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The GCN2-ATF4 pathway is critical for tumor cell survival and proliferation in response to nutrient deprivation

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1st Editorial Decision 12 October 2009

Thank you for submitting your manuscript to the EMBO Journal. I am sorry for the slight delay in getting back to you, but I have now heard back from the three referees. As you can see below, the referees appreciate the analysis on the role of the GCN2-eIF2a-ATF4 pathway in tumor development in response to nutrient deprivation and find it very interesting. However it is also clear that more work is needed in order to consider publication here. There are some different technical concerns raised, but the analysis also needs to be taken further in particular with respect to discern the role of GCN2 and PERK in tumor development. Should you be able to address the concerns raised in full, then we would be willing to consider a revised manuscript. Acceptance of your paper will be dependent upon persuading the referees that you have provided a sufficient amount of new data to answer all their criticisms. I should also add that it is EMBO Journal policy to allow a single round of revision only and it is therefore important to fully resolve the concerns at this stage if you wish the manuscript ultimately to be accepted.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This manuscript by Koumenis and colleagues discovers a major role for the GCN2-eIF2 α -ATF4 pathway in tumor development. The manuscript shows that knock-down of ATF4 expression upregulates p21 expression, prevents asparagine synthetase expression, and activates a cytoprotective autophagic response. The inability of tumor cells to upregulate this pathway in response to amino acid starvation results in apoptosis. Consistent with this important linkage between the GCN2-eIF2α-ATF4 pathway and tumor progression, human and mouse tumors were shown to overexpress GCN2 protein kinase resulting in increased eIF2 phosphorylation and increased ATF4 expression. Importantly, the manuscript provides evidence for a role in the nutrient-regulated eIF2 kinase GCN2 and provides mechanistic evidence that ATF4-mediated expression of asparagine synthetase is the major defect. The manuscript also incorporates several rescue experiments which in most cases only partially rescue phenotypic defects, suggesting that multiple mechanisms may be involved in the underlying block in cell growth. Overall, there is much enthusiasm for this manuscript. The study is significant, furthering our understanding of the role of eIF2 kinases and nutrient sensing in tumor development, and is of broad interest. There are only minor concerns for the authors' consideration.

Reviewer concerns:

1) Fig. 1: The authors showed that cell survival as measured by MTT assay following 48 hours of culture was reduced by ~60% in HT1080 and DLD1 cells and in the case of HT1080 cells, could be partially recovered by the addition of NEAA. The authors attribute this defect in cell survival to a G1/S arrest and reduced cell proliferation in ATF4 knock-down cells. However, knock-down of ATF4 only reduced proliferation as measured by EdU incorporation by 35%. Have the authors ruled out other possible defects such as adherence to plastic, etc? Do equal numbers of cells adhere to the plastic dish at the onset of the cell survival assay? A simple growth curve comparing control vs. ATF4 knock-down cells would also address this issue.

2) Fig. 3B: The resolution of the images makes it somewhat difficult to discern between increased formation of autophagosomes or increased vacuolarization. As has been described in other studies, incorporation of GFP-LC3 may more convincing.

3) Fig. 3D: The Knock-down of ATG7 reduces cell survival in ATF4 knock-down cells but no control is included showing that knock-down of ATG7 in HT1080 cells indeed affects autophagy. Does knock-down of ATG7 reduce the increased LC-3II levels in ATF4 knock-down cells? In an earlier figure, the knock-down of ATF4 results in reduced cell survival. Presumably knock-down of ATG7 in ATF4 knock-down cells reduced viability even further; although, this cannot be determined with the data presented as is.

4) In the discussion, the authors may wish to compare (differences/overlap) the consequence of phosphorylation of eIF2 induced by GCN2 during nutrient deficiency and that elicited by hypoxia. This laboratory has suggested both contribute to tumor progression and there are similar features, although the timing and participation of some key regulators may differ.

5) Typo-Page 12, 3rd line from bottom, sentence should read ...tumors and the samples from breast and lung tumors.

Referee #2 (Remarks to the Author):

Ye et al.

This manuscript concerns the role of ATF4, a transcription factor, in the proliferation and survival of transformed cells. Expression of ATF4 can be controlled through the regulated translation of its mRNA, which is enhanced under conditions where the translation initiation factor eIF2 is phosphorylation and consequently inhibited. GCN2 is one of several kinases that can phosphorylate

eIF2; GCN2 is activated by uncharged tRNA and may therefore be switched on under conditions of amino acid deficiency. This paper reports that the 'GCN2-eIF2 α -ATF4' pathway is critical for maintaining metabolic homeostasis in tumor cells' and that may therefore be 'a novel and attractive target for anti-tumor approaches'.

MAJOR POINTS:

1. Fig. 1B and S2A:are the NEAA already present in DMEM? If so, how the do concentrations used here compare to the concentrations already present in this medium? It would be surprising that, assuming these AA are already present in DMEM, further addition of these AA has the effects described here. Further discussion of this is required.

2. P. 11 and Fig. 7A: what is the evidence that glucose deprivation decreases amino acid levels in the cells used here? This is an important issue for the interpretation of the data and for the discussion.

3. in Fig. 9, it is important that the P-GCN2 antibody is completely for P-GCN2 for such histology analyses. How can the authors be sure that it does not detect any other proteins, phosphorylated or otherwise?

MINOR POINTS:

1. Fig. S2B: it is important that the authors quantitate the cell cycle data from multiple experiments to facilitate comparison between the conditions tested and provide evidence of reproducibility.

2. P. 8, line 3 up: what is 'Asn may also an important role of Gln' supposed to mean?

3. 'wide type' should be 'wild type': this error occurs in numerous places in this manuscript.

4. p. 11, line 2: is it true that the carbon backbone of ALL amino acids can enter glycolysis? I believe that some may be broken down to acetyl-CoA which cannot enter glycolysis (but does enter the TCA cycle).

5. p. 14, last line: the term 'antioxidant function' suggests that ATF4 itself acts as an antioxidant. A fuller explanation is required.

p. 17, line 16: is 'agent' an appropriate word to use here? Do the authors mean 'target'?

Referee #3 (Remarks to the Author):

Ye et al provide evidence that the GCN2-eIF2alpha-ATF4 axis is involved in cell survival in response to nutrient deprivation. The paper is of interest , but the authors need to perform more experiments to improve the paper. The work consists of 4 parts.

The first part shows that inactivation of ATF4 in HT1080 or DLD1 tumour cells by shRNA results in the induction of autophagy together with a decrease in cell survival, effects which are partially rescued by the provision of non-essential amino acids (NEAA). The authors conclude that induction of autophagy is a pro-survival effect, whose inhibition together with the elimination of ATF4 results in the cooperative enhancement of cell death. The authors' claim in the Abstract about an "initial" activation of autophagy by the loss of ATF4, is not supported by any of the experiments in the first part. Nevertheless, they convincingly show that ATF4 deficiency is associated with reduced levels of asparagine synthase (ASNS) and re-introduction of ASNS or supplementation with asparagines (Asn) increases survival of the ATF4 deficient tumour cells. A possible caveat of these experiments is the selection process for the isolation of clones deficient in ATF4. If elimination of ATF4 results in inhibition of cell survival, could it be possible that the selection results in ATF4-deficient cells that activated other endogenous control pathways to bypass the deleterious effects of the loss of ATF4? Perhaps the authors need to show that transient inactivation of ATF4 by shRNA can yield similar results.

The second part of the work investigates the GCN2-eIF2alpha-ATF4 arm in immortalized GCN2-/- and eIF2alphaA/A MEFs in response to amino acid deprivation. They provide good evidence that eIF2alpha phosphorylation is induced in ATF4 deficient HT1080 cells deficient in ATF4, and this anomaly is restored by the addition of Asn or NEAA. They provided further evidence that this is due to an induction of GCN2 activity. The authors need to verify the specificity of GCN2 and examine whether other eIF2alpha kinases, such as PERK or PKR, are aslo involved in this process. The authors further examined the role of GCN2 and eIF2alpha phosphorylation by employing MEFs deficient in GCN2 or in phosphorylation of eIF2alpha. Although GCN2 activation is clearly implicated in this process based on the data in Fig. 6B, the role of eIF2alpha phosphorylation is not fully supported by data in Fig. 6C, because there is a minimal induction of eIF2alpha phosphorylation in the absence of Cln and the effects on ASNS and p21 are hardly convincing. The authors need to quantify the blots in order to make a firm conclusion. Furthermore, the quality of data in Fig. 6E is not as good as in other experiments (see Fig. 3A), and the authors need to re-examine this effect. In addition to amino acid starvation, the authors also looked at the effect of glucose deprivation in GCN2-ATF4 arm. Although the data in Fig. 7 implicate GCN2 in this process, the possibility that other eIF2alpha kinases (e.g. PERK) are also involved can not be ruled and should be examined in order to conclude on the specificity of GCN2.

The third part of the work focuses on the effects of GCN2-ATF4 arm in tumour development. Using xenograft tuour assays, the authors show that HT1080 tumour cells with deficient GCN2 or ATF4 produce smaller tumours than cells with intact GCN2-ATF4. Although this is an interesting observation, it is not clear what sort of tumour microenvironment is implicated in this effect. Is it due to nutrient deprivation or hypoxic environment? Is it possible to correct this anomaly by providing essential amino acids at the tumor site or perhaps better, by overexpressing ASNA in the GCN2-ATF4-deficient cells? Because HT1080 cells (and DLD1 cells) contain activated Ras [Oncogene. 1999 Mar 11;18(10):1807-17], are their findings specific to Ras tumours or are they generally applicable to all tumour cells? The authors previously showed that the Ras-PERK pathway is involved in tumour promotion [EMBO J. 2005 Oct 5;24(19):3470-81], and the question is how one can distinguish, based on their data, between the roles of GCN2 and PERK in tumour promotion as much as stress in the tumour microenvironment is concerned?

The last part examines GCN2 activation and eIF2alpha phosphorylation in various tumour specimens in order to demonstrate the physiological significance of the findings. Although the data are of interest, the analysis would be strengthened if the authors could connect GCN2 and eIF2alpha to the lesions of the tumours. Also, it would be better to examine whether GCN2 levels correlate with GCN2 phosphorylation levels (Fig. 9A, B), because eIF2alpha phosphorylation levels differ between tumour samples with the same levels of GCN2. It is of interest that the tumour cells used in part I of the study contain activating mutations of K-ras, and the question is whether aberrant Ras signaling signals to GCN2 and ATF4.

1st Revision - authors' response 10 January 2010

Reviewer #1.

We thank the reviewer for expressing enthusiasm for our study.

1) "...Fig. 1: The authors showed that cell survival as measured by MTT assay following 48 hours of culture was reduced by ~60% in HT1080 and ... However, knock-down of ATF4 only reduced proliferation as measured by EdU incorporation by 35%. Have the authors ruled out other possible defects such as adherence to plastic, etc? Do equal numbers of cells adhere to the plastic dish at the onset of the cell survival assay? A simple growth curve comparing control vs. ATF4 knockdown cells would also address this issue".
We do observe a small defect in adherence of the ATF4 knockdown cells to plastic. However, our data also point to defects in proliferation and apoptosis (Figs. 1 and 2), which together could account for the difference in growth assayed by the 48 MTT. As suggested, we have also performed a long-term growth assay by plating equal number of cells and following their growth for 6 days (Fig. S1B). As seen in this figure, after 2 days there is already a growth defect (due primarily to apoptosis and decreased attachment to plastic), but also a proliferation defect, as evidenced by the much

steeper growth curve of the shNT control vs. the shATF4 cells.

2) "Fig. 3B: The resolution of the images makes it somewhat difficult to discern between increased formation of autophagosomes or increased vacuolarization. As has been described in other studies, incorporation of GFP-LC3 may more convincing".
We have performed transient transfections with GFP-LC3 in HT1080.shNT and HT1080.shATF4 cells. As shown in Fig. 3C, the GFP signal in the shNT cells is dispersed throughout the cytoplasm, whereas in the shATF4 cells it is punctate, suggesting vacuolar localization. Quantification of this data showsan over than 3-fold increase in basal autophagy which is substantially reversed upon NEAA supplementation. Together with the increased basal LC3-II signal (Fig. 3A and 3D) and the EM images (Fig. 3B), these results indicate that knockdown of ATF4 in these cells increases basal autophagy levels.

3) "Fig. 3D: The Knock-down of ATG7 reduces cell survival in ATF4 knock-down cells but no control is included showing that knock-down of ATG7 in HT1080 cells indeed affects autophagy. Does knock-down of ATG7 reduce the increased LC-3II levels in ATF4 knock-down cells? In an earlier figure, the knock-down of ATF4 results in reduced cell survival. Presumably knock-down of ATG7 in ATF4 knockdown cells reduced viability even further; although, this cannot be determined with the data presented as is".
Knockdown of Atg7 has been shown in several studies to block autophagy (reviewed in Klionksy et al., Autophagy. 2008 4:151-75). In our study, we showed that knockdown in Atg7 results in increased levels of cleaved PARP in shNT cells (Fig. 3D) which correlates with slightly decreased survival (Fig. 3E). These levels of apoptosis are further increased in the absence of ATF4, suggesting that basal ATF4 levels determine autophagic flux in these cells.

4) "In the discussion, the authors may wish to compare (differences/overlap) the consequence of phosphorylation of eIF2a induced by GCN2 during nutrient deficiency and that elicited by hypoxia. This laboratory has suggested both contribute to tumor progression and there are similar features, although the timing and participation of some key regulators may differ".
This is an excellent suggestion. We have also pondered whether GCN2 may contribute to hypoxic resistance and whether hypoxia regulates GCN2. The short answer, is that based on our data, there is little crosstalk between hypoxia and GCN2 regulation. First, in experiments using WT, GCN2-/- and PERK-/- MEFs shown in figure S5A, we show that while eIF2α phosphorylation in response to hypoxia is somewhat decreased in the GCN2-/- MEFs, it is completely absent in the PERK-/- MEFs. Since we have previously shown that eIF2α phosphorylation is required for hypoxic resistance, the contribution of GCN2 to this process is substantially less prominent than that of PERK. This is further corroborated by analysis of cell survival under extreme hypoxia, which shows no significant difference between GCN2+/+ vs. GCN2-/- MEFs. This is in contrast to the increased sensitivity of PERK-/- MEFs to hypoxia in vitro and in vivo (Koumenis et al, MCB, 2002 and Bi et al, MCB 2005). In vivo, we believe that GCN2 and PERK contribute to resistance to distinct stresses (though there may be some overlap) which exist I the tumor microenvironment and which may or may not be spatially distinct. We have also modified our current working model (Fig. 9D) to illustrate these differences.

5) "Typo-Page 12, 3rd line from bottom, sentence should read ...tumors and the samples from breast and lung tumors".
This has been corrected.

Reviewer #2:

1. "...Fig. 1B and S2A: are the NEAA already present in DMEM? If so, how the do concentrations used here compare to the concentrations already present in this medium? It would be surprising that, assuming these AA are already present in DMEM, further addition of these AA has the effects described here. Further discussion of this is required".
The concentrations of the amino acids in the NEAA mixture and in DMEM are shown in. Table S I, which is now included in the supplementary data. With the exception of Glycine and Serine, the other 5 amino acids in the NEAA mixture are not present in DMEM. Importantly, Asparagine (Asn) is not present in DMEM but present in the NEAA mixture. Our results in Fig. 4A indicate the Asn but not any other single AA form that mixture is sufficient to recapitulate the cell-survival benefit.

2."...P. 11 and Fig. 7A: what is the evidence that glucose deprivation decreases amino acid levels in the cells used here? This is an important issue for the interpretation of the data and for the discussion"
The reviewer raises a good point. We believe the best evidence is the induction of GCN2 phosphorylation which is the most sensitive and specific sensor of amino-acid deprivation in cells (Kilberg et al., Ann. Rev. Nutr. Vol. 25: 59-85, 2005). Analysis of the intracellular levels of aminoacids in cells is quite difficult and special instrumentation is required-which we do not possess.
However, to try to further address this question, we have used a kit that analyzes levels of glutamine (which is readily consumed by transformed cells) to measure glutamine concentration before and after glucose deprivation (thereby providing a measure of glutamine consumption). As shown in new Fig. 7C, Gln consumption more than doubled after 16h incubation of cells in low glucose (normalized to cell number). This result, together with our data showing increased eIF2α phosphorylation following glucose deprivation which is reversed by excess Gln, strongly support our model.

3."... in Fig. 9, it is important that the P-GCN2 antibody is completely for P-GCN2 for such histology analyses. How can the authors be sure that it does not detect any other proteins, phosphorylated or otherwise?"
We believe that the anti-phospho-GCN2 antibody does specifically recognize phosphorylated GCN2 for the following reasons: (a) in our immunoblot analyses, it fails to recognize unphosphorylated GCN2, and at least at high-MW range of the gel (GCN2 is a 250-kD protein) it does not cross-react with any other proteins. (b) We have performed control immunohistochemical analyses where the primary antibody is omitted-but the secondary is present. As shown in new Fig. 9C, we cannot detect significant staining in normal tissues. We believe that if there was cross-reaction with another protein, that antigen should also be present in normal tissue. (c) This Ab has been used in the past in other immunohistochemical studies and shown to detect P-GCN2 (Hao et al., Science, 307: 1776-78, 2005).

MINOR POINTS:

1. "Fig. S2B: it is important that the authors quantitate the cell cycle data from multiple experiments to facilitate comparison between the conditions tested and provide evidence of reproducibility."
We have quantitated the results from 3 independent experiments using the Cell-Quest analysis program. Based on this, there is a 40-50% increase in the G1/S ratio in the two shATF4 clones compared to control. This ratio is reduced by 20-25% when NEAA or Asn are added (New Figure S3C).

2. "P. 8, line 3 up: what is 'Asn may also an important role of Gln' supposed to mean?"
We have corrected this sentence to: "…suggesting that producing Asn may also be an important role of Gln,…"

3. "'wide type' should be 'wild type': this error occurs in numerous places in this manuscript".
This has been corrected.

4. "p. 11, line 2: is it true that the carbon backbone of ALL amino acids can enter glycolysis? I believe that some may be broken down to acetyl-CoA which cannot enter glycolysis (but does enter the TCA cycle)".
This is a valid point. We have changed the text to say: "…Given that the carbon backbone of amino acids can enter glycolysis or the citric acid cycle to produce ATP, and…"

5. "p. 14, last line: the term 'antioxidant function' suggests that ATF4 itself acts as an antioxidant. A fuller explanation is required."
We have altered the text to: "…that ATF4 plays an important role in cellular resistance to chemotherapeutic agents and genotoxic stress, perhaps through upregulation of target genes that promote production of reducing compounds."

6. p. 17, line 16: is 'agent' an appropriate word to use here? Do the authors mean 'target'?
L-Asparaginase is the enzyme (and thus the agent, or drug) that degrades the target L-Asparagine.

Reviewer #3:

1. ...The authors' claim in the Abstract about an "initial" activation of autophagy by the loss of ATF4, is not supported by any of the experiments in the first part. Nevertheless, they convincingly show that ATF4 deficiency is associated with reduced levels of asparagine synthase (ASNS) and reintroduction of ASNS or supplementation with asparagines (Asn) increases survival of the ATF4 deficient tumour cells. A possible caveat of these experiments is the selection process for the isolation of clones deficient in ATF4. If elimination of ATF4 results in inhibition of cell survival, could it be possible that the selection results in ATF4-deficient cells that activated other endogenous control pathways to bypass the deleterious effects of the loss of ATF4? Perhaps the authors need to show that transient inactivation of ATF4 by shRNA can yield similar results.
We respectfully disagree that we have not provided data to support a role for ATF4 in autophagy. The entire Fig. 3 is devoted to this finding. Using a several methods (e.g., LC3-II levels, electron microscopy and now accumulation of GFP-LC3 in punctate structures), we provide support to the model in which inhibition of basal ATF4 levels induce amino-acid deprivation which promotes autophagy. Our data do not exclude a potential role for stress-induced ATF4 in the induction of autophagy recently reported by some groups (e.g., Rouschop et al., JCI, 2010 and Milani et al., Cancer Res., 2008). We believe the two functions are distinct, and in our model, the role of ATF4 in preventing autophagy is indirect.
In terms of the role of the potential for clonal effects during selection, we believe this is unlikely for three reasons: (a) We have analyzed 2 different clones for HT1080 cells and another from DLD-1 cells with very similar results (Fig. 1B, C) thereby minimizing this likelihood. (b) Overexpression of mouse ATF4 restores a significant portion of cell survival (Fig. 2E). (c) As recommended by this reviewer, we have performed transient transfection with ATF4 siRNA. As shown in suppl. Figure S1C, there is a modest, but statistically significant decrease in survival by siRNA which is reversible by the addition of NEAA. The modest decrease is likely due to the fact that in a transient transfections ATF4 expression will not be completely inhibited in all HT1080 cells.

2. "...They provide good evidence that eIF2alpha phosphorylation is induced in ATF4 deficient HT1080 cells deficient in ATF4, and this anomaly is restored by the addition of Asn or NEAA. They provided further evidence that this is due to an induction of GCN2 activity. The authors need to verify the specificity of GCN2 and examine whether other eIF2alpha kinases, such as PERK or PKR, are aslo involved in this process. "
This is a valid point. Others have shown that of the four known eIF2α kinases (PERK, PKR, HRI and GCN2), only the latter responds to amino-acid deprivation to phosphorylate eIF2 α (Wek et al., Biochem Soc Trans. 34:7-11, 2006). Although there is apparently some cross-talk between the PERK and GCN2 pathways (especially with respect to glucose deprivation), amino-acid deprivation appears to specifically induce only GCN2 (at least short-term). We have performed an additional experiment to address this issue. As shown in new Fig. 7D, phosphorylation of eIF2 α in response to glutamine deprivation is completely blocked in GCN2-/- MEFs, but present in both WT and PERK-/- cells. We currently do not have PKR-/- cells in our disposal, but we believe it would be surprising if PKR (a kinase that responds to dsRNA) would be involved in this process.

2. "...The authors further examined the role of GCN2 and eIF2alpha phosphorylation by employing MEFs deficient in GCN2 or in phosphorylation of eIF2alpha. Although GCN2 activation is clearly implicated in this process based on the data in Fig. 6B, the role of eIF2alpha phosphorylation is not fully supported by data in Fig. 6C, because there is a minimal induction of eIF2alpha phosphorylation in the absence of Cln and the effects on ASNS and p21 are hardly convincing. The authors need to quantify the blots in order to make a firm conclusion".
We have re-scanned darker exposures of our ASNS and eIF2 α blots and have performed analysis with the Scion Image program (a PC-version of the NIH image shareware program). As shown in figure 6C, both eIF2 α and ASNS are clearly induced (2.2 and 1.9-fold respectively normalized to a-tub control) upon Gln deprivation in WT but not GCN2-/- MEFs. We have also re-run the gel suing the same extracts to obtain a darker exposure for p21. As shown in the same figure, there is clear induction of 21 levels, which is again absent from CCN2+/+ cells.

3. Furthermore, the quality of data in Fig. 6E is not as good as in other experiments (see Fig. 3A), and the authors need to re-examine this effect. In addition to amino acid starvation, the authors also looked at the effect of glucose deprivation in GCN2-ATF4 arm. Although the data in Fig. 7 implicate GCN2 in this process, the possibility that other eIF2alpha kinases (e.g. PERK) are also involved can not be ruled and should be examined in order to conclude on the specificity of GCN2.
We have also re-scanned the blot using a darker exposure with less background. We believe the new blot clearly shows the induction of autophagy in GCN2+/+ and less in the GCN2-/- MEFs and complete absence in the S51A MEFs. Moreover, following this reviewer's recommendation, we have looked at another marker of autophagy, the rapid degradation of p62, a protein with a long half-life, which actively participates in, and is rapidly degraded during autophagy (Klionsky et al., Autophagy, 2008). As shown in new Fig. 6F, this protein is readily degraded in control HT1080 shNT cells upon Gln deprivation but its degradation is blocked in HT1080 cells expressing shGCN2. Collectively, these results indicate that GCN2 and eIF2α participate in amino acid deprivation-induced autophagy.

4. "...it is not clear what sort of tumour microenvironment is implicated in this effect. Is it due to nutrient deprivation or hypoxic environment? Is it possible to correct this anomaly by providing essential amino acids at the tumor site or perhaps better, by overexpressing ASNA in the GCN2-ATF4-deficient cells? "
This is an issue we have also tried to address in more detail in the revised manuscript. Our results with both the ATF4 and GCN2 knockdown and knockout cells point towards a defective response to amino acid-and likely glucose deprivation which exists in tumors. Hypoxia may also contribute, but since GCN2-/- cells are as sensitive as WT cells to this stress, it is unlikely that it contributes to the defect in the latter cells. In vitro, ATF4 is required for both hypoxia and amino acid deprivation resistance. A more detailed analysis of contribution to resistance to specific stresses in vivo is difficult at this point, since it will require very sensitive analysis of immunohistochemical detection of phosphorylated GCN2, hypoxia, glucose deprivation and amino acid deprivation markers, and for the last two, there are no specific markers. However, as the reviewer suggested, we did test whether supplementation of amino acids in trans or expression of ASNS could rescue the tumor growth phenotype of ATF4 knockdown cells. The first approach-by daily injections of NEAA or Asn (100 microM) into the tumors-did not result in any significant effect. This could be due to inefficient delivery of the amino acids to all cells into the tumor, or a reflection of increased requirements of amin acids in vivo vs. in vivo. However, overexpression of ASNS in the tumor cells (by using HT1080 shATF4 cells stably transfected with human ASNS), resulted in partial rescue of tumor growth (Fig. 8C). Though in this experiment complete rescue of tumor growth was not achieved (due to time limitations we had to terminate the experiment early), it is important to point out that these tumors grew over 2.5 times the initial mass. In contrast, all the shATF4 tumors decreased in size after injection and some of them disappeared from the flanks of the mice. These results demonstrate that expression of ASNS is, at least partially, responsible for the growth defect in the ATF4 knockdown cells. The lack of complete rescue of the phenotype is likely due to (a) sub-optimal presence of glutamnine, the precursor for asparagine synthesis and ASNS substrate throughout the tumor growth or (b) contribution of ATF4 to other processes, such as angiogenesis. Indeed, an angiogenesis defect was also attributed to sub-optimal growth of PERK-/- tumors previously shown by our collaborators (Blais et al., MCB, 2006) and more recently suggested by another study (Gupta et al., PLoS One. 4: 2009). These issues are discussed in more detail in the Discussion section of our revised manuscript.

5. Because HT1080 cells (and DLD1 cells) contain activated Ras [Oncogene. 1999 Mar 11;18(10):1807-17], are their findings specific to Ras tumours or are they generally applicable to all tumour cells?
This is an interesting point. In vivo this may be the case, since by default, all our tumors we grew had mutant Ras. However, our in vitro results suggest that the phosphorylation of eIF2a and survival may be independent of Ras status. In response to this issue, we have performed analysis of eIF2 α phosphorylation and investigated the survival of GCN2+/+ and GCN2-/- MEFs with different transformation status (SV40 large-T only vs. SV40+K-RasV12). As seen in the figure provided (see file "Data for referees only"-not included in the manuscript), Ras status did not appreciably affected phosphorylation of eIF2α or overall survival under Gln deprivation (the mt-Ras expressing MEFs were more sensitive to the stress regardless of GCN2 status).

6. The authors previously showed that the Ras-PERK pathway is involved in tumour promotion

[EMBO J. 2005 Oct 5;24(19):3470-81], and the question is how one can distinguish, based on their data, between the roles of GCN2 and PERK in tumour promotion as much as stress in the tumour microenvironment is concerned?
Please see response to comment 5 above.

7. Although the data are of interest, the analysis would be strengthened if the authors could connect GCN2 and eIF2alpha to the lesions of the tumours. Also, it would be better to examine whether GCN2 levels correlate with GCN2 phosphorylation levels (Fig. 9A, B), because eIF2alpha phosphorylation levels differ between tumour samples with the same levels of GCN2.
We agree that this analysis would be of interest. Unfortunately, immunohistochemical detection of phospho-eIF2a is quite difficult and we, as well as others have not been able to detect P-eIF2α in vivo (to our knowledge there are no quality immunohistochemical analyses with P-eIF2α published). As for total vs. P-GCN2, our analysis shows that although not overlapping, there is some common staining by both antibodies (Fig. 9C). However, we noticed that staining for total GCN2 is more diffuse (as expected) but also less pronounced than that of P-GCN2. This is most likely due to differences in the reactivity of each antibody to the corresponding protein, something which would also make comparisons between the two rather unreliable. Neither antibody stained significantly any normal tissue, with the exception of endometrium and spleen (not shown), which showed rather extensive total GCN2 (but not for P-GCN2).

8. It is of interest that the tumour cells used in part I of the study contain activating mutations of K-ras, and the question is whether aberrant Ras signaling signals to GCN2 and ATF4.
Please see answer to question 5 and our "Figure for Reviewers". Although this is formally possible, comparison between the SV40-only and SV40/K-RasV12 MEfs does not reveal any apparent differences, at least in terms of eIF2 α phosphorylation. However, investigation of the effects of KRasV12 on these pathways is an ongoing area of interest for us and we plan to address this in more detail in the future.

2nd Editorial Decision 10 February 2010

Thank you for submitting your revised manuscript to the EMBO Journal. Your revised manuscript has now been seen by the three original referees and their comments are provided below. The referees appreciate the added data and all three are very supportive of publication in the EMBO Journal. Both referee #2 and 3 raise a few issues that should be resolved before publication here. I would like to ask you to address the last remaining points in a final revision.

Looking forward to seeing the revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This revised manuscript by Koumenis and colleagues discovers a major role for the GCN2-eIF2α - ATF4 pathway for tumor survival and proliferation. The study shows that lowered ATF4 expression alters p21 expression, prevents asparagine synthetase expression, and facilitates a cytoprotective autophagic response. The inability of tumor cells to upregulate this stress response during nutrient deficiency leads apoptosis. Further supporting this important linkage between the GCN2-eIF2α - ATF4 pathway and tumor progression, tumors were shown to overexpress GCN2 protein kinase, leading to increased eIF2α phosphorylation and increased ATF4 expression. Overall, the manuscript makes a convincing case for a role in the nutrient-regulated eIF2 kinase GCN2 in tumor progression and resistance to nutrient depletion. The prior reviewer concerns were satisfactorily addressed. This

is a significant study that will be of interest to a wide readership.

Referee #2 (Remarks to the Author):

The authors have responded to my initial set of comemnts by adding some additional data and/or providing further analysis of existing data, and by making a number of modifications to the text. However, two points do remain to be fully addressed, one major and one minor.

1. the authors were asked to provide data showing that amino acid levels fall in glucose deprived cells, to support their argument that this accounts for the increased phopshorylation of eIF2α (via GCN2, presumably). Glucose starvation could, directly or indirectly, exert a multiplicity of effects and indeed GCN2 (and other eIF2 kinases) can be activated by additional mechanisms.

While one accepts that analysis of amino acid levels does need specialist equipment (e.g., HPLC), it is neither complex nor unusual to make such measurements, nor are they very time-consuming. the authors should collaborate with a lab that routinely conducts such determinations and provide the missing data. They are important for understanding how starvation regulates eIF2 activity and ATF4 expression.

2. In Fig. S2B, the authors should provide information about the statistical significance of the differences they see in the % of cells in different stages of the cell cycle.

Referee #3 (Remarks to the Author):

The authors added a new experiment (Fig.S1B) in response to concerns about the xenograft tumours in nude mice. However, the data lack statistics.

Most of the new data is derived from re-scanning. It would have been nicer and preferable to show some new experiments.

I am not convinced by the data of the human tumour immunohistochemistry. That is, the authors claim that they cannot perform immunohistochemitry for phosphorylated eIF2alpha, which is indeed difficult, but was accomplished by others.

2nd Revision - authors' response 23 March 2010

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We have collaborated with Dr. Mark Yudoff, Director of the Mass Spectrometry facility at the UPennaffiliated Children's Hospital of Philadelphia to analyze amino acid levels in control and glucose-starved HT1080 cells. As we now show in Fig. 7C, a 2h glucose deprivation causes a

significant drop in the intracellular levels of Alanine and Serine (50% and 30%, respectively). At this time point, we did not observe any significant decreases in Asparagine, Glutamine or Leucine, though there was a trend towards decreased levels. By 4h, only the levels of Alanine were still repressed, while those of Glutamine and Asparagine showed an increase compared to those in cells replete with glucose (4.5 g/L). This increase can be attributed to the initiation of autophagy (and generation of amino acids from proteolysis), or the uptake of Glutamine from the media due to upregulation of transporters-which we show is also occurring in a GCN2-specific manner (Fig. 7E). Please note that a decrease in the levels of any of the amino acids should induce GCN2 phosphorylation, since it is a general amino acid sensor. Moreover, we did not analyze the levels of all amino acids, or looked at even earlier time points (e.g., 1h), due to excessive costs ($150 sample, which had to be done in triplicate), so it is possible that the observed effects may be even more pronounced. However, we strongly believe that the significant reduction in Ala and Ser levels is a strong candidate for GCN2 activation.

2. In Fig. S2B, the authors should provide information about the statistical significance of the differences they see in the % of cells in different stages of the cell cycle.
We have performed studentís t-test analysis and have found the differences in the G1/S ratios between scrambled ShRNA and shATF4 cells from 3 different biological replicates to be statistically significant (revised Fig. S3C)

Referee #3 (Remarks to the Author):

The authors added a new experiment (Fig.S1B) in response to concerns about the xenograft tumours in nude mice. However, the data lack statistics.

Please note that Fig. S1B shows tumor cell growth in vitro (MTT assay) and not tumor growth in nude mice. However, our mouse tumor growth data we provided in the last revision (Fig. 8C), indeed lacked statistics. We now provide statistics in the form of studentís t test on tumor size. Statistically significant differences ($p<0.05$) are indicated by asterisks in the figure. Most of the new data is derived from re-scanning. It would have been nicer and preferable to show some new experiments.
Please note that only one of the Figures we had submitted was from re-scanning of a blot (p-eIF2alpha in Figure 6C). The panels showing p21 and cleaved caspase-3 in the same figure were obtained by rerunning the protein samples on PAGE along with tubulin. We have also provided NIH image analysis of optical density of the bands in the specific figure.

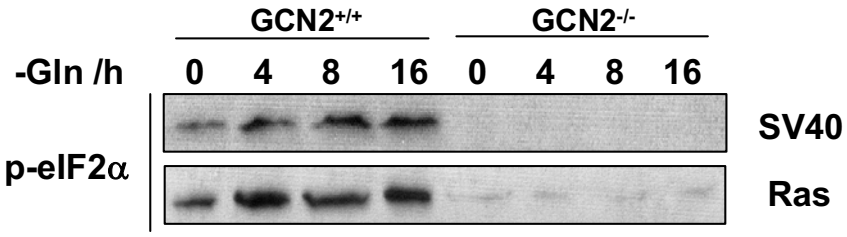
I am not convinced by the data of the human tumour immunohistochemistry. That is, the authors claim that they cannot perform immunohistochemitry for phosphorylated eIF2alpha, which is indeed difficult, but was accomplished by others.

We devoted considerable effort to extend our immunohistochemistry results as requested. After testing several commercially-available antibodies against phospho-eIF2α, we identified one (Cell Signaling; Ab 119A11) which gives a weak, but specific signal for phospho-eIF2α (see Figures 9D and S6). The specificity of this signal (as well as that of the more robust phopspho-GCN2) was tested by (a) incubating the tumor sections with lambda phosphatase prior to incubation with the antibody. This incubation, substantially reduced or completely abolished the signal from the phosphospecific antibodies. (b) No substantial signal was observed in sections incubated with secondary only (no primary) antibody. More importantly, our data with different sections form 2 human patient tumors show extensive co-localization of phospho-GCN2 and phospho-eIF2□ signals. It should be noted that we also performed staining for total eIF2α, which showed significant levels in the malignant cells in the liver, while the stroma showed reduced staining. This is not suprising, since other translation factors (e.g., eIF4E), have been reported to be overexpressed in tumor vs. normal tissues (e.g., Rosenwald et al., Oncogene, 18:2507-17, 1999).

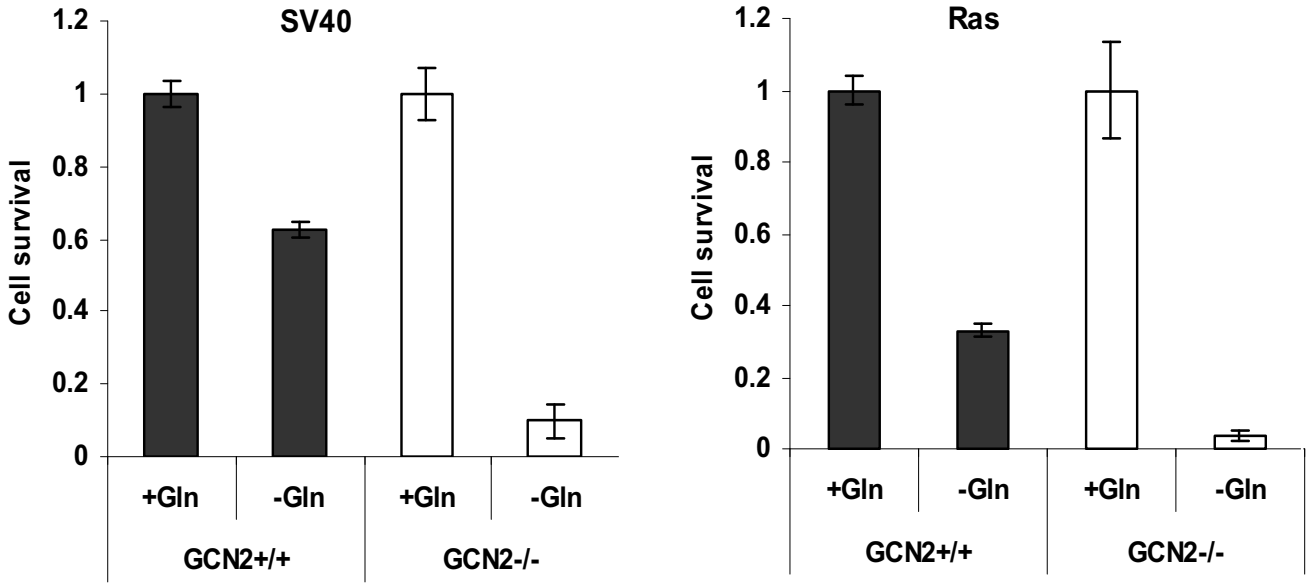
Additional Correspondence 06 April 2010

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referee # 3 to take a final look at the manuscript and I have now heard back from this referee. The referee is happy with the revised version and has not further comments to the authors. I am therefore very pleased to proceed with the acceptance of your paper for publication in the EMBO Journal. You will receive the " formal" acceptance letter tomorrow.

A.



B.



(A) SV40 immortalized or Ras transformed GCN2+/+ and GCN2-/- MEFs were incubated in Gln free media for 4,8 and 16h. cells were harvested for immunoblot. **(B)** SV40 immortalized (left) or SV40+Ras (right) transformed GCN2+/+ and GCN2-/- MEFs were incubated in media with or without Gln for 48h. Cell survival was analyzed using the MTT assay. Data represent mean ± SEM, n = 3.