Hairless promotes PPARgamma expression and is required for white adipogenesis

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Review timeline:

<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
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<tbody>
<tr>
<td>Submission date</td>
<td>06 March 2012</td>
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<tr>
<td>Editorial Decision</td>
<td>29 March 2012</td>
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<tr>
<td>Revision received</td>
<td>03 August 2012</td>
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<td>Editorial Decision</td>
<td>17 August 2012</td>
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<tr>
<td>Revision received</td>
<td>20 August 2012</td>
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<tr>
<td>Accepted</td>
<td>21 August 2012</td>
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</tbody>
</table>

Transaction Report:

(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 29 March 2012

Thank you for the submission of your manuscript to EMBO reports. I have taken over its handling, since I am responsible for manuscripts that fall into the subject categories "metabolism and transcription". We have now received the enclosed reports on your study.

As you will see, both referees 1 and 3 support the publication of your manuscript in our journal. They also suggest a few additions and changes to the study that I would like you to address in a revised version of the manuscript.

Both referees indicate that supplementary figure 8 is not conclusive and I therefore agree that it can be deleted. Referee 1 further suggests to move supplementary figure 9 into the main figure 4, and to modify the language of both the manuscript text and title in order to more accurately reflect the actual data. Referee 3 indicates that both Pparg1 and 2 expression should be analyzed in response to HR, and that the regions of the Pparg promoters used need to be mentioned and discussed in light of a possible direct regulation by HR. The referee further points out missing controls for lentiviral infection levels and HR protein knockdown, and indicates that brown adipose tissue-specific gene expression should be analyzed to support the observation that it is not affected by HR knockout.
Along these lines, the referee also suggests to investigate whether thermoregulation is indeed unaffected in HR knockout mice. Concerning the additional comments from referee 2, I would like to point out that they do not need to be addressed experimentally, since this would most certainly result in a manuscript that would no longer be suitable for a short format EMBO reports paper. I do agree with this referee, however, that the figure legends need to include the number (n) of samples/animals. Please also define the error bars in the relevant figure legends (1A, D; 2C, D; 3A; 4A, D, E, G; and supplementary figures) and include scale bars in all images of cells and tissues.

Given these evaluations, I would like to invite you to revise your manuscript, with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Also, the length of the revised manuscript may not exceed 30,000 characters (including spaces and references) and 5 figures. We usually also only allow a maximum of 5 supplementary figures, which should directly relate to one of the main figures. I suggest that you move SF2 (including HR protein knockdown) to main figure 1, that you delete SF8, and move SF9 to main figure 4. You will also have to shorten the manuscript text somewhat (currently around 32,000 characters), and to make this easier you can move parts of the materials and methods to the supplementary information. Please note, however, that materials and methods essential for the understanding of the experiments described in the main manuscript file must remain in the main text.

I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:
Referee #1:

1. Do the contents of this manuscript report a single key finding? YES

The manuscript by Kumpf et al defines a novel role for the JmjC domain-containing transcriptional co-factor Hairless in the regulation of adipogenesis.

2. Is the main message supported by compelling experimental evidence? YES

The authors use a variety of complementary experimental approaches to provide compelling evidence to support their main conclusion that Hairless is a critical regulator of adipocyte differentiation. First, they demonstrate that the shRNA-mediated knockdown of Hairless prevents the adipogenic differentiation of both established murine 3T3-L1 preadipocytes and primary murine preadipocyte cells. Second, the authors demonstrate that both preadipocytes and normal fibroblasts derived from Hairless-deficient mice are unable to undergo adipocyte differentiation in response to adipogenic stimuli. Third, they demonstrate that the in vivo development of adipocytes is significantly attenuated in Hairless deficient animals. Finally, they demonstrate that overexpression of Hairless in either non-adipogenic NIH3T3 fibroblasts or murine C2C12 myoblasts is sufficient to promote adipocyte differentiation in response to adipogenic stimuli and that this effect maybe mediated by a direct effect on the promoter of PPARg the well-established master regulator of adipogenesis. Taken together, these complementary gain-of-function and loss-of-function
experiments provide strong and compelling evidence that Hairless is a critical endogenous regulator of adipocyte differentiation.

3. Have similar findings been reported elsewhere (e.g. on a closely related protein; in another organism or context)? NO

Please elaborate:
The data and conclusions reported in the current manuscript are novel. As far as I am aware, a role for Hairless in the regulation of adipocyte differentiation has not previously been reported.

4. Is the main finding of general interest to molecular biologists? YES

Please justify:
The JmjC domain-containing family of proteins, of which Hairless is a member, has been strongly implicated as transcriptional regulators that play a role in the regulation of cell differentiation and development. Hairless has previously been shown to play a crucial role in the regulation of hair growth and formation. However, a role for Hairless in the regulation of adipocyte differentiation has not previously been postulated. The JmjC domain of many family members has previously been shown to encode a histone demethylase activity and it is widely believed that this function plays an important role in activity of this class of transcriptional co-factors. Interestingly, the current study demonstrates that the pro-adipogenic activity of Hairless appears to be independent of its JmjC domain, thereby suggesting that Hairless likely mediates its effects on adipocyte differentiation by an alternative mechanism. Given the novel functional insights into the role for Hairless in the regulation of adipocyte differentiation this study is likely to be of significant interest to molecular biologists, particularly those interested in the transcriptional regulation of cell differentiation.

5. After appropriate revision, would a resubmitted manuscript be most suited for publication:

[a] in EMBO reports

6. Please add any further comments you consider relevant:

In addition to an effect on the regulation of adipocyte differentiation, the authors also make the claim that Hairless is critical for adipocyte lineage commitment and preadipocyte identity. However, while the author's provide strong evidence that Hairless is involved in the regulation of adipocyte differentiation, their experiments do not really directly address a role in adipocyte lineage commitment. To make this claim it would be necessary to demonstrate that in Hairless-deficient mice there is a specific deficit in the generation of preadipocyte cells. At present the authors do not have such data, they are only able to show that there is a defect in the appearance of fully differentiated adipocytes in the Hairless mutant mice. Consequently, I would recommend modifying the language in both the title and the manuscript itself to more accurately reflect a critical role for Hairless in adipocyte differentiation.

The experiments in Supplementary Figure 8 are not particularly compelling. The authors report that overexpression of Hairless does not affect the methylation status of bulk histones. However, since one would presumably only expect those histones located in the immediate vicinity of chromatin-bound Hairless, this experiment may not be sufficiently sensitive enough to detect an effect of Hairless on histone modification. Accordingly, I would recommend deleting this data from the manuscript.

The author's observation that an Hairless mutant lacking the JmjC domain is still able to promote adipogenesis when overexpressed is interesting, as it affords potential insight into the mechanisms involved. Accordingly, I think that the authors should consider formally moving this data into the manuscript in Fig. 4. In any case, the authors need to provide more details on the nature of this mutant, either details of cloning, or a prior reference if it has been previously described. It would also be helpful to include a schematic representation of the domain structure of Hairless and the
location of the JmjC domain.

The authors should note that they have apparently inadvertently mixed up Fig. S5 and Fig S6 - these should be switched.

The authors should carefully proofread their manuscript, as there are a number of typos and grammatical errors.

Referee #2:

Hairless is crucial in white adipocyte lineage commitment and differentiation
Kumpf et al.

This manuscript presents supporting evidence that Hairless, a member of the jmjC protein family is required for subcutaneous adipose tissue development in vivo and normal adipocyte differentiation in cellular models.

The results presented including gain and loss of function experiments in vitro and in vivo are supportive of the claim presented by the authors. However to make this research more competitive it would be necessary to provide information about:

a) Tissue distribution including different adipose tissue depots, specific cell in adipose tissue and the normal regulation of Hairless in different depots in response to feeding, fasting, obesity and high fat diet and age
b) The animals deficient in hairless are supposed to have less adipose tissue mass and maintained body weight. Is there any evidence of increased lean mass in this model?
c) Forced expression of hairless in c2c12 cells increase the adipogenic programme. Is there any evidence of increased hairless in skeletal muscle in parallel with HFD and /or ageing?
d) How is the expression of PPArg1 and PPArg2 isoforms in the KO model
e) Is there any suggestion what may be the Transcription factors involved in the different regulation of promoters and its regulation by hairless?
f) Is the KO mouse more insulin resistant? What is the lipid biochemistry in the fed and fasted state. These data will lend support to a lipodystrophy phenotype which is what could expected from the function of hairless.
g) Characterise the BAT gene expression in vivo to determine the relevance of the thermoinsulation in this model.

Other the authors aware of lipodystrophy syndromes associated with problems in hair.

Minor

Legends should include information about number of samples/animals, gender, age

Referee #3:

Summary:

This paper exposes a new and important role for Hairless in adipocyte differentiation that is likely to be of interest across fields. The finding is supported through several experimental approaches but has minor gaps that should be easily addressed.

Comments:
Evidence is provided of a novel role for the jumonji-domain protein Hairless (HR) in adipocyte differentiation. Endogenous expression and gain- and loss-of-function data support a requirement for HR during the early stages of adipogenesis, and in vitro experiments are strongly supported by histology from HR-knockout mouse pups. The putative demethylating JmjC domain appears dispensable for adipogenesis. Using luciferase reporter assays, the authors argue that HR specifically activates Ppary1 rather than Ppary2 gene expression in order to produce its adipogenic effect. Taken together, the data are broadly consistent and support the major conclusion that HR is important in early adipocyte differentiation. However, specific weaknesses in the data should be addressed to solidify the conclusions, as described below. It should be possible to easily produce the requested data from their existing samples or with short in vitro experiments.

1. The authors have proposed that HR directly modulates Ppary1 but not Ppary2 expression based on their luciferase reporter assays. In each of Figs. 1D, 2C-D, and 4D-E, Ppary is shown only as “total” RNA. Furthermore, the Ppary Western blot in Fig. 1E does not distinguish or indicate the two isoforms, which should be visible. Additional RT-PCR from those samples with isoform-specific primers should be conducted to assess how each transcript behaves. The panel in Fig. 1E should be expanded in order to indicate both Ppary1 and γ2 proteins.

In asserting an early effect on Ppary (and presumed before C/EBPα), it would be helpful to show the expression of the upstream C/EBP's (β, δ) that activate Ppary, and whether they are properly expressed.

Additional description of the luciferase reporter plasmids would be helpful, such as the regions of Ppary1/2 promoters included. Are there any indications of the motifs in the Ppary1 promoter that are required for activity, and therefore might indicate whether HR action is direct or indirect?

2. 3T3-L1 adipocytes undergo a mitotic clonal expansion phase in the 48 hours following addition of cocktail that provides for proper Ppary expression and full differentiation. Since the authors see a strong effect of HR-knockdown, have they inhibited MCE? This could be addressed by counting the cells in control and shHR cultures between d0 and d2.

3. Figures 1, S3, and S4 show inhibition of adipocyte differentiation by lentiviral shRNA infection. However, lentivirus alone can inhibit differentiation with increasing titre. The phenotypic data should be supported with an RNA or protein result showing that similar levels of infection were produced.

As well, the shHR knockdown is shown solely with RT-PCR data. Loss of HR protein should also be demonstrated by adding an additional anti-HR blot to Fig. 1E.

4. Considering the 2/3 reduction in WAT mass, was it difficult to isolate primary pre-adipocytes? This point should be discussed as it may bear on whether or not the non-differentiating pre-ads are retained in the residual WAT depots through adulthood.

5. "The development of BAT was not impaired by HR loss of function." This statement should be referenced. The only evidence shown is Fig. 3A showing BAT weight; addition of basic gene expression analysis (eg. Ppary, Ucp1) would be supportive of a WAT-specific effect.

6. Fig. 4D, E: Additional myogenic gene expression data (eg. Myf5, MyoD, myogenin) should be added to Fig. S7 to indicate whether the C2C12 cells have been converted to adipocytes (and lost myogenic identity) or if the adipocyte program has superseded the muscle program. The myotube fusion deficit (Fig. 4C) suggests a loss of terminal differentiation but does not indicate identity.

7. Fig. 4D-E: Does ectopic HR produce any detectable gene expression changes pre-induction? Is expression of Ppary (1 or 2) dependent on HR + hormonal stimulation?

8. The assertion that the JmjC domain lacks histone demethylation activity is tenuous. Even with over-expression of HR, loss in global histone methylation may not be observable. However, this does not detract from the major conclusion since expression of the deletion mutant indicates that this domain is dispensable for adipogenesis. The text could be revised to emphasize the latter point.
whereas the former is supported but not conclusive.

9. The histology in Fig. 3 is compelling, if thermoregulation is indeed unaffected. Housing of litters in thermoneutral (30°C) conditions followed by histology would help to confirm that the morphological results are not related to energy expenditure.

Minor Corrections:

1. The opening sentence of the abstract should be revised. Suggest:

"Adipose tissue is the largest compartment in the mammalian body for storing energy as fat, providing an important reservoir of fuel for maintaining whole body energy homeostasis."

2. Fig. 1B: Repeat Western blots - LaminA loading control is wildly variable, in contrast to Fig. 1E (lamin A, tubulin).

3. Fig. 4B, C: Since GFP control and HR vectors were used, GFP panels could be added to this figure to visualize similar levels of infection.

4. Supplemental Fig. S5 and S6 are reversed in order.

5. In lieu of broken y-axes, possibly use logarithmic scales?

1st Revision - authors' response 03 August 2012

Important points highlighted by the editor:

1. We agree with reviewer 1 and 3 that supplementary figure 8 is not conclusive and we have therefore removed it from the manuscript.

2. We have moved supplementary figure 9 into the main figure 4 as suggested by reviewer 1. In collaboration with the laboratory of Prof. Christian Wolfrum, ETH Zurich, Switzerland – his laboratory is specialized in adipocyte biology – we have quantified numbers of preadipocytes as suggested by reviewer 1 and 3 (see below). In fact, the amount of pre-adipocytes is not affected by loss of hairless challenging its requirement in adipocyte lineage commitment in vivo. Nevertheless, changes in hairless expression in precursors may still be important in lineage specification in vivo. In other words, hairless is not required in lineage commitment but changes in its expression may still be sufficient to affect the latter. Even though we have no data in vivo for this assumption, experiments in non adipocyte cell lines hint at such a scenario. Rather than making strong statements (title and abstract) on lineage commitment, we discuss this issue more carefully in the main text based on all the data that are now available in the revised version of the manuscript.

3. We fully agree with reviewer 2 and 3 that investigation of expression of both isoforms, PPARγ1 and γ2 was important in this study. We have done this as requested and extended the discussion on the issue whether or not hairless directly regulates PPARγ (see below).

4. We fully agree with reviewer 3 that there were some controls missing. We have included all of them in the new version of the manuscript. Importantly, conclusions remain the same after adding these controls.

5. Unfortunately, we could not entirely address the issue on thermoregulation as suggested by reviewer 3. As it was and still is indicated in the manuscript, adult hairless mutant mice have no fur. Comparing mice without fur with mice with normal hair is indeed problematic but it remains doubtful at seemingly thermoneutral conditions. Mutant and control mice obviously remain different also when maintained at 30°C. In other words, it is accepted that 30°C is thermoneutral for normal mice but it is indeed highly questionable whether 30°C is thermoneutral for hairless mutant mice that do not have fur. Therefore, we favored to analyze mice before they lose hair, which is before
they become 18 days old. Striking differences between mutant and control mice at this age have been shown already in the previous version of the manuscript. Even though, there is no reason for differences in thermoregulation at this age, we transferred pups with their mothers to 30°C chambers for 5 days. Unfortunately, this experiments seemed to be associated with a lot of stress for the pups (and perhaps for the mother) and the mortality of the litters was indeed too high to raise conclusions. Nevertheless, we also used brown adipocyte markers to investigate its distribution and extent and found no differences between mutant and control mice. Overall, these results underscore our initial findings and suggest that thermoregulation in hairless mutant mice is unaffected and that hairless mainly acts to control adipocyte differentiation.

6. We agree that experiments suggested by reviewer 2 – even though they would provide important new mechanistic insights – would go beyond a manuscript in Embo Reports. Nevertheless, we discuss in detail her/his points in this response letter and made some changes in the manuscript text. We have added indications of experimental numbers (n) of samples/animals and define error bars in the figure legends. We have also added scale bars in all images of cells and tissues.

7. We have adapted the manuscript according to format outlines given by Embo Reports. The manuscript now contains 5 main figures and 5 supplemental figures.

Reviewer #1:

Reviewer 1 has favorably answered to formal questions by Embo reports on the suitability and scientific quality of our manuscript. He or she highlights the novelty of our study and its general importance to molecular biologists.

1. Nonetheless, reviewer 1 raised a valid and important point. While we have provided compelling evidence for a role of hairless in adipocyte differentiation, our data appear not to support its role in adipocyte lineage commitment. For example, experimental evidence for reduced preadipocyte numbers in hairless mutant mice as compared to control mice was lacking. We have now quantified preadipocyte numbers using a recently published flow cytometry protocol for isolation of adipocyte precursors (Sca1+/CD34+/CD29+/Lin-) from the adipose stromal vascular fraction (Rodeheffer et al. Cell 2008; 17;135(2):240-249). We have performed these experiments in collaboration with the laboratory of Prof. Christian Wolfrum, ETH Zurich, Switzerland. In fact, preadipocyte numbers were not affected in hairless mutant mice as compared to control animals (Supplementary Fig S3B). These new data thus indicate that hairless is not required for adipocyte lineage commitment in vivo. Based on experiments in non-adipocyte cell lines, in which forced expression of hairless resulted in enhanced adipogenesis (Fig 4F – I) and most strikingly expression of adipocyte marker genes prior induction of differentiation in NIH3T3 cells (new Fig 4D), hairless expression might still play a role in adipocyte fate determination. However, we have no data in vivo so far that support the latter hypothesis. We thus removed any straight claim on adipocyte identity in this manuscript and mainly focus on the role of hairless in adipocyte differentiation as suggested by reviewer 1. Nevertheless, we briefly and critically discuss a potential involvement of hairless in adipocyte lineage commitment based on data presented in the revised manuscript.

2. Reviewer 1 suggested removing experiments in supplementary figure 8. We fully agree with reviewer 1’s notion on limitations of this experiment. We have thus removed these data from the manuscript as suggested.

3. Reviewer 1 highlighted the importance of our experiment presented in supplementary figure 9 in the initial version of our manuscript. This experiment shows that the JmjC domain is not required to promote adipogenesis in NIH3T3 cells. As suggested, we have moved this panel to main figures (Fig 5B). We have also added a schematic view of the hairless protein (Fig 5A) and describe the truncation the JmjC domain mutant in methods in the supplementary information section.

4. Reviewer 1 correctly hinted at a mistake in the citation of supplementary figures 5 and 6 in the main text of our initial version of the manuscript. Following the policy of Embo Report, which is to restrict the number of supplementary figures to an amount of 5, figure labeling and citations needed
to be entirely changed. In any case, citations of main and supplementary figures in the manuscript text have been very carefully checked.

5. We have carefully proofread the manuscript to avoid typos and grammatical errors.

Reviewer #2:

Reviewer 2 acknowledged that conclusions raised in our study are supported by our data. However, she or he suggests a number of additional experiments that would extend the knowledge about hairless in adipocyte function. We are well aware that our study lacks investigation of adipocyte function under different metabolic challenges in mice and that it does not provide a detailed molecular mechanism downstream of hairless. Nevertheless, this study is first to show a role of hairless in adipogenesis and our data provide compelling evidence for this assumption. Extending beyond this key message would, to our opinion, not be in the scope of *Embo Reports* that aims at publishing one novel key finding that is supported by strong data.

a) Reviewer 2 suggested to address the mass and cellular composition of different adipose tissue depots and to investigate hairless expression in different fat depots in response to metabolic challenges as well as aging. We are well aware of distinct properties of different fat depots (for example subcutaneous versus visceral fat). We are also aware of the fact that adipocyte size and the presence of inflammatory cells changes these properties and that remodeling of adipose affecting these factors occurs in response to metabolic challenges listed by reviewer 2. We thus fully agree with this reviewer that results of such experiments will be very interesting and will provide additional important insights. We strongly feel, however, that these analyses would distract from the key message of this study, which is that hairless is a basic new regulator of white adipogenesis. Moreover, as currently available adult hairless mutant mice have no hair, a possible impact of metabolic challenges and/or aging on adipose tissue function in this model will not be conclusive. Generation of conditional adipocyte-specific hairless knockout mice that will allow addressing these questions independent of a phenotype in hair growth will thus be necessary. Unfortunately, these mice are currently not available.

b) Reviewer 2 asked whether there was evidence for increased lean mass in hairless mutant mice when comparing them to control mice as the body weight seemed to be maintained despite the fact that the fat mass was markedly diminished. We are not entirely sure, which data reviewer 2 is exactly referring to. We have measured fat pads in 10 weeks old mice as shown in supplementary figure 5 of the previous version of the manuscript (now supplementary Fig S4). At this age, fat mass is markedly reduced in hairless mutant mice, while the body weight is significantly increased as compared to control mice. We apologize that we have not indicated significance for body weight differences in the initial version of the manuscript. Even though we have not determined total lean mass in these mice, it is known that hairless mutant mice get progressively wrinkled skin (Catherine C. Thomson Nucl. Recept Signal 2009, Vol. 7). This expansion of skin accounts for the overall increased body weight despite reduced fat mass. As adult mice have no hair and this might impact on energy homeostasis, we also measured weights of subcutaneous fat pads in 18 days old mice, which are indistinguishable from control mice in terms of hair growth and appearance of skin. At this age, the body weight was indeed significantly reduced in hairless mutant mice due to reduced fat mass (Fig 3A of previous and new version of the manuscript).

c) Reviewer 2 asked whether hairless expression in skeletal muscle was enhanced upon high fat feeding and/or aging as forced expression of hairless in C2C12 cells results in a more efficient induction of the adipogenic expression program in these cells. For reasons delineated in response to point a) of reviewer 2, we have not assessed hairless expression in different organs in response to these challenges. Nevertheless, we agree with reviewer 2, if expression of hairless would be enhanced, it might hint at an important function in ectopic fat deposition potentially linking it to insulin resistance.

d) Reviewer 2 requested the assessment of the expression of PPARγ1 and γ2 in hairless mutant mice. We have assessed expression of PPARγ1 and γ2 at the RNA (quantitative RT-PCR) and
protein level (Western blot). We have measured expression of these two isoforms in 3T3-L1 cells (Fig 1F), in primary cells (Fig 2 C and D), in C2C12 cells (Fig 4E) and in NIH3T3 cells (Fig 4D and G). The bottom line of these experiments is that modulation of hairless expression affects expression of both PPARγ1 and γ2 at both the RNA as well as protein level. Compatibility of these results with our luciferase reporter assays that indicated specific and direct regulation of PPARγ1 by hairless is now discussed more thoroughly in the manuscript. The contribution of both isoforms to adipogenesis is controversially discussed. Both isoforms are induced upon adipogenic stimulation. While PPARγ1 is ubiquitously expressed in all tissues, PPARγ2 expression is restricted to adipocytes. While one team showed that expression of PPARγ2 but not PPARγ1 restored adipogenesis in cells devoid of “total” PPARγ (Ren D et al. G&D 2002; 16:27-32), another team demonstrated that both isoforms are capable to rescue adipocyte formation (Mueller E et al. JBC 2002; 277, 44-1;41925-41930). It has also been shown that ectopic PPARγ1 expression induced PPARγ2 transcription suggesting a potential cross-talk between these isoforms in differentiating adipocytes (Tontonoz, P et al. Cell 1994; 79, 1147-1156). Based on these previous observations, it was very unlikely that specific hairless-dependent regulation of PPARγ1 expression without affecting PPARγ2 underlies the observed phenotypes in adipogenesis in our study. This is also supported by the fact that addition of rosiglitazone, activating both PPARγ1 and γ2 through direct binding to their ligand binding domain, has no effect on adipogenesis if hairless is inactivated. Nonetheless, direct regulation of PPARγ1 transcription by hairless may indirectly affect PPARγ2 expression. However, PPARγ has also been shown to be regulated at the post-transcriptional level. Hence, our data do not exclude direct post-transcriptional regulation of PPARγ2 by hairless. We discuss this issue more thoroughly in the revised version of the manuscript.

e) Reviewer 2 hinted at the lack of a detailed mechanism as to how hairless regulates adipogenesis. We fully agree with this notion but strongly feel that further mechanistic insights should be subject of a subsequent study.

f) Reviewer 2 indicated that it would have been important to assess insulin sensitivity and to determine circulating lipids in fed and fasted mice to corroborate a lipodystrophy phenotype in hairless mutant mice. We fully agree with reviewer 2 that lipodystrophy leads to enhanced circulating lipids and ectopic fat deposition in lean tissues resulting in systemic insulin resistance. We found a significant reduction in adipose tissue mass in mutant mice as compared to control mice. Whether this phenotype is associated with features of lipodystrophy is indeed an interesting question but will be subject of subsequent studies preferentially with adipocyte-specific conditional knockout mice.

g) Reviewer 2 asked us to further characterize brown adipose gene expression and thermoinsulation in our mouse models. We have assessed the expression of two brown adipocyte-specific transcripts, Ucp1 and Lhx8 in white adipose tissue in vivo and did not observe any significant differences between control and mutant mice. Thus, there is no evidence for increased conversion of WAT into BAT. As shown already in the previous version of our manuscript for 18 days old mice (Fig 3A), BAT depots are not heavier in hairless mutant mice as compared to control mice (previous and new Fig 3A). We now show that expression levels of Ucp1 and Lhx8 in BAT are comparable between both genotypes (Fig 3C). Most importantly, differences in white adipose tissue mass are independent of hair loss. Altogether, there is thus no evidence for differences in thermoinsulation in hairless mutant mice as compared to control mice.

Minor comment

Reviewer 2 correctly hinted at the lack of indications of experimental numbers (n) of samples/animals. We have added this information in figure legends where applicable.

Reviewer #3:

Reviewer 3 emphasized the novelty and importance of our study and lists valid and important experimental gaps that we were able to address during revision.
1. Reviewer 3 correctly indicated that separate measurements of PPARγ isoforms, PPARγ1 and γ2 rather than “total” PPARγ was important to further analyze a specific hairless-dependent transcriptional regulation of PPARγ1 that was evidenced by our luciferase reporter assay. As requested, we have measured expression of these two isoforms in 3T3-L1 cells (Fig 1F), in primary cells (Fig 2 C and D), in C2C12 cells (Fig 4E) and in NIH3T3 cells (Fig 4D and G). Collectively, these new results indicate that expression of both isoforms are regulated by hairless. The contribution of both isoforms to adipogenesis is controversially discussed. Both isoforms are induced upon adipogenic stimulation. While PPARγ1 is ubiquitously expressed in all tissues, PPARγ2 expression is restricted to adipocytes. While one team showed that expression of PPARγ2 but not PPARγ1 restored adipogenesis in cells inactivated for “total” PPARγ (Ren D et al. G&D 2002; 16:27-32), another team demonstrated that both isoforms are capable to rescue adipocyte formation (Mueller E et al. JBC 2002; 277, 44-1:41925-41930). It has also been shown that ectopic PPARγ1 expression induced PPARγ2 transcription suggesting a potential cross-talk between these isoforms in differentiating adipocytes (Tontonoz, P et al. Cell 1994; 79, 1147-1156). Based on these previous observations, it was very unlikely that specific hairless-dependent regulation of PPARγ1 expression without affecting PPARγ2 underlies the observed phenotypes in adipogenesis in our study. This is also supported by the fact that addition of rosiglitazone, activating both PPARγ1 and γ2 through direct binding to their ligand binding domain, has no effect on adipogenesis if hairless is inactivated. Nonetheless, direct regulation of PPARγ1 transcription by hairless (evidenced by the luciferase reporter assay) may indirectly affect PPARγ2 expression. However, PPARγ1 as well as PPARγ2 was also shown to be strongly regulated at the post-transcriptional level. Hence, we cannot exclude direct post-transcriptional regulation of PPARγ2 by hairless. We discuss this issue more thoroughly in the revised version of the manuscript.

In this context, reviewer 3 also requested to assess expression of early C/EBP’s (β and δ) upstream of PPARγ. We added this new data to figures 1D, 2C and D as well as 4D and E. There is no significant difference in the expression of early C/EBP’s supporting our assumption that hairless may have a direct impact on PPARγ expression. This doesn’t exclude, however, that hairless modulates activity of C/EBP’s on the PPARγ promoter.

Reviewer 3 also requested additional information on the reporter plasmids to gain more insights into binding motifs of transcription factors. Plasmids containing human sequences of alternative promoters of PPARγ1 and γ2, respectively, have been obtained from the laboratory of Prof. Johan Auwerx, ETH Lausanne, Switzerland. Indeed, distinct transcription factor motifs have been described for these alternative promoters. Based on this information, it is, however, impossible to extrapolate whether hairless action is direct or indirect and why it is specific for PPARγ1 in this assay. We have put more information about constructs used for luciferase reporter assays into the methodology section of supplementary information.

2. Reviewer 3 asked us to assess clonal expansion of 3T3-L1 adipocytes. We have done this experiment and observed a minor effect on clonal expansion (Supplementary Fig S2B). This effect might contribute to impaired adipogenesis in hairless-depleted 3T3-L1 cells but our data provide evidence that hairless is likely to act more downstream, potentially at the level of PPARγ.

3. Reviewer 3 indicated that lentiviral infection alone might have an impact on adipocyte differentiation. We have indirectly assessed genomic integration of the lentiviral plasmid by quantifying transcription of the puromycine resistance gene of the viral plasmid in 3T3-L1 and primary cells using quantitative RT-PCR. Transcription of the puromycine resistance gene was clearly less for shRNA-infected cells as compared to control-infected cells strongly supporting that phenotypes seen in cells are due to the knockdown of hairless and not because of virus-induced off-target effects. As we are confronted with space restrictions in supplementary information (maximal 5 supplementary figures), we have attached these results directly to this cover letter (see annex).

In this context, reviewer 3 also wanted us to determine deletion of hairless at the protein level. We have added these data to Figure 1H as requested. Deletion by shRNA was indeed very efficient. We would like to emphasize, however, that shHr1 was more efficient than shHr2, which correlates well with a less strong inhibition of adipogenesis and suppression of adipogenic downstream genes obtained by shHr2 as compared to shHr1.
4. Reviewer 3 asked about preadipocyte numbers in hairless mutant mice as compared to control mice. We have assessed this in collaboration with the laboratory of Prof. Christian Wolfrum, ETH Zurich, Switzerland using a recently published flow cytometry protocol (Rodeheffer et al. Cell 2008; 17;135(2):240-249). Using this approach, we cannot observe differences in preadipocyte numbers (Supplemental Figure S3B). This challenges the requirement of hairless in adipocyte lineage commitment but does not exclude that modulation in expression of hairless might contribute to the latter as supported by experiments in non-adipogenic cell lines (Fig 4 and see see additional explanations in point 7). Nevertheless, we removed any straight claim on adipocyte identity and discuss this option more carefully in the new version of the manuscript.

5. Reviewer 3 correctly indicated that the only basis to conclude that brown adipose tissue (BAT) development was not affected in hairless mutant mice were unaltered BAT weights. We therefore followed the advice of reviewer 3 and measured two genes, UCP1 and Lxh8 in WAT and BAT (Fig 3C). No differences were observed supporting that BAT gene expression is not altered and that there is no obvious conversion from WAT into BAT in the absence of hairless.

6. Reviewer 3 requested myogenic gene expression data to evidence that C2C12 cells indeed loose their myogenic identity upon ectopic hairless expression. We differentiated C2C12 myoblasts into myotubes in cells ectopically expressing hairless-GFP or GFP alone. Hairless expression did not inhibit myogenic differentiation (new Fig 4J). This indeed challenges the potential of hairless to induce a switch of muscle into adipocyte fate, once permissive conditions for muscle differentiation are set in place. We added the later statement into the manuscript.

7. Reviewer 3 asked whether hairless regulates expression of PPAR’s prior hormonal induction of adipogenesis. As requested, we have included these data into Figure 4D and E. Indeed, there is significantly enhanced induction of both PPARγ1 and γ2 when hairless is ectopically expressed in NIH3T3 cells prior addition of the adipogenic cocktail (new Fig 4D). These effects were however not seen in C2C12 cells (new Fig 4E). Interestingly, hairless and PPARγ were expressed much higher in C2C12 as compared to NIH3T3. This may indicate that undifferentiated C2C12 cells are already more primed to become adipocytes and that differences in adipogenic gene expression under non-permissive conditions are not to be expected. Nevertheless, under adipogenic conditions, expression of hairless clearly potentiated adipogenesis in C2C12 cells (Fig 4H & 4I).

8. Reviewer 3 indicated that histone demethylation assays in the former supplementary figure 8 were not conclusive. We thus decided to remove these data from the manuscript as suggested by reviewer 1.

9. Reviewer 3 correctly objected the lack of evidence that altered thermoregulation does not contribute to the adipose phenotype seen in mutant mice. She or he therefore suggested repeating experiments under thermoneutral conditions. As it was and still is indicated in the manuscript, adult hairless mutant mice have no fur. Comparing mice without fur with mice with normal hair is indeed problematic but it remains doubtful at seemingly thermoneutral conditions. Mutant and control mice obviously remain different also when maintained at 30°C. In other words, it is accepted that 30°C is thermoneutral for normal mice but it is indeed highly questionable whether 30°C is thermoneutral for hairless mutant mice that do not have fur. Therefore, we favored to analyze mice before they lose hair, which is before they become 18 days old. Striking differences between mutant and control mice at this age have been shown already in the previous version of the manuscript. Even though, there is no reason for differences in thermoregulation at this age, we transferred pups with their mothers to 30°C chambers for 5 days. Unfortunately, this experiments seemed to be associated with a lot of stress for the pups (and perhaps for the mother) and the mortality of the litters was indeed too high to raise conclusions. Nevertheless, we also used brown adipocyte markers to investigate its distribution and extent and found no differences between mutant and control mice. Overall, these results underscore our initial findings and suggest that thermoregulation in hairless mutant mice is unaffected and that hairless mainly acts to control adipocyte differentiation.

Minor Corrections:

1. Reviewer 3 suggested to change the first sentence of the abstract. We have changed it as suggested.
2. Reviewer 3 indicated that the loading control, LaminA, in Figure 1B of the previous version of the manuscript was not stable during the course of adipogenesis. We repeated this experiment and consistently had fluctuations of LaminA during adipogenesis although sample concentrations have been adapted after Bradford measurements. We therefore redid the western blot and decided to use a different loading control, which is GCN5, a histone acetyltransferase (new Figure 1B).

3. Reviewer 3 asked us to add GFP panels for overexpression experiments shown in figure 4B and C in the previous version of the manuscript. We have added flow cytometry measurements and immunofluorescence pictures showing a comparable GFP expression for both, cells expressing GFP alone and cells expressing hairless-GFP (Figure 4B and C). However, we would like to indicate that overexpression of these constructs has been achieved by lipofection. So there was no infection involved in these experiments.

4. Reviewer 3 indicated that Fig. S5 and S6 were reversed in order. Following the policy of Embo Reports to restrict the number of supplementary figures to an amount of 5, figure labeling and citations needed to be entirely changed. In any case, citations of main and supplementary figures in the manuscript text have been very carefully checked.

5. Reviewer 3 advised us to avoid broken y-axes and to use logarithmic scales, which we did where applicable.

ANNEX
Quantification of transcription of the puromycin resistance gene of the viral plasmid in 3T3-L1 and primary preadipocytes using quantitative RT-PCR. PAC stands for puromycin N-acetyl-transferase.

2nd Editorial Decision 17 August 2012

Thank you for the submission of your revised manuscript. It was sent to referees 1 and 3 and as you will see, both support now publication of the study in EMBO reports. However, referee 3 still has a few suggestions for how the results and discussion section could be improved that I would like you to address before we can proceed with the official acceptance of your manuscript. The referee also suggests one more rescue experiment that, however, would not be essential for publication of the study here.

I also noticed that the legend for figure 1 does not define the error bars depicted in panels A, C, E, F, G and I. The same applies to figures 2C, D; 3A, C; 4A, D, E, G, I, J; 5D; SF2B; SF3, SF4, SF5. Please add this missing information. If the error bars are always the same (for example standard
error of the mean or standard differentiation) for each panel, you can also only add one sentence at the end of each figure legend.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

The revised manuscript by Kumpf et al fully addresses my initial criticisms and comments raised during the initial review process.

Referee #3:

The authors have made substantial efforts to address the reviewers' comments. Aside from several textual changes that are asked (see below), their results are acceptable for publication.

Revise:

As requested, the authors have distinguished Pparγ1 and γ2, bringing some clarity to their relative expression. However, their final "Results" section (Pparγ, JmjC domain) is confusing written and should be revisited. Furthermore, both there and in their "Concluding remarks", the authors continue to emphasize the idea that HR is primarily regulating Pparγ1, despite the new γ1/γ2 data. Indeed, figures 1(F), 2(C, D), and 4(D, G) all suggest a relatively modest effect on γ1 and a dramatic effect on γ2. Only the luciferase data in figure 5(D) supports a γ1-specific effect, and that could reflect a deficit in the ability of the γ2 reporter to recapitulate the endogenous gene expression. I suggest that they emphasize the effect on total Pparγ, in conjunction with a more streamlined discussion of their data for specific effects on the γ1 and/or γ2 promotors.

Other Comments:

- A rescue experiment of the HR-knockout primary adipocytes with ectopic HR expression (following Fig. 2) would be great confirmation that the remaining SVF cells are actually pre-adipocytes, and that they have a cell-autonomous differentiation defect. The manuscript is acceptable without it but this would be a very nice result to include.

- The C2C12 differentiation experiment addressed a different question than I had intended. While it does show that they remain myogenic in myogenic conditions, I meant to ask if the C2C12+HR cells maintained any myogenic gene expression in adipogenic conditions (ie. an incomplete fate switch, with the adipose program dominant). However, this question does not need to be addressed any further here.

2nd Revision - authors' response 20 August 2012

We indeed highly appreciate that our revision of the manuscript entitled “Hairless is crucial in white adipocyte differentiation” was satisfactory to reviewer 1 and 3.

We have successfully revised the last paragraph of the result section as well as concluding remarks as suggested by reviewer 3. We also added statements on error bars in figure legends as requested by you. We have not performed the rescue experiment as suggested by reviewer 3, as it was not considered essential for publication.
We hope that with these changes in the text, our manuscript can now be accepted for publication in *Embo Reports*.

Please do not hesitate to contact me if you require further information. We are looking forward to hearing from you.

3rd Editorial Decision 21 August 2012

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.

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Yours sincerely,

Editor
EMBO Reports