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Programmed mitophagy is essential for the glycolytic switch during cell differentiation

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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 November 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see, the referees appreciate your findings. However, they also think that the link between mitophagy and glycolysis needs to be strengthened (referee #1, points 1, 5; referee #2, points 4,5,7) and that further data on hypoxia during retina development and on glycolysis driving differentiation are needed (referee #1, point 3; referee #2, point 6). Furthermore, missing quantifications need to be added (referee #2, point 2) and the macrophage data need to better incorporated or removed/placed into supplementary data (referee #2, point 3; referee #3, last paragraph).

REFEREE REPORTS

Referee #1:

The manuscript by Esteban-Martinez et al reports that programmed mitophagy is essential for induction of glycolysis, needed for differentiation of RGCs during embryogenesis. Using pharmacological and genetic manipulations on embryonic retina ex vivo, the authors show the molecular basis of this pathway. The evidence is convincing, the experiments well-planned,

performed with proper controls and esthetically presented. The discussion is balanced. Taking the comments below into consideration would improve the manuscript.

MAJOR:
1. Pyruvate Kinase isoenzyme M2 (PKM2) was implicated in HIF1-dependent transcription and M1 transition in macrophages (Palsson-McDermott, 2015). Can mitophagy affect PKM2?
2. Described here mitophagy is not the only factor affecting RGC differentiation. Induction of RGC from stem cells in vitro using growth and differentiation factors supports this view (e.g. Ohlemacher et al., 2016); how does the need for growth/differentiation factors in RGC development reconcile with the mitophagy/glycolysis- dependent differentiation?
3. Is there in vivo evidence that local hypoxia in the developing eye occurs?
4. Is inhibition of glycolysis sufficient to block RGC differentiation in vivo?
5. Does the mitophagy-dependent metabolic switch occur in macrophages in vivo? For example, would Atg5-/- or Nix-/- mice have a skew towards M2- responses?

MINOR:
1. Spl. Figure 2e, Spl. Figure 3g - the error bars are not straight.
2. 'chondrocyte' instead of 'condrocyte', p.13

Referee #2:

In this paper, the authors provide an interesting connection between mitophagy and glycolysis in RGC differentiation. They show that NIX-dependent mitophagy is required for RGC development. The increase in mitophagy normally seen in this differentiation stage is accompanied by a glycolytic switch that may be caused by increased mitophagy. Published work had already implicated an important role for autophagy in neuronal differentiation, the current studies indicate that mitophagy is also important. The effect of NIX deletion on RGC differentiation is a strong direct evidence for a role of mitophagy in this experimental context. However, there are few weaknesses and inconsistencies that should be addressed (as detailed below).

Specific comments

1. The complete absence of RGC in NIX-/- mouse retina in Fig5f implies mitophagy is absolutely required for RGC development. If so, why do the authors see only a slight changes in mitochondrial content (Fig 2 c-d) and RGCs number (Fig 3c) when autophagy is inhibited? Could it be that either NIX has an additional function outside of mitophagy in RGC development or that autophagy/mitophagy is not the underlying driver of the glycolytic switch but that it may be some other mitochondrial defect? These caveats should be clarified and discussed.

2. The author should consider moving the data on NIX to earlier section/figures in the paper. The NIX genetic tool is the strongest argument and the most direct evidence for the role of mitophagy in the authors' experimental system. They should also provide actual quantifications for RGCs and TOMM20 in current Fig 5e-f. The use of CsA as a tool to inhibit mitophagy is questionable and data related to CsA can have other interpretations given inhibition of cyclophilin D in these experiments.

3. Is NIX also relevant for M1 polarization? If not, the M1 and M2 macrophage experiments do not add value to the central findings of the current paper and the genetic data on NIX regulation of RGC development.

4. Because glycolytic enzymes, in addition to NIX, are transcriptional targets of HIF1, increased mitophagy and glycolysis could be parallel events during RGC development. However, the authors propose that the glycolytic switch they observe is due to increased mitophagy based on the changes in lactate following CsA treatment. The rigorous test to this is examination of glycolysis in NIX -/- embryonic retina (also see point 2 above).

5. The authors should provide OCR data in addition to ECAR measurements to strengthen their argument of mitophagy-induced glycolytic switch. This will complement and strengthen their observations and assumptions of mitochondrial membrane potential changes.

6. What promotes HIF1 activation at early stage? The authors mention "a general agreement on the low oxygen tension during embryonic development". However, it is not clear from the explanations and data provided that there are actual changes in oxygen tension in the different stages of retinal development. It is important to clarify that HIF1 per se is changed, for example, by showing corresponding changes in HIF1 stability in the different stages.

7. If changes in glycolysis preference over time are lost postnatally, does an increase in mitochondrial content trigger this switch? Is mitochondrial content change during the same time periods as in Fig 2e?

8. The authors are encouraged to revise the discussion section of the paper for a more crisp and succinct summary of key findings, novel observations with relevant comparisons and contrasts with other models of metabolic reprogramming in development.

Additional points

9. Can the authors comment on why there are no differences in mitochondrial content in Atg5 KO cells at E13.5 even though a basal level of mitophagy is likely required at this stage to maintain healthy mitochondria pool, given the dependence on oxidative phosphorylation for ATP production?

10. A higher resolution and magnification for images in Fig 1g will be highly beneficial. The mitochondria highlighted by arrows are very difficult to see in the current images.

11. The authors should provide clear indication in the figure legends on sample sizes or number of cohorts that were analyzed, including all the imaging experiments.

12. The text description for Fig 2c does not match the data. The text reads: "and MTDR staining by flow cytometry was attenuated" in response to mitophagy and autophagy inhibitors, while the figure shows that CsA and 3MA increase the mitotracker signal at E15.5.

13. In the materials and methods, under metabolic determinations, 20ug of tissue is likely a typo, it should be 20mg?

Referee #3:

This work examines the role of mitophagy in the RGC differentiation in the mouse eye. the authors test the idea that a reduction in mitochondrial mass by mitophagy leads to a shift to glycolysis in the RGC which supports differentiation in the developing retina. There are some studies that were also performed on macrophages.

Overall the data are quite good and convincingly demonstrate that mitochondrial mass is altered during RGC development by mitophagy. Less convincing are data to demonstrate that without autophagy (e.g. Atg5-/-) there are developmental issues beyond the immunofluorescent microscopy for beta-III-Tub. There needs to be a demonstration that these Atg5 deficient RGC cells are defective in some way, perhaps electrophysiological recordings of the retina shortly after birth (before the Atg5 deficient mice die) would show that there are developmental issues that have functional consequences. Including more retina structural analysis would also go a long way in convincing the reader that this is important.

The macrophage data seem to detract from the RGC story. It is suggested that these data be omitted. The authors might want to comment on a recent paper (PMID: 27732846) concerning M1/M2 polarization and the role of OX-Phos.

1st Revision - authors' response 03 March 2017

Point by Point response to the referees

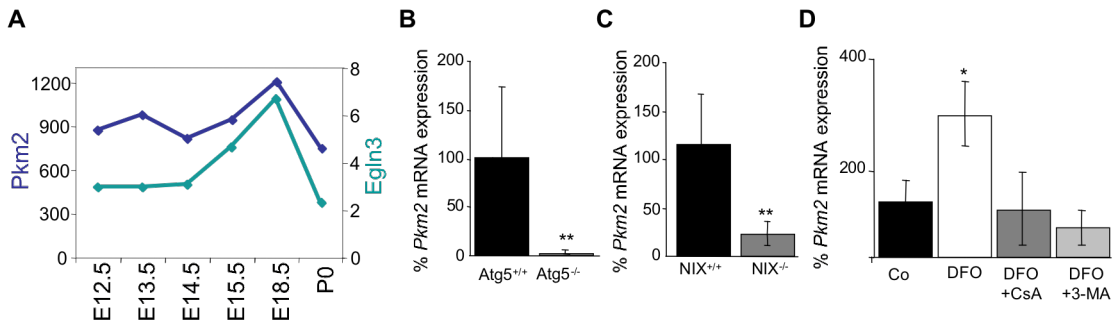
Referee #1:
The manuscript by Esteban-Martinez *et al* reports that programmed mitophagy is essential for induction of glycolysis, needed for differentiation of RGCs during embryogenesis. Using pharmacological and genetic manipulations on embryonic retina *ex vivo*, the authors show the molecular basis of this pathway. The evidence is convincing, the experiments well-planned, performed with proper controls and esthetically presented. The discussion is balanced. Taking the comments below into consideration would improve the manuscript.

We thank this reviewer for the enthusiastic evaluation of our work and for noticing our efforts to present a well-controlled and balanced story. We also appreciate very much the reviewer's suggestions that we agree have contributed to further improve our manuscript

MAJOR:

1.1 Pyruvate Kinase isoenzyme M2 (PKM2) was implicated in HIF1-dependent transcription and M1 transition in macrophages (Palsson-McDermott, 2015). Can mitophagy affect PKM2?

The results on macrophages described in the aforementioned study, as well as data on cancer cells reported by Luo (Luo et al, 2011), demonstrate that in addition to the "classical" metabolic activity of PKM2 in regulating pyruvate levels in glycolysis, PKM2 can form a complex with prolyl hydroxylase domain enzyme 3 (also known as PHD3 or Egln3) and HIF-1A to regulate gene expression. This complex acts as a protein transactivator, regulating several genes including PKM2. PKM2 thus participates in a positive feedback loop that promotes HIF-1-dependent transactivation and reprogramming of glucose metabolism. As the reviewer points out, it would be interesting to determine whether mitophagy affects PKM2. As shown in the figure below panel A, our transcriptomic analysis revealed a marked increase in the expression of *Pkm2* and *Egln3* (PHD3) mRNA, beginning at E15.5 and peaking at E18.5. This time period coincides with onset of mitophagy in the mouse embryonic retina, suggesting that a similar feedback mechanism may exist in the mouse retina. More importantly, we observed a reduction in *Pkm2* mRNA expression in Atg5 and NIX-deficient retinas (Fig. 4F, 6P in the manuscript and panel B and C, below). In agreement with those observations, inducing chemical hypoxia with DFO increased *Pkm2* mRNA expression in an autophagy and mitophagy-dependent manner (see figure below panel D). Taken together, these data show that *Pkm2* expression is regulated in a mitophagy-dependent manner in the mouse embryonic retina.



1.2. Described here mitophagy is not the only factor affecting RGC differentiation. Induction of RGC from stem cells in vitro using growth and differentiation factors supports this view (e.g. Ohlemacher et al., 2016); how does the need for growth/differentiation factors in RGC development reconcile with the mitophagy/glycolysis- dependent differentiation?

Cell differentiation is a highly regulated process that depends on cell-intrinsic and cell-extrinsic factors. Cell-intrinsic factors in the retina include the transcription factors *Atoh7, Pou4f1* (Brn3a), *Pou4f2* (Brn3b), *Isl1,* and *Myt1*, which regulate RGC differentiation in a coordinated manner. Although specific transcription factors mediate the early stages of retinal differentiation, RGC numbers are also regulated by extrinsic factors, such as neurotrophic factors. These neurotrophic factors protect RGCs from cell death during the differentiation process, and in disease conditions such as glaucoma. Ohlemacher and coworkers demonstrated the cytoprotective effects of BDNF and PEDF in RGCs differentiated from patient-derived IPs. In our manuscript, we show that hypoxia-induced mitophagy regulates RGC differentiation by inducing a metabolic shift towards glycolysis at E15.5. While we have not tested the specific effects of BDNF and PEDF on mitophagy during RGC differentiation or cell survival, data generated by our group and others indicate that growth factors such as insulin can modulate autophagy (unpublished observations), cell death, and RGC number (Diaz et al, 2000). These findings indicate that RGC differentiation and survival are regulated by multiple intrinsic and extrinsic factors.

1.3. Is there *in vivo* evidence that local hypoxia in the developing eye occurs?
We have addressed this issue in the revised version of the manuscript. We performed experiments using the Hypoxyprobe kit, which is widely used to label hypoxic areas in cells and tissues. This method is based in the reaction of pimonidazole with cellular proteins in conditions of low oxygen concentrations, and recognition of the adducts formed using a specific antibody. As shown now in Figure 5A, we detected positive staining that began at E13.5 and persisted in the RGCs in the adult mouse retina. These new data are in agreement with previous results from the literature where local hypoxia including HIF-1a staining was observed in the embryonic retina and in the inner retina in adult mice (Kurihara et al, 2010). We thank the referee for suggesting this analysis. The data have been added and discussed in the revised version of the manuscript.

1.4. Is inhibition of glycolysis sufficient to block RGC differentiation *in vivo*?

To better corroborate the link between glycolysis and cell differentiation we performed experiments in which we increased glycolysis. To this end, we used the mitochondrial pyruvate carrier inhibitor UK5099, which has been shown to decrease pyruvate entry into the mitochondria, resulting in a concomitant increase on glycolysis (Zhong et al, 2015). As shown in the new Figure 7D,E, UK5099 increased RGC differentiation but had no effect on the overall abundance of mitochondria, suggesting that mitophagy occurs upstream of the metabolic change essential for cell differentiation. We have also tried to block glycolysis *in vivo*, by injecting pregnant mothers with 2DG, but could not observe relevant changes in the number of RGCs in the embryonic retinas. However, our positive results obtained when upregulating glycolysis support the idea that

glycolysis is sufficient to drive RGC differentiation *in vivo*. We have included the data modulating glycolysis with UK5099 in the revised manuscript.

1.5. Does the mitophagy-dependent metabolic switch occur in macrophages *in vivo*? For example, would Atg5-/- or Nix-/- mice have a skew towards M2-responses?

We have addressed this question in NIX-deficient mice, since $Atg5^{-/-}$ animals die during the perinatal period. Our new data, presented in Figure 6P,Q and Appendix Figure S6B,C, show that NIX-deficient macrophages exhibit a decreased M1 response when challenged with proinflammatory stimuli such as LPS and IFN-γ, and display decreased mRNA expression of glycolytic enzymes, suggesting a reduced M1 phenotype. Our data thus support the idea that mitophagy regulates M1 polarization while it does not have any effects on M2 polarization. We thank this reviewer for suggesting the studies in the $Nix^{-/-}$ mice that are now included in Fig. 6 and S6.

MINOR:
1.6 Spl. Figure 2e, Spl. Figure 3g - the error bars are not straight.
1.7 'chondrocyte' instead of 'condrocyte', p.13

This has been corrected in the revised version of the manuscript.

Referee #2:

In this paper, the authors provide an interesting connection between mitophagy and glycolysis in RGC differentiation. They show that NIX-dependent mitophagy is required for RGC development. The increase in mitophagy normally seen in this differentiation stage is accompanied by a glycolytic switch that may be caused by increased mitophagy. Published work had already implicated an important role for autophagy in neuronal differentiation, the current studies indicate that mitophagy is also important. The effect of NIX deletion on RGC differentiation is a strong direct evidence for a role of mitophagy in this experimental context. However, there are few weaknesses and inconsistencies that should be addressed (as detailed below).

We thank this reviewer for considering our study interesting and that it shows strong direct evidence of a role for mitophagy in RGC development. We have taken the remaining minor concerns of this reviewer at heart and have addressed them as indicated below.

Specific comments

2.1. The complete absence of RGC in NIX-/- mouse retina in Fig5f implies

mitophagy is absolutely required for RGC development. If so, why do the authors see only a slight changes in mitochondrial content (Fig 2 c-d) and RGCs number (Fig 3c) when autophagy is inhibited? Could it be that either NIX has an additional function outside of mitophagy in RGC development or that autophagy/mitophagy is not the underlying driver of the glycolytic switch but that it may be some other mitochondrial defect? These caveats should be clarified and discussed.

The reviewer raises an important issue. The data presented in the original Figure 5g show increased mitochondrial mass in the NIX-deficient retina and an absence of staining with the neuronal marker β-III-tubulin at E15.5. In the original text, we stated that NIX-deficient retina display reduced neuronal differentiation. To further assess the altered phenotype in the NIX-deficient retina, we carried out several additional experiments in which we stained NIX-deficient retinas with the RGC-specific marker Brn3a in flatmounts (Fig 6G) and in cryosections (Fig 6I) together with another RGCs marker γ-sinuclein (Fig 6I). These data has also been quantified in Fig 6J and K. $NIX^{-/-}$ animals displayed reduced (but not absent) number of RGCs an effect that was also observed in adult mice (Fig 6L-N). Our data therefore support the view that differentiated RGCs are decreased in number in the NIX-deficient retina. We agree with the referee that the effect in NIX-deficient retinas on the number of RGCs is quite dramatic, an effect that could also be explained by the decreased mRNA expression of *Pou4f1* (Brn3a) in the NIX-deficient retinas (new Fig 6F). Interestingly, this decrease has not been observed in the Atg5-deficient retinas. We have now included the new data in the text and placed it in context in the discussion.

2.2. The author should consider moving the data on NIX to earlier section/figures in the paper. The NIX genetic tool is the strongest argument and the most direct evidence for the role of mitophagy in the authors' experimental system. They should also provide actual quantifications for RGCs and TOMM20 in current Fig 5e-f. The use of CsA as a tool to inhibit mitophagy is questionable and data related to CsA can have other interpretations given inhibition of cyclophilin D in these experiments.

We agree with the reviewer that the NIX data provides the strongest argument supporting the role of mitophagy in cell differentiation. Based on the reviewer's comment we have added quantitative data on RGC number and TOMM20 levels in NIX-deficient retinas (see Fig 6C and 6J-N). These data confirm that NIX-deficient retinas display increased mitochondrial mass and decreased neuronal differentiation.

Given that several studies, including our own, have used CsA to block mitophagy (Carreira et al, 2010; Domenech et al, 2015; Kim et al, 2007; Mauro-Lizcano et al, 2015) and that the results obtained are in line with those obtained following genetic and pharmacological inhibition of autophagy, we feel there are sufficient grounds to support the view that the phenotype obtained with CsA is related to its inhibition of mitophagy.

2.3. Is NIX also relevant for M1 polarization? If not, the M1 and M2 macrophage experiments do not add value to the central findings of the current paper and the genetic data on NIX regulation of RGC development.

To address this question we conducted additional experiments in which we isolated peritoneal macrophages from NIX-deficient animals (see also Response 5 to Referee #1). Our new data show that $NIX^{-/-}$ macrophages display decreased expression of glycolytic enzymes and reduced levels of M1 markers, suggesting that NIX-mediated mitophagy is essential for the glycolytic switch that regulates macrophage polarization. These new data are included and discussed in the revised version of the manuscript.

2.4. Because glycolytic enzymes, in addition to NIX, are transcriptional targets of HIF1, increased mitophagy and glycolysis could be parallel events during RGC development. However, the authors propose that the glycolytic switch they observe is due to increased mitophagy based on the changes in lactate following CsA treatment. The rigorous test to this is examination of glycolysis in NIX -/- embryonic retina (also see point 2 above).

We fully agree with the reviewer and have added the relevant data to the revised version of the manuscript. As shown in Fig. 6E, mRNA expression of glycolytic genes is reduced in NIX-deficient retinas, in support of our hypothesis. In agreement, Atg5-deficient retinas also showed a similar decrease in mRNA expression of glycolytic enzymes (Fig 4F). Taken together these data support the view that mitophagy occurs upstream of the observed glycolytic shift.

2.5. The authors should provide OCR data in addition to ECAR measurements to strengthen their argument of mitophagy-induced glycolytic switch. This will complement and strengthen their observations and assumptions of mitochondrial membrane potential changes.

Based on the reviewer's suggestions we have added the relevant data to the revised version of the manuscript.

2.6. What promotes HIF1 activation at early stage? The authors mention "a general agreement on the low oxygen tension during embryonic development". However, it is not clear from the explanations and data provided that there are actual changes in oxygen tension in the different stages of retinal development. It is important to clarify that HIF1 per se is changed, for example, by showing corresponding changes in HIF1 stability in the different stages.

We agree with the reviewer that it is important to demonstrate that actual changes in oxygen tension occur during retinal development. To this end we have performed pimonidazole immunostaining experiments, enabling assessment of the formed adducts between pimonidazole and proteins at low oxygen tension. As shown in Fig. 5A of the revised manuscript, hypoxia is evident as early as E13.5 and is clearly observed in RGCs at E15.5 and E18.5. In agreement we found a previous report from the literature where increased HIF1a staining in the mouse embryonic retina is observed (Kurihara et al, 2010). These data have now been added and discussed in the revised version of the manuscript.

2.7. If changes in glycolysis preference over time are lost postnatally, does an increase in mitochondrial content trigger this switch? Is mitochondrial content change during the same time periods as in Fig 2e?

The reviewer raises an important point, which we have now addressed in the revised manuscript by discussing our results in the context of retinal development and metabolism. Most studies of retinal metabolism have been performed using adult tissues. The mouse retina becomes postmitotic around day P9, where the proliferative marker PCNA dramatically decreases (Appendix Figure S7B). When the animal opens the eye around P12 phototransduction starts and the expression of recoverin (a protein implicated in the phototransduction cascade) can be clearly observed (Appendix Figure S7B).

As we have stated in the introduction of our manuscript, the mature retina is highly glycolytic even in the presence of oxygen, owing to the high metabolic requirements of photoreceptor cells, the main cell type in the adult mouse retina. The high polyunsaturated fatty acid content of outer segments results in rapid photo-oxidation by light during phototransduction. Thus to avoid the accumulation of damaged membranes, outer segment renewal involves daily shedding of the 10% distal outer segment tips, their phagocytosis by the retinal pigmented epithelial cells, and the growth of new discs from photoreceptor inner segments. Thus in the adult retina photoreceptor cells maintain a constant outer segment length by achieving a balance between disc shedding and the assembly of new discs. This is a very energetically demanding process, which requires the synthesis of new lipids and proteins in the inner segment. In this sense the photoreceptor is comparable to a rapidly proliferating cell (Ng et al, 2015). This metabolic demand starts when the animal opens its eyes (around P12) and phototransduction starts.

Thus two stages involving glycolytic metabolism are found in the retina: (i) during embryonic development, as described in the present manuscript; and (ii) in the mature retina, as has been previously described (Warburg, 1956). To further corroborate these findings, we have added new data to the revised version of the manuscript showing retinal lactate production at different stages of maturation, as well as data showing that the highest extracellular acidification rates (ECAR) are observed in the adult retina (P90) (see new Appendix Figure S7C). In line with these data, expression of the glycolytic enzymes GAPDH and PFKFB3 gradually increase after P9 (Appendix Figure S7B). As shown in the new Appendix Figure S7B the expression of several mitochondrial proteins is largely constant in the postnatal stages. It is important to note that the adult retina is composed of 1 glial and 6 neuronal cell types, and while glycolytic metabolism is observed throughout the entire adult retina, not all cells contribute to this metabolic profile.

We also show that mitochondrial mass increases in RGCs after differentiation, as it is known that adult RGCs display abundant mitochondria (Appendix Figure S7A, note the abundant mitochondria staining with TOMM20 in the adult (P90) retina in the RGCL). Accordingly, the highest levels of PKM2 (the main isoform regulating aerobic glycolsis) and LDH in the adult retina are found in photoreceptor cells, while inner retinal neurons such as RGCs predominantly display PKM1 labelling (Casson et al, 2016). Moreover, the levels of COX-IV expression in RGCs are higher than those observed in other retinal cell types. Together these data support the idea that the retina as a whole has mainly a glycolytic metabolism derived from photoreceptors, but that mitochondrion-rich RGCs display a preponderantly oxidative metabolism. The revised

manuscript contains a new paragraph at the end of the discussion section in which we comment these metabolic features of the embryonic and adult retina.

2.8. The authors are encouraged to revise the discussion section of the paper for a more crisp and succinct summary of key findings, novel observations with relevant comparisons and contrasts with other models of metabolic reprogramming in development.

The discussion section has been fully revised in accordance with the reviewer's suggestion.

Additional points

2.9. Can the authors comment on why there are no differences in mitochondrial content in Atg5 KO cells at E13.5 even though a basal level of mitophagy is likely required at this stage to maintain healthy mitochondria pool, given the dependence on oxidative phosphorylation for ATP production?

The reviewer raises an important point, based on which we have provided a better explanation of regional differences in retinal maturation at E13.5. At this stage the retina is composed of young RGCs in the central retina and undifferentiated neuroblasts in the peripheral retina, as shown in the cartoon in Appendix Fig S1. Flow cytometry analysis, which is performed by dissociating the entire retina, reveals no differences in MTDR levels at E13.5 (Fig. 4D). However, analysis of distinct regions (Fig. 4C) reveals differences in TOMM20 levels only in the young RGCs but no such differences in the Nb. We recognize that these data were not clearly presented in the original manuscript; no statistical indicators were displayed and the symbols used in Figure 4b where too small. We have corrected this oversight in the revised version of the manuscript, adding the relevant asterisks and clearly describing the findings in the results section. Moreoever we have now added the separate quantifications of TOMM20 levels in the Nb and the RGCs in new Fig. 5C and Appendix Fig. S4C.

2.10. A higher resolution and magnification for images in Fig 1g will be highly beneficial. The mitochondria highlighted by arrows are very difficult to see in the current images.

In the revised version of the manuscript we have substituted the images in question and added an additional higher magnification (3X) image in which the mitochondria are visible.

2.11. The authors should provide clear indication in the figure legends on sample sizes or number of cohorts that were analyzed, including all the imaging experiments.

In line with the reviewer's comment, we have included the relevant information in the revised version of the manuscript.

2.12. The text description for Fig 2c does not match the data. The text reads: "and

MTDR staining by flow cytometry was attenuated" in response to mitophagy and autophagy inhibitors, while the figure shows that CsA and 3MA increase the mitotracker signal at E15.5.
This error has been rectified in the revised version of the manuscript.

2.13. In the materials and methods, under metabolic determinations, 20ug of tissue is likely a typo, it should be 20mg?
This error has been rectified in the revised version of the manuscript.

Referee #3:

This work examines the role of mitophagy in the RGC differentiation in the mouse eye. the authors test the idea that a reduction in mitochondrial mass by mitophagy leads to a shift to glycolysis in the RGC which supports differentiation in the developing retina. There are some studies that were also performed on macrophages. Overall the data are quite good and convincingly demonstrate that mitochondrial mass is altered during RGC development by mitophagy.

We thank the referee for his/her positive comments.

3.1 Less convincing are data to demonstrate that without autophagy (e.g. Atg5-/-) there are developmental issues beyond the immunofluorescent microscopy for beta-III-Tub. There needs to be a demonstration that these Atg5 deficient RGC cells are defective in some way, perhaps electrophysiological recordings of the retina shortly after birth (before the Atg5 deficient mice die) would show that there are developmental issues that have functional consequences. Including more retina structural analysis would also go a long way in convincing the reader that this is important.

We fully agree with the reviewer that it would be interesting to determine whether this decrease in RGC number in the Atg5-deficient animals has physiological consequences, such as reduced visual function. Unfortunately, the Atg5-deficient animals die before the retina reaches maturity so visual function cannot be assessed in these animals.

Following the reviewer's suggestion we have now performed additional experiments that clearly show important alterations in RGCs in Atg5-deficient retinas. Figure 4I shows retinal flatmounts immunostained with the RGC-specific transcription factor Brn3a, in which a reduction in RGC number is clearly observed. Figure 4J shows the decrease in β-III-tubulin staining in cryosections from Atg5-deficient retinas. Figure 4H shows the quantification of RGC number, which reveals a significant decrease with respect to wt retinas. In Figure EV3, we now show decreased staining with Brn3a and γ-sinuclein staining at E13.5, E15.5 and E18.5 in retinas from Atg5-deficient mice. And finally, axons in the Atg5-deficient retina display morphological alterations (Fig 4K). We feel that these data are robust and clearly demonstrate a decrease in RGC differentiation induced by deficient autophagy.

3.2 The macrophage data seem to detract from the RGC story. It is suggested that these data be omitted. The authors might want to comment on a recent paper (PMID:27732846) concerning M1/M2 polarization and the role of OX-Phos.

The main goal of our study is to demonstrate that by eliminating mitochondria, mitophagy induces metabolic reprogramming towards glycolysis that has important consequences for cell differentiation. If this hypothesis holds true in 2 very different paradigms, namely RGC differentiation and macrophage activation, it would suggest that this mitophagy-dependent metabolic shift may constitute a general mechanism by which cell fate is coordinated with autophagy and metabolism. The revised manuscript includes additional evidence demonstrating that, at the molecular level, NIX-deficient macrophages display reduced M1 activation as compared with wild-type macrophages. In accordance with the reviewer's suggestion the aforementioned study is now cited in the revised discussion section. Considering the positive reaction of the other reviewers and editor to the macrophage studies, we have decided to kept the macrophage data, but have incorporated additional studies that we hope this reviewer agree contribute to strengthen the revised version of our manuscript.

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2nd Editorial Decision 21 March 2017

Thank you for submitting your revised manuscript for our consideration.
Your manuscript has now been seen once more by the original referees whose comments are provided below. As you will see they appreciate the revision, and I am thus happy to accept your manuscript in principle for publication in The EMBO Journal.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Patricia Boya
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2016-95916R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

USEFUL LINKS FOR COMPLETING THIS FORM

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http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum

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http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No power calculations were used.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No power calculations were used.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All data were included. No data were excluded.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Yes, we randomly distributed the samples (retinas from different embryos or macrophages from different animals) in the different experimental groups, trying that the number of samples per group was the same.
For animal studies, include a statement about randomization even if no randomization was used.	We randomly distributed the samples (retinas from different embryos or macrophages from different animals) in the different experimental groups, trying that the number of samples per group was the same.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA. The investigators were not blinded during experiments and outcome assessment.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The investigators were not blinded during experiments and outcome assessment.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, data meet the assumptions or non-parametric test (Mann-Whitney U test) were performed. Normality assesed with Kolmogorov-Smirnov (K-S) test and equal variances were assessed with Fisher F.
Is there an estimate of variation within each group of data?	We have no provided variation information.
Is the variance similar between the groups that are being statistically compared?	Yes, we assesed it with Fisher F test

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Immunostaining: TOMM20 (sc-11415, Santa Cruz), COXIV (459600, Invitrogen), Brn3a (MAB1585, Millipore), β-III-tubulin (MMS-435P, COVANCE), GLUT1 (AB400084, Abcam), and PAb2627AP (HP3-100 kit, Hypoxyprobe). The g-synuclein antibody {Nguyen, 2011 #8820} was kindly provided by Prof. Marsh-Armstrong (University of California at Davis). Western blot: anti-LC3 (L7543, Sigma), PCNA, (DeltaBiolabs C19), GAPDH (Ab8245, Abcam), TIMM23 (BD 611222), α-tubulin (sc-8035, Santa Cruz), b-actin (A5441, Sigma), TOMM20 (sc-11415), Cox-IV (459600, Invitrogen) and PFKFB3 (131238, Cell Signalling) .

7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All the animals used in this study are mice. Wild-type C57BL/6J and CD1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Atg5 mice were kindly provided by Dr. Noboru Mizushima (Department of Physiology and Cell Biology, Tokyo Medical and Dental University, Tokyo, Japan). NIX/BNIP3L mice were kindly provided by Dr. Gerald W. Dorn II (Washington University at St. Louis, St. Louis, MO, USA). Atg5 and NIX/BNIP3L deficient mice are both in C57BL/6J background. Male and female animals were equally used in this study. Mice were reared in a barrier-controlled facility (20ºC; 12-hour light/dark cycle) with ad libitum access to food and water. Animals were crossed and the morning on which the vaginal plug was detected was designated embryonic day (E) 0.5. Animals were euthanized by cervical dislocation and embryos removed by caesarean section. The embryos were staged and then placed in a Petri dish in 1X phosphate-buffered saline (PBS). The experiments have been performed with different embryonic - E - and postnatal - P - stages as indicated in each figure.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal procedures and study protocols were approved by the local ethics committee for animal experimentation and the ethics committees of the CSIC and the Albert Einstein College of Medicine, and were carried out in accordance with US and EU regulations and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	For hypoxia staining we used the Hypoxyprobe kit (Cat. # HP3-100kit). Pimonidazole at 60 mg/kg body weight was injected intraperitoneally. Three hours later, mice were euthanized by cervical dislocation and embryos removed by caesarean section. All euthanasia was performed at the same time to minimize the differences in embryo development and light in the case of adults

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	RNA-seq data has been deposited in the GEO repository, accession number GSE84299.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA