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Obesity resistance and increased hepatic expression of catabolism-related mRNAs in Cnot3+/- mice

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

1st Editorial Decision 09 May 2011

Thank you very much for submitting your research paper for consideration to The EMBO Journal editorial office. Having received consistent comments from two expert scientists, I am able to reach a decision on your study that should facilitate efficient proceedings. As you will see from the reports, the direct link of the Ccr4-Not complex to the regulation of metabolism and glucose tolerance is very appreciated. However, both scientists request better direct evidence for CNOT3's molecular function (and we do prefer here some more experimental evidence to address this point!).

Also, mRNA-stability and -length analyses of at least some of the affected RNA's would improve the significance and insight that can be derived from your study and would thus need your attention before returning a revised version of your paper for external re-assessment.

Please be reminded that it is EMBO_J policy to allow a single round of revisions only and that the final decision solely depends on the content and strength of this amended version of your manuscript.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

In this study, Morita et al reveal a role for the CNOT3 subunit of the Ccr4-Not complex, carrying as another subunit the major eukaryotic deadenylase, in the regulation of metabolism and glucose tolerance in liver and white adipose tissue in mice.

The authors determined that heterozygous CNot3+/- mice were smaller than the wild-type counterparts, with in particular reductions in liver and WAT tissue, and reductions of liver triglyceride and lipid accumulation. Food intake was similar, but oxygen consumption greater, fasting glucose levels were reduced, serum triglyceride levels were reduced at all times, and insulin sensitivity was greater, in the heterozygous mice. Deletion of a copy of the CNOT3 gene was able to render mice resistant to diet induced obesity, and to improve obesity and insulin resistance in ob/ob mice. Micro-array analysis of mRNAs expressed in livers from wild-type or heterozygous mice demonstrated a number of increased mRNAs (about 250 more than 2-fold up) and few decreased (less than 20 2-fold down). mRNAs involved in lipid metabolism were most enriched (19 mRNAs), some of which might contribute to leanliness of the heterozygous mice. For 2 of the mRNAs, an increase in Poly(A) tail length could be demonstrated, and the 3'UTR regions of the mRNAs rendered luciferase levels from a reporter fusion sensitive to copies of the CNOT3 gene when transfected into liver HepG2 cells. An interaction between CNot3 and one of these mRNAs could be demonstrated by RNA IP. Finally, the levels of CNot3 protein (but not mRNA) in liver and WAT, but not pancreas or brain, were shown to drop in response to fasting, whilst the levels of other subunits of the Ccr4-Not complex did not change, and micro-array analysis revealed common genes up-regulated in livers of fasting or Cnot3 heterozygous mice.

Based upon all of this data the authors suggest that CNot3 recruits the Ccr4-Not deadenylase to the 3'ends of specific mRNAs, and responds to feeding to regulate deadenylation of specific mRNAs and energy metabolism.

This is a very nice and exciting study that connects the Ccr4-Not complex very specifically to energy metabolism, and provides new knowledge about the CNot3 subunit, connecting it to deadenylation control in specific tissues.

The data is clean and well presented and all highly relevant.

Major point

The conclusion that CNot3 recruits the deadenylase to 3' ends of mRNAs however, is not supported by the data. The data shows that CNot3 associates with a specific mRNA, but does not show that the association of the deadenylase with the mRNA depends upon CNot3 (it could even be the other way around). CNot3 could have an impact on deadenylation by many possible ways, and no experiments in the paper address specifically by which way. This should either be addressed by additional experiments, or should be written differently in the manuscript.

Minor point

A control to show that not all poly(A) tail lengths are increased, but only upregulated mRNAs is needed.

Referee #2:

The authors nicely document the very interesting observation of a role for CNOT3 in metabolic regulation in CNOT3+/- mice. Several aspects of the underlying mechanism of CNOT3 regulation of analyzed genes, however, could be pursued in more detail to provide in depth insights into this area - particularly since it appears that the CNOT3 subunit helps target the CCR4/Caf1 deadenylase complex to very specific mRNAs. This would provide more overall impact to the study and increase its appeal to a broad audience Specific Points:

1. Fig. 6B is missing two controls. First, does the poly(A) tail length for an mRNA that is not regulated in CNOT3 +/- mice change or is the increase specific for Pdk4, Igfbp1? Second, how big are the poly(A) tails in the +/- samples compared to wt? The addition of size markers to the lanes would help answer this question.
2. Fig. 5D and Fig. 6: The authors assume that CNOT3 is regulating the levels of the targeted mRNAs through differential mRNA stability - but this is never directly shown. I think it is important to demonstrate differences in mRNA stability since the CNOT complex has been previously implicated in transcriptional regulation as well as mRNA decay. The addition of some half life analyses for the luciferase reporter constructs should readily address this concern.
3. Since the authors have the reporter constructs in hand, it would add additional impact to the study if they would further delineate the requisite cis-acting RNA elements for CNOT3-mediated regulation in the 921-1643 base fragment in Fig. 6D.

1st Revision – Authors' Response 04 August 2011

Referee #1:

Major point:

The conclusion that CNot3 recruits the deadenylase to 3' ends of mRNAs however, is not supported by the data. The data shows that CNot3 associates with a specific mRNA, but does not show that the association of the deadenylase with the mRNA depends upon CNot3 (it could even be the other way around). CNot3 could have an impact on deadenylation by many possible ways, and no experiments in the paper address specifically by which way. This should either be addressed by additional experiments, or should be written differently in the manuscript.

To address this important issue, we assessed whether CNOT6L, a catalytic subunit of the CCR4-NOT deadenylase complex, can be associated with *Pdk4* mRNA in a CNOT3-dependent manner. We carried out RNA-immunoprecipitation microarray (RIP-CHIP) analysis using the anti-CNOT6L antibody and the hepatocyte lysates. *Pdk4* mRNA was listed in the anti-CNOT6L immunoprecipitates, suggesting that CNOT6L associates with *Pdk4* mRNA, which we verified by immunoprecipitation-RT-qPCR analysis (added as Figure 6G). The association of *Pdk4* mRNA with the CNOT6L was less in *Cnot3*$^{+/-}$ hepatocytes than that in wild-type hepatocytes (Figure 6G). In addition, CNOT6L associates with CNOT proteins, including CNOT3 (Morita et al., 2007, MCB 27, 4980) and the expression level of CNOT6L is not affected by *Cnot*3 haplodeficiency (Figure 1B). These data suggest that CNOT3 is involved in the control of *Pdk4* mRNA stability and in the association of the CNOT6L-containing CCR4-NOT deadenylase complex with the *Pdk4* mRNA.

As discussed in the text, there is a report that the *Drosophila* CCR4-NOT complex is recruited to the Bic-C mRNA through an interaction between dNOT3 and the Bic-C mRNA-binding protein (Chicoine *et al*, 2007, Dev Cell 13, 691), indicating that dNOT3 recruits the deadenylase to Bic-C mRNA through its interaction with RNA binding protein. Mammalian CNOT3 may also interact with a *Pdk4* mRNA-binding protein(s) so that the CCR4-NOT deadenylase complex could target the *Pdk4* mRNA. Since our data do not explain precisely how CNOT3 and the CCR4-NOT deadenylase interact with the 3'UTR of *Pdk4* mRNA, we have modified the relevant description in the abstract (page 2, line 11-12). Namely, instead of saying that "we provide evidence that CNOT3 recruits the CCR4-NOT deadenylase to the 3' end of …", we stated that "We provide evidence that CNOT3 is involved in recruitment of the CCR4-NOT deadenylase to the 3' end of …". We also added the description mentioned above in the results and discussion sections (page 12, line 19-page 13, line 3, and page 17, line 22-24, respectively).

Minor point:

A control to show that not all poly(A) tail lengths are increased, but only upregulated RNAs is needed.

We compared the poly(A) tail length of *Gapdh* mRNA in wild-type and *Cnot3*$^{+/-}$ hepatocytes. We showed that expression of *Gapdh* mRNA is not affected by *Cnot3* haplodeficiency and detected virtually no difference in the poly(A) tail length of *Gapdh* mRNA between wild-type and *Cnot3*$^{+/-}$ hepatocytes (data added in Figure 6B), which is described in page 11, line 15-18 of the revised manuscript.

Referee #2:

The authors nicely document the very interesting observation of a role for CNOT3 in metabolic regulation in CNOT3+/- mice. Several aspects of the underlying mechanism of CNOT3 regulation of analyzed genes, however, could be pursued in more detail to provide in depth insights into this area - particularly since it appears that the CNOT3 subunit helps target the CCR4/Caf1 deadenylase complex to very specific mRNAs. This would provide more overall impact to the study and increase its appeal to a broad audience.

This comment, we believe, overlaps to the major concern raised by referee #1. As we wrote in response to the referee #1's comment, we examined whether CNOT3 plays a part in the association between the catalytic subunit of the CCR4-NOT complex and *Pdk4* mRNA (described in page 12, line 19-page 13, line 3). We provide data arguing that the CNOT6L catalytic subunit associates with *Pdk4* mRNA and the degree of association was significantly less in *Cnot3*$^{+/-}$ mice than in wild-type mice (added as Figure 6G). Accordingly, we propose that CNOT3 is involved in the association of the CNOT6L-containing CCR4-NOT deadenylase complex with the *Pdk4* mRNA. The relevant description was also added to the discussion section of the revised manuscript (page 17, line 22-24).

Specific Points:

1. Fig. 6B is missing two controls. First, does the poly(A) tail length for an mRNA that is not regulated in CNOT3 +/- mice change or is the increase specific for Pdk4, Igfbp1? Second, how big are the poly(A) tails in the +/- samples compared to wt? The addition of size markers to the lanes would help answer this question.

As we wrote in response to the referee #1's comment, we compared the poly(A) tail length of *Gapdh* mRNA that is not regulated in *Cnot3*$^{+/-}$ mice, and found that it is virtually the same between wild-type and *Cnot3*$^{+/-}$ mice (added in Figure 6B and described in page 11, line 15-18). We have also shown the estimated poly(A) tail length in the revised manuscript (Figure 6B). The data indicate that CNOT3 does not regulate the length of poly(A) tail of every mRNA but regulates that of specific mRNAs.

2. Fig. 5D and Fig. 6: The authors assume that CNOT3 is regulating the levels of the targeted mRNAs through differential mRNA stability - but this is never directly shown. I think it is important to demonstrate differences in mRNA stability since the CNOT complex has been previously implicated in transcriptional regulation as well as mRNA decay. The addition of some half life analyses for the luciferase reporter constructs should readily address this concern.

To address this issue, the luciferase reporter that contains the sequence corresponding the 3'UTR of *Pdk4* mRNA was transfected to wild-type and *Cnot3*$^{+/-}$ hepatocytes. The half-life of the luciferase reporter mRNA was measured by treating the reporter-transfected cells with actinomycin D. Time course analysis revealed that the rate of decline of the luciferase transcript levels is lower in *Cnot3*$^{+/-}$ hepatocytes than in wild-type hepatocytes (added as Figure 6D). The data argue that *Pdk4* mRNA is more stable in *Cnot3*$^{+/-}$ hepatocytes than in wild-type hepatocytes. These were described in page 12, line 1-7.

3. Since the authors have the reporter constructs in hand, it would add additional impact to the study if they would further delineate the requisite cis-acting RNA elements for CNOT3-mediated regulation in the 921-1643 base fragment in Fig. 6D.

Global analyses of mRNA decay have reproducibly linked AREs with instability and deadenylase-dependent degradation (Raghavan *et al*., 2002, NAR 30, 5529; Mukherjee *et al*., 2009, Mol Syst Biol 5, 288). The 921-1643 base fragment of 3'UTR of *Pdk4* mRNA has two possible AREs, and the first ARE termed ARE1 is conserved between human and mouse, but the other ARE is not conserved. Therefore, we examined whether the ARE1 is indispensable for the CNOT3-mediated regulation. We found that the luciferase activity of ARE1-deleted construct was not significantly affected by the reduction of CNOT3 (added in Figure 6E), which suggests that other cis-acting elements would be required for CNOT3-mediated regulation. Recent studies showed that GU-rich elements (GREs) influence decay of short-lived mRNAs (Lee *et al.*, 2010, PLoS ONE 5, e11201), and that GRE-binding protein recruits PARN deadenylase to a target mRNA (Moraes *et al*., 2006, RNA 12, 1084). We found one possible GRE site in the 921-1643 region (see Figure 6E). Furthermore, GW182 protein binds to the CCR4-NOT complex to promote the miRNA-dependent degradation (Fabian *et al*., 2009, Mol Cell, 24: 868-880). Database search revealed that there are six conserved miRNA binding sites within the 921-1643 region. It is likely that not only AREs but also these other sequences could play some roles in determining the stability of the *Pdk4* mRNA. Although we agree with the referee that delineation of the requisite cis-acting elements for CNOT3-mediated regulation would add additional impact, we believe that the issue belongs to the future study because we most likely need to deal with various combinations of the AREs, GREs, miRNA binding sites and possibly other sequences to find out the stability-controlling elements. Nevertheless, we have added the above description in the discussion (page 18, line 3-20).

2nd Editorial Decision 08 August 2011

I did receive final remarks from one of the original referees that will lead to eventual acceptance of your study (see below). Checking your paper carefully again at the editorial level, I would still like yo to attend to two issues:

- Please do provide an 'author's contribution statement' to be incorporated after the acknowledgements.

- In an effort to make original data more transparent and accessible at The EMBO Journal, we would kindly ask you to provide a more comprehensive presentation of the blots in figure 2K (particularly the Ser473-Akt blot as there is no harm in showing the higher mobility, presumably unspecific bands). This will become a more and more common procedure at the journal and I would also, on this occasion, be happy if you incorporate appropriate size-marker for this blot.

I am very much looking forward to these amendments before final and official acceptance of your study.

Yours sincerely,

Editor
The EMBO Journal

ref#2:

The authors have addressed the points raised in the previous critique with new data or thoughtful discussion. I find the revised manuscript to be improved and to contain an interesting mix of phenotypic and mechanistic insights regarding the role of Cnot3 in regulating a subset of metabolically-related mRNAs in these transgenic animals. I think that it will appeal to a broad audience.

