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EgIN2 Associates with the NRF-PGC1 Complex and Controls Mitochondrial Function in Breast Cancer

Jing Zhang, Mr. Chengyang Wang, Xi Chen, Mamoru Takada, Cheng Fan, Xingnan Zheng, Haitao Wen, Yong Liu, Chenguang Wang, Richard G. Pestell, Katherine M. Aird, William G. Kaelin, Jr., X. Shirley Liu, and Qing Zhang

Corresponding author: QING ZHANG, University of North Carolina-Chapel Hill

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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 29 April 2015

Thank you for submitting your manuscript for consideration by the EMBO Journal and my apologies for the unusual delay in the review process here. Your study has now been seen by three referees whose comments are shown below.

As you will see from the reports, all referees express interest in the findings reported in your manuscript, however they do also raise a number of rather critical concerns for the conclusiveness and significance of the reported findings that will have to be fully addressed before they can support publication in The EMBO Journal.

I realize that addressing all concerns raised will involve extensive experimental efforts of an uncertain outcome and I would therefore understand if you would rather prefer to seek more rapid publication at a less demanding venue at this stage. However, if you were to undertake the task of revising the manuscript as outlined by the referees we would be willing to consider such a revised version. We would also be happy to extend the revision time beyond the standard three months given the extensive need for extra experiments. If you should decide to rather publish the manuscript rapidly and without any significant changes elsewhere, please let us know so we can withdraw it from our system.

Given the referees' overall positive recommendations, I would thus invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your

manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

REFEREE REPORTS

Referee #1:

In this manuscript, Zhang et al., have investigated the control of the NRF-PGC-1alpha pathway in breast cancer by the EglN2 oxygen-sensing hydroxylase. The authors provide strong evidence that by interacting with NRF1 and PGC-1alpha (in a HIF-independent manner), Egln2 regulates mitochondrial function through the regulation of FDXR expression and propose a link between this and breast cancer development. This is a comprehensive and important piece of work with strong implications for the potential of targeting hydroxylases in breast cancer. However, the authors should address some concerns as outlined below:

Major Issues:

1) In Figure 1, the authors show a relationship between EglN2 depletion and mitochondrial DNA content / respiration. Is this specific for EglN2 or does it also happen with depletion of the other HIF hydroxylase isoforms (EglN1/3)?

2) Does control shRNA impact upon respiration and mitochondrial content in comparison to mock transfected cells? Is there a non-specific shRNA effect?

3) There appears to be a difference between the effects of EglN2 depletion on respiration and mitochondrial DNA between figures 1 and 2. In figure 2D, the effect of Egln2 depletion on oxygen consumption are somewhat less impressive than in Fig 1, is this difference statistically significantly different? Similarly in Figure 2E, is the difference between control and sh326 significantly different? The application of statistical analysis to the respiratometry data should be included to provide assurance.

4) Figures 4E and 4F demonstrate that while hypoxia enhances the interaction between EglN2 and NRF1, DMOG does not. Does this indicate that the interaction is independent of the hydroxylase activity of EglN2. This could be tested by using an enzymatically dead EglN2 and seeing if it bound to NRF1 under conditions of hypoxia.

5) The central observation would be strengthened by the demonstration using (for example) immunohistochemistry to demonstrate the formation of an endogenous NRF1/EglN2/PGC1alpha complex under conditions of hypoxia.

6) Is there a difference in cellular respiration / mt DNA in cells derived PHD1 knockout mice which are, to my knowledge, phenotypically normal?

7) It has previously been demonstrated that inhibition of mitochondrial respiration under oxygen-limited conditions results in increased oxygen availability to hydroxylases resulting in HIF

repression, an event which may potentially contribute to a reduction in tumor growth (Hagen et al., Science 2003). Have the authors considered this as a contributory mechanism to the anti-tumor effects of EglN2 KO?

8) In Figure 5B, the number of EglN2 binding sites greatly outnumbers those of NRF1. This begs the question of other EglN2 complexes on DNA. Have the authors considered this? Analysis of the EglN2 interactome under normoxia vs. hypoxia would make a nice addition to the manuscript which may provide insight into this.

Minor Issues:

1) Abstract, line 3 replace "playing" with "play".
Abstract line 9 replace "condition" with "conditions"

Referee #2:

Overall, I think this paper reports several interesting findings. In particular, that EglN2 binds chromatin and forms a complex with NRF1 and PGC1α that activates the transcription of FDXR. Upregulation of this target gene appears to mediates, at least some of the effects of EglN2 on mitochondrial function.

What is not clear is the extent to which these effects are HIF-independent (the authors only looked at HIF-1α and not HIF-2α or HIF-3 α -and some of the experiments proposed to support HIF-independence were performed in normoxia not hypoxia).

Finally, the authors show a correlation between mtDNA content, mitochondrial respiration and tumour growth, but do not show direct links between any of these processes.

In particular, the authors claim (in the abstract and introduction) that impaired mitochondrial respiration occurs as a result of the decrease in mtDNA content, but show no direct link between the two. I agree that both effects may be direct (or indirect / HIF mediated) consequences of EglN2 depletion; however, that does not definitely show that one occurs as a direct result of the other.

In terms of the links to breast cancer, it is unclear as to the extent to which any pro-tumorigenic effects of Egln2 in ERα+ breast cancer are mediated by its effects on the mitochondria - I am convinced that it is capable of upregulating FDXR, at least in certain circumstances, but FDXR may have consequences for tumour growth that are unrelated to the mitochondria.

Consequently, I'm not sure that I agree with their statement that the mitochondrial function of EglN2 can be exploited as a 'potential therapeutic target' - FDXR may well be a possible target for therapeutic intervention, but this would need to be explored in further detailed studies.

Below are comments relating to specific data sets / figures.

Specific points
Figure 1
The authors show that knockdown of EglN2 lowers the rate of mitochondrial oxygen consumption and decreases mitochondrial DNA content.
Query: comparing 1B with 1H: why is the basal OCR so different for the controls in these experiments? Is it correct that for the same cell line, the difference in basal OCR across experiments is greater than the changes caused by depletion/overexpression of EglN2.
Fig 1A OCR values: control = 400 EglN2 depletion = 250-300 (difference = 100-150)
Fig 1H OCR values: control = 870 EglN2 overexpression = 1200 (difference = 500)
Difference in basal OCR between experiments = 470

Figure 2
The authors show that depletion of EglN2 does not cause an increase in levels of HIF-1α protein in normoxia (if anything HIF-1α is decreased in MCF-7 cells). They also show that the effects of

EglN2 depletion on mitochondria are independent of HIF-1α and ARNT.

Specific Comments:
- FigE: If the effects of EglN2 depletion on mtDNA content are HIF-1α dependent, then the simultaneous depletion of Elgn2 and HIF-1α should cause the same / similar decrease in mtDNA content as EglN2 depletion alone. i.e. the important comparison in this figure is between the [Egnl2 shRNA + Ctrl] and [EglN2 shRNA + HIF1α shRNA] samples, yet there is not statistical analysis has been done for this comparison. Likewise for the comparison of [Egnl2 shRNA + Ctrl] and [EglN2 shRNA + ARNT shRNA] in Figure 1H?

- What about HIF-2α and HIF-3α ? Experiments to test their potential involvement need to be carried out.

- It is important that these experiments are repeated under hypoxic conditions - as this is when they see the greatest effects of EglN2 on the mitochondria.

Figure S2
Same comments as for Figure 2 (see above).

Figure 3
The authors show that EglN2 depletion also leads to decreased mitochondrial respiration and mtDNA content in hypoxia, while overexpression of EglN2 has the converse effect. These observations are similar to those made in normoxia, although the fold change caused by EglN2 over-expression is higher in hypoxia (4-fold compared to 1.5-fold in normoxia).
The authors go on to show that EglN2 is bound to chromatin in hypoxia but not normoxia, and that it has transactivation activity in TET reporter assays.

Specific comments:
The authors comment that EglN2 depletion has a greater effect on OCR and mtDNA in hypoxia than normoxia, which suggests "a distinct role of EglN2 on mitochondrial respiration under hypoxia."
- It is notable that in Figure 4J the authors perform a similar experiment, but in this case overexpression of Flag-Elgn2 (in combination with a control shRNA) in hypoxia/T47D cells causes only a 1.5-fold induction of mtDNA - similar to what was previously observed in normoxia. Comment?

- I don't like their use of the word 'distinct,' as EglN2 is clearly having a significant effect on the mitochondria in both normoxic and hypoxic conditions. There is no evidence to suggest that this occurs via a different mechanism. I assume this statement is made because the authors only see EglN2 on chromatin in hypoxia (and not in normoxia). However, if that's the case, then it would be nice if they could provide an explanation for how EglN2 is influencing the mitochondria in normoxia.

Furthermore, because of this discrepancy between normoxia and hypoxia, it is especially important for the authors to show that they hypoxia effects are also independent of HIF (i.e. repeat expts. from Fig2 under hypoxia).

- Why were the TET reporter assays performed in 293T cells and not ER+ breast cancer cells to be consistent with the rest of the paper? (Possibly because 293T are easier to transfect, but the experiments need to be done ER+ cells)

- The finding that EglN2 is chromatin-bound and has transactivation activity is one of the more novel findings in the paper - I think it's more interesting than the mitochondrial story.

Figure 4
The authors show that EglN2 is indeed bound to chromatin in hypoxia, and is present in the promoter regions of numerous genes. Genes that are positively regulated by EglN2 show enrichment of binding sites for NRF1. EglN2 interacts with GST-NRF1 in vitro, and with both PGC1α and NRF1 (HA-tagged and endogenous) in cells. Depletion of EglN2 by shRNA leads to decreased binding of NRF1 to PGC1α, suggesting that this interaction is mediated to some extent by EglN2 and that these three factors likely form a ternary complex. In further support of this, the increase in

mtDNA content caused by EglN2 overexpression is abrogated by knockdown of either NRF1 or PGC1α.

Specific Comments:
- I am confused as to why the authors did not perform their microarray experiment in normoxia as well as hypoxia. I would have thought it would be interesting (though not essential) to look for genes that are regulated by EglN2 specifically in hypoxia. Results in normoxia should be shown.
- Fig4E-G: NRF1 and PGC1α interact with EglN2 in both T47D/MCF-7 cells in normoxia (albeit weakly) as well as hypoxia. The authors should comment on this. Where is this interaction taking place? As their previous data indicates that EglN2 is not chromatin bound in normoxia. Does this argue against a 'hypoxia specific' mechanism of mitochondrial regulation? The authors comment here (and again in the discussion) that DMOG has "no effect" on these interactions, yet it clearly does (albeit to a lesser extent than hypoxia).
- Although EglN2 does not appear to regulate the stability of HIF-1α in hypoxia (Fig2), it could still be bound to HIF-1α (or 2α) in hypoxia, and this could be a mechanism by which it is recruited to gene promoters. Fig4B suggests that binding sites for HIF1α and ARNT (which they now refer to as HIF1β - should be consistent) are present in the promoter regions of genes that are up- and down-regulated by EglN2, yet they do not comment on this finding. Could Egnl2 be bound to DNA via HIF?
Fig4E-F: The authors should immunoblot for HIF-1α in the EglN2 IP. They show that it is present in the WCE but it is important to demonstrate that it does not bind EglN2.
- The authors state that "the interaction between NRF1 and EglN2 was not affected upon HIF1α depletion in these cells under hypoxia (data not shown)" - it is important to show this data, as it would support a HIF-independent mechanism.
In regard of this it would be interesting to show results for catalytically inactive EglN2.

Figure 5
The authors show that binding sites for EglN2 overlap with many of those identified for NRF2 in hypoxia. They identify a gene, FDXR, that shows decreased expression upon depletion of either NRF1 or EglN2 in hypoxia. Overexpression of EglN2 leads to elevated expression of FDXR, as effect that is dependent on NRF1 and PGC1α.
- It would be interesting to see how the expression level of FDXR in hypoxia compares to that in normoxia, and how the latter is affected by NRF1 or EglN2 depletion, especially since the authors believe this is a hypoxia-specific mechanism.
Figure S3
Fine - no comments.

Figure 6
The authors show that depletion of FDXR in hypoxia causes a decrease in mtDNA content similar to that observed following depletion of EglN2. Furthermore, overexpression of FDXR rescued the effects of EglN2 depletion on mtDNA content and mitochondrial respiration, indicating that the mitochondrial effects of EglN2 in hypoxia are at least partly mediated by FDXR. The authors show that FDXR depletion inhibits breast tumor growth in vivo, and find that FDXR expression is higher in ERα+ breast cancer cohorts than normal cohorts.
Specific comments
- The findings from the in vivo studies are interesting, but there is no direct link with the mitochondrial work presented in Figures 1-5. Are the authors suggesting that the decrease in mitochondrial mass observed with FDXR knockdown is linked with its effects on tumor growth. I'm not sure that it is possible to show this directly...

Referee #3:

The manuscript by Zhang et al. reports that the prolyl hydroxylase controls mitochondrial bioenergetics via interacting with transcriptional components of the mitochondrial biogenesis and OXPHOS pathway, NRF1 and PGC1a. It appears that one of the key targets to mediate EglN2-dependent mitochondrial function is the ferridoxin reductase protein. The authors agglutinate this mechanism and molecular components within the tumor biology of breast, showing that FDXR promotes breast tumorigenesis. Overall, these studies are novel and of broad interested and provide sufficient mechanism to explain, at least in part, the functional tumor experiments. There are

however, key experiments, that are needed to strengthen the manuscript and to support the conclusions raised by the authors.

1- Fig1. Authors convincingly showed that oxygen consumption is affected upon Egln2 knock down, however due to possible off-target effects it would be recommended to rescue the respiration by overexpressing Egln2 exogenously.
Moreover, alternative methods to assess the mitochondrial mass should be performed, such as mitotracker or mitored.

2- Fig1S. It is clear that mtDNA is downregulated when Egln2 is silenced, however not a single mitochondrial encoded protein has been blotted. For instance; is mtCOI (complex IV subunit) downregulated? If so, are other proteins (nuclear encoded) belonging to this complex also affected? If this scenario is true, is this leading to a mito-nuclear imbalance?

3- Fig2. Is Egln2 controlling HIF-2? It will be nice to se HIF-2 (EPAS) in these blots.

4- Fig3. Authors argued that Egln3 effects on mtDNA are more pronounced under hypoxic conditions. Have they measure oxygen consumption under hypoxia using shEgln2 and FLAG-Egln2 cells lines?

5- Fig4- 4. Authors declare that Egln2 control mitochondrial function by binding and activate NRF1/PGC1a complex, and this interaction is stronger under hypoxia and almost nonexistence in normoxia. However all the effects in oxygen consumption seem before have been done under normoxia. Moreover, it has been clearly reported that hypoxia decrease oxygen consumption in many cell lines as well as in vivo, which raises the question of how low oxygen condition is going to induce this pro-oxidative phenotype. Authors should clarify this conundrum.

6- Fig5. Authors should show normoxic data of this figure.
Base on ChIP experiments in Fig C, Egln2 binds to many genes at its promoter region, and control their transcriptional levels. However these very same genes do not change when NRF1 is knock down. How do Authors explain this Egln2 dependent but NRF1 independent regulation in gene expression?

7- Fig6. It is not clear how FDXR, which is a mitochondrial flavoprotein that initiates electron transport for cytochromes P450, is able to dramatically influence mtDNA levels. Authors should outline a plausible theory.

8- Authors showed how tumors formed from shFDXR were smaller, which could be reasonably attributed to the detoxification function of FDXR rather that mtDNA depletion. Are shEgln2 formed tumors also smaller? Is this rescue by overspressing FDXR? Does shNRF1 affect tumor growth in these cells?

9-Egln2 also known PHD1 function as an oxygen dependent prolyl hydroxylase, nevertheless authors never assessed whether this function is important for the mechanism and phenotypes described in this paper. It will be enlightening the use of a mutated version of the enzyme lacking prolyl hydroxylase activity to address such questions.

10- Pyrimidine de novo synthesis is directly connected with the mitochondria function an disruption of the mitochondrial electron transport chain may impair UTP, TTP, and CTP synthesis which will lead to mtDNA reduction without affecting mitochondrial mass. Have Authors consider this possibility to explain their observed phenotypes.

1st Revision - authors' response 06 August 2015

Referee #1:

In this manuscript, Zhang et al., have investigated the control of the NRF-PGC-1alpha pathway in breast cancer by the EglN2 oxygen-sensing hydroxylase. The authors provide strong evidence that by interacting with NRF1 and PGC-1alpha (in a HIF-independent manner), Egln2 regulates

mitochondrial function through the regulation of FDXR expression and propose a link between this and breast cancer development. This is a comprehensive and important piece of work with strong implications for the potential of targeting hydroxylases in breast cancer. However, the authors should address some concerns as outlined below:

Major Issues:

1) *In Figure 1, the authors show a relationship between EglN2 depletion and mitochondrial DNA content / respiration. Is this specific for EglN2 or does it also happen with depletion of the other HIF hydroxylase isoforms (EglN1/3)?*

Response: We depleted EglN1 or 3 expressions in T47D cells and examined the mitochondrial DNA content and oxygen consumption rate (new Fig EV1G). In contrast to EglN2, depletion of EglN1 or EglN3 modestly increased mtDNA content (new Fig EV1I). EglN3 depletion did not affect either basal or maximal oxygen consumption rate (new Fig EV1H). EglN1 depletion minimally affected basal but not maximal consumption rate (new Fig EV1H). In conclusion, EglN2 is the primary prolyl hydroxylase in breast cancer that regulate mitochondrial function positively.

2) *Does control shRNA impact upon respiration and mitochondrial content in comparison to mock transfected cells? Is there a non-specific shRNA effect?*

Response: To rule out the non-specific shRNA effect, we infected EglN2 knockdown cells with shRNA resistant EglN2 overexpression followed by examination for respiration/mtDNA content. The results showed the effect of EglN2 knockdown on respiration/mtDNA was rescued by EglN2 overexpression, indicating that there is a specific EglN2 shRNA effect. Please see new Fig EV1A, EV1B and EV1C.

3) *There appears to be a difference between the effects of EglN2 depletion on respiration and mitochondrial DNA between figures 1 and 2. In figure 2D, the effect of Egln2 depletion on oxygen consumption are somewhat less impressive than in Fig 1, is this difference statistically significantly different? Similarly in Figure 2E, is the difference between control and sh326 significantly different? The application of statistical analysis to the respiratometry data should be included to provide assurance.*

Response: As reviewer suggested, we have performed the statistical analysis for all of these comparisons of Fig2 and found the difference is statistically significant. Please see new Fig 2D, 2F and 2H, EV2A, EV2B and EV2C. The original Fig 2D and 2G have been moved as new Fig EV2A and EV2C, respectively. The original Fig 2E and 2H have been moved as new Fig 2D and 2H, respectively.

4) *Figures 4E and 4F demonstrate that while hypoxia enhances the interaction between EglN2 and NRF1, DMOG does not. Does this indicate that the interaction is independent of the hydroxylase activity of EglN2. This could be tested by using an enzymatically dead EglN2 and seeing if it bound to NRF1 under conditions of hypoxia.*

Response: Thank the reviewer for pointing that out. As the reviewer suggested, we used the EglN2 enzymatic dead mutant (EglN2 H358A) to examine its binding with NRF1 or PGC1α under hypoxia. The results showed that the EglN2 enzymatic dead mutant binding with NRF1 or PGC1a is as strong as EglN2 WT, indicating that the interaction is independent of the hydroxylase activity of EglN2. Please see the new Fig EV4E and EV4F.

5) *The central observation would be strengthened by the demonstration using (for example) immunohistochemistry to demonstrate the formation of an endogenous NRF1/EglN2/PGC1alpha complex under conditions of hypoxia.*

Response: Thanks for reviewer's good suggestion. We tried different antibodies and did not find workable ones for immunohistochemistry. So, we performed the cell fractionation experiments and isolated cytoplasmic, nuclear soluble and chromatin bound fractions followed by western blots and

co-IP to examine the endogenous NRF1/EglN2/PGC1α complex. The results showed that the complex mostly takes place in chromatin bound fractions. Please see the new Fig EV4G and EV4H.

6) *Is there a difference in cellular respiration / mt DNA in cells derived PHD1 knockout mice which are, to my knowledge, phenotypically normal?*

Response: We examined the cellular respiration/mtDNA for EglN2 (PHD1) knockout and wild type MEFs derived from littermates. There is no distinctive difference for basal respiration or mtDNA content between EglN2 knockout and wild type MEFs (Fig EV1J, EV1K and EV1L).

7) *It has previously been demonstrated that inhibition of mitochondrial respiration under oxygen-limited conditions results in increased oxygen availability to hydroxylases resulting in HIF repression, an event which may potentially contribute to a reduction in tumor growth (Hagen et al., Science 2003). Have the authors considered this as a contributory mechanism to the anti-tumor effects of EglN2 KO?*

Response: To examine whether EglN2 depletion affects HIFs in oxygen-limited conditions, we showed in new Fig EV3B that EglN2 depletion in MCF-7 cells does not affect HIF1α, HIF2α or ARNT protein levels under hypoxia. For T47D, as shown in new Fig EV3C, EglN2 knockdown led to decreased HIF1α and HIF2α but not ARNT expression under hypoxia. Our results suggest that inhibition of mitochondrial under hypoxia resulting in oxygen availability to hydroxylase and HIF destabilization might be a cell type specific phenomena. Our previous publication showed that EglN2 depletion decreased Cyclin D1 in breast cancer via HIF independent manner (Zhang et al, 2009). Our current study showed that the effect of EglN2 on mitochondrial function is HIF1/2α independent. However, we cannot exclude the possibility that decreased HIF1/2 α level may partially contribute the antitumor effect of EgLN2 knockdown at the current stage, which will be our future interest.

In Figure 5B, the number of EglN2 binding sites greatly outnumbers those of NRF1. This begs the question of other EglN2 complexes on DNA. Have the authors considered this? Analysis of the EglN2 interactome under normoxia vs. hypoxia would make a nice addition to the manuscript which may provide insight into this.

Response: Thanks for reviewer's good suggestion. We acknowledge that there exists the possibility of other EglN2 complexes on DNA. For example, we identified ARNT and HIF1α motif enrichment in both EglN2 positively and negatively regulated genes by integrated analyses of ChIP-seq and microarray (See new Fig 4B). There exist the possibilities that EglN2 might bind to DNA via HIF and regulate pathways other than mitochondrial function. We have revised our discussion part to reflect that. See page 22 starting from line 15.

For the EglN2 interactome under normoxia versus hypoxia, as an alternative approach, we have examined the interaction complex between EglN2, PGC1α and NRF1 under normoxia and hypoxia as shown in new Fig EV4G and EV4H. We showed that EglN2, NRF1 and PGC1α form a stronger interaction in chromatin fractions under hypoxia. Combined with our data showed that EglN2 and NRF1 may co-regulate some transcriptional targets under hypoxia, including FDXR, our accumulative evidence provide some mechanism insight with regard to the role of EglN2 on regulating mitochondrial function. We plan to purify EglN2 interactome under normoxia vs. hypoxia to identify other EglN2 complexes on DNA in the future.

Minor Issues:

1) *Abstract, line 3 replace "playing" with "play".*
Abstract line 9 replace "condition" with "conditions"

Response: We corrected these oversights in the revised abstract.

Referee #2:

Overall, I think this paper reports several interesting findings. In particular, that EglN2 binds chromatin and forms a complex with NRF1 and PGC1α that activates the transcription of FDXR. Upregulation of this target gene appears to mediates, at least some of the effects of EglN2 on mitochondrial function.

What is not clear is the extent to which these effects are HIF-independent (the authors only looked at HIF-1α and not HIF-2α or HIF-3α -and some of the experiments proposed to support HIF-independence were performed in normoxia not hypoxia).

Response: We have revised our paper and dedicated the Figure 2 as well as Fig EV2 to address the HIF-independence issues. In addition to original HIF1α and ARNT (HIF1β) data, we also depleted HIF-2α in MCF-7 and T47D cells and the results showed that the effect of EglN2 on mitochondrial OCR or mtDNA are independent of HIF1/2α (Please see new Figure 2E, 2F, EV2C, EV2D, EV2I and EV2J). For HIF-3α, it was reported to dimerize with HIF1/2α and thereby impairs interaction between HIF1/2α and hypoxia response elements of the target genes, acts as a dominant-negative regulator of the HIF signaling (Makino et al, 2001; Torii et al, 2011). So far, there is very few publication showing that HIF3α is involved in regulating mitochondrial function. In addition, its expression in T47D or MCF-7 is extremely low (RMA Log2 value =4.5) versus HIF-1α (RMA Log2=12) or HIF-2α (RMA Log2=8.5) based on the mRNA expression obtained from cancer cell line encyclopedia. Consistently, we did not detect HIF3α protein expression under normoxia or hypoxia in T47D and MCF-7 cells. Therefore, we did not test that in our current revised manuscript. To be more rigorous, we used "HIF1/2α-independent" instead of 'HIF-independent" in revised manuscript. Please see the new abstract as well as main text page 9 starting from line 3.

As the reviewer suggested, we performed these experiments under hypoxia and the results showed these effects are also HIF1/2α-independent. Please see new Fig 3F and Fig EV3D.

Finally, the authors show a correlation between mtDNA content, mitochondrial respiration and tumour growth, but do not show direct links between any of these processes. In particular, the authors claim (in the abstract and introduction) that impaired mitochondrial respiration occurs as a result of the decrease in mtDNA content, but show no direct link between the two. I agree that both effects may be direct (or indirect / HIF mediated) consequences of EglN2 depletion; however, that does not definitely show that one occurs as a direct result of the other.

Response: Regarding the connection between mitochondrial respiration and mtDNA content, in our new Fig EV1D, we showed that cells depleted of EglN2 displayed decreased levels of some mitochondrial-encoded protein involved in complex I and IV, which are essential for mitochondrial respiration function. We revised the abstract and introduction to reflect the effect of EglN2 on mitochondrial respiration and mtDNA content and we did not claim the one occurs as the direct result of the other. Please see the revised abstract for the following statement "Here we show that EglN2 depletion decreases mitochondrial respiration in breast cancer under normoxia and hypoxia, which correlates with decreased mitochondrial DNA in a HIF1/2α independent manner"

In terms of the links to breast cancer, it is unclear as to the extent to which any pro-tumorigenic effects of Egln2 in ERα+ breast cancer are mediated by its effects on the mitochondria - I am convinced that it is capable of upregulating FDXR, at least in certain circumstances, but FDXR may have consequences for tumour growth that are unrelated to the mitochondria. Consequently, I'm not sure that I agree with their statement that the mitochondrial function of EglN2 can be exploited as a 'potential therapeutic target' - FDXR may well be a possible target for therapeutic intervention, but this would need to be explored in further detailed studies.

Response: Thank the reviewer for pointing this out. We agree with reviewer and revised the statement that mitochondrial function of EglN2 can be exploited as a " potential therapeutic target". We have changed our statement to "Our findings suggest that EglN2 regulates mitochondrial function in ERα positive breast cancer." Please see new abstract.

Below are comments relating to specific data sets / figures.

Specific points
Figure 1
The authors show that knockdown of EglN2 lowers the rate of mitochondrial oxygen consumption and decreases mitochondrial DNA content.
Query: comparing 1B with 1H: why is the basal OCR so different for the controls in these experiments? Is it correct that for the same cell line, the difference in basal OCR across experiments is greater than the changes caused by depletion/overexpression of EglN2.
Fig 1A OCR values: control = 400 EglN2 depletion = 250-300 (difference = 100-150)
Fig 1H OCR values: control = 870 EglN2 overexpression = 1200 (difference = 500)
Difference in basal OCR between experiments = 470

Response: We have repeated the OCR experiment by using newly generated EglN2 overexpression cell lines and found that the basal OCR levels for T47D control cells are consistent, around 400. Please see Fig 1B and new Fig 1H.

Figure 2
The authors show that depletion of EglN2 does not cause an increase in levels of HIF-1α protein in normoxia (if anything HIF-1α is decreased in MCF-7 cells). They also show that the effects of EglN2 depletion on mitochondria are independent of HIF-1α and ARNT.
Specific Comments:

- *FigE: If the effects of EglN2 depletion on mtDNA content are HIF-1α dependent, then the simultaneous depletion of Egln2 and HIF-1α should cause the same / similar decrease in mtDNA content as EglN2 depletion alone. i.e. the important comparison in this figure is between the [Egnl2 shRNA + Ctrl] and [EglN2 shRNA + HIF1α shRNA] samples, yet there is not statistical analysis has been done for this comparison. Likewise for the comparison of [Egnl2 shRNA + Ctrl] and [EglN2 shRNA + ARNT shRNA] in Figure 1H?*

Response: We thank for the reviewer's comment. Simultaneous depletion of EglN2 and HIF1α/ARNT caused the similar decrease in mtDNA as EglN2 alone suggested that the effect of EglN2 on mtDNA is independent of HIF1α/ARNT. In addition, in the cells depleted HIF1α, EglN2 depletion still led to decreased mtDNA, suggesting that the effect of EglN2 on mtDNA is HIF1α/ARNT independent. Since HIF1α or ARNT depletion robustly increased mtDNA, the important comparison would be Ctrl+ HIF1α shRNA/ARNT shRNA to EglN2 sh326+ HIF1α shRNA/ARNT shRNA. We have added these comparisons and statistical analyses for new Fig 2D and 2H. The original Fig 2E has been moved as new Fig 2D.

Furthermore, as the reviewer pointed out, depletion of EglN2 does not cause an increase level of HIF1α protein (if anything, decreased HIF1α protein). Since HIF1α depletion led to increased mtDNA content in these cells, this suggests that decreased mtDNA content by EglN2 depletion is not due to HIF1α.

- What about HIF-2α and HIF-3α? Experiments to test their potential involvement need to be carried out.

Response: Please see the response above in the first response session for reviewer #2.

- *It is important that these experiments are repeated under hypoxic conditions - as this is when they see the greatest effects of EglN2 on the mitochondria.*

Response: We have included experiments under hypoxic conditions in new Fig 3 F, EV3B and EV3C.

Figure S2
Same comments as for Figure 2 (see above).

Response: We have modified our figures and added these statistical analyses and comparisons in revised Fig EV2E and EV2I. We also included HIF2α in new Fig EV2B and EV2G and performed experiment under hypoxic condition showed in new Fig EV3D. The original Fig S2A and S2C have been moved as Fig EV2D and EV2H, respectively. The original Fig S2B and S2D have been moved as newly revised Fig EV2E and EV2I, respectively.

Figure 3
The authors show that EglN2 depletion also leads to decreased mitochondrial respiration and mtDNA content in hypoxia, while overexpression of EglN2 has the converse effect. These observations are similar to those made in normoxia, although the fold change caused by EglN2 over-expression is higher in hypoxia (4-fold compared to 1.5-fold in normoxia).
The authors go on to show that EglN2 is bound to chromatin in hypoxia but not normoxia, and that it has transactivation activity in TET reporter assays.

Specific comments:
The authors comment that EglN2 depletion has a greater effect on OCR and mtDNA in hypoxia than normoxia, which suggests "a distinct role of EglN2 on mitochondrial respiration under hypoxia."

- *It is notable that in Figure 4J the authors perform a similar experiment, but in this case overexpression of Flag-EglN2 (in combination with a control shRNA) in hypoxia/T47D cells causes only a 1.5-fold induction of mtDNA - similar to what was previously observed in normoxia. Comment?*

Response: We appreciate the reviewer's comment. In Figure 4J, during the second round transfection of control siRNA, PGC1□siRNA or NRF1siRNA, these cell lines (Ctrl and FLAG-EglN2 stable cell lines) have been kept in the culture for a longer period of time than those in Fig 3D. It is possible that these cell lines have an adaptive response with FLAG-EglN2 overexpression during the longer term of culture. We included this possibility in the paper text (Please see page 17 starting from line 2). This is an independent experiment done at different time, Ctrl and EglN2 overexpression cell lines still displayed statistically significant difference in mtDNA in Figure 4J.

- *I don't like their use of the word 'distinct,' as EglN2 is clearly having a significant effect on the mitochondria in both normoxic and hypoxic conditions. There is no evidence to suggest that this occurs via a different mechanism. I assume this statement is made because the authors only see EglN2 on chromatin in hypoxia (and not in normoxia). However, if that's the case, then it would be nice if they could provide an explanation for how EglN2 is influencing the mitochondria in normoxia.*

Response: We deleted the word " distinct". We did cell fractionation followed by endogenous IP between EglN2 and NRF1/PGC1α, which suggested that EglN2/NRF1/PGC1α formed the complex in chromatin bound compartment under both normoxia and hypoxia, albeit showing stronger binding under hypoxia (Fig 4D, Fig 4E, Fig EV4E, EV4F, Fig EV4G and EV4H).

Furthermore, because of this discrepancy between normoxia and hypoxia, it is especially important for the authors to show that they hypoxia effects are also independent of HIF (i.e. repeat expts. from Fig2 under hypoxia).

Response: As discussed above, we did not claim there was discrepancy between normoxia and hypoxia. As the reviewer suggested, we have included these experiments under hypoxic conditions to show the HIF independence in new Fig 3F and EV3D.

- *Why were the TET reporter assays performed in 293T cells and not ER+ breast cancer cells to be consistent with the rest of the paper? (Possibly because 293T are easier to transfect, but the experiments need to be done ER+ cells)*

Response: Thanks for reviewer's suggestion. We have repeated the TET reporter assay in T47D cells and included the data in new Figure 3I. The original Figure 3D and Figure 3E were moved as new Fig 3G and Fig 3H, respectively. The original Figure 3F, 3G and 3H were moved as new Fig

EV3E, EV3F and EV3G, respectively.

- *The finding that EglN2 is chromatin-bound and has transactivation activity is one of the more novel findings in the paper - I think it's more interesting than the mitochondrial story.*

Response: We appreciate the reviewer for pointing that out.

Figure 4
The authors show that EglN2 is indeed bound to chromatin in hypoxia, and is present in the promoter regions of numerous genes. Genes that are positively regulated by EglN2 show enrichment of binding sites for NRF1. EglN2 interacts with GST-NRF1 in vitro, and with both PGC1α and NRF1 (HA-tagged and endogenous) in cells. Depletion of EglN2 by shRNA leads to decreased binding of NRF1 to PGC1α, suggesting that this interaction is mediated to some extent by EglN2 and that these three factors likely form a ternary complex. In further support of this, the increase in mtDNA content caused by EglN2 overexpression is abrogated by knockdown of either NRF1 or PGC1α.
Specific Comments:

- *I am confused as to why the authors did not perform their microarray experiment in normoxia as well as hypoxia. I would have thought it would be interesting (though not essential) to look for genes that are regulated by EglN2 specifically in hypoxia. Results in normoxia should be shown.*

Response: We have performed the microarray for EglN2 under normoxia and included the data in new Fig EV4A, EV4B and Table EV3.

- *Fig4E-G: NRF1 and PGC1α interact with EglN2 in both T47D/MCF-7 cells in normoxia (albeit weakly) as well as hypoxia. The authors should comment on this. Where is this interaction taking place? As their previous data indicates that EglN2 is not chromatin bound in normoxia. Does this argue against a 'hypoxia specific' mechanism of mitochondrial regulation? The authors comment here (and again in the discussion) that DMOG has "no effect" on these interactions, yet it clearly does (albeit to a lesser extent than hypoxia).*
-

Response: Thanks for the reviewer's suggestion. We added comment in the revised text in Page 16 starting from line 22. We examined the localization of these interactions by using cell fractionation followed by endogenous EglN2 IP. As shown in new Fig EV4G and EV4H, the interaction of EglN2 with NRF1 and PGC1α mainly takes place in chromatin bound compartment. In addition, as shown in new Fig EV4G and EV4H, EglN2 also binds with NRF1 and PGC1α in chromatin bound fractions under normoxia, albeit much weaker than hypoxic condition. Therefore, as suggested by the reviewer, we did not state " hypoxia specific" mechanism of mitochondrial regulation in the revised paper text. With regard to DMOG, we have revised our statement. Please see revised paper text Page 15 starting from Line 18.

- *Although EglN2 does not appear to regulate the stability of HIF-1α in hypoxia (Fig2), it could still be bound to HIF-1α (or 2α) in hypoxia, and this could be a mechanism by which it is recruited to gene promoters. Fig4B suggests that binding sites for HIF1α and ARNT (which they now refer to as HIF1β - should be consistent) are present in the promoter regions of genes that are up- and down- regulated by EglN2, yet they do not comment on this finding. Could Egnl2 be bound to DNA via HIF?*

Response: We tested the binding between EglN2 and HIF1α under hypoxia. Under our experiment condition, we did not find the distinctive binding of EglN2 to HIF1α in MCF-7 cells (new Fig 4F), albeit very weak binding in T47D cells (new Fig 4E). On the other hand, we found more robust binding between EglN2 and NRF1 or PGC1α under hypoxia (new Fig 4E and 4F). We acknowledge that potential HIF involvement in EglN2 binding to some gene promoter. However, we are mainly interested in how EglN2 was recruited to target gene promoters involved in its role in mitochondrial regulation. Our data in new Figure 2 and 3 showed this function regulated by EglN2 is independent

of HIF1α/2α.

In addition, NRF1 motif was uniquely enriched in EglN2 positively regulated gene list but not negatively regulated gene list (Fig 4B). For HIF1α and ARNT (HIF1β) motifs, they are both enriched in either upregulated or downregulated gene lists (Fig 4B). Since the TET reporter assay (new Fig 3I and Fig EV3G) indicated that EglN2 preferably acts as a transcriptional co-activator under hypoxic condition, we mainly focus on the role of NRF1 in this process. We have incorporated some of these comments in the main text in page 14 starting from line 13 and page 22 starting from line 21.

It remains possible that EglN2 binds to DNA via HIF, which will need further study by using HIF1α, HIF2α and ARNT knockout cell lines to perform EglN2 ChIP-Seq, which will be our interests of future investigation.

To be consistent, we changed HIF1β to ARNT in new Fig 4B.

Fig4E-F: The authors should immunoblot for HIF-1α in the EglN2 IP. They show that it is present in the WCE but it is important to demonstrate that it does not bind EglN2.
- The authors state that "the interaction between NRF1 and EglN2 was not affected upon HIF1α depletion in these cells under hypoxia (data not shown)" - it is important to show this data, as it would support a HIF-independent mechanism.
In regard of this it would be interesting to show results for catalytically inactive EglN2.

Response: For HIF1α blot in the EglN2 IP, please see newly revised Fig 4E and 4F. Under our experiment condition, we did not find the distinctive binding of EglN2 to HIF1α in MCF-7 cells (new Fig 4F), albeit very weak binding in T47D cells (new Fig 4E). On the other hand, we found more robust binding between EglN2 and NRF1 or PGC1α under hypoxia (new Fig 4E and 4F).

For the interaction between NRF1 and EglN2 was not affected upon HIF1α depletion, please see new Fig EV4D. In support of this, as suggested by the reviewer, we also performed the CO-IP experiments with EglN2 catalytically inactive mutant (H358A) in new Fig EV4E and EV4F.

Figure 5
The authors show that binding sites for EglN2 overlap with many of those identified for NRF1 in hypoxia. They identify a gene, FDXR, that shows decreased expression upon depletion of either NRF1 or EglN2 in hypoxia. Overexpression of EglN2 leads to elevated expression of FDXR, as effect that is dependent on NRF1 and PGC1α.

- *It would be interesting to see how the expression level of FDXR in hypoxia compares to that in normoxia, and how the latter is affected by NRF1 or EglN2 depletion, especially since the authors believe this is a hypoxia-specific mechanism.*

Response: As the reviewer suggested, we examined the expression of FDXR in hypoxia compared to normoxia and observed a modest but consistent upregulation of FDXR under hypoxia (new Fig EV5C). In addition, we also showed the FDXR expression was affected by NRF1 or EglN2 under normoxia in new Fig EV5A and EV5B. We did not claim this is a hypoxia-specific mechanism, EglN2 interacts with NRF1 and PGC1α on DNA and promotes the transcription of FDXR, the interaction happened under both normoxia and hypoxia, albeit normoxia with weaker binding than hypoxia (Fig 4D, Fig 4E, Fig EV4E, EV4F, Fig EV4G and EV4H).

Figure S3
Fine - no comments.

Figure 6
The authors show that depletion of FDXR in hypoxia causes a decrease in mtDNA content similar to that observed following depletion of EglN2. Furthermore, overexpression of FDXR rescued the effects of EglN2 depletion on mtDNA content and mitochondrial respiration, indicating that the

mitochondrial effects of EglN2 in hypoxia are at least partly mediated by FDXR. The authors show that FDXR depletion inhibits breast tumor growth in vivo, and find that FDXR expression is higher in ERα+ breast cancer cohorts than normal cohorts.
Specific comments
- *The findings from the in vivo studies are interesting, but there is no direct link with the mitochondrial work presented in Figures 1-5. Are the authors suggesting that the decrease in mitochondrial mass observed with FDXR knockdown is linked with its effects on tumor growth. I'm not sure that it is possible to show this directly...*

Response: Fig 6D and 6E showed that FDXR mediated the effect of EglN2 on mtDNA and OCR. In addition, new Fig 6F showed that FDXR partially mediated the effect of EglN2 on anchorage independent growth, an important indicator for tumor growth.

Referee #3:

The manuscript by Zhang et al. reports that the prolyl hydroxylase controls mitochondrial bioenergetics via interacting with transcriptional components of the mitochondrial biogenesis and OXPHOS pathway, NRF1 and PGC1a. It appears that one of the key targets to mediate EglN2-dependent mitochondrial function is the ferridoxin reductase protein. The authors agglutinate this mechanism and molecular components within the tumor biology of breast, showing that FDXR promotes breast tumorigenesis. Overall, these studies are novel and of broad interested and provide sufficient mechanism to explain, at least in part, the functional tumor experiments. There are however, key experiments, that are needed to strengthen the manuscript and to support the conclusions raised by the authors.

1- Fig1. Authors convincingly showed that oxygen consumption is affected upon Egln2 knock down, however due to possible off-target effects it would be recommended to rescue the respiration by overexpressing Egln2 exogenously.
Moreover, alternative methods to assess the mitochondrial mass should be performed, such as mitotracker or mitored.

Response: To rule out the possible off-target effects of EglN2 shRNA, we have performed EglN2 overexpression rescue experiments. Please see new Fig EV1A, EV1B and EV1C. The results indicated that overexpressed EglN2 could rescue the effect of EglN2 shRNA on OCR/mtDNA. As the reviewer suggested, we also included mitotracker green experiment results. Please see new Fig EV1F.

2- Fig1S. It is clear that mtDNA is downregulated when Egln2 is silenced, however not a single mitochondrial encoded protein has been blotted. For instance; is mtCOI (complex IV subunit) downregulated? If so, are other proteins (nuclear encoded) belonging to this complex also affected? If this scenario is true, is this leading to a mito-nuclear imbalance?

Response: We have examined the effect of EglN2 depletion on some mitochondrial-encoded proteins (such as in complex I and IV). As shown in new Fig EV1D, all of tested markers in complex I and some of markers (such as Cox1) in complex IV were downregulated by EglN2 depletion. In addition, we also examined some nuclear encoded mitochondrial protein in these complexes and did not find their protein expressions were affected. Mito-nuclear imbalance is an imbalance between the expression of nuclear and mitochondrial encoded mitochondrial proteins (Karpac & Jasper, 2013; Mouchiroud et al, 2013). The results we got so far showed that depletion of EglN2 may lead to a mito-nuclear imbalance, we also revised our paper text to reflect that. Please see page 7 starting from line 8.

3- Fig2. Is Egln2 controlling HIF-2? It will be nice to see HIF-2 (EPAS) in these blots.

Response: As the reviewer suggested, we included the data suggesting that EglN2 did not control HIF2α protein levels in new Fig 2A and 2B.

4- Fig3. Authors argued that Egln2 effects on mtDNA are more pronounced under hypoxic conditions. Have they measure oxygen consumption under hypoxia using shEgln2 and FLAG-Egln2

cells lines?

Response: As suggested by the reviewers, we measured the oxygen consumption under hypoxia using shEglN2 and FLAG-EglN2 cell lines. Please see results in new Fig 3C, 3E and EV 3A. It is important to point out that because the XF24 extracellular flux analyzer cannot fit in our hypoxia chamber for measuring oxygen consumption rate under hypoxia, we obtained a Hansatech Oxygen Electrode system to measure the total cellular oxygen consumption of indicated cell lines (new Fig 3C, 3E and EV 3A) under hypoxia as described previously (Zhang et al, 2012).

5- Fig4- 4. Authors declare that Egln2 control mitochondrial function by binding and activate NRF1/PGC1a complex, and this interaction is stronger under hypoxia and almost nonexistence in normoxia. However all the effects in oxygen consumption seem before have been done under normoxia. Moreover, it has been clearly reported that hypoxia decrease oxygen consumption in many cell lines as well as in vivo, which raises the question of how low oxygen condition is going to induce this pro-oxidative phenotype. Authors should clarify this conundrum.

Response: Our further experiments indicated that EglN2 interacted with NRF1/PGC1α under both normoxia and hypoxia, albeit the binding under normoxia is much weaker than hypoxia. As suggested by the reviewers, we have included oxygen consumption data in new Fig 3C, Fig 3E and EV 3A.

We acknowledge that hypoxia decrease oxygen consumption in many cell lines as well as *in vivo* according to published literatures (Denko, 2008). So, we clarified in our text that under low oxygen condition, there is a stronger binding between EglN2 and NRF1/PGC1α to sustain mitochondrial function. We provided some literature evidence supporting that even under hypoxic condition, oxygen consumption and mitochondrial function is still important for cancer cells. For example, glutamine-driven oxidative phosphorylation is a major means of ATP production even in hypoxic cancer cells (Fan et al, 2013). Another example is that mitochondrial enzyme SHMT2 is induced upon hypoxia and is critical for maintaining NADPH production and redox balance to support cancer cell growth (Ye et al, 2014). In addition, previous research showed that hypoxia activates transcription via a mitochondria-dependent signaling (Chandel et al, 1998). Our results suggest that by binding with NRF1 and PGC1α complex, EglN2 serves to maintain the mitochondrial function under hypoxia in ERα positive breast cancer. We have revised our discussion part to explain this in detail. Please see page 23 starting from line 3.

6- Fig5. Authors should show normoxic data of this figure.
Base on ChIP experiments in Fig C, Egln2 binds to many genes at its promoter region, and control their transcriptional levels. However these very same genes do not change when NRF1 is knock down. How do Authors explain this Egln2 dependent but NRF1 independent regulation in gene expression?

Response: As suggested by the reviewer, we showed normoxic data of this figure in new Fig EV5A and EV5B. We have repeated the NRF1 knockdown experiments by using more efficient NRF1 siRNAs in T47D cells and showed that NRF1 depletion decreased all of these gene expression either under normoxia or hypoxia (New Fig 5G, Fig 5H and EV5B), indicating the co-regulation of these target genes by EglN2 and NRF1.

7- Fig6. It is not clear how FDXR, which is a mitochondrial flavoprotein that initiates electron transport for cytochromes P450, is able to dramatically influence mtDNA levels. Authors should outline a plausible theory.

Response: There are a few possibilities. One possibility is that our metabolomics analysis showed the decreased Glutamine to Glutamate conversion upon FDXR depletion (Zhang J and Zhang Q, unpublished). Since Glutamine to Glutamate conversion is important for generation of intermediates important for production of pyrimidine de novo synthesis (Newsholme et al, 2003; Pearce et al, 2013), FDXR depletion could lead to decreased pyrimidine synthesis and mtDNA reduction without affecting mitochondrial mass. The other possibility is that mitochondrial dNTP imbalance has been reported to decrease mtDNA content in various cells or mouse tissues (Akman

et al, 2008; Lopez et al, 2009; Song et al, 2003). Our preliminary results show that FDXR depletion led to unbalanced dNTP production (Zhang J and Zhang Q, unpublished), which could contribute to the mtDNA reduction. We have added these plausible theories into the discussion part in the revised paper text. Please see page 23 starting from line 17 (paragraph 2).

8- Authors showed how tumors formed from shFDXR were smaller, which could be reasonably attributed to the detoxification function of FDXR rather that mtDNA depletion. Are shEgln2 formed tumors also smaller? Is this rescue by overspressing FDXR? Does shNRF1 affect tumor growth in these cells?

Response: According to our paper published previously, EglN2 knockdown showed smaller tumor growth (Zhang et al, 2009). In our new Fig 6F, we also confirmed that EglN2 depletion decreased anchorage independent growth, an important indicator for tumor growth. We performed FDXR overexpression experiments and found that FDXR overexpression rescued the effect of EglN2 on anchorage independent growth (new Fig 6F).

In addition, we also tested the effect of NRF1 depletion on anchorage independent growth as shown in the new Fig EV4C. Depletion of NRF1 decreased soft agar growth.

9-Egln2 also known PHD1 function as an oxygen dependent prolyl hydroxylase, nevertheless authors never assessed whether this function is important for the mechanism and phenotypes described in this paper. It will be enlightening the use of a mutated version of the enzyme lacking prolyl hydroxylase activity to address such questions.

Response: We have used EglN2 (PHD1) H358A catalytic dead mutant to repeat some of the phenotype and mechanism experiments and showed that its enzymatic activity is not necessary for its regulation on mitochondrial function. Please see new Fig 1G, 1H and 1I, new Fig 3D and 3E, new Fig EV4E and EV4F.

10- Pyrimidine de novo synthesis is directly connected with the mitochondria function an disruption of the mitochondrial electron transport chain may impair UTP, TTP, and CTP synthesis which will lead to mtDNA reduction without affecting mitochondrial mass. Have Authors consider this possibility to explain their observed phenotypes.

Response: Please see the response to question No.7 above.

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2nd Editorial Decision 04 September 2015

Thank you for submitting your revised manuscript to The EMBO Journal and sorry for the slightly extended duration of the re-review process. Your study has now been seen by the three original referees (comments included below) and as you will see they all find that the criticisms raised have been sufficiently addressed and they therefore support publication. However, before we can proceed to officially accept your manuscript I have to ask you to address the following minor points:

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your revision.

REFEREE REPORTS
Referee #1:

The authors have satisfactorily addressed my concerns with regard to this manuscript and I believe it is now acceptable for publication in the EMBO Journal.

Referee #2:

Overall, I believe the authors have made substantial efforts to address the issues raised by all the referees. More could be asked in terms of mechanisms, but I think this would be unreasonable and I'm happy to recommend publication of an interesting story.

Referee #3:

The authors have adequately address the concerns and critiques raised in the previous review.

2nd Revision - authors' response 04 September 2015

- Our figures are typeset in portrait rather than landscape format, so could you please adopt them accordingly (both for main and EV figures). In addition, we have to currently limit the number of expanded view figures to 5 so I would be grateful if you could combine figures EV5 and EV6 into a single figure.

Response: We changed all figures into portrait format and combined Fig EV5 and Fig EV6 into a single figure. In addition, we also revised the text accordingly.

- I would also such modifying the title of your study along the following lines: 'EglN2 associates with the NRF-PGC1α complex and controls mitochondrial function in breast cancer'. Could you let me know if you agree to this or if you would have another suggestion for an alternative?

Response: Thanks for your suggestion. We agree to this and have changed the title.

- We encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format. The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". The source data files can be sent to me directly by email and we will then upload them in house.

Response: Since the revised paper already contains large amount of data figures, we don't intend to put extra burden on the readers for all of source data. However, we have all of source data available and will be more than happy to provide upon request.

- Papers published in The EMBO Journal include a 'Synopsis' to further enhance its discoverability. The synopsis consists of a short standfirst - written by the handling editor - as well as 2-5 one sentence bullet points that summarise the paper and are provided by the authors. I would therefore ask you to include your suggestions for bullet points.

Response: We have included the bullet point synopsis for the revised manuscript.

- In addition, I would encourage you to provide an image for the synapsis. This image should

provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

Response: We have provided an image for the synapsis. Because of the character limitation (55,000 characters), we moved some part of materials and methods to 'Appendix Supplementary Methods'.

