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FoxO3A promotes metabolic adaptation to hypoxia by antagonizing Myc function

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Transaction Report:

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

1st Editorial Decision 21 April 2011

Thank you very much for submitting your paper that proposes FOXO3A to participate in hypoxic regulation myc-dependent mitochondrial proteins for consideration to The EMBO Journal editorial office.
I received comments from three scientists enclosed for your information below. Though expressing some interest in the study, their support remains in light of the very preliminary status of the study rather vague. As you can see, ref#1 is not convinced that the presented dataset drives your main hypothesis. Ref#2 requests a much better analysis of the FOXO3A-dependent metabolic phenotypes and thorough establishment of the myc-antagonism, concerns shared by ref#3 that need to be dissolved by the suggested experiments.

I am very much aware that these are demanding tasks amounting to significant further experimental efforts. I would thus suggest that you first evaluate your options and let me know whether you prefer to take the paper for rapid publication to a less stringent alternative journal OR attempt revisions for The EMBO Journal. Please understand that we do have to demand definitive molecular mechanism and thorough functional evaluation according to the aim and scope of The EMBO Journal. This proposal is therefore solely in yours- and the papers' best interest and intends to avoid later disappointments.

Finally, I do have to formerly remind you that it is EMBO Journal policy to allow a single round of revisions only and that the ultimate decision depends on the content and strength of a potential revised version.

Please do not hesitate to contact me in case of further questions (preferably via E-mail).

I am very much looking forward to your response and remain with best regards.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1:

The authors provide evidence in support of the hypothesis that FOXO3A participates in the hypoxic downregulation of nuclear genes encoding mitochondrial proteins by inhibiting MYC binding.

1. The authors provide additional information beyond what is already published regarding the activation of FOXO3A gene expression by HIF-1 in hypoxia. They show evidence for binding of HIF-1alpha by chromatin immunoprecipitation (ChIP) assays and by a reporter gene assay. This analysis would be strengthened by investigating whether HIF-2alpha also binds to the same site and the relative abilities of HIF-1alpha and HIF-2alpha to drive reporter gene expression. The putative HIF binding sites in the hypoxia response element should also be mutated to establish their function.

2. The authors provide microarray data regarding genes that are downregulated under hypoxic conditions in a FoxO3A-dependent manner. Since the authors propose that FoxO3A expression is dependent on HIF-1, then expression of shRNA against HIF-1alpha should give similar results to those obtained using shRNA against FoxO3A.

3. The authors overinterpret the data in Figure 2A. The term "slightly more" is unacceptable. Either the results are significantly different or not (no statistical tests appear to have been performed on the data in 2A or 2C).

4. The data in Fig. 4 do not support the authors' model. They show that their knockdown of Myc is complete. If FoxO3A is acting via inhibition of Myc, then knockdown of FoxO3A should have no effect in the absence of Myc, yet expression of all of the mitochondrial genes tested is increased by FoxO3A knockdown in Myc-knockdown cells.

5. In Fig. 5, the authors perform ChIP analysis on several genes encoding mitochondrial proteins. They show clear evidence for Myc binding to these genes by ChIP, with enrichment of PCR by several logs when anti-Myc antibody is used for ChIP compared to IgG. In contrast, ChIP experiments using anti-FoxO3A antibody provide results that are not compelling, with at best only several-fold difference in enrichment. It is not at all clear how to interpret these data. Is FoxO3A binding directly to DNA? If so, what is the sequence to which it is binding and how does this binding interfere with the binding of Myc? Putative MYC/FOXO3A binding regions need to be analysed in reporter assays to further clarify their mechanisms of action.

Conclusion: The data presented in the manuscript do not adequately support the hypothesis that FoxO3A represses mitochondrial gene expression in hypoxic cells by competing with Myc for binding to gene promoters.

Referee #2:

Jensen et al "FoxO3A promotes metabolic adaptation to hypoxia by antagonizing Myc function". The authors identify HIF1-dependent induction of the FoxO3A transcription factor in cells treated with hypoxia or DFO. They then determine gene expression changes in hypoxic cells with or without FoxO3A expression. Analysis of this data identifies a group of genes that are repressed by hypoxia in a FoxO3A-dependent manner. Many of these genes are involved in mitochondrial

biogenesis and mitochondrial function. They functionally characterize the metabolic consequence of FoxO3A activity in hypoxia, and establish a significant mitochondrial inhibition. This metabolic phenotype appears to be advantageous to the survival of cells treated with hypoxia in vitro, and to the growth of modle tumors. They propose a potential mechanism by which FoxO3A antagonizes myc function to establish this metabolic program. The findings offer a novel mechanism of gene regulation in response to hypoxia, that may have significant implications in physiologic and pathologic conditions. While it would be very interesting to conclusively establish the mechanism of myc antagonism, it is outside the scope of this manuscript.

Major points.

1. The authors should present a more thorough analysis of the metabolic phenotype described in figure 2. The differences in mitochondrial mass and oxygen consumption seem significant, but it would help to understand more about these differences. For example, myc has been implicated in regulating mitochondrial consumption of glutamine. Is the difference seen in figure 2B due to altered glutamine metabolism, or glucose, or both? These experiments should be repeated using media without glucose or glutamine to determine the relative contributions. In addition, glucose uptake and lactate production should be performed on these cells in vitro in normoxia and hypoxia to determine if the reduction in mitochondrial function is compensated for by an increase in glycolysis.
2. The immunohistochemical data showing induction of FoxO3A in perinecrotic regions of tumors (fig 6A), where there is CA9 expression, is important. However, this observation needs to be quantitated. How many tumors were observed, what fraction showed co-localization, how much of the section showed co-localization.
3. The model tumor data needs to be repeated. Were there only the tumors shown in the pictures (ie three of each)? A significant number of tumors needs to be grown in duplicate experiments. Vector controls and both knockdown lines need to be run in replicate experiments. Tumor volumes should be carried out to volumes of several hundred mm3, so that measurements can be taken more accurately. Accurate growth rates and volumes can help determine if there is an absolute reduction in growth rate, or simply a growth delay. Tumors should be harvested and protein extracts run to determine level of FoxO3A knockdown, and capase activation, post tumor growth.
4. FDG uptake in tumors needs to be normalized for tumor volumes. Growth rate differences in the control versus knockdown tumors suggest that knockdown tumors were smaller at the time of scanning. Larger tumors typically have higher FDG uptake, so SUV needs to be plotted (or tumors should be measured of equal volume) as a function of tumor volume.

Minor comments

1. If there is increased apoptosis in knockdown cells treated with hypoxia in vitro (figure 3), is there incrased apoptosis in the tumors grown in vivo?
2 The authors should comment on the potential contribution of other gene expression changes that may contribute to the tumor growth phenotype.

Referee #3:

This is an interesting paper in which the authors characterize a mechanism that FoxO3A is required for hypoxic suppression of mitochondrial mass, oxygen consumption, and ROS production and promotes cell survival in hypoxia. The authors show that FoxO3A is recruited to the promoters of nuclear-encoded mitochondrial genes where it directly antagonizes c-Myc function. Furthermore, the author observe that FoxO3A is activated in human hypoxic tumor tissue in vivo and that FoxO3A shRNA-expressing xenograft tumors are decreased in size and metabolically changed.

Overall, this study presents novel findings that should be of wide interest. I do have a number of comments that the authors should address to strengthen the study prior to publication.

1. The authors should perform more experiments in the normal diploid cells, such BJ, WI38 or IMR90 cells, to define the competitive relationship between FOXO3a and c-Myc under the hypoxia condition.

2. The RNAi experiments should be strengthened with additional shRNAs or rescue controls in Figure 5 and Figure 6C-E.

3. The authors should include more experiments to characterize the interaction between c-Abl and MST1 under normoxia and hypoxia conditions.

4. In Figure 5, another FOXO3 RNAi or FOXO3 knockout cells to strengthen the conclusion that the direct action of FoxO3A on the promoters of hypoxia-repressed nuclear-encoded mitochondrial genes applies to a specific subclass of Myc target genes.

5. To rule out the possibility that FOXO3a represses c-Myc transcription through Mxi induction, the authors should perform the similar experiments in the background of MXI knockdown.

6. Comments on the Figure 6:
(1) The authors should describe how many tumor samples were immunostained? Statistical analysis is required for the conclusion.
(2) From the Figure S1G, the nuclear FOXO3a is not significantly increased even thought he total protein levels are increased under hypoxia treatment. The authors claim that FOXO3a in tumor samples becomes activated only basing on that the FOXO3a is co-stained with HIF alpha target gene-CA9. The authors should carefully calculate the ratio of nuclear FOXO3a vs cytoplasmic FOXO3a since FOXO3a protein levels are increased in the hypoxia area, and use the expression of FOXO3a targets to indicate the transcriptional activation of FOXO3a .
(3) The cell death should be analyzed in the clinic samples as well as the tumor blocks from the transplanted tumor mass.
(4) As stated in point 2, additional FOXO3a shRNA should be used in the xenograft experiments.
(5) The expression of c-Myc target genes (Aco2 and LARS2) that related with hypoxia response should be examined in the transplanted tumor mass.

Additional Author Correspondence 08 May 2011

Thank you very much for your mail concerning our manuscript. After some intense and unfortunately also time-consuming discussions with our collaborators we have decided to attempt revisions for The EMBO Journal.

This decision is based on:

1) Recently obtained data that could not be included into the first version of the manuscript.

2) Our dedication to fulfill the reviewers' requirements by further experimental studies and by the clarification of points that were expressed ambiguously in the current version. (e.g. Referee #2: "3. The model tumor data needs to be repeated. Were there only the tumors shown in the pictures (i.e. three of each)?"
- No, this series comprised 12 animals, which means 12 control tumors and 12 knock-down tumors. These 12 (of each) are the basis of the growth curve in Fig. 6C.)

3) The in general positive attitude of Referees #2 and #3, despite their detailed criticism and their requests of additional experiments.
Referee #2: "The findings offer a novel mechanism of gene regulation in response to hypoxia, that may have significant implications in physiologic and pathologic conditions. While it would be very interesting to conclusively establish the mechanism of myc antagonism, it is outside the scope of this manuscript."
Referee #3: "Overall, this study presents novel findings that should be of wide interest. I do have a number of comments that the authors should address to strengthen the study prior to publication."

However, we are a bit puzzled about one point of criticism made by referee #3:
"3. The authors should include more experiments to characterize the interaction between c-Abl and MST1 under normoxia and hypoxia conditions."

Neither of the two kinases is mentioned in our manuscript, nor did we perform any experiments with these. Could you please comment on how to deal with such a point?

Additional Editorial Correspondence 09 May 2011

Thank you for your note outlining that you are going to revise your paper for The EMBO Journal. Related to referee #3's point 3, I did receive the following note that I'm happy to pass on for your information:

.....
I found that there is mistake in my point 3, which should be:
3. The authors should include more experiments to characterize the interaction between FOXO3a and Myc under normoxia and hypoxia conditions. Could you please pass the corrected point to the authors? Sorry for the mistake.
.....

This should clarify the point.

Yours sincerely,

Editor
The EMBO Journal

1st Revision - Authors' Response 04 August 2011

Response to reviewers

Referee 1

1. The authors provide additional information beyond what is already published regarding the activation of FOXO3A gene expression by HIF-1 in hypoxia. They show evidence for binding of HIF-1alpha by chromatin immunoprecipitation (ChIP) assays and by a reporter gene assay. This analysis would be strengthened by investigating whether HIF-2alpha also binds to the same site and the relative abilities of HIF-1alpha and HIF-2alpha to drive reporter gene expression. The putative HIF binding sites in the hypoxia response element should also be mutated to establish their function.

We have extended our reporter gene analysis by including HIF-2alpha overexpression (Supplementary Figure S2B). Reporter gene constructs with mutated HIF binding sites have been included. Based on these experiments, we conclude that both the single HRE element as well as the tandem element located closer to the start site of transcription contribute to transcriptional activation by HIF. In addition, both HIF1alpha and HIF2alpha can activate the reporter gene. siRNA-mediated knockdown of either HIF-1alpha or HIF-2alpha indicates that both factors can contribute to the induction of FoxO3A expression in hypoxia (Figures S1B and S1C). Accordingly, a combined knockdown of HIF-1alpha/2alpha in HeLa cells abrogates hypoxic induction of FoxO3A (Figure S1D).

2. The authors provide microarray data regarding genes that are downregulated under hypoxic conditions in a FoxO3A-dependent manner. Since the authors propose that FoxO3A expression is dependent on HIF-1, then expression of shRNA against HIF-1alpha should give similar results to those obtained using shRNA against FoxO3A.

As expected, a knockdown of HIF-1alpha by siRNA significantly compromises the hypoxic repression of mitochondrial genes as illustrated in Figure S3. The remaining effect may be attributed to HIF-2alpha and residual HIF-1alpha expression in the knockdown samples as indicated by the presence of the HIF-1alpha mRNA.

3. The authors overinterpret the data in Figure 2A. The term "slightly more" is unacceptable. Either the results are significantly different or not (no statistical tests appear to have been performed on the data in 2A or 2C).

We have changed the phrasing in the manuscript and performed statistical analysis on replicate experiments analogous to the one presented in Figure 2A, which indicates a statistically significant and reproducible difference in the NAO staining in both knockdown clones (Fig. 2B). Moreover, treatment of FoxO3A knockdown cells with the antioxidant Trolox reduces cell death and the amount of cleaved Caspase 3 (Figures 3F and 3G), indicating that increased hypoxic ROS production as detected by DCFDA staining in Figure 2F contributes to reduced cell viability in hypoxic conditions.

4. The data in Fig. 4 do not support the authors' model. They show that their knockdown of Myc is complete. If FoxO3A is acting via inhibition of Myc, then knockdown of FoxO3A should have no effect in the absence of Myc, yet expression of all of the mitochondrial genes tested is increased by FoxO3A knockdown in Myc-knockdown cells.

We disagree with the notion that the knockdown of Myc is complete in this experiment, since this is a siRNA-mediated effect and not a knockout. Residual expression of Myc is detected in the Western blot shown. Moreover, we do not claim that the counteraction of Myc activity on these promoters by FoxO3A in hypoxia is the only mechanism in place and have re-worded this in the manuscript.
We have extended our investigations of the antagonism of FoxO3A and Myc with the help of a stably expressed hormone-inducible allele of Foxo3A in U2OS cells. These results are presented in Figure 6 of the revised manuscript.
Figure 6C shows that the activation of FoxO3A(A3)-ER exerts a strong repressive effect on the mitochondrial genes that is dependent on the presence of Myc. Along the same lines, experiments using siRNA oligonucleotides against FoxO3A and Myc in WI38 and TIG3 immortalized human fibroblasts indicate that the repressive effect of FoxO3A in hypoxia largely depends on Myc (Figures S4 and S5).

5. In Fig. 5, the authors perform ChIP analysis on several genes encoding mitochondrial proteins. They show clear evidence for Myc binding to these genes by ChIP, with enrichment of PCR by several logs when anti-Myc antibody is used for ChIP compared to IgG. In contrast, ChIP experiments using anti-FoxO3A antibody provide results that are not compelling, with at best only several-fold difference in enrichment. It is not at all clear how to interpret these data. Is FoxO3A binding directly to DNA? If so, what is the sequence to which it is binding and how does this binding interfere with the binding of Myc? Putative MYC/FOXO3A binding regions need to be analyzed in reporter assays to further clarify their mechanisms of action.

We agree with the reviewer that the enrichment of endogenous Foxo3A in our ChIP experiments is relatively small. However, given that our ChIP protocol works for other transcription factors such as Myc and HIF-1, we would exclude a general problem with these experiments. Moreover, as we show in Figures 5 and S9, knockdown of FoxO3A by two different shRNA sequences significantly reduces the amount of bound FoxO3A in the ChIP experiments on all promoters investigated. In addition, ChIP experiments employing the hormone inducible allele FoxO3A(A3)-ER in U2OS cells confirm binding of FoxO3A to the studied promoter sequences in a hormone-dependent fashion.
We agree that it is interesting whether FoxO3A needs a specific binding site on DNA to exert its repressive effect. To address this question, we employed the H212R mutant version of FoxO3A that cannot bind to the consensus FHRE element. This is documented by the ChIP results for the human p27 promoter in Figure 6A and consequently, this mutant fails to induce transcription of p27 (Figure 6B).
In ChIP experiments, we find this mutant to be enriched on the repressed mitochondrial gene promoters to a similar extent as the wild-type FoxO3A (Figures 6D, S11, and S12). Moreover, it is equally capable to repress these genes (Figure 6C). These results are in accordance with reports in the literature (Czymai et al., 2010; Ramaswamy et al., 2002) that describe gene regulation by DNA-binding deficient mutant forms of FoxO proteins.
In addition, we investigated the possibility of a direct interaction of FoxO3A and Myc in co-immunoprecipitation experiments as illustrated in Figure S13. However, we could not find any evidence for a stable complex formation of the two proteins, which does not exclude the possibility of a transient or indirect interaction in the context of the chromatin environment.
Taken together, our data are compatible with a mechanism that involves the recruitment of FoxO3A to specific sites within the chromatin of the repressed loci that is independent of FHRE elements in the DNA. This might occur via protein-protein interactions with another DNA binding factor, whose identification would be very interesting but in our opinion beyond the scope of this manuscript.

Referee 2

1. The authors should present a more thorough analysis of the metabolic phenotype described in figure 2. The differences in mitochondrial mass and oxygen consumption seem significant, but it would help to understand more about these differences. For example, myc has been implicated in regulating mitochondrial consumption of glutamine. Is the difference seen in figure 2B due to altered glutamine metabolism, or glucose, or both? These experiments should be repeated using media without glucose or glutamine to determine the relative contributions. In addition, glucose uptake and lactate production should be performed on these cells in vitro in normoxia and hypoxia to determine if the reduction in mitochondrial function is compensated for by an increase in glycolysis.

We have extended our analysis according to the suggestions made by referee 2. Indeed, we find that glutamine-free medium reduces oxygen consumption of FoxO3A knockdown cells in hypoxia, and that the absence of glucose reveals a much higher capacity of these cells to consume oxygen even in hypoxic conditions (Figure 2C). Accordingly, FoxO3A knockdown cells show reduced glucose uptake (Figure 2D) and lactate secretion (Figures 2D and 2E).

2. The immunohistochemical data showing induction of FoxO3A in perinecrotic regions of tumors (fig 6A), where there is CA9 expression, is important. However, this observation needs to be quantitated. How many tumors were observed, what fraction showed co-localization, how much of the section showed co-localization.

We have added a statistical analysis of the immunohistochemical data presented in Table S7, which was conducted by an experienced pathologist (Göran Landberg). An example as to how this was performed for the individual tumor nodules is illustrated in Figure S15. The statistical analysis demonstrated a significant correlation between tumors having increased nuclear expression of FoxO3A in perinecrotic areas and tumors showing perinecrotic CA9 expression as an indicator of an oxygen gradient across the tumor (Table S7).

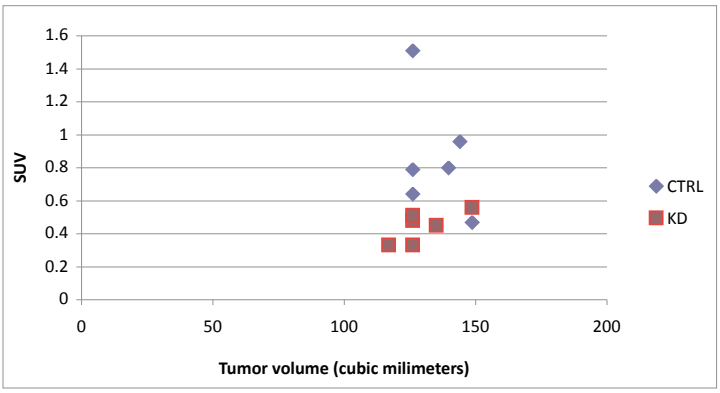
3. The model tumor data needs to be repeated. Were there only the tumors shown in the pictures (ie three of each)? A significant number of tumors needs to be grown in duplicate experiments. Vector controls and both knockdown lines need to be run in replicate experiments. Tumor volumes should be carried out to volumes of several hundred mm3, so that measurements can be taken more accurately. Accurate growth rates and volumes can help determine if there is an absolute reduction in growth rate, or simply a growth delay. Tumors should be harvested and protein extracts run to determine level of FoxO3A knockdown, and capase activation, post tumor growth.

We apologize for a misleading illustration of the data in the previous version of the manuscript. The growth curves were based on 12 pairs of tumors (CTRL and FoxO3A KD#1), of which three representative examples after tumor resection were shown.
We have replaced the figure with the results of a new experiment, in which 12 animals were injected with both control and KD#1 cells, and additional 12 animals with control and KD#2 cells, resulting in 24 control tumors and each 12 tumors of both knockdown cell lines. In order to obtain bigger tumors for a more precise monitoring of tumor sizes and of glucose uptake, we doubled the amount of injected cells per site to $8x10^6$ cells.
The results of these experiments confirm or previous finding of a reduced tumor growth rate (Figure 8A). Both knockdown cell lines gave rise to tumors that showed reduced glucose uptake at the endpoint of the experiment (Figure 8B). Four matched pairs of control and knockdown tumors were analyzed for FoxO3A, MRPL12 and cleaved Caspase 3 expression by Western blotting (Figure 8C) and mRNA expression of mitochondrial genes by qPCR (Figure 8E). This was performed for both FoxO3A knockdown clones.

4. FDG uptake in tumors needs to be normalized for tumor volumes. Growth rate differences in the control versus knockdown tumors suggest that knockdown tumors were smaller at the time of scanning. Larger tumors typically have higher FDG uptake, so SUV needs to be plotted (or tumors should be measured of equal volume) as a function of tumor volume.

We acknowledge the concern that the differences in tumor size could potentially impact the glucose uptake. However, for the sake of clarity we should stress that the FDG uptake displayed in Figure

8B is already normalized to tumor volume/mass as estimated by the PET (presented as %ID/g). Although it was inherently difficult to obtain evenly sized tumors of both genotypes due to the growth differences shown in Figure 8A, a few tumors of the Ctrl and knockdown groups were in the same size range of 120 to 150 cubic millimeters. For these tumors we plotted SUV versus tumor volume (see diagram below). The results for these evenly sized tumors reflect those obtained for the entire groups showing an on average increased FDG uptake of the Ctrl tumors compared with FoxO3A knockdown tumors and thus indicate that the observed differences in FDG uptake are unlikely to be a consequence of differences in tumor volume.



Minor comments
1. If there is increased apoptosis in knockdown cells treated with hypoxia in vitro (figure 3), is there increased apoptosis in the tumors grown in vivo?
2. The authors should comment on the potential contribution of other gene expression changes that may contribute to the tumor growth phenotype.

1. We have analyzed the apoptotic marker cleaved Caspase-3 by western blotting (Figure 8C upper and lower panel for FoxO3A-KD#1 and –KD#2, respectively) and by immunofluorescence staining (Figure S15) and found increased levels in FoxO3A knockdown tumors compared to control.

2. We agree that this is important to comment on and have added the following comment to the discussion:
"Although the experiments performed in cell culture show an important correlation between dysregulation of metabolism, ROS production, and cell death, it should be stressed that we cannot exclude that other hypoxic gene expression changes caused by knockdown of FoxO3A, such as the reduced activation of many hypoxia-induced genes, might contribute to the observed cell- and tumor growth phenotypes."

Referee 3

1. The authors should perform more experiments in the normal diploid cells, such BJ,WI38 or IMR90 cells, to define the competitive relationship between FOXO3a and c-Myc under the hypoxia condition.

We have investigated the antagonistic role of FoxO3A and Myc in human diploid immortalized WI38 and TIG-3 cells. siRNA-mediated knockdown of FoxO3A and Myc in these cells confirmed our previous findings in Hela: We observed a hypoxia-mediated repression of nuclear-encoded mitochondrial genes that depended on FoxO3A. Knockdown of Myc inhibited a further repression of these gens by FoxO3A (see Figures S4 and S5).

2. The RNAi experiments should be strengthened with additional shRNAs or rescue controls in Figure 5 and Figure 6C-E.

We have repeated all relevant experiments with a second knockdown clone expressing a different shRNA construct targeting FoxO3A. Results are shown in Figures 5, S9, and 8 of the revised manuscript.

3. The authors should include more experiments to characterize the interaction between c-Myc and FoxO3A under normoxia and hypoxia conditions.

We have investigated the possibility of a direct interaction of FoxO3A and Myc in normoxia and hypoxia in co-immunoprecipitation experiments as illustrated in Figure S13. However, we could not find any evidence for a stable complex formation of the two proteins under IP conditions. However, in our opinion this does not exclude the possibility of a transient or indirect interaction in the context of the chromatin environment.

4. In Figure 5, another FOXO3 RNAi or FOXO3 knockout cells to strengthen the conclusion that the direct action of FoxO3A on the promoters of hypoxia-repressed nuclear-encoded mitochondrial genes applies to a specific subclass of Myc target genes.

We have included a second knockdown clone expressing a different shRNA construct targeting FoxO3A. Results are shown in Figures 5 and S9. In addition we have used the hormone inducible alleles FoxO3A(A3)-ER and FoxO3A(A3)-H212R-ER stably expressed in U2OS. We find that these proteins bind to the investigated promotes in a hormone-dependent fashion. Moreover, the ability of FoxO3A(A3)-H212R-ER to bind to the promoters and to repress transcription indicates that a FHRE consensus element is not required for the repressive effect (Figures 6, S11 and S12)

5. To rule out the possibility that FOXO3a represses c-Myc transcription through Mxi induction, the authors should perform the similar experiments in the background of MXI knockdown.

We performed knockdown of Mxi1 in HeLa cells and did not find any significant contribution of Mxi1 to the hypoxia-mediated repression of the investigated genes (Figure S7).

6. Comments on the Figure 6:
(1) The authors should describe how many tumor samples were immunostained? Statistical analysis is required for the conclusion.
(2) From the Figure S1G, the nuclear FOXO3a is not significantly increased even Though the total protein levels are increased under hypoxia treatment. The authors claim that FOXO3a in tumor samples becomes activated only basing on that the FOXO3a is co-stained with HIF alpha target gene-CA9. The authors should carefully calculate the ratio of nuclear FOXO3a vs cytoplasmic FOXO3a since FOXO3a protein levels are increased in the hypoxia area, and use the expression of FOXO3a targets to indicate the transcriptional activation of FOXO3a .
(3) The cell death should be analyzed in the clinic samples as well as the tumor blocks from the transplanted tumor mass.
(4) As stated in point 2, additional FOXO3a shRNA should be used in the xenograft experiments.
(5) The expression of c-Myc target genes (Aco2 and LARS2) that related with hypoxia response should be examined in the transplanted tumor mass.

(1 and 2)
We disagree that the amount of FoxO3A in the nuclear compartment is not clearly increased in hypoxia (Figure S1E of the revised manuscript). This finding is also in agreement with the findings of others (Bakker et al., Molecular cell 2007).
We completely agree that a statistical analysis of the immunohistochemical data is required and this was performed on 86 tumor nodules by an experienced pathologist (Göran Landberg) and is shown in Table S7. An example as to how this was performed for the individual tumor nodules is illustrated in Figure S14. Despite our efforts we were not able to get reliable ratios of the nuclear versus the cytoplasmic fractions of FoxO3A in perinecrotic cells in the tumors. However, only cells that

showed clear nuclear localization of FoxO3A such as the ones illustrated in Figure 7A (please see higher magnifications of indicated regions to the right) were considered positive in our analysis. The statistical analysis demonstrated a significant correlation between tumors having increased nuclear expression of FoxO3A in perinecrotic areas and tumors showing perinecrotic CA9 expression as an indicator of an oxygen gradient across the tumor (Table S7).
Adjacent sections of the DCIS tumors were stained for expression of the well-characterized FoxO3A target gene p27/kip1 and for MMP13, which has recently been linked to FoxO3A-mediated tumor cell invasiveness (Storz et al., MCB 2009). Despite the fact that we tested two different antibodies for MMP-13, we did not obtain a satisfactory quality of staining (not shown). In contrast, we found elevated p27 expression within regions in the DCIS lesions that showed concomitant high signals for CA9 and FoxO3A (Figure 7 A and B).

(3) We have analyzed the apoptotic marker cleaved Caspase-3 by western blotting (Figure 8C upper and lower panel for FoxO3A-KD#1 and –KD#2, respectively) and by immunofluorescence staining (Figure S15) and found increased levels in FoxO3A knockdown tumors compared to control.
Göran Landberg and colleagues have previously investigated the frequency of apoptotic cells in DCIS lesions that show hypoxic gradients (Helczynska et al., Cancer Res 2003) and detected only few cells undergoing apoptosis by TUNEL staining (see picture taken from the publication below). Accordingly, cell lines derived from DCIS do not respond to hypoxic culture conditions with increased apoptotic rates (Helczynska et al., Cancer Res 2003).

(4) According to the suggestion, we have used an independently derived cell line expressing an alternative shRNA hairpin sequence in all relevant experiments (Figure 8).

(5) The mRNA expression of LARS2, ACO2, MRPL12, and OXNAD1 in the xenograft tumors has been analyzed. These data have been added as Figure 8E for both knockdown lines.

Additional information for the referees (from: Helczynska et al., Cancer Res 2003).

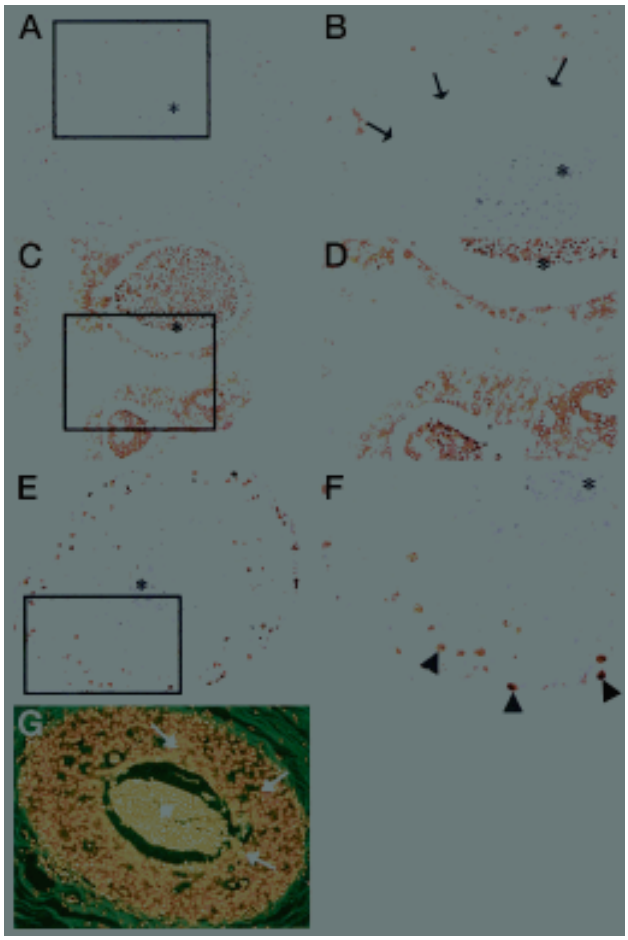


Fig. 2. ER, CK19, and Ki-67 immunoreactivity and TUNEL labeling in DCIS. *A* and *B*, staining for ER is positive in all but the innermost cell layers (indicated by *arrows*), which show lack of signal. C and D, CK19 staining intensity is increased in the inner cell layers surrounding the central necrosis (_). E and F, Ki-67 staining shows the highest prevalence of Ki-67-positive cells in the outer cell layers (indicated by arrowheads). G, TUNEL staining of a DCIS lesion. Note the few green, apoptotic cells indicated by the arrows. B, D, and F are magnifications of the framed areas in A, C, and E.

2nd Editorial Decision 15 August 2011

The paper has been re-reviewed by referee # 3 with no further comments.

