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PRDM16 represses the type I Interferon response in adipocytes to promote mitochondrial and thermogenic programing

Megan Kissig, Jeff Ishibashi, Matthew Harms, Hee-Woong Lim, Rachel Stine, Kyoung Jae Won and Patrick Seale

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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 19 September 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see, the referees appreciate your findings and provide clear and constructive reports on how to better support your conclusions. Importantly, they all point out that your findings need to be further substantiated in an in vivo and physiological relevant setting.

REFEREE REPORTS

Referee #1:

Interferon signaling and interferon regulatory factors are emerging as regulators of lipid homeostasis and thermogenesis. The authors present a new for PRDM16 in suppressing the type I IFN response. PRDM16 is shown to repress transcription of ISGs by directly binding to promoter regions in pre-adipocytes. The authors also show that activating type I IFN response in beige/brown differentiated adipocytes and adipose tissue leads to repression of thermogenic and mitochondrial genes. These studies are interesting and rigorous, but some issues should be addressed prior to publication.

Major Comments:
1) There is a disconnect between the study of preadipocytes and the ultimate function of thermogenesis is mature adipocytes. The manuscript provides no direct evidence linking repression of the IFN signal by PRMD16 in preadipocytes and promotion of thermogenic programing in adipocytes.
2) Fig. 3 and 4 are the only experiments done in adipocytes and adipose tissue, respectively, to test the consequence of IFN stimulation. In Fig. 3 the authors should include experiments with PRDM16 gain- or loss-of-function to test if PRDM16 reverses IFNa signal-mediated gene repression.
3) Does IFNAR and IRFs change during adipocyte differentiation?
4) IRF7 was analyzed throughout the paper and its expression was highest in Fig EV2B, but the authors chose to focus on IRF1. Does IRF1 level change in Figs. 1-5. Does IRF1 overexpression or knockdown change thermogenic genes in adipocytes?
5) Does IFNa treatment change PRDM16 occupancy on ISGs and/or thermogenic genes promoter/enhancer regions?

Minor Comments:
1) The authors should include UCP1 immunoblot in Fig. 4A.
2) PRDM16 level seems to increase upon IFNa treatment in Fig. 2D. Is it significantly upregulated? Could this be a negative feedback mechanism?
3) Some IRFs are also known to activate thermogenic genes. The authors should discuss and/or introduce IRFs role and specificity in regulating adipose genes.
4) What are the expression levels of thermogenic genes in iWAT of mice treated with IFNa? Maybe repression of ISGs by PRDM16 in WAT preadipocytes is a way of priming cells to become more beige-like adipocytes.

Referee #2:

Summary
In this manuscript, Kissig et al. explore the regulation of the type I interferon response in adipocytes by PRDM16, the latter of which has been previously identified by the authors as a critical determinant of brown adipocyte-specific transcriptional programs. Here, the authors demonstrate that PRDM16 functions as a repressor of type I interferon-stimulated genes (ISG) in beige and brown adipocytes. ISG activation in turn impaired mitochondrial activity and oxygen consumption rates in isolated adipocytes. Treatment of wild-type and brown adipose tissue (BAT)-specific PRDM16 KO mice with IFNalpha led to an increase expression of ISG in KO only, correlating with reduced levels of BAT identity genes. Mechanistically, the authors show that PRDM16 controls ISG through direct chromatin contact in relevant promoter regions of ISG, counteracting the action of IFN regulatory factor (IRF) 1 at these sites. Overall, the authors conclude that PRDM16 counteracts the IFN response in brown adipocytes to maintain their functional integrity.

General comments
The expansion and functional activation of BAT has emerged as a promising approach to counteract obesity and related metabolic complications. In this respect, the current manuscript by Kissig et al. describes an interesting and relevant topic by identifying mechanisms helping to protect brown adipocyte integrity. Overall, the manuscript is well written, clearly structured and comprises a multi-level approach to support the author's conclusions, including cellular, animal, and state-of-the-art molecular tools. However, the manuscript would still greatly benefit from three major improvements: a) While the molecular and cellular mechanisms have been worked out in detail, there seem to be discrepancies between the cellular and animal systems, e.g. IFN alpha treatment did not change ISG in wild-type mice which could have been expected based on the in vitro results. The authors should strengthen their conclusions by performing ChIP experiments in wild-type and PRDM16 KO mice to demonstrate PRDM16 recruitment to ISG also in vivo. Also, data on energy expenditure/oxygen consumption in wild-type and KO animals in response to IFN alpha treatment should be added to further support the overall relevance of their findings. b) In the same regard, any relevance for the human situation remains completely unclear. As a minimum approach, the authors should recapitulate their key findings in a human adipogenic cell line, e.g. the hMADS system. c) The relative importance of ISG repression vs. direct activation of BAT-specific programs by

PRDM16 remains to be determined, i.e. to which degree does the lack of DNA binding capacity of PRDM16 impair BAT function as compared with the inability to directly stimulate BAT-specific gene expression? What happens if one restores PRDM16 KO cells with either wild-type or mutant PRDM16 versions, monitoring both adipocyte differentiation and function in the mature state? Which functional PRDM16 features are more relevant/dominant to maintain BAT integrity? Specific comments are listed below:

Specific comments
1. Figure 1F: Please include data on Stat 1 mRNA levels. Same holds true for Figure 2D.
2. Figure 2E: Please include groups treated with IFN AND antibody. How effective does the antibody counteract IFN signaling?
3. Figure 3: How do you explain the specificity of the IFN response towards specific ISG? Please discuss.
4. Figure 4: Tubulin seems to change in a similar manner as Stat 1. Please provide a densitometrical analysis to verify the effect.
5. Figure 6B: Wild-type control cells are missing. What is the effect of IRF shRNA treatment in PRDM16-proficient cells?

Referee #3:

This manuscript identifies a role for PRDM16 in suppressing IFN-stimulated gene expression in vitro and in vivo. The authors show that IFN signaling causes mitochondrial dysfunction and PRDM16 knockout exaggerates IFNα-induced dysfunction of brown adipocyte tissue. Finally, the authors propose a direct competition between PRDM16 and IRF1 at the promoter of various IFN-stimulated genes.

The experiments appear to be carefully performed, with several loss-of-function and gain-of-function models. The quality of the data is excellent. Overall, the manuscript presents convincing mechanistic data about the known conflict between the immune response and thermogenesis.

1. The authors may want to show their findings in a more physiological setting. For example, does cold exposure activate PRDM16 and suppress IFN-stimulated genes in BAT? Conversely, does warmth (thermoneutrality) increase the immune response in BAT or beige cells while suppressing PRDM16?

2. Does IFNα stimulation cause reduced PRDM16 binding and increased IRF1 binding in the promoter regions of IFN-stimulated genes, consistent with the gene expression change?

3. In Fig 6A, two shIRF1 hairpins are used, with b showing better knockdown efficacy than a. However, in Fig 6B, the effects of those shIRF1s on gene expression were not consistent with the KD efficiency. This should be explained. A PRDM16 add-back to the knock-down cells would help reassure us that this is not an off-target effect.

1st Revision - authors' response 13 January 2017

Point-by-point response to reviewers:

Reviewer #1:

We thank the reviewer for his/her positive general remarks on our study.

1) *There is a disconnect between the study of preadipocytes and the ultimate function of thermogenesis is mature adipocytes. The manuscript provides no direct evidence linking repression of the IFN signal by PRMD16 in preadipocytes and promotion of thermogenic programing in adipocytes.*

The reviewer raises an interesting question. To address this, we performed new experiments:

We tested the effects of IFNa-treatment at different stages of adipocyte differentiation. We found that treatment only during the first few days of differentiation (day 0 to 4) led to persistent reduction in brown fat and mitochondrial gene levels at day 9. This early treatment reduced thermogenic programing more so than late treatment. These new results are shown in Figure EV3D.

We also found that suppression of the Interferon response in wildtype and *Prdm16*-knockout preadipocytes by neutralizing antibody led to increased levels of thermogenic genes in mature adipocytes. These data are now presented in Figure 2E-F.

2) *Fig. 3 and 4 are the only experiments done in adipocytes and adipose tissue, respectively, to test the consequence of IFN stimulation. In Fig. 3 the authors should include experiments with PRDM16 gain- or loss-of-function to test if PRDM16 reverses IFNa signal-mediated gene repression.*

We performed the experiment suggested by the reviewer. As shown in Fig 3I, PRDM16-expression in preadipocytes reversed the effects of IFNa on thermogenic gene expression in mature adipocytes.

3) *Does IFNAR and IRFs change during adipocyte differentiation?*

As recommended, we measured the expression of *Ifnar1* and all the *Irf* genes in preadipocytes and mature brown adipocytes. These results are now shown in Figure EV6C. We also cited a paper from Evan Rosen's lab that previously investigated the expression levels of Irfs during adipocyte differentiation (Eguchi *et al*., *Cell Metab*, 2008)

4) *IRF7 was analyzed throughout the paper and its expression was highest in Fig EV2B, but the authors chose to focus on IRF1. Does IRF1 level change in Figs. 1-5. Does IRF1 overexpression or knockdown change thermogenic genes in adipocytes?*

We focused our studies on IRF1 because it is known to function upstream in the IFN-signaling cascade and can activate many of the downstream ISGs. IRF7 is a canonical ISG and its levels are strongly decreased by *Prdm16*. We thus reasoned that IRF1 was a good candidate to function at the top of the cascade and to be functionally regulated by PRDM16. As noted below, PRDM16 does not regulate *Irf1* levels but does affect IRF1 activity. However, we cannot exclude additional roles for functional interactions between PRDM16 and other IRFs, including IRF7. We have included a comment on this in the discussion:

"While the interaction between PRDM16 and IRF1 plays an important role in regulating the IFN-response in adipogenic cells, whether PRDM16 also functionally interacts with other IRFs remains to be determined."

Additionally, to address the reviewer's specific questions about *Irf1* expression, we measured IRF1 levels under a variety of experimental conditions (See Figure EV6). Briefly, *Irf1* expression does not change during adipocyte differentiation or in response to PRDM16-expression.

We also tested whether *Irf1* knockdown reduces thermogenic genes in adipocytes as suggested. Knockdown of *Irf1* in wildtype brown adipocytes did not affect ISG levels or thermogenic genes. This result is consistent with our model that endogenous PRDM16 acts in these cells to restrain the IFN-response and thus IRF1-function at these genes is low under normal conditions.

5) *Does IFNa treatment change PRDM16 occupancy on ISGs and/or thermogenic genes promoter/enhancer regions?*

We attempted this experiment but were unable to reliably ChIP endogenous PRDM16 (which is expressed at low levels) in preadipocytes.

Minor Comments:

1) *The authors should include UCP1 immunoblot in Fig. 4A.*

We examined UCP1-expression by immunostaining in Figure 4C.

2) *PRDM16 level seems to increase upon IFNa treatment in Fig. 2D. Is it significantly upregulated? Could this be a negative feedback mechanism?*

We looked into this more and don't believe there is any significant up-regulation of PRDM16 levels by IFNa-treatment. As now shown in Figure EV3A, IFNa-treatment of cells does not significantly affect PRDM16 protein levels. Consistent with this, we did not observe changes in PRDM16 protein levels in BAT upon IFNa-treatment of mice.

3) *Some IRFs are also known to activate thermogenic genes. The authors should discuss and/or introduce IRFs role and specificity in regulating adipose genes.*

This is a good point. In the results section where we begin to focus on IRFs, we state that various IRFs have been implicated in adipocyte differentiation and function and cite the relevant papers. We also comment on this point in the last paragraph of the discussion.

4) *What are the expression levels of thermogenic genes in iWAT of mice treated with IFNa? Maybe repression of ISGs by PRDM16 in WAT preadipocytes is a way of priming cells to become more beige-like adipocytes.*

To answer this, we analyzed the ingWAT of the *Prdm16* KO (*Myf5-Cre*) and control mice. Under our conditions, we did not observe any significant effects of IFNa-treatment on the expression of *Ucp1* or *Cidea* in ingWAT. Interestingly however, we did find that IFNa-treatment caused a significant reduction in the expression of various mitochondrial genes, including *mt-Co1* and *Mt-Cytb*. These experiments are shown in Figure EV4C,D.

Reviewer #2:

We thank the reviewer for his/her positive comments on our paper. The following questions were raised.

1) *While the molecular and cellular mechanisms have been worked out in detail, there seem to be discrepancies between the cellular and animal systems, e.g. IFN alpha treatment did not change ISG in wild-type mice which could have been expected based on the in vitro results. The authors should strengthen their conclusions by performing ChIP experiments in wild-type and PRDM16 KO mice to demonstrate PRDM16 recruitment to ISG also in vivo. Also, data on energy expenditure/oxygen consumption in wild-type and KO animals in response to IFN alpha treatment should be added to further support the overall relevance of their findings.*

These are good points. We speculate that the reason we don't observed ISG increases in the BAT of control (wildtype) mice is that in our protocol we collected tissues 3 days after the last injection of IFNa. ISG induction in wildtype mice may occur acutely as the IFNa has a short half-life *in vivo*.

To address the important question about energy expenditure effects of IFNa, we measured oxygen consumption in vehicle and IFNa-treated *Prdm16* KO (*Myf5-Cre*) and control mice (4 groups). Basal and norepinephrine (NE)-induced oxygen consumption was measured in mice housed at thermoneutrality in metabolic cages. This method is considered as the gold-standard approach to measure BAT-thermogenesis (Cannon & Nedergaard, JEB 2011). Notably, we found that KO mice treated with IFNa had a very significant reduction in NE-stimulated oxygen consumption as compared to the other groups (KO-vehicle, WT-vehicle, WT-IFNa) (Figure 4E). These data indicate that the loss of *Prdm16* synergizes with IFNa-activation to reduce BAT-thermogenic function.

2) *In the same regard, any relevance for the human situation remains completely unclear. As a minimum approach, the authors should recapitulate their key findings in a human adipogenic cell line, e.g. the hMADS system.*

We have not yet explored whether this PRDM16/IFN pathway regulates mitochondrial and thermogenic activity in human adipocytes. We agree that this is an important question, but

we feel that it is beyond the scope of the current studies. Dedicated studies are needed to address this question in a thorough and meaningful way.

Indeed, the role of PRDM16 itself in different types of human adipocytes has not yet been tested. We plan to do a comprehensive analysis of PRDM16 function (including its role in suppressing IFN-responses) in a separate study using a variety of human adipocyte systems. However, we do note that Chad Cowan's lab recently reported that JAK-kinase inhibitors, which block IFN-signaling, promote the browning of human white adipocytes. In their studies, JAK-inhibition and the browning response was associated with reduced expression of IRF1 and many ISGs. We have commented on this question in the discussion section as follows:

"Importantly, blocking JAK-STAT signaling in human adipocytes decreases IFN signaling and induces brown fat-like characteristics (Moisan et al, 2015), suggesting a potentially important role for this pathway in human metabolism."

3) *The relative importance of ISG repression vs. direct activation of BAT-specific programs by PRDM16 remains to be determined, i.e. to which degree does the lack of DNA binding capacity of PRDM16 impair BAT function as compared with the inability to directly stimulate BAT-specific gene expression? What happens if one restores PRDM16 KO cells with either wild-type or mutant PRDM16 versions, monitoring both adipocyte differentiation and function in the mature state? Which functional PRDM16 features are more relevant/dominant to maintain BAT integrity?*

This is a good question. As pointed out by the reviewer, we propose that PRDM16 regulates the brown fat program through at least two mechanisms: (1) direct binding and transcriptional activation of brown fat genes, and (2) repression of the type 1 IFN pathway.

We found that expression of the DNA-binding mutant form of PRDM16 (R998Q) into *Prdm16* KO cells was unable to reduce the expression of ISGs. However, these $PRDM16^{R998Q}$-expressing cells activated the expression of *Ucp1* and other thermogenic markers to a similar degree as wildtype (WT) PRDM16. These data suggest that the direct-action of PRDM16 on thermogenic genes is likely to be dominant. These new data are presented in Figure EV5A-B.

In the context of *Prdm16*-depletion, blocking the IFN-pathway led to recovery in the expression of many mitochondrial genes, indicating an important role for PRDM16-mediated repression of this pathway (Figure 2E-F).

Additional comments:

4) *Figure 1F: Please include data on Stat 1 mRNA levels. Same holds true for Figure 2D.*

We limited our analysis in Figure 1 to the ISGs which were part of the blue cluster identified by GO analysis. As requested, comprehensive data on *Stat1* regulation by PRDM16 are now provided in many of the panels in Figure 2.

5) *Figure 2E: Please include groups treated with IFN AND antibody. How effective does the antibody counteract IFN signaling?*

We have added new studies to address this question. As now shown in Figure EV2B, the IFNAR-antibody very effectively blocks basal and IFN-a stimulated ISG expression. We also show in Figure EV3B that the suppressive effects of IFNa on thermogenic gene expression are prevented by co-treatment with IFNAR-antibody.

6) *Figure 3: How do you explain the specificity of the IFN response towards specific ISG? Please discuss.*

IFNa activates a variety of ISGs in a cell-specific manner. We measured the expression of many (but not all the) ISGs that were strongly expressed in adipogenic cells. Future unbiased studies will be needed to determine the full set of ISGs regulated by IFNa and PRDM16 in adipocytes.

7) *Tubulin seems to change in a similar manner as Stat 1. Please provide a densitometrical analysis to verify the effect.*

Thank you for this comment. After further analysis in various samples, we indeed found that Tubulin (as well as Actin) protein levels change along with changes in the structure of adipose tissue (whitening). Thus, these proteins are not appropriate loading controls. We have now stripped and re-probed these blots with GAPDH, which we believe is a much better loading control (see Figure 4A).

8) *Figure 6B: Wild-type control cells are missing. What is the effect of IRF shRNA treatment in PRDM16-proficient cells?*

We performed this experiment as suggested by the reviewer. As shown in Figure EV6D, knockdown of *Irf1* did not affect the levels of ISGs or brown fat-specific genes in wildtype brown adipocytes that express PRDM16. This result is consistent with our model that PRDM16 naturally functions to suppress IRF1-action under basal conditions.

Reviewer #3:

1) *The authors may want to show their findings in a more physiological setting. For example, does cold exposure activate PRDM16 and suppress IFN-stimulated genes in BAT? Conversely, does warmth (thermoneutrality) increase the immune response in BAT or beige cells while suppressing PRDM16?*

This is an interesting question. Over the course of many experiments, we do not observe activation of PRDM16 by cold-exposure. PRDM16 is highly expressed in BAT under basal and cold-induced states. PRDM16-function is not directly linked to the acute cold-response. PRDM16 is required to make adipocytes competent for thermogenic activation, but it is not directly involved in the acute effects of cold/b-agonists in brown fat. Consistent with this, we do not see any cold-induced changes in PRDM16 or ISG levels in the interscapular BAT. We did however find that cold-induced browning of ingWAT depots was associated with a slight increase in *Prdm16* (presumably heterogeneous) and lower levels of many ISGs; these data are provided in Figure EV1D.

2) *Does IFNα stimulation cause reduced PRDM16 binding and increased IRF1 binding in the promoter regions of IFN-stimulated genes, consistent with the gene expression change?*

We found that IRF1 levels were increased by IFNa-treatment (Figure EV6G), which confounds analysis of IRF1 binding activity. We also attempted but failed to reliably detect endogenous binding of PRDM16 in preadipose cells, since it is expressed at low levels.

3) *In Fig 6A, two shIRF1 hairpins are used, with b showing better knockdown efficacy than a. However, in Fig 6B, the effects of those shIRF1s on gene expression were not consistent with the KD efficiency. This should be explained. A PRDM16 add-back to the knock-down cells would help reassure us that this is not an off-target effect.*

We found that two independent shRNAs cause effective knockdown of IRF1 levels and resulting reduction of ISGs. The data from these experiments were from 3-independent biological pools for each treatment condition, which could account for some level of variability. However, the extent of ISG repression is qualitatively similar with both shRNA-treatments. PRDM16 suppresses IRF1 and ISGs, so adding back PRDM16 would only be expected to reduced ISG levels even further than their already low levels in sh cells.

2nd Editorial Decision 01 February 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by the three original referees again, whose comments are enclosed.

As you will see, all referees appreciate your revision and support publication should you be able to address some remaining issues. I would thus like to ask you to address the remaining concerns of referee #2 and #3 and to provide a final version of your manuscript.

REFEREE REPORTS

Referee #1:

The authors have addressed all concerns raised. I recommend publication.

Referee #2:

The authors have significantly improved their manuscript and addressed most of the original concerns by the referees.

However, one missing result remains the induction of ISG by INF alpha in wild-type BAT as originally requested. I still feel that the induction of ISG in BAT should be demonstrated in vivo to solidify the basis of the current study. If more acute ISG responses are to be expected as stated by the authors I would suggest to perform a time course experiment in wt animals to monitor ISG responses in this setting.

Referee #3:

Overall, the work is pretty thoroughly done. I wasn't crazy about the way the group responded to each of my concerns, however.

1. I am satisfied with the issue of cold exposure-I think the new data and the explanation provided suffice.

2. I asked whether IRF1 binding is increased after IFN exposure, and the authors stated that this can't be assessed because IRF1 levels increase, thus precluding an easy analysis of any independent effect on binding affinity. But that's not what I was driving at: the question I am asking is whether the increased IRF1 levels translates into increased binding at the promoters of IFN-inducible genes. It's a pretty simple experiment.

3. The issue of non-linearity of the extent of knockdown and the response in terms of gene expression between the two shIRF1 hairpins is obvious and real, and the proposed explanation makes no sense. Where I erred was in asking for a PRDM16 add-back; that was a typo. What is needed to confirm that this is not an off-target effect is add-back of IRF1 to the KD cells.

2nd Revision - authors' response 27 February 2017

Referee #1:

The authors have addressed all concerns raised. I recommend publication.

Referee #2:

The authors have significantly improved their manuscript and addressed most of the original concerns by the referees.

However, one missing result remains the induction of ISG by INF alpha in wild-type BAT as originally requested. I still feel that the induction of ISG in BAT should be demonstrated in vivo to solidify the basis of the current study. If more acute ISG responses are to be expected as stated by the authors I would suggest to perform a time course experiment in wt animals to monitor ISG responses in this setting.

In Figure EV4A, you can see that *Irf7* is activated in the BAT and inguinal WAT of both wildtype and *Prdm16* KO mice by IFNa treatment. This is the only ISG increased by IFNa in both groups of mice, demonstrating that WT animals are responsive to IFNa.

Additionally, in the experiment presented below, WT Black6 male mice were injected with PBS or IFN for 3 days and analyzed 12 hours after the last injection. ISG levels are very significantly increased at this acute time point, demonstrating responsiveness.

One of the key messages of our paper is that PRDM16 expression in the BAT is required to oppose type 1 IFN-responses in the tissue. Loss of PRDM16 from BAT (and cells in earlier Figs), results in hyper activation of ISG responses. For the studies included in the paper, animals were treated 6 times over a 2 week period with the last injection being 3 days before analysis. The injection period was chosen to show long-term effects of increased IFN signaling. We avoided daily treatment in order to avoid refractoriness of response that has been shown in cells and liver tissue (Sarasin-Filipowicz *et al*. 2009. *Mol. Cell. Biol*. 29: 4841-4851)

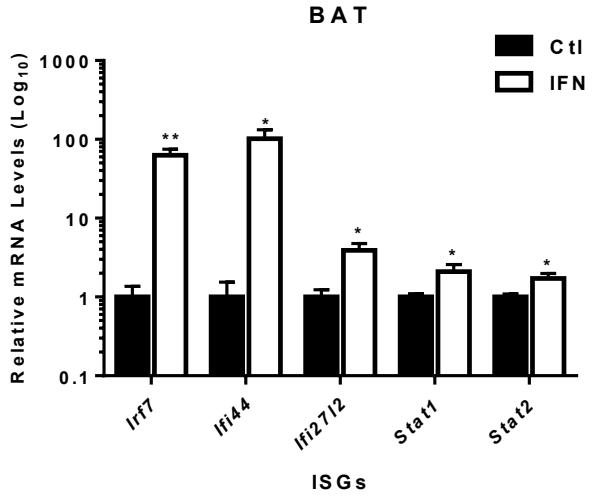

Figure: Wildtype C57Black6 mice were treated with vehicle (PBS, Ctl) or recombinant IFNa for 3 consecutive days. BAT was analyzed 12 h after the last treatment for expression of type 1 ISGs.

Referee #3:

Overall, the work is pretty thoroughly done. I wasn't crazy about the way the group responded to each of my concerns, however.

1. I am satisfied with the issue of cold exposure-I think the new data and the explanation provided suffice.

2. I asked whether IRF1 binding is increased after IFN exposure, and the authors stated that this can't be assessed because IRF1 levels increase, thus precluding an easy analysis of any independent effect on binding affinity. But that's not what I was driving at: the question I am asking is whether the increased IRF1 levels translates into increased binding at the promoters of IFN-inducible genes. It's a pretty simple experiment.

In Figure **EV6J**, we now show that IFNa treatment of brown preadipocytes significantly increases IRF1 binding at *Ifi44*. There is also higher IRF1 binding levels in *Prdm16* KO relative to that in wildtype cells.

3. The issue of non-linearity of the extent of knockdown and the response in terms of gene expression between the two shIRF1 hairpins is obvious and real, and the proposed explanation makes no sense. Where I erred was in asking for a PRDM16 add-back; that was a typo. What is needed to confirm that this is not an off-target effect is add-back of IRF1 to the KD cells.

We performed additional experiments to address this question regarding the specificity of the shIRF1 treatments. As shown in Figure **EV6D**, the reduced ISG expression caused by shIrf1 was reversed by co-expression of a shRNA-resistant human IRF1 cDNA. Additionally, as now shown in Figure **EV6F**, we observed that knockdown of IRF1 using a CRISPR/Cas9 strategy results in a similar reduction of ISGs in *Prdm16* KO cells (as seen with shRNAs).

3rd Editorial Decision 15 March 2017

Thank you for submitting your revised manuscript to us. As you can see below, the referees appreciate the introduced changes, and I am happy to accept your manuscript in principle for publication in The EMBO Journal.
Congratulations!

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Patrick Seale
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-95588

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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 χ^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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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?	No formal mathematical power calculations were performed prior to the study; however, previous experience in the lab indicated that detectection of physiological effects with underlying biological variances were able to be accomplished with n greater than 5.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For animal studies, a sample size greater than 5 was attempted for each experimental group.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	There were no samples/animals excluded from analysis in experiments from this study.
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.	In animal studies, sibling-matched controls were used for treatment groups. Mice were age-matched and all treatments were done at the same time of day. These controls were used to eliminate any bais in animal/treatment allocation. During NE-stimulation experiment, genotypes were randomly assigned to CLAMS chambers to eliminate any effect change due to timing of injection.
For animal studies, include a statement about randomization even if no randomization was used.	Randomization was not used in animal studies due to limiting numbers of age-matched mice. However, all appropriate controls were used for genotype and treatments.
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.	No, investigator wasn't blinded, only genotype of the mice was used as a stratification for treatment groups.
4.b. For animal studies, include a statement about blinding even if no blinding was done	For animal studies, theresearcher administering treatment injections was blinded to genotype of mice.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	For ANOVA calculations, D'Agostino-Pearson test was performed for normality with deviations significant at p-value less than 0.05. For Student' T-test, data were visualized and appeared approximately normal; no formal testing was performed.
Is there an estimate of variation within each group of data?	Yes, sample variances were estimated from data, as no population parameters were known.
Is the variance similar between the groups that are being statistically compared?	For data shown as log scale, statistical tests were performed that did not assume equality of underlying variances. For non-log scale, equal variance was assumed.

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).	anti-PRDM16 (Seale et al, 2007), anti-FLAG (Sigma, F1804), anti-pSTAT1 (Santa Cruz, sc7988), anti-STAT1 (Santa Cruz, sc-346), anti-pSTAT2 (Millipore, 07-224), anti-STAT2 (Cell Signaling Technology, 4597S), anti-STAT3 (Cell Signaling Technology, 9139S), anti-Tubulin (Sigma, T6199), anti-UCP1 (R&D Systems, MAB6158), anti-Actin (Millipore), total OXPHOS antibody cocktail (Abcam, ab110413), anti-MT-CO1 (Abcam, ab14705), anti-IRF1 (Cell Signaling Technology, 8478S; Figure 6).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines within the lab are tested for mycoplasma contamination every 3 months. Brown adipocyte (wildtype and Prdm16 KO) cells lines were developed in the Seale lab (Harms et al. 2014).

* 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.	Rosa26CreER, Prdm16flox mice were maintained on a mixed 129Sv/C57Black6 genetic background (Harms et al, 2014). Myf5Cre;Prdm16flox mice were backcrossed into the C57Black6 background for 10 generations (Harms et al, 2014). Male mice between 6-10 weeks old were used for all experiments. Mice were maintained on normal chow diet at room temperature.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were approved by the University of Pennsylvania's Institutional Animal Care and Use Committee.
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.	Study is in compliance.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	n/a
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.	n/a
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	n/a
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	n/a
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	n/a
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.	n/a
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.	n/a

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	Microarrary data (GSE86018), ChIP-Seq data (GSE86017)
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).	n/a
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).	n/a
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	n/a
22. 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.	n/a

G- Dual use research of concern

23. 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.	n/a