Lack of TRPV2 impairs thermogenesis in mouse brown adipose tissue

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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 07 July 2015

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

As you will see, the referees acknowledge that a role for TRPV2 in brown thermogenesis is interesting. However, they also note that the study is mainly descriptive and that it remains unclear how TRPV2 induces the expression of UCP1 and PGC1alpha and promotes thermogenesis. Insight into this mechanism would certainly be welcome but is not strictly required for publication of the manuscript by EMBO reports, however, possible mechanisms should be discussed in the manuscript. It will be essential to address the following concerns and suggestions: Both referees 1 and 2 remark that it should be investigated whether TRPV2 regulates adipogenesis and in which tissues it is expressed. This is important, given that the TRPV2 KO mouse is a global KO model. It should also be examined which physiological stimuli regulate TRPV2 expression or function. In his/her cross-comments, referee 3 agrees with referee 2 that evidence for a role of TRPV2 in obesity would strengthen the study. Please let me know if you disagree and we can discuss this issue further. The study should also be better placed in the current literature, the data discussed in light of the literature, missing controls added and statistical analyses performed. While detailed mechanistic insight into how the absence of TRPV2 induces whitening of brown adipocytes does not need to be provided, effects on PRDM16 expression could be analyzed.

Given these constructive 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.

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

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

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REFEREE REPORTS

Referee #1:

In this study Sun et al. describe a potential role for the TRPV2 channel in brown adipocyte thermogenesis. The authors present compelling evidence for TRPV2 function ranging from single adipocyte, BAT and whole animals. This is an exciting report. I do have several suggestions and points that should be clarified.

1. TRPV2 expression is analyzed in differentiated brown adipocytes and BAT. It is important to document expression in pre-adipocytes as well. Is TRPV2 up-regulated upon adipogenesis?

2. Similarly, is there differential expression of TRPV2 between BAT versus WAT?

3. Further, it is important to compare expression of TRPV2 in BAT versus TRPM8 previously implicated in BAT thermogenesis.

4. TRPV2-KO animals have impaired adrenergic triggered thermogenesis. Yet, NE evoked Ca2+ responses appear similar in WT and TRPV2-deficient adipocytes. Why? This does not seem to support the hypothesis that TRPV2 is a transduction channel downstream of BAR signaling?

5. Authors state that data presented in Figure 1 conclusively support functional TRPV2 responses. I think more decisive support is established later in the manuscript (Fig. 2.)

Referee #2:

In this manuscript, the authors demonstrate a cell-autonomous role for TRPV2 in the regulation of thermogenesis by murine brown adipocytes. Lack of TRPV2 reduces Ucp1 and Pgc1α mRNA expression, and impairs the induction of these genes by adrenergic stimulation. Ucp1 and PGC1α expression was also reduced in BAT of TRPV2KO mice, and brown adipocytes in TRPV2KO BAT showed white-adipocyte-like morphology. Cold-induced and adrenergic-agonist induced upregulation of Ucp1 and PGC1α expression was reduced in TRPV2KO BAT. TRPV2KO mice also showed a blunted increase in BAT and rectal temperature in response to adrenergic agonist treatment. Overall these data suggest a role of TRPV2 in brown cells to maintain brown thermogenesis, so TRPV2 could be considered as a potential target to promote brown fat expansion in the context of obesity.

The manuscript is predominantly a descriptive account of the thermogenic phenotype of TRPV2KO brown adipocytes and BAT of TRPV2KO mice, although there is an insightful section in the discussion that describes mechanisms by which TRPV2 could be transported to the brown adipocyte plasma membrane and activated in the context of BAT sympathetic stimulation, and how TRPV2 activation could regulate thermogenic gene expression. I think this discussion is important because TRPV2 is a heat-activated channel, so it is not logical that activation of a heat-activated channel would increase thermogenic gene expression - unless heat is not the physiological activator.
Major comments:
1. The authors use a global KO mouse mode. What is the tissue distribution of TRPV2? This would be important to justify that the phenotype of TRPV2KO mice is due to a defect specifically in BAT. Additionally, authors mentioned in the introduction page 4 that macrophages also express TRPV2. Because macrophages, and particularly, alternative macrophages "M2" play a role in brown adipocyte activation, I would ask the authors to compare TRPV2 gene/protein expression in the different BAT cell fractions: mature adipocytes, endothelial cells, and particularly macrophages. Although the authors focused on BAT, it is well known that subcutaneous fat depot is prone to browning in response to cold and high fat diet. Did authors analyse the subcutaneous fat depot in TRPV2KO mice in response to cold? What is the gene expression profile in white adipose tissue regarding brown markers?

2. Could the authors demonstrate the physiological relevance of TRVP2 regulation of brown adipocyte thermogenic gene expression, for example by showing that TRVP2 expression in BAT is regulated by diet, cold-exposure, and obesity? A major concern about this study is the lack of pathophysiological relevance of TRPV2 in the context of obesity. It is well characterised that BAT can be activated in response to high fat diet. Considering the potential role of this channel in the maintenance of BAT thermogenic response, it would be interesting to phenotype TRPV2 KO mice during the development of obesity. The authors suggest that TRPV2 positively regulates oxidative metabolism and brown adipocyte function. If so, we would expect an aggravation of obesity in TRPV2 KO mice. Moreover, treatment with TRPV2 agonists (if suitable in vivo) would ameliorate obesity related complications by activating BAT thermogenesis.

3. There is a lack of background literature on TRVP channels in adipose tissue. Recent studies highlighted a role of TRPV1 in regulation of food intake and glucose homeostasis in white fat during obesity (Lee E et al, FASEB 2015). A role of TRPV3 has been highlighted in regulation of adipogenesis; TRPV3 agonist inhibits white adipogenesis (Cheung SY et al, Endocrinology, 2015). The work of Ma, et al (J Mol Cell Biol 2012 - Activation of the cold-sensing TRPM8 channel triggers UCP1-dependent thermogenesis and prevents obesity) should be cited, as this paper sets the precedent for TRP channels modulating thermogenesis in brown adipocytes in a cell-autonomous manner. Finally, Li Ye et al (Cell, 2012), identified TRPV4 as a regulator of adipose oxidative metabolism, inflammation and energy homeostasis. Is this study the authors showed that TRPV4 KO mice were protected from diet-induced obesity, a mechanism involving increased browning and activation of PGC1α. In this paper, they showed in vitro that depletion of TRPV4 in white adipocytes resulted in increased PGC1α expression whereas depletion of TRPV2 provoked a decreased expression (results in line with the present study). Taken together, this last study suggest that i) TRPV4 and TRPV2 would act in opposite directions to regulate brown activity in adipose cells and ii) TRPV2 would play a relevant role in white adipocytes and might be involved in subcutaneous fat "browning" in vivo.

4. Could the authors complement their loss-of-function experiments using TRVP2KO brown adipocytes with experiments using the TRPV2 agonists utilized in Figure 1D and E? Does treatment of brown adipocytes with these agonists reduced Ucp1 and PGC1α gene expression? Additionally, as mentioned above, TRPV3 plays a role in white adipogenesis and TRPV3 agonists inhibit cell differentiation. I suggest that the authors test the influence of TRPV2 agonists on brown adipocyte differentiation capacity. What is the impact of TRPV2 depletion in brown adipocyte adipogenesis? Decreased responsiveness to β-adrenergic response could result from lower differentiation state of primary brown adipocytes from KO mice. What is the expression profile of the TRPV1-4 during adipogenesis? Is TRPV2 only expressed in differentiated adipocytes?

Minor comments
1. Please could the authors revise the sentence in the introduction page 3 "The functions of these two types...the opposite of another". I found this statement too cathegorical considering the fact that in both depots, adipocytes are the main cells producing adipokines, responding to insulin and catecholamines to promote lipolysis. Moreover, white adipocytes can express UCP-1 in condition of cold/diet-induced browning.

2. Page 3 in the introduction, the authors should precise that in addition to be present in adult, BAT
can be activated. Both activation capacity and amount of tissue are negatively correlated with adiposity.

3. In figure 1A, the expression of Trpv family members is measured in brown adipocytes, with beta-actin shown as a housekeeper. In figure 1B, the data is normalized to a different housekeeping gene, 36b4. Is there a particular reason for using one house-keeper in the gel analysis, and another for quantification?

4. In Figure 2A, The authors should also examine the protein expression of the TRVP1, 3 and 4 in brown adipocytes and tissue, in order to see any potential compensatory response.

5. In Figure 2B, whereas the response to 2APB is completely abolished in brown adipocytes from TRPV2 KO mice, response to NE is not statistically modified suggesting that other channels are involved in the NE response. Please could the authors discuss this.

6. Figure 2D: I would also suggest to examine the functional response of WT and KO brown adipocytes to isoproterenol by measuring glycerol or free fatty acid release. Moreover, the authors should test different pharmacological activators of lipolysis (Isoproterenol, Forskolin and DeAMP); this would to distinguish if the impairment of the β-adrenergic response results from decreased expression/functionality of β-adrenergic receptors or alteration to mitochondrial function.

7. The fact that TRPV2KO mice are lighter than wild-types (Supplementray figure EV2A) must be considered when interpreting the data from figure 3-6. For example, are these animals just lighter (i.e. have less fat mass) than wild-types, or are they shorter i.e. is there a growth defect? I suspect this is a growth defect, as TRPV2KO mice had heavier WAT depot weights. The data in Figure 3B should be normalized to body weight because WTs and TRPV2KOs had different body weights. This would actually reveal a more significant increase in WAT depot weight in TRPV2KOs. Analysing body composition using DEXA or Body Composition Analyzer based on TD-NMR that would constitute a more precise method for measuring lean and fat mass in WT and KO mice.

8. In the section "Lack of TRPV2 impaired the expression of mitochondrial oxidative metabolism-related genes in brown adipocytes," Ucp1 and PGC1α are the genes referred to as "mitochondrial oxidative metabolism-related genes." As someone in the field, I would use that label for PGC1α, mitochondrial transporters, and electron transport complex components. I think a better description of the function encompassed by Ucp1 and PGC1α is "thermogenic genes."

9. Please could the authors provide statistical analysis in Table 1. KO mice seem to display increased plasma insulin levels conversely with decreased serum cholesterol.

Referee #3:

In this study, authors show that TRPV2 expressed in brown adipocytes, furthermore TRPV2 mediated mitochondrial oxidative metabolism-related function was impaired in absence of TRPV2 gene. TRPV2 KO mice was susceptible to obesity and showed cold intolerance and reduced responses to β3-adrenergic receptor activation. Authors concluded that TRPV2 plays a crucial role in the maintain of thermogenesis of brown adipose tissue. Although authors report some novelties in this study, most results are phenomenon description and lack deep deciphering in mechanism. I have following concerns

Major comments:

1. UCP1 accounts for the thermogenesis by mitochondria and is a widely used BAT marker. Pgc1α is a crucial modulator of mitochondrial biogenesis. How does TRPV2 affect these two key molecules? Authors should provide more solid evidences to explain their links rather than only showing their expression in BAT in the absence of TRPV2.

2. The configuration of results seems poor of logic. For example, the core results of the whole work showing the impaired cold-induced thermogenesis in Trpv2 KO mice appear in Figure 5. The results on the expression levels of Ucp1 and Pgc1α, which seem to be the critical point explaining the
phenotype, scatter in Figures 2, 3, 5 and 6. It is better to rearrange Figures 2, 3, 4 and 5 following a more logical order like this: the core novelty and phenomenon, the tissue or cell behaviors explaining the phenotype, the critical molecular changes accounting for those behaviors and finally, the detailed molecular mechanism.

3. In detailed section, the authors predict that TRPV2 activation modulates brown adipocyte function through Ca2+ influx. However, there is no experimental evidence supporting this presumption. Trpm8, Trpv1 and Trpv4 are reported to play critical roles in modulation of Ca2+ influx in response to stimulus, and all of them involve in BAT function. Why do different TRP channels play a similar role or opposite role (such as TRPV4) in BAT mediated thermogenesis? Authors should discuss this point. Current discussion has more repetitive description as results.

4. The results showing impaired β3-adrenergic receptor stimulation-induced thermogenesis in TRPV2KO mice is the consequence of low thermogenic function by mitochondria and could not explain the mechanism of the cold intolerance of TRV2 KO mice. As the increased expression of UCP1 and PGC1a by ISO treatment or cold stimulus was almost totally blocked by TRPV2 knockout, the detailed experiments of the pro-whitening effect of TRPV2 KO should be provided to explain the key point of novelty. For example, the mRNA level, protein stability or binding activity of a critical transcription factor of differentiation of BAT, PRDM16, should be considered to be involved in search for this mechanism.

Minor comments:
1. In figure 1, the expression levels of the four TRPV channels could not be determined merely by quantitative RT-PCR because the efficiency of PCR primers were different. Therefore, it could not be concluded that the expression of Trpv2 is the highest in all four Trpv members. In addition, the importance of the other TRP channels, such as TRPV1, TRPM8, TRPV4, should not be neglected.

2. In figure 4, the authors showed that the cell size of iBAT was much larger in Trpv2 KO mice than WT controls because more lipid droplets were accumulated. However, in Figure 3B, there was no difference of iBAT weight between the two groups. Was the cell number in iBAT of Trpv2 KO mice less than WT controls?

3. In Figure 5E, the statistical result of UCP1 protein levels of iBAT of Trpv2 KO mice was much lower than WT mice after cold exposure. But the tendency of UCP1 expression could hardly be reflected in the representative images of Western blot in Figure 5D. The authors should replace the images or present all six blots per group in supplementary materials.

4. How TRPV2 is activated upon sympathetic nerve activation is not closely related to the main scientific question of the study and there is no experimental evidence to solve this issue, which is needless to discuss.

5. One related article should be cited "Zhang LL, et al. Activation of transient receptor potential vanilloid type-1 channel prevents adipogenesis and obesity, Cir Res, 2007, 100:1063-1070"

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1st Revision - authors' response

Referee #1:

1. TRPV2 expression is analyzed in differentiated brown adipocytes and BAT. It is important to document expression in pre-adipocytes as well. Is TRPV2 up-regulated upon adipogenesis?

Response:

*In accord with the referee’s suggestion, we examined Trpv2 mRNA expression in pre-adipocytes, and we found that TRPV2 was up-regulated upon adipogenesis. Trpv2 mRNA expression was significantly higher in differentiated brown adipocytes than in pre-adipocytes, and such increased expression was not observed in Trpv1, Trpv3 or Trpv4. Accordingly, we have included the data in the revised Figure 2A.*

2. Similarly, is there differential expression of TRPV2 between BAT versus WAT?
Response:
We examined the mRNA level of Trpv2 in BAT and WAT, and there was no significant difference. Accordingly, we have included the data in the revised Figure EV1.

3. Further, it is important to compare expression of TRPV2 in BAT versus TRPM8 previously implicated in BAT thermogenesis.

Response:
In accord with the referee’s suggestion, we examined the mRNA levels of Trpv2 and Trpm8 in BAT. The Trpv2 mRNA level was significantly higher than Trpm8 mRNA level with primers that we used in our experiments. Accordingly, we have added these data as Figure EV1B.

4. TRPV2-KO animals have impaired adrenergic triggered thermogenesis. Yet, NE evoked Ca2+ responses appear similar in WT and TRPV2-deficient adipocytes. Why? This does not seem to support the hypothesis that TRPV2 is a transduction channel downstream of BAR signaling?

Response:
Thermogenesis is triggered by catecholamines through the activation of β3-adrenergic receptors in adipocytes. NE-evoked increase in [Ca2+]i in adipocytes shown in the previous Figure 1D and E is supposed to be due to activation of the Gq-coupled α1-adrenergic receptor. We examined NE effects because NE responses are known to be increased in the differentiated adipocytes. In other words, NE responses are not related to BAR signaling.

5. Authors state that data presented in Figure 1 conclusively support functional TRPV2 responses. I think more decisive support is established later in the manuscript (Fig. 2.)

Response:
Based upon the referee’s suggestion, we have changed Figure 1 by moving the previous Figure 2A and B to the revised Figure 1. Then, we now more clearly conclude the functional TRPV2 expression in brown adipocytes.

Referee #2:
The manuscript is predominantly a descriptive account of the thermogenic phenotype of TRPV2KO brown adipocytes and BAT of TRPV2KO mice, although there is an insightful section in the discussion that describes mechanisms by which TRPV2 could be transported to the brown adipocyte plasma membrane and activated in the context of BAT sympathetic stimulation, and how TRPV2 activation could regulate thermogenic gene expression. I think this discussion is important because TRPV2 is a heat-activated channel, so it is not logical that activation of a heat-activated channel would increase thermogenic gene expression - unless heat is not the physiological activator.

Response:
We discussed the possible mechanisms of TRPV2 involvement in thermogenesis in the previous manuscript. As the referee pointed out, TRPV2 was initially identified as a high temperature-activated ion channel with a temperature threshold above 52°C. However, TRPV2 expression is found in many tissues not normally exposed to such high temperatures. Those results have suggested the existence of other physiological stimuli that could include some endogenous molecules or mechanical stimulus. Indeed, there are several reports regarding candidate endogenous activators that could be involved in the phenomenon observed in our study with brown adipocytes.

Major comments:
1. The authors use a global KO mouse mode. What is the tissue distribution of TRPV2? This would be important to justify that the phenotype of TRPV2KO mice is due to a defect specifically in BAT. Additionally, authors mentioned in the introduction page 4 that macrophages also express TRPV2. Because macrophages, and particularly, alternative macrophages "M2" play a role in brown adipocyte activation, I would ask the authors to compare TRPV2 gene/protein expression in the
different BAT cell fractions: mature adipocytes, endothelial cells, and particularly macrophages. Although the authors focused on BAT, it is well known that subcutaneous fat depot is prone to browning in response to cold and high fat diet. Did authors analyse the subcutaneous fat depot in TRPV2 KO mice in response to cold? What is the gene expression profile in white adipose tissue regarding brown markers?

Response:

TRPV2 is widely expressed in various cell types, such as neurons, macrophages, pancreatic β cells, muscles, astrocytes, microglia, white adipocytes and epithelial cells. As the referee pointed out, it would be important to examine which BAT cell fractions predominantly express TRPV2. However, we could not conduct the analysis because currently available anti-TRPV2 antibodies are not good enough to be used for immunohistochemical analysis. We also could not examine the fat depot in TRPV2 KO mice because of the low reproduction rate of TRPV2 KO mice (perinatal lethality) (Park U et al. J Neuroscience, 2011). In terms of browning markers, we examined the mRNA expression of Ucp1 in inguinal WAT (iWAT) of WT mice upon cold exposure for 3 days, and we found that Ucp1 was significantly increased as previously reported. We also found that Trpv2 in iWAT was significantly increased upon cold exposure. These data suggest that TRPV2 might be involved in iWAT “browning”. Accordingly, we have included the data as the revised Figure EV2A and B.

2. Could the authors demonstrate the physiological relevance of TRPV2 regulation of brown adipocyte thermogenic gene expression, for example by showing that TRPV2 expression in BAT is regulated by diet, cold-exposure, and obesity? A major concern about this study is the lack of pathophysiological relevance of TRPV2 in the context of obesity. It is well characterised that BAT can be activated in response to high fat diet. Considering the potential role of this channel in the maintenance of BAT thermogenic response, it would be interesting to phenotype TRPV2 KO mice during the development of obesity. The authors suggest that TRPV2 positively regulates oxidative metabolism and brown adipocyte function. If so, we would expect an aggravation of obesity in TRPV2 KO mice. Moreover, treatment with TRPV2 agonists (if suitable in vivo) would ameliorate obesity related complications by activating BAT thermogenesis.

Response:

We thank the reviewer for his/her important suggestion that led us to very exciting findings. We examined Trpv2 mRNA levels in iBAT of obese mice treated with a high fat diet (HFD) and db/db mice, and we observed that Trpv2 mRNA levels were significantly increased in the iBAT of these obese mice. We also found that Trpv2 mRNA levels were increased in mouse iBAT upon cold exposure. These results indicate that TRPV2 is involved in BAT thermogenesis in these specific conditions. Moreover, we found that TRPV2 KO mice had heavier body and fat weights than WT mice upon HFD treatment for 8 weeks as the referee expected. Unfortunately, we were unable to examine activation of TRPV2 in vivo to see the effects on obesity because of a lack of specific TRPV2 agonists. Nevertheless, the above new results clearly indicate the importance of TRPV2 in BAT thermogenesis and possible prevention of obesity upon TRPV2 activation. Accordingly, we have included the new data in the revised Figures 6, 8 and EV3.

3. There is a lack of background literature on TRPV channels in adipose tissue. Recent studies highlighted a role of TRPV1 in regulation of food intake and glucose homeostasis in white fat during obesity (Lee E et al, FASEB 2015). A role of TRPV3 has been highlighted in regulation of adipogenesis; TRPV3 agonist inhibits white adipogenesis (Cheung SY et al, Endocrinology, 2015). The work of Ma, et al (J Mol Cell Biol 2012 - Activation of the cold-sensing TRPML8 channel triggers UCP1-dependent thermogenesis and prevents obesity) should be cited, as this paper sets the precedent for TRP channels modulating thermogenesis in brown adipocytes in a cell-autonomous manner. Finally, Li Ye etal (Cell, 2012), identified TRPV4 as a regulator of adipose oxidative metabolism, inflammation and energy homeostasis. Is this study the authors showed that TRPV4 KO mice were protected from diet-induced obesity, a mechanism involving increased browning and activation of PGC1α. In this paper, they showed in vitro that depletion of TRPV4 in white adipocytes resulted in increased PGC1α expression whereas depletion of TRPV2 provoked a decreased expression (results in line with the present study). Taken together, this last study suggest that i) TRPV4 and TRPV2 would act in opposite directions to regulate brown activity in adipose...

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cells and ii) TRPV2 would play a relevant role in white adipocytes and might be involved in subcutaneous fat "browning" in vivo.

Response:
We have revised and cited all of the suggested references in our revised Introduction section. We have also changed the Discussion regarding the apparently opposite actions of TRPV2 and TRPV4 and TRPV2’s involvement in subcutaneous fat "browning" according to the referee’s suggestion. In terms of the second point, we found that Trpv2 mRNA was increased not only in BAT but also in iWAT upon a 3-day cold exposure together with iWAT “browning” that was indicated by the Ucp1 mRNA increase. Accordingly, we have included the new data in the revised Figure EV2.

4. Could the authors complement their loss-of-function experiments using TRVP2KO brown adipocytes with experiments using the TRVP2 agonists utilized in Figure 1D and E? Does treatment of brown adipocytes with these agonists reduced Ucp1 and PGC1α gene expression? Additionally, as mentioned above, TRPV3 plays a role in white adipogenesis and TRVP3 agonists inhibit cell differentiation. I suggest that the authors test the influence of TRPV2 agonists on brown adipocyte differentiation capacity. What is the impact of TRPV2 depletion in brown adipocyte adipogenesis? Decreased responsiveness to β-adrenergic response could result from lower differentiation state of primary brown adipocytes from KO mice. What is the expression profile of the TRPV1-4 during adipogenesis? Is TRPV2 only expressed in differentiated adipocytes?

Response:
We again thank the referee for these good suggestions. We previously showed the increases in 
\[Ca^{2+}\]i upon treatments with two known TRPV2 agonists, 2APB and LPC (previous Figure 1D and E). The 2APB-evoked responses were drastically reduced in TRPV2KO adipocytes (previous Figure 2B). Then, we examined the effects of another TRPV2 agonist, LPC, on brown adipocytes lacking TRPV2. LPC-evoked responses were also significantly reduced in TRPV2KO cells. We also examined the effects of the two TRPV2 agonists (after a 6 day treatment) on brown adipocyte differentiation in accordance with the referee’s suggestion. We found that 2APB and LPC significantly reduced adipocyte differentiation similar to the reported TRPV3 function in adipocytes. Moreover, we observed significant increases in differentiated brown adipocyte numbers and triglyceride levels in TRPV2KO cells compared with WT brown adipocytes. These data indicate that TRPV2 is involved in brown adipocyte differentiation, at least in vitro. Although the referee speculated a lower differentiation capacity of primary brown adipocytes from TRPV2KO mice, the results were the opposite. We interpreted that the decreased responsiveness to β-adrenergic response in TRPV2KO brown adipocytes is not related to the level of differentiation. In terms of Trpv1, Trpv2, Trpv3 and Trpv4 mRNA expression, we found that Trpv2 is expressed in both pre-adipocytes and differentiated adipocytes and that only Trpv2 expression was significantly upregulated in differentiated brown adipocytes compared with pre-adipocytes. Accordingly, we have included the data in the revised Figure EV1 and Figure 2 and changed the Results and Discussion.

Minor comments:
1. Please could the authors revise the sentence in the introduction page 3 " The functions of these two types...the opposite of another". I found this statement too categorical considering the fact that in both depots, adipocytes are the main cells producing adipokines, responding to insulin and catecholamines to promote lipolysis. Moreover, white adipocytes can express UCP-1 in condition of cold/diet-induced browning.

Response:
We have revised the Introduction section in accordance with the referee’s suggestions.

2. Page 3 in the introduction, the authors should precise that in addition to be present in adult, BAT can be activated. Both activation capacity and amount of tissue are negatively correlated with adiposity.

Response:
We have revised the Introduction accordingly.
3. In figure 1A, the expression of Trpv family members is measured in brown adipocytes, with beta-actin shown as a housekeeper. In figure 1B, the data is normalized to a different housekeeping gene, 36b4. Is there a particular reason for using one house-keeper in the gel analysis, and another for quantification?

Response:
We examined TRPV family members in differentiated mouse brown adipocytes with β-actin as a housekeeping gene. When we did quantification analysis of mRNAs, we found that the mRNA levels of β-actin were not stable in some adipose tissues. In addition, 36B4 (RPLP0), an acidic ribosomal phosphoprotein P0, which forms tight associations with the smaller 40S unit, is commonly used in the analysis of adipocytes because it is not affected by adipogenesis. Accordingly, we decided to use 36B4 as a control in the mRNA quantification.

4. In Figure 2A, the authors should also examine the protein expression of the TRVP1, 3 and 4 in brown adipocytes and tissue, in order to see any potential compensatory response.

Response:
We did not examine the protein expression levels of TRPV1, TRPV3 and TRPV4 in brown adipocytes and tissues due to the antibody availability. Instead, we examined the mRNA levels of Trpv1, Trpv3 and Trpv4 in brown adipose tissue from WT and TRPV2KO mice. We found that Trpv1, Trpv3 and Trpv4 mRNAs in TRPV2KO iBAT were not different from those of WT iBAT. Accordingly, we have included the data in the revised Figure EV4.

5. In Figure 2B, whereas the response to 2APB is completely abolished in brown adipocytes from TRPV2 KO mice, response to NE is not statistically modified suggesting that other channels are involved in the NE response. Please could the authors discuss this.

Response:
Thermogenesis is triggered by catecholamines through the activation of β3-adrenergic receptors in adipocytes. NE-evoked increase in [Ca²⁺]i in adipocytes (shown in the previous Figure 1D and E) is supposedly due to activation of a Gq-coupled α1-adrenergic receptor. We examined NE effects because NE responses are known to be increased in the differentiated adipocytes. In order to minimize the misunderstanding, we have changed the manuscript.

6. Figure 2D: I would also suggest to examine the functional response of WT and KO brown adipocytes to isoproterenol by measuring glycerol or free fatty acid release. Moreover, the authors should test different pharmacological activators of lipolysis (Isoproterenol, Forskolin and DcAMP); this would to distinguish if the impairment of the β-adrenergic response results from decreased expression/functionality of β-adrenergic receptors or alteration to mitochondrial function.

Response:
Following the referee’s suggestions, we examined the effects of forskolin on Ucp1 mRNA expression and free fatty acid release of brown adipocytes from WT and TRPV2KO mice. We found that forskolin caused Ucp1 mRNA induction and free fatty acid release in WT brown adipocytes, and such increases were significantly reduced in the cells from TRPV2KO mice. These data together with the data shown in previous Figure 2D and E suggest that the β-adrenergic pathway is impaired in brown adipocytes lacking TRPV2. We previously showed that beta adrenergic receptor mRNA did not differ between WT and TRPV2KO iBAT (previous Figure 3C). Thus, mechanisms other than expression/functionality of β-adrenergic receptors would more likely be affected by Trpv2 deletion. We have included the new data in revised Figure 3.

7. The fact that TRPV2KO mice are lighter than wild-types (Supplementray figure EV2A) must be considered when interpreting the data from figure 3-6. For example, are these animals just lighter (i.e. have less fat mass) than wild-types, or are they shorter i.e. is there a growth defect? I suspect this is a growth defect, as TRPV2KO mice had heavier WAT depot weights. The data in Figure 3B
should be normalized to body weight because WTs and TRPV2KOs had different body weights. This would actually reveal a more significant increase in WAT depot weight in TRPV2KOs. Analysing body composition using DEXA or Body Composition Analyzer based on TD-NMR that would constitute a more precise method for measuring lean and fat mass in WT and KO mice.

**Response:**

*It has been reported that TRPV2 is involved in mouse prenatal viability and axon outgrowth. As the referee suggested, smaller body weight in TRPV2KO mice could be a growth defect. However, the body weight of TRPV2KO mice caught up to WT mice at 12 weeks of age. Nevertheless, we normalized tissue weight data to their body weight as the referee suggested. In the analysis, we found that iBAT in addition to iWAT and eWAT in TRPV2KO was significantly heavier than WT. We have accordingly changed the tissue weight data (revised Figure 4D) in the revised manuscript. In terms of the analysis of body composition, we unfortunately were not able to do it because we didn’t have the apparatus to perform the analysis.*

8. In the section "Lack of TRPV2 impaired the expression of mitochondrial oxidative metabolism-related genes in brown adipocytes," Ucp1 and Pgc1α are the genes referred to as “mitochondrial oxidative metabolism-related genes.” As someone in the field, I would use that label for PGC1α, mitochondrial transporters, and electron transport complex components. I think a better description of the function encompassed by Ucp1 and PGC1α is “thermogenic genes.”

**Response:**

*In accordance with the referee’s suggestion, we have revised the description.*

9. Please could the authors provide statistical analysis in Table 1. KO mice seem to display increased plasma insulin levels conversely with decreased serum cholesterol.

**Response:**

*None of the parameters shown in Table I exhibited a significant difference. We have added the results of statistical analysis to the revised Table I.*

**Referee #3:**

**Major comments:**

1. UCP1 accounts for the thermogenesis by mitochondria and is a widely used BAT marker. Pgc1α is a crucial modulator of mitochondrial biogenesis. How does TRPV2 affect these two key molecules? Authors should provide more solid evidences to explain their links rather than only showing their expression in BAT in the absence of TRPV2.

**Response:**

*Since TRPV2 is a non-selective calcium-permeable cation channel, activation of TRPV2 leads to [Ca$$^{2+}$$], increase as previously shown (Figure 1D and E, and Figure 2B). We therefore examined the effects of BAPTA-AM, a cell-permeant calcium chelator, on the expression of thermogenic genes. Isoproterenol-induced increases in Ucp1 and Pgc1α mRNA expression were significantly suppressed by a BAPTA-AM treatment, indicating that [Ca$$^{2+}$$], changes are involved in the induction of the two genes downstream of β-adrenergic receptor activation, and that TRPV2 could be possibly involved in the Ca$$^{2+}$$-mobilization pathway. Note that we observed a similar reduction of Ucp1 and Pgc1α mRNA expression in TRPV2KO brown adipocytes. Accordingly, we have included the new data to the revised Figure 3F and G.*

2. The configuration of results seems poor of logic. For example, the core results of the whole work showing the impaired cold-induced thermogenesis in Trpv2 KO mice appear in Figure 5. The results on the expression levels of Ucp1 and Pgc1a, which seem to be the critical point explaining the phenotype, scatter in Figures 2, 3, 5 and 6. It is better to rearrange Figures 2, 3, 4 and 5 following a more logical order like this: the core novelty and phenomenon, the tissue or cell behaviors explaining the phenotype, the critical molecular changes accounting for those behaviors and finally, the detailed molecular mechanism.
Response:
We thank the referee for this kind suggestion. However, we conducted many experiments suggested by the three referees, and we obtained considerable amounts of new data that support the concept of involvement of TRPV2 in the thermogenic function of brown adipocytes. Because of the amount of new data, we minimized the changes in Figure orders, and we hope that the referee understands our decision.

3. In discussion section, the authors predict that TRPV2 activation modulates brown adipocyte function through Ca2+ influx. However, there is no experimental evidence supporting this presumption. Trpm8, Trpv1 and Trpv4 are reported to play critical roles in modulation of Ca2+ influx in response to stimulus, and all of them involve in BAT function. Why do different TRP channels play a similar role or opposite role (such as TRPV4) in BAT mediated thermogenesis? Authors should discuss this point. Current discussion has more repetitive description as results.

Response:
The present data suggest that TRPV2 functions downstream of b-3 adrenergic receptor activation. We examined the changes in mRNA expression of Ucp1 and Pgc1a upon isoproterenol stimulation without and with a membrane-permeant calcium chelator, BAPTA-AM. Increases in the two genes were significantly smaller in the presence of BAPTA-AM, suggesting the importance of [Ca2+]i in the induction of the two genes. Such [Ca2+]i increases (possibly through calcium-permeable channels including TRP channels) could be important for adipocyte functions. Because adipocytes play many pivotal functions, multiple calcium influx pathways could exist although we still do not know the mechanisms of apparently opposing functions of TRPV4 and others.

4. The results showing impaired β3-adrenergic receptor stimulation-induced thermogenesis in TRPV2KO mice is the consequence of low thermogenic function by mitochondria and could not explain the mechanism of the cold intolerance of TRV2 KO mice. As the increased expression of UCP1 and PGC1a by ISO treatment or cold stimulus was almost totally blocked by TRPV2 knockout, the detailed experiments of the pro-whitening effect of TRPV2 KO should be provided to explain the key point of novelty. For example, the mRNA level, protein stability or binding activity of a critical transcription factor of differentiation of BAT, PRDM16, should be considered to be involved in search for this mechanism.

Response:
In accordance with the referee’s suggestion, we examined the mRNA level of Prdm16 in the iBAT from WT and TRPV2KO mice. However, we did not observe any significant difference, indicating that brown adipocyte differentiation is not different between the two genotypes at the tissue level. Related to this issue, we found that brown adipocyte differentiation is facilitated in the cells lacking TRPV2 and that TRPV2 activation by its chemical agonists facilitated brown adipocyte differentiation, indicating that TRPV2 activation could prevent brown adipocyte differentiation at least in vitro.

Minor comments:
1. In figure 1, the expression levels of the four TRPV channels could not be determined merely by quantitative RT-PCR because the efficiency of PCR primers were different. Therefore, it could not be concluded that the expression of Trpv2 is the highest in all four Trpv members. In addition, the importance of the other TRP channels, such as TRPV1, TRPM8, TRPV4, should not be neglected.

Response:
Based upon the referee’s suggestion, we examined the mRNA level of Trpm8 in iBAT and found the normalized Trpm8 level was also significantly smaller than Trpv2, suggesting that mRNA expression of Trpv2 is higher than Trpv1, Trpv3, Trpv4 and Trpm8 in iBAT. However, we cannot conclude that the expression of Trpv2 is the highest among the TRP channel mRNAs examined upon quantitative RT-PCR alone as the referee pointed out. Accordingly, we have revised the manuscript by adding the Trpm8 expression data in the revised Figure EV1B.
2. In figure 4, the authors showed that the cell size of iBAT was much larger in Trpv2 KO mice than WT controls because more lipid droplets were accumulated. However, in Figure 3B, there was no difference of iBAT weight between the two groups. Was the cell number in iBAT of Trpv2 KO mice less than WT controls?

Response:
TRPV2KO mouse body weights were significantly lower than those of WT mice from 3 weeks of age to 8 weeks of age. Therefore, we normalized tissue weight data to body weights (this is a suggestion by referee #2), and found that iBAT/body weight ratios in TRPV2KO were significantly higher than those in WT mice, which could reflect larger sizes of TRPV2KO brown adipocytes. Accordingly, we have replaced the previous Figure 3B with the revised Figure 4D.

3. In Figure 5E, the statistical result of UCP1 protein levels of iBAT of Trpv2 KO mice was much lower than WT mice after cold exposure. But the tendency of UCP1 expression could hardly be reflected in the representative images of Western blot in Figure 5D. The authors should replace the images or present all six blots per group in supplementary materials.

Response:
As the referee pointed out, the difference in UCP1 protein levels between WT and TRPV2KO did not look large in the previous Figure 5D. We have replaced the representative Western blot data with a better one in the revised Figure 6E.

4. How TRPV2 is activated upon sympathetic nerve activation is not closely related to the main scientific question of the study and there is no experimental evidence to solve this issue, which is needless to discuss.

Response:
We conducted many experiments to establish the data that connect beta adrenergic receptor stimulation with TRPV2 activation. Unfortunately, however, we failed to solve the issue. Accordingly, we have deleted the discussion in the revised manuscript as the referee suggested.

5. One related article should be cited "Zhang LL, et al. Activation of transient receptor potential vanilloid type-1 channel prevents adipogenesis and obesity, Cir Res, 2007, 100:1063-1070"

Response:
We thank the referee for this suggestion and have cited this article in the revised manuscript.
Authors have more than adequately addressed concerns. This is a very nice study.

Referee #2:

Review 2: Lack of TRPV2 impaired thermogenesis in mouse brown adipose tissue.
I thank the authors for the substantially revised manuscript. They addressed most of the comments raised by the reviewer and added convincing data that support their conclusion. However some points raised still need to be addressed.

In particular they did not address the first comment regarding tissue/cell distribution which I think is an important question. Despite the lack of working antibodies, the authors still have the possibility to measure mRNA TRPV2 expression in the different tissues and cell fractions, in particular macrophages fraction.

Moreover, they did not address the question of phenotyping white adipose tissue of the TRPV2 KO mice. This is an important issue since the authors then provide interesting evidence of a role of TRPV2 in tissue "browning" upon cold exposure. Considering the fact that this study is based on the TRPV2 KO model, it is difficult to believe that the authors cannot have access anymore to white fat pad for histology. In addition, the authors now presented in additional figures mRNA expression of TRPV2 in WAT/BAT showing high expression in WAT. I think it remains important to examine (histology and gene expression) the white tissue of the KO at room temperature and upon cold exposure.

Referee #3:

I am overall pleased with the revised manuscript since the authors have addressed most of my questions and performed a lot of experiments in the revised manuscript. However, the length of this manuscript is too long, and the authors should further concise the description of results and discussion.

1. The authors should emphasize the key novelty (regulation of Ca influx by TRPV2 is critical to BAT function) and delete some redundant description of TRPV2 expression and TRP channels in introduction section, as well as the unnecessary citations.

2. The description of experimental process in result section should be concised to the greatest extent, excluding all unnecessary presentation of dosage, raw data and p values.

3. The key novelty should be clearly presented and discussed instead of only explaining the results again.

Cross-comments from referee 3:

I think that reviewer's comments are reasonable. It is better to include these data if authors have sample of fat tissue and experimental conditions.

2nd Revision - authors’ response 30 December 2015

Referee #2:

1. They did not address the first comment regarding tissue/cell distribution which I think is an important question. Despite the lack of working antibodies, the authors still have the possibility to measure mRNA TRPV2 expression in the different tissues and cell fractions, in particular macrophages fraction.

Response:
We thank the referee’s comment. According to the referee’s suggestion, we first separated brown adipocytes and stromal vascular fractions (SVFs) using collagenase digestion. We realized that SVFs were very small compared with brown adipocytes. Then, SVFs were further divided into two populations using a magnetic cell sorting system. We confirmed that only CD-11b-positive fraction showed high Cd11b mRNA expression. Then, we examined the expression of Ucp1 and Trpv2 mRNAs in adipocytes, CD11b-negative and CD-11b-positive cells. Ucp1 mRNA expression was extremely high in adipocytes while negligible in CD11b-negative and positive cells. On the other hand, Trpv2 mRNA expression was not different among the three fractions. About 0.7% of SVFs were CD-11b-positive cells, indicating that CD11b-positive macrophages were very few in iBAT, suggesting the little contribution of macrophages to the phenomenon which we observed in the study. Accordingly, these results have been added as the revised Figure EV1B-D.

2. Moreover, they did not address the question of phenotyping white adipose tissue of the TRPV2 KO mice. This is an important issue since the authors then provide interesting evidence of a role of TRPV2 in tissue "browning" upon cold exposure. Considering the fact that this study is based on the TRPV2 KO model, it is difficult to believe that the authors cannot have access anymore to white fat pad for histology. In addition, the authors now presented in additional figures mRNA expression of TRPV2 in WAT/BAT showing high expression in WAT. I think it remains important to examine (histology and gene expression) the white tissue of the KO at room temperature and upon cold exposure.

Response:
According to the referee’s suggestion, we compared the iWAT histology between WT and TRPV2KO mice. We found that there was no apparent difference in sizes of white adipocytes. In addition, we compared Ucp2 and Lpl mRNAs between the two genotypes, and again no difference was observed. Furthermore, we examined the mRNA levels of Ucp1, Pgc1a and Pparg in WT and TRPV2KO iWAT upon without and with cold exposure. Increase in Pgc1a mRNA upon cold exposure was significantly smaller in TRPV2KO iWAT than in WT iWAT while Pparg mRNA levels were not changed upon cold exposure both in WT and TRPV2KO iWAT. Increase in Ucp1 mRNA upon cold exposure tended to be smaller in TRPV2KO iWAT while no statistical significance was observed. These data suggest that TRPV2 plays some roles in iWAT similar to iBAT upon cold exposure. Accordingly, we have added the data as the revised Figure EV2B, E-H.

Referee #3:
1. The authors should emphasize the key novelty (regulation of Ca influx by TRPV2 is critical to BAT function) and delete some redundant description of TRPV2 expression and TRP channels in introduction section, as well as the unnecessary citations.

Response:
According to the referee’s suggestion, we have deleted the redundant description of TRPV2 expression and TRP channels in introduction section.

2. The description of experimental process in result section should be concised to the greatest extent, excluding all unnecessary presentation of dosage, raw data and p values.

Response:
We have deleted all unnecessary description in result section accordingly.

3. The key novelty should be clearly presented and discussed instead of only explaining the results again.

Response:
We have revised and discussed the novelty of our findings in discussion section accordingly.

Cross-comments from referee 3:
I think that referee #2’s comments are reasonable. It is better to include these data if authors have sample of fat tissue and experimental conditions.

Response:
According to the suggestion, we performed additional experiments and have added the iWAT data in the revised manuscript.

3rd Editorial Decision 13 January 2016

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.