EMBO reports

Regulator of Calcineurin 1 (RCAN1) helps coordinate whole body metabolism and thermogenesis

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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 11 July 2017

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

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also point out that significant revisions are required to strengthen the study. Several concerns and suggestions by referees 1 and 3 overlap, and these and all other concerns need to be addressed for publication of the study here.

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

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. Given the 7 main figures, I suggest that you layout your manuscript as a full article with separate results and discussion sections. The entire materials and methods must be included in the main manuscript file. Please note that supplemental material is called Expanded View (EV) now at EMBO press. However, we can only offer a maximum of 5 EV figures, in addition to EV movies and tables, for each paper. Please upload EV figures as individual files and add the EV figure legends to the end of the main manuscript file. Additional supplementary data will need to be moved to an Appendix file. You can find more information in our guide to authors online.

Regarding data quantification, please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the

respective figure legends. This information is currently incomplete and must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, 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 PDF file per figure or per figure panel.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where in the manuscript the requested information can be found. The completed author checklist will also be part of the RPF (see below).
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution

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

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. 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.

You are able to opt out of this by letting the editorial office know (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."

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

REFEREE REPORTS

Referee #1:

Comments to the manuscript: EMBOR-2017-44706V1
The manuscript entitled "Regulator of Calcineurin 1 (RCAN1) helps coordinate whole body metabolism and limit energy expenditure" by Rotter D etal, investigates the impact of Rcan1 deletion on non-shivering thermogenesis in white adipose tissue and muscle. They demonstrated that Rcan-1 KO mice were resistant to diet-induced obesity as a result of higher metabolic rate due 1/ increased adipocyte response to adrenergic stimuli and subsequent browning of the subcutaneous fat, and 2/increased sarcolipin expression and subsequent activation of NST in skeletal muscle.
The novelty of the manuscript is in the identification of Rcan1 as a thermoregulatory factor in both adipose tissue and muscle linking calcineurin-dependent signalling to cold-induced PGC1α expression and resulting NST. However, the part focused on the muscle is minimal with respect to adipose tissue, what is a disappointing because this is the main novelty of the study
The manuscript is well written making it easy to read. However, few more experiment and validations are requested for their results to support completely their conclusions.

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tissue and muscle.
Additionally, authors mentioned in the introduction page 4 that calcineurin inhibitors play a direct role in immunosuppression. Please could the authors provide additional information about the potential mechanism by which Rcan1 could control immune response. Is Rcan1 also expressed in immune cells? I would ask the authors to compare Rcan1 gene/protein expression in the different adipose tissue cell fractions: mature adipocytes, endothelial cells, and particularly macrophages.
Although the authors focused on ScWAT, they totally neglected the principal organ of NST. Is there any reasons why the authors omitted BAT analysis in the manuscript? Did authors analyse BAT in Rcan1 KO mice in basal status and in response to cold? What is the gene expression profile in BAT regarding brown markers?
Could the authors demonstrate the physiological relevance of Rcan1 regulation in WAT and beige adipocyte thermogenic gene expression, for example by showing that Rcan1 expression in WAT is regulated by diet, cold-exposure, and obesity?
Rcan1 seems to play a major role in energy storage as a "thrifty gene" which could enable efficiently lipid storage during periods of food abundance in order to provide for periods of food shortage. The role of these "thrifty genes" have been particularly demonstrated in some population such as Pima Indians who, exposed to a western diet, develop a massive obesity and insulin resistance. Is there any GWAS data in different population associating SNPs in Rcan1 to susceptibility to TD2 for example?
Moreover, many animal species including mice employ natural hypothermia in seasonal (hibernation) and daily (torpor) strategies to save energy. Considering the role of Rcan1 in the suppression of energy mobilization, we could hypothesize a role of this protein in the regulation of circadian rhythm. Please the authors discuss
The authors mentioned in the introduction page 5 that high glucose is associated to both increased Rcan1 expression and mitochondrial dysfunctions. Considering the strong phenotype of the Rcan1 KO mice regarding tissue browning and increase PGC1a expression, and electron transport complex component, the authors should provide addition information regarding mitochondria biology in their animal/cell models.
The authors suggest higher adrenergic sensitivity in Rcan1KO mice attested by increased expression of PGC1 and ucp1 in response to adrenergic agonists. However, the authors should provide additional in vivo/exvivo functional validation to support their conclusion in analysing 1/ the maximal thermogenic capacity of the mice in response to noradrenaline, 2/ lipolytic activity in 3T3L1/primary cells.
Moreover, thermogenesis in BAT and browning of the WAT are mainly regulated by the sympathetic nervous system. Is Rcan1 expressed in the brain? How the authors can discard a central role of Rcan1 in the phenotype of the mice?
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In Figure 2, the authors presented a significant reduction of area under the curve regarding the ITT. However, they authors did not considered the initial glucose level which is significantly lower in the KO mice. It is likely that the significance disappear when the results are normalized to the baseline. Moreover, the slop in decreased blood glucose level in the 30 first minutes is not different among the genotype indicating that the insulin sensitivity is not different.

Other Comments:
The statistical analysis section is not complete. The authors should have use a one-way and two-way ANOVA in their analysis (see figure 2). Please the authors consider appropriate statistical analysis.
The authors should give more details about the animal models and indicate if they used male, female, control littermates (which is important since the authors used a mix background for the KO). And at which age where performed the experiments
Why the authors used 6C when they acclimated mice for 5 days and not 4C like the acute experiment of 24h exposition?

Referee #2:

This is an interesting manuscript suggesting that RCAN1 plays a homeostatic role in energy expenditure and whole body metabolism. The experiments are rigorously performed and the data are

well presented. The data are of good quality and also confirmed by different approaches and methods . The results strongly support their general conclusions that RCAN1 might play a negative role in systemic energy expenditure by suppressing NST mechanisms in skeletal muscle and adipose tissue.
The manuscript is well written and the discussion is balanced and the limitations are also addressed .

Minor comments:
There is no such thing as "resting muscle" all posture muscles do function even when an animal is not moving .
The authors suggest that resting muscle energy expenditure is high .This will require direct measurements of oxygen consumption(Oxygraph studies)in muscle.

It will also help performing phenotyping of muscle to show higher proportion of oxidative muscle (enhanced oxidative capacity). It can be easily done by SDH staining a marker of high oxidative capacity.

Referee #3:

In the manuscript titled "regulator of Calcineurin 1 (RCAN1) helps coordinate whole body metabolism and limit energy expenditure", Rotter et al. reported the observation that RCAN1 KO mice are protected against HFD induced obesity. The authors claim that the metabolic benefits of RCAN1-deficiency is caused by removing the repression of RCAN1 on NST in both fat and skeletal muscle. The observation is potentially interesting, however several aspects of the study need to be further investigated and clarified before any conclusion can be substantiated.

Major points:

1. Given RCAN1 expresses in many tissues and the KO model used here is a whole body knockout, the authors should include the data demonstrating the expression level of RCAN1 in the liver, skeletal muscle, brown fat, inguinal subcutaneous white adipose tissue and epididymal visceral white adipose tissue. The model the authors proposed suggests that RCAN1 plays this central regulatory role of NST in both fat and muscle and these regulations are cell-autonomous, then the expression level of RCAN1 in these tissues are very relevant.
2. The authors should investigate whether and how much brown fat might contribute to the phenotype of RCAN1 KO phenotype. When the topic is about "whole body metabolism" and "energy expenditure", not to present data from brown fat is not ok.
3. It has recently been suggested that thermogenic beige adipocytes exist in visceral white fat as well (Cell metabolism, 2017, 25(4):811-822), the authors should include more molecular analysis of the visceral fat.
4. If RCAN1 does play an important role regulating NST, then it is conceivable, its expression may be regulated during cold exposure (CE) and/or HFD. Given the authors actually did these experiments in Figure 5, they should test Rcan1 level in fat and skeletal muscle after both CE and HFD.

Minor points:

1. The authors clearly stated in the "material and methods" the Rcan1-KO lines are maintained on a mixed 129SvJ X C57BL/6 genetic background. However, throughout the paper, they did not specify that the WT control mice were littermate control. In particular, when describing experiments in figure 4f, (txt on page 8), the authors call WT c57BL/6. The authors need to clarify what control they were using.
2. In figure 6 legend, the authors write "...and epididymal adipose (sWAT) (B)...", why would they call epiddidymal white adipose tissue "sWAT"? They defined in the first paragraph of "introduction" that sWAT is subcutaneous WAT.
3. In Figure S1, the authors measured blood parameters in "two-month old" mice "after 8 weeks 60% HFD". Since in the first three weeks of a mouse life, they fed on milk not solid food, clearly this statement is wrong.
4. In Figure 1F, KO mice body weight at point "start" and after 6weeks on "HFD", is not changed, or maybe trending go down. However, in Fig1B, after 6 weeks on HFD, KO mice body weight

actually goes up, not as much as the control mice, but clearly increased. The authors should clarify this discrepancy.
5. In Figure 1C, the authors present food intake after normalization to body weight. They did not clarify this weekly food intake data was the average throughout the 25weeks of HFD, or the food intake at certain specific time points. Given the WT mice on HFD at week 20, BW is 1.7 folds higher than the KO, the data actually demonstrate the actual amount of food that WT mice on HFD ate, is much, much more than the amount consumed by the KO on HFD. The way the authors presented the data is misleading.
6. In Fig5c and Fig 5B, the authors tested more thermogenic gene expression in sWAT of mice after HFD than the sWAT of mice after cold exposure, which really does not make any sense.

All these minor errors, collectively cast doubts on the overall carefulness how the study was carried out.

1st Revision - authors' response 12 September 2018

RESPONSE TO REVIEWER #1:

Comments to the manuscript: EMBOR-2017-44706V1
The manuscript entitled "Regulator of Calcineurin 1 (RCAN1) helps coordinate whole body metabolism and limit energy expenditure" by Rotter D etal, investigates the impact of Rcan1 deletion on non-shivering thermogenesis in white adipose tissue and muscle. They demonstrated that Rcan-1 KO mice were resistant to diet-induced obesity as a result of higher metabolic rate due 1/ increased adipocyte response to adrenergic stimuli and subsequent browning of the subcutaneous fat, and 2/increased sarcolipin expression and subsequent activation of NST in skeletal muscle.

The novelty of the manuscript is in the identification of Rcan1 as a thermoregulatory factor in both adipose tissue and muscle linking calcineurin-dependent signalling to cold-induced PGC1α expression and resulting NST. However, the part focused on the muscle is minimal with respect to adipose tissue, what is a disappointing because this is the main novelty of the study

The manuscript is well written making it easy to read. However, few more experiment and validations are requested for their results to support completely their conclusions.

Major Comments:

Query 1: The authors use a global KO mouse mode. What is the tissue distribution of Rcan1? This would be important to justify that the phenotype of Rcan1KO mice is due to a defect specifically in adipose tissue and muscle.

Our response*: The point the reviewer has raised is indeed a fundamental question, as it is important to know whether RCAN1 is present in relevant tissues. To address this, we have added a new figure (Figure EV 3A) showing RCAN1 protein levels in BAT, sWAT, gWAT, liver, and skeletal muscle. RCAN1 was present in all three adipose depots at similar levels in both males and females. Levels were higher in adipose than in liver, but lower than those found in skeletal muscle. Thus, RCAN1 is present in each of the tissues relevant to our studies. Because skeletal muscle is a mosaic of different muscle fiber types, we have also added a figure comparing RCAN1 protein levels in muscle groups of varying fiber type composition (Figure 5E). RCAN1.1 levels were similar throughout, whereas RCAN1.4 levels depended on fiber type composition, with higher levels present in more highly oxidative muscle such as soleus and plantaris. Extracts from heart and brain are included in these figures for comparison. We would like to point out that RCAN1 is not restricted to the tissues examined in our study and likely carries out functions in other organs, tissues and cell types that could contribute to the metabolic phenotype. We have added additional text to the discussion to highlight this point.*

Query 2: Additionally, authors mentioned in the introduction page 4 that calcineurin inhibitors play a direct role in immunosuppression. Please could the authors provide additional information about the potential mechanism by which Rcan1 could control immune response. Is Rcan1 also expressed

in immune cells? I would ask the authors to compare Rcan1 gene/protein expression in the different adipose tissue cell fractions: mature adipocytes, endothelial cells, and particularly macrophages.

***Our response**: The reviewer makes an extremely relevant point regarding the potential contribution of calcineurin/RCAN1 activity in the immune system to the lean phenotype and adaptive NST response. There is indeed a growing literature regarding RCAN1 in the immune system, most of which points to an enhanced immune response in KO animals. We do not therefore believe that the lack of inflammatory infiltrate in the gWAT of the KO mice on a high fat diet (Figure 1L) is due to a suppressed immune response, but rather reflects the lean phenotype of the KO. In the course of our studies, we carried out pilot PCR studies to look for evidence of type-two macrophages (M2) in adipose tissue of the* KO, *but found no indication of an accelerated M1 to M2 conversion (data not shown). In endothelial cells, RCAN1 has been shown to be important for maintaining barrier integrity, thus, loss of RCAN1 function in endothelial cells of adipose depots would more likely enable, rather than block tissue inflammation. We have added the following text and references to our discussion to address this important point raised by the reviewer.*

Calcineurin signaling is important in immune responses and indeed *Rcan1* is present in a variety of cell types of both the innate and adaptive immune systems [1-4]. RCAN1 can suppress NF-κβ activation [5] and *Rcan1 KO mice* show enhanced inflammatory responses [6,7]. Thus, the reduction in inflammatory markers in the gWAT of the *KO* mice on HFD is likely secondary to the lean phenotype rather than due to suppression of an immune response in the *KO*. Along these same lines, endothelial cell dysfunction is a prominent feature of diabetes [8], whereas, RCAN1 promotes barrier integrity [9], thus, loss of *Rcan1* in endothelial cells is not likely the driving mechanism behind the lean phenotype in our studies.

Query 2: Although the authors focused on ScWAT, they totally neglected the principal organ of NST. Is there any reasons why the authors omitted BAT analysis in the manuscript? Did authors analyse BAT in Rcan1 KO mice in basal status and in response to cold? What is the gene expression profile in BAT regarding brown markers?

***Our response**: The reviewer is of course right; we should have included BAT analysis in our initial submission but removed it because of concerns over space constraints and a lack of significant findings in KO BAT. However, the lack of effect on BAT of Rcan1 KO animals may itself be of interest, so we have added data showing that transcript levels for Ucp1, Pgc1α, and Adrb3 are similar in BAT of WT and KO animals under basal conditions (Figure EV 4A). Cold-induced activation of Ucp1 and Pgc1α-1b expression in BAT is likewise similar in WT and KO (Figure EV 4B), suggesting that in BAT, the ability to respond to an adrenergic stimulus is the same. Consistent with this, western blot analysis likewise shows little difference in UCP1 protein levels in BAT when comparing KO with WT (Figure EV 4C), although the age of the animals influenced UCP1 levels, which were higher in the 14-week old animals compared to 8-week old animals in either genotype. As BAT is the primary source of thermogenic activity under acute stimulation, we compared the ability of WT and KO to maintain body temperature following cold exposure and found them to be similar (Figure EV 4D). We also assessed basal and stimulated rates of lipolysis in BAT tissue explants and found no difference between KO and WT (Figure EV 4E). Taken together, these findings suggest that loss of RCAN1 has minimal impact on lipolysis and thermogenic responses in BAT.*

Although based on the parameters we measured we found no differences in the BAT of KO animals compared to WT. We do not interpret this to mean that RCAN1 does not influence calcineurin activity in BAT, rather that calcineurin/RCAN1 do not play a major role in activation of Ucp1 expression in BAT under the conditions we assayed. However, given the fundamental role calcineurin plays in many cellular signaling events, it is likely that we would find changes in calcineurin-dependent signaling in KO BAT if the appropriate parameters were assayed.

Query 3: Could the authors demonstrate the physiological relevance of Rcan1 regulation in WAT and beige adipocyte thermogenic gene expression, for example by showing that Rcan1 expression in WAT is regulated by diet, cold-exposure, and obesity?

***Our response:** Here we have added new western data showing that levels of both RCAN1 isoforms increase in white adipose and livers of WT mice after 8 weeks on a HFD (Figure EV 5E). In*

contrast, RCAN1 protein levels did not change in skeletal muscle in response to either 8 weeks of HFD or short-term cold exposure (Figure EV 5F). It may be significant that RCAN1 protein levels increase in response to HFD feeding only in the tissues showing a positive association between Rcan1 transcript levels and HOMA-IR (adipose and liver but not skeletal muscle) (Figure 6 and EV 5D). These findings are consistent with the idea that the mechanism of RCAN1 action in adipose tissue is different than its mode of action in skeletal muscle.

Query 4: Rcan1 seems to play a major role in energy storage as a "thrifty gene" which could enable efficiently lipid storage during periods of food abundance in order to provide for periods of food shortage. The role of these "thrifty genes" have been particularly demonstrated in some population such as Pima Indians who, exposed to a western diet, develop a massive obesity and insulin resistance. Is there any GWAS data in different population associating SNPs in Rcan1 to susceptibility to TD2 for example?

***Our response**: Although we have looked extensively, we are not aware of any human GWAS studies associating SNPs in RCAN1 with metabolic phenotypes. We are aware of one study that showed a strong association between RCAN1 (DSCR1) and adaptation to climate a*[10]*. The goal of this study was to use climate adaptation as a method to identify potential candidate genes for metabolic disorders. Interestingly, in this paper it appears that RCAN1 (DSCR1) may have initially been selected as a control SNP that ended up showing a strong association. We have now added a reference to this paper in our introduction.*

The reviewer makes a valid point that RCAN1 appears to be functioning as a "thrifty gene", a gene selected over the course of evolution to favor energy storage [11]*. Following the reviewer's suggestion, we have looked closely at a number of the potential "thrifty" genes identified in the Pima Indian population. Intriguingly, a number of these do have connections to calcineurin, such as ASK1* [12]*, which is a known calcineurin substrate* [13] *and HNF4α* [14]*, which has been implicated in cyclosporine induced post-transplantation diabetes* [15]*. However, given that neither of these relationships have been explored directly in this manuscript, and there are many other genes identified in this population for which there are no obvious calcineurin connections, we felt it premature to add this to the discussion at this juncture.*

Query 5: Moreover, many animal species including mice employ natural hypothermia in seasonal (hibernation) and daily (torpor) strategies to save energy. Considering the role of Rcan1 in the suppression of energy mobilization, we could hypothesize a role of this protein in the regulation of circadian rhythm. Please the authors discuss

***Our response**: This is an interesting question, particularly in light of reports of seasonal changes in Rcan1 transcript levels in hibernating animals* [16] *and circadian patterns of Rcan1.4 expression in striated muscle from our lab and others* [17-19]*. In the revised manuscript we have expanded our discussion of these changes and their potential implications.*

Query 6: The authors mentioned in the introduction page 5 that high glucose is associated to both increased Rcan1 expression and mitochondrial dysfunctions. Considering the strong phenotype of the Rcan1 KO mice regarding tissue browning and increase PGC1a expression, and electron transport complex component, the authors should provide addition information regarding mitochondria biology in their animal/cell models.

***Our response**: This is indeed an important question that likely goes beyond simply increased mitochondrial content in the sWAT of KO mice following cold exposure. Calcineurin has many substrates in addition to the transcription factors NFAT and CRTC that we discuss in the manuscript. Dephosphorylation of the pro-mitochondrial fission protein DRP1 by calcineurin promotes its translocation to mitochondria and initiation of mitochondrial fission* [20]*. We have recently published work showing that calcineurin/DRP1-dependent mitochondrial fission is increased in myocytes and fibroblasts isolated from the Rcan1 KO mice* [21]*. Consistent with increased fission, we documented a decreased in mitochondrial membrane potential, lower O_2 consumption, decreased ATP synthesis, a lower respiratory reserve, and a decrease in MCU-dependent mitochondrial calcium uptake. Importantly, we found no change in mitochondrial content or coupling efficiency.*

Taken at face value, such changes would be predicted to reduce whole body metabolic rate rather than increase it, thus, increased mitochondrial fragmentation is unlikely to be the direct cause of the lean phenotype of the KO mouse. However, given that mitochondria are a fundamental unit of metabolism, we have added a reference to this work in the discussion. We have also added reference to a recent paper that reports a connection between PKA-dependent activation of mitochondrial fission and the white-to-beige conversion of human adipocytes, although the specific mechanism of action was not explored [22]. *It would be very exciting if in the future we were able to make a mechanistic link between altered mitochondrial dynamics and transcriptional activation of adaptive NST that we have documented here.*

Query 7: The authors suggest higher adrenergic sensitivity in Rcan1KO mice attested by increased expression of PGC1 and ucp1 in response to adrenergic agonists. However, the authors should provide additional in vivo/exvivo functional validation to support their conclusion in analysing 1/ the maximal thermogenic capacity of the mice in response to noradrenaline, 2/ lipolytic activity in 3T3L1/primary cells.

***Our response**: Here, we have added new experiments comparing the ability of the mice to maintain body temperature following cold exposure (Figure EV 4D). Measured hourly using a rectal thermometer there was no difference between KO and WT animals in their ability to maintain body temperature following a shift to 4°C. Although many factors go into body temperature regulation, BAT is the primary source for acute, UCP1-dependent heat generation. Similar maintenance of body temperature upon cold exposure is consistent with similar acute, adrenergic activation of NST in WT and KO BAT.*

To assess depot-specific adrenergic responses, we used tissue explants from young animals raised at normal temperatures. The basal rates of lipolysis in BAT and gWAT were similar in explants from WT and KO, as were the fold increases in lipolysis following adrenergic stimulation with isoproterenol (Figure EV 4E). In contrast, the basal rate of lipolysis in explants of sWAT from the KO was significantly higher than in sWAT explants from WT. Although maximal activities following the addition of isoproterenol were similar, fold activation was significantly lower in the KO. This suggests that sWAT in the Rcan1 KO may more actively burn lipids in the basal state without an additional adrenergic stimulus. Taken together, these findings further support the idea that the primary impact of RCAN1 deficiency on adipose tissues is focused on subcutaneous adipose.

Query 8: Moreover, thermogenesis in BAT and browning of the WAT are mainly regulated by the sympathetic nervous system. Is Rcan1 expressed in the brain? How the authors can discard a central role of Rcan1 in the phenotype of the mice?

***Our response**: We apologize for sounding as if we discounted the potential for RCAN1 functions in the CNS to impact the metabolic phenotype of the KO mice. Our intent was to contrast the feeding behavior of the RCAN1 KO with that of the RCAN2 KO. RCAN1 protein is abundant in the brain* [23] *at much higher levels than in other tissues (see also Figure EV 3A). We have previously described a number of distinct behavioral and learning phenotypes in RCAN1 KO animals* [23,24] *as well as changes in synaptic vesicle trafficking* [25,26]. *Therefore, we are well aware that RCAN1 has a clear impact on CNS function and agree that this may in some, as yet unknown way impact metabolic regulation. However, the lean phenotype of the KO animal is not caused by a change in satiety (Fig 1C) or physical activity (Fig 3D), both processes in which the CNS plays an important role. Our studies, while not eliminating a role for RCAN1 in the CNS, provide evidence for RCAN1 acting in at least two peripheral tissues (adipose and skeletal muscle) and that in the case of adipocytes, we provide evidence that RCAN1 can act cell autonomously. Rcan2 KO animals are also reported to be lean, but this has been shown to be due to reduced caloric intake caused by loss of RCAN2 function in the hypothalamus* [27,28]. *In response to the reviewer's query we have revised the discussion to try and make it clearer that we do not discount the potential of there being a CNS role of RCAN1 in the lean phenotype.*

Query 9: The authors mentioned in the discussion p12 that Cna1 inhibits glucose stimulated insulin secretion in pancreatic cells. Please the authors provide more detail about the mechanism.

***Our response**: We are presuming that the reviewer meant to write Rcan1, rather than Cna1 (often used as the gene symbol for the catalytic subunit of calcineurin) as most evidence indicates that*

calcineurin facilitates insulin secretion from beta cells rather than inhibiting it. The impact of calcineurin appears to be on multiple levels including transcription, β-cell proliferation and survival, vesicle loading, and vesicle release. We have expanded this point in our discussion by referencing the studies of Heit et al [29] *who used a beta cell specific knockout of calcineurin to demonstrate its effect on the transcription of a number of genes important for beta cell proliferation. They further demonstrated that this is due directly to the effect of calcineurin on NFAT transcriptional pathways in beta cells. We also add reference to studies showing RCAN1's impact on vesicle cycling. Despite these diverse mechanisms through which calcineurin has been shown to impact pancreatic function, we do not see significant differences in plasma insulin levels in young KO animals on a normal diet. It may be that compensatory mechanisms are mobilized in the KO mice to maintain homeostasis.*

Query 10: Is there any change in glucose transport in adipocytes from Rcan1KO mice that could also explain the reduced cell size in condition of HFD?

Our response*: We have added new data showing that there is no difference in Glut1 and Glut4 transcript levels in gWAT, sWAT and BAT of the KO compared to WT (Figure EV 1B). Thus, there is no transcriptional evidence that glucose uptake is deficient in the adipose depots of the KO and thus responsible for the smaller adipocyte size. We postulate that the higher metabolic rate of the KO animals is the primary cause of smaller adipocytes size in WAT, as a larger proportion of calories is being consumed rather than stored in white adipocytes.*

Query 11: In Figure 2, the authors presented a significant reduction of area under the curve regarding the ITT. However, they authors did not considered the initial glucose level which is significantly lower in the KO mice. It is likely that the significance disappear when the results are normalized to the baseline. Moreover, the slop in decreased blood glucose level in the 30 first minutes is not different among the genotype indicating that the insulin sensitivity is not different.

Our response*: The reviewer makes a very relevant point that in the ITT tests in figure 2 H, the primary difference influencing the area under the curve is a lower starting glucose concentration in the KO. This is why we were careful in the text not to make any claims regarding major differences in rates of glucose uptake in the ITT, simply pointing out that blood glucose levels were lower in the KO throughout. We tried fasting the animals for a longer period of time prior to the assay so that starting blood glucose levels were the same in the WT and KO (as in the GTT assay) but found that the KO mice frequently died of hypoglycemic shock when injected with insulin, particularly those on the high fat diet. In the revised manuscript we now also call attention to the similarity in the shape of the ITT response curves.*

As recommended by the reviewer, we have also replotted the ITT data from figure 2 such that it is normalized to the baseline starting levels. These can be found in Figure EV 1D. We have left the original plot of actual changes in blood glucose levels in Figure 2 because we feel it provides more information to the reader as well as making easier to see the similarity between the shapes of the two curves, whereas the normalized plots in EV 1D make it look as though the rate of glucose is faster in the KO, and this may not be an accurate representation of the absolute rate of glucose uptake.

However, we would like to point out that, in contrast to the younger mice used in the HFD studies in Figure 2, 12 month old KO mice maintained on normal chow do manifest a more rapid initial rate of glucose uptake compared to WT in an insulin tolerance test (Figure EV 2B). Thus, over time, KO mice appear to be able to maintain better insulin sensitivity.

We have also added additional data showing that phosphorylation of AKT in skeletal muscle of young mice in response to insulin is similar in the two genotypes (Figure EV 5C). Given the prominent role of skeletal muscle in insulin-stimulated glucose uptake, this would be consistent with the similarity in the shapes of the ITT response of the WT and KO.

Other Comments:

Query 12: The statistical analysis section is not complete. The authors should have use a one-way and two-way ANOVA in their analysis (see figure 2). Please the authors consider appropriate statistical analysis.

***Our response**: We apologize profusely for this error. It was completely due to oversights on the part of the corresponding author when compiling data and methods from multiple sources. All original Prism and Excel spreadsheets have now been consulted to verify the method of analysis used and these are now reported in each figure.*

Query 13: The authors should give more details about the animal models and indicate if they used male, female, control littermates (which is important since the authors used a mix background for the KO). And at which age where performed the experiments

***Our response**:* More detailed information has been provided throughout the manuscript, in figure legends, and in methods.

Unless specifically stated otherwise, all animal experiments were carried out on a 129SvJ X C57BL/6 mixed genetic background mice, from two distinct populations: one in the United States, the other in Australia. The Rcan1 KO alleles were independently derived as described. Both populations displayed the same metabolic phenotype and resistance to HFD obesity. Initially, all KO and WT animals were littermates from Het by Het crosses. Later, to control costs and increase yields, sibling mice were used to set up (Het x KO) crosses to generate KO mice and (Het x Het) crosses to generate WT to use for (WT x WT) crosses to yield WT. New breeding pairs were periodically set up from the offspring of (Het x Het) crosses. Experimental animals that were not littermates were age matched and always only one generation away from the founding (Het x Het) cross. We have expanded our description of this in the methods section.

Query 14: Why the authors used 6C when they acclimated mice for 5 days and not 4C like the acute experiment of 24h exposition?

***Our response**: We used 4°C for acute exposures (< 24 hours) and 6°C for more prolonged periods of time out of concern for the animal's welfare. We also consulted the literature and often found 6°C to be the choice of temperature upon longer exposures.*

RESPONSE TO REVIEWER #2:

This is an interesting manuscript suggesting that RCAN1 plays a homeostatic role in energy expenditure and whole body metabolism. The experiments are rigorously performed and the data are well presented. The data are of good quality and also confirmed by different approaches and methods . The results strongly support their general conclusions that RCAN1 might play a negative role in systemic energy expenditure by suppressing NST mechanisms in skeletal muscle and adipose tissue.
The manuscript is well written and the discussion is balanced and the limitations are also addressed.

Minor comments:

Query 1: There is no such thing as "resting muscle" all posture muscles do function even when an animal is not moving .

***Our response**: The reviewer makes an important point. We have removed the word "resting" from the abstract and have used the term "skeletal muscle, in animals at rest" instead of "resting skeletal muscle" elsewhere in the text to try and indicate we are referring to the metabolic activity of a sedentary animal.*

Query 2: The authors suggest that resting muscle energy expenditure is high. This will require direct measurements of oxygen consumption(Oxygraph studies)in muscle.

***Our response**: The reviewer is correct that our experiments do not directly test whether skeletal muscle is a site of increased energy expenditure in vivo. Although we have shown that SLN*

expression and protein levels are higher in the skeletal muscle of KO mice, as far as we can tell SLN levels are still quite low, and whether they are elevated sufficiently to have a significant impact on VO_2 and VCO_2 measured in Figure 3 remains to be determined.

As mentioned above in our response to Reviewer 1, we recently published a paper showing that calcineurin-dependent mitochondrial fission is increased in myocytes and fibroblasts isolated from the Rcan1 KO mice [21]*. Consistent with increased fission, we documented a decreased in mitochondrial membrane potential, lower O_2 consumption, decreased ATP synthesis, a lower respiratory reserve, and a decrease in MCU-dependent mitochondrial calcium uptake in isolated cardiomyocytes. Importantly, we found no change in mitochondrial content or decrease in coupling efficiency.*

Taken at face value, such changes would be predicted to reduce whole body metabolic rate rather than increase it, thus, increased mitochondrial fragmentation is unlikely to be the direct cause of the lean phenotype of the KO mouse. However, given that mitochondria are a fundamental unit of metabolism, we have added a reference to this work in the discussion of our revised manuscript. We have also added reference to a recent paper that reports a connection between PKA-dependent activation of mitochondrial fission and the white-to-beige conversion of human adipocytes, although the specific mechanism of action was not explored [22]*. It would be very exciting if in the future we were able to make a mechanistic link between altered mitochondrial dynamics and transcriptional activation of adaptive NST mechanisms that we have documented here.*

Query 3: It will also help performing phenotyping of muscle to show higher proportion of oxidative muscle (enhanced oxidative capacity). It can be easily done by SDH staining a marker of high oxidative capacity.

Our response*: A number of new items have been added expanding our analyses of the skeletal muscle phenotype:*

1. We have added a figure comparing RCAN1 protein levels in muscle groups of varying fiber type composition (Figure 5E). RCAN1.1 levels are similar throughout, whereas RCAN1.4 levels vary with fiber type composition. Higher levels of RCAN1.4 were present in more highly oxidative muscle such as soleus and plantaris. As Rcan1.4 expression is under the control of calcineurin/NFAT [30] *this is consistent with the documented association between calcineurin activity and oxidative muscle fibers* [31].

2. We have added metachromatic staining for muscle fiber type composition [32]*(Figure EV 5A) as well as high resolution SDS-PAGE analysis of myosin heavy chain composition* [33] *(Figure EV 5B). Both findings indicate that the skeletal muscle of the Rcan1 KO animal is not shifted toward a more oxidative fiber type composition. If anything, there appears to be a reduction in highly oxidative type I fibers. Thus, a shift in fiber type composition cannot explain the elevated metabolic rate of these animals.*

3. We compared the ability of skeletal muscle to respond to insulin by assessing AKT phosphorylation following an insulin injection. The responses of KO and WT muscle were similar (Figure EV 5C). Although this by no means provides a comprehensive assessment of insulin sensitivity, it does suggest that there are no gross differences in the insulin response of the two genotypes, it is also consistent with the similarity in the shapes of the ITT glucose response curves reported in Figure 2H.

RESPONSE TO REVIEWER #3:

In the manuscript titled "regulator of Calcineurin 1 (RCAN1) helps coordinate whole body metabolism and limit energy expenditure", Rotter et al. reported the observation that RCAN1 KO mice are protected against HFD induced obesity. The authors claim that the metabolic benefits of RCAN1-deficiency is caused by removing the repression of RCAN1 on NST in both fat and skeletal muscle. The observation is potentially interesting, however several aspects of the study need to be further investigated and clarified before any conclusion can be substantiated.

Major points:

Query 1: Given RCAN1 expresses in many tissues and the KO model used here is a whole body knockout, the authors should include the data demonstrating the expression level of RCAN1 in the liver, skeletal muscle, brown fat, inguinal subcutaneous white adipose tissue and epididymal visceral white adipose tissue. The model the authors proposed suggests that RCAN1 plays this central regulatory role of NST in both fat and muscle and these regulations are cell-autonomous, then the expression level of RCAN1 in these tissues are very relevant.

***Our response**: As detailed in our response to Reviewer 1, to address this, we have added a new figure (Figure EV 3A) showing that RCAN1 protein is present in BAT, sWAT, gWAT, liver, and skeletal muscle. RCAN1 was present in all three adipose depots at similar levels in both males and females. Levels were higher in adipose than in liver, but lower than those found in skeletal muscle. Thus, RCAN1 is present in each of the tissues relevant to our studies. Because skeletal muscle is a mosaic of different muscle fiber types, we have also added a figure comparing RCAN1 protein levels in muscle groups of varying fiber type composition (Figure 5E). RCAN1.1 levels were similar throughout, whereas RCAN1.4 levels depended on fiber type composition, with higher levels present in more highly oxidative muscle such as soleus and plantaris. Extracts from heart and brain where included for comparison.*

Query 2: The authors should investigate whether and how much brown fat might contribute to the phenotype of RCAN1 KO phenotype. When the topic is about "whole body metabolism" and "energy expenditure", not to present data from brown fat is not ok.

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***Our response**: The reviewer is of course right; we should have included BAT analysis in our initial submission but removed it because of concerns over space constraints and a lack of significant findings in KO BAT. However, the lack of effect on BAT of Rcan1 KO animals may itself be of interest, so we have added data showing that transcript levels for Ucp1, Pgc1α, and Adrb3 are similar in BAT of WT and KO animals under basal conditions (Figure EV 4A). Cold-induced activation of Ucp1 and Pgc1α-1b expression in BAT is likewise similar in WT and KO (Figure EV 4B), suggesting that in BAT, the ability to respond to an adrenergic stimulus is the same. Consistent with this, western blot analysis likewise shows little difference in UCP1 protein levels in BAT when comparing KO with WT (Figure EV 4C), although the age of the animals influenced UCP1 levels, which were higher in the 14-week old animals compared to 8-week old animals in either genotype. As BAT is the primary source of thermogenic activity under acute stimulation, we compared the ability of WT and KO to maintain body temperature following cold exposure and found them to be similar (Figure EV 4D). We also assessed basal and stimulated rates of lipolysis in BAT tissue explants and found no difference between KO and WT (Figure EV 4E). Taken together, these findings suggest that loss of RCAN1 has minimal impact on lipolysis and thermogenic responses in BAT.*

Although based on the parameters we measured we found no differences in the BAT of KO animals compared to WT. We do not interpret this to mean that RCAN1 does not influence calcineurin activity in BAT, rather that calcineurin/RCAN1 do not play a major role in activation of Ucp1 expression in BAT under the conditions we assayed. However, given the fundamental role calcineurin plays in many cellular signaling events, it is likely that we would find changes in calcineurin-dependent signaling in KO BAT if the appropriate parameters were assayed.

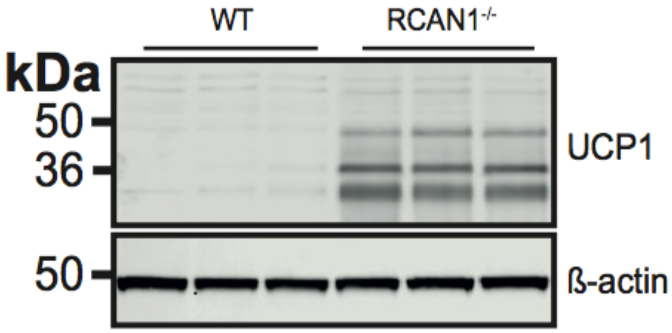
Query 3: It has recently been suggested that thermogenic beige adipocytes exist in visceral white fat as well (Cell metabolism, 2017, 25(4):811-822), the authors should include more molecular analysis of the visceral fat.

***Our response**: We have expanded our analysis of visceral fat to include:*

1. *Comparison of RCAN1 protein levels in gWAT compared to other adipose stores (Figure EV 3A)*
2. *Changes in RCAN1 protein levels in WT animals on NC and HFD showing that both RCAN1 isoforms increase on a HFD (Figure EV 5E).*

3. *Measures of basal lipolysis and stimulated lipolysis in response to an adrenergic stimulus (Figure EV 4E) showing that there are no significant differences in the gWAT from WT and KO. The response of BAT is likewise the same. Only the response of sWAT was altered in the Rcan1 KO tissue explants, showing evidence of increased basal lipolysis. Thus, relative to adipose depots, RCAN1 appears to have its primary impact in sWAT.*
4. *PCR of Glut1 and Glut4 transcript levels showing that they are not significantly different between WT and iKO Figure EV 1B).*
5. *We also used PCR to look for cold induction of Ucp1 and Pgc1α-1b in gWAT. Transcript levels were below the level of detection both at base line and following cold exposure. Increasing cycle number eventually generated some product, but the melt curves indicated that they were not the target transcripts, rather the product of mis-priming at some other site when the target sequence was not available. As this is a null result, it is simply mentioned in the text rather than depicted graphically in a figure.*
6. *Despite not being able to detect cold-induction of UCP1 expression in gWAT, we have been able to detect low levels of UCP1 protein in gWAT of older KO males (6-8 months old) (Reviewer Figure 1) but not younger animals, or females. This suggests that loss of RCAN1 may also increase the capacity of gWAT to activate Ucp1 expression, although not to the extent of the response seen in sWAT of the KO. It also suggests that the increase in RCAN1 levels that we observe in gWAT of the HFD animals (Figure EV 5E) may contribute to suppression of a beiging response in this depot.*

Figure 1 for Reviewer: *Western blot analysis of gWAT from 6-8 month old WT and KO males shows an increase in UCP1 signal in RCAN1-/- KO animals. In addition to the expected UCP1 signal at around 33 kDa, there are two additional bands around*



25 and 50 kDa. These we believe are detecting IgG heavy and light chains respectively. This could be an indication of increased vascularization of gWAT in these animals. UCP1-positive adipose is known to be more highly vascularized and innervated.

Query 4: If RCAN1 does play an important role regulating NST, then it is conceivable, its expression may be regulated during cold exposure (CE) and/or HFD. Given the authors actually did these experiments in Figure 5, they should test Rcan1 level in fat and skeletal muscle after both CE and HFD.

Our response*: We have added new western data showing that levels of both RCAN1 isoforms increase in white adipose and livers of WT mice after 8 weeks on a HFD (Figure EV 5E). In contrast, RCAN1 protein levels did not change in skeletal muscle in response to either 8 weeks of HFD or short-term cold exposure (Figure EV 5F). Thus, only the tissues showing a positive association between Rcan1 transcript levels and HOMA-IR (Figure 6) showed increased protein levels on a HFD. These findings are consistent with the idea that the mechanism of action of RCAN1 relative to NST is different in skeletal muscle compared to adipose (suppression of Sln expression in skeletal muscle verses suppression of Pgc1α expression in adipose).*

Minor points:

Point 1: The authors clearly stated in the "material and methods" the Rcan1-KO lines are maintained on a mixed 129SvJ X C57BL/6 genetic background. However, throughout the paper, they did not specify that the WT control mice were littermate control. In particular, when describing experiments in figure 4f, (txt on page 8), the authors call WT c57BL/6. The authors need to clarify what control they were using.

***Our response**: We apologize for any confusion over this point. The Ing-svf cells used in Fig 4F were isolated from WT, inbred C57BL/6 mice. Unless specifically designated, all other experiments were carried out on 129SvJ X C57BL/6 mixed genetic background mice, from two distinct populations; one in the United States, the other in Australia. The Rcan1 KO alleles were independently derived as described. Both populations displayed the same metabolic phenotype and resistance to HFD obesity. Initially, all KO and WT animals were littermates from Het by Het crosses. Later, to control costs and increase yields, sibling mice were used to set up (Het x KO) crosses to generate KO mice and (Het x Het) crosses to generate WT to use for (WT x WT) crosses to yield WT. New breeding pairs were periodically set up from the offspring of (Het x Het) crosses. Experimental animals that were not littermates were age matched and always only one generation away from the founding (Het x Het) cross. We have expanded our description of this in the methods section.*

Point 2: In figure 6 legend, the authors write "...and epididymal adipose (sWAT) (B)...", why would they call epiddidymal white adipose tissue "sWAT"? They defined in the first paragraph of "introduction" that sWAT is subcutaneous WAT.

***Our response**: Thank you so much for catching this mistake. The Keller* [34] *and Parks* [35] *studies analyzed transcript levels in epididymal/gonadal adipose. We made a mistake by using "sWAT" in the figure legend and have corrected this to gWAT in the figure legend as well as designating the adipose source as "gonadal" rather than epididymal to avoid confusion.*

Point 3: In Figure S1, the authors measured blood parameters in "two-month old" mice "after 8 weeks 60% HFD". Since in the first three weeks of a mouse life, they fed on milk not solid food, clearly this statement is wrong.

***Our response**: We apologize and have changed this to 18-week-old mice as they were 10-weeks-old when this experiment began.*

Point 4: In Figure 1F, KO mice body weight at point "start" and after 6weeks on "HFD", is not changed, or maybe trending go down. However, in Fig1B, after 6 weeks on HFD, KO mice body weight actually goes up, not as much as the control mice, but clearly increased. The authors should clarify this discrepancy.

***Our response**: These data are from a different groups of animals than those reported in Figure 1B. The starting age for this set of animals was older and this may be the reason for the slower trajectory in weight gain. Note the higher starting body weights in 1F (starting age of 15 weeks old compared to 6-8 weeks old in 1A and B). Our main goal for the MRI analysis was to assess differences in fat/lean body composition which showed the primary difference was in fat mass whether on NC (starting measures) or after HFD feeding.*

Point 5: In Figure 1C, the authors present food intake after normalization to body weight. They did not clarify this weekly food intake data was the average throughout the 25weeks of HFD, or the food intake at certain specific time points. Given the WT mice on HFD at week 20, BW is 1.7 folds higher than the KO, the data actually demonstrate the actual amount of food that WT mice on HFD ate, is much, much more than the amount consumed by the KO on HFD. The way the authors presented the data is misleading.

***Our response**: The reviewer makes an important point. We thought long and hard about what would be the most relevant way to present the data and decided that the most informative way would be to divide the total grams of food consumed throughout the duration of the study by the change in body weight over the course of the study.*

In other words, how much food is required for the animal to gain a gram of body weight? Revised Figure 1C shows that the KO mice consume significantly more food to achieve gram increase in body weight. To provide a more comprehensive comparison of food consumption we have also added two graphs to the EV material that rack weekly food consumption over the course of the study, normalized to body weight during that week (grams consumed per day per kg body weight)) and on a per animal basis (grams consumed per day per mouse) (Figure EV 1A). Throughout the study, the KO animals consume more food than WT on ether diet when normalized to body weight. Whereas the WT do indeed consume more per animal because of their greater body weight as predicted by the reviewer.

Please note that during the first week on NC, food intake by WT animals was depressed compared to subsequent weeks. We found that the WT animals consistently consumed a lower amount of NC during the first week of a feeding study whereas the KO animals did not. We speculate that this may relate to the differences in anxiety behaviors we have previously reported [24]. *As a result, the WT animals take longer to adjust to the stress of a new environment and increased handling, whereas the low anxiety KO mice start eating right away. Interestingly, we did not observe this on the HFD, a food source so appealing that we found the animals would refuse to eat for several days when switched back to NC (personal observation).*

Also relevant to the reviewer's query is the fact that mice placed on a HFD tend to lose the normal daily pattern of fasting and feeding, choosing instead to eat throughout the 24-hour day. A growing number of studies have now demonstrated that maintaining appropriate fasting/feeding rhythms is protective against developing metabolic disorders even on a high fat diet [36]. *We therefore felt it important to know whether the feeding pattern of the Rcan1 KO mice differed from that of WT on the HFD. A group of three WT and three KO males were place on HFD or NC, starting at 10 weeks of age. After eight weeks, mice were moved to individual metabolic cages with the capacity for continuous monitoring of food consumption. After three days acclimation to the new environment, hourly food consumption was tracked over three days. The animals on NC displayed typical circadian patterns of food consumption with peak food intake occurring during the night when mice are normally most active (Reviewer's figure 2A). In contrast, on the HFD, both genotypes ate continuously, irrespective of time of day (Reviewer's figure 2B). Thus, differences in feeding patterns were not responsible for the differences in metabolic phenotypes. We provide these figures for the benefit of the reviewers to provide a more complete comparison of food intake of the two genotypes. We have not added them to the text of the paper. Although fasting/feeding cycles are very important, they are not an aspect of the current manuscript, and we feel would not benefit the flow of the paper.*

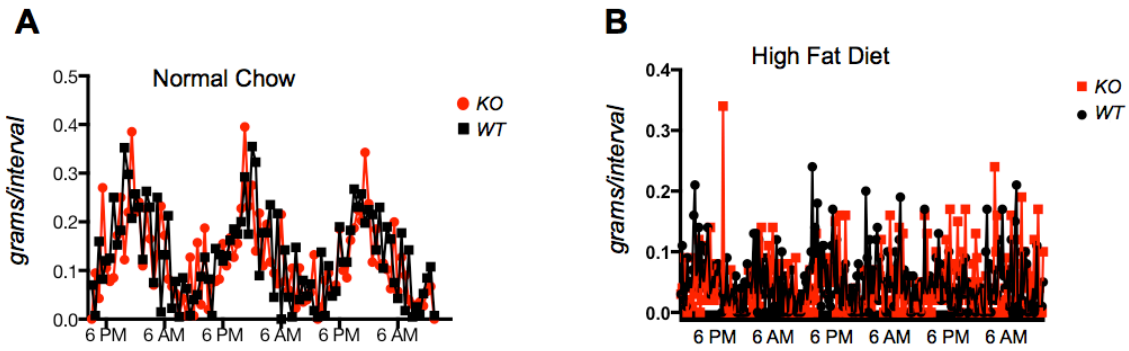


Figure 2 for Reviewer: *Shows that daily pattern of food consumption is similar in KO and WT animals on normal chow (A) and that both genotypes lose the normal circadian patter of food consumption when placed on a high fat diet (B).*

Point 6: In Fig5c and Fig 5B, the authors tested more thermogenic gene expression in sWAT of mice after HFD than the sWAT of mice after cold exposure, which really does not make any sense.

Our response*: Our initial studies were done in the context of HFD and so we screened for differences in expression of a wide range of genes. This allowed us to identify the Pgc1α-1b/Ucp1*

axis as a key difference between the two genotypes. Thus, we focused on this pathway in the cold exposure studies rather than on genes that did not change in the HFD studies. In the paper the PCR results form the HFD sWAT (Figure 5C) are presented out of chronological order relative to when they were carried out, but in the order that we felt was most appropriate to the flow of the paper.

The reviewer's will note that Adrb3 transcripts are higher in the sWAT of KO mice on HFD (Figure 5C), and in the differentiated Rcan1-depleted Ing-svf cells in Figure 4G. We therefore tried to compare Adrb3 transcripts in sWAT following cold exposure. The results were profoundly variable both within WT and KO. Because of this huge variability there was no significant difference between the two genotypes. We have now added the following text to the manuscript indicating this.

"Following cold exposure, there was not a significant difference in *Adrb3* transcript levels in the sWAT of *KO* mice compared to *WT* due to profound variability within each genotype (data not shown). The cause of this extreme variability is unknown, however, the data in figure 4G suggests that the mechanisms controlling *Adrb3* transcript levels are likely very different from those controlling *Ucp1* and *Pgc1α*"

Point 7: All these minor errors, collectively cast doubts on the overall carefulness how the study was carried out.

***Our response**: We apologize for any errors made in our initial submission and would like to thank all of the reviewers for the careful attention they gave to our manuscript. We have tried our best to address and correct all points raised. We believe that the resulting product has substantially elevated the quality of the manuscript.*

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Acceptance 4 October 2018

Thank you for the submission of your revised manuscript. We have now received the comments from the referees, and I am happy to tell you that both support its publication now. We can therefore accept your manuscript.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

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Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-44706V2

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	As these studies had never been carried out before with RCAN1 KO mice, no information was available to asign a pre-specified effect size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	sample size was based on previous experience with similar studies.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Older animals were excluded from the the cold-induction experiments due to high veriability in their responses. Food consumption data for 3 wt and 3 KO mice at week 7 was excluded as food was inadvertantly added to the cages without weighing.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	With the exception of the order of western blot loading, sample identity (genotype and treatment) was blinded.
For animal studies, include a statement about randomization even if no randomization was used.	Animals were randomized to control or treatment in cold exposure and HFD experiments except for in an initial HFD studies where a group of WT and KO animals were all placed on a high fat diet.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Investigators were blinded as to the genotype of the animal when ever possible.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Animal samples were handled in a double-blinded manner. With the exception of western blot loading.
5. For every figure, are statistical tests justified as appropriate?	Yes, number n and statistical method are included in each figure.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	For experiments where the number of independent experiments is too small to assess normal distribution (ie, n=3) we assumed normal distribution.
Is there an estimate of variation within each group of data?	reported as SD or SEM as indicated
Is the variance similar between the groups that are being statistically compared?	Equal varience was tested usig a Brown-Foresythe test.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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

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

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

http://datadryad.org

http://figshare.com

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

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

http://biomodels.net/

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

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Provided in Table 2
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The original source of the 3T3-L1 and C2C12 cells was ATCC. Amplified stocks are maintained by the Gupta and Rothermel labs respectively. Active lines are checked for mycoplasma contamination annually. The lines are regularly authenticated by their ability to differentiat to either mature adipocytes or mature myotubes. Derivation of the Jag cuf cells is described in the

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Two independently derived Rcan1-KO lines were used in our studies; one lacking exons 5 and 6 and the other deficient for the coding regions of exon 6 and 7 (references are provided in manuscript). Mice were maintained on a mixed 129SvJ x C57BL/6 genetic background, homozygous for a wild type allele of the nicotinamide nucleotide transhydrogenase locus (Nnt). The two lines were indistinguishable relative to metabolic phenotype. Whenever possible, littermates from (Het x Het) crosses were used. In addition, to increase yields, sibling mice from (Het x Het) crosses were used to set up (Het x KO) and (WT x WT) crosses, to generate KO and WT animals respectively. New breeding pairs were periodically set up from the offspring of (Het x Het) crosses. Experimental animals were age matched and always only one generation away from the founding (Het x Het) cross. Unless otherwise noted, animals were housed at 23°C under standard vivarium conditions under a 12:12 hour light:dark cycle. Animals were allowed free access to water and were fed ad libitum either a normal chow diet (NC), in which 4% of the caloric content derived from fat (LabDiet 5001), a high fat diet (HFD) in which 60% of the calories derived from fat (Research diet D12492), or a 35% fat diet of similar composition produced by Specialty Feeds (WA, Australia). Food consumption and body weight were recorded weekly. For cold-induction experiments, mice were housed at 6°C under a 12:12 hour light:dark cycle with free access to food and water. Age and gender of animals is reported for each experiment.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal procedures were carried out with the oversight and approval of the University's Institutional Animal Care and Use Committee and conformed to the current Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Done

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	References to two data sets usded in the paper are provided both in the text and in methods.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA