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BNIP3 promotes HIF-1α-driven melanoma growth by curbing intracellular iron homeostasis

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1st Editorial Decision 31st Aug 2020

Dear Patrizia,

Thank you for the submission of your manuscript (EMBOJ-2020-106214-T) to The EMBO Journal. Please again accept my apologies for the unusual delay with the peer-review of your manuscript due to delayed referee input at this time of the year and detailed discussions in the team. We have sent your manuscript to three reviewers for evaluation and now received reports from all of them, which I copy below. I am afraid that in light of their comments we decided that we cannot offer publication in The EMBO Journal.

As you will see, the referees appreciate the potential interest of your results for the field. However they also raise major concerns with the analysis that I am afraid preclude publication here. In more detail, referee #3 states major issues with the advance of the current work given reported overall links between BNIP3 and HIF1a, and points to a number of unsupported claims, which in his-her view undermine the impact of your results. Referee #2 agrees in that the novelty of the findings is compromised by earlier work (ref#2 standfirst, pt.3). Referee #1 is concerned about the level of molecular depth and finds the mechanistic insights provided too premature (Ref#1, pt.1; see also ref#3).

Given these negative opinions from good experts on the field, and considering that the journal only offers one concise round of major revisions, I am afraid we have concluded that we cannot offer to publish your study in The EMBO Journal.

Thank you in any case for the opportunity to consider this manuscript. I regret we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful. I again apologise for the unusual protraction.

Kind regards,

Daniel

Daniel Klimmeck, PhD
Editor
The EMBO Journal

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Referee #1:

In this manuscript, Vara-Perez et al. found positive correlation between the increased BNIP3 expression in melanoma and poor prognosis. They show that in melanoma cells, an adaptor protein for mitophagy, BNIP3 prevents NCOA4-mediated ferritinophagy and in turn stabilizes HIF1α, supporting tumor metabolism and proliferation. Mechanistically, BNIP3 interacts with NCOA4, inhibits the degradation of ferritin and decreases intracellular Fe^{2+} level. As a result, the activity of prolylhydroxylase 2 declined, the level of hydroxylated HIF1α decreased, and then degradation of

the HIF1α through the ubiquitin-proteasome pathway was impaired. Thus, the ablation of BNIP3 abrogated not only mitochondria clearance but also anaerobic glycolysis through the stabilization of HIF1α in melanoma, resulted in decreased melanoma growth. While this manuscript contains interest findings, substantial issues that should be addressed remained.

Comments
1. Most problematic issue is that the regulation as well as detail molecular mechanism of the NCOA4-mediated ferritinophagy by BNIP3 are unclear. The authors should investigate the timing and the way that BNIP3 binds to NCOA4. What factor(s) controls the interaction? Why does the constitutive binding occur in melanosome? Is the BNIP3 bound to NCOA4 able to act as a mitophagy adaptor? Is the NCOA4 bound to BNIP3 unable to serves as a ferritin adaptor? If so, the mechanism? Is the negative regulation of NCOA4-mediated ferritinophagy by BNIP3 general, or specific in melanosome? The authors should show the (mitochondrial) localization of NCOA4 in melanosome.
2. Another serious problem is the metabolome analyses. Are the data statistically significances? In particular, the tracer experiments with 13C-glucose in this manuscript did not support the author's claim at all.
3. In Figure 2F, how does loss of BNIP3 increase autophagy-flux? In Figure 3A, the number of GFP-LC3 puncta in BNIP3-depleted cells was comparable to that in control cells, which was inconsistent with the data presented in Fig. 2F. The LC3B-positive structures shown in Fig. 2F (BNIP3KD) are too large, and they seem not to be autophagosomes.
4. NCOA4 is mainly degraded by macroautophagy, and BNIP3-mediated mitophagy is dependent on macroautophagy. Nevertheless, the amount of NCOA4 in ATG5-deleted cells (defective both mitophagy and NCOA4-turnover) was comparable to that in control cells (Fig. 5). Why?
5. In Fig. S2D, quantification is needed.

Referee #2:

In this manuscript, Vara-Perez et al. have investigated the role of BNIP3 in melanoma tumor cell growth. They provide evidence that increased BNIP3 expression correlates with poorer patient survival and that BNIP3 depletion decreases HIF-1 expression and HIF-dependent glycolysis via altered intracellular iron levels which impacts tumor growth. The authors conclude that their study identifies a new and unexpected role for BNIP3 in the regulation of HIF. A number of previous studies have identified a role for BNP3 in tumor progression and BNIP3 expression has previously been shown to be a heavily HIF-dependent. While there is a good case for investigating the interactions between BNIP3 and HIF-1alpha, their interaction in the context of cancer has been well studied detracting somewhat from the novelty of this study which lies mainly in the demonstration of BNIP3-dependent HIF activity.

Major Points:
1) In Figure 1C and 1D, how is the BNIP3 stained? What is the colour of the BNIP3 staining versus the background H&E stain? It is difficult to discern the BNIP3-specific staining on these tissues. Overall, figure 1 is quite descriptive and adds little in the way on new insight as the association of high BNIP3 levels and low cancer survival is known.

2) The effects of BNIP3 KD on tumor volume in Fig 2B is impressive. The authors do not address the apparently striking impact of BNIP3KD on the rate of tumor cell death (Fig 2D). This does not tally with a protective effect of BNIP3 KD in terms of tumor growth. Can the authors look at another measure of tumor cell apoptosis to further investigate significance of this.

3) The data presented in Figure 3A is largely confirmatory of previous work. Some of the metabolomic data is not very convincing. For example, the reported trend towards reduction in extracellular acidification rate in shBNIP3 cells reported in Figure 3D is not at all clearly different from control (even though an unlikely statistical significance is reported). The authors conclude from their BNIP3 KD experiments that BNIP3 promotes glycolysis. Is the converse true? Does overexpressing BNIP3 induce an increase in glycoloysis.

4) In Figure 4A, does BNIP3 KD also affect HIF-2alpha expression (the isoform more usually associated with cancer). The data in figure 4B should be quantified. Does BNIP3 overexpression enhance HIF-1/2 expression? The data demonstrating increased HIF hydroxylation in BNIP3 depleted cells could be strengthened by for example using a hydroxylation (CO2-release) assay or mass spectrometry. Furthermore, the authors should demonstrate the effects of BNIP expression levels on HIF ubiquitination by the pVHL protein as this is key to it's post-translational stability.

5) The authors focus on the impact of BNIP3 on iron content as a link to HIF-1alpha stabilization. In Figure 3C, knockdown of BNIP3 appears to increase OCR which would result in less oxygen being available in the cell which would favour HIF stabilization. Have the authors considered this as a possible contributory link between BNIP3 and HIF-1alpha stabilization (similar to the mechanism propose din Hagen et al, Science 2003).

Minor Points:

1) In "the paper explained", the authors refer to HIF-1 as an oncoprotein. Many patients are receiving hydroxylase inhibitors which stabilize and activate the HIF` pathway, however there is no evidence for increased melanoma growth in these patients. It is possibly an oversimplification to render HIF an oncoprotein. The authors could consider this point.

Referee #3:

In this manuscript the authors examine the role of BNIP3 in melanoma. BNIP3 is a protein associated with the mitochondrial outer membrane and functions as a cargo receptor for mitophagy. BNIP3 has been identified as having a role in the progression of various forms of cancers, presumably through it activity in promoting mitophagy. Multiple types of cancers have been found to depend on mitophage and autophagy to maintain their metabolism in changing cancer environments. Here the authors present evidence that higher levels of BNIP3 are associated with reduced survival time in patients with melanoma. Using in vitro and in vivo mouse models of melanoma, the authors show that tumor growth is specifically reduced when expression of BNIP3 is reduced and that depletion of BNIP3 is associated with metabolic changes and altered mitochondrial energy metabolism. Previous studies have shown that BNIP3 is both responsive to changes in HIF1a activity and promotes HIF1a activity that can account for the metabolic changes observed. Here the authors confirm that HIF1a levels and activity are reduced in melanoma cells depleted of BNIP3. These changes in HIF1a could be traced to increased activity of the prolyl hydroxylase (PHD2) that targets HIF1 for ubiquitin-mediated degradation. Evidence is presented examining the role of altered iron homeostasis to account for increased PHD activity.

The question of how altered mitophagy could affect the turnover of HIF1a is interesting and highly relevant to cancer biology. Hypoxia and the accumulation of inhibitory substrates for PHDs have been implicated in HIF-mediated tumor formation because oxygen and the intermediate metabolite 2-oxoglutarate are required for PHD activity. Iron is also a required cofactor for PHD activity and the availability of cellular iron can affect the activity of PHD in cells and tissues in vivo. The evidence the authors present here for altered PHD activity is fairly persuasive and largely rests on the observation of reduced levels of HIFa in BNIP-depleted cells and tumor tissue. Although the authors try to assert that the relative amounts of hydroxylated HIF are increased, they don't really show this. Instead, the small amount of detectable OH-HIF that they detect doesn't really change, only the amount of total HIF. If the authors wanted to show more OH-HIF, they would need to block HIF degradation with a proteasome inhibitor or a VHL inhibitor. However, the use of mutant HIF1 that cannot be hydroxylated does tend to rescue the HIF phenotypes observed in their BNIP3-depleted cells.

The authors argue that increases in intracellular Fe(II) are responsible for an increase in PHD activity. This would suggest that there is a pool of un-metallated PHD in the cell. While NCOA4 levels do increase in BNIP3-depleted cells, the authors have not shown how this could affect iron. NCOA4 directs ferritin to the lysosome for turnover. That does not necessarily equate with elevated cytosolic iron. The authors present no data regarding ferritin levels, which would surely be affected by alterations in NCOA4 that are great enough to produce elevated intracellular iron. What happens to ferritin? Can these effects on PHD be mimicked by simply adding iron to the culture medium or animal chow? The ferroOrange reagent needs some additional controls before these data regarding iron can be reliably interpreted. Furthermore, it is more likely that only cytosolic iron (II), rather than total intracellular iron, is changed with BNIP3 knockdown. But the authors have not measured this.

What remains poorly addressed is why the levels of PHD2 increase in BNIP-depleted cells. This effect is clear in Fig. 5A, but less so in Fig. 5E. This effect may have nothing to do with iron availability, but instead reflect a more direct effect on PHD turnover.

In short, the evidence that intracellular iron is increasing the activity of PHD is weak, although the evidence that HIF reduced in clear and the change in PHD activity is a reasonable explanation, although not directly demonstrated.

Minor points:

1. Why is LC3-II so abundant after BNIP depletion in Fig. 2F, there is much less LC3-II in the images in Fig. 3A?
2. The increase in NCOA4 after bafilomycin treatment is clear, but why does it go up more in baf-treated BNIP knockdown? Is BNIP sending NCOA4 to the proteasome? Would proteasome inhibition make any of this clearer?

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Rebuttal and experimental plan with respect to Reviewer 1 comments:

1. Reviewer 1's comment 1: *Most problematic issue is that the regulation as well as detail molecular mechanism of the NCOA4-mediated ferritinophagy by BNIP3 are unclear. The authors should investigate the timing and the way that BNIP3 binds to NCOA4. What factor(s) controls the interaction? Why does the constitutive binding occur in melanosome? Is the BNIP3 bound to NCOA4 able to act as a mitophagy adaptor? Is the NCOA4 bound to BNIP3 unable to serves as a ferritin adaptor? If so, the mechanism? Is the negative regulation of NCOA4-mediated ferritinophagy by BNIP3 general, or specific in melanosome? The authors should show the (mitochondrial) localization of NCOA4 in melanosome.*

Answer. NCOA4 was coined as a ferritinophagy receptor in 2014, when two independent studies reported the ability of NCOA4 to bind LC3, hereby targeting ferritin for lysosomal degradation (1,2). Since then, different groups have been making great efforts to understand how NCOA4-mediated ferritinophagy works, what are the stress pathways triggering ferritinophagy, the involvement of components of the autophagic or proteasomal machinery. However, only very limited knowledge on the molecular mechanisms regulating ferritinophagy is currently available.

We are quite excited therefore by the finding that BNIP3 is a novel interactor of NCOA4. Our data suggest that BNIP3-NCOA4 binding limits the engagement of NCOA4 in ferritinophagy in melanoma cells, thereby restricting PHD2-mediated hydroxylation activity.

However, we feel that addressing the full mechanistic underpinnings of the BNIP3 mediated control of ferritinophagy and its cross-talk with mitophagy, as the Reviewer1 asks, is a full study on its own, the extent of which is clearly beyond the scope of this work. Moreover, there are several questions raised by the Reviewer1 that remain rather unclear to us, more in particular related to the interaction between BNIP3 and NCOA4 in the melanosomes.

This is puzzling to us since we do not show, nor we claim, the involvement of melanosomes in the BNIP3-mediated effects on ferritinophagy or HIF-1α signaling, nor we are aware of possible recruitment of BNIP3 to melanosomes.

Experimental work proposed: We are currently generating (lentivirally-transduced) stably cell lines rescuing in BNIP3 silenced cells steady-state expression level of (myc-tagged) BNIP3 full length (BNIP3FL) and a mitophagy-defective BNIP3 mutant (BNIP3deltaTM) lacking the transmembrane domain, thus disabling BNIP3 from docking to the outer mitochondrial membrane. By performing subcellular fractionation (mitochondria/cytosol) and IPs in these cell lines, we will be able to differentiate: **i) if NCOA4 binding depends on BNIP3's docking to the outer mitochondrial membrane, and on BNIP3's transmembrane domain** and **ii) whether (rescuing or not) BNIP3-mitophagy affects ferritinophagy, intracellular iron levels and HIF-1α signaling**, **altogether adding important and novel mechanistic insights to this BNIP3 regulated pathway** (together with the data already present in the main figure 5 of the submitted manuscript)**.**

2.Reviewer 1's comment 2: *Another serious problem is the metabolome analyses. Are the data statistically significances? In particular, the tracer experiments with 13C-glucose in this manuscript did not support the author's claim at all.*

Answer. We believe we would be able to answer effectively this 'serious concern' since this seems just a misinterpretation of the data.

We would like to highlight that not only the data shown in Figure 3F **but also those of Figure 3D and sup. Figure 4C-D-E-F-G together show -by using different approaches**, **namely GC-MS metabolomics, Seahorse and ^{1}H-NMR**- that melanoma cells with defective BNIP3 expression accumulate glucose and produce and secrete lower levels of lactate, both readouts of impaired glycolysis. As the glycolytic reduction comes from the transcriptional downregulation of several HIF-1α targets and key enzymes of the glycolytic pathway, the glucose that enters in the glycolytic pathway is effectively converted into lactate/pyruvate by the reduced levels of the enzymes (again, we have lower levels but the enzymes are active). Therefore, although lower levels of glucose are entering glycolysis, this is effectively converted and thus not significantly impacting the labelling of the downstream metabolites; as it could be expected if only one particular step of the glycolytic cascade was impaired and not all the ^{13}C glucose reached the end of the pathway.

Changes in the manuscript: to avoid future misunderstandings, additional metabolomics experiments (which were shown separately just because of their different baselines) will be repeated so that they can be pulled together into a single graph and statistically analyzed. Moreover, the representation of the ^{13}C labelling will be changed into a graph where only the labelled species will be represented and statistically analyzed.

3.Reviewer 1's comment 3: *In Figure 2F, how does loss of BNIP3 increase autophagy-flux? In Figure 3A, the number of GFP-LC3 puncta in BNIP3-depleted cells was comparable to that in control cells, which was inconsistent with the data presented in Fig. 2F. The LC3B-positive structures shown in Fig. 2F (BNIP3KD) are too large, and they seem not to be autophagosomes.*

Answer. We believe we would be able to effectively answer these concerns.

The image in Figure 3A represents one plane of a z-stack confocal image whereas Figure 2F is standard widefield fluorescence microscopy. Moreover, the confocal imaging was performed with a GFP-LC3 plasmid whereas the tissue staining was performed through antibody-based immunohistochemistry, as detailed in the methods. As a technical note, we tried the same antibody in immunocytochemistry for the colocalization with unsuccessful results. For this reason, we decided to use the tagged plasmid for the colocalization. It should also be noted that, because of the transient transfection, the plasmid might not be always expressed to the same extent in all the cells, causing variability in the absolute number of autophagosomes: for this reason, we express our colocalization results as a percentage of autophagosomes colocalizing and not absolute numbers of autophagosomes. Lastly, LC3 can decorate other vesicles beyond the autophagosomes (3)

Regarding the mechanism through which BNIP3 loss affects autophagy, we suspect that upon loss of BNIP3 (because of the glycolytic defect) mTORC1 pathway is affected, which may keep autophagic flux ongoing in BNIP3 compromised cells.

Experimental work proposed: to strengthen the imaging data we can image the tumor tissue with a confocal microscope instead of using a widefield microscope, this will allow us to define better LC3-associated structures. We will perform western blot and qPCR to assess mTORC1 status and ULK1 phosphorylation, in BNIP3 proficient and silenced melanoma cells.

4.Reviewer 1's comment 4: *NCOA4 is mainly degraded by macroautophagy, and BNIP3-mediated mitophagy is dependent on macroautophagy. Nevertheless, the amount of NCOA4 in ATG5-deleted cells (defective both mitophagy and NCOA4-turnover) was comparable to that in control cells (Fig. 5). Why?*

Answer. As already commented above, NCOA4-mediated ferritinophagy is a very novel selective autophagy pathway. Our data shows that treatment with the lysosomal blocker BafA recovers NCOA4 levels and in the case of shBNIP3, NCOA4 is degraded in the lysosome to a higher extent than in shCntl and shATG5 conditions, suggesting that this effect might be independent of macroautophagy. Recent studies have highlighted that NCOA4 can also be degraded through different autophagic pathways (studies that are mentioned and discussed in our submitted manuscript: in the results section (lines 321-329), in the discussion section (lines 477-490) and references 24, 25 and 26). Addressing why ATG5 is not involved in the NCOA4 turnover is a matter of future more mechanistic studies (as we state in the discussion), which goes beyond the main message of this manuscript, showing that HIF-1α is controlled by BNIP3 through pathways that are not phenocopied by ATG5.

5.Reviewer 1's comment 5: *In Fig. S2D, quantification is needed*

Answer. We believe we would be able to effectively answer this concern.

Experimental work proposed: confocal quantification of autophagosomes.

Rebuttal and experimental plan with respect to Reviewer 2 comments:

6. Reviewer 2's initial comment: *In this manuscript, Vara-Perez et al. have investigated the role of BNIP3 in melanoma tumor cell growth. They provide evidence that increased BNIP3 expression correlates with poorer patient survival and that BNIP3 depletion decreases HIF-1 expression and HIF-dependent glycolysis via altered intracellular iron levels which impacts tumor growth. The authors conclude that their study identifies a new and unexpected role for BNIP3 in the regulation of HIF.*
A number of previous studies have identified a role for BNP3 in tumor progression and BNIP3 expression has previously been shown to be a heavily HIF-dependent. While there is a good case for investigating the interactions between BNIP3 and HIF-1alpha, their interaction in the context of cancer has been well studied detracting somewhat from the novelty of this study which lies mainly in the demonstration of BNIP3-dependent HIF activity.

Answer. While we agree with the Reviewer2's comment about existing literature on the contextual role of BNIP3 in cancer, **we humbly disagree with the view that this has been studied in the context of the HIF-1α signaling**. **Clearly, BNIP3 is a known HIF-1α target but whether and how HIF-1α and BNIP3 cooperate or crosstalk to control tumor growth is understudied.** A recent report using a **breast cancer model** ((4), also cited in our manuscript, reference 7) investigated in great mechanistic details the role of BNIP3-HIF-1α axis and found **that BNIP3 repressed HIF-1α pro-tumorigenic signaling** (by inciting mitophagy and inhibition of glycolysis). Here, we provide compelling evidence that **instead, BNIP3 in melanoma supports HIF-1α signaling**. Quite interestingly, these data are very much in line with reports in clinical samples that show that in breast cancer BNIP3 is downregulated and associated with poor prognosis (4,5) whereas in uveal melanoma (as reported in our introduction, lines 92-95, reference 8) as well as in our cutaneous melanoma cohorts, BNIP3 is upregulated and associated with poor prognosis. **In fact, to our knowledge no study has shown that BNIP3 supports HIF-1α pro-tumorigenic activity by keeping PHD2 activity at bay.** We also are not aware of other studies exploring the role of BNIP3-HIF-1α in melanoma growth *in vivo*.

7. Reviewer 2's comment 1: *In Figure 1C and 1D, how is the BNIP3 stained? What is the colour of the BNIP3 staining versus the background H&E stain? It is difficult to discern the BNIP3-specific staining on these tissues. Overall, figure 1 is quite descriptive and adds little in the way on new insight as the association of high BNIP3 levels and low cancer survival is known.*

Answer. For the procedure of staining, we refer to our methods section (lines 870-881 of the submitted manuscript). As stated in the methods, **these tissues have not been counterstained; therefore, everything highlighted as pink/red is BNIP3 specific staining**.

Regarding the descriptiveness of the figure, other retrospective studies have shown similar procedures to show BNIP3 in cancer patient samples and to our knowledge, only one study has explored BNIP3 levels in a cutaneous melanoma cohort (6) as part of a hypoxic signature and related to PD-1 treatment in a small patient cohort (n=19). Therefore, we believe that the patient data that we show is novel and relevant in this context.

8. Reviewer 2's comment 2: *The effects of BNIP3 KD on tumor volume in Fig 2B is impressive. The authors do not address the apparently striking impact of BNIP3KD on the rate of tumor cell death (Fig 2D). This does not tally with a protective effect of BNIP3 KD in terms of tumor growth. Can the authors look at another measure of tumor cell apoptosis to further investigate significance of this.*

Answer. We believe we would be able to effectively answer this concern.

In figure 2D we show overall cell death (necrotic) area, which is reduced. When we performed the staining of **cleaved Caspase 3** (an apoptotic marker) we **could see a very subtle yet statistically significant increase in the number of positive cells per field,** bearing in mind that an imaging field harbors on average 700 cells (see **Figure 1** at the end of this rebuttal). However, the drastic drop in cell proliferation (measured by Ki67, figure 2E of the manuscript) points towards a drop in proliferation as the major cause behind the reduction in tumor growth of shBNIP3 cells.

Experimental work proposed: inclusion in the paper of images showing representative ki67 staining and cleaved caspase 3 as well as quantification of the cleaved Caspase staining as a percentage of positive cells versus total number of cells per imaging field.

9. Reviewer 2's comment 3: *The data presented in Figure 3A is largely confirmatory of previous work. Some of the metabolomic data is not very convincing. For example, the reported trend towards reduction in extracellular acidification rate in shBNIP3 cells reported in Figure 3D is not at all clearly different from control (even though an unlikely statistical significance is reported). The authors conclude from their BNIP3 KD experiments that BNIP3 promotes glycolysis. Is the converse true? Does overexpressing BNIP3 induce an increase in glycoloysis.*

Answer. We believe we would be able to effectively answer this concern.

As already mentioned in our previous comments, in breast cancer BNIP3 by stimulating mitophagy keeps mitochondria-ROS levels down thus impairing HIF-1α stabilization and glycolysis (4). Instead here we show that while BNIP3 still operates as mitophagy receptor, when BNIP3 is silenced, mitochondria are forced to respire (even if unhealthily) because the glycolytic pathway is transcriptionally downregulated by the reduced levels of HIF-1α. **Hence, BNIP3 in breast cancer or melanoma exerts a rather opposite effect on HIF-1α signaling. We believe that this is an important conclusion of our study, which sheds new light on the still controversial role of BNIP3 in cancer and delineate BNIP3 as potential biomarker of early melanoma progression.**

Experimental work proposed: together with the proposed experiments to the comment 2 of Reviewer1, repetition of the Seahorse experiments in basal conditions to reduce the difference between experiments; assessment of the ECAR and glycolytic readouts in the BNIP3-FL and BNIP3deltaTM stable cell lines that we are currently generating.

10.Reviewer 2's comment 4: *In Figure 4A, does BNIP3 KD also affect HIF-2alpha expression (the isoform more usually associated with cancer). The data in figure 4B should be quantified. Does BNIP3 overexpression enhance HIF-1/2 expression?*

Answer. We believe we would be able to effectively answer this interesting point in support of which we do have preliminary data, that we did not add initially to the manuscript to avoid increasing the complexity of the study. However, upon request of the Reviewer they can be added.

Indeed, preliminary data in B16-F10 cells under basal conditions showed that **knocking down BNIP3, but not ATG5, downregulates also HIF-2α protein levels** (see **Figure 2** at the end of this rebuttal). **This is associated with a corresponding downregulation of cyclin D1, one of the HIF-2α main targets** (7). As in the case of HIF-1α, decreased protein levels of HIF-2α in BNIP3 silenced cells are not explained by an effect on *Hif2a* mRNA level. Since HIF-2α protein stability is also regulated by iron-dependent PHD2 activity (8), **this observation further supports the proposed mechanism that BNIP3 feeds pro-tumorigenic HIF-signaling by regulating the availability of intracellular iron, as the key co-factor controlling PHD2 hydroxylating activity towards HIF-1α and HIF-2α under conditions of oxygen availability**.

Experimental work proposed :As a rescue strategy -rather than overexpressing BNIP3, which may affect cell death or other pathways, we are currently generating stably transduced cell lines (shCntl/shBNIP3) rescuing steady-state expression level of (myc-tagged) BNIP3 full length (BNIP3FL) and a mitophagy-defective BNIP3 mutant (BNIP3deltaTM) lacking the transmembrane domain, thus disabling BNIP3 from docking to the outer mitochondrial membrane. **We will then analyze HIF-1α and HIF-2α protein and RNA levels in these cell lines, which will validate BNIP3 as the molecular mediator and will further reveal whether mitophagy is relevant in this process (see also answer to comment 1 from Reviewer 1).**

11.Reviewer 2's comment 5. *The data demonstrating increased HIF hydroxylation in BNIP3 depleted cells could be strengthened by for example using a hydroxylation (CO2-release) assay or mass spectrometry. Furthermore, the authors should demonstrate the effects of BNIP expression levels on HIF ubiquitination by the pVHL protein as this is key to it's post-translational stability.*

Answer: We thank the Reviewer for his/her comment. We have indeed data confirming the classical pathway of PHD-mediated HIF-1α degradation through HIF-1α ubiquitination (see **Figure 3** at the end of this rebuttal). We also checked total pVHL levels (see **Figure 3** at the end of this rebuttal) but since HIF-1α ubiquitination cannot occur without hydroxylation, we decided not to include it so that we would not overwhelm the reader. In the figure below, you can find our preliminary data, which can be easily included in our manuscript.

Experimental work proposed: repeat MG132 treatments to be able to show in the same blot HIF-1α (hydroxylated and total) as well as pVHL recovery after proteasomal blockade (not explored in our preliminary data).

12.Reviewer 2's comment 6: *The authors focus on the impact of BNIP3 on iron content as a link to HIF-1alpha stabilization. In Figure 3C, knockdown of BNIP3 appears to increase OCR which would result in less oxygen being available in the cell which would favour HIF stabilization. Have the authors considered this as a possible contributory link between BNIP3 and HIF-1alpha stabilization (similar to the mechanism propose din Hagen et al, Science 2003).*

Answer: This is an interesting remark. However, in our model and despite the increased OCR, BNIP3 silenced cells display **less HIF-1α stabilization/levels**, **not increased HIF-1α levels**. Therefore, the proposed mechanism -which we are aware of- does not seem to explain the BNIP3 phenotype we observe in melanoma cells. BNIP3 silencing indeed increases OCR (while other studies show the opposite, (4)) and also increases PHD2 activity thereby favoring HIF-1α destabilization, and both phenotypes have been linked in literature with an increase in intracellular iron (9), which we also have upon BNIP3 silencing. **We can further highlight this point in the main text.**

13.Reviewer 2's Minor point 1: *In "the paper explained", the authors refer to HIF-1 as an oncoprotein. Many patients are receiving hydroxylase inhibitors which stabilize and activate the HIF` pathway, however there is no evidence for increased melanoma growth in these patients. It is possibly an oversimplification to render HIF an oncoprotein. The authors could consider this point.*

Answer: We thank the Reviewer for highlighting this point. It is true that HIF-1α has not been described as an initiating event in many cancer types (except in renal cancer, where it has been associated with a loss of pVHL), but several studies have correlated high HIF signaling with poor prognosis (10,11), therefore our use of 'oncoprotein' (especially in a section aimed to non-scientific public) but considering all the effects described for HIF-1α (as well comparing HIF-1α vs HIF-2α), we agree that it is definitely an oversimplification.
The revised manuscript, unless we are told otherwise, it will not include "the paper explained" section because it was meant for *EMBO Molecular Medicine* and not for *The EMBO Journal*.

Rebuttal and experimental plan with respect to Reviewer 3 comments

14.Reviewer 3's comment 1: *In this manuscript the authors examine the role of BNIP3 in melanoma. BNIP3 is a protein associated with the mitochondrial outer membrane and functions as a cargo receptor for mitophagy. BNIP3 has been identified as having a role in the progression of various forms of cancers, presumably through it activity in promoting mitophagy. Multiple types of cancers have been found to depend on mitophage and autophagy to maintain their metabolism in changing cancer environments. Here the authors present evidence that higher levels of BNIP3 are associated with reduced survival time in patients with melanoma. Using in vitro and in vivo mouse models of melanoma, the authors show that tumor growth is specifically reduced when expression of BNIP3 is reduced and that depletion of BNIP3 is associated with metabolic changes and altered mitochondrial energy metabolism. Previous studies have shown that BNIP3 is both responsive to changes in HIF1a activity and promotes HIF1a activity that can account for the metabolic changes observed. Here the authors confirm that HIF1a levels and activity are reduced in melanoma cells depleted of BNIP3.*

Answer: To our knowledge this evidence has not been shown before, thus it cannot be confirmatory. As explained above (see the answer to the comment 6 from Reviewer 2), while there is existing literature on the contextual role of BNIP3 in cancer, **we humbly disagree with the view that this has been studied in the context of the HIF-1α signaling**. **Clearly, BNIP3 is a known HIF-1α target but whether and how HIF-1α and BNIP3 cooperate or crosstalk to control tumor growth is understudied.** A recent report using a **breast cancer model** (also cited in our manuscript, reference 7) investigated in great mechanistic details the role of BNIP3-HIF-1α axis and found **that BNIP3 repressed HIF-1α pro-tumorigenic signaling** (by inciting mitophagy and inhibition of glycolysis). Here, we provide compelling evidence that **instead, BNIP3 in melanoma supports HIF-1α signaling**. Quite interestingly, these data are very much in line with reports in clinical samples that show that in breast cancer BNIP3 is downregulated and associated with poor prognosis (4,5) whereas in uveal melanoma (as reported in our introduction, lines 92-95, reference 8) as well as in our cutaneous melanoma cohorts, BNIP3 is upregulated and associated with poor prognosis. **In fact, to our knowledge no study has shown that BNIP3 supports HIF-1α pro-tumorigenic activity by keeping PHD2 activity at bay.** We also are not aware of other studies exploring the role of BNIP3-HIF-1α in melanoma growth *in vivo*.

15.Reviewer 3's comment 2: *These changes in HIF1a could be traced to increased activity of the prolyl hydroxylase (PHD2) that targets HIF1 for ubiquitin-mediated degradation. Evidence is presented examining the role of altered iron homeostasis to account for increased PHD activity. The question of how altered mitophagy could affect the turnover of HIF1a is interesting and highly relevant to cancer biology. Hypoxia and the accumulation of inhibitory substrates for PHDs have been implicated in HIF-mediated tumor formation because oxygen and the intermediate metabolite 2-oxoglutarate are required for PHD activity. Iron is also a required cofactor for PHD activity and the availability of cellular iron can affect the activity of PHD in cells and tissues in vivo. The evidence the authors present here for altered PHD activity is fairly persuasive and largely rests on the observation of reduced levels of HIFa in BNIP-depleted cells and tumor tissue. Although the authors try to assert that the relative amounts of hydroxylated HIF are increased, they don't really show this. Instead, the small amount of detectable OH-HIF*

that they detect doesn't really change, only the amount of total HIF. If the authors wanted to show more OH-HIF, they would need to block HIF degradation with a proteasome inhibitor or a VHL inhibitor. However, the use of mutant HIF1 that cannot be hydroxylated does tend to rescue the HIF phenotypes observed in their BNIP3-depleted cells.

Answer: We thank the Reviewer for highlighting some critical findings of our study. Indeed as the Reviewer mentions, **the use of mutant HIF-1α that cannot be hydroxylated does tend to rescue the HIF-1α phenotype observed in the BNIP3-depleted cells**, thereby **supporting our proposed model that the effects of BNIP3 are mediated by changes in HIF-1α-OH both *in vitro* (rescue of the glycolysis) and most importantly *in vivo* (rescue of tumor growth).** Data with proteasomal inhibitors rescuing HIF-1α-OH and HIF-1α levels in BNIP3 silenced cells are available (see **Figure 3** at the end of this rebuttal) and can be added.

Experimental work proposed: repeat MG132 treatments to be able to show in the same blot HIF-1α (hydroxylated and total) as well as VHL recovery after proteasomal blockade (not explored in our preliminary data). This was already proposed in the answer to the comment 11 of Reviewer 2.

16.Reviewer 3's comment 3: *The authors argue that increases in intracellular Fe(II) are responsible for an increase in PHD activity. This would suggest that there is a pool of un-metallated PHD in the cell. While NCOA4 levels do increase in BNIP3-depleted cells, the authors have not shown how this could affect iron.*

Answer: We humbly disagree and find this Reviewer's comment. **In Fig 5F, I and J we do show that silencing NCOA4 in BNIP3 compromised cells, reduces the free iron pool to levels of the control cells.** Perhaps this critical experiment escaped the Reviewer's attention.

17.Reviewer 3's comment 4: *NCOA4 directs ferritin to the lysosome for turnover. That does not necessarily equate with elevated cytosolic iron. The authors present no data regarding ferritin levels, which would surely be affected by alterations in NCOA4 that are great enough to produce elevated intracellular iron. What happens to ferritin?*

Answer. We have checked levels of ferritin light chain (FTL), and it is indeed degraded by ferritinophagy, as indicated by the NCOA4 rescue upon BafA treatment (see **Figure 4** at the end of this rebuttal) and we can also see a higher rescue for FTL in the shBNIP3 condition, hereby supporting our idea that shBNIP3 promotes ferritinophagy. We should be aware, though, that HIF-1α can also transcriptionally modulate ferritin light chain (12)(ref) and that cytosolic ferritin can also be degraded by the proteasome (13), which add additional levels of complexity to this loop.

Experimental work proposed: add FTL to the representative blots of ferritinophagy, explore the proteasomal degradation of FTL with MG132 inhibition as well as FTL transcriptional levels on qPCR.

18.Reviewer 3's comment 5: *Can these effects on PHD be mimicked by simply adding iron to the culture medium or animal chow? The ferroOrange reagent needs some additional controls*

before these data regarding iron can be reliably interpreted. Furthermore, it is more likely that only cytosolic iron (II), rather than total intracellular iron, is changed with BNIP3 knockdown. But the authors have not measured this.

Answer: We thank the reviewer for his/her constructive comments. However, adding iron would not reveal further the BNIP3-PHD2 link since BNIP3 compromised cells already display higher intracellular iron. Adding iron to WT cells will probably affect PHD2 activity but would not tell us more on the mechanism mediated by BNIP3.

Experimental work proposed: We plan to measure the Fe^{2+}/Fe^{3+} ratio by quantitative CE-ICP-MS analysis (14). This "state of the art" quantitative methodology will confirm the increase in the total pool of free iron and provide quantitative measurement of the Fe^{2+}/Fe^{3+} ratio in these melanoma cells.

19.Reviewer 3's comment 6: What remains poorly addressed is why the levels of PHD2 increase in BNIP-depleted cells. This effect is clear in Fig. 5A, but less so in Fig. 5E. This effect may have nothing to do with iron availability, but instead reflect a more direct effect on PHD turnover.

Answer: The effect of PHD2 turnover can be easily checked experimentally.

Experimental work proposed: We plan to explore PHD2 protein turnover upon BafA (lysosomal degradation) or MG132 (proteasomal degradation) treatment in the shCntl and shBNIP3 cell lines.

20.***Reviewer 3's comment 7:*** In short, the evidence that intracellular iron is increasing the activity of PHD is weak, although the evidence that HIF reduced in clear and the change in PHD activity is a reasonable explanation, although not directly demonstrated.

Answer: As already commented, the knockdown of NCOA4 recovered iron levels in BNIP3 compromised cells and partially rescued HIF-1α levels (figures 5I-J of the submitted manuscript). However, to satisfy Reviewer's 3 curiosity, we plan to provide further evidence about the link iron-PHD2.

Experimental work proposed: As already proposed in the comment 19 to Reviewer 3, we plan to provide further evidence about the link iron-PHD2 by further co-IP experiments, expression of BNIP3 mutants, using iron chelator strategies and by measuring Fe^{2+}/Fe^{3+} ratio in BNIP3 silenced cells.

21.Reviewer 3's minor comment 1: Why is LC3-II so abundant after BNIP depletion in Fig. 2F, there is much less LC3-II in the images in Fig. 3A?

Answer: We believe we would be able to effectively answer this minor concern. The image in Figure 3A represents one plane of a z-stack confocal image whereas Figure 2F is standard widefield fluorescence microscopy (see answer and proposed work to the comment 3 from Reviewer 1).

22.Reviewer 3's minor comment 2: *The increase in NCOA4 after bafilomycin treatment is clear, but why does it go up more in baf-treated BNIP knockdown? Is BNIP sending NCOA4 to the proteasome? Would proteasome inhibition make any of this clearer?*

Answer: The increase in NCOA4 seen in the BNIP3 knockdown is indicative of an increased ferritinophagic flux. We believe that BNIP3, through NCOA4 binding, limits its engagement in ferritinophagy. When BNIP3 is absent (shBNIP3 condition), then NCOA4 is free to engage in ferritinophagy. Perhaps this critical information escaped the Reviewer's attention. Since we will be including MG132 treatments in our experimental plan (see experimental plan proposed to comment 17, which comments a similar phenomenon for FTL), we can always check for NCOA4 degradation as well.

Figures for referees removed

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1st Revision - Editorial Decision 21st Sep 2020

Dear Patrizia,

Thank you for contacting me regarding our decision and for your patience with my response, which got delayed due to internal discussions in the team regarding your point-by-point response.

We realise that you would - judging from the information provided in the rebuttal letter - be potentially able to address the issues raised by the referees in a revised version of the manuscript.

Overall, we would thus invite you to work towards a re-review. Accordingly, we would - given that you addressed the experimental issues with compelling data - be prepared to ask the referees for further input.

Please note in particular that we consider your response to the mechanistic interrogation of the link between BNIP3 and NCOA4 (ref#1 pt.1) as appropriate and would be satisfied with your addressing this point accordingly as detailed in your response.

Please note however, that we would need the additional major concerns of the referees regarding statistical significance and conclusiveness of your metabolomics findings as well as complementary experiments to consolidate the BNIP3-NCOA4-PHD2-HIF1a axis to be addressed satisfactorily. In that sense, it would be essential to provide a definitive and accurately described dataset in the revised version.

Please contact me if you have any questions, need further input on the referee comments or if you consider engaging in a compelling revision, in which case we would not close the file.

Kind regards,

Daniel

Daniel Klimmeck, PhD
Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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Rebuttal to Reviewer 1 comments:

1. Reviewer 1's comment 1: *Most problematic issue is that the regulation as well as detail molecular mechanism of the NCOA4-mediated ferritinophagy by BNIP3 are unclear. The authors should investigate the timing and the way that BNIP3 binds to NCOA4. What factor(s) controls the interaction? Why does the constitutive binding occur in melanosome? Is the BNIP3 bound to NCOA4 able to act as a mitophagy adaptor? Is the NCOA4 bound to BNIP3 unable to serves as a ferritin adaptor? If so, the mechanism? Is the negative regulation of NCOA4-mediated ferritinophagy by BNIP3 general, or specific in melanosome? The authors should show the (mitochondrial) localization of NCOA4 in melanosome.*

Answer. We appreciate Reviewer 1's comments and questions. It should be noted that NCOA4 was coined as a ferritinophagy receptor in 2014, when two independent studies reported the ability of NCOA4 to bind LC3, hereby targeting ferritin for lysosomal degradation (Mancias *et al*, 2014; Dowdle *et al*, 2014). Since then, different groups have been making great efforts to understand how NCOA4-mediated ferritinophagy works, what are the stress pathways triggering ferritinophagy or the involvement of components of the autophagic or proteasomal machinery. However, only very limited knowledge on the molecular mechanisms regulating ferritinophagy is currently available. We are quite excited therefore by the finding that BNIP3 is a novel interactor of NCOA4. Our data suggest that BNIP3-NCOA4 binding limits the engagement of NCOA4 in ferritinophagy in melanoma cells, thereby restricting PHD2-mediated hydroxylation activity.

However, we feel that addressing the full mechanistic underpinnings of the BNIP3-mediated control of ferritinophagy and its cross-talk with mitophagy, as the Reviewer 1 asks, is a full study on its own, the extent of which is clearly beyond the scope of this work. Moreover, there are several questions raised by the Reviewer 1 that remain rather unclear to us, more in particular related to the interaction between BNIP3 and NCOA4 in the melanosomes. This is puzzling to us since we do not show, nor we claim, the specific involvement of melanosomes in the BNIP3-mediated effects on ferritinophagy or HIF-1α signaling.

Since we share Reviewer 1's curiosity about the interaction between BNIP3 and NCOA4, we still tried to shed some extra light into the molecular mechanisms involved in this interaction. For this reason, we generated new shBNIP3 cell lines where we reintroduced (through lentiviral transduction) either murine (myc-tagged) BNIP3 full length (BNIP3FL) or a mitophagy-defective (Kanzawa *et al*, 2005; Prabhakaran *et al*, 2007) BNIP3 mutant (BNIP3deltaTM) in the shBNIP3 cell lines. Co-immunoprecipitation from lysates of BNIP3-FL and BNIP3deltaTM expressing cells showed that both BNIP3 proteins were able to bind NCOA4 (Rebuttal Figure 1A, see below

this answer), identifying the cytosolic domain of BNIP3 as the domain mediating the interaction with NCOA4. Further analysis of the subcellular localization of both BNIP3-FL and BNIP3deltaTM by subcellular fractionation that segregated the cytosolic from the mitochondrial cellular compartments (Rebuttal Figure 1B-C, see below this answer), showed that BNIP3-FL is enriched in the mitochondrial fraction as expected, alike the endogenous BNIP3 from the shCntl control. However, the truncated mutant BNIP3deltaTM, despite being enriched in the cytosolic fraction, was still detectable in the mitochondrial fraction as well, both in basal and BafA treated conditions (Rebuttal Figure 1B-C, see below this answer). The presence of BNIP3deltaTM in the mitochondrial fraction could be caused by the interaction of BNIP3 with other mitochondrial proteins through its cytosolic domain, thereby explaining its partial localization to the mitochondria. An example of these putative interactors could be the NIX-MIEAP complex, which binds to BNIP3 through the BNIP3's cytosolic region but the whole complex is located at the outer mitochondrial membrane (Nakamura *et al*, 2012). This unexpected finding did not allow us to separate completely the mitochondrial versus the non-mitochondrial action of BNIP3, and clearly requires a more detailed characterization of the molecular effects of BNIP3deltaTM in melanoma cells that unfortunately we were not able to carry out during the 3 months-period allocated for the revision.

We then chose to prioritize the set of experiments suggested by the reviewers themselves. To address the effect of the re-expression of BNIP3 (please note that the overexpression of BNIP3 can also be toxic to the cells (Prabhakaran *et al*, 2007; Kanzawa *et al*, 2005), so we did not pursue this strategy) on HIF-1α and glycolysis requested by Reviewer 2 (comment 3), we used the aforementioned shBNIP3 cells where we re-expressed BNIP3-FL. However, we only succeeded in rescuing the endogenous level of BNIP3 to a partial extent (Figure S3A-B of the revised manuscript). The exact reasons for the lack of full recovery are not completely clear and could be due to residual activity of the shRNA against the mutated BNIP3-FL and/or to an increased susceptibility of the myc-tagged BNIP3-FL mutant to proteasomal degradation, since BNIP3 steady state levels are controlled by both the lysosome (via mitophagy, see Figure EV2D) but also by the proteasome, and BNIP3-FL has been shown indeed to be targeted to and degraded by the proteasome (Park *et al*, 2013) (Rebuttal Figure 1D, see below this answer). In spite of this, partial recovering of BNIP3 expression in the shBNIP3 cells proportionally rescued HIF-1α levels and reduced its hydroxylation (Figure S3B of the revised manuscript), to a similar degree. Together with the co-IP experiments shown below confirming the NCOA4 binding to BNIP3-FL (and the mutant) and the new determination of the effects of BNIP3 on the cytoplasmic Fe^{2+}/Fe^{3+} ratio (see new Figure 6) that are mediated by NCOA4, we hope to have

convinced the reviewer that the effects of loss of BNIP3 on HIF-1 signaling in melanoma are specific and involve a new BNIP3-NCOA4 regulatory axis, which deserves to be fully explored in future studies.

Figure for referees removed

2.Reviewer 1's comment 2: *Another serious problem is the metabolome analyses. Are the data statistically significances? In particular, the tracer experiments with 13C-glucose in this manuscript did not support the author's claim at all.*

Answer. Answer. We appreciate the Reviewer 1's remarks. We would also like to highlight that our manuscript contains, several complementary readouts based on Seahorse and ^{1}H-NMR analysis (Figure 3D-E-F-G and Figure S2 together), that show how melanoma cells with defective BNIP3 expression accumulate glucose and produce and secrete lower levels of lactate, both readouts of impaired glycolysis.

Secondly, we apologize for the confusion regarding the ^{13}C-glucose tracer experiment, as it was not properly stated in the text that it referred to steady state labelling (see now lines 225-232). We now run 4 additional ^{13}C labelling experiments (total of 7), which all clearly and significantly show that the amount of lactate labelled (m_{0+3}) from 13C labeled glucose is significantly reduced in shBNIP3 cells when compared to both shCntl and shATG5 conditions. These data thus indicate that less glucose is contributing to lactate production, as expected from cells with impaired glycolysis. Together with the reduced secretion of lactate these results confirm that in BNIP3 deficient cells glucose that enters glycolysis, is not efficiently converted into lactate because of the downregulation of in the levels of glycolytic enzymes, catalyzing its conversion. We hope that with the additional metabolomics experiments (Figure 3E-H, EV4C-D, S2C of the revised manuscript), the adapted representation of the labelling data (Figure 3E of the revised

manuscript) and the rephrased explanation in the main text (lines 225-232), we have clarified the metabolic effect of shBNIP3 in melanoma cells.

3.Reviewer 1's comment 3: *In Figure 2F, how does loss of BNIP3 increase autophagy-flux? In Figure 3A, the number of GFP-LC3 puncta in BNIP3-depleted cells was comparable to that in control cells, which was inconsistent with the data presented in Fig. 2F. The LC3B-positive structures shown in Fig. 2F (BNIP3KD) are too large, and they seem not to be autophagosomes.*

Answer. This is an interesting point and we thank the Reviewer for raising this question. Because of the accumulation of LC3B-I and LC3B-II in the shBNIP3 condition, we suspected that LC3B upregulation was elevated at the transcriptional level. Indeed, we confirmed transcriptional upregulation of the autophagy mediators LC3B (*Map1lc3b*) and p62 (*Sqstm*) in the shBNIP3 condition *in vitro* (Figure EV1G of the revised manuscript) and *in vivo* (Figure 2H of the revised manuscript), thereby sustaining the higher autophagy capacity observed in the shBNIP3 cells. Since both LC3B and p62 have been described as targets of TFEB in murine cells (Wang *et al*, 2019), we also checked TFEB protein levels and indeed, higher TFEB protein levels were also observed in shBNIP3 cells *in vitro* (Figure EV1H of the revised manuscript). The transcriptional upregulation of LC3B can easily explains the differences in puncta number between Figure 3A and Figure 2F (now figure 2G in the revised manuscript), since Figure 3A was performed with a GFP-LC3 plasmid whereas the tissue staining was performed through antibody-based immunohistochemistry, as detailed in the methods section. As a technical note, we tried the same antibody in immunocytochemistry for the colocalization with unsuccessful results. For this reason, we decided to use the tagged plasmid for the colocalization (Figure 3A). While the tissue staining in Figure 2F (now figure 2G) displays the endogenous LC3B levels in the three conditions, the expression of GFP-LC3 through a plasmid is driven by a constitutive promotor sequence that will not reflect changes in endogenous levels of LC3B. It should also be noted that, because of the transient transfection, the plasmid might not be always expressed to the same extent in all the cells, causing variability in the absolute number of autophagosomes: for this reason, we express our colocalization results as a percentage of autophagosomes colocalizing and not absolute numbers of autophagosomes.

Regarding the size of the autophagosomes, we thank the Reviewer's for the remark. The differences between the two images are the result of the different microscopes used for their imaging: Figure 3A represents one plane of a z-stack confocal image whereas the original Figure 2F is standard widefield fluorescence microscopy. To address this concern, we imaged the LC3B-stained tumors with a confocal microscope and the representative images are shown in the new version of Figure 2F (now figure 2G).

4.Reviewer 1's comment 4: *NCOA4 is mainly degraded by macroautophagy, and BNIP3-mediated mitophagy is dependent on macroautophagy. Nevertheless, the amount of NCOA4 in ATG5-deleted cells (defective both mitophagy and NCOA4-turnover) was comparable to that in control cells (Fig. 5). Why?*

Answer. We appreciate the Reviewer #1's observation and it may have escaped to Reviewer's attention that this very relevant point was already addressed in our submitted manuscript: both in the results section (lines 327-338 of the original manuscript, now lines 361-373) and in the discussion section (lines 490-504 of the original manuscript, now lines 526-540), reporting data from the following references (Santana-Codina & Mancias, 2018; Mejlvang *et al*, 2018; Goodwin *et al*, 2017) also in the original manuscript.

As already commented above, NCOA4-mediated ferritinophagy is a very novel selective autophagy pathway. Our data shows that treatment with the lysosomal blocker BafA recovers NCOA4 levels and in the case of shBNIP3, NCOA4 is degraded in the lysosome to a higher extent than in shCntl and shATG5 conditions, suggesting that this effect might be independent of ATG5. Recent studies (that were mentioned and discussed in our submitted manuscript) have highlighted that NCOA4 can also be degraded through different autophagic pathways. Addressing why ATG5 is not involved in the NCOA4 turnover is a matter of future more mechanistic studies (as we state in the discussion), which goes beyond the main message of this manuscript, showing that HIF-1α is controlled by BNIP3 through pathways that are not phenocopied by ATG5.

5.Reviewer 1's comment 5: *In Fig. S2D, quantification is needed*

Answer. Following the Reviewer #1's kind suggestion, we incorporated the quantification (Figure EV1E of the revised manuscript) corresponding to figure S2D (now Figure EV1D of the revised manuscript).

Rebuttal and experimental plan with respect to Reviewer 2 comments:

6. Reviewer 2's initial comment: *In this manuscript, Vara-Perez et al. have investigated the role of BNIP3 in melanoma tumor cell growth. They provide evidence that increased BNIP3 expression correlates with poorer patient survival and that BNIP3 depletion decreases HIF-1 expression and HIF-dependent glycolysis via altered intracellular iron levels which impacts tumor growth. The authors conclude that their study identifies a new and unexpected role for BNIP3 in the regulation of HIF. A number of previous studies have identified a role for BNP3 in tumor progression and BNIP3 expression has previously been shown to be a heavily HIF-dependent. While there is a good case for investigating the interactions between BNIP3 and HIF-1alpha, their interaction in the context of cancer has been well studied detracting somewhat from the novelty of this study which lies mainly in the demonstration of BNIP3-dependent HIF activity.*

Answer. While we agree with the Reviewer 2's comment about existing literature on the contextual role of BNIP3 in cancer, we respectfully disagree with the view that this has been studied in the context of HIF-1α signaling. Clearly, BNIP3 is a known HIF-1α target, but whether and how HIF-1α and BNIP3 cooperate or crosstalk to control tumor growth is understudied**.** A recent report using a breast cancer model ((Chourasia *et al*, 2015), also cited in our submitted manuscript) investigated in great mechanistic details the role of BNIP3-HIF-1α axis and found that BNIP3 repressed HIF-1α pro-tumorigenic signaling (by inciting mitophagy and inhibition of glycolysis). Here, we provide compelling evidence that, BNIP3 by regulating ferritinophagy in normoxic melanoma cells, is upstream to HIF-1α signaling instead. Quite interestingly, these data are very much in line with reports in clinical samples that show that in breast cancer BNIP3 is downregulated and associated with poor prognosis (Chourasia *et al*, 2015; Vara-Perez *et al*, 2019) whereas in uveal melanoma (as reported in our introduction, lines 96-99, reference (Jiang *et al*, 2018)) as well as in our cutaneous melanoma cohorts, BNIP3 is upregulated and associated with poor prognosis. In fact, to our knowledge no study has shown that BNIP3 supports HIF-1α pro-tumorigenic activity by keeping PHD2 activity at bay. We also are not aware of other studies exploring the role of BNIP3-HIF-1α in melanoma growth *in vivo*.

7. Reviewer 2's comment 1: *In Figure 1C and 1D, how is the BNIP3 stained? What is the colour of the BNIP3 staining versus the background H&E stain? It is difficult to discern the BNIP3-specific staining on these tissues. Overall, figure 1 is quite descriptive and adds little in the way on new insight as the association of high BNIP3 levels and low cancer survival is known.*

Answer. For the procedure of staining, we refer to our methods section (lines 883-895 of the submitted manuscript, now lines 953-965 of the revised manuscript). As stated in the methods, these tissues have not been counterstained; therefore, everything highlighted as pink/red is BNIP3 specific staining.

Regarding the descriptiveness of the figure, other retrospective studies have shown similar procedures to investigate BNIP3 in cancer patient samples (3) and this TCGA analysis was also performed by Chourasia and co-workers in breast cancer patients, where a positive correlation between *BNIP3* levels and improved patient's survival was found ((Chourasia *et al*, 2015). To our knowledge, only one study has explored BNIP3 levels in a cutaneous melanoma cohort (Buart *et al*, 2017), as part of a hypoxic signature and related to PD-1 treatment in a small patient cohort (n=19). Therefore, we believe that the patient data that we show here is novel and relevant for melanoma, and further supports the contextual role of BNIP3 in different cancer types.

8. Reviewer 2's comment 2: *The effects of BNIP3 KD on tumor volume in Fig 2B is impressive. The authors do not address the apparently striking impact of BNIP3KD on the rate of tumor cell death (Fig 2D). This does not tally with a protective effect of BNIP3 KD in terms of tumor growth. Can the authors look at another measure of tumor cell apoptosis to further investigate significance of this.*

Answer. We appreciate the Reviewer #2's comment. In figure 2D we show that overall cell death (necrotic) area in shBNIP3 tumors is reduced. This is in line with the reports that high BNIP3 levels within the tumor, especially associated with hypoxia, promote cell death (Mellor & Harris, 2007) and therefore, removing BNIP3 from melanoma cells might have a small protective effect. When we performed the staining of cleaved Caspase 3 (a known apoptotic marker) in the tumor sections, we could observe a very subtle (yet statistically significant) increase in the number of positive cells per field (see Rebuttal Figure 3 for the Reviewer 2, below this answer). However, the drastic drop in cell proliferation seen in shBNIP3 tumors, from 64% to 25% as measured by Ki67 positive staining (Figure 2E of the revised manuscript, for which now we added a representative image, see figure 2F of the revised manuscript), points towards the drop in proliferation as the major cause behind the reduced ability of shBNIP3 cells to grow *in vivo*. Because of the very subtle changes in cleaved caspase 3 tumoral staining, we believe that the inclusion of these data in the main manuscript is of little added value, and hence we suggest to not add it.

Figure for referees removed

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9. Reviewer 2's comment 3: *The data presented in Figure 3A is largely confirmatory of previous work. Some of the metabolomic data is not very convincing. For example, the reported trend towards reduction in extracellular acidification rate in shBNIP3 cells reported in Figure 3D is not at all clearly different from control (even though an unlikely statistical significance is reported). The authors conclude from their BNIP3 KD experiments that BNIP3 promotes glycolysis. Is the converse true? Does overexpressing BNIP3 induce an increase in glycoloysis.*

Answer. We consider the Reviewer 2's remark. We respectfully disagree with the Reviewer comment that "*The data presented in Figure 3A is largely confirmatory of previous work".* The canonical pro-autophagic role of BNIP3 -as described in breast cancer- posits that BNIP3 by stimulating mitophagy keeps mitochondria-ROS levels down, thus impairing HIF-1α stabilization

and glycolysis -rather than promoting it- as we report here (Chourasia *et al*, 2015). Indeed, here we show that, when BNIP3 is silenced, mitochondria are forced to respire (even if unhealthily) because the glycolytic pathway is transcriptionally downregulated by the reduced levels of HIF-1α. We further discovered that this 'unconventional' role of BNIP3 is explained by the elevation in the Fe^{2+}/Fe^{3+} ratio of melanoma cells lacking BNIP3, which then fostered PHD2-mediated HIF-1α downregulation. To our knowledge, the ability of BNIP3 to maintain the free iron pool by interacting with the ferritinophagy receptor NCOA4 has never been reported before. We believe that the unprecedented mechanism through which BNIP3 controls melanoma growth, unraveled by our study, helps shedding new light on BNIP3- HIF-1α axis in cancer and delineates BNIP3 as a potential biomarker of early melanoma progression.

Following the Reviewer 2's kind suggestion, we repeated the Seahorse ECAR experiments to reduce the standard deviation among experiments (see updated Figure 3D in the revised manuscript). Moreover, and as already mentioned in the answer to Reviewer1's comment 2, we also performed additional Seahorse and GC-MS metabolomics experiments to be able to pull the GC-MS data in a single graph (see updated Figure 3D and 3H in the revised manuscript).

To address Reviewer's 2 question regarding BNIP3 overexpression, by using a lentiviral vector approach we introduced a murine (myc-tagged) BNIP3 full length (BNIP3-FL) construct in the BNIP3 silenced cells (see Figure S3A-B of the revised manuscript), resulting in a partial rescue of the endogenous BNIP3 protein levels. It should be noted that we did not pursue overexpression of BNIP3 in wild type cells as a strategy, since forcing BNIP3 expression to a higher extent has been shown to be lethal for the cells (Prabhakaran *et al*, 2007; Kanzawa *et al*, 2005), due to enforcing the BNIP3 pro-death activity.

We only succeeded in rescuing the endogenous level of BNIP3 to a partial extent (Figure S3A of the revised manuscript). The exact reasons for the lack of full recovery are not completely clear and could be due to residual activity of the shRNA against the mutated BNIP3-FL or to an increased susceptibility of the myc-tag BNIP3-FL mutant to proteasomal degradation, since BNIP3 steady state levels are controlled by both the lysosome (via mitophagy, figure EV2D of the revised manuscript) but also by the proteasome (Park *et al*, 2013) (Rebuttal figure 1D, above this answer). In spite of this, partial recovering of BNIP3 expression in the shBNIP3 cells proportionally rescued HIF-1α levels (by approx. 30%) and reduced its hydroxylation (Figure S3B of the revised manuscript), to a similar degree.

10.Reviewer 2's comment 4: *In Figure 4A, does BNIP3 KD also affect HIF-2alpha expression (the isoform more usually associated with cancer). The data in figure 4B should be quantified. Does BNIP3 overexpression enhance HIF-1/2 expression?*

Answer: Preliminary data in B16-F10 cells under basal conditions showed that knocking down BNIP3, but not ATG5, downregulates also HIF-2α protein levels (see Rebuttal Figure 4, below this answer). This effect is associated with a corresponding downregulation of cyclin D1 *in vitro* (Rebuttal Figure 4A), one of the HIF-2α main targets (Gordan *et al*, 2007). As in the case of HIF-1α, decreased protein levels of HIF-2α in BNIP3 silenced cells are not explained by an effect on *Hif2a* mRNA level, while the reduced protein levels of cyclin D1 are accompanied by a significant reduction in the transcript levels of this HIF-2α target, thus suggesting that BNIP3 loss also affect HIF-2α signaling (Rebuttal Figure 4B).

However, in contrast to HIF-1α, we did not find evidence for a decrease in the expression of the HIF-2α target cyclin D1 from tumor extracts (Rebuttal Figure 4C), suggesting that -at least *in vivo*- BNIP3 mainly affects HIF-1α signaling. We thus believe that showing these *in vitro* data in the main manuscript would distract from the main message of the manuscript.

Figure for referees removed

11.Reviewer 2's comment 5. *The data demonstrating increased HIF hydroxylation in BNIP3 depleted cells could be strengthened by for example using a hydroxylation (CO2-release) assay or mass spectrometry. Furthermore, the authors should demonstrate the effects of BNIP*

expression levels on HIF ubiquitination by the pVHL protein as this is key to it's post-translational stability.

Answer: We thank the Reviewer for his/her comment. We have indeed data confirming the classical pathway of PHD-mediated HIF-1α degradation through HIF-1α ubiquitination and proteasomal degradation, which we have now added to the main manuscript (see Figure S3E of the revised manuscript). We then as suggested also checked total pVHL levels (see Rebuttal Figure 5, below this answer). However, since no significant changes in pVHL levels could be observed and blocking the proteasome rescued HIF-1α but not its transcriptional activity (as shown by LDHA levels), we decided not to include it so that we would not overwhelm the reader with additional information.

Figure for referees removed

12.Reviewer 2's comment 6: *The authors focus on the impact of BNIP3 on iron content as a link to HIF-1alpha stabilization. In Figure 3C, knockdown of BNIP3 appears to increase OCR which would result in less oxygen being available in the cell which would favour HIF stabilization. Have the authors considered this as a possible contributory link between BNIP3 and HIF-1alpha stabilization (similar to the mechanism propose din Hagen et al, Science 2003).*

Answer: This is an interesting remark. However, in our model and despite the increased OCR, BNIP3 silenced cells display less HIF-1α stabilization/levels, not increased HIF-1α levels. Therefore, the proposed mechanism -which we are aware of- does not seem to explain the BNIP3 phenotype we observe in melanoma cells. BNIP3 silencing indeed increases OCR (while other studies show the opposite, (Chourasia *et al*, 2015)) and also increases PHD2 activity

thereby favoring HIF-1α destabilization, and both phenotypes have been linked in literature with an increase in intracellular iron (Walter *et al*, 2002), which we also have upon BNIP3 silencing.

13.Reviewer 2's Minor point 1: *In "the paper explained", the authors refer to HIF-1 as an oncoprotein. Many patients are receiving hydroxylase inhibitors which stabilize and activate the HIF` pathway, however there is no evidence for increased melanoma growth in these patients. It is possibly an oversimplification to render HIF an oncoprotein. The authors could consider this point.*

Answer: We thank the Reviewer for highlighting this point. It is true that HIF-1α has not been described as an initiating event in many cancer types (except in renal cancer, where it has been associated with a loss of pVHL), but several studies have correlated high HIF signaling with poor prognosis (Pezzuto & Carico, 2018; Schito & Semenza, 2016), therefore our use of 'oncoprotein' (especially in a section aimed to non-scientific public) but considering all the effects described for HIF-1α (as well comparing HIF-1α vs HIF-2α), we agree that it is definitely an oversimplification.

This revised manuscript, unless we are told otherwise by the editorial board, does not include "the paper explained" section because it was meant for *EMBO Molecular Medicine* and not for *The EMBO Journal*.

Rebuttal and experimental plan with respect to Reviewer 3 comments

14.Reviewer 3's comment 1: *In this manuscript the authors examine the role of BNIP3 in melanoma. BNIP3 is a protein associated with the mitochondrial outer membrane and functions as a cargo receptor for mitophagy. BNIP3 has been identified as having a role in the progression of various forms of cancers, presumably through it activity in promoting mitophagy. Multiple types of cancers have been found to depend on mitophage and autophagy to maintain their metabolism in changing cancer environments. Here the authors present evidence that higher levels of BNIP3 are associated with reduced survival time in patients with melanoma. Using in vitro and in vivo mouse models of melanoma, the authors show that tumor growth is specifically reduced when expression of BNIP3 is reduced and that depletion of BNIP3 is associated with metabolic changes and altered mitochondrial energy metabolism. Previous studies have shown that BNIP3 is both responsive to changes in HIF1a activity and promotes HIF1a activity that can account for the metabolic changes observed. Here the authors confirm that HIF1a levels and activity are reduced in melanoma cells depleted of BNIP3.*

Answer: To our knowledge this evidence has not been shown before, thus it cannot be confirmatory. As explained above (see the answer to the comment 6 from Reviewer 2), while there is existing literature on the contextual role of BNIP3 in cancer, we respectfully disagree with the view that this has been studied in the context of the HIF-1α signaling. Clearly, BNIP3 is a known HIF-1α target, but whether and how HIF-1α and BNIP3 cooperate or crosstalk to control tumor growth is understudied. A recent report using a breast cancer model (also cited in our submitted manuscript, (Chourasia *et al*, 2015) investigated in great mechanistic details the role of BNIP3-HIF-1α axis and found that BNIP3 repressed HIF-1α pro-tumorigenic signaling (by inciting mitophagy and inhibition of glycolysis). Here, we provide compelling evidence that, BNIP3 by regulating ferritinophagy in normoxic melanoma cells, is upstream to HIF-1α signaling instead. Quite interestingly, these data are very much in line with reports in clinical samples that show that in breast cancer BNIP3 is downregulated and associated with poor prognosis (Vara-Perez *et al*, 2019; Chourasia *et al*, 2015) whereas in uveal melanoma (as reported in our introduction, lines 96-99,(Jiang *et al*, 2018)) as well as in our cutaneous melanoma cohorts, BNIP3 is upregulated and associated with poor prognosis. In fact, to our knowledge no study has shown that BNIP3 supports HIF-1α pro-tumorigenic activity by keeping PHD2 activity at bay through a mechanism involving the intracellular iron pool. We also are not aware of other studies exploring the role of BNIP3-HIF-1α in melanoma growth *in vivo*.

15.Reviewer 3's comment 2: *These changes in HIF1a could be traced to increased activity of the prolyl hydroxylase (PHD2) that targets HIF1 for ubiquitin-mediated degradation. Evidence is presented examining the role of altered iron homeostasis to account for increased PHD activity. The question of how altered mitophagy could affect the turnover of HIF1a is interesting and highly relevant to cancer biology. Hypoxia and the accumulation of inhibitory substrates for PHDs have been implicated in HIF-mediated tumor formation because oxygen and the intermediate metabolite 2-oxoglutarate are required for PHD activity. Iron is also a required cofactor for PHD activity and the availability of cellular iron can affect the activity of PHD in cells and tissues in vivo. The evidence the authors present here for altered PHD activity is fairly persuasive and largely rests on the observation of reduced levels of HIFa in BNIP-depleted cells and tumor tissue. Although the authors try to assert that the relative amounts of hydroxylated HIF are increased, they don't really show this. Instead, the small amount of detectable OH-HIF that they detect doesn't really change, only the amount of total HIF. If the authors wanted to show more OH-HIF, they would need to block HIF degradation with a proteasome inhibitor or a VHL inhibitor. However, the use of mutant HIF1 that cannot be hydroxylated does tend to rescue the HIF phenotypes observed in their BNIP3-depleted cells.*

Answer: We thank the Reviewer for highlighting some critical findings of our study. Indeed, as the Reviewer mentions, the use of mutant HIF-1α that cannot be hydroxylated (HIF-1α-AA) rescues the HIF-1α phenotype observed in the BNIP3-depleted cells (data shown now in Figures 7 and EV5 of the revised manuscript), thereby supporting our proposed model that the effects of BNIP3 are mediated by changes in HIF-1α-OH both *in vitro* (rescue of the glycolysis) and most importantly *in vivo* (rescue of tumor growth). Moreover, data with the proteasomal inhibitor MG132 rescuing HIF-1α-OH and HIF-1α levels in BNIP3 silenced cells are available and have been added to the revised manuscript (Figure S3E).

16.Reviewer 3's comment 3: *The authors argue that increases in intracellular Fe(II) are responsible for an increase in PHD activity. This would suggest that there is a pool of un-metallated PHD in the cell. While NCOA4 levels do increase in BNIP3-depleted cells, the authors have not shown how this could affect iron.*

Answer: We respectfully disagree with the Reviewer's comment. In the original manuscript, Figures 5F, I and J (now figures 6A, EV4J and 6F of the revised manuscript) already showed that silencing NCOA4 in BNIP3 compromised cells reduces the free iron (Fe^{2+}) pool to levels of the control cells. Perhaps this critical experiment escaped the Reviewer's attention. Moreover, in this revised version of our study, we have added more compelling data using the very

sensitive high-end CE-ICP-MS analysis (Michalke *et al*, 2019). This quantitative analysis, allowing the determination of the Fe^{2+}/Fe^{3+} speciation analysis, clearly showed that the Fe^{2+}/Fe^{3+} ratio is dramatically elevated in BNIP3 silenced cells (but not after ATG5 silencing, see figure 6B and Table S4 of the revised manuscript). Moreover, this elevation in Fe^{2+}/Fe^{3+} ratio is completely abolished by the knock down of NCOA4 in the BNIP3 depleted cells (see Figure 6E and Table S5 of the revised manuscript). These new results, together with the co-IP revealing the interaction between BNIP3 and NCOA4 (originally Figure 5H, now figure 6D of the revised manuscript), are not only in line with our previous data using the Fe^{2+} specific dye FerroOrange (now Figures 6A and EV4J of the revised manuscript), but further proves that the effects of BNIP3 on the intracellular iron pool are strongly dependent of NCOA4-mediated ferriitinophagy.

17.Reviewer 3's comment 4: *NCOA4 directs ferritin to the lysosome for turnover. That does not necessarily equate with elevated cytosolic iron. The authors present no data regarding ferritin levels, which would surely be affected by alterations in NCOA4 that are great enough to produce elevated intracellular iron. What happens to ferritin?*

Answer. We thank the reviewer for this question. We checked the levels of ferritin light chain (FTL) and, upon BafA treatment (see Figure 6C of the revised manuscript), we can also see a higher rescue for FTL in the shBNIP3 condition, in line with the NCOA4 data, hereby supporting our idea that shBNIP3 promotes ferritinophagy. It should be noted that ferritin can also be degraded by the proteasome (De Domenico *et al*, 2006; Gammella *et al*, 2020; Du *et al*, 2019).The fact that FTL levels are lower upon BafA than in the untreated condition can be explained by studies showing that upon inhibition of lysosomal degradation by BafA, ferritin is directed towards the proteasome for degradation (De Domenico *et al*, 2006; Gammella *et al*, 2020; Du *et al*, 2019), which adds additional levels of complexity to this loop. As already mentioned, the FTL data has been incorporated to the main manuscript (Figure 6C, Table S2) and the main text has been adapted accordingly (lines 361-373).

18.Reviewer 3's comment 5: *Can these effects on PHD be mimicked by simply adding iron to the culture medium or animal chow? The ferroOrange reagent needs some additional controls before these data regarding iron can be reliably interpreted. Furthermore, it is more likely that only cytosolic iron (II), rather than total intracellular iron, is changed with BNIP3 knockdown. But the authors have not measured this.*

Answer: We thank the reviewer for his/her constructive comments. However, adding iron would not reveal further the BNIP3-PHD2 link since BNIP3 compromised cells already display higher intracellular iron. Adding iron to shCntl cells will probably affect PHD2 activity (and mitochondria energetics as well) but would not tell us more on the mechanism mediated by BNIP3.

As mentioned above in answer to the comment 3 of the Reviewer, we have added more compelling data using the very sensitive high-end CE-ICP-MS analysis (Michalke *et al*, 2019) which showed that the Fe^{2+}/Fe^{3+} ratio is dramatically elevated in BNIP3 silenced cells (but not after ATG5 silencing, figure 6B and Table S4 of the revised manuscript) and this elevation is completely abolished by the knock down of NCOA4 in the BNIP3 depleted cells (see Figure 6E and Table S5 of the revised manuscript). This is in line with our previous data using FerroOrange, which only binds Fe^{2+} (see Figures 6A and EV4J from the revised manuscript).

19.Reviewer 3's comment 6: *What remains poorly addressed is why the levels of PHD2 increase in BNIP-depleted cells. This effect is clear in Fig. 5A, but less so in Fig. 5E. This effect may have nothing to do with iron availability, but instead reflect a more direct effect on PHD turnover.*

Answer: The effect of BNIP3 on PHD2 levels seems to be exerted at the transcriptional level (see figure EV4A of the revised manuscript) and PHD2 remained unaffected after proteasomal inhibition (see Rebuttal Figure 6, below this answer). While PHD2 has been reported as a HIF-1α target (Meneses & Wielockx, 2016), in our cancer model it is unlikely that this transcription is driven by HIF-1α itself, since HIF-1α is downregulated and HIF-1α reintroduction did not affect PHD2 levels (figure EV5C). It remains thus unclear which alternative transcriptional mechanism underlies this effect.

Figure for referees removed

20.***Reviewer 3's comment 7:*** *In short, the evidence that intracellular iron is increasing the activity of PHD is weak, although the evidence that HIF reduced in clear and the change in PHD activity is a reasonable explanation, although not directly demonstrated.*

Answer: As already commented, the knockdown of NCOA4 recovered iron levels in BNIP3 compromised cells and partially rescued HIF-1α levels (Figures 5I-J of the submitted manuscript, now Figures 6E-F, EV4J and table S4 of the revised manuscript). Moreover, we also showed that knockdown of PHD2 recovers HIF-1α levels and transcriptional activity (Figures 5E and S6B of the submitted manuscript, now Figures 5F and EV4B of the revised manuscript). In the light of all these data, we propose that the effects of BNIP3 silencing on HIF-1α are driven by a combination of PHD2 upregulation and the increase in the Fe^{2+} levels. We apologize if this was not clear enough in the main manuscript.

21.Reviewer 3's minor comment 1: *Why is LC3-II so abundant after BNIP depletion in Fig. 2F, there is much less LC3-II in the images in Fig. 3A?*

Answer. This is an interesting point and we thank the Reviewer for raising this question. Because of the accumulation of LC3B-I and LC3B-II in the shBNIP3 condition, we suspected that LC3B upregulation was elevated at the transcriptional level. Indeed, we confirmed transcriptional upregulation of the autophagy mediators LC3B (*Map1lc3b*) and p62 (*Sqstm*) in the shBNIP3 condition *in vitro* (Figure EV1G of the revised manuscript) and *in vivo* (Figure 2H of the revised manuscript), thereby sustaining the higher autophagy levels observed in the shBNIP3 cells. Since both LC3B and p62 have been described as targets of TFEB in murine cells (Wang *et al*, 2019), we also checked TFEB protein levels and indeed, higher TFEB protein levels were also observed in shBNIP3 cells *in vitro* (Figure EV1H of the revised manuscript). The transcriptional upregulation of LC3B easily explains the differences in puncta number between Figure 3A and Figure 2F (now Figure 2G in the revised manuscript), since Figure 3A was performed with a GFP-LC3 plasmid whereas the tissue staining was performed through antibody-based immunohistochemistry, as detailed in the methods section. As a technical note, we tried the same antibody in immunocytochemistry for the colocalization with unsuccessful

results. For this reason, we decided to use the tagged plasmid for the colocalization (Figure 3A). While the tissue staining in Figure 2F (now Figure 2G) displays the endogenous LC3B levels in the three conditions, the expression of GFP-LC3 through a plasmid is driven by a constitutive promotor sequence that will not reflect changes in endogenous levels of LC3B. It should also be noted that, because of the transient transfection, the plasmid might not be always expressed to the same extent in all the cells, causing variability in the absolute number of autophagosomes: for this reason, we express our colocalization results as a percentage of autophagosomes colocalizing and not absolute numbers of autophagosomes.

Regarding the size of the autophagosomes, we thank the Reviewer 's for the remark. The differences between the two images are the result of the different microscopes used for their imaging: Figure 3A represents one plane of a z-stack confocal image whereas the original Figure 2F is standard widefield fluorescence microscopy. To address this concern, we imaged the LC3B-stained tumors with a confocal microscope and the representative images are shown in the new version of Figure 2F (now Figure 2G).

22.Reviewer 3's minor comment 2: *The increase in NCOA4 after bafilomycin treatment is clear, but why does it go up more in baf-treated BNIP knockdown? Is BNIP sending NCOA4 to the proteasome? Would proteasome inhibition make any of this clearer?*

Answer: The increase in NCOA4 seen in the BNIP3 knockdown is indicative of an increased ferritinophagy flux. We believe that BNIP3, through NCOA4 binding, limits NCOA4 engagement in ferritinophagy. When BNIP3 is absent (shBNIP3 condition), then NCOA4 is more available to engage in ferritinophagy. Moreover, following the reviewer's suggestion, we checked NCOA4 protein levels after a 6h treatment with the proteasomal inhibitor MG132 (Rebuttal Figure 7, see below this answer) and we only detect a subtle NCOA4 accumulation in the presence of the proteasomal inhibitor. Of note, NCOA4 levels are very low under basal conditions in B16 cells, being this the reason why a BafA treated lysate was included in our IP blot as a reference (see figure 6D of the revised manuscript). Together with our data after BafA treatment (Figure 6D, EV4H and 7E of the revised manuscript), we can state that NCOA4 is preferentially degraded by the lysosome and not by the proteasome in the B16 cells under normoxic conditions.

Figure for referees removed

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2nd Revision - Editorial Decision 11th Feb 2021

Dear Patrizia,

Thank you for submitting your revised manuscript (EMBOJ-2020-106214R) to The EMBO Journal. Please accept my sincere apologies for the unusual protraction due to delayed reviewer input during re-review as well as detailed discussions here in the team. Your amended study was sent back to the referees, and we have received comments from one of them, which I enclose below. The other referees were despite repeatedly promised reports in the end not able to provide feedback at this time due to other obligations. Please note however that we have carefully evaluated your response to their critique and found the issues raised to be satisfactorily addressed.

As you will see referee #3 stated that the manuscript has been significantly improved, and that s/he is now broadly in favour of publication.

As detailed earlier, while per se well taken, we concur that further mechanistic interrogation of the molecular link between BNIP3 and NCOA4 is beyond the scope of the current study.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

We still need you to take care of a number of points related to formatting and data representation as detailed below, which should be addressed at re-submission.

Further, I will share additional changes and comments from our production team during the next days to be considered.

Please contact me at any time if you have additional questions related to below points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal.
I look forward to your adjusted manuscript files.

Again, we are happy to swiftly move forward with acceptance of this work upon re-submission. Please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

>> Please rename the 'Competing interests' section to 'Conflict of Interest'.

>> Recheck callouts and their correct order in the main text for Fig. 2I.

>> Rename the 'Supplementary Materials' file to 'Appendix' ; rename figures and tables to 'Appendix Figure S1, S2...', 'Appendix Table S1, S2...', and adjust the callouts nomenclature throughout legends and manuscript text. Add tables directly to the appendix PDF.

>> Please remove figures from the manuscript.

>> Omit the 'EV materials' and 'Supplementary Materials' lists from the manuscript.

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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 11th May 2021.

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Referee #3:

Knockdown of BNIP3 show increase in FtnL consistent with increased ferritin synthesis in the setting of elevated cytosolic Fe (II) and reduced lysosomal turnover. This would tend to support the authors' hypothesis and other direct measurements of Fe(II). The authors do not consider that the cell-autonomous iron regulatory system is also in operation, complicating interpretation of these data. Prolonged lysosomal/endosomal inhibition with BafA typically causes cytosolic iron deficiency due to reduced iron uptake as well as impaired ferritin turnover. See" Impaired lysosomal acidification triggers iron deficiency and inflammation in vivo" eLife. 2019; 8: e51031.

After considering the additional data and the responses of the authors, my concerns about this manuscript have largely been addressed.

I would have liked to see more controls related to the use of Ferroorange, as it is a fairly new and untested reagent, but there are so many data in this paper that I think it should be published as is.

3rd Authors' Response to Reviewers 23rd Feb 2021

The authors performed the requested editorial changes.

3rd Revision - Editorial Decision 24th Feb 2021

Dear Patrizia,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. As discussed before, I kindly ask you to confirm that the additional reviewer figures should be excluded from this file.

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On a different note, I would like to alert you that EMBO Press is currently developing a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work. This has proven to offer a nice opportunity for exposure i.p. for the first author of the study. Please see the following link for representative examples:
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Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

If you have any questions, please do not hesitate to call or email the Editorial Office.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

Kind regards,

Daniel

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Corresponding Author Name: Patrizia Agostinis

Journal Submitted to: The EMBO Journal

Manuscript Number: EMBOJ-2020-106214-T

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For human data, depended on availability. For mice experiments, at least 4 mice per condition. For in vitro experiments, at least three biologically independent experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For mice experiments, at least 4 mice per condition.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Exclusion criteria followed the ethical guidelines of our institution: if the animal had to be sacrificed because of a humane endpoint (for example, lost more than 10% of body weight) that was not reaching 1000m3 of tumor size, then it was automatically excluded of the experiment and not further considered.
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.	All the animals received the same treatment, independently of the cells injected, to minimize bias.
For animal studies, include a statement about randomization even if no randomization was used.	For this animal study, no randomization was used.
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.	No.
4.b. For animal studies, include a statement about blinding even if no blinding was done	For this animal study, no blinding was used.
5. For every figure, are statistical tests justified as appropriate?	Yes, as indicated in the corresponding figure legends and the subsection "Statistics" in the section Materials and Methods.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, as indicated in the corresponding figure legends and the subsection "Statistics" in the section Materials and Methods.

Is there an estimate of variation within each group of data?	Yes, as indicated in the corresponding figure legends and the subsection "Statistics" in the section Materials and Methods.
Is the variance similar between the groups that are being statistically compared?	Yes, as indicated in the corresponding figure legends and the subsection "Statistics" in the section Materials and Methods.

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).	For all the antibodies, catalog numbers and dilution factors are provided in the Materials and Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Melanoma cell line B16-F10 (murine) and WM35 (human) were obtained from ATCC. WM35 was validated by STR profiling. Murine cell lines cannot be reliably validated by STR profiling at this moment. Patient-derived early stage human melanoma cell line 530 was a kind gift from Dr. G. N. P. van Muijen (Radboud University, The Netherlands).

* 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.	Species, strain, gender, age, source, housing and husbandry conditions of the animals used in this publication are provided in the subsection "Mice experiments" of the section Materials and Methods.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal procedures were approved by the Institutional Animal Care and Research Advisory Committee of the KU Leuven (project ECD P237/2015) and performed in accordance with the institutional and national guidelines and regulations.
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.	Yes, we have followed the ARRIVE guidelines where applicable.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	TMA: The study was approved by the Medical Ethics Committee of the University Hospital of Leuven (project number S58320).
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.	TMA: Biopsies from benign nevi, primary and metastatic melanoma were collected at the Unversity Hospital of Leuven with patient's informed consent, in accordance with the Declaration of Helsinki and with the approval of the Medical Ethics Committee of the University Hospital.
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.	TCGA:From the original 469 SKCM cases, 15 samples were discarded because of lack of available data and one was discarded due to data disparity, resulting in 453 cases. TMA: From the original 181 cases, 16 did not have tissue available and other 7 did not have complete survival data. These 23 cases were excluded from the analysis, resulting in 158 cases.
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.	Yes, we have followed the REMARK guidelines where applicable.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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	NA
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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
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G- Dual use research of concern

22. 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