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Temporal orchestration of circadian autophagy rhythm by C/EBPβ

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Transaction Report:

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

1st Editorial Decision 01 April 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been now been evaluated by three referees and I enclose their reports below. As you will see the referees provide mixed recommendations although they find the links between C/EBPbeta, autophagy and the clock to be potentially interesting. They raise a number of significant concerns that need to be experimentally addressed before a revised manuscript can be considered for The EMBO Journal. The referees seem to be in agreement with concerns that there is currently insufficient evidence that autophagy is rhythmic and synchronized, as a consequence they require that this analysis is extended. In addition they are all concerned that there is no direct evidence linking the clock directly to these changes in gene expression and autophagy. Should you be able to address these issues, we would consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses in this revised version. I do realize that addressing all the referees' criticisms will require a lot of additional time and effort and be technically challenging. I would therefore understand if you wish to publish the manuscript rapidly and without any significant changes elsewhere.

When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

In this article, Ma and Lin described for the first time a diurnal autophagy in mouse liver. Indeed, they showed that different markers of autophagy (LC3-II, p62, autophagosome density and expression of autophagy-related genes mRNA) present a diurnal pattern in mouse liver with a tendency for a maximum level during the day. By screening a selected pool of transcription factors involved in circadian clock and lipid metabolism, they characterized C/EBPβ as a regulator of autophagy in cultured hepatocytes. C/EBPβ expression presents a diurnal pattern and seems to be regulated by the circadian clock and feeding/fasting rhythms. Finally, they showed that C/EBPβ regulates directly transcription of autophagy-related genes and is required for rhythmic regulation of autophagy in mouse liver.
If it was observed by morphometric analysis that a rhythmic autophagy presumably exists in mouse liver, this article presents the first molecular evidences of this phenomenon. In addition, this is the first demonstration of a role of C/EBPβ in this process. However, some conclusions seem to be not completely supported by the described experiments which need to be completed.

1- The argument that autophagy is rhythmic is difficult to follow as the pattern of p62 and LC3 expression seems to present a period which is not 24h. This is due in part because only a single cycle is displayed, and this is under LD conditions. This may be improved if 2 cycles-ie 48h-were presented in LD but also in Dark-Dark conditions. For the same reason, the 6h time span between measures of autophagosome density did not allow a correlation between these measures and autophagy markers expression. In addition, the presentation of the LC3-I/LC3-II ratio on Figure 1B will support more the data.
2- If most of the autophagy related genes present a rhythmic expression with a 24h period, they are not synchronized to each other (peaks of expression are spread from ZT4 to ZT16) and not synchronized to the autophagy markers which present a period which is not 24h. Please comment this discrepancy. In addition how Authors explain the diurnal pattern of LC3-I expression whereas LC3 mRNA appears not rhythmic (at least in the heart)?
3- Authors show that starvation induces autophagy and C/EBPβ expression. First, the time of starvation is not indicated (presumably one complete night) and has to be indicated. Second, it will be interesting to show if, as circadian genes expression, C/EBPβ rhythmic expression is sustained under longer (24h) starvation, as well as expression of autophagy markers.
4- On Fig. 5C, Authors showed that the rhythmic mRNA expression of some autophagy-related genes is disrupted in mice with a liver-specific deletion of the circadian regulator Bmal1. However, considering the poor correlation between this rhythmic expression and autophagy, these data are not sufficient to prove a regulation of autophagy by the circadian clock. At least expression of LC3 isoforms and p62 have to be added.

Referee #2:

In this manuscript the authors describe diurnal gene expression in liver, which is paralleled by diurnal autophagy rhythms.
Although the authors make interesting observations, there is no evidence provided that the observed rhythms are driven by clock mechanisms. A molecular link to the clock is completely missing and the observed rhythms may be of systemic nature (Vollmers et al., 2010). Furthermore, there is lack of consistency in the data (e.g. Fig. 4A peak of C/EBP expression is at ZT15, whereas in Fig. 5 D it is at ZT10). In Fig. 1B there is lack of statistical power and some target genes of C/EBP are

expressed earlier than C/EBP itself (Fig. 5D Ulk1). Overall the presented data are not convincing to me.

Referee #3:

The work examines a novel mechanistic relationship between C/EBPβ and macroautophagy. Autophagy is regulated in mouse liver in cyclic fashion by C/EBPβ that is under both nutritional and circadian control. This is a potentially significant study that suggests a novel mechanism of autophagy regulation by C/EBPβ. I have some concerns over weaknesses in the data that attempt to establish that autophagy is increased by C/EBPβ. However, overall this is a very interesting and well-performed investigation.

Specific comments
1. The data in Figure 1 demonstrate changes in autophagosome number but not function. The number could increase as the result of a decrease in autophagic function caused by decreased lysosomal fusion. p62 levels are not always totally regulated by autophagy and therefore not an ideal measure of function. Autophagic flow should be assessed by comparing LC3 II levels in untreated and chloroquine- or leupeptin-injected mice at selected times.
2. The specific "autophagy genes" chosen for study in Fig. 2 are not justified and somewhat arbitrary. For example, why was cathepsin L examined but not more central factors like Atg4, Atg5 or Atg7? These findings also cannot be used as an indication of changes in the regulation of the rate of autophagosome function. For example, cathepsin L mediates fusion and not autophagosome formation. Finally, some of the gene changes are relatively modest and no protein analysis provided.
3. The PCR data in Fig. 3B are again a selective list and should be validated by protein analysis. Lemasters and colleagues have demonstrated a marked increase in autophagy in primary hepatocytes with culture which may have influenced the findings. What is the level of C/EBPβ expression in this cells (i.e. are the levels physiologic or is it massively overexpressed)?
4. Fig.3E presents the critical autophagic flow experiments but the results do not reflect the conclusions in the text. The LC3 II:I ratio is said to be increased in C/EBPβ-expressing cells but the blot shown exhibits the opposite finding - the ratio of lanes 3 and 5 compared to lane 1 (flow for control) appears greater than that for lanes 4 rand 6 compared to lane 2 (flow for C/EBPβ overexpressing cells). Therefore autophagic function seems decreased with C/EBPβ overexpression. The densitometric results of the blot shown and data combined from 3 or more independent experiments are needed.
5. In the long-lived protein degradation experiments to assess autophagic function (Fig. 3C), the amount of basal autophagy is surprisingly almost completely macroautophagy independent (very minor inhibition by 3MA) raising concerns over the validity of the experiment. More than one time point should be examined.
6. No consistent effect of the C/EBPβ knockdown is seen on LC3 in Fig. 7B and 8B in contrast to the massive changes in p62. Why is this? This discrepancy and the fact that p62 levels are not totally dependent on autophagy raises concern over the conclusion that autophagy is inhibited. What does EM of the liver show in terms of autophagosome number? Protein degradation studies should be carried out in knockdown cells.
7. Studies of mTOR levels are needed.
8. The data in Fig. 8B indicate that C/EBPβ does not only regulate the diurnal induction of autophagy but also basal levels as well.
9. The changes clearly rely on the peripheral clock but it is unclear if the clock is mediating nutritional effects only, or exerts effects independent of nutrition. In the end, it is not apparent whether C/EBPβ is a mechanism of nutritional effects on autophagy or there is a separate circadian influence unrelated to feeding status.

Minor points. For Fig. 1B times should be shown on the x axis of the p62 blot. Fig. 1B should contain error bars. The number of independent experiments and statistical significance should be provided for this figure and Fig. 1D. Fig. 3B needs statistical analysis. Panels in Fig. 3 should be rearranged to correspond to their order of presentation in the text. Fig. 2 and 3C lack information on the number of independent experiments. Methods lack a description of how the gene arrays were performed and the bioinformatics employed to analyze them.

1st Revision - Authors' Response 14 July 2011

Responses to the reviewers' comments *(original comments are in italics)*

Reviewer #1

In this article, Ma and Lin described for the first time a diurnal autophagy in mouse liver. ... If it was observed by morphometric analysis that a rhythmic autophagy presumably exists in mouse liver, this article presents the first molecular evidences of this phenomenon. In addition, this is the first demonstration of a role of C/EBPβ in this process. However, some conclusions seem to be not completely supported by the described experiments which need to be completed.

We appreciate this reviewer's enthusiasm on the novelty of our findings and his/her thoughtful comments on improving the quality of this manuscript. Two major questions raised concern the pattern of autophagy rhythm and the influence of nutritional and clock signals on this rhythm. We have now performed *in vivo* autophagy flux analysis using a new method recently developed by Choi and colleagues (Haspel et al., 2011). Our results indicate that autophagy flux exhibits a strong diurnal rhythm that peaks during the late light phase (Fig. 1B-C). With regard to the nutritional regulation on autophagy rhythm, we have examined temporal expression of C/EBPβ and autophagy genes under starvation state. Detailed responses are summarized below.

1. The argument that autophagy is rhythmic is difficult to follow as the pattern of p62 and LC3 expression seems to present a period which is not 24h. This is due in part because only a single cycle is displayed, and this is under LD conditions. This may be improved if 2 cycles-ie 48h-were presented in LD but also in Dark-Dark conditions. For the same reason, the 6h time span between measures of autophagosome density did not allow a correlation between these measures and autophagy markers expression. In addition, the presentation of the LC3-I/LC3-II ratio on Figure 1B will support more the data.

It has been recognized there are limitations of using steady-state p62 and LC3 levels to infer autophagy activity. To address this, we employed a recently developed method to directly assess autophagy flux in mouse liver using lysosomal protease inhibitor. We injected a single dose of PBS or leupeptin into mice kept under DD conditions at six different time points and harvested tissues three hours later. We examined the accumulation of LC3-II during the 3-hr periods to measure autophagy flux. Consistent with the EM data, liver autophagy flux peaks during late light phase and is suppressed in the dark phase. These new data have been added to Fig. 1B-C.

We have also included autophagy gene expression profiles generated in a high-temporal resolution liver microarray study performed by Hogenesch and colleagues (Hughes et al., 2009). In this study, liver gene expression was profiled hourly for a total of 48 hrs under DD conditions. Similar to our qPCR results, mRNA expression of C/EBPβ, LC3B, Ulk1, Gabarapl1, Bnip3, and Ctsl exhibits robust circadian rhythms (Fig. S2). These results provide further support to the existence of functional and molecular autophagy rhythms in the liver.

2. If most of the autophagy related genes present a rhythmic expression with a 24h period, they are not synchronized to each other (peaks of expression are spread from ZT4 to ZT16) and not synchronized to the autophagy markers which present a period which is not 24h. Please comment this discrepancy. In addition how Authors explain the diurnal pattern of LC3-I expression whereas LC3 mRNA appears not rhythmic (at least in the heart)?

There are several categories of autophagy related genes that undergo circadian oscillation, including those involve in autophagosome formation (Ulk1, LC3B, Gabarapl1), mitophagy (Bnip3), and lysosomal acidification and degradation (Atp6v1d, Ctsl). As they likely perform distinct molecular functions, it is not completely surprising that they display different phases of rhythm. Similar phenomenon has been observed for hepatic gluconeogenic genes, such as PEPCK and G6Pase, which exhibit opposite phase.

Steady-state LC3-I protein levels are determined by its translation, conversion to LC3-II, and regeneration from LC3-II. It is unknown at present the exact mechanisms responsible for the differential regulation of LC3 mRNA and LC3-I protein expression in the heart.

3. Authors show that starvation induces autophagy and C/EBPβ expression. First, the time of starvation is not indicated (presumably one complete night) and has to be indicated. Second, it will be interesting to show if, as circadian genes expression, C/EBPβ rhythmic expression is sustained under longer (24h) starvation, as well as expression of autophagy markers.

Per reviewer's suggestion, we performed additional studies to examine the mRNA expression of C/EBPβ and autophagy genes at different time points after 24hrs of starvation. As shown in Fig. S5, starvation significantly elevates the expression of Gabarapl1, Ulk1, Bnip3, Ctsl, LC3B, and C/EBPβ at all time points. Interestingly, the expression of Ulk1, Bnip3, Ctsl, and C/EBPβ still appears to be rhythmic, although the phase and amplitude are slightly altered. These results are consistent with our model that both nutritional and circadian signals impinge on the autophagy pathway. We added these results on page 7 and Fig. S5. In addition, we have included detailed information about the starvation experiment in the figure legend of Fig. S5.

4. On Fig. 5C, Authors showed that the rhythmic mRNA expression of some autophagy-related genes is disrupted in mice with a liver-specific deletion of the circadian regulator Bmal1. However, considering the poor correlation between this rhythmic expression and autophagy, these data are not sufficient to prove a regulation of autophagy by the circadian clock. At least expression of LC3 isoforms and p62 have to be added.

We have included immunoblots for autophagy markers (LC3, p62, and Ulk1). To further address the functional impact of clock deficiency on autophagy, we performed autophagy flux assay and found that peak autophagy activity is significantly reduced in Bmal1 liver-specific knockout mouse livers. These new data have been added to Fig. 7B-C and discussed on page 13.

References:

Haspel J, Shaik RS, Ifedigbo E, Nakahira K, Dolinay T, Englert JA, Choi AM (2011) Characterization of macroautophagic flux in vivo using a leupeptin-based assay. *Autophagy* 7(6): 629-642

Hughes ME, DiTacchio L, Hayes KR, Vollmers C, Pulivarthy S, Baggs JE, Panda S, Hogenesch JB (2009) Harmonics of circadian gene transcription in mammals. *PLoS Genet* 5(4): e1000442

Reviewer #2

In this manuscript the authors describe diurnal gene expression in liver, which is paralleled by diurnal autophagy rhythms. Although the authors make interesting observations, there is no evidence provided that the observed rhythms are driven by clock mechanisms. A molecular link to the clock is completely missing and the observed rhythms may be of systemic nature (Vollmers et al., 2010). Furthermore, there is lack of consistency in the data (e.g. Fig. 4A peak of C/EBP expression is at ZT15, whereas in Fig. 5 D it is at ZT10). In Fig. 1B there is lack of statistical power and some target genes of C/EBP are expressed earlier than C/EBP itself (Fig. 5D Ulk1). Overall the presented data are not convincing to me.

We appreciate the reviewer's comments and have provided additional new data to address his/her concerns. First, we have performed *in vivo* autophagy flux analysis in mice under constant darkness and observed the persistence of robust autophagy rhythm (Fig. 1B-C). In addition, we analyzed the expression of C/EBPβ and autophagy genes in fasted mice and found that while their expression levels are elevated in the starvation state, the oscillatory nature of gene expression remains (Fig. S5). We have also performed in vivo autophagy flux analysis in Bmal1 liver-specific knockout mice during autophagy peak hours and found significantly decreased autophagy flux in the liver lacking a function clock. As such, it is likely that circadian signals exert a significant regulatory function on the autophagy pathway. We agree with the reviewer that the exact molecular mechanism underlying clock regulation of C/EBPβ is an important question to be addressed in future studies. With regard

to peak C/EBPβ expression, there is general agreement among many independent experiments that it reaches highest expression level near the onset of dark phase. The apparent difference may be due to limited sampling density. Finally, it is highly likely that additional physiological signals other than C/EBPβ may participate in the circadian regulation of Ulk1 expression. We have added one sentence in discussion to reflect this.

Reviewer #3

The work examines a novel mechanistic relationship between C/EBPβ and macroautophagy. Autophagy is regulated in mouse liver in cyclic fashion by C/EBPβ that is under both nutritional and circadian control. This is a potentially significant study that suggests a novel mechanism of autophagy regulation by C/EBPβ. I have some concerns over weaknesses in the data that attempt to establish that autophagy is increased by C/EBPβ. However, overall this is a very interesting and well-performed investigation.

We thank reviewer #3 for his/her insightful comments and suggestions. The questions raised concern the methods used to assess the autophagy activity in the liver and the interpretations of these results. Per reviewer's suggestions, we have performed *in vivo* autophagy flux analysis at different time points in mice and demonstrate that autophagy flux is highly rhythmic in the liver. We have also reassessed C/EBPβ overexpression in primary hepatocytes upon treatments with lysosomal inhibitors. Further, we performed new studies to explore the potential crosstalk between C/EBPβ and the mTOR pathway. These new data have been incorporated in the revised manuscript, as detailed below.

1. The data in Figure 1 demonstrate changes in autophagosome number but not function. The number could increase as the result of a decrease in autophagic function caused by decreased lysosomal fusion. p62 levels are not always totally regulated by autophagy and therefore not an ideal measure of function. Autophagic flow should be assessed by comparing LC3 II levels in untreated and chloroquine- or leupeptin-injected mice at selected times.

We greatly appreciate this reviewer's suggestion for assessing *in vivo* autophagy flux following leupeptin injection. We have now performed this experiment using the protocol established by Haspel et al. Briefly, we injected a single dose of PBS or leupeptin into mice kept under constant darkness conditions at six different time points and harvested tissues three hours later. We examined the accumulation of LC3-II during the 3-hr period to measure autophagy flux and found that, consistent with the EM data, liver autophagy flux peaks during late light phase and is suppressed in the dark phase. These new data have been added to Fig. 1B-C.

2. The specific "autophagy genes" chosen for study in Fig. 2 are not justified and somewhat arbitrary. For example, why was cathepsin L examined but not more central factors like Atg4, Atg5 or Atg7? These findings also cannot be used as an indication of changes in the regulation of the rate of autophagosome function. For example, cathepsin L mediates fusion and not autophagosome formation. Finally, some of the gene changes are relatively modest and no protein analysis provided.

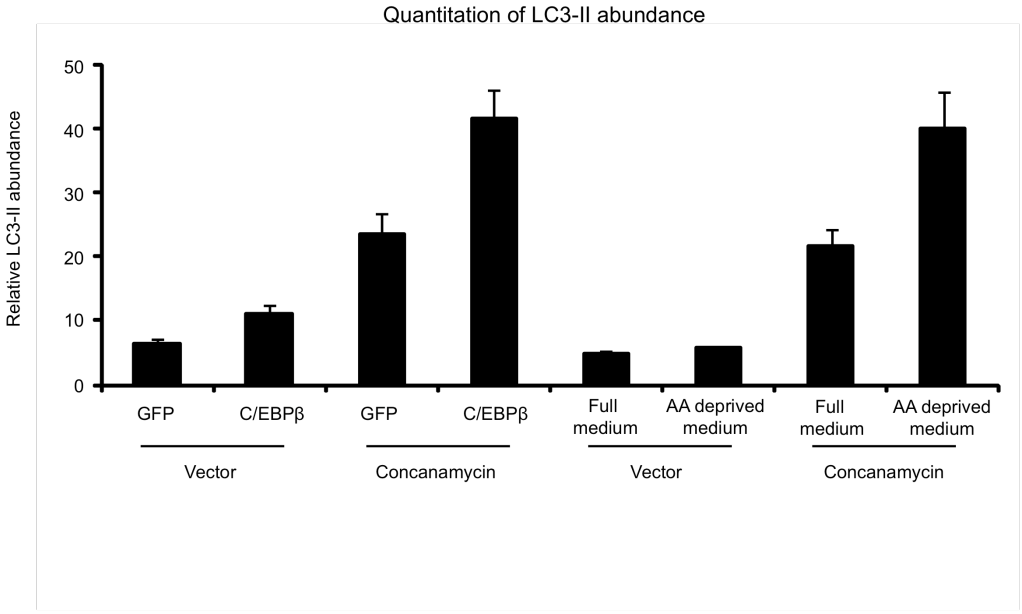
We agreed with the reviewer that genes such as Atg4, Atg5, or Atg7 are critical for autophagosome formation and are central to the autophagy processes. We have now included mRNA and protein expression data for these genes. As shown in Fig. 2 and S5, the expression of these factors appears modestly affected by circadian and nutritional states. In contrast, the expression of Gabarapl1, Ulk1, Bnip3, and Ctsl displays circadian rhythmic pattern (Fig. 2) and is highly responsive to starvation in the liver (Fig. 5E and S5). These results suggest that only a subset of autophagy genes is regulated at the transcriptional level. We discussed this result on page 7.

3. The PCR data in Fig. 3B are again a selective list and should be validated by protein analysis. Lemasters and colleagues have demonstrated a marked increase in autophagy in primary hepatocytes with culture which may have influenced the findings. What is the level of C/EBPβ expression in this cells (i.e. are the levels physiologic or is it massively overexpressed)?

We validated mRNA expression of autophagy genes by immunoblotting and included new data in Fig. 3C. We used C/EBPβ adenoviruses at a moiety of infection from 2 to 5 in our studies and recognized that C/EBPβ is expressed at higher than normal levels. The loss-of-function studies using RNAi knockdown provide critical additional evidence to support gain-of-function studies.

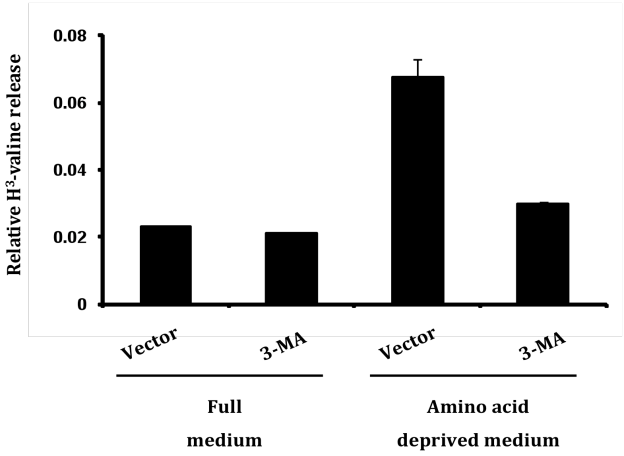
4. Fig.3E presents the critical autophagic flow experiments but the results do not reflect the conclusions in the text. The LC3 II:I ratio is said to be increased in C/EBPβ-expressing cells but the blot shown exhibits the opposite finding - the ratio of lanes 3 and 5 compared to lane 1 (flow for control) appears greater than that for lanes 4 rand 6 compared to lane 2 (flow for C/EBPβ overexpressing cells). Therefore autophagic function seems decreased with C/EBPβ overexpression. The densitometric results of the blot shown and data combined from 3 or more independent experiments are needed.

NH_4Cl and chloroquine raise lysosomal pH but have significant side effects (Seglen, 1983). We have now replaced this study using concanamycin A, an inhibitor of vacuolar ATPase, to more specifically address the regulation of autophagy flux by C/EBPβ. As shown in Fig. 3D and quantitated in Fig. 3E, we found that C/EBPβ significantly increases LC3-I to LC3-II conversion in hepatocytes. In addition, we have performed parallel studies following amino acid deprivation and found that C/EBPβ stimulates LC3-II accumulation in a similar manner compared to starvation-induced autophagy (please see the graph below).



5. In the long-lived protein degradation experiments to assess autophagic function (Fig. 3C), the amount of basal autophagy is surprisingly almost completely macroautophagy independent (very minor inhibition by 3MA) raising concerns over the validity of the experiment. More than one time point should be examined.

We had validated our assay with protein degradation following amino acid starvation. As shown in the representative figure below, 3-MA does not significantly alter protein degradation rate under nutrient-rich conditions whereas it potently decreases autophagy-mediated degradation under starvation conditions. Similar results have been reported by other investigators (Fig. S5 in Settembre C et al., 2011 and Fig. 1B in Seglen PO and Gordon PB, 1982). It is possible that the sensitivity of this assay is insufficient to detect low levels of autophagy-mediated protein degradation under nutrient-rich conditions.



6. No consistent effect of the C/EBPβ knockdown is seen on LC3 in Fig. 7B and 8B in contrast to the massive changes in p62. Why is this? This discrepancy and the fact that p62 levels are not totally dependent on autophagy raises concern over the conclusion that autophagy is inhibited. What does EM of the liver show in terms of autophagosome number? Protein degradation studies should be carried out in knockdown cells.

The reviewer correctly pointed out the limitations of using steady-state p62 and LC3 levels to infer autophagy activity. To address the discrepancy, we examined *in vivo* autophagy flux during peak hours of autophagy in control and C/EBPβ knockdown livers. As shown in Fig. 9C, autophagy flux, as revealed by leupeptin-induced LC3-II accumulation, is nearly abolished in siC/EBPβ mouse livers. These results support our conclusion that C/EBPβ plays a significant role in physiological regulation of autophagy gene expression and flux.

7. Studies of mTOR levels are needed.

We examined the relationships between mTOR and C/EBPβ in two new studies. First, we assessed whether C/EBPβ modulates the mTOR activity and found that adenoviral-mediated expression of C/EBPβ does not appear to affect nutritional regulation of mTOR (Fig. S7). In the second study, we addressed whether mTOR regulates C/EBPβ expression and found that Torin1 treatments of hepatocytes do not affect C/EBPβ expression. We note these results on page 9-10.

8. The data in Fig. 8B indicate that C/EBPβ does not only regulate the diurnal induction of autophagy but also basal levels as well.
Yes.

9. The changes clearly rely on the peripheral clock but it is unclear if the clock is mediating nutritional effects only, or exerts effects independent of nutrition. In the end, it is not apparent whether C/EBPβ is a mechanism of nutritional effects on autophagy or there is a separate circadian influence unrelated to feeding status.

This is an excellent point that pertains to the complex crosstalk between the clock and nutritional signaling pathways in the whole animal setting. Our data suggests that the liver clock exerts a tissue-autonomous effect on C/EBPβ expression and the autophagy pathway. At the same time, it is also clear that nutritional signals regulate autophagy via transcriptional and post-translational mechanisms, as depicted in our model.

Minor points. For Fig. 1B times should be shown on the x axis of the p62 blot. Fig. 1B should contain error bars. The number of independent experiments and statistical significance should be provided for this figure and Fig. 1D. Fig. 3B needs statistical analysis. Panels in Fig. 3 should be rearranged to correspond to their order of presentation in the text. Fig. 2 and 3C lack information on the number of independent experiments. Methods lack a description of how the gene arrays were performed and the bioinformatics employed to analyze them.

We thank the reviewer for his/her careful reading and have made relevant changes in the text and figures.

References:

Haspel J, Shaik RS, Ifedigbo E, Nakahira K, Dolinay T, Englert JA, Choi AM (2011) Characterization of macroautophagic flux in vivo using a leupeptin-based assay. *Autophagy* 7(6): 629-642

Seglen PO (1983) Inhibitors of lysosomal function. *Methods in Enzymology* 96:737-64

Settembre C, Di Malta C, Polito VA, Garcia Arencibia M, Vetrini F, Erdin S, Erdin SU, Huynh T, Medina D, Colella P, Sardiello M, Rubinsztein DC, Ballabio A (2011) TFEB links autophagy to lysosomal biogenesis. *Science* 332(6036):1429-33

Seglen PO, Gordon PB (1982) 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. *Proc Natl Acad Sci U S A* 79(6):1889-92

2nd Editorial Decision 28 July 2011

Thank you for submitting your revised manuscript for consideration by the EMBO Journal. It has now been seen by two of the original referees who both find that the study is significantly improved and recommend publication pending some minor text and figure changes.

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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

In this revised article, Ma et al. made an important effort to answer the points highlighted by the reviewers. Notably, measures of autophagy flux increase considerably the relevance and the consistency of the presented data. In addition, important efforts have been made to highlight the role of feeding and the TOR signaling pathway on this phenomenon. Addition of data on Bmal1 ko animals is also important to show the role of the clock on this process.
However, if this revised article convincingly shows that autophagy is diurnal and controlled by the clock on mouse liver and that CEBP is an important regulator of autophagy, the link between the two phenomena is not evident as the maximum of autophagy is observed when the level of CEBP is at its minimum level, and vice-versa. At least a discussion about this fact is required to conclude this nevertheless interesting article.

Referee #3:

The autophagic flow has been calculated incorrectly in Fig. 1C. The authors subtracted the PBS-treated LC3-II level from the concanamycin-treated one. The flow should be calculated as the ratio of the concanamycin-treated to PBS-treated LC3-II ratio.

2nd Revision - Authors' Response 04 August 2011

Responses to the reviewers' comments *(original comments are in italics)*

Reviewer #1

In this revised article, Ma et al. made an important effort to answer the points highlighted by the reviewers. Notably, measures of autophagy flux increase considerably the relevance and the consistency of the presented data. In addition, important efforts have been made to highlight the role of feeding and the TOR signaling pathway on this phenomenon. Addition of data on Bmal1 ko animals is also important to show the role of the clock on this process.

However, if this revised article convincingly shows that autophagy is diurnal and controlled by the clock on mouse liver and that CEBPβ is an important regulator of autophagy, the link between the two phenomena is not evident as the maximum of autophagy is observed when the level of CEBPβ is at its minimum level, and vice-versa. At least a discussion about this fact is required to conclude this nevertheless interesting article.

We appreciate the reviewer 1's enthusiasm about our manuscript. We have added one sentence on page 11 to discuss about the apparent phase discrepancy of autophagy rhythm and *CEBPβ* expression rhythm.

Reviewer #3

The autophagic flow has been calculated incorrectly in Fig. 1C. The authors subtracted the PBS-treated LC3-II level from the concanamycin-treated one. The flow should be calculated as the ratio of the concanamycin-treated to PBS-treated LC3-II ratio.

We appreciate the reviewer's comment. However, as shown in previous studies by other investigators (Haspel J et al.), we believe that subtracted LC3-II levels in leupeptin-treated samples more appropriately reflect autophagy flux. All LC3-II values were normalized to β-actin to ensure equal loading.

Reference:

Haspel J, Shaik RS, Ifedigbo E, Nakahira K, Dolinay T, Englert JA, Choi AM (2011) Characterization of macroautophagic flux in vivo using a leupeptin-based assay. *Autophagy* 7(6): 629-642

