), comprising 24 independent labeling measurements of citrate alone. In our experience, other fragments of citrate have not proven reliable, and we cannot accurately correct for natural abundance based on formulas in unlabeled abstracts. We perform extensive validation on all metabolite fragments used for GC/MS-based MFA, as inclusion of questionable metabolite data compromises model fit. Please also see Antoniewicz et al. Analytical Chemistry 2007 and J Biol Chem, 2009: Vol. 284 no. 48 pp. 33425-33436. Effect of anaplerotic fluxes and amino acid availability on hepatic for some of these validations.

B) It appears in reading the paper that the quantitative TCA fluxes are derived solely using 13C-glutamine labeling data. Hopefully this is just a misunderstanding, but it is essential to see supplementary data showing fit of glucose labeling between model and data, as is shown now for glutamine.

In cultured cell lines (in particular transformed cells) glutamine is a major anaplerotic substrate and provides rich data for TCA cycle labeling. As a result we chose to focus especially on 13C-glutamine as a tracer, since most glucose is diverted to lactate. To
provide supporting evidence for the observed decrease in PDH flux upon K-Ras transformation, we initially included labeling data obtained from cells treated with glucose tracers, which was entirely consistent. We now provide a complete MFA model fit using a combination of [1-13C]glucose and [U-13C6]glucose tracers. Although the absolute fluxes estimated from glucose and glutamine tracers differ slightly, the trends in fluxes are very similar. In particular we see a significant decrease in PDH flux and the oxidative TCA cycle. To our knowledge, no publication has successfully performed equivalent MFA estimations with two independent tracers, and it is not expected that flux sets should match when using such a stringent model. Furthermore, we have previously shown that different tracers allow resolution of specific fluxes in the model, with glutamine providing better confidence intervals within the TCA cycle as compared to glucose tracers (Journal of biotechnology,

2009: Vol. 144 pp. 167-174. Evaluation of 13C isotopic tracers for metabolic flux analysis in mammalian cells.).

Minor comments:
A) "Table 1 is not readily readable due to small size. It is also hard to digest due to information on many unconstrained exchange fluxes which add no useful information to a general reader".

We have clarified Table 1, accordingly by improving resolution and omitting unobservable exchange fluxes. For completeness the entire set of model results is included in the supplement.

B) "In Figure 5, how is decreased gln uptake compatible with unchanged flux from gln to glu from FBA perspective"?

MFA results demonstrate that flux from gln to glu is less in transformed cells. Although less glutamine is taken up by the cells, less glutamate is secreted by the cells (see Figure 1A and MFA results). Therefore, the remaining glu can be metabolized to AKG, and these values are equivalent between the cells tested.

C) "The word robust is misused in the text".

We agree with the suggestion of the reviewer and have corrected the text.

D) "The authors claim in the abstract that they "surprisingly" see non-canonical labeling of aspartate, although actually there are a spate of careful recent papers (starting from seminal work of Kelleher and Stephanopoulos) that have made this pathway (and more recently its significance in cancer) quite clear. Thus, the results regarding IDH2 flux are not surprising at all".

We agree with the suggestion of the reviewer and modified the sentence.

In conclusion, we have made our best effort to consider and answer to all Editor and Referees remarks. A number of experiments have been added to the manuscript to address the refereesí main issues: dependence from K-Ras and from p53, role of this re-routing of mitochondrial metabolism in supporting cellular growth in transformed cells. We feel that the bulk of the obtained results will be found to give stronger ground to the results describing K-Ras-mediated metabolic transformation in this manuscript. With the help of the reviewers, we have also resolved the ambiguities or discrepancies present in the original submission. We hope therefore that now the revised manuscript will be found by you and by the Referees suitable for publication on Molecular Systems Biology. If you have any additional questions or require any clarifications please do not hesitate to contact us. Yours sincerely, Greg Stephanopoulos & Lilia Alberghina

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2nd Editorial Decision   27 May 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the two referees who accepted to evaluate the revised study. As you will see, referee #2 is now supportive. Reviewer #3 continues to find your study interesting but still raises a series of concerns related to the transcriptional analysis and some of the reported flux results:

- The correlation between changes in gene expression and metabolic fluxes seems to be largely overstated. It would therefore be necessary to either document this overall correlation in a quantitative unbiased way or to considerably tone down (in abstract, main text & synopsis) the claims related to the impact of the reorganization of transcriptional activity on the observed metabolic changes. Alternatively, experimental support for the functional relevance of some of the reported gene expression changes may also contribute to address this point.

- Some important discrepancies are noted between several of the metabolic analysis results. These issues should be convincingly explained and resolved.

- The potential limitations associated with some of the methods used should be made clear.

On a more editorial note, we would also ask you to deposit the new microarray data in one of the major public databases and include the respective accession number in the Materials & Methods section.

Thank you for submitting this paper to Molecular Systems Biology.

Yours sincerely,

Editor
Molecular Systems Biology

Referee reports:

Reviewer #2 (Remarks to the Author):

The investigators have done an excellent job of responding to the previous critique. This study is likely to generate lively discussion in the Cancer Metabolism community.

Reviewer #3 (Remarks to the Author):

This interesting paper applies for the first time metabolic flux analysis to examine the effects of an oncogene on intracellular fluxes. This yields the important new conclusion that K-Ras increases dependence of the TCA cycle on glutamine relative to glucose.

Unfortunately, however, the paper continues to contain unsupported conclusions about gene expression - metabolic flux correlation. Moreover, there are persistent methodological issues in both MFA and metabolomic analyses.

1. The abstract claims: "MFA data strongly correlate with gene expression profiling." Actually no correlation is published in the paper, only qualitative description which seems to me highly misleading. Looking at Figure 4A, it is clear that most genes of TCA cycle are up even though decreased TCA flux is a major claim of the paper. Moreover, in Figure 2C under the category "glutamine metabolism" the most strongly up-regulated genes are Anpep (alanine aminopeptidase), Enpp1/3 (ectonucleotide phosphorylases), and Mgst1/2/3 (microsomal glutathione s-transferases). These have nothing obvious to do with glutamine! The MGST genes, for example, are involved in detoxification of electrophilic compounds and relate to the reactive thiol, not nitrogen content, of glutathione.

2. Regarding the MFA, the following concerns were evident in the revision:
(a) In Table 1, there is a zero value reported for pyruvate carboxylase flux. How is M0 aspartate made without any PC activity?
(b) In Figure 1, it looks like ~ 50% of the glutamine uptake is excreted as glutamate. In Table 1, the value is 90%. This is a major discrepancy.
(c) Via the AOA experiments and N labeling studies, a strong argument is made for the importance of aminotransferase activity. However, the MFA indicates that these reaction are inactive. Please explain.

3. The metabolomics assays are poorly described. In the response to reviewers, it is admitted that these assays involved complete (undialyzed serum) and a washing step. Both are likely to perturb metabolome measurements. Minimally being clear about these limitations is necessary.

Please find enclosed the second revision of the manuscript "Oncogenic K-ras decouples glucose and glutamine metabolism to support cancer cell growth" by Daniela Gaglio, Christian M. Metallo, Paulo A. Gameiro, Karsten Hiller, Lara Sala Danna, Chiara Balestrieri, Lilia Alberghina, Gregory Stephanopoulos and Ferdinando Chiaradonna to be re-submitted as a Research Article to Molecular Systems Biology. We have addressed the reviewers concerns as detailed below. In addition, the microarray data have been deposited in the NCBI GEO database (accession ID from GSM741354 to GSM741361 and from GSM741368 to GSM741375). All the samples are included on GSE29962 series with a restricted access. We decided to restrict the access to the data for a few months, because only a portioin of the deposited full transcriptional dataset (4 samples out of 14 samples) is discussed in this manuscript. The complete dataset is available to the editors and referees at the address below:
http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=zhmdvuysekcogrq&acc=GSE29962.
However, if MSB policy requires the data to be freely available we will be happy to release all the data after your communication.
We hope that the revised manuscript will be found by you and the Referees to be suitable for publication in Molecular Systems Biology.

If you have any additional questions or require any clarifications please do not hesitate to contact us.

Response to reviewers:
We thank the reviewers for their comments and have revised the manuscript to address all concerns. Briefly, we have eliminated definitive claims regarding the causative nature of transcriptional changes and toned down comments that alluded to a correlative analysis. We have also removed some of the genes in question from the text and heat map in Figure 2.

We have clarified issues with MFA interpretation and modified Table 1 to improve accessibility to reader, and we have cleared up the discrepancy in extracellular glutamine uptake depicted in Figure 1A. Finally, we have added more detail to the Methods describing metabolomics assays.

Detailed descriptions and explanations for all changes are listed below in blue typed Times font.

Reviewer #2 (Remarks to the Author):

The investigators have done an excellent job of responding to the previous critique. This study is likely to generate lively discussion in the Cancer Metabolism community.

We thank the reviewer for the positive comment.

Reviewer #3 (Remarks to the Author):

This interesting paper applies for the first time metabolic flux analysis to examine the effects of an oncogene on intracellular fluxes. This yields the important new conclusion that K-Ras increases dependence of the TCA cycle on glutamine relative to glucose.

Unfortunately, however, the paper continues to contain unsupported conclusions about gene expression - metabolic flux correlation. Moreover, there are persistent methodological issues in both MFA and metabolomic analyses.

1. The abstract claims: "MFA data strongly correlate with gene expression profiling." Actually no correlation is published in the paper, only qualitative description which seems to me highly misleading. Looking at Figure 4A, it is clear that most genes of TCA cycle are up even though

decreased TCA flux is a major claim of the paper. Moreover, in Figure 2C under the category "glutamine metabolism" the most strongly up-regulated genes are Anpep (alanine aminopeptidase), Enpp1/3 (ectonucleotide phosphorylases), and Mgst1/2/3 (microsomal glutathione s-transferases). These have nothing obvious to do with glutamine! The MGST genes, for example, are involved in detoxification of electrophilic compounds and relate to the reactive thiol, not nitrogen content, of glutathione.

We have considerably toned down the claims in the manuscript regarding the impact of transcriptional changes on the metabolic reprogramming in cells transformed with oncogenic K-Ras. We have changed the depiction of our transcriptional profiling study throughout the manuscript and eliminated specific comments that may have alluded to a correlation analysis or "integration." Some minor grammatical and cosmetic changes have also been made but not listed in detail for brevity.

Abstract: We have removed the statement: "MFA data strongly correlate with gene expression profiling, as cells expressing oncogenic K-Ras exhibited elevated expression of glycolytic, glutamine metabolism and nucleotides synthesis genes as well as deregulation of several TCA cycle related genes."

This sentence now reads: "Transcriptional profiling detected elevated expression of several genes associated with glycolysis, glutamine metabolism, and nucleotide biosynthesis in cells expressing oncogenic K-Ras."

Introduction: In the third paragraph we now highlight the "combined application of metabolic and transcriptional analyses" as opposed to an "integration."

Results:
Transcriptional profiling… section, paragraph 3, page 7
"Taken together these results indicate an important role of gene expression in oncogenic-K-ras driven metabolic reprogramming…" was changed to: "Taken together these these results indicate that numerous genes regulated by oncogenic K-ras may drive the observed metabolic reprogramming, although we cannot exclude the possibility that other mechanisms (e.g. post-translational modifications, mass action, allosteric enzyme regulation) also participate in the observed metabolic alterations of transformed cells."

Transcriptional analysis of glutamine entry into TCA cycle section, paragraph 1, page 8:
We note the following sentence (unchanged) does not suggest such a strong correlation.
"However, while transcriptional data are for the most part consistent with the reduction of glutamine uptake and its use for biosynthesis, not all metabolic fluxes are expected to be only regulated at the transcriptional level."

Transformed cells increase their use of glutamine nitrogenÖ section, paragraph 2, page 9
We have removed genes not associated with GSH metabolism and simplified the claims. We have toned down the wording regarding transcriptional changes: Now we write: "Such transcriptional changes suggest increased activityÖ"

K-Ras transformed human cells show metabolic alterationsÖ section, paragraph 3 and 4, page 11
We have removed one sentence claiming that our transcriptional analysis "confirmed the role of gene expression in cancer cell metabolic alterations." We have revised the following sentence to say that our findings "suggest" that these transcriptional programs "play a role in" rather than "cause coherent metabolic alterations." We have removed the claim of "integration of MFA and transcriptional data" and the claim that flux is "strongly" supported by gene expression changes.

Discussion:
Second paragraph: rather than say that metabolic reprogramming is "substantially linked to a large reorganization of transcriptional activityÖ" we now say "Our analysis also suggests that transcriptional changes contribute to the observed metabolic reprogramming in mouse and human transformed cells."

We have also removed the Anpep, Enpp1/3, and Mgst1/2/3 genes from Figure 2 and the text. These were mentioned on page 9, paragraph 2.

2. Regarding the MFA, the following concerns were evident in the revision:
(a) In Table 1, there is a zero value reported for pyruvate carboxylase flux. How is M0 aspartate made without any PC activity?

We understand the confusion but note to the reviewer that malic enzyme (ME) is a reversible enzyme, and the reverse (exchange) flux executes a reaction identical to PC. Thus, M0 malate, oxaloacetate, and aspartate can be generated.

We have been careful throughout the manuscript to avoid making conclusions regarding "unresolvable" fluxes such as those involved in pyruvate cycling. With the tracers we have utilized it is difficult or impossible to definitively estimate fluxes along this pathway. In actuality, confidence intervals rather than absolute fluxes provide the true reading of our model fits and the PC flux confidence intervals are non-zero. Notably, the confidence intervals for reverse ME flux are identical to that of PC. While we can designate the values for reverse ME as PC, this would be somewhat arbitrary.

(b) In Figure 1, it looks like ~ 50% of the glutamine uptake is excreted as glutamate. In Table 1, the value is 90%. This is a major discrepancy.

We thank the reviewer for identifying this major error and apologize for the confusion. During the initial calculation of the glutamine consumption flux an error was made. Specifically, the time zero measurement was abnormally high (reading over 4.00mM). In actuality the value is approximately 3.6 due to dilution from the serum. Thus, we overestimated the amount consumed and the calculated flux value. All other values remain the same in Figure 1A. This error was identified during the course of our flux modeling, as we were unable to obtain statistically acceptable fits (too much carbon entering the system). Unfortunately Figure 1A was not updated.

We now provide for the reviewer data from an independent experiment highlighting the reproducibility of these results. Extracellular fluxes for glucose, lactate, glutamine, and glutamate are presented below in Reviewer Figure. In addition, we have calculated the ratios of lactate secretion/glucose uptake and glutamate secretion/glutamine uptake for both experiments. The values are quite similar (data from manuscript is on left).

While the data in Table 1 is similar to that in Figure 1A, the values should not necessarily match exactly. Flux data in Table 1 are estimated values obtained from the fit and confidence intervals. The measured data (Figure 1A) are incorporated to the model, but the ultimate flux values are adjusted to minimize the sum of all squared errors, including all labeling and measured flux values. Therefore, the values may differ slightly to obtain the best fit while taking into account penalties in the form of residual errors.

(c) Via the AOA experiments and N labeling studies, a strong argument is made for the importance of aminotransferase activity. However, the MFA indicates that these reactions are inactive. Please explain.

The aminotransferase reactions are highly active in our model, as evidence by the high exchange flux between Glu and AKG. The AOA experiment in particular implicates aminotransferase activity involved in the malate-aspartate shuttle. Operation of this shuttle involves no net change of carbon (or net flux reactions); rather, simply high forward and reverse fluxes are required (in the cytosol and mitochondria). However, the exchange flux should be quite high. In our network we capture the reversibility of aminotransferase reactions with the following reaction: (Glu   AKG), as several amino acids can be used for this reaction. The flux values (and confidence intervals) for these reactions are quite high, indicative of the importance of aminotransferase activity. Our model is not compartmentalized so we simply observe high exchange fluxes of the overall reaction, which is consistent with our findings in the AOA experiment.

The net fluxes to Asp and Ala provide a readout of flux to biomass, and the values for these biosynthetic fluxes are low relative to other fluxes. In reformatting Table 1 during the last revision we made a rounding error such that the values read zero (and did not match the full data set in the

supplement). We have now corrected these values.

3. The metabolomics assays are poorly described. In the response to reviewers, it is admitted that these assays involved complete (undialyzed serum) and a washing step. Both are likely to perturb metabolome measurements. Minimally being clear about these limitations is necessary.

We apologize for the omission. We have now included these details in the Methods section of the manuscript, highlighting the inclusion of dialyzed serum, use of a norvaline internal standard, and washing step, which is important to minimize the contribution of spent culture medium to the intracellular metabolite signals.