Glucose deprivation activates a metabolic and signaling amplification loop leading to cell death

Nicholas A. Graham, Martik Tahmasian, Bitika Kohli, Evangelia Komisopoulou, Maggie Zhu, Igor Vivanco, Michael A. Teitell, Hong Wu, Antoni Ribas, Roger S. Lo, Ingo K. Mellinghoff, Paul S. Mischel, Thomas G. Graeber

Corresponding author: Thomas G Graeber, UCLA

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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 22 November 2011

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 find the topic of your study of potential interest. They raise, however, substantial concerns on your work, which, I am afraid to say, preclude its publication in its present form.

Each of the reviewers recognized that this work could potentially provide some important insights into the molecular systems underlying the glucose deprivation response in cancer cells. They each indicated, though, that key aspects of this work would need to be supported with additional experiments and controls before this work would provide the kind of conclusiveness that would be expected in Molecular Systems Biology. One important issue appears to be the strength of the evidence supporting ROS production from NADPH oxidases. The third reviewer felt that a role for mitochondrial ROS production had not been rigorously excluded, and the other two reviewers both raised important points that seem to be related to this issue (point #2 from Reviewer #2 and Reviewer #1's major point). Some of the other points raised by the reviewers may also require additional experimental work (e.g. point #1 from Reviewer #2, and points 3-5 from Reviewer #3).

On a more editorial note, when submitting your revised work please provide the figures as higher-resolution image files. The text and line-art within the figures is noticeably blocky/blurry in several cases when zooming in. You will get the best results if the figures are made directly in a professional quality vector graphics program like Illustrator or the free, opensource alternative Inkscape, and saved directly as EPS files.

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.

PLEASE NOTE As part of the EMBO Publications transparent editorial process initiative (see http://www.nature.com/msb/journal/v6/n1/full/msb201072.html), Molecular Systems Biology now publishes online a Review Process File with each accepted manuscript. Please be aware that in the event of acceptance, your cover letter/point-by-point document will be included as part of this file, which will be available to the scientific community. Authors may opt out of the transparent process at any stage prior to publication (contact us at msb@embo.org). More information about this initiative is available in our Instructions to Authors.

Sincerely,

Editor - Molecular Systems Biology
msb@embo.org

Referee reports:

Reviewer #1 (Remarks to the Author):

This manuscript by Graham et al. is a nice study advancing our understanding of cell death caused by glucose deprivation. The novelty of this study is that the authors reveal how the inflexible dependence of cancer cells on glucose makes cancer cells vulnerable to glucose deprivation. They identify tyrosine hyperphosphorylation as a common phenomenon using a nicely designed experimental strategy to carry out phosphoproteome profiling. This manuscript should be of interest to multiple areas in cancer including apoptosis, autophagy, glucose metabolism and systems modeling of homeostasis. Overall, I think this paper is suitable for publication in Molecular Systems Biology after the following issues are addressed:

Major point:

The correlation between tyrosine hyperphosphorylation and NADPH oxidase is demonstrated only in one cell line sensitive to glucose deprivation (Figure 5C and Supplemental Figure S8B). It would be nice to see this important finding validated in other cell lines of the similar properties such as LIN18, TC32 and M202 used in this manuscript.

Minor points:

1. In Figure 1G, the loading control (e.g. actin) is not provided.
2. In Figure 3D, the error bars for EPHA2 pY588 & pY594 are not symmetric.

Reviewer #2 (Remarks to the Author):

General comments
The findings presented in the manuscript by Graham et al. contribute to the understanding of the mechanisms that lead to cell death in glucose-addicted cancer cells. The authors describe a general increase in p-Tyr levels in glucose-dependent cancer cells deprived of glucose but not in those that do not need glucose for survival. In glucose-dependent cells ROS levels seem higher and somehow contribute to cell death. The findings are interesting, but in its present form the study raises some concerns.

Specific comments:
1. Figure 4. The probe DCFDA can be activated during apoptosis and therefore is not necessarily

appropriate for measuring ROS levels in dying cells. The gating performed prior to the analysis of the fluorescent signal might not remove cells in early phases of apoptosis from the analysis. The authors should demonstrate differential ROS generation in both contexts by using an additional method not biased by cell death, or at least demonstrate that the increased levels of fluorescence persist after inhibition of cell death.

2. Figure 5. It is unclear why only a p-Tyr band of unknown identity is shown in B and C. Since DPI is not a NOX specific inhibitor, in order to demonstrate that Nox enzymes are mediating the increase ROS generation a more direct approach should also be used, for instance targeting of p22phox or Nox1/4.

3. The authors propose that the cross-talk between metabolic regulation and cellular signaling in glucose sensitive cells is at the level of the PPP, but they do not address that hypothesis experimentally. It would add interest to the paper if an experimental connection between NADPH depletion, ROS generation and glucose withdrawal is made. And the authors should also explain how depletion of the essential Nox cofactor NADPH can activate Nox.

4. The manuscript describes the interesting finding that glucose deprivation induces a p-Tyr signature associated with focal adhesions, but the authors do not connect this finding with the induction of cell death. Are these two phenomena related? Does it have anything to do with anoikis?

5. In several figure legends the authors explain that cells used in the experiment were deprived of pyruvate in addition to glucose. This is confusing and raises questions that are only addressed in the Material and Methods section. The reason for concomitant pyruvate and glucose deprivation should be addressed in the main text rather than the M&M section.

Reviewer #3 (Remarks to the Author):

Overall:
The authors report very interesting and provocative data to support the hypothesis that Glucose Deprivation activates a signaling loop that involves metabolic oxidative stress mediated by NADPH oxidases that is selectively cytotoxic to a variety of cancer vs. normal cell types including GBM cells. This is a potentially very significant finding since GBM prognosis is dismal and these observations could lead to the development of new therapies targeted at fundamental differences in oxidative metabolism between cancer vs, normal cells. Once the authors have rigorously responded to the following specific comments this work could represent a very significant contribution to the literature.

Specific Comments:
1) Tyrosine kinase activation by glucose deprivation in breast cancer cells accompanied by increased ERK1/2, JNK, and Lyn kinase activation as been reported in human breast cancer cells (J. Biol. Chem. 1998, 273:5294-5299; Free Radic. Biol. Med. 1999, 26:419-430; Free Radic. Biol. Med. 2000, 28:575-584). The current work is unique in that these observations can now be extended to several different and important human cancer cell types including GBM, but some reference to the previous literature should be included in the discussion.
2) In the recent literature the notion that Glucose deprivation-induced oxidative stress is at least partially mediated by mitochondrial ROS is firmly established (J. Biol. Chem. 2005, 280:4254-4263; Cancer Biol Ther 2009, 8:1228-36). In the current report the authors argue the source of ROS responsible for glucose deprivation-induced killing and oxidative stress is an NADPH oxidase enzyme based on data using 10 uM of the non-specific flavin oxidase inhibitor (DPI) and the fact that rotenone (a complex I blocker of electron transport) did not did not protect. This argument is flawed because at 10 uM, DPI will also inhibit flavins in mitochondria and rotenone enhances (not inibits) ROS production from complex I (Cancer Biol Ther 2009, 8(13):1228-36; J. Biol. Chem. 2001, 276:29251-29256). It is possible that both NADPH-oxidases and mitochondria contribute to a feed forward loop of prooxidant production during glucose deprivation similar to what has been suggested for H2O2 (J.Biol.Chem. 2001, 276:29251-29256), but the authors need to use a more specific method of inhibiting NADPH-oxidase activity by targeting the P22phox or specific NOX subunits using antisense oligonucleotides or siRNA (J. Biol. Chem. 2001, 276:29251-29256; Cancer Res. 2011, 71(11):3932-40) to clearly show the involvement of NADPH oxidase. As it stands now,

it is equally likely that mitochondria are involved as NADPH oxidases, and the authors need to recognize this and design experiments to differentiate between these possibilities or state the uncertainty.
3) Did superoxide in mitochondria as determined by mitoSOX oxidation change?
4) Did Peg-SOD plus Peg-catalase protect better than either enzyme alone? When non-pegylated forms of catalase were used did it enter cells? MnTMPyP scavenges both superoxide and hydrogen peroxide (Arch Biochem Biophys. 1997, 347:256-62), so the authors should use the pegylated SOD and catalase enzymes to determine specifically the involvement of superoxide and H2O2 in the observed effects.
5) Did the authors get similar results when clonogenic cell survival was utilized to assess toxicity?

1st Revision - authors' response 10 April 2012

We would like to thank the reviewers for their highly constructive comments regarding our manuscript. We are encouraged that they found our data "very interesting and provocative," and we appreciate Reviewer #3's comment that our observations are "a potentially very significant finding… [which] could lead to the development of new therapies targeted at the fundamental differences in oxidative metabolism between cancer and normal cells." The experiments that they suggested have provided valuable new data to support and extend our model, substantially improving the manuscript.

First, we have strengthened the evidence that NADPH oxidase (NOX)-mediated reactive oxygen species (ROS) mediate glucose withdrawal-induced phospho-tyrosine signaling. Specifically, we demonstrate using siRNA that knockdown of the NOX subunit p22phox attenuates glucose withdrawal-induced phospho-tyrosine signaling (Fig. 6B). In addition, we now show in multiple cell lines that glucose withdrawal-induced phospho-tyrosine signaling is completely abrogated by as little as 1 micromolar of the flavo-protein inhibitor DPI (Fig. 6A).

Next, as suggested by Reviewer #3, we have explored the contribution of mitochondrial ROS to glucose withdrawal-induced phospho-tyrosine signaling. Using the mitochondrial superoxide sensor mitoSOX, we found that glucose withdrawal-sensitive but not -insensitive cells exhibit a dramatic upregulation of mitochondrial superoxide production in response to glucose withdrawal (Fig. 4D-F). We also show that rho-zero cells, which lack a functional mitochondrial electron transport chain, do not exhibit tyrosine hyper-phosphorylation following glucose starvation, whereas the parental cells do (Fig. 6D). Taken together, our data support a model whereby both NOX and mitochondria contribute to glucose withdrawal-induced signaling feedback loop (Fig. 8).

Finally, because our model describes a positive feedback loop between ROS generation, tyrosine phosphatase inhibition and increased tyrosine kinase signaling, we tested whether chemical inhibition of tyrosine phosphatases (ie, vanadate) could *synergize* with glucose withdrawal to kill glucose withdrawal-sensitive cancer cells. Indeed, in U87 glioblastoma cells, we demonstrate a synergistic cell death in response to vanadate treatment and glucose withdrawal (Fig. 7C-D). This synergistic effect illustrates the highly interconnected nature of the metabolic and signaling network underlying glucose deprivation-induced cell death.

Reviewer #1

This manuscript by Graham et al. is a nice study advancing our understanding of cell death caused by glucose deprivation. The novelty of this study is that the authors reveal how the inflexible dependence of cancer cells on glucose makes cancer cells vulnerable to glucose deprivation. They identify tyrosine hyperphosphorylation as a common phenomenon using a nicely designed experimental strategy to carry out phosphoproteome profiling. This manuscript should be of interest to multiple areas in cancer including apoptosis, autophagy, glucose metabolism and systems modeling of homeostasis. Overall, I think this paper is suitable for publication in Molecular Systems Biology after the following issues are addressed:

Major point:

The correlation between tyrosine hyperphosphorylation and NADPH oxidase is demonstrated only in one cell line sensitive to glucose deprivation (Figure 5C and Supplemental Figure S8B). It would be nice to see this important finding validated in other cell lines of the similar properties such as LIN18, TC32 and M202 used in this manuscript.

As suggested, we have validated the effect of DPI on two additional glucose withdrawal-sensitive cell lines (LN18 and T98) (Fig. 6A). Please also note that upon a ten-fold reduction in the concentration of DPI (from 10 micromolar in our initial submission to 1 micromolar in this revised manuscript), the substantial inhibition of tyrosine hyperphosphorylation following glucose withdrawal in U87 cells is retained (Fig. 6A).

In addition to this pharmacological data, we have used siRNA targeting p22phox to validate the role of NOX in glucose withdrawal-mediated tyrosine hyperphosphorylation. In cells with reduced p22phox expression (>90% knockdown), we demonstrate the attenuation of glucose withdrawal-induced tyrosine phosphorylation, confirming a role for NOX in our system (Fig. 6B).

Minor points:

1. In Figure 1G, the loading control (e.g. actin) is not provided.

We have re-done the blot for Fig. 1G and included actin as a loading control.

2. In Figure 3D, the error bars for EPHA2 pY588 & pY594 are not symmetric.

This errors bars on the previous Fig. 3D were the standard deviation of the non-log transformed data. Because the y-axis of Fig. 3D is plotted logarithmically, the error bars did not appear symmetric. However, because the data is more Guassian upon log transformation, we have recalculated the error bars (ie, the standard deviation) using the log transformed data, which does make them symmetric in the revised Fig. 3D.

We have made the same changes to Supplemental Figs. S6C and S12B.

Reviewer #2

General comments
The findings presented in the manuscript by Graham et al. contribute to the understanding of the mechanisms that lead to cell death in glucose-addicted cancer cells. The authors describe a general increase in p-Tyr levels in glucose-dependent cancer cells deprived of glucose but not in those that do not need glucose for survival. In glucose-dependent cells ROS levels seem higher and somehow contribute to cell death. The findings are interesting, but in its present form the study raises some concerns.

Specific comments:
1. Figure 4. The probe DCFDA can be activated during apoptosis and therefore is not necessarily appropriate for measuring ROS levels in dying cells. The gating performed prior to the analysis of the fluorescent signal might not remove cells in early phases of apoptosis from the analysis. The authors should demonstrate differential ROS generation in both contexts by using an additional method not biased by cell death, or at least demonstrate that the increased levels of fluorescence persist after inhibition of cell death.

The ROS induction that we observe following glucose and pyruvate starvation in glucose withdrawal-sensitive cells is detectable at early time points at which cells are not irreversibly committed to cell death (eg, 1-3 h). At these early times, one can rescue viability by re-supplementing starved cells with glucose and pyruvate (Supplemental Fig. S7B). We have made a note of this in the manuscript (page 9, first full paragraph):

"Notably, increased ROS levels occurred at times when cells could be rescued from glucose withdrawal-induced cell death by re-supplementation with glucose and pyruvate (Supplemental Fig. S7B)."

This result supports that the ROS generation at the reported times (3 h in Fig. 4 and Supplemental Fig S7) is independent of cell death.

In the cell death process invoked by glucose withdrawal, we have detected neither activation of caspases 3, 7 and 9 nor cleavage of PARP (not shown). Additionally, the caspase inhibitor Z-VAD-FMK did not rescue cells from glucose withdrawal-induced cell death (not shown). These results suggest that the cells are dying through a non-apoptotic mechanism. The successful mechanisms that we have found for protecting cells against glucose withdrawal-induced death all directly quench ROS (eg, catalase, MnTMPyP), and thus we have not identified an approach to test for increased DCF-DA fluorescence after inhibition of cell death.

Finally, we have now included a second oxidation sensitive probe, the mitochondrially-targeted superoxide sensor mitoSOX, which shows differential ROS generation in glucose withdrawal-sensitive cell lines (eg, LN18, T98, U87). The ROS induction trends measured using mitoSOX are consistent with DCF-DA (Fig. 4 and Supplemental Fig. S7D).

2. Figure 5. It is unclear why only a p-Tyr band of unknown identity is shown in B and C. Since DPI is not a NOX specific inhibitor, in order to demonstrate that Nox enzymes are mediating the increase ROS generation a more direct approach should also be used, for instance targeting of p22phox or Nox1/4.

As suggested, we used siRNA targeting p22phox to validate the role of NOX in glucose withdrawal-mediated tyrosine hyperphosphorylation. In cells with reduced p22phox expression (>90% knockdown), we demonstrate the attenuation of glucose withdrawal-induced tyrosine phosphorylation, confirming a role for NOX in our system (Fig. 6B).

In the previous Fig. 5B and 5C, the band of unknown identity is EGFRvIII (approx 155 kDa). In U87-EGFRvIII cells, this is the most highly phosphorylated protein in both basal and glucose-starved conditions (Fig 1C and 1E). To avoid confusion, we have added a) an arrow on Fig. 5B and b) specific text in the figure legend to denote that the band is EGFRvIII. The added text can be found on page 35 in the figure legend for Figure 5:

"Western blotting revealed that MnTMPyP treatment reduced tyrosine phosphorylation of EGFRvIII (~155 kDa, the most prominent band in the phospho-tyrosine Western blot),following glucose withdrawal."

For the previous Fig. 5C, we have re-done the experiment using LN18, T98 and U87 to confirm our initial experiments using U87-EGFRvIII cells. Western blotting with an anti-phospho-tyrosine antibody shows that multiple phospho-tyrosine bands are induced by glucose withdrawal and inhibited by DPI treatment. These Western blot results are now presented in Fig. 6A.

3. The authors propose that the cross-talk between metabolic regulation and cellular signaling in glucose sensitive cells is at the level of the PPP, but they do not address that hypothesis experimentally. It would add interest to the paper if an experimental connection between NADPH depletion, ROS generation and glucose withdrawal is made. And the authors should also explain how depletion of the essential Nox cofactor NADPH can activate Nox.

In our initial submission, we had proposed that NADPH depletion could play a critical role in the observed tyrosine hyperphosphorylation following glucose withdrawal. This assertion was based on other reports that glucose withdrawal can reduce NADPH levels (Ahmad et al, J. Biol. Chem (2005); 280; 4254-4263); however, because we do not have any direct measurements of NADPH to support this conclusion, we have removed the PPP from our model (Fig. 8) and removed references to NADPH depletion from the text.

4. The manuscript describes the interesting finding that glucose deprivation induces a p-Tyr signature associated with focal adhesions, but the authors do not connect this finding with the induction of cell death. Are these two phenomena related? Does it have anything to do with anoikis?

The reviewer makes an insightful point here. We are also intrigued by the potential connection between glucose withdrawal-induced phospho-tyrosine signaling at focal adhesions and cell death following matrix detachment (ie, anoikis). To highlight these issues, we have included a reference in the discussion to a recent publication describing how matrix detachment can induce the loss of glucose transporters and oxidative stress in normal epithelial cells (page 16, first full paragraph):

"In light of our data demonstrating that glucose withdrawal-induced phospho-tyrosine signaling is driven by focal adhesions, it is interesting to note that normal epithelial cells exhibit loss of glucose transporters and oxidative stress following detachment from extracellular matrix (Schafer et al, 2009)."

A full examination of the potential connection between glucose withdrawal-mediated cell death and anoikis is beyond the scope of this manuscript.

5. In several figure legends the authors explain that cells used in the experiment were deprived of pyruvate in addition to glucose. This is confusing and raises questions that are only addressed in the Material and Methods section. The reason for concomitant pyruvate and glucose deprivation should be addressed in the main text rather than the M&M section.

To avoid confusion, we have added the details regarding pyruvate starvation to the main text of the manuscript (page 5, first paragraph):

"To investigate the signaling mechanisms that underlie rapid cell death following glucose withdrawal (ie, glucose "addiction"), we tested the response of four GBM cell lines (LN18, LN229, T98, U87MG) to withdrawal of glucose and the glycolytic product pyruvate which may serve as an alternate substrate for the TCA cycle (Yang et al, 2009). Within 24 h of glucose and pyruvate withdrawal, LN18, T98 and U87MG exhibited rapid cell death, whereas LN229 cells exhibited only a minor (~15%) loss of viability (Fig. 1A), consistent with previous reports (Elstrom et al, 2004). In our systems, standard pyruvate levels (110 mg/L) did not rescue glucose starvation-induced cell death (not shown). "

Additionally, we have been careful to state where possible (eg, figure legends) that cells were starved of both glucose and pyruvate.

Reviewer #3

Overall:
The authors report very interesting and provocative data to support the hypothesis that Glucose Deprivation activates a signaling loop that involves metabolic oxidative stress mediated by NADPH oxidases that is selectively cytotoxic to a variety of cancer vs. normal cell types including GBM cells. This is a potentially very significant finding since GBM prognosis is dismal and these observations could lead to the development of new therapies targeted at fundamental differences in oxidative metabolism between cancer vs, normal cells. Once the authors have rigorously responded to the following specific comments this work could represent a very significant contribution to the literature.

Specific Comments:
1) Tyrosine kinase activation by glucose deprivation in breast cancer cells accompanied by increased ERK1/2, JNK, and Lyn kinase activation as been reported in human breast cancer cells (J. Biol. Chem. 1998, 273:5294-5299; Free Radic. Biol. Med. 1999, 26:419-430; Free Radic. Biol. Med. 2000, 28:575-584). The current work is unique in that these observations can now be extended to several different and important human cancer cell types including GBM, but some reference to the previous literature should be included in the discussion.

We have included these references in the discussion section of the manuscript (page 14, first paragraph):

"Building on the unexpected observation that glucose withdrawal induces supra-physiological levels of phospho-tyrosine signaling, our systems-level feedback amplification loop model integrates the observations that a) glucose withdrawal induces oxidative stress (Aykin-Burns et al, 2009; Spitz et al, 2000) and can activate diverse intracellular kinases including ERK, JNK and Lyn (Blackburn et al, 1999; Lee et al, 1998b; Lee et al, 2000)…"

And also a second time on page 15, last full paragraph:

"The diminished activity of PTPs following glucose starvation (Fig. 7) is also consistent with the observation by us and others that serine/threonine MAPK signaling is induced by glucose withdrawal (Fig. 3B) (Blackburn et al, 1999; Lee et al, 1998b; Lee et al, 2000), as dual-specificity phosphatases (DUSPs), which dephosphorylate MAPK enzymes, can be inhibited by oxidation of the catalytic cysteine residue (Kamata et al, 2005)."

2) In the recent literature the notion that Glucose deprivation-induced oxidative stress is at least partially mediated by mitochondrial ROS is firmly established (J. Biol. Chem. 2005, 280:4254-4263; Cancer Biol Ther 2009, 8:1228-36). In the current report the authors argue the source of ROS responsible for glucose deprivation-induced killing and oxidative stress is an NADPH oxidase enzyme based on data using 10 uM of the non-specific flavin oxidase inhibitor (DPI) and the fact that rotenone (a complex I blocker of electron transport) did not did not protect. This argument is flawed because at 10 uM, DPI will also inhibit flavins in mitochondria and rotenone enhances (not inibits) ROS production from complex I (Cancer Biol Ther 2009, 8(13):1228-36; J. Biol. Chem. 2001, 276:29251-29256). It is possible that both NADPH-oxidases and mitochondria contribute to a feed forward loop of prooxidant production during glucose deprivation similar to what has been suggested for H2O2 (J.Biol.Chem. 2001, 276:29251-29256), but the authors need to use a more specific method of inhibiting NADPH-oxidase activity by targeting the P22phox or specific NOX subunits using antisense oligonucleotides or siRNA (J. Biol. Chem. 2001, 276:29251-29256; Cancer Res. 2011, 71(11):3932-40) to clearly show the involvement of NADPH oxidase. As it stands now, it is equally likely that mitochondria are involved as NADPH oxidases, and the authors need to recognize this and design experiments to differentiate between these possibilities or state the uncertainty.

To address the reviewer's concerns regarding whether NOX and/or mitochondria are responsible for ROS generation following glucose withdrawal, we have pursued three lines of experimentation:

1. Using siRNA, we demonstrate that knockdown of the NOX subunit p22phox (>90% efficiency) attenuated phospho-tyrosine signaling in U87 cells following glucose withdrawal (Fig. 6B).
2. We tested whether rho-zero cells, which cannot generate mitochondrial superoxide due to a defective electron transport chain, can induce tyrosine hyper-phosphorylation following glucose and pyruvate starvation. Notably, three cell lines (T98, U87 and 143B.TK-) showed that whereas parental cells exhibit ROS-mediated cell death and glucose withdrawal-induced phospho-tyrosine signaling, their rho-zero derivatives did not (Fig. 6D), demonstrating the contribution of mitochondrial superoxide.
3. We show that a ten-fold reduction in the concentration of DPI (1 micromolar) ablated glucose withdrawal-induced phospho-tyrosine signaling as effectively as the 10 micromolar dose used in our initial submission (Fig. 6A).

Taken together, this data validates the reviewer's hypothesis that both NOX and mitochondria are involved in ROS-mediated cell death and phospho-tyrosine signaling following glucose and pyruvate starvation. We have updated our model (Fig. 8) to reflect the contribution of both sources of ROS to tyrosine hyper-phosphorylation following glucose withdrawal.

We would like to thank Reviewer #3 for pointing out reports that rotenone can increase mitochondrial superoxide production in some cell systems (Cancer Biol Ther 2009, 8(13):1228-36; J. Biol. Chem. 2001, 276:29251-29256). Since the response to rotenone cannot be unambiguously

interpreted and because our experiments demonstrate that glucose withdrawal increases mitochondrial ROS production, we have removed the rotenone-treatment data from the Supplemental Figures.

3) Did superoxide in mitochondria as determined by mitoSOX oxidation change?

Using the mitochondrial superoxide probe mitoSOX, we found that glucose withdrawal-sensitive cells exhibit upregulation of mitochondrial ROS superoxide levels following glucose withdrawal whereas glucose withdrawal-insensitive cells exhibit only a minor increase (Fig. 4D-F and Supplemental Fig. S7D). This result is consistent with the observation that rho-zero cells do not exhibit upregulation of phospho-tyrosine signaling (Fig. 6D).

4) Did Peg-SOD plus Peg-catalase protect better than either enzyme alone? When non-pegylated forms of catalase were used did it enter cells? MnTMPyP scavenges both superoxide and hydrogen peroxide (Arch Biochem Biophys. 1997, 347:256-62), so the authors should use the pegylated SOD and catalase enzymes to determine specifically the involvement of superoxide and H2O2 in the observed effects.

PEG-catalase effectively protected U87 cells against glucose withdrawal-induced cell death (data not shown), consistent with the effects of non-pegylated catalase (Fig. 5C). Addition of PEG-SOD, however, did not increase cell survival following glucose withdrawal (data not shown). We have not explicitly tested whether non-pegylated catalase entered cells, but the literature suggests that it does not (Beckman et al, J. Biol. Chem. (1998); 263**;** 6884-6892).

We have added the following text regarding the effects of PEG-catalase (page 11, first paragraph):

"PEG-conjugated catalase, which can enter cells by endocytosis (Beckman et al, 1988), also protected cells against glucose withdrawal-induced cell death (data not shown)."

We have altered the text of the manuscript to reflect that MnTMPyP is a non-specific antioxidant, rather than a superoxide dismutase mimetic (page 10, second paragraph):

"We next tested the effects of a redox active manganic porphyrin (MnTMPyP) that can protect cells against oxidative stress and found that glucose withdrawal-induced activation of EGFRvIII was strongly attenuated in U87-EGFRvIII cells (Fig. 5B)."

And also in the legend of Figure 5 (page 35):

"U87-EGFRvIII cells were starved of glucose and pyruvate for 3 h in the presence of either DMSO or the antioxidant MnTMPyP (25 mM)."

5) Did the authors get similar results when clonogenic cell survival was utilized to assess toxicity?

For our experiments, we have used trypan blue exclusion to demonstrate differential sensitivity to glucose withdrawal in sensitive (eg, LN18, T98, U87) and insensitive cell lines (eg, LN229, M229) at 24 h following glucose and pyruvate withdrawal. Notably, even the cell lines that are most resistant to glucose withdrawal do exhibit loss of viability 3-6 days after glucose and pyruvate starvation, thus precluding the implementation of relatively long-term (7-14 days) clonogenic assays. Because of this limitation, we have not pursued these experiments.

2nd Editorial Decision 04 May 2012

Thank you again for submitting your revised work to Molecular Systems Biology. We have now heard back from the two referees who agreed to evaluate the study. As you will see, the referees felt that the changes made to this work had satisfied their concerns and they are now supportive of publication. Before we can formally accept this work for publication, however, we have a few minor issues related to data presentation and formatting which we would ask you to address in a final revision of this work.

1. Journal policy generally discourages the presentation of error bars derived from technical replicates. Please see our policies on the presentation of statistical analyses in our Instructions for Authors for more information (www.nature.com/msb/authors/index.html#a3.4.3).

2. In addition to our capacity to host datasets in our supplementary information section, we provide a new functionality that allows readers to directly download the 'source data' associated with selected figure panels (e.g. <http://tinyurl.com/365zpej>). This sort of figure-associated data may be particularly appropriate for some of the figures in this work, and would be a good way to provide replicate measurements (both technical and biological, please label each clearly in the source files). Please see our Instructions of Authors for more details on preparation and formatting of figure source data (<http://www.nature.com/msb/authors/index.html#a3.4.3>).

3. Please provide a single Supplementary Information pdf file that includes all of the Supplementary Figures, with the figure legends immediately below the appropriate figures. This file should begin with a Table of Contents listing the Supplementary Figures. The Supplementary figure legends can then be removed from the main manuscript.

4. Please include the PRIDE accession number for the proteomic data in the Methods section of the main manuscript.

Thank you for submitting this paper to Molecular Systems Biology.

Sincerely,

Editor - Molecular Systems Biology
msb@embo.org

Referee reports:

Reviewer #2 (Remarks to the Author):

The authors have satisfactorily addressed all of my concerns.

Reviewer #3 (Remarks to the Author):

The authors have satisfactorily responded to the previous critique by including appropriate new data. The manuscript is now acceptable for publication.

2nd Revision - authors' response 10 May 2012

1. Journal policy generally discourages the presentation of error bars derived from technical replicates. Please see our policies on the presentation of statistical analyses in our Instructions for Authors for more information (www.nature.com/msb/authors/index.html#a3.4.3).

We have removed the technical replicate error bars from Figures 3D, S6C and S12B.

2. In addition to our capacity to host datasets in our supplementary information section, we provide a new functionality that allows readers to directly download the 'source data' associated with selected figure panels (e.g. <http://tinyurl.com/365zpej>). This sort of figure-associated data may be particularly appropriate for some of the figures in this work, and would be a good way to provide replicate measurements (both technical and biological, please label each clearly in the source files). Please see our Instructions of Authors for more details on preparation and formatting of figure source data (<http://www.nature.com/msb/authors/index.html#a3.4.3>).

We have provided source data for Figures 3C, 7A, 7B and 7C. Note that the source data for Figures 3D, S6C and S12B is included in the source data for Figure 3C, since these figures are particular examples from the full data set.

3. Please provide a single Supplementary Information pdf file that includes all of the Supplementary Figures, with the figure legends immediately below the appropriate figures. This file should begin with a Table of Contents listing the Supplementary Figures. The Supplementary figure legends can then be removed from the main manuscript.

We provided this Supplementary Information file as requested.

4. Please include the PRIDE accession number for the proteomic data in the Methods section of the main manuscript.

We have added the PRIDE accession numbers on page 22 of the Methods:
"Mass spectra have been deposited in the PRIDE database (http://www.ebi.ac.uk/pride/, accession number 19835-19854)."

