THE EMBO JOURNAL

Manuscript EMBO-2015-91385

The transcription factor Cabut coordinates energy metabolism and the circadian clock in response to sugar sensing

Osnat Alsheich-Bartok, Mari Teesalu, Reut Ashwall-Fluss, Varun Pandey, Mor Hanan, Bohdana Rovenko, Minna Poukkula, Essi Havula, Arieh Moussaieff, Sadanand Vodala, Yaakov Nahmias, Sebastian Kadener and Ville Hietakangas

Corresponding author: Ville Hietakangas, University of Helsinki

Review timeline:

****Submission date:	20 June 2014
Editorial Decision:	24 July 2014
Appeal:	31 July 2014
Additional correspondence - editor:	17 September 2014
Resubmission:	25 February 2015
Editorial Decision:	27 March 2015
Revision received:	31 March 2015
Accepted:	01 April 2015

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: Anne Nielsen

1st Editorial Decision 24 July 2014

Thank you for submitting your manuscript for consideration by The EMBO Journal and my apologies for the slight delay in communicating our decision to you. Your study has now been seen by three referees whose comments are shown below. As you will see, while the referees all express interest in the work and topic in principle, they all do not offer strong support for publication in The EMBO Journal.

I will not repeat all their individual points of criticism here, but it becomes clear that the referees find the depth of analysis to be too limited and that the study is thus too premature for them to support its publication here. While I recognise that referee #3 is more positive about the study, ref#2 finds that further functional insight - especially on the intersection with circadian regulation - would be neeeded to make the manuscript a strong candidate for publication in The EMBO Journal. At the same time ref#1 argues that the specific interplay between PEPCK expression and glyceroneogenesis would have to be more conclusively shown and extended to a much broader metabolite analysis. Clearly, an extensive amount of further experimentation would be required to address these issues and to bring the study to the level of insight and significance required for

publication here. Furthermore, the outcome of such experiments cannot be predicted at this point and would thus lie outside the scope and the timeframe of a revision.

Given these negative opinions and extensive requests from the referees and the fact that the EMBO Journal can only afford to accept papers which receive enthusiastic support from a majority of referees, I am afraid we are unable to offer further steps towards publication in The EMBO Journal at the current stage.

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

****

Referee #1:

The manuscript by Bartok and colleagues characterizes the transcriptional network downstream of the fly Klf-10 orthologue cabut (cbt). Although previous studies have reported links between several of the genes, physiological processes and metabolites described in this manuscript, the authors' emphasis in the network's nutritional regulation and physiological significance is novel and leads to the identification of a metabolically significant downstream gene: PEPCK. My only major issue concerns the link between PEPCK and glyceroneogenesis in the context of sugar adaptations (which, in my opinion, is the most novel/unexpected finding in the manuscript):

Firstly, the phenotypic analysis of PEPCK mutants is too superficial. Are these mutants developmentally delayed? Do they attain a normal size/eclose at normal numbers? Any development al effects could lead to secondary effects on lipid and carbohydrate metabolism.

Secondly, the authors largely ignore the effect of PEPCK loss on glucose levels (PEPCK is after all the main rate-limiting enzyme in gluconeogenesis). Any glycolysis/gluconeogenesis imbalances could impact on glycerol 3 phosphate availability in a glyceroneogenesis and Gyk-independent way. Hence, the statement that "cabut regulates glyceroneogenesis" is not supported by the available data. More extensive metabolic profiling might support this statement.

Related to this, even if the constitutive role of PEPCK is to maintain steady-state glycerol levels, the nutritional regulation of PEPCK downstream of mlx/cbt may serve a different function. The most compelling experiment arguing against this possibility is that the abnormal glycerol levels of mlx mutants are restored in the mlx, pepck double mutant (which is very nice), but did the authors look at other metabolites (e.g. glucose, trehalose)? How specific is this phenotype to the glyceroneogenesis pathway? The metabolomics experiment suggested above might hopefully show changes in, for example, OAA/phosphoenolpyruvate ratio, but they would always be correlative by definition. The authors should also test whether targeted genetic manipulation of other enzymes in the glyceroenogenesis pathway can modulate the mlx/cbt phenotypes.

Minor points
1. None of the dietary experiments control for nutrient quality rather than quantity - for example, no isocaloric controls were used in refeeding experiments so it is unclear whether gene expression changes result from exposure to sugars or, more generally, to nutrients after a period of starvation. These controls should ideally be provided. Failing that, statements about "sugar responses" should be replaced with more general statements about diet.
2. The lack of knowledge about the metabolic roles of cabut-like proteins as ChREBP targets is overstated - see, for example, references in Iizuka et al Endocrine Journal 2013, 60 (5), 543-555.
3. Genotypes for all control and RNAi experiments as well as n numbers need to be provided in either the figure panels or legends. In the methods, the authors should also provide information about age, sex etc of the adult flies used.
4. Page 7. I assume that the first refeeding experiment was done in adults?
5. Page 11. Please provide reference for the statement "circulating glucose is derived from dietary intake". This is an important point that should be discussed given that only glucose (and not trehalose) is affected in the mutants. I would have also liked to see some discussion about the

apparent lack of mitochondrial PEPCK in flies (and expected metabolic consequences). In general, the discussion is a bit weak - What have we learned? What is unexpected and why might this be?
6. The reference to Figure 3E (page 8) needs to be amended (there is no Figure 3E).
7. The genetic setup for the microarray/RNAseq experiments is odd to say the least - rather than confining cbt downregulation to a couple of random tissues, ubiquitous (or fat body), temporally restricted gene downregulation in adult flies by means of a tub-Gal4 (or r4-Gal4), tub-Gal80ts transgene would have been more appropriate, especially given that 1) developmental and circadian phenotypes were expected and 2) mlx has been shown to act primarily in the fat body. More than one RNAi line should have been used to confirm the phenotypes. It is unclear why some experiments were carried out in larvae and some in adults. That said, the transcriptional data is supported by multiple gain- and loss-of-function experiments so the main findings are probably sufficiently supported. I am not going to ask the authors to repeat all their transcriptional profiling, but some discussion of the strengths/weaknesses of the current approaches would be appreciated.
8. Figs 6A and 6B (and related to the previous point). Why was the cbt RNAi experiment not carried out using the same FB driver used to rescue the mlx mutants in the previous panel? Can the glycerol levels of an mlx mutant be rescued by FB-specific reintroduction of cbt? Also, in order to make those two experiments more comparable the same scale should be used for both bar graphs.
9. "Group 2" seems to be absent from Figure 3B.

Referee #2:

Comments to the authors
Bartok et al. investigate the mechanisms of transcriptional repression by sugars in Drosophila. Previously, the authors showed that Mondo/ChREBP-Mlx controls glucose and lipid metabolism and also promotes expression of the Krüppel-like transcription factor cabut (cbt). Here, the authors show a repressive branch of the sugar-sensing transcriptional network, i.e., CBT is a repressor. Upon sugar feeding, cbt expression is enhanced in an Mlx-dependent manner. Using a genome wide analysis, the authors show that CBT represses metabolic genes including pepck, encoding the rate-limiting enzyme for gluconeogenesis and glyceroneogenesis. Using genetic approaches, they also show that Mondo/Mlx negatively regulates pepck and that Mlx controls glycerol homeostasis via PEPCK.

Major points:

1. A major concern of the current study is that it does not significantly extent prior knowledge, albeit from rodents. An experiment that could be performed to extend the current work is to look at Mondo/ChREBP-Mlx, CBT and carbon metabolism in the context of circadian rhythm given that CBT is also regulated by CLOCK. Other suggested experiments (not all of which are necessary) that could also extend the current study are proposed below.

2. To overexpress or knockdown cbt, the authors use the tim-gal4 driver where a gene of interest is widely expressed in cells harboring an active circadian clock. Since the authors previously reported that Mondo/Mlx is functionally important in the fat body (Havula et al., 2013), why did the author not use the fat body (r4)-specific gal4 driver?

3. How does CBT expression-mediated regulation of glycerol and triglyceride homeostasis affect fat body mass?

4. Figure 2: The authors describe 2 putative carbohydrate -responsive elements (ChoRE) in the cbt promoter region. Is Mlx binding to ChoRE dependent on sugar availability? The authors should perform a luciferase reporter assay and determine whether one or both ChoREs are necessary/sufficient for Mlx-dependent cbt transcription.

5. Figure 3 and S3: For the genome wide approaches, the design of the experiment does not include a proper control. Fly heads from fed and starved flies should be collected at the same time of day due to circadian changes in metabolism.

6. The authors show that CBT represses the expression of genes involved in glycerol biosynthesis such as pepck and glycerol kinase (Gck) but then focus exclusively on PEPCK in the Mlx/CBT-mediated regulation of glycerol metabolism. How much does GCK contribute to the glycerol metabolism phenotype?

7. Figure 4C: How do the authors explain that pepck expression in not repressed by sugar in a dose dependent manner in controls? Can this be explained by the refeeding time (18 hours)?

8. Figure 4E: To directly assess whether Mondo-Mlx represses pepck expression in a CBT-dependent manner, can the authors also overexpress cbt in mlx1 mutants in addition to measuring pepck expression in a mlx1 mutant and cbt RNAi flies?

9. Figure 4F: Is CBT binding to pepck promoter dependent on sugar availability? Is CBT exclusively localized in the nucleus or does it shuttles upon sugar feeding? What are the pepck promoter regions required for the CBT-mediated transcriptional repression? These experiments would extend previous knowledge on Krüppel/CBT.

10. Figure 5D: It is surprising that upon fasting, pepck mutants display similar trehalose levels as compared to controls. Does glycogenolysis compensate for the defective gluconeogenesis in the PEPCK mutants? The authors could measure different metabolites involved in these processes.

11. The authors state that cbt RNAi flies display enhanced triglyceride levels while CBT overexpression (cbt OE) leads to reduced triglyceride levels. The conclusion that "cbt RNAi flies display enhanced triglyceride levels" is not accurate since the statistical difference exists between cbt RNAi vs cbt OE and controls vs cbt OE. The appropriate comparison would be cbt RNAi vs controls. The authors must provide an explanation why the downregulation of cbt does not enhance triglyceride levels (Figure 6F).

Minor points:
12. Page 8 Line 11: The authors refer to Figure 3E which does not exist. The authors must refer to Figure 3B.

13. Page 9 Line 9: The authors refer to Figure 1B-1D but describe the Figure 3B-3D.

14. Page 20 Line 17: Typo.

Referee #3:

This manuscript studies the underlying transcriptional network regulating expression of gluconeogenic and glyceroneogenic genes (in particular PEPCK) in Drosophila in response to sugar feeding. This is interesting in the context of our current pandemic of metabolic syndrome, and the overabundance of sugars in our diets. Overall, the data are of good quality, and the conclusions are likely of broad interest. In particular since Cabut has a homolog in humans, klf10, this work suggests that the metabolic aspects of klf10 function should be examined in mammalian models.

Minor Issues:
1. Figure 1B - are these the top enriched GO categories? Because the p-values for 'metabolism' aren't that great. If yes, please specify this in the figure legend. If no, please additionally provide a list of the top enriched GO categories as supplemental.
The figure legend should also indicate what program was used for the GO term enrichment analysis.

2. Figure 1C - same. Are these the top misregulated genes? If not, how were they selected?

3. Figure 2D should also show equivalent data for some negative control regions, not expected to be bound by Mlx, to exclude the possibility that the Mlx pull-downs were simply more 'dirty' and pulled down more of everything.

4. Since Cabut function is studies by RNAi, and RNAi is notorious for off-target effects, some of the key findings need to be confirmed with a second independent Cabut RNAi construct
-effect of Cbt on PEPCK expression
-effect of Cbt RNAi on circulating glycerol levels
-effect of Cbt RNAi on TAG levels

5. On page 8 "Of these 51 genes, twenty-two mRNAs were downregulated
in a CBT-dependent manner (Figure 3E..."
-> should refer to Figure 2E

6. Data in Fig 2E and Suppl Fig S3B - How reproducible are these data? From the experiment scheme, it is unclear if each condition was only done in 1 biological replicate, or several biological replicates. If the latter, the authors should include some information about variance between biological replicates, and statistical significance in Suppl Fig 3B, to show the reader that the differences in fold-change between control and CbtRNAi flies are consistent amongst biological replicates. In case only 1 biological replicate was used, then the authors should confirm by Q-RT-PCR the differential regulation of these 22 genes in control vs CbtRNAi flies in 1 or 2 more biological replicates.

7. page 9 "We identified ten transcriptional modules with different temporal patterns of expression following sugar intake (Figures 1B-1D..."
-> should probably refer to Figures 3B-3D?

8. page 9 "In mammals, PEPCK exists as cytoplasmic (PEPCK-C) and mitochondrial (PEPCK-M) isoforms, and since Drosophila PEPCK lacks a mitochondrial targeting sequence it is a likely ortholog of PEPCK-C."
There seem to be 2 genes with phosphoenolpyruvate carboxykinase (GTP) activity in the fly genome, and there seems to be some confusion between them:
One is Flybase ID FBgn0003067, corresponds to CG17725, with official gene symbol PEPCK. Under "also known as", it says "CG10924".
However, the neighboring gene with Flybase ID FBgn0034356 and official gene symbol CG10924 is a different gene, also with phosphoenolpyruvate carboxykinase (GTP) activity.
The authors should clarify in the main text which gene they're referring to as PEPCK (I assume CG17725).
Is the other gene (CG10924) also transcriptionally regulated by Cabut?
Could the presence of CG10924 be the reason why PEPCK knockout flies are not lethal?

9. Figure 5A: the genomic coordinates are probably incorrect. The gene annotated as PEPCK is on 2R at 14425k, and not 1425k as indicated in the figure.

10. "circulating glucose levels were moderately, but significantly, downregulated (Figures 5D and 5E). The latter suggests more efficient utilization of diet-derived glucose in the absence of PEPCK"
In Fig 5D there is indeed a drop in trehalose levels, and the lack of statistical significance is borderline and could simply be technical (ie due to the large error bars). The experiment should be repeated either to reduce the error bars, or to see whether the averages become closer to each other.
The reduced circulating glucose (fig 5E) could be more efficient use of glucose, as authors write, but it could also be due to reduced intake/eating, especially since also trehalose is reduced and TAG is reduced (ie all forms of stored energy seem to be reduced in PEPCK mutants, so it's hard to imagine this is due to increased efficiency of energy use?) The rate of eating of PEPCK mutants should be tested to exclude this likely explanation.

11. Fig 6b and 6c - since the magnitude of the effect is small, both parental genotypes should be tested as controls (ie for 6B CbtRNAi x control and GAL4 x control; for 6C Tub-Gal4 x control and EP-PEPCK x control)

12. CbtRNAi doesn't really seem to increase TAGs compared to controls (Fig 6F). Is the difference in females significant? Because if not, it seems unlikely that the reason why CbtRNAi flies don't increase their glycerol as much as mlx1 mutants is due to triglyceride biosynthesis (as the authors write), if CbtRNAi flies don't make more TAGs that controls...

Maybe the reason could be that the mlx1 mutants have 7-fold elevated pepck levels, compared to 5-fold elevated in the Cbt RNAi flies (Fig 4E)?

13. In Intro, "Dietary sugars reduce glyceroneogenesis (Chen et al, 2005), but it remains poorly understood how this regulation is achieved and what is the physiological impact of such control." PEPCK is a classical FOXO target, and dietary sugar would presumably activate insulin signaling, inhibiting FOXO and hence repressing PEPCK expression.
What is the role of foxo in regulating pepck in response to sugar refeeding in drosophila?

Appeal 31 July 2014

Thank you for the feedback on our manuscript.

Although it is not my habit to appeal on negative decisions, this time I wanted to make an exception. This is because I sincerely believe that the reviewer comments can be addressed in the timeframe of a revision.

Especially, I would like to point out that the only major concern of Reviewer 1 bears some signs of misunderstanding and in my opinion can be easily addressed. He/she concludes that the statement that "cabut regulates glyceroneogenesis" is not supported by the available data and suggests a series of complex and time-consuming experiments to address this issue (e.g. analysis of "OAA/phosphoenolpyruvate ratio" and "genetic manipulation of other enzymes in the glyceroneogenesis pathway"). We have carefully thought through these suggestions and think that none of the suggested experiments will provide a rigorous test to our conclusion. It is critical to understand that glyceroneogenesis is not a separate pathway composed of specific enzymes. It is rather a description of a Pepck-dependent metabolic flow that incorporates carbon into glycerol-3-P from other sources than glucose - Pepck being the only enzyme that is not shared by pathways mediating glycolysis/glycolytic synthesis of glycerol-3-P. Therefore we think that the only relevant genetic experiment to test our hypothesis is the one we have done, i.e. suppression of mlx phenotype by pepck mutant (which also the Reviewer acknowledges). For the very same reasons, measurement of OAA/phosphoenolpyruvate ratio is likely to yield inconclusive data.

However, to relieve the doubts of Reviewer 1 we are now proposing an additional biochemical assay, which directly addresses the flow of carbon from pyruvate to glycerol (i.e. glyceroneogenesis). This is based on the use of 14C-labeled pyruvate on isolated fat bodies to measure the impact of Mondo-Mlx/Cabut on carbon flow from pyruvate to triglyceride glycerol. This assay has been previously utilized in the Drosophila system (Okamura et al., 2007) and should therefore be technically feasible. We expect that by successfully performing this assay and by better explaining the nature of the glyceroneogenesis pathway, the major concern of Reviewer 1 will be satisfyingly addressed.

Reviewer 2 gives suggestions to expand the manuscript, for example towards circadian regulation. Indeed, we do have data on Cabut overexpressing flies, in which circadian rhythms are abolished, but we feel that expanding the study into this direction would dilute the main message. However, if you feel that adding data on circadian regulation would be necessary to make the paper suitable to EMBO Journal, we are happy to reconsider this matter. Reviewer 2 also asks for additional insight into how glycerol homeostasis affects the fat

body mass. In fact, we have recently obtained data showing that pepck activity is linked to lipogenic gene expression, which provides a mechanistic link between pepck regulation and lipid homeostasis. Adding this data would in our opinion increase the novelty and mechanistic insight, without shifting the focus of the manuscript, and therefore we would be happy to include this data into the amended manuscript, along with the other revisions suggested by Reviewer 2.

Should you have any questions or concerns, please do not hesitate to contact me.

Thank you for your consideration.

Additional correspondence - editor 17 September 2014

Thanks again for your patience in this matter. I'm very sorry for the unusual delay in my reply to you!

I have now finally had the chance to sit down and go through both your manuscript and the referee comments in detail once more, in light of the points you raise in your letter to me. I am afraid the outcome is that we are not in a position to revert our decision for your manuscript.

Regarding the comments from ref #1, I realize that you are addressing a complex metabolic process, but at the same time I am sympathetic to the request from the referee to gain more definite insight on the broader metabolic consequences of Cabut deletion. As such, I do appreciate your suggested experiment to measure the carbon flow and I agree that it would strengthen your manuscript. However, we would also need you to include additional genetic data, if possible, to address the putative contribution from other pathway enzymes.

Regarding the comments from ref #2, I understand your desire to keep the story focused on Cabut in glyceroneogenesis but since the referee specifically states that this aspect of the story does not in his/her view raise the manuscript to the level of advance required for publication in The EMBO Journal, you would have to expand the study to include the functional interaction between Cabut and circadian control.

On this note, I do also have to mention that both referee #1 and #2 rated the overall novelty and general interest of your findings as low and medium in their recommendations to our editorial office. A putative revised version of the manuscript would therefore need to not only address the technical concerns raised but also to significantly expand the scope of the study in order to provide the level of advance required for its publication here.

Given these concerns - and in light of the still unclear nature of the outlined experiments and additional data - I am afraid we have to maintain our initial decision not to invite a revised manuscript in this case. If you were to extensively revise the study to address the concerns raised by the referees - and expand the link between Cabut, glyceroneogenesis and circadian control - we could be willing to look at a new version of the manuscript at a later stage and as an independent submission. However, I fully understand if you would at this stage rather pursue rapid publication at a less demanding venue elsewhere.

I am sorry to disappoint you again, but I hope these comments can at least be helpful to you in deciding the future strategy for this work. Thanks again for contacting me about this and my apologies for the delay.

Resubmission 25 February 2015

Point-by-point response, Bartok, Teesalu et al.

Referee #1:

The manuscript by Bartok and colleagues characterizes the transcriptional network downstream of the fly Klf-10 orthologue cabut (cbt). Although previous studies have reported links between several of the genes, physiological processes and metabolites described in this manuscript, the authors' emphasis in the network's nutritional regulation and physiological significance is novel and leads to the identification of a metabolically significant downstream gene: PEPCK. My only major issue concerns the link between PEPCK and glyceroneogenesis in the context of sugar adaptations (which, in my opinion, is the most novel/unexpected finding in the manuscript):
Firstly, the phenotypic analysis of PEPCK mutants is too superficial. Are these mutants developmentally delayed? Do they attain a normal size/eclose at normal numbers? Any development al effects could lead to secondary effects on lipid and carbohydrate metabolism.

As suggested, we have now performed a more thorough phenotypic analysis of *pepck* mutant flies. Our data shows that while *pepck* mutants display modestly reduced feeding, they do not display developmental delays and their size is similar to controls. The metabolic changes we observe reflect a specific shift in metabolic homeostasis, since the levels of glutamine and glutamate remain unchanged in the *pepck* mutant larvae. Thus, we conclude that the metabolites downstream of the PEPCK-mediated cataplerosis pathway (OAA => PEP) are present at lower levels in the *pepck* mutants. We have added the new data as Supplementary Figures S14, S15, and S17.

Secondly, the authors largely ignore the effect of PEPCK loss on glucose levels (PEPCK is after all the main rate-limiting enzyme in gluconeogenesis). Any glycolysis/gluconeogenesis imbalances could impact on glycerol 3 phosphate availability in a glyceroneogenesis and Gyk-independent way. Hence, the statement that "cabut regulates glyceroneogenesis" is not supported by the available data. More extensive metabolic profiling might support this statement.

Related to this, even if the constitutive role of PEPCK is to maintain steady-state glycerol levels, the nutritional regulation of PEPCK downstream of mlx/cbt may serve a different function. The most compelling experiment arguing against this possibility is that the abnormal glycerol levels of mlx mutants are restored in the mlx, pepck double mutant (which is very nice), but did the authors look at other metabolites (e.g. glucose, trehalose)? How specific is this phenotype to the glyceroneogenesis pathway? The metabolomics experiment suggested above might hopefully show changes in, for example, OAA/phosphoenolpyruvate ratio, but they would always be correlative by definition. The authors should also test whether targeted genetic manipulation of other enzymes in the glyceroenogenesis pathway can modulate the mlx/cbt phenotypes.

We thank the Reviewer for this thoughtful insight. In response to these issues, we have now performed additional experiments and modified our conclusions. Because we observed strong phenotypes on circulating glycerol levels, our original model had an emphasis on glyceroneogenesis. As stated by the Reviewer, PEPCK is not specific to glyceroneogenesis. Instead, it is the gatekeeper enzyme for one of the cataplerotic pathways regulating the carbon flux from the TCA cycle towards glycolytic intermediates. Depending on the activity of other metabolic enzymes, these carbons can end up in various destinations, including glycerol and trehalose (in insects), through glycero- and gluconeogenesis, respectively. Therefore, and following the comment of the Reviewer, we have now modified the text of the manuscript to better convey this concept. Notably, for the glyceroneogenesis pathway, PEPCK is the only

enzyme, which is not common with the glycolysis pathway, therefore testing other enzymes than PEPCK in the context of glyceroneogenesis is not feasible. However, we have now performed additional experiments to achieve a more complete picture of the metabolic changes caused by Cabut-mediated repression of *pepck*. As suggested by the Reviewer, we have analyzed the PEP and OAA levels and have observed a significant elevation in the PEP/OAA ratio upon *cabut* knockdown as well as in *mlx* mutants. The results of these experiments constitute a direct confirmation of the conclusion that Mondo-Mlx/Cabut regulates PEPCK-mediated conversion of OAA to PEP. We have added this data as Figure 5A, B in the amended manuscript.

As suggested by the Reviewer, we have analyzed the consequences of loss of *pepck* on glucose and trehalose. By adding more replicates, we have now shown that loss of *pepck* does lead to a modest, but significant reduction in steady-state trehalose levels. Furthermore, we now show that loss of *pepck* partially rescues the elevated trehalose levels of *mlx* mutants. Thus, we conclude that the carbon channeled through the cataplerotic route upon high *pepck* expression contribute to the elevation of trehalose levels in *mlx* mutants, in addition to the earlier observed effects on circulating glycerol. We also measured levels of circulating glucose in *mlx* mutants in the presence and absence of *pepck*, but observed no significant differences. We have added these data as Figures 5E and S18 in the amended manuscript and we have modified our conclusions accordingly. We think that these revisions have significantly improved our manuscript.

Minor points

1. None of the dietary experiments control for nutrient quality rather than quantity - for example, no isocaloric controls were used in refeeding experiments so it is unclear whether gene expression changes result from exposure to sugars or, more generally, to nutrients after a period of starvation. These controls should ideally be provided. Failing that, statements about "sugar responses" should be replaced with more general statements about diet.

We have added new evidence to show that feeding the animals with protein causes no upregulation of *cbt* and very limited downregulation of *pepck* expression, thus providing further evidence for a sugar-specific regulatory mechanism. These data have been added as Supplementary Figures S2 and S10 to the amended manuscript.

2. The lack of knowledge about the metabolic roles of cabut-like proteins as ChREBP targets is overstated - see, for example, references in Iizuka et al Endocrine Journal 2013, 60 (5), 543-555.

We have now included the original study by Iizuka and coworkers (Iizuka et al., 2011) into the Discussion of the amended manuscript. By doing overexpression of Klf-10, Iizuka and coworkers suppressed the activation of ChREBP target genes (incl. *FAS* and *ACC*) concluding that Klf-10 constitutes a negative feedback loop for ChREBP. We have tested this hypothesis by analyzing *FAS* and *ACC* expression in CBT loss-of-function animals and found no evidence to support the model of Iizuka and coworkers (Supplementary Figure S21). We have discussed this accordingly.

3. Genotypes for all control and RNAi experiments as well as n numbers need to be provided in either the figure panels or legends. In the methods, the authors should also provide information about age, sex etc of the adult flies used.

We have now added the requested information.

4. Page 7. I assume that the first refeeding experiment was done in adults?

We have now better clarified the developmental stages used in the experiments.

5. Page 11. Please provide reference for the statement "circulating glucose is

derived from dietary intake". This is an important point that should be discussed given that only glucose (and not trehalose) is affected in the mutants. I would have also liked to see some discussion about the apparent lack of mitochondrial PEPCK in flies (and expected metabolic consequences). In general, the discussion is a bit weak - What have we learned? What is unexpected and why might this be?

We have eliminated the statement and amended the Discussion. Moreover, we have included data on the regulation and function of the other *Drosophila* PEPCK isoform (CG10924), which is likely a mitochondrial isoform. We show that CG10924 (which we now call Pepck2) is regulated by CBT and also contributes to circulating glycerol levels. The new data is presented as Figure 3E and Supplementary Figure S16.

6. The reference to Figure 3E (page 8) needs to be amended (there is no Figure 3E).

Corrected.

7. The genetic setup for the microarray/RNAseq experiments is odd to say the least - rather than confining cbt downregulation to a couple of random tissues, ubiquitous (or fat body), temporally restricted gene downregulation in adult flies by means of a tub-Gal4 (or r4-Gal4), tub-Gal80ts transgene would have been more appropriate, especially given that 1) developmental and circadian phenotypes were expected and 2) mlx has been shown to act primarily in the fat body. More than one RNAi line should have been used to confirm the phenotypes. It is unclear why some experiments were carried out in larvae and some in adults. That said, the transcriptional data is supported by multiple gain- and loss-of-function experiments so the main findings are probably sufficiently supported. I am not going to ask the authors to repeat all their transcriptional profiling, but some discussion of the strengths/weaknesses of the current approaches would be appreciated.

We have now confirmed the regulation of *pepck* by CBT in larvae by using an independent RNAi line (Supplementary Figure S12). We understand the point raised by the Reviewer. In the original manuscript we explained that the tim-gal4 driver was chosen due to lack of lethality as well as to the fact that it is highly expressed in the fat body. We have now added significant amount of additional data linking CBT function to the circadian system, which also makes more relevant the use of GAL4 driver. One reason for performing experiments in the larvae was the pupal lethality of *mlx* mutants, which we also state in the manuscript.

8. Figs 6A and 6B (and related to the previous point). Why was the cbt RNAi experiment not carried out using the same FB driver used to rescue the mlx mutants in the previous panel? Can the glycerol levels of an mlx mutant be rescued by FB-specific reintroduction of cbt? Also, in order to make those two experiments more comparable the same scale should be used for both bar graphs.

Genetic manipulation of *cbt* is challenging, because one needs to find the right balance between too weak and too strong depletion. We have tried many drivers and have concluded that the genetic combinations used in the manuscript give the most robust phenotypes with minimal adverse effects on the health of the animals. We have tried fat body specific overexpression of Cabut in *mlx* mutant background, but this inhibited larval growth significantly and thus performing the experiment (requires 3rd instar larvae) was not feasible. Instead, we measured *pepck* expression and observed a clear rescue of *pepck* repression by fat body specific overexpression of Cabut in the *mlx* mutants (Figure 3D).

9. "Group 2" seems to be absent from Figure 3B.

We display figures just for selected groups (now displayed in in Figures 2E and S8). Data for all 10 groups (including group 2 and 7) is presented in the Supplementary file 5.

Referee #2:

Comments to the authors

Bartok et al. investigate the mechanisms of transcriptional repression by sugars in Drosophila. Previously, the authors showed that Mondo/ChREBP-Mlx controls glucose and lipid metabolism and also promotes expression of the Krüppel-like transcription factor cabut (cbt). Here, the authors show a repressive branch of the sugar-sensing transcriptional network, i.e., CBT is a repressor. Upon sugar feeding, cbt expression is enhanced in an Mlx-dependent manner. Using a genome wide analysis, the authors show that CBT represses metabolic genes including pepck, encoding the rate-limiting enzyme for gluconeogenesis and glyceroneogenesis. Using genetic approaches, they also show that Mondo/Mlx negatively regulates pepck and that Mlx controls glycerol homeostasis via PEPCK.

Major points:
1. A major concern of the current study is that it does not significantly extent prior knowledge, albeit from rodents. An experiment that could be performed to extend the current work is to look at Mondo/ChREBP-Mlx, CBT and carbon metabolism in the context of circadian rhythm given that CBT is also regulated by CLOCK. Other suggested experiments (not all of which are necessary) that could also extend the current study are proposed below.

Although we completely understand the point raised by the Reviewer (and indeed we have followed exactly his/her suggestion, see below), we would like to state that we believe our findings significantly extend prior knowledge. For example, the current literature does not contain unbiased genome-wide data on the contribution of Cabut/Klf-10 in the dietary sugar controlled gene expression. Moreover, there is no available data on the mechanism by which intracellular sugar sensing controls the *pepck*-mediated cataplerosis and limited insight into the physiological consequences of such regulation. Thus we believe our study makes an important addition to the literature on sugar sensing in animals. However, we have followed the suggestion of the Reviewer and performed extensive genome-wide analyses on the impact of CBT on circadian gene expression. Our new data reveals that CBT represses the cycling of a metabolic subset of circadian output genes, while having no influence on the core clock components. Moreover, overexpression of CBT has profound effects both on circadian gene expression and on behavior. Altogether, the new circadian data adds a new dimension to the manuscript by revealing the regulatory input of *cbt* into the circadian system in *Drosophila*. We thank the Reviewer for the suggestion, which has helped us to significantly improve the manuscript.

2. To overexpress or knockdown cbt, the authors use the tim-gal4 driver where a gene of interest is widely expressed in cells harboring an active circadian clock. Since the authors previously reported that Mondo/Mlx is functionally important in the fat body (Havula et al., 2013), why did the author not use the fat body (r4)-specific gal4 driver?

We have indeed tested this. However, the use of r4 driver to overexpress CBT causes developmental delay. Therefore it can only be reliably used in the first instar larvae, which limits its use significantly.

3. How does CBT expression-mediated regulation of glycerol and triglyceride homeostasis affect fat body mass?

Due to technical challenges related to CBT overexpression (see above), we have not achieved conclusive data on the effects of CBT on triglyceride homeostasis and fat body mass. Since this data is not essential in terms of the core conclusions of the study, we have decided not to include this analysis.

4. Figure 2: The authors describe 2 putative carbohydrate -responsive elements (ChoRE) in the cbt promoter region. Is Mlx binding to ChoRE dependent on sugar availability? The authors should perform a luciferase reporter assay and determine whether one or both ChoREs are necessary/sufficient for Mlx-dependent cbt transcription.

Following these comments, we have now performed additional experiments and demonstrate that Mlx binding to *cabut* promoter is indeed glucose inducible. We have also used a luciferase reporter to show that ChoRE 2 (located in the first intron of *cbt*) is the main mediator of the sugar-dependent promoter activation. We have added these data as Figure 1D and Figure 1E.

5. Figure 3 and S3: For the genome wide approaches, the design of the experiment does not include a proper control. Fly heads from fed and starved flies should be collected at the same time of day due to circadian changes in metabolism.

We understand the concern of the Reviewer and we agree that there are no controls for possible circadian effects. However, as stated in the methods, we have eliminated from our analysis those genes that show fluctuations through the 18 hour time course and hence could potentially be circadian. Therefore we believe circadian effects are not a cause for concern. Furthermore, our circadian time point studies (in Figure 6) address the issue of CBT-controlled genes more comprehensively.

6. The authors show that CBT represses the expression of genes involved in glycerol biosynthesis such as pepck and glycerol kinase (Gck) but then focus exclusively on PEPCK in the Mlx/CBT-mediated regulation of glycerol metabolism. How much does GCK contribute to the glycerol metabolism phenotype?

We have analyzed the consequences of glycerol kinase (Gyk) RNAi and observed elevated levels of circulating glycerol, which is in line with the fact that Gyk phosphorylates glycerol into glycerol-3-phosphate and makes it available for triglyceride biosynthesis (Figure R1). We have chosen not to include the data, in order to keep the study in focus. If the Reviewer prefers this data to be included as a Supplementary Figure, we are happy to do so.

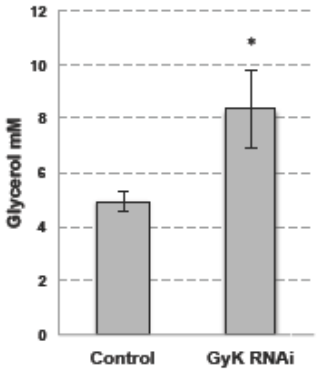

Figure R1. Gyk RNAi leads to elevated circulating glycerol levels. Error bars show SD. * $p<0.05$.

7. Figure 4C: How do the authors explain that pepck expression in not repressed by sugar in a dose dependent manner in controls? Can this be explained by the refeeding time (18 hours)?

It appears that *pepck* expression is highly sensitive to glucose and maximal inhibition can be achieved already by 1,25% glucose. Notably, upon Cabut depletion even much

higher glucose levels (5%) are insufficient to cause similar inhibition, providing a strong argument for the essential role of Cabut in the dietary sugar-dependent repression of *pepck*.

8. Figure 4E: To directly assess whether Mondo-Mlx represses pepck expression in a CBT-dependent manner, can the authors also overexpress cbt in mlx1 mutants in addition to measuring pepck expression in a mlx1 mutant and cbt RNAi flies?

This is indeed a valid experiment. We have now performed the experiment, which demonstrates that Cabut overexpression in the *mlx* mutant background rescues the repression of both *pepck* isoforms. This is consistent with our model that Cabut acts downstream of Mlx to regulate *pepck1* and *pepck2*. We have added this data as Figure 3D and Supplemental Figure S13.

9. Figure 4F: Is CBT binding to pepck promoter dependent on sugar availability? Is CBT exclusively localized in the nucleus or does it shuttles upon sugar feeding? What are the pepck promoter regions required for the CBT-mediated transcriptional repression? These experiments would extend previous knowledge on Krüppel/CBT.

We agree with the Reviewer that these experiments would reveal additional details on the function of CBT. However, in our opinion they would not provide major conceptual advancements for our study, which already is rather voluminous in its current form. Therefore, we think that these experiments go beyond the scope of the present manuscript.

10. Figure 5D: It is surprising that upon fasting, pepck mutants display similar trehalose levels as compared to controls. Does glycogenolysis compensate for the defective gluconeogenesis in the PEPCK mutants? The authors could measure different metabolites involved in these processes.

We have now performed additional analyses for trehalose levels and adding more replicates to this assay has allowed us to detect a modest and but a significant reduction of trehalose levels in *pepck* mutants (Figure 4D). However, as the Reviewer suggests, it is indeed possible that glycogenolysis contributes in maintaining the circulating trehalose levels in the *pepck* mutants.

11. The authors state that cbt RNAi flies display enhanced triglyceride levels while CBT overexpression (cbt OE) leads to reduced triglyceride levels. The conclusion that "cbt RNAi flies display enhanced triglyceride levels" is not accurate since the statistical difference exists between cbt RNAi vs cbt OE and controls vs cbt OE. The appropriate comparison would be cbt RNAi vs controls. The authors must provide an explanation why the downregulation of cbt does not enhance triglyceride levels (Figure 6F).

Due to technical challenges we have been unable to confirm these data and therefore will not include it into the amended manuscript.

Minor points:
12. Page 8 Line 11: The authors refer to Figure 3E which does not exist. The authors must refer to Figure 3B.

Corrected.

13. Page 9 Line 9: The authors refer to Figure 1B-1D but describe the Figure 3B-3D.

Corrected.

14. Page 20 Line 17: Typo.

Corrected.

Referee #3:

This manuscript studies the underlying transcriptional network regulating expression of gluconeogenic and glyceroneogenic genes (in particular PEPCK) in Drosophila in response to sugar feeding. This is interesting in the context of our current pandemic of metabolic syndrome, and the overabundance of sugars in our diets. Overall, the data are of good quality, and the conclusions are likely of broad interest. In particular since Cabut has a homolog in humans, klf10, this work suggests that the metabolic aspects of klf10 function should be examined in mammalian models.

Thank you.

Minor Issues:

1. Figure 1B - are these the top enriched GO categories? Because the p-values for 'metabolism' aren't that great. If yes, please specify this in the figure legend. If no, please additionally provide a list of the top enriched GO categories as supplemental. The figure legend should also indicate what program was used for the GO term enrichment analysis.

Yes, these are the top enriched GO categories. Due to manuscript reorganization, we have now moved this analysis to the Supplementary Figures. We have indicated the requested information in the figure legend.

2. Figure 1C - same. Are these the top misregulated genes? If not, how were they selected?

Yes, they are. We have stated this clearly in the Figure legend now.

3. Figure 2D should also show equivalent data for some negative control regions, not expected to be bound by Mlx, to exclude the possibility that the Mlx pull-downs were simply more 'dirty' and pulled down more of everything.

We have added *actin* as a negative control to rule out unspecific binding. The new data is included into Figure 1D.

4. Since Cabut function is studies by RNAi, and RNAi is notorious for off-target effects, some of the key findings need to be confirmed with a second independent Cabut RNAi construct
-effect of Cbt on PEPCK expression
-effect of Cbt RNAi on circulating glycerol levels
-effect of Cbt RNAi on TAG levels

As suggested by the Reviewer, we have now used an independent RNAi line to confirm the repressive effect of Cbt on *pepck*. The new data is presented as Supplementary Figure S12. Due to technical challenges, we have decided to omit data on circulating glycerol and triglycerides upon CBT knockdown.

5. On page 8 "Of these 51 genes, twenty-two mRNAs were downregulated in a CBT-dependent manner (Figure 3E..."
-> should refer to Figure 2E

Corrected

6. Data in Fig 2E and Suppl Fig S3B - How reproducible are these data? From the experiment scheme, it is unclear if each condition was only done in 1 biological

replicate, or several biological replicates. If the latter, the authors should include some information about variance between biological replicates, and statistical significance in Suppl Fig 3B, to show the reader that the differences in fold-change between control and CbtRNAi flies are consistent amongst biological replicates. In case only 1 biological replicate was used, then the authors should confirm by Q-RTPCR the differential regulation of these 22 genes in control vs CbtRNAi flies in 1 or 2 more biological replicates.

We apologize for the oversight on this important matter. We have now made it clear that these data come from two biological replicas of RNA seq and that the analysis was performed utilizing all the samples. We also stated clearly in the Methods, which statistical tests were performed to test the significance.

7. page 9 "We identified ten transcriptional modules with different temporal patterns of expression following sugar intake (Figures 1B-1D..."
-> should probably refer to Figures 3B-3D?

Revised.

8. page 9 "In mammals, PEPCK exists as cytoplasmic (PEPCK-C) and mitochondrial (PEPCK-M) isoforms, and since Drosophila PEPCK lacks a mitochondrial targeting sequence it is a likely ortholog of PEPCK-C."
There seem to be 2 genes with phosphoenolpyruvate carboxykinase (GTP) activity in the fly genome, and there seems to be some confusion between them: One is Flybase ID FBgn0003067, corresponds to CG17725, with official gene symbol PEPCK. Under "also known as", it says "CG10924". However, the neighboring gene with Flybase ID FBgn0034356 and official gene symbol CG10924 is a different gene, also with phosphoenolpyruvate carboxykinase (GTP) activity. The authors should clarify in the main text which gene they're referring to as PEPCK (I assume CG17725). Is the other gene (CG10924) also transcriptionally regulated by Cabut? Could the presence of CG10924 be the reason why PEPCK knockout flies are not lethal?

As suggested we have now analyzed the role of the other *Drosophila* PEPCK homolog CG10924, which is located next to PEPCK in the fly genome. We have analyzed the expression of CG10924 by CBT and show that it is also derepressed upon *cbt* knockdown. Furthermore, we have depleted CG10924 by RNAi and have observed reduced levels of circulating glycerol, similar to that caused by PEPCK (CG17725) RNAi. It is possible that the lack of lethality in *pepck* mutants can be explained by the presence of CG10924 (we have used a deficiency which deletes CG10924 in *trans* with the *pepck* deletion). However, it is notable that loss of PEPCK (CG17725) is sufficient to cause metabolic phenotypes as well as rescue *mlx* phenotypes (glycerol, trehalose, lethality), demonstrating that these two PEPCK genes are not completely functionally redundant. We have added the new data as Figure 3E and Supplementary Figure S16. For clarity, we now call the *pepck* homologs as *pepck1* (CG17725) and *pepck2* (CG10924).

9. Figure 5A: the genomic coordinates are probably incorrect. The gene annotated as PEPCK is on 2R at 14425k, and not 1425k as indicated in the figure.

Corrected.

10. "circulating glucose levels were moderately, but significantly, downregulated (Figures 5D and 5E). The latter suggests more efficient utilization of diet-derived glucose in the absence of PEPCK"
In Fig 5D there is indeed a drop in trehalose levels, and the lack of statistical significance is borderline and could simply be technical (ie due to the large error bars). The experiment should be repeated either to reduce the error bars, or to see whether the averages become closer to each other. The reduced circulating glucose

(fig 5E) could be more efficient use of glucose, as authors write, but it could also be due to reduced intake/eating, especially since also trehalose is reduced and TAG is reduced (ie all forms of stored energy seem to be reduced in PEPCK mutants, so it's hard to imagine this is due to increased efficiency of energy use?) The rate of eating of PEPCK mutants should be tested to exclude this likely explanation.

As suggested by the Reviewer, we have now repeated the experiment with more replicates and found that the modest reduction in trehalose levels reaches statistical significance. We have included the new data as Figure 4D and have modified our conclusion accordingly. As suggested by the Reviewer, we have analyzed the food intake and observed a minor, but significant, reduction. However, we do not think that the observed reductions in the metabolite levels are solely due to reduced food intake, since the developmental kinetics and pupal volume of the *pepck* mutant animals are indistinguishable from those of controls, which one would not expect if the mutant animals were starving. Furthermore, levels of glutamine and glutamate are unchanged in the *pepck* mutants, suggesting that the phenotypes we are observing are reflecting a true metabolic imbalance, not just a general downregulation of all metabolites. We have added the new data as Supplementary Figures S14, S15 and S17.

11. Fig 6b and 6c - since the magnitude of the effect is small, both parental genotypes should be tested as controls (ie for 6B CbtRNAi x control and GAL4 x control; for 6C Tub-Gal4 x control and EP-PEPCK x control).

We thank the Reviewer for pointing this out. We performed the experiments suggested and based on the results cannot conclusively rule out the impact of genetic background. Therefore we have decided to omit these data from the revised manuscript. Instead, we now show that depletion of Cabut leads to elevated PEP/OAA ratio, providing direct evidence for increased PEPCK-mediated cataplerosis.

12. CbtRNAi doesn't really seem to increase TAGs compared to controls (Fig 6F). Is the difference in females significant? Because if not, it seems unlikely that the reason why CbtRNAi flies don't increase their glycerol as much as mlx1 mutants is due to triglyceride biosynthesis (as the authors write), if CbtRNAi flies don't make more TAGs that controls...
Maybe the reason could be that the mlx1 mutants have 7-fold elevated pepck levels, compared to 5-fold elevated in the Cbt RNAi flies (Fig 4E)?

Due to technical difficulties we could not achieve conclusive confirmation on TAG levels upon CBT knockdown and we have decided to omit these data. As new evidence we present data showing elevated PEP/OAA ratio upon CBT knockdown. This is consistent with elevated cataplerosis in CBT loss-of-function animals.

13. In Intro, "Dietary sugars reduce glyceroneogenesis (Chen et al, 2005), but it remains poorly understood how this regulation is achieved and what is the physiological impact of such control." PEPCK is a classical FOXO target, and dietary sugar would presumably activate insulin signaling, inhibiting FOXO and hence repressing PEPCK expression. What is the role of foxo in regulating pepck in response to sugar refeeding in drosophila?

We have now mentioned the known role of FOXO as a regulator of *pepck* in the Introduction. While we fully agree with the Reviewer on the importance of this question, we think the interplay between FOXO and CBT on *pepck* regulation (and beyond) should be studied with great care in a forthcoming study.

2nd Editorial Decision 27 March 2015

Thank you for submitting your manuscript for consideration by the EMBO Journal and sorry for the slight delay in communicating our decision to you. There were a few issues raised by the referees that required additional discussions in-house and on top of that I was away from the office for the first half of the week.

We have now heard back from two of the three original referees (ref#1 and #2) and their comments are included below.

As you will see from the reports, ref#2 - who was originally the most critical of the three referees - finds that the study has been sufficiently extended and consequently supports publication. However, ref #1 still has lingering concerns on the conclusiveness of the gluceroneogenesis effects that you will have to discuss/clarify further in a final revision of your study.

Given the already extensive revisions that have gone into the current version we will not insist on the inclusion of additional epistasis experiments as suggested by ref #1 (point 1) - although if you have data at hand that could be included to strengthen this aspect we would certainly encourage you to include it. However, I would ask you to comment on/clarify the basis for the dietary scheme used, the possible contribution from pepck2 and the PEP/OAA ratio in pepck mutant flies (points 2,3,4).

I would also at this stage already encourage you to include the following editorial points in the revised manuscript

-> Please include a short statement on author contributions and conflict of interest at the end of the manuscript.

-> As of Jan 1st 2014 every paper published in The EMBO Journal includes a 'Synopsis' to further enhance its discoverability. The synopsis consists of a short standfirst - written by the handling editor - as well as 2-5 one sentence bullet points that summarise the paper and are provided by the authors. These bullet points should be complementary to the abstract - i.e. not repeat the same text. I would therefore ask you to include your suggestions for bullet points.

-> In addition, I would encourage you to provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the final comments of the reviewers. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://emboj.embopress.org/about#Transparent_Process

Thank you for the opportunity to consider your work for publication and please feel free to contact me with any questions. I look forward to receiving the final revision of your study.

Referee #1:

In this manuscript, Bartok and colleagues extend their previous characterisation of the Mondo/ChREBP transcription factor complex, an important mediator of dietary sugar-triggered metabolic adaptations. The authors focus on the targets and functions of cabut, the Drosophila homologue of klf10. Their most compelling finding is the identification of cabut-mediated pepck repression as a key downstream process, nicely shown by the rescue of Mlx mutant phenotypes by mutation of pepck. Together, this and other findings in the manuscript shed light on mechanisms of transcriptional repression downstream of intracellular sugar sensing, and suggest that cell-intrinsic nutrient sensing mechanisms contribute to pepck regulation. The manuscript also provides a few

other interesting, but somewhat fragmentary, observations. These include comprehensive RNAseq analyses of cbt targets and metabolic phenotyping of relevant mutants.

Major comments
1. Although I agree that most of the data is consistent with the model that the authors propose in Figure 7, most of the experiments are descriptive (e.g. metabolic profiling of mutants) and, with the exception of the experiment described above, do not probe the epistatic relationship between members of this proposed sugar sensing pathway. More detailed analysis of the cbt regulation of pepck transcription in response to sugar and/or epistasis experiments between cbt and pepck would strengthen the functional links between these genes.

2. The dietary paradigm used in this study (starvation following by a sugar-only diet) is very different from the excess dietary sugars that the authors keep referring to as major contributors to adiposity in mammals. Why did the authors not use a previously established high sugar diet that also leads to adiposity, insulin resistance etc in flies. Does this diet lead to any of the reported phenotypes and/or changes in gene expression?

3. The use of pepck1 over a deficiency that removes both pepck1 and 2 is not appropriate - pepck2 compensatory effects are still possible with one copy of pepck2, and it makes it difficult to ascribe phenotypes to pepck1 loss and/or pepck2 haploinsufficiency. The authors should confirm one or two of the key reported pepck1 phenotypes in pepck1-only mutants.

4. Is the PEP/OAA ratio affected as expected in pepck mutants? I may have missed this, but only cbt RNAi and mlx1 flies seem to have been profiled. Related to this, although Glu and Gln are not affected in pepck mutants, pretty much everything else is - including food intake and carbohydrate metabolism. In light of this, I do not feel that the discussion claims regarding specific effects on glyceroneogenic as opposed to gluconegenic pathways are justified and should be toned down/explained further.

Minor comments
1. The authors should consistently refer to pepck1 as pepck1 and not pepck (e.g. Fig 4).

2. I do not see the need to have so many one-panel supplementary figures - they could be consolidated into fewer figures.

3. How were the glutamate/glutamine quantifications (fig S17) carried out?

4. The authors should add labels to figures to clarify whether the measurements were conducted in larvae or adults.

5. Page 15. Please provide the reference for the statement that cabut regulates many clk-regulated genes (i.e. the reference with the list of clk-regulated genes that the authors used to compare their data against).

Referee #2:

The authors have significantly modified the manuscript. They intergrated the suggested experiments especially in the context of circadian rhythm. Overall, the authors extensively improved their study. We are in favor of publication.

1st Revision **31 March 2015**

Referee #1:

In this manuscript, Bartok and colleagues extend their previous characterisation of the Mondo/ChREBP transcription factor complex, an important mediator of dietary sugar-triggered metabolic adaptations. The authors focus on the targets and functions of cabut, the Drosophila homologue of klf10. Their most compelling finding is the identification of cabut-mediated pepck repression as a key downstream process, nicely shown by the rescue of Mlx mutant phenotypes by mutation of pepck. Together, this and other findings in the manuscript shed light on mechanisms of transcriptional repression downstream of intracellular sugar sensing, and suggest that cell-intrinsic nutrient sensing mechanisms contribute to pepck regulation. The manuscript also provides a few other interesting, but somewhat fragmentary, observations. These include comprehensive RNAseq analyses of cbt targets and metabolic phenotyping of relevant mutants.

Major comments
1. Although I agree that most of the data is consistent with the model that the authors propose in Figure 7, most of the experiments are descriptive (e.g. metabolic profiling of mutants) and, with the exception of the experiment described above, do not probe the epistatic relationship between members of this proposed sugar sensing pathway. More detailed analysis of the cbt regulation of pepck transcription in response to sugar and/or epistasis experiments between cbt and pepck would strengthen the functional links between these genes.

While we agree with the Reviewer that adding more data would strengthen the study, we believe that the main conclusions are already now sufficiently supported by experimental evidence. Our data shows direct binding of CBT to pepck promoter, reduced pepck expression upon CBT overexpression and inhibition of sugar-dependent repression of pepck upon CBT depletion. Depletion of CBT leads to elevated PEP/OAA ratio, which is the expected outcome for increased PEPCK activity. Furthermore we show that overexpression of CBT in the mlx mutant background restores the repression of pepck, which is in full agreement with our model that CBT acts downstream of Mondo-Mlx to repress pepck.

2. The dietary paradigm used in this study (starvation following by a sugar-only diet) is very different from the excess dietary sugars that the authors keep referring to as major contributors to adiposity in mammals. Why did the authors not use a previously established high sugar diet that also leads to adiposity, insulin resistance etc in flies. Does this diet lead to any of the reported phenotypes and/or changes in gene expression?

We understand the concern of the Reviewer. Indeed, some research groups in the field use experimental diets with superphysiological sugar levels (>30%), which leads to a pathophysiological state in wild type flies. Instead, we have chosen to use sugar concentrations that are commonly found in the natural diet of D. melanogaster. As shown in Figure 3B, the regulation of pepck1 expression by sugar is observed already at relatively low levels of sugar feeding (1,25 – 5%). In

agreement, our earlier study (Havula et al., 2013) showed that the mlx mutants display a diabetic phenotype already on 5% dietary sucrose. This is in line with the idea that the Mondo-Mlx-mediated sugar sensing is indeed operational and essential in physiological range of dietary sugar intake. Figure S6A further shows that the kinetics of pepck1 repression is similar on 5 and 10% sucrose diets, implying that the moderate sugar levels are already saturating in this experimental setting. Thus, there is no need to use superphysiological dietary sugar levels to study CBT-dependent gene regulation.

3. The use of pepck1 over a deficiency that removes both pepck1 and 2 is not appropriate - pepck2 compensatory effects are still possible with one copy of pepck2, and it makes it difficult to ascribe phenotypes to pepck1 loss and/or pepck2 haploinsufficiency. The authors should confirm one or two of the key reported pepck1 phenotypes in pepck1-only mutants.

We agree with the Reviewer that this is a valid point. We have now added new data showing reduced circulating glycerol levels in the pepck1 mutant homozygote larvae. The new data is displayed in Figure 4F of the amended manuscript.

4. Is the PEP/OAA ratio affected as expected in pepck mutants? I may have missed this, but only cbt RNAi and mlx1 flies seem to have been profiled. Related to this, although Glu and Gln are not affected in pepck mutants, pretty much everything else is - including food intake and carbohydrate metabolism. In light of this, I do not feel that the discussion claims regarding specific effects on glyceroneogenic as opposed to gluconegenic pathways are justified and should be toned down/explained further.

Only cbt RNAi and mlx1 were measured for PEP/OAA ratio. We do not think adding more genotypes would conceptually advance our study. Our intention was not to make claims regarding specific effects on glyceroneogenic as opposed to gluconeogenic pathways. To avoid such misunderstanding, we have modified the Discussion.

Minor comments
1. The authors should consistently refer to pepck1 as pepck1 and not pepck (e.g. Fig 4).

We have now modified the manuscript to refer consistently either to pepck1 or both pepck isoforms.

2. I do not see the need to have so many one-panel supplementary figures - they could be consolidated into fewer figures.

As suggested by the Reviewer, we have now combined the supplementary figures into multi-panel figures.

3. How were the glutamate/glutamine quantifications (fig S17) carried out?

These measurements are detailed in the Supplementary text.

4. The authors should add labels to figures to clarify whether the measurements were conducted in larvae or adults.

We have now clearly indicated the stage of the animals in the Figure legends.

5. Page 15. Please provide the reference for the statement that cabut regulates many clk-regulated genes (i.e. the reference with the list of clk-regulated genes that the authors used to compare their data against).

In fact we describe the comparison with clk targets already in page 9. We have now added the reference into page 9 and into page 15 we have added a notification for the reader to look into the earlier chapter for more details.

Referee #2:

The authors have significantly modified the manuscript. They intergrated the suggested experiments especially in the context of circadian rhythm. Overall, the authors extensively improved their study. We are in favor of publication.

Thank you. *c*