EMBO reports

Manuscript EMBOR-2011-35430

Loss of autophagy in hypothalamic POMC neurons impairs lipolysis

Susmita Kaushik, Esperanza Arias, Hyokjoon Kwon, Nuria Martinez Lopez, Diana Athonvarangkul, Srabani Sahu, Gary J. Schwartz, Jeffrey E. Pessin and Rajat Singh

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Review timeline:

Submission date:	20 September 2011
Editorial Decision:	18 October 2011
Revision received:	06 December 2011
Correspondence:	14 December 2011
Editorial Decision:	15 December 2011
Revision received:	16 December 2011
Accepted:	16 December 2011

Transaction Report:

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

1st Editorial Decision 18 October 2011

Thank you for your patience while your study has been under peer-review at EMBO reports. We have now received reports from three referees that were asked to evaluate your study, which you will find at the end of this email. Please note that there is no referee 1; there was an error in our system and thus there were numbered 2-4. As you will see, all the referees find the topic of interest and referee 2 asks just for minor modifications. However, referee 1 brings up very similar points to those raised previously and referee 3, although very supportive of the study, also considers that it needs further work to become acceptable.

I feel that the three issues brought up by referee 3 (regarding quantitation throughout the study, the quality of LC3 blots and measure of p62 flux) should be addressed. Regarding the issues brought up by reviewer 1, as I mentioned when you first approached me, they seem relevant to the conclusiveness of the study. As you were already working on solving these issues, you probably will be in a position to address them. Referee 1's point 2 would not be crucial to publication in EMBO reports, but it would be important to clarify points 1 (the nature of the stained structures, why they differ, and the possible bleedthrough of the fluorescent signal), 3 and 4. Point 5 seems redundant with 1, and points 6-7 go in the direction of referee 3's request for a more thorough quantification. Do let me know where you stand on these issues, particularly if the ones brought up by referee 1 are a problem, and we can decide how to proceed.

If the referee concerns can be adequately addressed, we would be happy to accept your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of

revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

I hope the revision does not seem too problematic and look forward to seeing a revised form of your manuscript when it is ready. In the meantime, do not hesitate to get in touch with me if I can be of any assistance.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:
Referee #2:

Dr. Kaushik and colleagues used the Cre/loxP system to delete Atg7 from POMC cells in mice. They report that loss of ATG7 from POMC cells causes altered glucose/insulin homeostasis and increased visceral adiposity owing, in part, to reduced adipose tissue lipolysis. The results are potentially important as they identify a novel pathway by which POMC neurons control energy balance. This reviewer raises the following criticisms:

1) Authors suggest that autophagy is lost in POMC neurons of mutant mice and convey this concept throughout the narrative. However, evidence supporting this contention is lacking. Data shown in Fig. 1C are provided to support authors' claim that "loss of Atg7 led to reduced functional autophagy as reflected by increased accumulation of the autophagy substrate p62 within POMC neurons". Unfortunately, these data are not convincing because a) POMC neurons cannot be visualized: what does the punctuated green staining represent? Does it represent axonal terminals? Are these fibers of passage?, b) the morphological feature of whatever is stained in green is obviously different between the upper (control, punctuated pattern) and lower (KO, pseudo-circular pattern) panels - why? c) it looks like that the yellow and green have exactley the same pattern suggesting that whatever the nature of the fluorescent signal may be it passes through the 2 different color filters used (it is very unlikely that both POMC and p62 have identical cellular distribution). What are the evidences demonstrating that the antisera used to visualize POMC and p62 are specific to those 2 proteins? Authors must clarify these serious issues.
2) POMC cells include pituitary corticotrophs. Thus it is important to evaluate if cortiocosterone levels are altered/normal in mutants because the consequences of loss of Atg7 in pituitary POMC cells on energy and glucose balance need to be evaluated. Data shown in Fig. S1 are really not convincing because in rodents corticosterone (not cortisol as the authors write; indeed cortisol is made by humans) levels are typically 3-fold higher during the PMs compared to the AMs periods. Here, authors show almost the opposite. Maybe the AMs samples were collected from stressed mice? Thus, this reviewer is not convinced that the possibility that the phenotypes displayed by mutants have a pituitary origin is ruled out.
3) How authors can distinguish ACTH from alpha-MSH?
4) How authors validated the western blot results using the ACTH antisera as this reagent seems not to be guaranteed for mouse use?
5) Data shown in Fig. 1E: I cannot see POMC neurons - what does the punctuated green staining represent?
6) Please, provide the percentage of POMC neurons that express Cre in controls and mutants?
7) Please, provide the percentage of POMC neurons that express ATG7 in controls and mutants?

Referee #3:

Kaushik and colleagues present inriguing evidence that autophagy in POMC neurons maybe required for metabolic effects in the periphery. Alpha-MSH levels seem to mediate these effects. Of course, e blackbox remains: How does Alpha-MSH mechanistically trigger the observed metabolic effects? Is it secreted or not? And if not, why? Nonetheless, the paper contains suficent interesting and surprising data to be published, in case the authors are willing to take into consideration the following points:

-It should be made clear in the abstract, that a quite unvonvetional form of autophagy is probably responsable for the observed effects.

-Please speculate at the end of the paper where alpha-MSH is located and how this localisation matches with the observed effects. In other words. Suggest a mechanism.

-A huge number of data is not shown but could be shown within the supplemental part. In particular the enhanced triglyceride content and decreased macrophage proportion of the KO mice

Referee #4:

This paper studies a mouse model where the key autophagy gene Atg7 is conditionally deleted in POMC neurons. This causes decreased a-MSH levels due to impaired processing of POMC preprotein. This leads to expected metabolic abnormalities. The interesting additional observation is that the autophagy deficiency in the POMC neurons and the associated abnormalities seem also to be seen in aged normal mice.

In general the study has been performed well. The obvious conceptual gap is to address how autophagy modulates a-MSH production. Is this direct or indirect? However, I would not consider this a showstopper for this paper, as there are plenty of other interesting data.

I think there are two areas that could be improved from a technical perspective:
1. Sometimes the data in micrographs and gels are not quantitated. This should be addressed where relevant - e.g. 1C, s2G, much of S2 etc.
2. For me, a key set of data are those showing decreased autophagy with age. While people often say this is the case in the literature, this paper has the potential to provide the most robust data for this assertion in the brain. The authors will know that Atg7 levels in themselves are not a marker for autophagy although this is interesting data.

The LC3 blots are not convincing. There is very little effect of the lysosomal inhibitors on LC3-II levels in all cases except for the first 3 month pair. Are the authors using sufficient concentrations of inhibitor? Maybe they should inhibit for longer? - even the second 3 month pair shows no effect. There appears to be no statistical difference between the 3 month and 22 month mice on the LC3 assay statistics. This makes the claim very fragile. I think the LC3 experiment needs to be redone and the authors need to show they are using "saturating" levels of inhibitors and show more convincing data and statistics. For me, clarification of this issue would really provide important data for the field that has until now largely claimed age effects on autophagy on the basis of uncertain assays - this group have the potential to sort the issue out at least for these neurons.

The p62 flux - Since the pool size of p62 is lower in older mice one cannot make direct comparisons by simply subtracting the levels in the presence of inhibitors from those in the absence of inhibitors. This is incorrect -one needs to calculate fractional clearance - in other words, what fraction of the pool that is affected by the inhibitors. (In other words, this is analogous to a half-life experiment where the total pools are different - if the the total pool in the younger mice is much larger that of the older mice, then the amount that the younger mice clear in a given time will be much greater than that cleared by the older mice even if the half-lives are identical.) I am not sure that the inhibitor approach will be most powerful - it may be better to use pulse-chase experiments with and without 3MA as well as inhibitors. Also, since the pool sizes of the p62 differ with age, there will be concerns about linearity of the blotting chemiluminescence response - this could be avoided if the authors had access to a Licor machine or something similar (or if they used radioactive secondary antibodies. At worst, they should try to examine different blot exposures so they can compare the younger and older samples at similar baseline exposure levels. One other concern - it is possible that the higher levels of p62 in the younger mice may result in more oligomerisation - this may affect the clearance kinetics and create situations where the p62 clearance (or the inverse as determined with inhibitors) deviates from first order assumptions.

1st Revision - authors' response 06 December 2011

Reply to Referees

Referee #1:

Dr. Kaushik and colleagues used the Cre/loxP system to delete Atg7 from POMC cells in mice. They report that loss of ATG7 from POMC cells causes altered glucose/insulin homeostasis and increased visceral adiposity owing, in part, to reduced adipose tissue lipolysis. The results are potentially important as they identify a novel pathway by which POMC neurons control energy balance. This reviewer raises the following criticisms:

We are extremely thankful to Referee #1 for his/her comments that our findings of loss of autophagy in POMC neurons in control of glucose homeostasis are potentially important and novel. We are also very thankful for his/her questions and viewpoints, which we feel has helped us prepare a better manuscript.

1) Authors suggest that autophagy is lost in POMC neurons of mutant mice and convey this concept throughout the narrative. However, evidence supporting this contention is lacking. Data shown in Fig. 1C are provided to support authors' claim that "loss of Atg7 led to reduced functional autophagy as reflected by increased accumulation of the autophagy substrate p62 within POMC neurons". Unfortunately, these data are not convincing because a) POMC neurons cannot be visualized: what does the punctuated green staining represent? Does it represent axonal terminals? Are these fibers of passage?, b) the morphological feature of whatever is stained in green is obviously different between the upper (control, punctuated pattern) and lower (KO, pseudo-circular pattern) panels - why? c) it looks like that the yellow and green have exactley the same pattern suggesting that whatever the nature of the fluorescent signal may be it passes through the 2 different color filters used (it is very unlikely that both POMC and p62 have identical cellular distribution). What are the evidences demonstrating that the antisera used to visualize POMC and p62 are specific to those 2 proteins? Authors must clarify these serious issues.

Answer for 1 a) and b): We thank the referee for these questions, and we do agree with the referee that these are important issues that need to be verified. While we cannot textually address these specific questions, we have repeated these experiments in new cohorts of control and KO mice and we feel confident that the better quality of the new images clearly shows reduced autophagy in POMC neurons from KO mice **(New Fig 1C)**. Please see below:

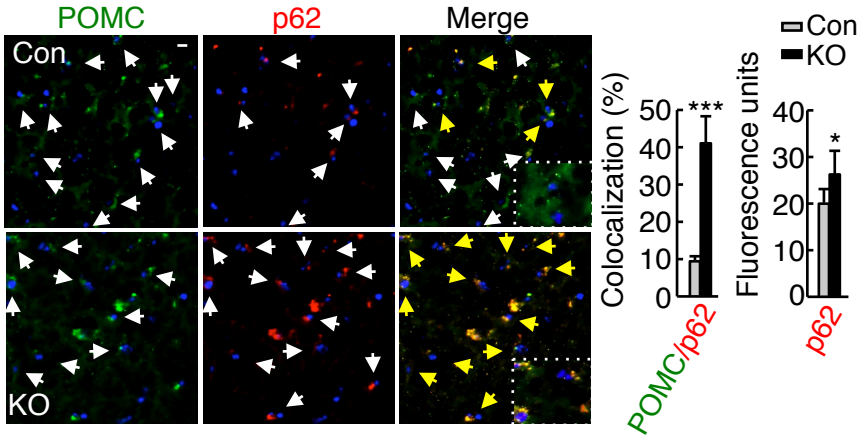


Fig. 1 (C) *Indirect immunofluorescence showing POMC (green) and p62 staining (red), and colocalization of POMC with p62 in MBH sections from Con and KO mice (n=6). Nuclei are in blue (DAPI). Scale insert: 10μm. White arrows indicate individual POMC, and p62 signals, and yellow arrows indicate merged signal. *p<0.05, ***p<0.001.*

Answer for 1 c): The quantifications for **new Fig 1C** reveal marked increases in colocalization of POMC with p62, and significantly raised p62 levels in POMC-positive cells from KO mice. These results are identical to those presented in the primary submission. Our arguments against antibody cross reactivity between POMC and p62 are as follows:

• The KO POMC neurons consistently display significantly higher POMC levels (please see Fig 1A with POMC quantification, Fig 1B, new Fig 1C, new Fig 1E, Fig 2I, and Fig 2G with POMC quantification) (please also see figure below). A second *expected* effect of loss autophagy is the accumulation of autophagy substrate, p62 that we also observe in KO POMC neurons **(New Fig 1C),** thus increases in both POMC and p62 within POMC neurons from KO mice appear to contribute to an intense merged signal. Furthermore, we would like to respectfully disagree with the assumption that POMC and p62 may not interact closely in the cell simply because there is no current data indicating the same. P62 is also a cellular adaptor molecule that regulates cellular signaling, e.g., ERK signaling, (EMBO Rep. 2010; 11(3): 226-32), and may regulate specific aspects of POMC processing, trafficking, etc, through close interactions with POMC and LC3-II (unpublished results from our lab).

• We have taken every effort to ensure that the antisera do not cross react in between these two proteins. We have verified these in control hypothalamic N41 cells and in those with an acute siRNA knockdown of atg7 (please see below), and these results from N41 cells not only support our *in vivo* findings **(new Fig 1C),** but also demonstrate the absence of nonspecific interactions. ***The results below are not included in the manuscript***:

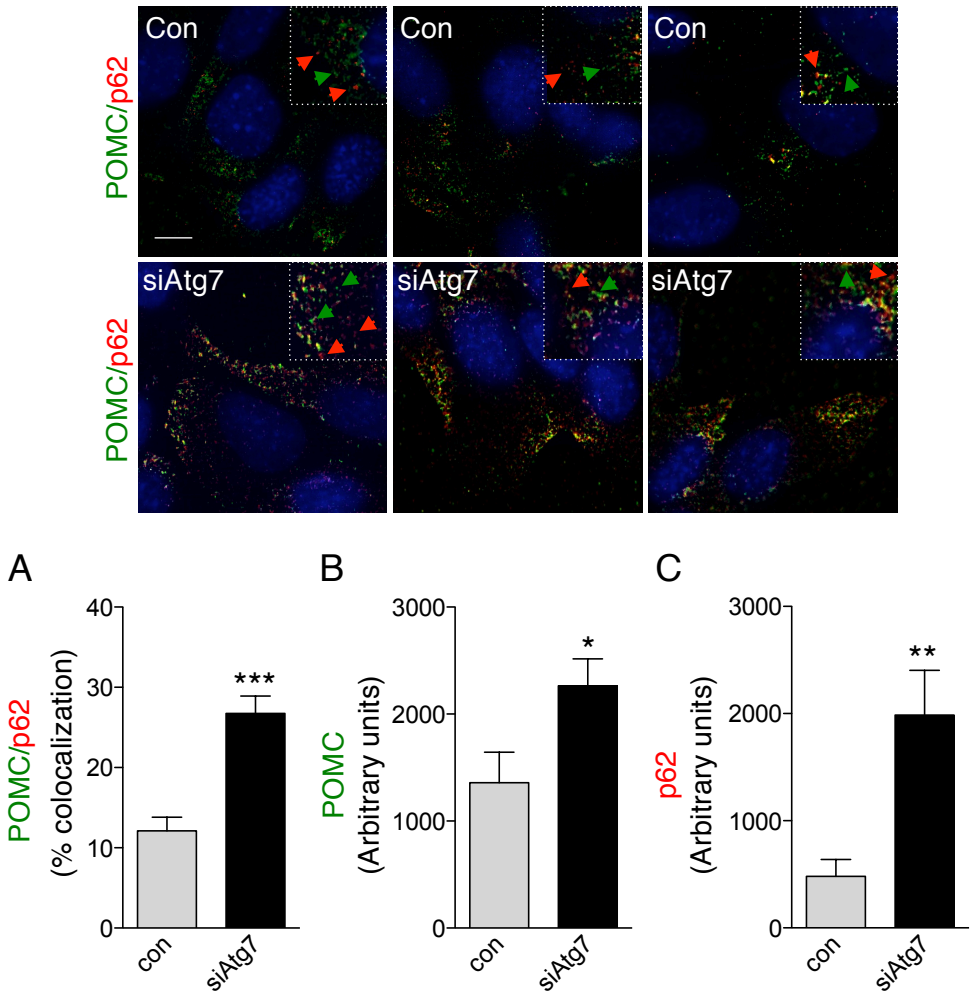


Figure Legend. *Indirect immunofluorescence showing colocalization of POMC (Green; green arrow) and p62 (Red; red arrow) in control hypothalamic N41 cells (Con) and in those silenced for Atg7 (siAtg7). Quantification indicates (A) % colocalization, and arbitrary values for (B) POMC and (C) p62. Three independent fields are shown. *p<0.5, **p<0.01, ***p<0.001. Results are from at least 20 independent fields each from 2 distinct experiments.*

• In addition, the details of the primary antibodies for POMC and p62 co-staining, as well as corresponding secondary antibodies (including their species of origin are included) for the referee to examine.

POMC: Goat primary
Anti-Goat secondary, (**Alexa Fluor® 488**, *chicken* anti-goat)

p62: Rabbit primary
Anti-Rabbit secondary, (**Alexa Fluor® 647**, *donkey anti-rabbit*)

Verified with:

POMC: Goat primary
Anti-Goat secondary, (**Alexa Fluor® 488**, *donkey anti-goat*)

p62: Rabbit primary
Anti-Rabbit secondary, (**Alexa Fluor® 555**, *donkey anti-rabbit*)

2) POMC cells include pituitary corticotrophs. Thus it is important to evaluate if cortiocosterone levels are altered/normal in mutants because the consequences of loss of Atg7 in pituitary POMC cells on energy and glucose balance need to be evaluated. Data shown in Fig. S1 are really not convincing because in rodents corticosterone (not cortisol as the authors write; indeed cortisol is made by humans) levels are typically 3-fold higher during the PMs compared to the AMs periods. Here, authors show almost the opposite. Maybe the AMs samples were collected from stressed mice? Thus, this reviewer is not convinced that the possibility that the phenotypes displayed by mutants have a pituitary origin is ruled out.

Answer for 2): We thank the reviewer for bringing this to our attention that we should mention corticosterone for rodents, and not cortisol (in humans). We have now repeated these experiments in fresh 8AM and 6PM plasma samples collected from new aged-matched groups consisting of 5 controls and 6 KO rodents using a new commercial EIA kit for corticosterone (Cayman Chemicals, catalog #500655), and we have presented this new data in **New Fig S1.** Our results reveal no difference in levels of corticosterone in Con and KO mice excluding defective corticotroph function as a reason for adiposity in the KO mice. The data is shown below:

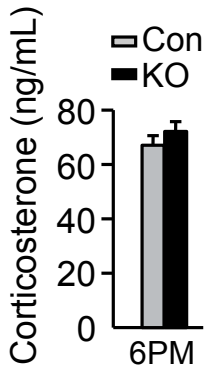


Figure Legend. *Corticosterone levels in serum from 6mo old RD-fed control (Con) and KO mice, collected at 6PM (n=5-6).*

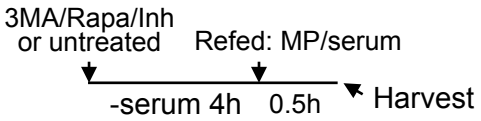
3) How authors can distinguish ACTH from alpha-MSH?

Please see below.

4) How authors validated the western blot results using the ACTH antisera as this reagent seems not to be guaranteed for mouse use?

Answer for 3) and 4): To demonstrate that antibodies for ACTH and α-MSH do not cross-react with each other, we used indirect immunofluorescence for ACTH and α-MSH in untreated hypothalamic N41 cells (Con), methylpyruvate (MP)-treated cells (MP), and in MP-treated cells pretreated with autophagy inhibitor 3-methyladenine (3MA), lysosomal inhibitor ammonium chloride/leupeptin (Inh), or the autophagy activator rapamycin (Rapa). The experiments were performed according to scheme detailed below **(Fig S2C in manuscript)**.

Scheme:



Our results clearly indicate (please see on page 5) predominantly distinct ACTH and α-MSH signals **(magnified inserts included)** in untreated controls (Con), MP-treated cells, and in MP-treated cells pretreated with autophagy inhibitors, with ~10% colocalization between ACTH/α-MSH under all of these conditions.

Additionally, we observed increased levels of ACTH, and reduced levels of α-MSH upon inhibiting autophagy with 3MA or Inh (please see data on the page 5). These results are consistent with N41 data in **Fig S2F and S2G,** and with *in vivo* data presented in **Fig 2G, 2I, and 2J.** In the event of a possible cross-reactivity between antibodies for ACTH and α-MSH, these increases in ACTH upon autophagy inhibition would have associated with similar increases in α-MSH. In contrast, and in agreement with our *in vivo* data (**Fig 2G, 2I, and 2J**), inhibiting autophagy in N41 cells remarkably reduces α-MSH levels, while ACTH accumulates. **Observing these dynamic changes in ACTH and α-MSH upon blocking autophagy would not have been possible with two cross-reacting antibodies.** The addition of rapamycin to MP-treated cells (MP+Rapa) remarkable increased (i) colocalization between ACTH/α-MSH, and (ii) localized ACTH and α-MSH to cellular projections. These results demonstrate that components of the autophagic machinery are required for α-MSH production, and suggest that autophagy may also be required to mobilize ACTH and α-MSH-containing vesicles to cellular projections.

Overall, these results not only confirm our present findings but also provide support that antibodies for ACTH and α-MSH do not cross-react with each other.

Figure follows:

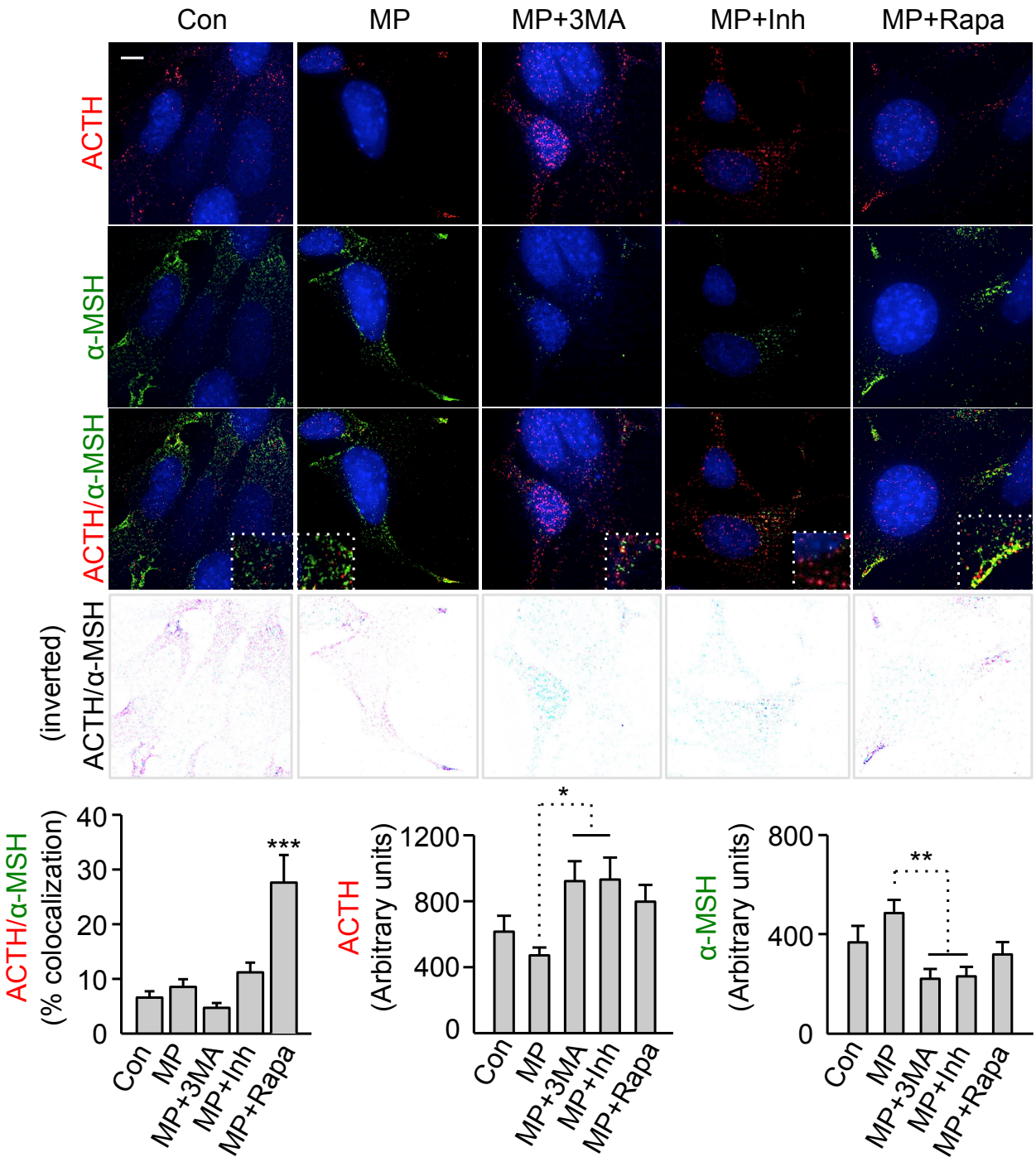
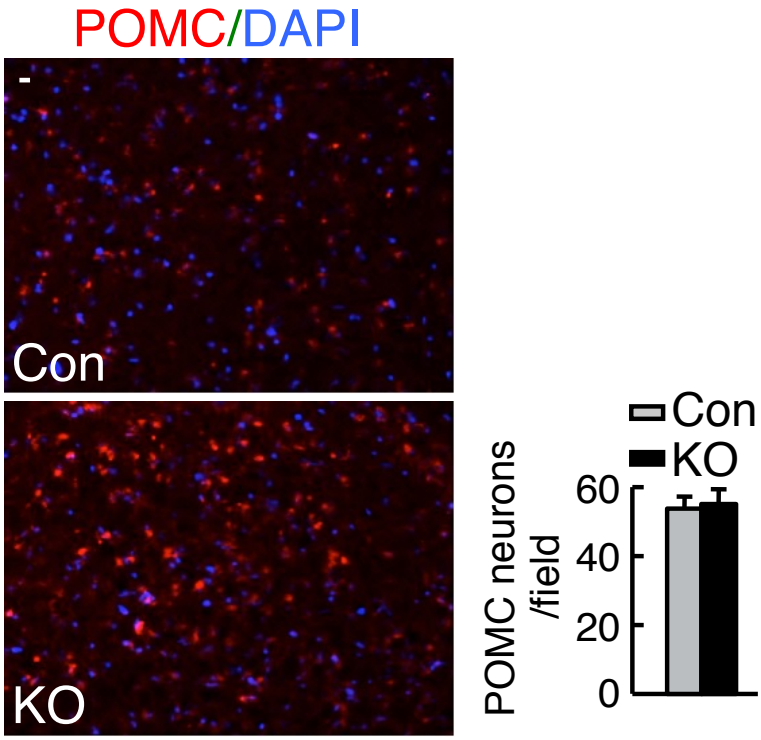


Figure Legend. *Indirect immunofluorescence for ACTH (Red) and α-MSH (green) in untreated N41 hypothalamic cells (Con), and in those treated with methylpyruvate (MP) or in MP-treated cells exposed to 3-methyladenine (MP+3MA), lysosomal inhibitors (MP+Inh), and rapamycin (MP+Rapa). Quantification indicates % colocalization between ACTH and α-MSH, and arbitrary fluorescence units for ACTH and α-MSH. *p<0.05, **p<0.01, ***p<0.001. Results are from at least 20 distinct fields from two independent experiments.* ***These images are not included in manuscript supplementary figures.***

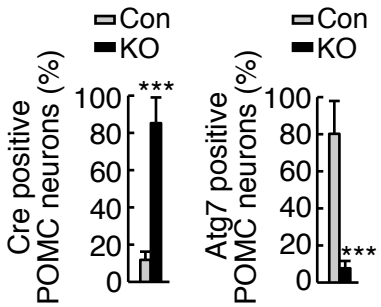
5) Data shown in Fig. 1E: I cannot see POMC neurons - what does the punctuated green staining represent?

Answer for 5): We thank the referee for his/her question. To satisfactorily address the referee's question we have repeated POMC immunostaining in new groups of control and KO mice, and we are confident of the enhanced quality of these new images that are now included in the revised manuscript **(New Fig 1E)** (please see below). We find no difference in the number of POMC-positive cells in between controls and KO rodents (**Alexa Fluor® 555**, donkey anti-goat). However, in the figure below KO mice display increases in POMC staining in agreement with results in the manuscript (please also see images for POMC in green in Fig 1A with POMC quantification, Fig 1B, new Fig 1C, new Fig 1E, Fig 2I, and immunoblots for POMC in Fig 2G with POMC quantification).



6) Please, provide the percentage of POMC neurons that express Cre in controls and mutants?

7) Please, provide the percentage of POMC neurons that express ATG7 in controls and mutants?



Answer for 6 and 7). We apologize to the referee for not having included this information in the primary submission.

% Control POMC neurons positive for Cre: 11.65+4%
% KO POMC neurons positive for Cre: 84.9%+18.2%

% Control POMC neurons positive for Atg7: 79.3+18.9%
% KO POMC neurons positive for Atg7: 7.4%+3.9%

Referee #2:

Kaushik and colleagues present inriguing evidence that autophagy in POMC neurons maybe required for metabolic effects in the periphery. Alpha-MSH levels seem to mediate these effects. Of course, e blackbox remains: How does Alpha-MSH mechanistically trigger the observed metabolic effects? Is it secreted or not? And if not, why? Nonetheless, the paper contains suficent interesting and surprising data to be published, in case the authors are willing to take into consideration the following points:

We are immensely grateful to Referee 2 for his/her comments that our findings are intriguing and of sufficient interest to merit publication. We are also thankful for these suggestions that will help us present a better manuscript. We have included the following changes as per the referee's advice.

-It should be made clear in the abstract, that a quite unvonvetional form of autophagy is probably responsable for the observed effects.

Answer for 1): It has been made clear in the abstract, and in the manuscript that an unconventional form of autophagy is probably responsible for these observed effects.

-Please speculate at the end of the paper where alpha-MSH is located and how this localisation matches with the observed effects. In other words. Suggest a mechanism.

Answer for 2): We have now speculated at the very end of the manuscript of what might be the possible mechanism by which autophagy controls POMC processing. To project these in a better manner, we have modified our model in Supplementary Figure 6.

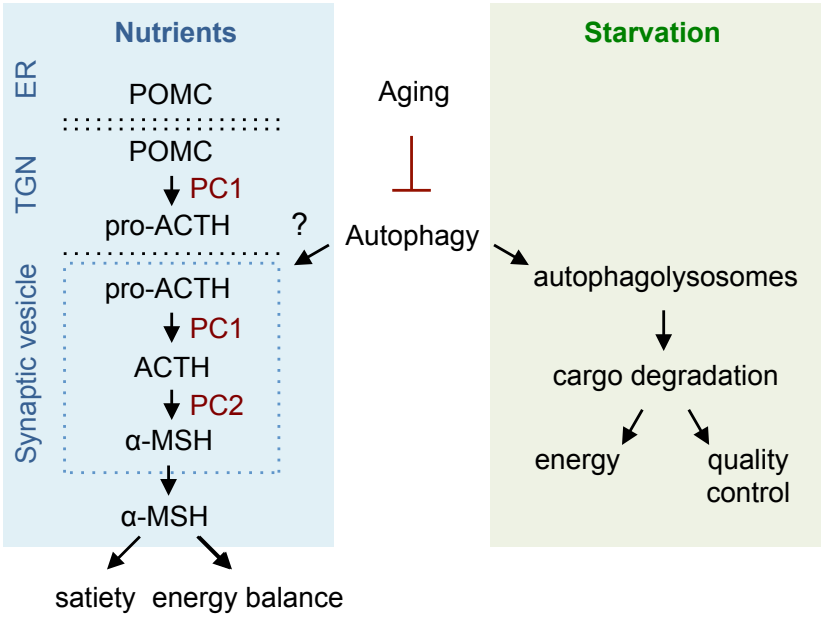


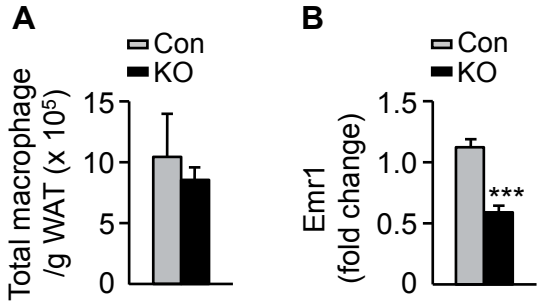
Fig S6 POMC precursor is processed to generate α-MSH via successive steps in the endoplasmic reticulum (ER), trans-golgi network (TGN) and in synaptic vesicles, and requires a number of peptidases, including proprotein convertases-1 (PC1), and -2 (PC2). Nutrient signals may initiate an unconventional form of autophagy in POMC neurons wherein autophagy components are functionally diverted toward POMC processing and α-MSH production, and possibly, α-MSH secretion to generate satiety, whereas starvation activates the canonical autophagy pathway resulting in turnover of cellular contents.

-A huge number of data is not shown but could be shown within the supplemental part. In particular the enhanced triglyceride content and decreased macrophage proportion of the KO mice.

Answer for 3): We thank the reviewer for raising this issue.

- We have now included the triglyceride data in the text on page 4 of the manuscript. The text now reads, "*KO mice displayed increased liver weights (Fig 2F) and triglyceride content (µg/mg tissue wt: Con, 19.3+/-4.6; KO, 35.5+/-8.3, n=6, p<0.05) suggesting aberrant hepatic lipid deposition*".

- Additionally, we have included the macrophage data that indicates reduced macrophage infiltration in adipose tissues from KO mice. This forms **new Fig S5A and S5B**. The text (page 6) now reads, "*Surprisingly, increases in the M1 macrophage proportion occurred despite reduced total amount of adipose tissue infiltrated macrophages (ATM) in the lipolysis-deficient KO mice (Fig S5A), as confirmed by decreased adipose expression of macrophage-specific gene Emr1 (Fig S5B)*". **New Fig S5A and S5B** are shown below:



Referee #3:

This paper studies a mouse model where the key autophagy gene Atg7 is conditionally deleted in POMC neurons. This causes decreased a-MSH levels due to impaired processing of POMC preprotein. This leads to expected metabolic abnormalities. The interesting additional observation is that the autophagy deficiency in the POMC neurons and the associated abnormalities seem also to be seen in aged normal mice.

In general the study has been performed well. The obvious conceptual gap is to address how autophagy modulates a-MSH production. Is this direct or indirect? However, I would not consider this a showstopper for this paper, as there are plenty of other interesting data.

We are immensely thankful to Referee 3 for his/her comments that our findings are of interest, in particular those related to aged mice. We are also thankful for his/her suggestions that will help us present a stronger manuscript. We have tried our best to incorporate all changes suggested by the referee.

I think there are two areas that could be improved from a technical perspective:
1. Sometimes the data in micrographs and gels are not quantitated. This should be addressed where relevant - e.g. 1C, s2G, much of S2 etc.

Answer for 1): We thank the reviewer for raising the issue of the missing quantifications. The missing quantifications for **Fig 1A, 1B, 1C,** and those for **Fig S2A, S2B, S2D, S2E, S2F,** and **S2G** have now been included.

2. For me, a key set of data are those showing decreased autophagy with age. While people often say this is the case in the literature, this paper has the potential to provide the most robust data for this assertion in the brain. The authors will know that Atg7 levels in themselves are not a marker for autophagy although this is interesting data.

The LC3 blots are not convincing. There is very little effect of the lysosomal inhibitors on LC3-II levels in all cases except for the first 3 month pair. Are the authors using sufficient concentrations of inhibitor? Maybe they should inhibit for longer? - even the second 3 month pair shows no effect. There appears to be no statistical difference between the 3 month and 22 month mice on the LC3 assay statistics. This makes the claim very fragile. I think the LC3 experiment needs to be redone and the authors need to show they are using "saturating" levels of inhibitors and show more convincing data and statistics. For me, clarification of this issue would really provide important data for the field that has until now largely claimed age effects on autophagy on the basis of uncertain assays - this group have the potential to sort the issue out at least for these neurons.

Answer for 2): We appreciate very much the words of Referee 3 that our group has the potential to sort the issue whether autophagy indeed decreases with age, at least in these neurons. We completely agree with the referee that this is an important issue, and we have felt very inspired to try our best to support our initial findings of reduced hypothalamic autophagy with age. We agree that the LC3 blots are not convincing, and therefore we have analyzed several more hypothalami from aged and young rodents for LC3-II flux. In fact, we have now performed a comprehensive in-depth analysis of autophagy activity in hypothalami from aged mice. We now include (i) better LC3-II blots and provide three different LC3-II-based quantification approaches to represent autophagy status in aged hypothalami, include (ii) NBR1 (neighbor of BRCA1) flux (please see details below) in aged hypothalami, and (iii) imaging data from mediobasal hypothalamic (MBH) sections of aged rodents to demonstrate remarkable accumulation of autophagy substrate p62 in POMC neurons with age, and (iv) net p62 flux, and steady state p62 levels in hypothalami from new cohorts of aging mice. We now feel confident that with the available resources at our hands, and with the currently available techniques to analyze autophagy flux *in vivo,* we have been able to demonstrate reduced hypothalamic autophagy, at least in POMC neurons, during aging.

i. As mentioned, we have now measured **(a)** steady state LC3-II levels from aged (22mo) and young (3mo) old rodents, which reflects total cellular autophagic vacuole content in a given moment, **(b)** "rate of autophagolysosome fusion", calculated by dividing lysosomal inhibitor-treated LC3-II by untreated LC3-II values, and **(c)** "Net LC3-II flux" by subtracting untreated LC3-II values from lysosomal inhibitor-treated LC3-II values. These results **(new Fig 5B and 5C)** reveal significantly reduced autophagosome content, and reduced autophagic flux in normally aged mice compared to young mice. Figures for LC3-II assays are shown below:

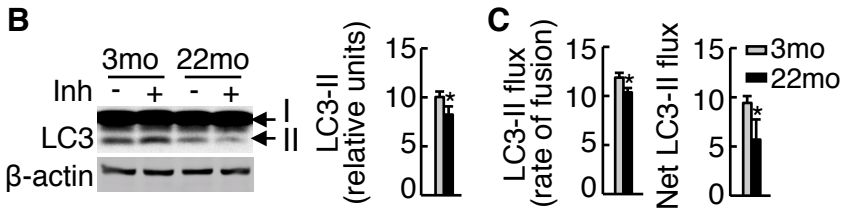


Figure Legend. (5B) *Steady-state LC3-II levels, and* ***(5C)*** *rate of autophagolysosome fusion (left), and net LC3-II flux (right) from hypothalamic explants of young (3mo) and aged (22mo) rodents cultured in presence or absence of lysosomal proteolysis inhibitors (Inh). *p<0.05 (n=11-13).*

ii. Next we measured NBR1 (neighbor of BRCA1) flux **(part of new Fig 5D)**, a recently elucidated autophagy adapter that gains access to lysosomes through its interaction with LC3 (Kirkin V et al. A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. *Mol Cell* 2009; 33**:** 505-516). Thus, reduced lysosomal accumulation of NBR1 in presence of lysosomal inhibitors in aged rodents supports our findings of reduced autophagy/LC3-II flux during aging. Indeed, our results show reduced NBR1 flux in aged rodents without differences in basal NBR1 levels in between young and old mice. Figures for NBR1 flux assays are shown below:

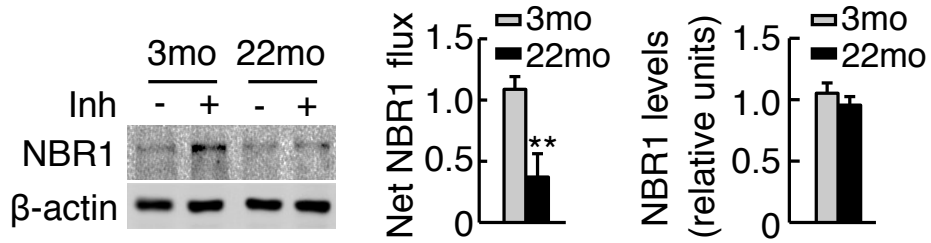


Figure Legend. *Immunoblots for NBR1 in hypothalamic explants from young (3mo) and aged (22mo) rodents cultured in presence or absence of lysosomal proteolysis inhibitors (Inh). Quantification for net NBR1 flux (left), and basal NBR1 levels (right) are shown. **p<0.01 (n=11-13).*

iii. Third, we present imaging data **(New Fig 5E)** in mediobasal hypothalamic (MBH) sections from aged rodents showing remarkable accumulation of autophagy substrate p62 in POMC neurons during aging. Additionally, aging-induced accumulation of p62 occurs, for most parts, in POMC neurons as indicated by increased colocalization of p62 with POMC **(New Fig 5E)**, and only modestly in AgRP neurons (preliminary unpublished data from ongoing studies – not shown). These suggest a selective sensitivity of POMC neurons to reduction of autophagy with age, which is consistent with speculations from others that POMC neurons may be more sensitive in general to dysfunction from reactive oxygen species (ROS, etc) as compared to AgRP/NPY neurons due to presence of ROS-scavenging mechanisms (UCP2) in AgRP neurons (Andrews et al. Nature. 2008; 454(7206): 846-51). Aged rodents with reduced autophagy also demonstrate increased POMC accumulation (please also see Fig 5F) consistent with our findings of increased POMC in MBH from KO mice (Fig 1A, Fig 1B, new Fig 1C, new Fig 1E, Fig 2I, and Fig 2G). Images, and quantification are shown below:

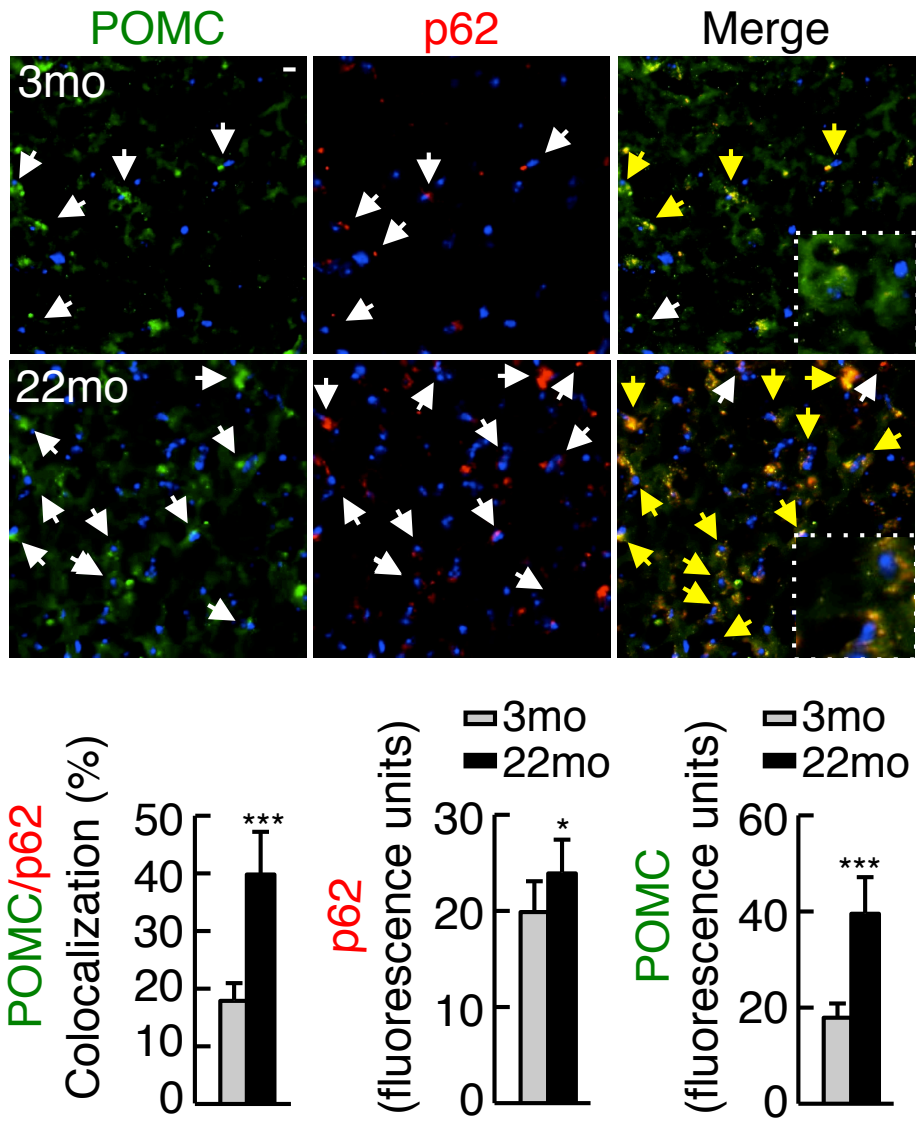


Figure Legend. *Immunofluorescence for POMC (green) and p62 staining (red), and colocalization of POMC with p62 in MBH sections from 3mo and 22mo mice (n=6). Nuclei are in blue (DAPI). Scale insert: 10μm. White arrows indicate individual channels, and yellow indicates merged signal. *p<0.05, ***p<0.001.*

The p62 flux - Since the pool size of p62 is lower in older mice one cannot make direct comparisons by simply subtracting the levels in the presence of inhibitors from those in the absence of inhibitors. This is incorrect -one needs to calculate fractional clearance - in other words, what fraction of the pool that is affected by the inhibitors. (In other words, this is analogous to a half-life experiment where the total pools are different - if the the total pool in the younger mice is much larger that of the older mice, then the amount that the younger mice clear in a given time will be much greater than that cleared by the older mice even if the half-lives are identical.) I am not sure that the inhibitor approach will be most powerful - it may be better to use pulse-chase experiments with and without 3MA as well as inhibitors. Also, since the pool sizes of the p62 differ with age, there will be concerns about linearity of the blotting chemiluminescence response - this could be avoided if the authors had access to a Licor machine or something similar (or if they used radioactive secondary antibodies. At worst, they should try to examine different blot exposures so they can compare the younger and older samples at similar

baseline exposure levels. One other concern - it is possible that the higher levels of p62 in the younger mice may result in more oligomerisation - this may affect the clearance kinetics and create situations where the p62 clearance (or the inverse as determined with inhibitors) deviates from first order assumptions.

Answer for 3):

In depth analyses that included analyzing additional hypothalamic samples from aged and younger mice has verified that aged mice have a very minor reduction, if any, in steady-state p62 levels **(new Fig 5D)**. We would understand the referee's concerns regarding the need for measuring fractional clearance in case steady state levels of p62 in aged mice were dramatically less compared to young mice. We also agree with the referee that pulse chase experiments with or without inhibitors would have been a useful second approach to verify results from the flux assays (as generally the case with experiments using cultured cells), and in fact we have successfully used that approach in cultured hypothalamic GT1-7 cells in an earlier publication (Kaushik et al. *Cell Metab* **14;**173-183:2011), however, we have been unable to standardize this pulse chase assay in tissue explants. Moreover, we cannot perform a pulse and chase experiment in cultured cells in the context of the present question since, at present, an aging cell culture model is not available. Unfortunately, the other approaches (radioactive secondaries or Licor) suggested by the reviewer could not be performed due to feasibility issues.

However, taking into account all of the biochemical (LC3-II, p62, NBR1) flux analyses, and the new imaging data **(New Fig 5E),** we feel very confident about our results that autophagy in the hypothalamus indeed decreases with aging. Below, we present updated p62 data **(part of new Fig 5D)** including the quantification for p62 flux, and for the steady-state p62 levels in young and old animals.

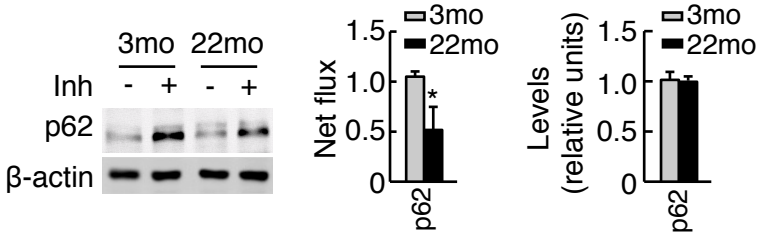


Figure Legends. *Net p62 flux (left), and steady state p62 levels (right) in hypothalamic explants from young (3mo) and aged (22mo) rodents cultured in presence or absence of lysosomal proteolysis inhibitors (Inh). *p<0.01 (n=11-13).*

Correspondence 14 December 2011

Just a short note to let you know, that I have now heard back from the referees that were asked to assess the revised version. They are supportive of publication and have no further comments. Thus, we will be happy to accept your study for publication in our March issue (online as soon as possible).

Tomorrow I will go through the piece in detail in preparation for acceptance and be in touch with more detailed instructions on how to proceed.

Yours sincerely,

Editor
EMBO reports

2nd Editorial Decision 15 December 2011

As I mentioned, we will now be happy to accept your study for publication, once a few minor issues have been attended to. In going through your file prior to acceptance I have noted a few minor things that need to be addressed, and I am therefore writing with an 'accept in principle' decision, which means that I will be happy to officially accept your manuscript for publication once they have been taken care of.

- I have noticed that a few figure legends are not complete with respect to statistical information. Most are, but the number of mice is missing from Fig 1 A and B, and the identity of the columns and error bars for the whole figure (mean +- SEM?). Likewise, some info is missing in the legend to Supp Fig 1, 2 and 5. Please ensure that all have the identity of the columns, error bars, the number of samples and the p values, if applicable.

- I feel that the part of the discussion regarding the unconventional protein secretion should cite all the related studies, as they are not that many and you will have enough space (see below comment on new reference format). The recent work of the Deretic and Virgin labs, the back-to-back study with the Malhotra one your cite from the Subramani lab, and the recent Malhotra study describing CUPS should all be included, in addition to the existing citation.

- Please merge all supplementary info (figures and legends) into one pdf file

- Please ensure that all subheadings are a maximum of 50 characters long

- As a standard procedure, we edit the title and abstract of manuscripts to make them more accessible to a general readership. In this case, I think the title is good and I have only done minor editing to the abstract. Please find the edited version at the end of this email and let me know if you do NOT agree with any of the changes. I feel that this new form of secretion cannot be called autophagy (as its name implies degradation) but it is mediated by compartments positive for some autophagy molecules.

In addition, your article is scheduled to appear in our March issue, which will be the first one to feature a new EMBO reports reference style. Please find attached the EndNote style file and instructions on how to install it below. Essentially, articles will be cited using numbers in the text, which will save a lot of characters. I am sorry for the inconvenience of having to reformat your references at this stage, but this will also allow you to slightly extend the discussion of unconventional secretion and incorporate the references mentioned above.

Thank you for your contribution to EMBO reports. I am happy to be the bearer of good news!

Yours sincerely,

Editor
EMBO Reports

2nd Revision - authors' response 16 December 2011

We have now submitted using the online system, a revised version of our manuscript entitled "**Loss of autophagy in hypothalamic POMC neurons impairs lipolysis**" for consideration for publication in *EMBO reports*. In this revised version, we have incorporated all of the changes that you have very kindly suggested. Briefly, we have:

1. Included all missing statistical information for Fig 1, Suppl Fig 1, 2 and 5, as well as ensured that all column identities, error bars, sample numbers and *P* values have been included.

2. Incorporated all of the references suggested, including 2 papers from Dr. Malhotra's lab, and 1 each from the laboratories of Dr. Deretic, Dr. Subramani, and Dr. Virgin.

3. Merged all supplementary information into 1 PDF file.

4. Shortened all subheadings (except 2) to less than 50 characters with spaces. Two subheadings are at 52 and 51 characters (with spaces) despite every attempt to shorten these.

Loss of POMC neuronal *atg7* impairs glucose tolerance (52 characters)

Aging reduces POMC neuronal autophagy and lipolysis (51 characters).

We are open to any suggestions from the Editorial office regarding this.

5. We agree with the Editor that this unconventional form of secretion may not be called autophagy, and we find that the edited abstract fits well with the contents on this manuscript. The changes suggested have been incorporated. We have followed this theme in other parts of the manuscript as well.

6. We have incorporated the new EMBO Reports reference style into this revised version.

Again, we thank you very much for your kind consideration of our work, and we do hope that you will now find our manuscript suitable for publication in *EMBO Reports*.

3rd Editorial Decision 16 December 2011

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

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

Yours sincerely,

EMBO Reports
Editorial office

