THE
EMBO
JOURNAL

NUTRIENT-SENSITIVE TRANSCRIPTION FACTORS TFEB AND TFE3 COUPLE AUTOPHAGY AND METABOLISM TO THE PERIPHERAL CLOCK

Nunzia Pastore, Anna Vainshtein, Niculin J. Herz, Tuong Huynh, Lorenzo Brunetti, Tiemo J. Klisch, Margherita Mutarelli, Patrizia Annunziata, Kenichiro Kinouchi, Nicola Brunetti-Pierri, Paolo Sassone-Corsi, and Andrea Ballabio.

Review timeline:

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Editorial Decision:	1st April 2019
Revision received:	3rd April 2019
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Editor: Elisabetta Argenzio

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 31st January 2019

Thank you for submitting your manuscript on clock-independent activation of transcription factors TFEB and TFE3 by food for the rhythmic induction of genes involved in autophagy and cell metabolism to The EMBO Journal. Please accept my apologies for the extended duration of the review process due to the seasonal holidays. Your study has been seen by three referees and their comments are enclosed below for your information.

As you can see, the referees find your work potentially interesting and appreciate the quality of the data. However, they also raise several points that need to be addressed before they can support publication in The EMBO Journal. In particular, referee #1 requests you to further discuss your model in the context of the existing literature. Referee #2 stresses that experiments have to be quantified and analyzed with proper statistical methods. Referee #3 is the most critical one and asks you to clarify the effects of TFEB/TFE3 loss of function on gene expression, as well as to test the expression of other key circadian clock components.

Addressing these issues as suggested by the referees would be essential to warrant publication in The EMBO Journal. Given the overall interest of your study, I would like to invite you to submit a revised version of the manuscript according to the referees' requests.

REFEREE REPORTS

Referee #1:

The manuscript "Nutrient-sensitive transcription factors TFEB and TFE3 couple autophagy and

metabolism to the peripheral clock" from Pastore and colleagues demonstrates a novel mechanism of circadian gene transcription through combination of nutrient-sensitive transcription factors TFEB and TFE3 and clock component Rev-erbα. The authors show that nuclear accumulation and transcriptional activity of TFEB and TFE3 are directly regulated by feeding rhythms resulting in rhythmic activation of autophagy. Consistently, autophagy oscillations are impaired in TFEB and TFE3 mutant mice. Along with this, TFEB and TFE3 activate expression of Rev-erbα via E-boxes modulating its rhythmic expression. Interestingly, TFEB/TFE3 and Rev-erbα are independently recruited to many other targets, involved in regulation of autophagy and metabolism, thus revealing a direct connection of these pathways to the circadian clock.

This work will be interesting for the broad readership and results presented here are relevant for the circadian field. However, there are some weak points in the final model, which are specified below:

Major points:

- Fig 6. The authors suggest a model where TFEB and TFE3 activate and Rev-erbα represses autophagy genes yielding their circadian expression. However, they do not provide any substantial evidence to their assumption that Rev-erbα repression occurs during the night (active phase). Rather, Preitner et al (2002) clearly demonstrated that Rev-erbα abundance in the liver peaks at ZT 6, which does not support the suggested model. Thus, Rev-erbα appears to be active at the same time as TFEB and TFE3. The authors should comment on this or revise their model.

Minor points:

- Fig 4D. Cropped ChIP-seq images contain some numbers in the corner, but respective figure legends lack a description.
- Fig 4E. "Numbers in parenthesis indicate the number from transcriptional start site. The sequences of the elements are highlighted in grey." These labels are not presented on the respective figure.
- The authors focus their work specifically on Rev-erbα and do not mention the contribution of Rev-erbβ. However, both Rev-erbα and β are required to maintain rhythmicity in mice (Cho et al, 2012). Therefore it will be interesting to know whether Rev-erbβ also contributes to this pathway.

Referee #2:

Pastore et. al demonstrated that TFEB and TFE3, the known transcriptional factors of autophagy and lysosome biogenesis, display a circadian activation during 24-hour cycle and may modulate the rhythmic oscillation of autophagy genes expression in a food-dependent/clock-independent manner. In addition, TFEB and TFE3 were implicated in direct regulation of REV-ERBα (the transcriptional repressor component of the core clock machinery, previously implicated as regulator of autophagy) expression by binding its promoter region. The newly discovered association between TFEB/TFE3 and REV-ERBα and the fact that both are regulating the expression of autophagy genes served the authors to link it to the circadian clockwork. Based on this and the extensive overlap of TFEB/TFE3 and REV-ERBα binding sites, the authors suggested a model of a dynamic balance between TFEB/TFE3 (activation of autophagy genes) and REV-ERBα (repression of autophagy genes) which is responsible for the circadian oscillation of autophagy.
This research provides interesting observations on autophagy circadian oscillations mediated by an interplay between TFEB/TFE3 and REV-ERBα. Overall, the manuscript is well written and the presented data are mostly clear and reliable. However, some issues indicated in comments below should be clarified by the authors.
Comments:
Figure 1D - The authors should relate to the fact that the GABARAP level at ZT5 dropped in contrast to Ctsl and RragD. In addition, the information regarding statistics significance is missing. Generally, the authors should provide the missing information regarding statistics significance for figures 1F, 1G, 2B, 3E. Moreover, it would be much clearer if in most cases the authors will use first the ANOVA test and if needed the Student t-test.
Figure 4F - The plot is difficult to follow. Moreover, the authors should test their hypothesis by looking at the suggested changes in the autophagy proteins level using western blots. The usages of a lysosomal inhibitor may be required in the case of LC3B and Gabarap.

Figure 5 - the authors should relate to the fact that the results of TFE3KO presented in panels B and C are different. Moreover, the Y axes scales for the data presented in the left panels of B and C are different. The meaning of the significance star in the left panel of C remain unclear.
Some of the supplementary figures are missing legends to explain the different bars.
In the page 8 line 27 of the text the (Figures 4D and EV6C) should be (Figures 4F and EV6C).

Referee #3:

The manuscript by Pastore et al. describes the circadian oscillation of the TFE3/TFEB autophagic transcription factors and their link to circadian metabolic alterations in vivo and the circadian clock itself through direct activation of REV-ERBα. In series of experiments, particularly using genetically modified mouse models of loss-of-function (LOF) of TFE3 and/or TFEB in liver or muscle, the authors document the oscillation of TFE3/TFEB which peaks during day-time (sleep for mice), contrasting with the known peaking of mTOR signaling during the active phase or night time. Further, the authors show that oscillation of autophagic genes was blunted with TFE3/TFEB LOF, which was also associated with perturbed circadian activity in TFE3/TFEB deficient (muscle/liver) mice. Through examining the genomic effects of TFE3/TFEB, the authors also demonstrate that TFE3/TFEB could activate REV-ERBa, linking these autophagic transcription factors directly to the clock machinery - independent of BMAL1. Overall, this is a paper that could significantly adds to our understanding of the links between different helix-loop-helix transcription factor networks, the circadian clock, and control of oscillatory maintenance metabolism. However, there are a number of important issues that should be addressed.

Issues:
1. Do TFE3 and TFEB also regulate REV-ERBbeta.
2. "Circadian" oscillation of nuclear TFE3/TFEB: Figures 1A and 1E appear to show 12-hour oscillation of nuclear TFE3/TFEB. Most apparent for 12-h cycle is nuclear TFEB (Fig 1E) The authors need to expand on this. Further, the quantitations in Figure 1F do not seem to match immunoblots in Fig 1E; at zero time point the immunoblot shows low level for TFEB (for example), but the graph shows a high level.
3. Blots for Figure 1F and others should NOT be in double-plotted format, which is misleading to readers. While technically more difficult, the appropriate studies would be performed over a 48h time-course instead of "double-plotting."
4. Figure 2: The authors should clarify the effect of LOF of TFE3/TFEB on specific autophagic gene expression with some displaying phase shifting (ATG5-12) while others were severely dampened (GABARAP). The expression of other key circadian clock components (REV-ERBalpha, REV-ERBbeta, BMAL1, CLOCK, CRYs and PERs) should be shown so that the basis for phase-shifting could be understood.
5. Figure 3H: The authors should define "starvation." TORC1 activity status should be provided (also for Fig 3I).
6. For Figure EV5A, the IP for TFE3 seemed to pulldown TFEB, but not vice versa. Further, the IP for BMAL1 weakly pulled down for CLOCK. Hence, caution should be exercised regarding the lack of robustness of the assay for protein-protein association (ie, known associations between TFE3 and TFEB or BMAL1 and CLOCK are not clear evident) and hence the limitation should be discussed. In this regard, the mechanism proposed by Luo et al. 2016 should not be superficially dismissed in the Discussion.
7. For TFEB mKO, the distinction between the effects on muscle physiology (which is not measured) and on the circadian clock are not distinguished. Hence, this caveat should be considered.
8. In Figure EV7C, the data do seem to support the assertion that "resetting was impaired in TFE3KO mice" for Bmal1 and Clock. In TFE3KO, BMAL1 phase switches to perhaps a larger degree in TFE3KO than in WT in Day-Fed, and the same is true for Clock. It is difficult to interpret the REV-ERBa data since the Authors assert that REV-ERBa is a TFE3 target.
9. The description of the molecular clock in Page 3 should be updated with reference to not only protein level but protein localization and multimer formation, which are important in governing the clock.
10. For Figure 4, why are the list of genes examined in 4D different from those in 4F? Did not all the genes with strong REV-ERB/ TFE3 peaks change in 4F? Or are some of the genes in 4F indirectly regulated by REV-ERB / TFE3? Please clarify this.

11. Figure 5B is missing labels
12. For Figure 3D, the Authors should define which E-Box they are looking at by noting this in Figure 3C.

1st Revision - authors' response 11th March 2019

Referee #1:

The manuscript "Nutrient-sensitive transcription factors TFEB and TFE3 couple autophagy and metabolism to the peripheral clock" from Pastore and colleagues demonstrates a novel mechanism of circadian gene transcription through combination of nutrient-sensitive transcription factors TFEB and TFE3 and clock component Rev-erbα. The authors show that nuclear accumulation and transcriptional activity of TFEB and TFE3 are directly regulated by feeding rhythms resulting in rhythmic activation of autophagy. Consistently, autophagy oscillations are impaired in TFEB and TFE3 mutant mice. Along with this, TFEB and TFE3 activate expression of Rev-erbα via E-boxes modulating its rhythmic expression. Interestingly, TFEB/TFE3 and Rev-erbα are independently recruited to many other targets, involved in regulation of autophagy and metabolism, thus revealing a direct connection of these pathways to the circadian clock.

This work will be interesting for the broad readership and results presented here are relevant for the circadian field. However, there are some weak points in the final model, which are specified below:

Major points:

- Fig 6. The authors suggest a model where TFEB and TFE3 activate and Rev-erbα represses autophagy genes yielding their circadian expression. However, they do not provide any substantial evidence to their assumption that Rev-erbα repression occurs during the night (active phase). Rather, Preitner et al (2002) clearly demonstrated that Rev-erbα abundance in the liver peaks at ZT6, which does not support the suggested model. Thus, Rev-erbα appears to be active at the same time as TFEB and TFE3. The authors should comment on this or revise their model.

As pointed out by the reviewer, in Preitner et al (2002) (Fig 2B and 2C), as well as in other publications, *Rev-erbα* mRNA peaks around ZT6. However, REV-ERBα protein accumulates at ZT8, corresponding to its maximum activity (as shown by the strong inhibition of its target *Bmal1*). This is consistent with our model in which autophagy peaks at ZT5 (when TFEB and TFE3 are completely active) and is reduced around ZT9 (**Figure EV1B**), likely due to the inhibitory effect of REV-ERBα. Our model was misleading because it was showing that REV-ERBα was active during the dark phase. As suggested by the reviewer, we modified the model (**Figure 6**) by moving the position of REV-ERBα. In the modified model, REV-ERBα becomes active right before the beginning of the dark phase.

Minor points:

- Fig 4D. Cropped ChIP-seq images contain some numbers in the corner, but respective figure legends lack a description.

We thank the reviewer for pointing this out. We included the description of the numbers in the corner in the figure legend.

- Fig 4E. "Numbers in parenthesis indicate the number from transcriptional start site. The sequences of the elements are highlighted in grey." These labels are not presented on the respective figure.

We apologize for this mistake. We fixed the figure legend.

- The authors focus their work specifically on Rev-erbα and do not mention the contribution of Rev-erbβ. However, both Rev-erbα and β are required to maintain rhythmicity in mice (Cho et al, 2012). Therefore, it will be interesting to know whether Rev-erbβ also contributes to this pathway.

We thank the reviewer for raising this interesting point. We did not mention Rev-erbβ in our experiments because Rev-erbβ is not a transcriptional target of TFEB/TFE3. Therefore, it is unlikely that it contributes to the mechanism that we describe in our paper.

Referee #2:

Pastore et. al demonstrated that TFEB and TFE3, the known transcriptional factors of autophagy and lysosome biogenesis, display a circadian activation during 24-hour cycle and may modulate the rhythmic oscillation of autophagy genes expression in a food-dependent/clock-independent manner. In addition, TFEB and TFE3 were implicated in direct regulation of REV-ERBα (the transcriptional repressor component of the core clock machinery, previously implicated as regulator of autophagy) expression by binding its promoter region. The newly discovered association between TFEB/TFE3 and REV-ERBα and the fact that both are regulating the expression of autophagy genes served the authors to link it to the circadian clockwork. Based on this and the extensive overlap of TFEB/TFE3 and REV-ERBα binding sites, the authors suggested a model of a dynamic balance between TFEB/TFE3 (activation of autophagy genes) and REV-ERBα (repression of autophagy genes) which is responsible for the circadian oscillation of autophagy.
This research provides interesting observations on autophagy circadian oscillations mediated by an interplay between TFEB/TFE3 and REV-ERBα. Overall, the manuscript is well written, and the presented data are mostly clear and reliable. However, some issues indicated in comments below should be clarified by the authors.

Comments:

-Figure 1D - The authors should relate to the fact that the GABARAP level at ZT5 dropped in contrast to Ctsl and RragD. In addition, the information regarding statistics significance is missing.

We thank the reviewer for pointing this out. We have now included a more precise description of the results reported in Fig. 1D. We have also added the statistics, as suggested by the referee.

Generally, the authors should provide the missing information regarding statistics significance for figures 1F, 1G, 2B, 3E. Moreover, it would be much clearer if in most cases the authors will use first the ANOVA test and if needed the Student t-test.

We agree with the reviewer. We have now provided statistical analysis for figures 1F, 1G, 2B and 3E. In most cases we have performed all the statistical analysis using the ANOVA test.

Figure 4F - The plot is difficult to follow.

We agree with the reviewer that the plot may be a bit difficult to follow. However, the experiment contemplates several conditions and we found that this was the only way to compare all perturbations performed in the different settings.

Moreover, the authors should test their hypothesis by looking at the suggested changes in the autophagy proteins level using western blots. The usages of a lysosomal inhibitor may be required in the case of LC3B and Gabarap.

We thank the reviewer for suggesting this experiment. As shown in **Figure 4G**, we analyzed LC3II protein levels by western blotting in cells treated with the lysosomal inhibitor bafilomycin. In these cells inhibition of endogenous *Rev-erbα* with the concomitant overexpression of TFEB/TFE3 resulted in a small increase of LC3II protein.

Figure 5 - the authors should relate to the fact that the results of TFE3KO presented in panels B and C are different. Moreover, the Y axes scales for the data presented in the left panels of B and C are different. The meaning of the significance star in the left panel of C remain unclear.

We are grateful to the referee for pointing this out. We changed the scale in **Figure 5B**. Moreover, we repeated the statistics using the ANOVA test.

Some of the supplementary figures are missing legends to explain the different bars. In the page 8 line 27 of the text the (Figures 4D and EV6C) should be (Figures 4F and EV6C).

We have fixed the text according to the reviewer's indications.

Referee #3:

The manuscript by Pastore et al. describes the circadian oscillation of the TFE3/TFEB autophagic transcription factors and their link to circadian metabolic alterations *in vivo* and the circadian clock itself through direct activation of REV-ERBα. In series of experiments, particularly using genetically modified mouse models of loss-of-function (LOF) of TFE3 and/or TFEB in liver or muscle, the authors document the oscillation of TFE3/TFEB which peaks during day-time (sleep for mice), contrasting with the known peaking of mTOR signaling during the active phase or night time. Further, the authors show that oscillation of autophagic genes was blunted with TFE3/TFEB LOF, which was also associated with perturbed circadian activity in TFE3/TFEB deficient (muscle/liver) mice. Through examining the genomic effects of TFE3/TFEB, the authors also demonstrate that TFE3/TFEB could activate REV-ERBa, linking these autophagic transcription factors directly to the clock machinery - independent of BMAL1. Overall, this is a paper that could significantly add to our understanding of the links between different helix-loop-helix transcription factor networks, the circadian clock, and control of oscillatory maintenance metabolism. However, there are a number of important issues that should be addressed.

Issues:

1. Do TFE3 and TFEB also regulate REV-ERBβ.

No, Rev-erbβ does not appear to be a transcriptional target of TFEB/TFE3. Therefore, we have not mentioned Rev-erbβ in our manuscript.

2. "Circadian" oscillation of nuclear TFE3/TFEB: Figures 1A and 1E appear to show 12-hour oscillation of nuclear TFE3/TFEB. Most apparent for 12-h cycle is nuclear TFEB (Fig 1E) The authors need to expand on this.

In Figures 1A and 1E we show the nuclear/cytosolic distribution of TFEB and TFE3 in mice fed *ad libitum* (**Figure 1A**) and in mice under restricted feeding conditions (**Figure 1E**). The experiment demonstrates that TFEB and TFE3 translocate to the nucleus, thus are active, when mice are "fasted" and in particular at ZT1-9 in mice fed *ad libitum*, at ZT4-8 in mice fed exclusively during the night (NF) and at ZT16-20 in mice fed only during the day (DF).

Further, the quantitations in Figure 1F do not seem to match immunoblots in Fig 1E; at zero time point the immunoblot shows low level for TFEB (for example), but the graph shows a high level.

In Figure 1F, we reported the quantification of the ratio between the nuclear and the cytosolic fraction of TFEB and TFE3. Since at ZT0 the cytosolic TFEB/TFE3 protein levels are low, the resulting ratio is high.

3. Blots for Figure 1F and others should NOT be in double-plotted format, which is misleading to readers. While technically more difficult, the appropriate studies would be performed over a 48h time-course instead of "double-plotting."

In the field of circadian rhythm, several groups use “double-plotting” to better show the oscillation of gene expression. We found many papers in which the authors used double plotting (as an example see the attached figure from *Astafev et al*., Sci Rep 2017). In the figure legends we clearly indicated that we used double plotting. We agree with the reviewer that a 48h time-course would be ideal for circadian studies. However, restarting the study to perform a 48h time-course would require considerable amount of time and a huge number of animals and we believe that this is not justified. We hope that the reviewer agrees with us.

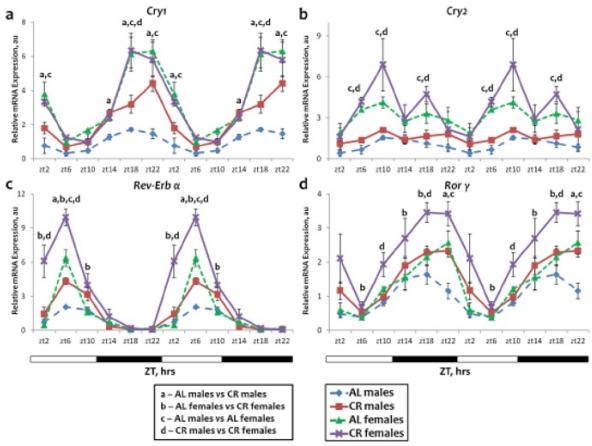


Figure 2. Sexual dimorphism in the expression and response to CR for some circadian clock genes. The daily rhythms in expression of mRNA for *Cry1* (**a**), *Cry2* (**b**), *Rev-Erb α* (**c**) and *Ror γ* (**d**) in the liver: blue diamonds and dashed lines – AL male mice; red squares and solid lines – CR male mice, green triangles and dashed lines – AL female mice; purple x and solid lines – CR female mice. For all panels graphs are double plotted. Light is on at ZT0 and off at ZT12. a, b, c, d – statistically significant difference ($p < 0.05$) between indicated groups at time point. Open bars represent light and black bars represent dark phase of the day. Food for CR group was provided at ZT14. At every time of the day 3 mice of each sex were used for each diet.

4. Figure 2: The authors should clarify the effect of LOF of TFE3/TFEB on specific autophagic gene expression with some displaying phase shifting (ATG5-12) while others were severely dampened (GABARAP).

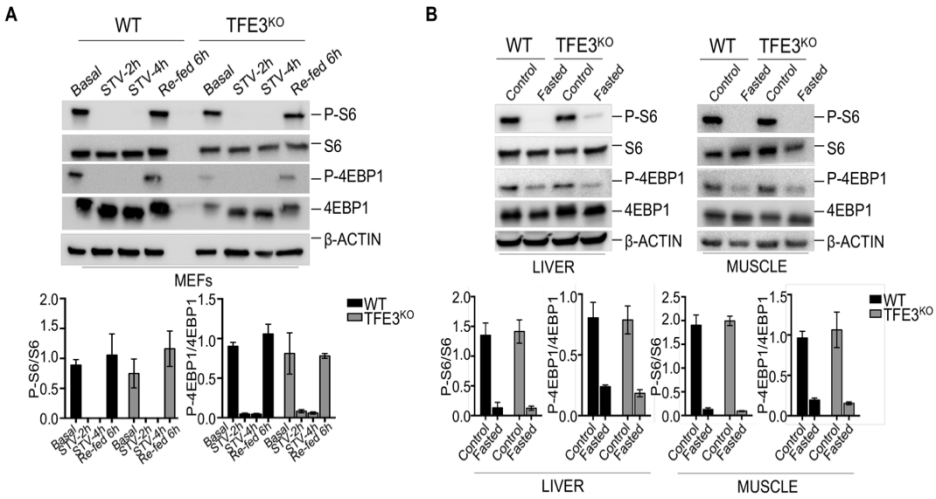
We thank the reviewer for this insightful comment. Our data show that some genes are strongly responsive to TFEB and TFE3 (e.g. *Gabarap* and *Lc3*) and, therefore, their expression is dampened in TFEB/TFE3 KO mice during the light phase compared to normal mice. Interestingly, *Ulk1*, *Atg5* and *Bnip3*, which are known targets of REV-ERBα, show a phase shifting. We postulate that the reduced expression of *Rev-erbα* at ZT5 in TFE3;TFEB^{cKO} mice results in increased expression of its preferential targets at later time points. We have now included this sentence in the discussion.

The expression of other key circadian clock components (REV-ERBα, REV-ERBβ, BMAL1, CLOCK, CRYs and PERs) should be shown so that the basis for phase-shifting could be understood.

As suggested by the reviewer, we have now included gene expression data for key components of the circadian clock machinery (**new Figure 5A**).

5. Figure 3H: The authors should define "starvation." TORC1 activity status should be provided (also for Fig 3I).

We apologize for not making clear the starvation conditions. We included the protocol used for the experiment in the method section. Moreover, we have now measured mTOR activity in the experimental conditions reported in Figures 3H and 3I (See **Reviewer Figure 1**). As shown in reviewer figure 1, mTOR activity was completely inhibited during starvation, as expected.



Reviewers Figure 1:

A. mTOR pathway status in WT and TFE3^{KO} MEFs in response to nutrient starvation (HBSS)/stimulation at the indicated time points.
B. mTOR pathway status in liver and muscle tissues isolated from WT and TFE3^{KO} mice fasted for 24hours.

6. For Figure EV5A, the IP for TFE3 seemed to pulldown TFEB, but not vice versa. Further, the IP for BMAL1 weakly pulled down for CLOCK. Hence, caution should be exercised regarding the lack of robustness of the assay for protein-protein association (ie, known associations between TFE3 and TFEB or BMAL1 and CLOCK are not clear evident) and hence the limitation should be discussed. In this regard, the mechanism proposed by Luo et al. 2016 should not be superficially dismissed in the Discussion.

We thank the reviewer for pointing this out. We recognize the limitation of the co-IP experiment, which is strongly dependent on the efficiency of the antibodies used as well as on the abundance of the proteins studied. We are aware that the TFE3 antibody is more efficient in pulling down TFEB compared to the TFEB antibody. Moreover, we believe that the weakness of the band for CLOCK pulled down using BMAL1 antibody depends on the efficiency of CLOCK antibody in immunoblots in tissue lysate (the whole-lysate band is also weak). As suggested by the reviewer, we have discussed these limitations in the text. However, we believe that the co-IP results that we obtained in the other experiments strongly suggest that TFEB and TFE3-mediated regulation of *Rev-erbα* expression does not rely on BMAL1 and CLOCK. Concerning the Luo et al. paper, we have now added a sentence in the discussion in which we state that we could not replicate the data, but we do not exclude the possibility that our inability to replicate those data was due to the limitations related to the quality of the antibodies used.

7. For TFEB mKO, the distinction between the effects on muscle physiology (which is not measured) and on the circadian clock are not distinguished. Hence, this caveat should be considered.

We thank the reviewer for this important comment. Our previous studies (Mansueto et al, Cell Metabolism 2016 and Pastore et al, EMM 2017) showed the reduced locomotor activity and endurance in $TFEB^{mKO}$ and $TFE3^{KO}$;$TFEB^{mKO}$ mice. Thus, we cannot exclude that the effects of TFEB and TFE3 on muscle physiology could play a role in the results obtained in the wheel-running experiment. In the present manuscript we show that TFEB and TFE3 depletion results in impaired adaptation to the dark-dark conditions (**Figure 5B**). Indeed, wild-type mice exhibit a free-running period of approximately 23.7h in constant darkness, whereas the free-running period is approximately 24.0h in $TFE3^{KO}$, $TFE3^{KO}$;$TFEB^{LiKO}$ and $TFE3^{KO}$;$TFEB^{mKO}$ mice (**Appendix Figures S2A and S2B**). This result strongly suggests that TFEB and TFE3 regulate locomotor behavior in mice and are required for maintaining normal circadian periods in constant darkness. We included this comment in the result section.

8. In Figure EV7C, the data do seem to support the assertion that "resetting was impaired in TFE3KO mice" for Bmal1 and Clock. In TFE3KO, BMAL1 phase switches to perhaps a larger degree in TFE3KO than in WT in Day-Fed, and the same is true for Clock. It is difficult to interpret the REV-ERBα data since the Authors assert that REV-ERBα is a TFE3 target.

We apologize with the reviewer for not being clear enough in the description of the results reported in figure EV7C. We repeated the statistics using the ANOVA test (as suggested by the other reviewer) and modified the text accordingly. Based on these data, we can conclude that TFE3 regulates preferentially *Rev-erbα* expression in response to environmental cues, such as the feeding switch. The increased expression of *Bmal1* and *Clock* that we observed (although not significant) is

consistent with the impaired expression of *Rev-erbα* at ZT0 in the DF conditions. We decided to move these data in the section on the direct regulation of *Rev-erbα* expression by TFEB/TFE3.

9. The description of the molecular clock in Page 3 should be updated with reference to not only protein level but protein localization and multimer formation, which are important in governing the clock.

We have now included a more detailed description of the molecular circadian clock in the introduction section.

10. For Figure 4, why are the list of genes examined in 4D different from those in 4F? Did not all the genes with strong REV-ERB/ TFE3 peaks change in 4F? Or are some of the genes in 4F indirectly regulated by REV-ERB / TFE3? Please clarify this.

We thank the reviewer for this comment. We have modified Figure 4D by including the same genes we reported in the Figure 4F.

11. Figure 5B is missing labels

We fixed the figure.

12. For Figure 3D, the Authors should define which E-Box they are looking at by noting this in Figure 3C.

We fixed the figure.

2nd Editorial Decision 1st April 2019

Thank you for submitting a revised version of your manuscript. It has now been seen by two of the original referees whose comments are appended below.

As you will see, while referee #2 finds that criticisms have been sufficiently addressed and recommend the manuscript for publication, referee #3 remains concerned that the use of double-plots is not appropriate here and reiterates his/her suggestion that a 48h time-course experiment would be a better solution.

In light of your comment on the high number of animals and the time required to address this issue from reviewer #3, I went back to him/her and asked for additional input on how to satisfy this request. S/he stated that "The plots could be presented as single-plots rather than double-plots. I feel strongly that double-plots are poor practice, and the data should just be presented as they are acquired. Double-plots assume that the trajectory of oscillation the second 24h period is identical to the first 24h and is misleading. Indeed, experimental data for the second 24h is almost never 'identical' as the first 24h. See Krishnaiah et al. Cell Metabolism 25:961-974 (2017) for an example of full 48h sampling (every hour) and how oscillations of metabolites are not identical between the first and second 24h window. I would not want the authors to do the 48h time course; however, they could present their data as single plots".

In addition to resolving this remaining point from referee #3, there are a few editorial issues concerning text and figures that I need you to address before we can officially accept the manuscript for publication.

REFEREE REPORTS

Referee #2:

The authors nicely addressed the comments fro m the first round and in its present form the manuscript meets Embo J merit.

Referee #3:

The authors are unresponsive to the following concern. It is inappropriate to justify using because double-plots by citing others who have inappropriately used them. Double-plots are misleading, despite explicitly stating their use in the figure legends.

3. Blots for Figure 1F and others should NOT be in double-plotted format, which is misleading to readers. While technically more difficult, the appropriate studies would be performed over a 48h time-course instead of "double-plotting."

2nd Revision - authors' response 3rd April 2019

We have addressed the reviewers' concerns by modifying the figures. Now we present the data as single plots. For a better visualization of the oscillation, we double-plotted only one time point in the graph and we clearly indicate it in the figure legend.

3rd Editorial Decision 15th April 2019

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

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Nunzia Pastore, Andrea Ballabio
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2018-101347

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.

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was chosen based on previous experience, on the type of experiment and on the anticipated variation according to previous experience from studies using related methods.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Statistical analysis used in each figure are specified in figure legends.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Male mice were used for all the studies to avoid additional variability due to the gender.
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.	No randomization was used. Animals were grouped based on their age or type of treatment.
For animal studies, include a statement about randomization even if no randomization was used.	Animals were assigned to the experimental groups based on the genotype and the data randomly collected and processed as well.
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.	Animals were assigned to the experimental groups based on the genotype by the technitian of the lab.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No bliding was done
5. For every figure, are statistical tests justified as appropriate?	Yes, they are.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The range of variability and the standard error were comparable among groups.
Is there an estimate of variation within each group of data?	Standard error. See figure legends.
Is the variance similar between the groups that are being statistically compared?	Yes, it is.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

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

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

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

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).	All commercially available antibodies have a data sheet reporting the validity for the species and application tested. Company and catalog numbers have been provided in the appendix table.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	See methods

* 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.	See methods
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	See methods
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.	I confirm the compliance of these recoomentations.

E- Human Subjects

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

F- Data Accessibility

18: Provide 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
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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.	No, it could not