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Deficiency in mTORC1-controlled C/EBPβ-mRNA translation improves metabolic health in mice

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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.)

Editor: Esther Schnapp

1st Editorial Decision 26 November 2014

Thank you for the submission of your research manuscript to EMBO reports. We have now received the enclosed referee reports on it.

As you will see, while the referees acknowledge that the findings are potentially interesting, both referees 1 and 2 point out that no direct evidence is provided to support the hypothesis that reduced LIP expression mediates the beneficial metabolic effects of CR, and both referees suggest the same experiment to address this concern. Referee 1 is further concerned about the small effects on transcription and also remarks that it should be examined whether BAT activity or browning of WAT could account for the increased energy expenditure of the mutant mice. Referee 2 indicates that an effect on mTOR signaling should be examined, and that the role of reduced LIP versus increased LAP expression in the metabolic phenotypes should be clarified. However, point 1 of referee 2 does not need to be experimentally addressed, as referee 1 agrees with us in his/her cross-comments that this is beyond the scope of this study. All 3 referees finally pinpoint missing quantifications and statistics, missing information on the experimental procedures and inaccurate interpretations.

Given these constructive comments and the potentially interesting findings, we would like to invite

you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. The most important concerns are mentioned above, but please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further. Also, the revised manuscript should not exceed 35,000 characters (including spaces and references) and 5 main plus 5 supplementary figures. Please let me know if you would like to include more than 5 main figures, as we recently decided that we will also publish longer articles from now on. Shortening of the manuscript text may be made easier by combining the results and discussion section which may help to eliminate some redundancy that is inevitable when discussing the same experiments twice. Commonly used materials and methods can further be moved to the supplementary information, however, please note that materials and methods essential for the understanding of the experiments described in the main text must remain in the main manuscript file.

Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS:

Referee #1:

Zidek et al. investigate the role of C/EBPbeta-LIP in metabolism. The authors found that mTORC1 signaling specifically regulates the expression of the C/EBPbeta-LIP but not -LAP isoform. Moreover, mice with reduced C/EBPbeta-LIP expression are metabolically healthier as compared to control littermates, suggesting that beneficial metabolic effects due to reduction of mTORC1 activity, for example upon caloric restriction (CR), might be mediated via regulation of C/EBPbeta-LIP levels. The results presented in this study are of interest although a bit on the descriptive side. The most worrying aspect of the study is that the transcriptional effects are quite small and it is therefore questionable whether the whole body effects are in fact due to the transcriptional defects. Finally, the manuscript would be improved if they examined whether reduced C/EBPbeta-LIP levels indeed account for the beneficial metabolic effects observed upon CR, although this might be beyond the scope of the paper.

Specific comments:
1. In figure 2C the authors state that mTORC1 activity and LIP levels remain relatively constant throughout the day. However, from the image provided it is not clear whether the samples from the two time points (3h and 14h) were run on the same gel. Only samples that were loaded on the same gel can be compared to each other. In order to make a statement about changes of mTORC1 activity and LIP levels throughout the day in fed ad libitum mice, both time points should be run on the same gel and compared to each other.

2. In figure 2D the signal for LIP is very weak. The authors should show a higher exposure to make LIP levels more visible.

3. In supplementary figure 3A the authors state that C/EBPalpha and PPARgamma expression is increased in visceral adipose tissue of C/EBPbeta delta uORF/Bl6 mice. However, according to the p-value the difference between the mutant and the control mice is not statistically significant. The

authors should change this sentence in the text and state that expression of adipogenic genes is unaltered in C/EBPbeta delta uORF/Bl6 mice.

4. In figure 4E expression levels of genes involved in lipogenesis and lipolysis in white adipose tissue (WAT) are shown. In general the observed changes in expression are relatively small. A Western blot for some of the tested genes should be performed, for example for ACC, FAS, GLUT4, and HSL to investigate whether these small changes in expression are reflected in changes in protein level. Moreover, for LPL, aP2 and ATGL the p-value does not indicate a significant change between control and C/EBPbeta delta uORF/Bl6 mice. The authors should correct the interpretation in the text.

5. The authors should determine mRNA and protein expression levels of genes involved in beta-oxidation in WAT.

6. In figure 4F the expression levels of genes involved in lipogenesis and beta-oxidation in liver are shown. As for the results presented in figure 4e, the presented changes are relatively small. It would be of importance to investigate the protein levels of some of the presented genes, for example CD36, LCAD, ACC, and FAS to investigate whether these small changes in expression are reflected in changes in protein level.

7. The authors conclude that the increased energy expenditure of C/EBPbeta delta uORF/Bl6 mice can be explained by an increase in locomotor activity. To this reviewer the presented differences in activity seem rather small and therefore unlikely the main reason for the increased energy expenditure. Increased activity of brown adipose tissue (BAT) or browning of WAT could account for the observed increase in energy expenditure in the mutant mice. The authors should investigate BAT function and WAT browning, for example by measuring mRNA and protein expression levels of genes involved in uncoupling and beta-oxidation, such as UCP1, Dio2, and PGC-1a in BAT and by measuring BAT markers, such as UCP1, Dio2 and Cidea in subcutaneous WAT.

8. In figure 5C insulin responsiveness of skeletal muscle is shown. To understand whether increased insulin responsiveness can be observed also in other metabolic tissues it would be of interest to investigate insulin responsiveness in WAT and liver of control and C/EBPbeta delta uORF/Bl6 mice.

9. In figure 5D and 5E only fed glucose and fasting insulin levels are presented. Also fasting glucose and fed insulin levels should be shown.

10. The authors speculate that the beneficial metabolic effects elicited by CR are caused by decreased C/EBPbeta-LIP expression. To test this hypothesis, the authors could perform a CR experiment with control and C/EBPbeta delta uORF/Bl6 mice and monitor body weight, glucose homeostasis, metabolic rate and gene expression profile in metabolic tissues. This would reveal whether CR still exerts additional beneficial metabolic effects in C/EBPbeta delta uORF/Bl6 mice or whether CR mediates its beneficial metabolic effects mainly via decreasing C/EBPbeta LIP expression.

Referee #2:

Zidek et al. report that translation of the truncated LIP isoform of CCAAT/Enhancer Binding Protein beta (C/EBPbeta/LAP) is suppressed by inhibiting mTORC1 signaling. Although mTORC signaling was previously reported to regulate the LAP/LIP ratio, the current study extends this observation to show that interventions such as rapamycin treatment or calorie restriction (CR), which lead to reduced mTOR activity, suppress LIP translation. Of note, the authors also used a genetic approach to demonstrate that deletion of the uORF element within the C/EBPbeta mRNA, which is required for translation of the LIP polypeptide, confers a metabolic phenotype that mimics calorie restriction in vivo.

The authors have made interesting observations regarding the physiological consequence of deleting the C/EBPbeta uORF, particularly their finding that the mutant at least partially phenocopies the effects of CR or reduced mTOR signaling. The study would be improved by including a more

thorough analysis of the mechanism by which LIP regulates metabolic function. As it stands, this part of the paper is somewhat vague and descriptive. For example, it is unclear whether the effects of the del uORF mutant result from reduced LIP per se or whether the increased LAP:LIP ratio leads to elevated LAP activity and thus improved metabolic function. These and other issues listed below should be addressed to strengthen the paper.

Specific comments:

1. It would be informative to compare the phenotypes of the del uORF mice and the complete knockout of C/EBPbeta. The latter mice were reported to display decreased body weight and less fat mass, somewhat similar to the del uORF animals. A comparison of the two mutants would give some insight into the function of LAP (which is retained in the del uORF strain) in regulation of energy metabolism.
2. A direct, side by side comparison of mTOR signaling in WT and del uORF cells would be helpful. This might rule out the possibility that mTOR signaling is affected in the mutant cells and is partially or largely responsible for the observed phenotype. The paper implies that LIP regulates metabolic genes downstream of mTORC, but this is not directly demonstrated. There could be some feedback effects from LIP that in turn control the mTORC pathway. For example, it has been reported that C/EBPbeta negatively regulates mTOR signaling under stress conditions through induction of Redd1 (Ho et al., Free Radic Biol Med. 2009 46:1158-67).
3. The authors should provide some data to demonstrate that LIP and/or LAP directly regulate some of the metabolic genes that are affected in the del uORF mutant. ChIP experiments or analysis of published genome-wide protein interaction data (e.g., ENCORE) might be presented to support a direct regulatory relationship. Also, the authors could determine the effect of over-expressing LIP in del uORF cells on metabolic parameters such as expression of putative target genes (assuming these effects are seen in the mutant MEFs), or on the ability of these cells to undergo adipogenesis (Supplementary Figure 3).
4. There are some discrepancies regarding the identity of LAP bands among the various gels. For example, the LAP bands depicted in Fig. 2a and b appear to be different (also in Supplementary Figure 1). Since the LAP/LIP ratio is central to the biochemical analysis of the present work, it is essential to accurately identify the bands and include proper controls. e.g., it would be informative to use C/EBPbeta knockout MEFs or RNAi ablation as negative controls or C/EBPbeta over-expression samples as positive controls for the Western blots.
5. According to the authors' model, the del uORF mice are not expected to show further metabolic improvement in response to rapamycin or CR. Has this been tested?
6. The levels of p4-EBP1 reduction in response to rapamycin treatment seem to be variable. For example, in Figure 2b rapamycin treatment did not affect the level of p-4EBP1 in adipose tissue, yet LIP levels were decreased. This result appears to contradict the authors' conclusion that the effects of rapamycin on LIP synthesis occur via 4EBP1 and not levels of pS6K1.

Minor comments:

1. The 4EBP1 Western blots show multiple bands that change upon treatment. What are the identities of the various species?
2. Since inhibition of mTORC signaling apparently reduces LIP levels through 4EBP1 rather than S6K1, it is necessary to show the level of p-4EBP1 in Figure 2a.
3. It would be useful to independently demonstrate the lack of involvement of S6K1 in regulating LIP using an alternative approach such as RNAi.

Referee #3:

This is an incredibly interesting and generally well executed paper by the authors. The authors have identified a novel indicator of mTORC1 signaling that can be used both in vitro and in vivo, and serves as a functional readout of 4EBP function. The authors have gone on to address the role of this effect on in vivo metabolism. With minor revisions, this manuscript will likely be suitable for publication, and will likely be highly cited in the field.
Major revisions:
1. For all figures with LAP/LIP quantification, error bars and statistical differences should be

indicated in each figure. N value should also be indicated in figure legend. Particularly missing in Figure 1.
2. The authors should explain if CEBP is expressed only in the liver, and if not, what other tissues it it is expressed in. The results suggest it is in fat, but muscle could also be examined if expressed there.
Minor revisions:
3. Figure 1: F1- Which is the n on this experiment? It would be helpful to also quantify at least 4EBP1, and present stats in every graph. It would be helpful to include the length of the treatments in the footnote of the figure.
4. Figure 1b: As total LAP is increased in 4EBP double KO MEF cells, it might be helpful to include quantification of LAP and LIP and stats on them.
5. Figure 2a: The feeding status of the mice should be indicated (ie, fasted overnight?). Also 4EBP1 phosphorylation should be shown.
6. Figure 2b - Why was fat tissue was harvested 12 h after treatment and not 24 h as liver?
7. Figure 4c: LDL is indicated as increased in the figure, is the figure or the text correct?
8. Figure 5d - At what time of the day was glucose measured? How many hours were the mice fasted before measuring blood insulin? Fasting glucose would be more informative as HOMA2-IR could be calculated.
9. Figure S1b - Are 4EBP-1 and LIP significantly reduced by rapamycin in the three cell types?

1st Revision - authors' response 29 March 2015

Thank you for considering our manuscript for publication in EMBO Reports and for your thoughtful comments. We thank the reviewers for their constructive criticism. We have dealt with all their comments; please find our replies below. All changes and additions in the main text are marked in red. Figure legends and supplementary/expanded view information were completely revised.

Referee #1:

Zidek et al. investigate the role of C/EBPbeta-LIP in metabolism. The authors found that mTORC1 signaling specifically regulates the expression of the C/EBPbeta-LIP but not -LAP isoform. Moreover, mice with reduced C/EBPbeta-LIP expression are metabolically healthier as compared to control littermates, suggesting that beneficial metabolic effects due to reduction of mTORC1 activity, for example upon caloric restriction (CR), might be mediated via regulation of C/EBPbeta-LIP levels. The results presented in this study are of interest although a bit on the descriptive side. The most worrying aspect of the study is that the transcriptional effects are quite small and it is therefore questionable whether the whole body effects are in fact due to the transcriptional defects. Finally, the manuscript would be improved if they examined whether reduced C/EBPbeta-LIP levels indeed account for the beneficial metabolic effects observed upon CR, although this might be beyond the scope of the paper.

We agree that the transcriptional effects are moderate. We have now added immunoblots in figure S4A and D showing that changes in expression on protein level generally follow the observed changes in transcription. We describe these new results at lines 255-258/263/287- 289. We also discuss this at lines 385-398 in the discussion session of the manuscript.

Specific comments:

1. In figure 2C the authors state that mTORC1 activity and LIP levels remain relatively constant throughout the day. However, from the image provided it is not clear whether the samples from the two time points (3h and 14h) were run on the same gel. Only samples that were loaded on the same gel can be compared to each other. In order to make a statement about changes of mTORC1 activity and LIP levels throughout the day in fed ad

libitum mice, both time points should be run on the same gel and compared to each other.

We agree with the reviewer that only samples can be compared which are loaded on the same gel. Therefore we compared the LAP and LIP levels of samples from mice fed ad libitum and under caloric restriction loaded on the same gel (Fig 2C, E). We chose to compare fed ad libitum versus caloric restriction for each time point on a single blot since this is what we were interested in. We used the signal quantification of the LAP/LIP ratio for overall comparison because this is the main focus of our study. We agree that a comparison of the two time points from two different blots is not possible. Since we see a clear phosphorylation signal of S6K1 after 3h in AL and in CR animals but after 14h only in the AL fed mice but not in the CR mice we believe that there is a more pronounced difference in mTORC1 signaling between the differentially fed animals after 14h compared to the 3h time point. This is also reflected by the LAP/LIP ratio that is clearly increased only under CR conditions at 14h.
Therefore we changed the text accordingly. The statement the reviewer refers to “remain relatively constant” is therefore changed to: “AL fed mice display more moderate diurnal cycles of fuel selection with a relative high mTORC1 activity and a LAP/LIP ratio that stays constant at 3h and 14h” (lines 154-156).
Although interesting, also because of possible circadian rhythm involvement, we hope that leaving out the direct comparison of mTORC1 signaling and expression of the single C/EBPβ isoform levels of AL fed mice at the different time points may not negatively affect the paper.
We are sorry that we have not fully fulfilled this first point. However, we tried to compensate this with all the new data that we have added.

2. In figure 2D the signal for LIP is very weak. The authors should show a higher exposure to make LIP levels more visible.

The uORF-mutation causes LIP protein expression to be strongly reduced; the small amount of signal is probably due to leaky scanning of ribosomes over the LAP*/LAP translation initiations sites. We now included a blot (Suppl. Fig S2B) showing wt and delta-uORF on a single blot for direct comparison, we also used a longer exposure as requested by the reviewer.

3. In supplementary figure 3A the authors state that C/EBPalpha and PPARgamma expression is increased in visceral adipose tissue of C/EBPbeta delta uORF/Bl6 mice. However, according to the p-value the difference between the mutant and the control mice is not statistically significant. The authors should change this sentence in the text and state that expression of adipogenic genes is unaltered in C/EBPbeta delta uORF/Bl6 mice.

We thank the reviewer for pointing this out and have now changed the text accordingly (line 192-193).

4. In figure 4E expression levels of genes involved in lipogenesis and lipolysis in white adipose tissue (WAT) are shown. In general the observed changes in expression are relatively small. A Western blot for some of the tested genes should be performed, for example for ACC, FAS, GLUT4, and HSL to investigate whether these small changes in expression are reflected in changes in protein level. Moreover, for LPL, aP2 and ATGL the pvalue does not indicate a significant change between control and C/EBPbeta delta uORF/Bl6 mice. The authors should correct the interpretation in the text.

We have now added immunoblots in Suppl. Fig S4A and D showing that expression on protein level generally follow the transcriptional changes. We describe this at lines 255-258/263. We also discuss this at line 385-398 in the discussion session.
We also changed the text for interpretation of the LPL-, aP2- and ATGL-expression (line 244-246 and 254-255).

5. The authors should determine mRNA and protein expression levels of genes involved in

beta-oxidation in WAT.

We added data showing mRNA expression of beta-oxidation genes (Fig 4F) and an immunoblot of the only enzyme we found to be upregulated (MCAD, also upregulated) (Suppl. Fig S4A).

6. In figure 4F the expression levels of genes involved in lipogenesis and beta-oxidation in liver are shown. As for the results presented in figure 4e, the presented changes are relatively small. It would be of importance to investigate the protein levels of some of the presented genes, for example CD36, LCAD, ACC, and FAS to investigate whether these small changes in expression are reflected in changes in protein level.

We included data showing protein levels by immunoblotting demonstrating that FAS and ACC protein levels follow transcript levels, while protein levels of MCAD, AOX or CD36 did not (Suppl. Fig S4D). Data are described at lines 287-289.

7. The authors conclude that the increased energy expenditure of C/EBPbeta delta uORF/Bl6 mice can be explained by an increase in locomotor activity. To this reviewer the presented differences in activity seem rather small and therefore unlikely the main reason for the increased energy expenditure. Increased activity of brown adipose tissue (BAT) or browning of WAT could account for the observed increase in energy expenditure in the mutant mice.
The authors should investigate BAT function and WAT browning, for example by measuring mRNA and protein expression levels of genes involved in uncoupling and beta-oxidation, such as UCP1, Dio2, and PGC-1a in BAT and by measuring BAT markers, such as UCP1, Dio2 and Cidea in subcutaneous WAT.

We agree that information about potential browning is interesting and therefore analyzed browning of WAT in C/EBPbeta delta uORF/Bl6 mice. The new data in Fig S4E show that subcutaneous inguinal WAT and visceral epididymal WAT have very low to no expression levels of browning genes or uncoupling genes and that no increase is observed in C/EBPbeta delta uORF/Bl6 mice. Therefore we conclude that no browning is taking place. Description of the results can be found in lines 300-306.

8. In figure 5C insulin responsiveness of skeletal muscle is shown. To understand whether increased insulin responsiveness can be observed also in other metabolic tissues it would be of interest to investigate insulin responsiveness in WAT and liver of control and C/EBPbeta delta uORF/Bl6 mice.

We performed the proposed experiments, and now added the data for liver in Suppl. Fig S5A, and changed the text accordingly in line 326. For the experiments with WAT we could not obtain a reliable p-Akt signal, therefore we did not include those data.

9. In figure 5D and 5E only fed glucose and fasting insulin levels are presented. Also fasting glucose and fed insulin levels should be shown.

We now included the requested data for glucose and insulin in the Figure 5D and E, and added description at lines 329-331.

10. The authors speculate that the beneficial metabolic effects elicited by CR are caused by decreased C/EBPbeta-LIP expression. To test this hypothesis, the authors could perform a CR experiment with control and C/EBPbeta delta uORF/Bl6 mice and monitor body weight, glucose homeostasis, metabolic rate and gene expression profile in metabolic tissues. This would reveal whether CR still exerts additional beneficial metabolic effects in C/EBPbeta delta uORF/Bl6 mice or whether CR mediates its beneficial metabolic effects mainly via decreasing C/EBPbeta LIP expression.

This is truly an interesting question, however, it is not feasible to do the required

experiments within the scope and time of this study.

Referee #2:

Zidek et al. report that translation of the truncated LIP isoform of CCAAT/Enhancer Binding Protein beta (C/EBPbeta/LAP) is suppressed by inhibiting mTORC1 signaling. Although mTORC signaling was previously reported to regulate the LAP/LIP ratio, the current study extends this observation to show that interventions such as rapamycin treatment or calorie restriction (CR), which lead to reduced mTOR activity, suppress LIP translation. Of note, the authors also used a genetic approach to demonstrate that deletion of the uORF element within the C/EBPbeta mRNA, which is required for translation of the LIP polypeptide, confers a metabolic phenotype that mimics calorie restriction in vivo.
The authors have made interesting observations regarding the physiological consequence of deleting the C/EBPbeta uORF, particularly their finding that the mutant at least partially phenocopies the effects of CR or reduced mTOR signaling. The study would be improved by including a more thorough analysis of the mechanism by which LIP regulates metabolic function. As it stands, this part of the paper is somewhat vague and descriptive. For example, it is unclear whether the effects of the del uORF mutant result from reduced LIP per se or whether the increased LAP:LIP ratio leads to elevated LAP activity and thus improved metabolic function. These and other issues listed below should be addressed to strengthen the paper.

The question whether the effects are due to reduced LIP per see or increased LAP:LIP ratio is very interesting. This relates to the question whether LIP is merely a negative competitor for LAP functions (which taken all literature into account certainly is a main function), or if LIP has specific functions different from LAP functions. In our opinion this can only be answered at the animal-physiological level by comparing delta-uORF, LAP-only, LIP-only and KO mice for metabolic parameters. We feel that this is beyond the scope of the manuscript.

Specific comments:

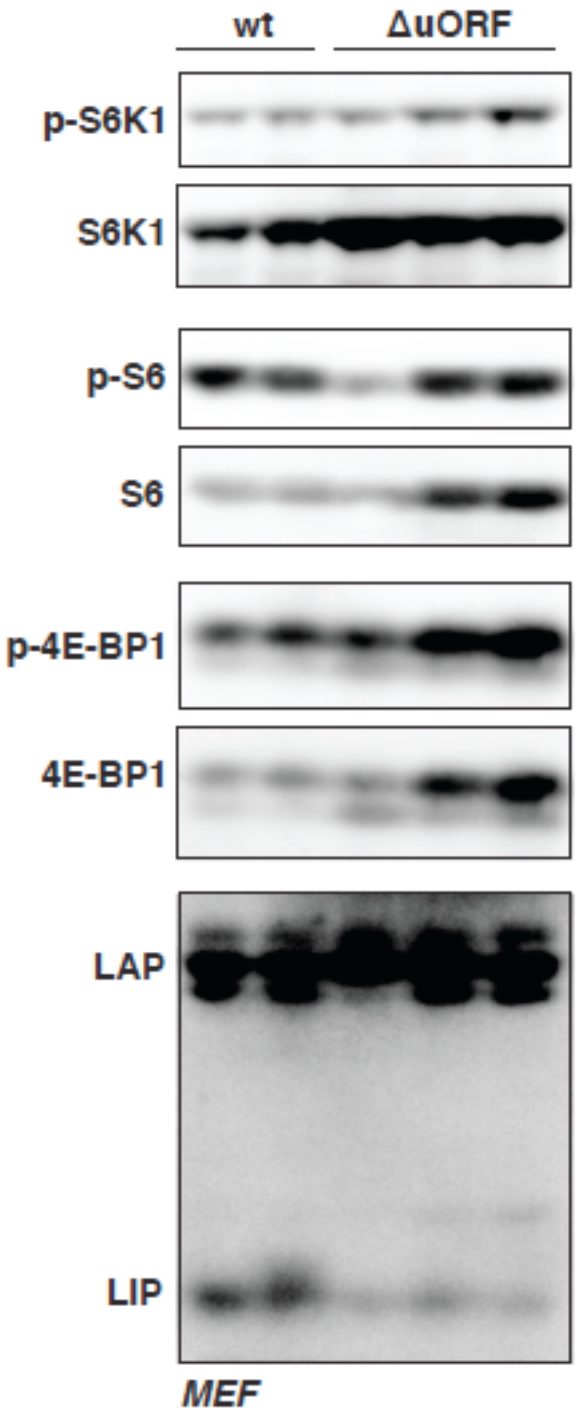
1. It would be informative to compare the phenotypes of the del uORF mice and the complete knockout of C/EBPbeta. The latter mice were reported to display decreased body weight and less fat mass, somewhat similar to the del uORF animals. A comparison of the two mutants would give some insight into the function of LAP (which is retained in the del uORF strain) in regulation of energy metabolism.

We added a brief discussion about the phenotype of the C/EBPbeta KO studies at lines 399-414.

2. A direct, side by side comparison of mTOR signaling in WT and del uORF cells would be helpful. This might rule out the possibility that mTOR signaling is affected in the mutant cells and is partially or largely responsible for the observed phenotype. The paper implies that LIP regulates metabolic genes downstream of mTORC, but this is not directly demonstrated.
There could be some feedback effects from LIP that in turn control the mTORC pathway. For example, it has been reported that C/EBPbeta negatively regulates mTOR signaling under stress conditions through induction of Redd1 (Ho et al., Free Radic Biol Med. 2009 46:1158-67).

We now show samples from wt and delta-uORF mice on the same immunoblot (Suppl. Fig S2C). The data from livers of wt and delta-uORF mice (3h post feeding) demonstrated no significant difference in p-4E-BP1/4E-BP1 or p-S6/S6, at least under these (non-stress) conditions (Suppl. Fig S2C); lines 175-179.
In addition, we present here the analysis of mTORC1 signaling in (immortalized) MEFs vs. delta-uORF MEFs. In MEFs we see an up-regulation of particular S6K1 (lowering the p-/pan ratio). Similar changes for S6 and upregulation of both 4E-BP1 and P-4E-BP1 (similar

p-/pan ratios) were obtained for the delta-uORF MEFs. We are not sure if it would be helpful to present this data within the manuscript. We think that it will further complicate the study, especially since these changes were only found in cultured cells. Therefore we ask the reviewer to advise whether or not to include and describe the data in the paper.



3. The authors should provide some data to demonstrate that LIP and/or LAP directly regulate some of the metabolic genes that are affected in the del uORF mutant. ChIP

experiments or analysis of published genome-wide protein interaction data (e.g., ENCORE) might be presented to support a direct regulatory relationship. Also, the authors could determine the effect of over-expressing LIP in del uORF cells on metabolic parameters such as expression of putative target genes (assuming these effects are seen in the mutant MEFs), or on the ability of these cells to undergo adipogenesis (Supplementary Figure 3).

Using the ENCODE data base we have in silico analysed the promoter occupation (ChIP-seq) by C/EBPbeta of all target genes used and found that all genes have been found to be associated with C/EBPbeta by ChIP-seq. Supplementary Table 1 lists the results. We explain the results in line 289-293.
We used delta uORF MEFs and delta uORF MEFs with ectopic expression of LIP-FLAG and analyzed their adipogenic gene expression profile (LPL, CD36, SREB1c, ACC, FAS, SCD1, HSL). Ectopic expression of LIP-FLAG inhibits the adipogenic gene expression (figure S4B).
We also generated a 3T3-L1 cell line with inducible expression of LIP (cumate system). The 3T3-L1 system was used to examine adipogenesis (INS/DEX/IBMX induced) (figure S3C) and adipogenic gene expression. Induction of LIP inhibits adipogenesis and fat accumulation (Suppl. Fig S4C); we describe our findings at lines 265-279.

4. There are some discrepancies regarding the identity of LAP bands among the various gels.
For example, the LAP bands depicted in Fig. 2a and b appear to be different (also in Supplementary Figure 1). Since the LAP/LIP ratio is central to the biochemical analysis of the present work, it is essential to accurately identify the bands and include proper controls. e.g., it would be informative to use C/EBPbeta knockout MEFs or RNAi ablation as negative controls or C/EBPbeta over-expression samples as positive controls for the Western blots.

The expression of LAP and LAP* appear differently in different tissues and cell lines as well as under different conditions. LAP* looks often very differently in levels and appearance. In an earlier paper we have extensively examined the identity and origin of the isoforms (Calkhoven et al 2000 Genes & Dev 14, 1920). We have no doubt about the specificity of the antibody as it is widely used for many years by the community including ourselves. To specify the isoforms we now included blots from MEF: wt, C/EBPbeta knockout and C/EBPknockout with overexpression of LAP*, LAP and LIP in Suppl Fig S1A.

5. According to the authors' model, the del uORF mice are not expected to show further metabolic improvement in response to rapamycin or CR. Has this been tested?

Although this is definitely interesting we have not tested this.

6. The levels of p4-EBP1 reduction in response to rapamycin treatment seem to be variable.
For example, in Figure 2b rapamycin treatment did not affect the level of p-4EBP1 in adipose tissue, yet LIP levels were decreased. This result appears to contradict the authors' conclusion that the effects of rapamycin on LIP synthesis occur via 4EBP1 and not levels of pS6K1.

There is some reduction in the p-4E-BP1 signal, which correlates with a shift to more alpha- 4E-BP1 signals in the 4E-BP1 blot referred to by the reviewer. We added a quantification to make this clearer to the reader. We agree that is a moderate regulation. (4E-BPs also seem be subject of rapamycin-resistant phosphorylation depending on the cell type (published by Thoreen et al (2009) JBC 284, 8023-8032), additionally the kinetics of 4E-BP1-phosphorylation and C/EBPbeta regulation maybe different but still related.)
We improved the labeling for the 4E-BP1 and p-4E-BP signals in all relevant figures (1, 2, S1, S2) and added quantification of relative alpha/beta/gamma-4EBP1 signals.

Minor comments:

1. The 4EBP1 Western blots show multiple bands that change upon treatment. What are the identities of the various species?

We improved the labeling for the 4E-BP1 and p-4E-BP signals in the Figures 1, 2 and S1 and now explain the identity of the bands in the text (lines 92-96).

2. Since inhibition of mTORC signaling apparently reduces LIP levels through 4EBP1 rather than S6K1, it is necessary to show the level of p-4EBP1 in Figure 2a.

We added p-4E-BP1 and 4E-BP1 blots and quantifications for 2A, B and D. The alterations in phosphorylation are moderate but visible (see also reply at point 6).

3. It would be useful to independently demonstrate the lack of involvement of S6K1 in regulating LIP using an alternative approach such as RNAi.

We have generated MEFs with knockdown of S6K1 and observed that S6K1 KD does not reduce LIP expression but rather enhances it. So this is very similar to the DG2 treatment (Fig 1C). Data are added in Supplemental Fig S1D. Text was added at lines 105/ 112-114.

Referee #3:

This is an incredibly interesting and generally well executed paper by the authors. The authors have identified a novel indicator of mTORC1 signaling that can be used both in vitro and in vivo, and serves as a functional readout of 4EBP function. The authors have gone on to address the role of this effect on in vivo metabolism. With minor revisions, this manuscript will likely be suitable for publication, and will likely be highly cited in the field.

We thank the reviewer for this positive comment on our manuscript.

Major revisions:

1. For all figures with LAP/LIP quantification, error bars and statistical differences should be indicated in each figure. N value should also be indicated in figure legend. Particularly missing in Figure 1.

We now provide quantification of LAP/LIP levels for Fig 1A, B, C and Suppl. Fig S1B calculated from independent experiments (see legend for n values). For all experiments n values are now added in the figure legends and statistical differences are stated in the graphs whenever appropriate.

2. The authors should explain if CEBP is expressed only in the liver, and if not, what other tissues it it is expressed in. The results suggest it is in fat, but muscle could also be examined if expressed there.

C/EBPbeta mRNA is broadly expressed in many tissues including liver, muscle and adipose tissues (http://www.genecards.org/cgi-bin/carddisp.pl?gene=CEBPB). We added information about C/EBPbeta expression in the introduction at line 43-45. The role of C/EBPbeta in muscle is currently under investigation and will be subject of another manuscript.

Minor revisions:

3. Figure 1: F1- Which is the n on this experiment? It would be helpful to also quantify at least 4EBP1, and present stats in every graph. It would be helpful to include the length of the treatments in the footnote of the figure.

We now provide quantifications for LAP/LIP (see point 1 and quantification of the

percentages alpha, beta and gamma 4E-BP1 bands on the blots). Length of treatments is now included in the figure legends.

4. Figure 1b: As total LAP is increased in 4EBP double KO MEF cells, it might be helpful to include quantification of LAP and LIP and stats on them.

We thought that it would be more relevant in the context of this manuscript to show quantification of the LAP/LIP ratios. Therefore we included those in the figures. However, separate quantification of LAP and LIP can still be included if the reviewer wishes. We felt that figures might get too crowded if we add more quantifications.

5. Figure 2a: The feeding status of the mice should be indicated (ie, fasted overnight?).

We included the feeding status of the mice in the figure legends.
Also 4EBP1 phosphorylation should be shown.

Please see reply at reviewer minor point 2: We added P-4E-BP1 and 4E-BP1 blots and quantifications for 2A, B and D. The alterations in phosphorylation are moderate but significant (see also reply at point 6).

6. Figure 2b - Why was fat tissue was harvested 12 h after treatment and not 24 h as liver? We performed for both time points in both tissues.

The effect was more prominent in WAT at 12h compared to 24h, in the liver it was the other way around. Therefore we included the data for 12h for WAT and 24h for liver in the figure. The reasons may be plural, e.g. diffusion differences of the drug, different uptake in tissues, differences in signaling.

7. Figure 4c: LDL is indicated as increased in the figure, is the figure or the text correct?

We corrected the text in lines 229-230.

8. Figure 5d - At what time of the day was glucose measured?
How many hours were the mice fasted before measuring blood insulin?

We added the information in the figure legends.

Fasting glucose would be more informative as HOMA2-IR could be calculated.

We added fasting glucose levels (Fig. 5D) and calculated HOMA2-IR, now shown in Fig 5F.

9. Figure S1b - Are 4EBP-1 and LIP significantly reduced by rapamycin in the three cell types?

See also point 1. Considering Figure 1A and S1B we can conclude that the catalytic inhibitor pp242 reliably inhibits S6K-phosphorylation, 4EBP1-phosphorylation and LIP expression.
Rapamycin inhibits S6K-phosphorylation in all cell types tested, however it shows cell type dependent variation on 4E-BP1-phosphorylation and LIP expression, which are correlated (we describe this in line 96-99). This is a known effect of rapamycin as was described in Thoreen et al (2009) JBC 284, 8023-8032. We have now extended Fig 1A and S1B with quantification graphs for p-4E-BP1/4E-BP1 and LAP/LIP.

2nd Editorial Decision 24 April 2015

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees that were asked to assess it. Referee 2 still has a few suggestions that need to be addressed and incorporated before we can proceed with the official acceptance of your manuscript. Referee 1 agrees with these remaining concerns, as indicated in her/his cross-comments pasted below.

The legends for figures 2B, 5F and EV4B say that n=2. In this case, no error bars can be shown. Please either repeat the experiment one more time, or remove the error bars and show instead the 2 individual data points in the graphs. EV3 and 4 are missing the panel labels.

I noticed that the manuscript is rather long for a short report, and I apologize for not explaining well enough our 2 different formats: normal articles versus short reports. For a short report, a maximum of 5 main figures can be included, the text may not exceed 35.000 characters, the results and discussion section must be combined, and parts of the materials and methods can be moved to the supplementary information. Your manuscript is rather in an article format, with separate results and discussion sections, all materials and methods in the main file and more than 64.000 characters. In order to change it into an article, we need to include one more main figure. You could either move one of the expanded view figures to the main article, or split one of the current main figures. If you rather publish the manuscript as a short format, you need to shorten the manuscript text, move commonly used materials and methods to the expanded view file and combine the results and discussion sections.

I look forward to seeing a final version of your manuscript as soon as possible. Please let me know if you have any questions.

REFEREE REPORTS:

Referee #1:

The authors performed most of the requested experiments and provided a satisfying response to all my concerns. The quality of the manuscript has improved and I can therefore recommend acceptance.

Minor comment:
The authors mention in line 77/78 of the introduction that glucose and insulin levels are "better". The authors should change "better" to, for this context, a more appropriate word, for example reduced or decreased.

Referee #2:

In their revised manuscript the authors have responded to many of the points raised by this referee and the two other reviewers. However, a few issues remain and should be addressed prior to acceptance of the manuscript.

Comments:

1. Some questions persist about the effects of the DuORF mutant on expression of lipogenic and lipid oxidation genes and how these alterations lead to the metabolic phenotype observed in vivo. The mutant cells/tissue show elevated expression of lipogenic as well as beta oxidation genes, and over-expression of LIP in MEFs reduces both. The authors need to articulate a model for how these changes cause decreased adiposity in the DuORF mutant, since increased lipogenesis would tend to enhance, not decrease, fat deposition. Furthermore, DuORF MEFs show increased differentiation

into adipocytes, and LIP suppresses adipogenesis when over-expressed in MEFs. The authors should also explain these effects, as they appear to contradict the observation that mutant mice with reduced LIP levels exhibit less WAT.

2. The quantification of p-4E-BP1 levels and phosphorylation is somewhat unclear. The associated graphs show the relative levels of the 3 isoforms and presumably do not represent the phosphorylated to unphosphorylated ratios. The latter parameter seems to be the most important for LIP regulation and should be shown in the figures rather than the ratios of the different isoforms. Because the effects of rapamycin on 4E-BP1 are subtle at best and are not always consistent with the effects on LIP expression, the authors should consider removing these data from the paper.

3. The data presented to the reviewers on mTORC1 signaling in WT and DuORF MEFs show significant sample to sample variation and lack loading controls such as beta-actin. In their current form results do not add much to the paper and should not be included. The variability between cell preparations is a concern; could this be due to differences in immortalization between individual cell lines?

4. The ENCODE "data" presented in Supplemental Table 1 are not illuminating as they consist only of web links to genes of interest in the ENCODE database. The authors should summarize the pertinent data for each putative target gene in a table or figure, indicating information such as where C/EBPbeta binds in the gene, whether the site is located in a known regulatory region, and what tissue/cell line was used for the analysis. These data will help the reader evaluate the importance of C/EBPbeta binding in relation to the authors' hypothesis.

Minor comments:

1. In some places the authors define RER as respiration exchange "rate," when it should be "ratio."

2. The text should be carefully edited for proper English, clarity, and grammar, particularly in the newly added sections.

Cross-comments from Referee #1:

1. We agree that the authors could discuss this point better to improve the understanding of their results. It seems that in general metabolic activity is increased in WAT of the mutant mice, which could account for decreased adiposity.
The results on in vitro adipocyte differentiation are also confusing to us. The authors should provide an explanation for their observations.

2. The authors should quantify p4E-BP/total 4E-BP.

3. We are not sure to which result the other reviewer is referring.

4. We agree with this point. The ENCODE "data" presented do not provide additional information regarding C/EBPbeta binding in the way they are presented.

2nd Revision - authors' response 19 May 2015

Please find the final version of our manuscript uploaded in the online submission system. We have made additions and changes according to the requests made by you and the reviewers and hope that these are sufficient to allow for official acceptance of the manuscript.

- we now show individual data points for Figure 2B and EV4B.
- we changed the manuscript into the article format with 6 main figures. Data from Figure 4, panels F, G and H, and from Figure EV4E are now combined in new Figure 5. Old

Figure 5 has become Figure 6.

Referee #2:
point 1: we now explained and discussed our theory on fat turnover in more detail at lines 360-381
point 2: we feel that quantification of the relative levels of alpha-hypophosphorylated, beta-hyperphosphorylated and gamma-hyperphosphorylated is the best way to quantify p-4E-BP1/4E-BP1, because signals originate from the same blot and antibody. The p-4E-BP1 antibody blots confirm that beta and gamma bands represent the hyperphosphorylated isoforms. We now marked the beta and gamma legends as phosphorylated in the figure and explain the experiment now better at lines 93-97.
Point 3: These data were presented in the point-to-point reply. The omission of the loading control is an unfortunate mistake but loading was comparable (we can provide the blot). Also our interpretation is that differences are due to differences in immortalization. We agree with the reviewer not to include these data into the manuscript.
Point 4: We changed Supplementary Table EV1 according to the referee's suggestion and added description at lines 295-298:" This analysis revealed that all genes analysed are associated with C/EBPβ at regions that are in most cases associated with the histone H3 lysine 27 acetylation (H3K27Ac) mark characterising active enhancers (Table EV1)."

Minor point 1: we exchanged "rate" for "ratio".
Minor point 2: We have edited the text for clarity and grammar. In the abstract it was falsely stated that genetic elimination of 4E-BPs would suppress LIP. To correct this we changed the text at lines 33-37.
The discussion was more extensively edited particularly at lines 356-381 dealing with point 1 to improve clarity without changing the concept or information content.

Cross- comments from Referee #1:
Point 1: see point 1 above
Point 2: see point 2 above
Point 3: see Point 3 above
Point 4: see point 4 above

We would like to thank you and the reviewers very much for helping us to improve the manuscript.

3rd Editorial Decision 21 May 2015

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.

