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Clusterin/ApoJ enhances central leptin signaling through Lrp2-mediated endocytosis

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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.)

Editor: Esther Schnapp

1st Editorial Decision 31 January 2014

Thank you very much for your patience while your manuscript was peer-reviewed at EMBO reports. I apologize for the delay in getting back to you; we have only now received the full set of referee reports on your study, which is copied below.

As you will see, all referees acknowledge that the findings are interesting. Given that they only raise a few concerns, all of them need to be addressed for publication of the manuscript by EMBO reports. Especially the mentioned discrepancy between changes in hypothalamic leptin signaling and body weight in clusterin deficient mice should be addressed.

Given these referee comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. 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. Also, the revised manuscript may not exceed 30,000 characters (including spaces, references and figure legends) and 5 main plus 5 supplementary figures, which should directly relate to their corresponding main figure. Please note that EMBO reports papers use a numbered reference style, and please change the current style accordingly.

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 tests used to calculate p-values in all the relevant figure legends? This information is currently incomplete and must be provided in the figure legends. Please also add clearly visible scale bars to all microscope images and define their length in the figure legend.

We recently decided to offer the authors the possibility to submit "source data" with their revised manuscript that will be published in a separate source data file online along with the accepted manuscript. If you would like to use this opportunity, please submit the source data (for example entire gels or blots, data points of graphs, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one file per figure or per figure panel.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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

REFEREE REPORTS:

Referee #1:

This manuscript provides evidence for novel mechanistic aspects of leptin signaling in the hypothalamus to control energy metabolism. The most intriguing aspect of the work is the emphasis on endocytosis. While the mechanistic aspects of this process is highly speculative,it is clearly an idea that needs to be available for field. The authors should look into the literature that has shown similar ideas, i.e., that leptin can be picked up by cells of the CNS and discuss those findings (J Neuroendocrinology, 2002 14:429-34.)

Referee #2:

This manuscript by Kim and colleagues reports that the novel neuropeptide clusterin/ApoJ is a key regulator of hypothalamic leptin signaling. The authors show that simultaneous administration of clusterin potentiates the anorexigenic effect of leptin and enhances Stat3 activation. They provide additional ex vivo evidence indicating that clusterin enhances leptin receptor binding and subsequent endocytosis through Lrp2. Notably, inhibition of hypothalamic clusterin, Lrp2 and endocytosis abrogated anorexia and hypothalamic Stat3 activation caused by leptin. Since these findings represent an entirely novel regulatory mechanism in central leptin signaling pathways they mean an important advance for the field. This is a well conceptualised set of elegant studies presenting mechanistic data on an important pathway with novel aspects and insights. The experiments were conducted with appropriate attention to detail and cutting edge methodology was used. There is only one criticism by this reviewer which relates to the relative disconnect between relatively substantial signalling changes and molecular consequences in the hypothalami of clustering deficient mice and the normal body weight of these mice (even though there is a small fat mass phenotype). It seems that comparable loss of leptin signal in the CNS using for example a direct virus assisted knock down would result in a more substantial body weight differences as shown by several labs in the past. Could the authors speculate as to why in this admittedly complex situation there is such a disconnect between hypothalamic leptin signaling and the metabolic

phenotype?

Referee #3:

The authors examined the interaction between clusterin/apoJ and leptin signaling. They demonstrated that clusterin potentiated leptin-induced hypothalamic STAT3 activation as well as leptin induced hypophagia and decreased body weight. The authors further propose a model in which a Leptin-clusterin complex binds leptin receptors as well as Lrp2. The study appears well done and the manuscript clearly written. Minor comments related to points of discussion as well as textual changes are below:

1. Does clusterin/apoj regulate food intake/body weight independent of leptin? What is the effect of inhibition or loss of clusterin/apoj in Ob/Ob mice?

2. How does endocytosis of leptin potentiate leptin signaling? Is there a functional activity of leptin in the cytoplasm?

3. How did the authors validate injection of siRNA targeting murine clusterin in the bilateral mediobasal hypothalamus?

4. The authors describe the effects of leptin or clusterin on food intake, body weight, and hypothalamic STAT3 signaling (pg 3 ln 19-22, pg 4 ln 16-18, pg 5 ln 1-5). In each case it is unclear as written whether food intake, body weight, and stat3 signaling are all reduced or if there is a differential regulation. These sentences should be rewritten.

1st Revision - authors' response 09 April 2014

Response to Referee #1

This manuscript provides evidence for novel mechanistic aspects of leptin signaling in the hypothalamus to control energy metabolism. The most intriguing aspect of the work is the emphasis on endocytosis. While the mechanistic aspects of this process are highly speculative, it is clearly an idea that needs to be available for field.

Comments:
The authors should look into the literature that has shown similar ideas, i.e., that leptin can be picked up by cells of the CNS and discuss those findings (J Neuroendocrinology, 2002 14:429-34.)
Response: We thank the reviewer for helpful comments. The paper published in J Neuroendocrinology convincingly demonstrated that ICV administered leptin was internalized and accumulated in serotonergic neurons of the raphe nuclei. This paper provides an *in vivo* evidence for leptin endocytosis and supports our hypothesis that leptin endocytosis may be a critical signaling event in central leptin actions. We discussed these findings in our revised manuscript (page 6, line 12).

Response to Referee #2

This manuscript by Kim and colleagues reports that the novel neuropeptide clusterin/ApoJ is a key regulator of hypothalamic leptin signaling. The authors show that simultaneous administration of clusterin potentiates the anorexigenic effect of leptin and enhances Stat3 activation. They provide additional ex vivo evidence indicating that clusterin enhances leptin receptor binding and subsequent endocytosis through Lrp2. Notably, inhibition of hypothalamic clusterin, Lrp2 and

endocytosis abrogated anorexia and hypothalamic Stat3 activation caused by leptin. Since these findings represent an entirely novel regulatory mechanism in central leptin signaling pathways they mean an important advance for the field. This is a well conceptualised set of elegant studies presenting mechanistic data on an important pathway with novel aspects and insights. The experiments were conducted with appropriate attention to detail and cutting edge methodology was used.

Comments:
There is only one criticism by this reviewer which relates to the relative disconnect between relatively substantial signaling changes and molecular consequences in the hypothalami of clusterin deficient mice and the normal body weight of these mice (even though there is a small fat mass phenotype). It seems that comparable loss of leptin signal in the CNS using for example a direct virus assisted knock down would result in a more substantial body weight differences as shown by several labs in the past. Could the authors speculate as to why in this admittedly complex situation there is such a disconnection between hypothalamic leptin signaling and the metabolic phenotype?
Response: We totally agree with the reviewer's concern. Despite a significant impairment in hypothalamic leptin signaling, general clusterin deficient mice display mild obesity phenotype. Although we could not provide a clear explanation for this discrepancy in this paper, some compensatory processes might occur during the development of generalized clusterin-deficient mice. Since clusterin is widely expressed in peripheral tissues, peripheral metabolic effects of clusterin deficiency might compensate central effects of clusterin deficiency, thus minimizing changes in adiposity. Tissue-specific clusterin knockout mice are needed to test this possibility. These mice are currently generated and under investigation. We discussed this important issue in the discussion (page 9, the 2nd paragraph).

Response to Referee #3

The authors examined the interaction between clusterin/apoJ and leptin signaling. They demonstrated that clusterin potentiated leptin-induced hypothalamic STAT3 activation as well as leptin induced hypophagia and decreased body weight. The authors further propose a model in which a Leptin-clusterin complex binds leptin receptors as well as Lrp2. The study appears well done and the manuscript clearly written.

Minor comments related to points of discussion as well as textual changes are below:

1. Does clusterin/apoj regulate food intake/body weight independent of leptin?
Response: As shown in Expended View Fig. 1, ICV administration of clusterin reduced food intake in ob/ob mice. Thus, clusterin can regulate feeding without leptin. The leptin-independent signaling pathways in clusterin-mediated feeding regulation need to be further identified. This data is described in the last paragragh in page 9.

What is the effect of inhibition or loss of clusterin/Apo-J in Ob/Ob mice?
Response: In ob/ob mice, suppression of hypothalamic clusterin expression by injecting clusterin siRNA increased food intake (Expended View Fig. 2). Unexpectedly, we found that ob/ob mice had higher clusterin expression levels in the hypothalamus compared to normal mice (Expended View Fig. 3). Thus increased clusterin expression in ob/ob mice may be a compensatory mechanism to reduce hyperphagia.

2. How does endocytosis of leptin potentiate leptin signaling?
Response: Although we did not demonstrate the mechanisms in detail, clusterin and endocytic receptor Lrp2 may form a complex with leptin and LepRb. These ligand-receptor complexes might interact with signaling molecules (e.g. Jak2, Stat3, etc) on the membrane of endosomal vesicles during the endosomal shuttling from the plasma membrane to the perinuclear area. Endosome-associated bulk shuttling of ligand-receptor-signaling molecules may offer a more efficient and

accelerated signal transduction compared to shuttling of individual signaling molecules. We discussed a role for endocytosis in leptin signaling in the discussion (page 8, 2nd paragragh).

Is there a functional activity of leptin in the cytoplasm?
Response: According to our new model, leptin exists inside endosomal vesicles where leptin might remain bound to Leprb (as in the case of EGF-EGFR) or dissociate from Leprb and lose its biological activity when endosomal pH becomes acidic. Although further studies are required to prove leptin activity in different endosomal compartments, leptin in early and late endosomes may be functionally active so as to activate Stat3 signaling. These points are described in page 8, 2nd paragragh.

3. How did the authors validate injection of siRNA targeting murine clusterin in the bilateral mediobasal hypothalamus?
Response: At the beginning of siRNA experiments, we injected siRNA particles mixed with a small amount of GFP-Adenovirus and verified our injection skill by examining the GFP expression in MBH. After obtaining a confident injection skill, to avoid possible non-specific effects of GFP-Ad, we injected siRNA particles alone in the experiments we presented in this paper. Adequate siRNA injection in individual mouse was verified by demonstrating a successful knockdown of target gene expression in MBH tissue blocks. If the mRNA expression was less than 30% of the average expression levels of control siRNA-injected mice, it was considered as a successful knockdown.

4. The authors describe the effects of leptin or clusterin on food intake, body weight, and hypothalamic STAT3 signaling (pg 3 ln 19-22, pg 4 ln 16-18, pg 5 ln 1-5). In each case it is unclear as written whether food intake, body weight, and stat3 signaling are all reduced or if there is a differential regulation. These sentences should be rewritten.
Response: We are sorry for the confusion we raised in the description of our findings due to a tight word limit (< 25,000 characters). We now carefully described our findings in our revised manuscript.

2nd Editorial Decision 11 April 2014

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the comments from referee 3, who was asked to assess it, and who supports publication of your work. Your manuscript can therefore in principle be accepted.

However, some information on the statistics needs to be added to the figure legends, and the reference style needs to be adapted to the EMBO reports numbered style. This will also help to reduce the character count, that currently exceeds our limits. Can you please specify the statistical tests used to calculate p-values in all figure legends, including supplementary figures, and specify the bars and error bars in the supplementary figure legends? This information must be provided in the figure legends. Please also add a scale bar to figure 3F and define its length in the figure or legend.

I would also like to suggest some changes to the abstract, that needs to be written in present tense:

Hypothalamic leptin signaling plays a central role in maintaining body weight homeostasis. Here we show that clusterin/ApoJ, recently identified as an anorexigenic neuropeptide, is an important regulator in the hypothalamic leptin signaling pathway. Coadministration of clusterin potentiates the anorexigenic effect of leptin and boosts leptin-induced hypothalamic Stat3 activation. In cultured neurons, clusterin enhances receptor binding and subsequent endocytosis of leptin. These effects are mainly mediated through the LDL receptor-related protein-2 (Lrp2). Notably, inhibition of hypothalamic clusterin, Lrp2 or endocytosis abrogates anorexia and hypothalamic Stat3 activation caused by leptin. These findings propose a novel regulatory mechanism in central leptin signaling

pathways.

I look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORT:

Referee #3:

The authors have satisfactorily addressed my comments.

2nd Revision – Revised manuscript received 14 April 2014

3rd Editorial Decision 15 April 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

