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Novel repressor regulates insulin sensitivity through interaction with Foxo1

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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 June 2011

Thank you very much for submitting your research paper on a novel Foxo1-regulator for consideration to The EMBO Journal editorial office. Having received comments from two expert scientists, I am able to reach a decision on your study to facilitate efficient proceedings. As you will see from the enclosed reports, both scientists emphasize potential novelty and thus interest in P13. However, both experts struggle with physiological support and actual function of P13. While ref#2 asks for clarifications on the transgenic phenotypes, ref#1 goes as far as to demand further molecular studies to better elucidate the mechanisms as well as thorough corroboration that should include loss-of function approaches (though not generation of a knock-out mouse at this point!). Given their constructive comments and recognizing the potential of your study, we would be willing to offer you the chance to significantly extend the current dataset during major amendments. We do however realize that this entails significant and challenging further experimentation. We would thus be prepared to offer more time than the limited amount of three month for revision upon your explicit request.

I do urge you to take these demands serious and carefully consider your options of EITHER pursuing revisions for The EMBO Journal OR seeking alternative publication elsewhere.

Please do not hesitate to contact me with in case of further questions or indeed outlining possible experiments and timeline in case you plan to revise for The EMBO Journal (preferably via E-mail).

Finally, I do have to formerly remind you that it is EMBO_J policy to allow one round of revisions only and that the final decision on acceptance or rejection depends on the content and strength of the future, revised version of your study.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

These studies by Nakae and Takahashi report the identification of a novel corepressor of FoxO1 through a yeast two-hybrid screening. While the identification and characterization is novel and of interest, this manuscript covers too much information lacking sufficient depth and/or adequate experimental approaches to support all the conclusions indicated. The authors try to include too much data that at this point is not clear what is the global conclusion and how P13 function in a metabolic context. In order to provide a clear message, mechanism and physiological context these studies should be presented focusing at one particular tissue and mechanism, for example the effects on adipose tissue and FoxO1 repression.

There are additional important points that should be addressed:

1- Specificity of the corepressor P13. How specific is P13 towards other FoxO proteins and other transcription factors. This should be answer with reporter assays and also include gene array analysis.
2- The studies in vivo using transgenic mice have been performed using gain-of-function, it would be necessary to include additional loss-of-function in vivo.
3- Along the same lines, knock-down experiments in adipose cell lines only use one hairpin; it would be necessary to use different shRNAs to avoid hairpin artifacts.
4- The expression of P13 in liver is very, very small; the hepatic biological confirmed showing endogenous P13 protein.
5- As in 4, it would be necessary to show endogenous P13 protein in adipose tissue as well as nuclear localization.
6- It is unclear when P13 repress FoxO1 and whether this is a regulatory event.
7- Related to the adipose function, the authors should investigate how affect FoxO1 function and its connection to PPARg. How much of the effects of P13 in adipose tissue are through FoxO1 inhibition of PPARg.

Referee #2:

In this manuscript, Nakae et al present the cloning of a novel Foxo1-interacting factor. The authors present evidence that their new molecule, cryptically named P13, can interfere with the Foxo1-Sirt1 interaction and leads to acetylated, inactive Foxo1. P13 can also regulate Crtc2, although the significance of this finding is not elaborated. These claims are supported by strong and elegant in vitro evidence and somewhat more tenuous in vivo data. However, within this one manuscript the authors both identify a novel gene and elucidate its biochemical function; an impressive feat in this post-genomic era. The northern blotting demonstrating tissue distribution and regulation of the endogenous transcript by nutritional status as well as the addition of a new actor in an important metabolic pathway are crucial new advances.

Major points:
1. In this reviewer's opinion, the central point of this manuscript should be the identification of a new gene and to describe its initial characterization. Currently the best defined role for Foxo1 is as a regulator of hepatic glucose production. The most straightforward in vivo evidence presented is

that knockdown of P13 can exacerbate HGP, presumably through Foxo1 regulation (Fig 7). Yet in the authors wade into the ambiguous territory of Foxo1-mediated control of adipocyte differentiation, a puzzle which does not have a clear solution. For example, no clear explanation is presented for why either aP2-WP13 or aP2-WP13 LepRdb/db transgenic mice should be lean. While the implications for P13 in the liver are easily accessible.

2. Numerous studies (including the work under review and earlier work from these authors) have implicated Foxo1 as a potent repressor of PPAR in adipose tissue. Consistent with this model, knockdown of P13 (a state of increased Foxo1 activity) completely blocks PPAR activity and adipogenesis (Figure 4). The converse experiment would be informative: does overexpression of P13 in cultured adipocytes lead to enhanced adipogenesis? Analysis of adipocyte size in aP2-WP13 mice of different body weights is not a valid comparison; younger mice should be examined.

3. Similarly, the interpretation of the transgenic phenotype is troublesome. Overexpression of a foxo1 inhibitor would be expected to increase PPAR activity leading to an increase in adipocyte differentiation and possible body weight gain. Instead the authors show transgenic mice with less adipose tissue mass. This decreased body weight cannot simply be explained due to decreased adipose tissue differentiation. Body weight is the balance between food intake and caloric expenditure. This point must be addressed. In addition, effects on insulin sensitivity appear to be secondary to body weight. Why does the body weight of the transgenics suddenly diverge?

Minor points:

1. The preponderance of data might be clarified by presenting a model.
2. P13 is not a very memorable name. Perhaps a new name should be proposed. for p13: Foxo1 Corepressor (FCoR)
3. Figure 5c requires error bars.
4. Primer sequences should be provided as well as the sequence of the shRNA construct.

1st Revision - Authors' Response 20 December 2011

Response to Referee #1

We would like to thank Referee #1 for the insightful and constructive comments. These comments helped us strengthen and clarify our report. Before responding to the comments from Referee #1, we would like to note that in response to the suggestions of another reviewer (Referee #2), we now refer to "P13" as "Foxo1 CoRepressor (FCoR)". Accordingly, we replaced "P13" with "FCoR" in the revised manuscript, including in the title, abstract, and figures. Furthermore, we added several new authors, including Drs. Yoshinaga Kawano and Risa Sekioka, who have done some experiments, and Dr. Hiroshi Kiyonari, who generated FcorKO mice, and Drs. Toshiya Tanaka and Juro Sakai, who constructed PPARg and RXRa expression vectors. And also we changed order of author list (Shin-Ichiro Takahashi and Hiroshi Itoh) because Dr. Hiroshi Itoh supervised and supported all studies of *FcorKO* mice. As suggested by Referee #1, we generated and analyzed *Fcor* knockout mice and now describe our findings in the last paragraph of the Results section and in Figure 8. We also generated a second adenovirus that encodes another shRNA (shRNA2) and then analyzed the effects of shRNA2 on adipocyte differentiation. We described our findings in the revised manuscript and in Supplementary Figure S5. Furthermore, the revised manuscript now includes data regarding the phosphorylation of FCoR by PKA and the direct acetylation of Foxo1 by FCoR, which was determined using an *in vitro* acetylation assay. We have revised several figures and made changes in the manuscript in response to the suggestions of Referee #1 as noted below.

Q1. Specificity of the corepressor P13. How specific is P13 towards other FoxO proteins and other transcription factors. This should be answer with reporter assays and also include gene array analysis.

A1. In response to this suggestion, we constructed the PM-Foxo3a and PM-FOXO4 vectors (page 11, lines 6-21 in the revised Supplementary Information) and performed the 5XGAL4-luciferase

assay. This assay showed that FCoR inhibited Foxo1- and Foxo3a-induced 5XGAL4 activity but not FOXO4-induced 5XGAL4 activity (page 7, line 21-page 8, line 7 and Figure 2F in the revised manuscript). To confirm these findings, we constructed the pCMV5/cMyc-FOXO4 expression vector (page 3, lines 11-18 in the revised Supplementary Information) and performed co-immunoprecipitation studies with Foxo and FCoR. These experiments demonstrated that FLAG-FCoR could bind to cMyc-Foxo1 and cMyc-Foxo3a but not to cMyc-FOXO4 (page 7, line 21-page 8, line 3 and Figure 2E in the revised manuscript). We concluded that FCoR was a Foxo- and Foxo3a-specific coreppressor, and we now describe it as such in the title of a section in the revised manuscript (page 7, line 7).

Q2. The studies in vivo using transgenic mice have been performed using gain-of-function, it would be necessary to include additional loss-of-function in vivo.

A2. In response to this suggestion, we generated and analyzed *FcorKO* mice (page 8, line 18-page 9, line 13 in the revised Supplementary information and Supplementary Figure S12). The *FcorKO* mice exhibited a lean, glucose-intolerant, and insulin resistant phenotype. Furthermore, they had larger adipocytes than control mice and showed increased expression of Foxo-target genes, including *Cdkn1b* and *Ccng2*, and inflammatory genes, including *Emr1* and *Ccr2*. These phenotypes are the opposite of the phenotypes that result from inactivation of Foxo1; in the latter, there is haploinsufficiency of Foxo1 and overexpression of a transactivation-defective form of Foxo1 (D256Foxo1) in adipose tissue. We now report these data in the revised manuscript (page 18, lines 2-15 and Figure 8). Furthermore, oxygen consumption and the respiratory quotient were significantly increased in *FcorKO* mice compared to control mice (Figure 8K and 8L in the revised manuscript), and PGC1a protein and *Ppargc1a* expression were also increased (Figure 8M and 8N in the revised manuscript). Notably, these data contrast with data from FCoR transgenic mice, which show decreased expression of PGC1a expression. Thus, the data obtained in *FcorKO* mice support the idea that the FCoR-Foxo1 axis serves as a novel metabolic regulator (page 18, line 16-page 19, line 4 in the revised manuscript).

Q3. - Along the same lines, knock-down experiments in adipose cell lines only use one hairpin; it would be necessary to use different shRNAs to avoid hairpin artifacts.

A3. In response to this suggestion, we generated another FCoR shRNA (shRNA2) (page 5, lines 8-18 in the revised Supplementary Information) and performed transduction studies of shRNA2 in 3T3-F442A cells. Specifically, we performed Oil-red O staining and real-time PCR as for the shRNA described in the original manuscript (shRNA1). These experiments demonstrated that infection of cells with an adenovirus encoding shRNA2 inhibited adipocyte differentiation and was accompanied by modified gene expression (page 12, lines 3-16 in the revised manuscript and Supplementary Figure S5).

Q4. The expression of P13 in liver is very, very small; the hepatic biological confirmed showing endogenous P13 protein.

A4. In response to this comment as well as to a comment by Referee #3, we removed the data pertaining to the liver and to Crtc in the revised manuscript. We now include just the data showing the extremely low expression level of FCoR in the liver (Figure 1E and 1I in the revised manuscript).

Q5. As in 4, it would be necessary to show endogenous P13 protein in adipose tissue as well as nuclear localization.

A5. As suggested, we performed Western blotting of the WAT and BAT from wild type mice in the fed and fasting states (page 6, lines 17-20 and Figure 1A and 1J in the revised manuscript). We also performed immunohistochemistry to detect WAT and BAT and demonstrated that an anti-FCoR antibody stained the nucleus of cells in these tissues (page 7, lines 3-5 and Figure 1M in the revised manuscript).

Q6. It is unclear when P13 repress FoxO1 and whether this is a regulatory event.

A6. We agree that this was originally unclear. In the revised manuscript, we added data that show the phosphorylation of FCoR by forskolin or by the catalytic subunit of cAMP protein kinase. We identified the phosphorylation site of FCoR, T93, by PKA using site-directed mutagenesis and a phospho-T93-specific antibody and demonstrated that phosphorylation of T93 caused the nuclear translocation of FCoR. We constructed a phosphorylation-mimic mutant of FCoR, T93D, and then performed the 5XGAL4 luciferase assay and examined acetylation by transfected Foxo1. We demonstrated that the T93D mutant localized to the nucleus and inhibits Foxo1-induced GAL4 activity in the absence of forskolin (page 10, line 1-page 12, line 2 and Figure 4 in the revised manuscript). We also propose a model for the roles of FCoR in Figure 9 in the revised manuscript. We think that during fasting or when exposed to the cold, increased cAMP levels result in the phosphorylation of FCoR, leading to nuclear transport, interruption of the association between nuclear Foxo1 and Sirt1, and increased acetylation and inhibition of Foxo1. In the fed state, both FCoR and Foxo1 are in the cytosol, where FCoR acetylates Foxo1 directly (page 9, lines 4-13 and Figures 3D and 3E in the revised manuscript).

Q7. Related to the adipose function, the authors should investigate how affect FoxO1 function and its connection to PPARg. How much of the effects of P13 in adipose tissue are through FoxO1 inhibition of PPARg.

A7. To address this question, we performed a luciferase assay using the J3-tk-Luc reporter vector, PPARg2, RXRa, CNFoxo1, and the FCoR expression vector in the presence of rosiglitazone (page 13, lines 3-9 and Supplenmentary Figure S6B in the revised manuscript; also see page 11, line 22-page 12, line 9 in the revised Supplementary Information). This experiment demonstrated that the effects of FCoR in adipose tissue were not due to Foxo1 inhibition of PPARg.

Response to Referee #2

We would like to thank Referee #2 for the insightful and constructive comments. These comments helped us strengthen and clarify our report. Before responding to the comments from Referee #2, we would like to note that we now refer to "P13" as "Foxo1 CoRepressor (FCoR)." Accordingly, we replaced "P13" with "FCoR" in the revised manuscript, including in the title, abstract, and figures. Furthermore, we added several new authors, including Drs. Yoshinaga Kawano and Risa Sekioka, who have done some experiments, and Dr. Hiroshi Kiyonari, who generated FcorKO mice, and Drs. Toshiya Tanaka and Juro Sakai, who constructed PPARg and RXRa expression vectors. And also we changed order of author list (Shin-Ichiro Takahashi and Hiroshi Itoh) because Dr. Hiroshi Itoh supervised and supported all studies of *FcorKO* mice. As suggested by Referee #1, we generated and analysed *Fcor* knockout mice and now describe our findings in the last paragraph of the Results section and in Figure 8. We also generated a second adenovirus that encodes another shRNA (shRNA2) and then analysed the effects of shRNA2 on adipocyte differentiation. We describe our findings in the revised manuscript and in Supplementary Figure S5. Furthermore, the revised manuscript now includes data regarding the phosphorylation of FCoR by PKA and the direct acetylation of Foxo1 by FCoR (which was determined using an *in vitro* acetylation assay). We have revised several figures and made changes in the manuscript in response to the suggestions of Referee #2 as noted below.

Major points:
Q1. In this reviewer's opinion, the central point of this manuscript should be the identification of a new gene and to describe its initial characterization. Currently the best defined role for Foxo1 is as a regulator of hepatic glucose production. The most straightforward in vivo evidence presented is that knockdown of P13 can exacerbate HGP, presumably through Foxo1 regulation (Fig 7). Yet in the authors wade into the ambiguous territory of Foxo1-mediated control of adipocyte differentiation, a puzzle which does not have a clear solution. For example, no clear explanation is presented for why either aP2-WP13 or aP2-WP13 LepRdb/db transgenic mice should be lean. While the implications for P13 in the liver are easily accessible.

A1. When we began this study, we investigated the roles of FCoR in adipose tissue because FCoR is expressed mainly in adipose tissues, including WAT and BAT. We agree with Referee #2 but FCoR

expression in the liver is faint in wild type mice under the normal chow diet. Therefore, we focused on adipose tissues and generated adipose tissue-specific transgenic mice.
In response to the second part of the comment, we now provide an explanation for the lean phenotype of aP2-WFCoR or aP2- WFCoR LepRdb/db transgenic mice (page 14, lines 4-7 in the revised manuscript).

Q2. Numerous studies (including the work under review and earlier work from these authors) have implicated Foxo1 as a potent repressor of PPARin adipose tissue. Consistent with this model, knockdown of P13 (a state of increased Foxo1 activity) completely blocks PPAR activity and adipogenesis (Figure 4). The converse experiment would be informative: does overexpression of P13 in cultured adipocytes lead to enhanced adipogenesis? Analysis of adipocyte size in aP2-WP13 mice of different body weights is not a valid comparison; younger mice should be examined.

A2. As suggested by Referee #2, we performed the J3-tk-luc reporter assay using PPARg, RXRa, CNFoxo1 and FCoR in the presence of rosiglitazone. These studies demonstrated that FCoR could not prevent inhibition of PPARg byFoxo1. We think that the effects of knockdown of FCoR in terms of inhibition of adipocyte differentiation are not mediated by the PPARg-Foxo1 interaction. We described and discuss this data in the revised manuscript (page 13, lines 3-9 and page 21, lines 3-8 and Supplementary Figure S6B).

In response to the first suggestion, we infected 3T3-F442A cells with adenoviruses encoding cMyc-FCoR and then performed Oil-red O staining and examined *Pparg* expression. These experiments demonstrated that although Oil-red O staining of FCoR-infected 3T3-F442A cells was similar to staining of LacZ-infected cells, the *Pparg* expression level was significantly increased at day 10. We report these data in the revised manuscript and in a Supplementary Figure (page 12, lines 20-page 13, line 2, Figure 5A, and Supplementary Figure S6A in the revised manuscript).

In response to the second suggestion, we measured the adipocyte size in younger mice and found that the adipocyte size was similar to that in older mice. This is noted in the revised manuscript (page 13, lines 19-page 14, line 3 and Supplementary Figure S7A and S7B).

Q3. Similarly, the interpretation of the transgenic phenotype is troublesome. Overexpression of a foxo1 inhibitor would be expected to increase PPAR activity leading to an increase in adipocyte differentiation and possible body weight gain. Instead the authors show transgenic mice with less adipose tissue mass. This decreased body weight cannot simply be explained due to decreased adipose tissue differentiation. Body weight is the balance between food intake and caloric expenditure. This point must be addressed. In addition, effects on insulin sensitivity appear to be secondary to body weight. Why does the body weight of the transgenics suddenly diverge?

A3. We agree that it is important to address these questions. Accordingly, we investigated the effect of FCoR on PPARg activity as described in A2 (above). Using the J3-tk-luc reporter assay, we demonstrated that FCoR didn't enhance PPARg activity and that overexpression of FCoR increased *Pparg* expression only on day 10. We concluded that FCoR doesn't affect PPARg activity (page 12, lines 3-9 and Suplementary Figure S6B in the revised manuscript).

Also in response to this comment, we evaluated food intake, energy expenditure, and locomotor activity in transgenic and control mice. We demonstrated that food intake, oxygen consumption and locomotor activity were similar in transgenic and control mice, but the respiratory quotient was significantly decreased in transgenic mice. This is reported in the revised manuscript (page 14, lines 4-7 and Figure 6H).

We agree with Referee #2 that the sudden decline in body weight in transgenic mice merits further investigation. We think that one of the reasons for the decreased weight is the decreased respiratory quotient of transgenic, which leads to increased usage of fat as an energy source. We now discuss this in the revised manuscript (page 14, lines 4-7). However, this is just speculation based on preliminary unpublished observations. For example, the present data show that FCoR has intrinsic acetyltransferase activity. Therefore, FCoR may acetylate not only Foxo1 but also other transcription factors or cofactors *in vivo* and affect their physiological function, leading to a sudden decline in body weight. This is also discussed in the revised manuscript (page 19, line 20-page 20, line 8).

Minor points:

Q1. The preponderance of data might be clarified by presenting a model.

A1. We agree that a model would be a good addition to our report. Accordingly, we now present a model for the roles that FCoR plays in fine-tuning Foxo1 activity (see Figure 9 in the revised manuscript). In this model, we describe FCoR as a protein that fine-tunes Foxo1-mediated transcription repression. We also describe the actions of FCoR in the revised manuscript (page 19, line 20-page 20, line 8).

Q2. P13 is not a very memorable name. Perhaps a new name should be proposed. for p13: Foxo1 Corepressor (FCoR)

A2. We thank the referee for this excellent suggestion and have changed the name P13 to Foxo1 CoRepressor (FCoR) in the revised manuscript. We agree that this name is more descriptive and thus more appropriate.

Q3. Figure 5c requires error bars.

A3. We added error bars to Figure 6C in the revised manuscript (this was Figure 5C in the original manuscript).

Q4. Primer sequences should be provided as well as the sequence of the shRNA construct.

A4. We now provide all primer sequences used in this study in Supplementary Table 1 in the revised manuscript. For Fcor, we list the primer sequences in the Supplementary Information (page 2, lines 15-17 in the revised Supplementary Information). We also note the sequences of shRNA1 and shRNA2 in the Supplemental Experimental Procedures in the Supplementary Information (page 5, lines 8-16).

Response to Referee #3

We would like to thank Referee #3 for providing many insightful and constructive comments. These comments helped us strengthen and clarify our report. Before responding to comments of Referee #3, we would like to note that in response to the suggestions of another reviewer (Referee #2), we now refer to "P13" as "Foxo1 CoRepressor (FCoR)." Accordingly, we replaced "P13" with "FCoR" in the revised manuscript, including in the title, abstract, and figures. Furthermore, we added several new authors, including Drs. Yoshinaga Kawano and Risa Sekioka, who have done some experiments, and Dr. Hiroshi Kiyonari, who generated *FcorKO* mice, and Drs. Toshiya Tanaka and Juro Sakai, who constructed PPARg and RXRa expression vectors. And also we changed order of author list (Shin-Ichiro Takahashi and Hiroshi Itoh) because Dr. Hiroshi Itoh supervised and supported all studies of *FcorKO* mice. In response to a suggestion by Referee #3, we removed Figure 7 and Figure 8 as well as the description about P13 and Crtcs from the original manuscript. We focused on the interaction between FCoR (P13) and Foxo1 in the revised manuscript. As suggested by Referee #1, we generated and then analysed FCoR knockout mice and now describe our findings in the last paragraph of the Results section and in Figure 8. Also in response to a suggestion by Referee #1, we generated a second adenovirus that encodes another shRNA (shRNA2) and analysed the effects of shRNA2 on adipocyte differentiation. We describe our findings in the revised manuscript and in Supplementary Figure S5. Furthermore, the revised manuscript now includes data regarding the phosphorylation of FCoR by PKA and the direct acetylation of Foxo1 by FCoR (which was determined using an *in vitro* acetylation assay). We have revised several figures and made changes in the manuscript according to the suggestions of Referee #3, as specified below.

Major points:
Q1. Re-iterating the point above, it is this reviewer's major suggestion that the authors leave the liver and CRTC2 data in Figure 7 and 8 out of this manuscript and save for a different manuscript.

Though they add in terms of interesting functions for P13, to this reviewer their inclusion confuses and diminishes the initial impact of the abundant data connecting P13 to FOXO function in adipocytes. If the authors, editors, or other reviewers believe this data is best kept in this same manuscript, further analysis is needed to distinguish effects P13 may be having on CRTC/CREB from effects on FOXO.

A1. In response to the suggestion by Referee #3 (and as noted above), we removed Figure 7 and Figure 8, the description and data regarding P13 in the liver, and the Crtcs data from the manuscript.

Q2. The identification of P13 as a novel FOXO interactor that modulates FOXO activity in metabolic tissues is very interesting. The authors work in figure 1 verifying the initial 2-hybrid isolation and narrowing the region of interaction are important. However, given that the authors note the presence of a predicted "Forkhead associated ligand domain from amino acid 78 to 84, they should directly address whether this region is needed for Foxo interaction. It would be particularly striking if the authors could abolish FOXO binding with a mutation in this region. Examining whether such a FOXO-defective binding mutant was still capable of regulating CRTC2 would be an important future goal.

A2. We agreed with the referee that this line of investigation would be interesting. Accordingly, we performed site-directed mutagenesis and constructed several mutant FCoR variants (I78A, T80A, L81A, L85A, and L87A) (page 3, line 19-page 4, line 18 in the revised Supplementary Experimental Procedures in the Supplementary Information) and performed the 5XGAL4 luciferase assay. These experiments demonstrated that all replacements with alanine except for L81A abolished inhibition of Foxo1 activity. We concluded that this region is important for inhibition of Foxo1 activity. Furthermore, this region shows sequence similarity to several acetyltransferases. As shown in Figure 3D and 3E in the revised manuscript, this region overlaps with homologous regions of other acetyltransferases and includes a nuclear export signal. These finding are described in the revised manuscript (page 9, lines 13-21, page 10, line 22-page 11, line 17, Figure 3D-3F, and Supplementary Figure S4A-S4C).

Although we did not add data about the Crtc2 and FCoR mutants described above to the revised manuscript, these mutants suppressed Crtc2-induced activity by 50% (wtFCoR suppressed Crtc2-induced activity by 75%).

Q3. Authors need to validate that their P13 antibody is detecting endogenous P13 and the correct proteins by immunoprecipitation in Figure 1C, immunoblot in Figure 1H, and chromatin IP in Figure 4D by using P13 RNAi to verify the signal goes away. This is particularly important not only as a critical control, but also to verify that P13 protein levels go up during differentiation as the effects on P13 mRNA in Figure 1G are much more compelling that the immunoblot data in Figure 1H. Perhaps a darker exposure of the immunoblot in Figure 1H for P13 would improve this, especially when one considers how small such a panel would ultimately be in a journal.

A3. In response to this suggestion about using FCoR shRNA, we performed Western blotting of FCoR (Supplementary Figure S4B in the original manuscript). This figure is now Figure 5C in the revised manuscript. We also constructed another shRNA (shRNA2) and performed the same experiment. We added data to Supplementary Figure S5C in the revised manuscript.

As suggested, Figure 1H in the original manuscript has been revised.

Q4. Authors need to examine the expression levels of P13 in BAT, WAT, and liver in B6 mice as well as the db/db mice used throughout this study. The regulation of many genes by fasting and refeeding is aberrant in db/db mice. Indeed the authors show here in Fig IJ that P13 levels are lower in db/db mice than "wild-type" mice, but they never look at the fed, fasting, and refed conditions of Fig. 1I in "wild-type" mice.

A4. We agree with Referee #3 that this was confusing, mainly due to our unclear description in the original manuscript (page 6, lines 11-15). We apologize to the referees for this mistake. Notably, we investigated *Fcor* expression as shown in Figure 1I in the original manuscript using C57BL/6J mice, not db/db mice. We have changed the description in the revised manuscript (page 6, line 17-page 7, line 1) and in the legend to Figure 1I.

Q5. The authors results that P13 overexpression regulates FOXO1 acetylation in HEK293 cells in Figure 3 are interesting, but should be examined in some adipocyte derived cell type (.e. 3T3-F442A), since the authors have adenoviruses for P13 shRNA and overexpression. If endogenous FOXO acteylation cannot be detected, the authors could co-infect with a FOXO1 expression construct. Also FOXO phosphorylation on Akt sites has been shown to be increased when FOXO is hyperacetylated, so the authors could use existing commercial Phospho-Foxo antibodies that do readily detect endogenous FOXO to look at effect of P13 in adipocytes.

A5. In response to this suggestion, we infected 3T3-F442A cells with adenoviruses encoding LacZ and FCoR on day 0 and harvested the cells on days 1, 2, 4, and 6. We performed immunoprecipitation on the cell lysates using an anti-FOXO1 antibody and performed Western blotting with an anti-acetyl lysine antibody. The results demonstrated that on day 4, Foxo1 was hyperacetylated in FCoR-infected 3T3-F442A cells (described on page 12, line 20-page 13, line 2 and shown in Figure 5G in the revised manuscript).

Q6. Is the co-immunoprecipitation of Foxo family members with P13 regulated by insulin or forskolin? This is critical where the data with CRTC stays in this manuscript or not.

A6. We agree that this is an interesting and important question and thank Referee #3 for bringing this up. To address it, we performed a series of co-immunoprecipitation studies. In the absence of serum and in the presence of forskolin, FCoR binds to Foxo1 in the nucleus. In the presence of serum and in the absence of forskolin, FCoR binds to Foxo1 (probably in the cytosol). However, in the presence of both serum and forskolin, FCoR does not bind to Foxo1 because, at that time, FCoR is in the nucleus and Fxo1 is in the cytosol. From these data, we concluded that the interaction between these two molecules depends on their subcellular localization. We describe these results in the revised manuscript (page 7, lines 21-page 8, line 3, page 11, lines 18-page 12, line 2, and Figures 2E and 4l in the revised manuscript). We performed these experiments using only Foxo1 because, as shown in Figure 2E in the revised manuscript, FOXO4 does not bind to FCoR and Foxo3a binds to FCoR more weakly than Foxo1.

Q7. Do the authors also observe upregulation of P13 mRNA during week 9 in db/db mice? What is the authors' explanation for the temporal expression of P13 only at this timepoint? It is unclear how much P13 induction might contribute to regulation of FOXO as compared to the insulin resistance and impact on insulin and glucagon dependent signaling perhaps already awry at this timepoint. Authors should also determine if P13 is elevated in the liver of B6 mice on a high fat diet or other insulin resistant or metabolic syndrome rodent models.

A7. Although we removed the liver data from the revised manuscript, we observed increased *Fcor* mRNA expression during week 9 in db/db mice. In addition, *Fcor* mRNA expression was decreased after that time. At this time point (9 weeks), db/db mice are not diabetic, although they have insulin resistance. We think that FCoR in the liver at 9 weeks inhibits gluconeogenesis and prevents the onset of diabetes.

In response to the referee's suggestion, we investigated Fcor expression in the livers of HFD B6 mice and found that Fcor expression was increased 2-fold at 8 weeks of a HFD. However, these data are not included in the revised manuscript.

Q8. The co-immunoprecipitation data with CRTC2 in Figure 8F is not very convincing. Can the authors at least reproduce this better with co-expression of tagged CRTC2 cDNA and immunoprecipitation +/- forskolin?

A8. We agree that Figure 8F could be more convincing. If we submit another manuscript that addresses Crtc and FCoR, we will try to perform this experiment as suggested by Referee #3.

Q9. Given that the authors suggest forskolin regulates the interaction of P13 with CRTC2, it begs the question of whether P13 can bind to CRTC and FOXO at the same time and whether the association of all three is regulated by forskolin. Can the author define a region of interaction in P13 for CRTC2 and does that overlap with the FOXO interaction region in P13 (see point #2 above)?

A9. We think this is a good suggestion and one that we will keep in mind in future studies.

Q10. The observations with CRTC2 in figure 8 are interesting but raise a number of additional questions that would need to be addressed if this data is to be left in the paper. How are CRTCs regulated in adipose and could P13 regulation of some CRTC family members be responsible for any of the effects in the P13 adipose transgenic mice? One of the CRTC family members, CRTC3, was recently connected to adipose function.

A10. Again, we agree that this is an interesting question and one that we would like to address in a future study.

Minor points:
Q1. Authors need to show expression of all three FOXO family members in WAT and BAT, at least put in supplement.

A1. In response to this comment, we added data of western blotting of Foxo family members in WAT and BAT. At this time, we used two kinds of antibody of FOXO1, including L27 from Cell Signaling and ab12161 from Abcam. L27 detected both Foxo1 and Foxo4 from our experiment (Supplementary figure 2B in the revised manuscript). In contrast, epitope (LPNQSFPHSVKTTTHS) of ab12161 is conserved not only in Foxo1 but also in Foxo3a weakly. However, this epitope is never conserved in Foxo4. Therefore, we think that this antibody (ab12161) can detect both Foxo1 and Foxo3a but not Foxo4. From these data, we demonstrated that both Foxo1 and Foxo3a are expressed in WAT but not Foxo4 and that all Foxos are expressed in BAT. We described these data in the revised manuscript and added a new supplementary figure (page 5, line 21-page 6, line 1 and Supplementary figure S2 in the revised manuscript).

Q2. Do authors see any changes in localization of P13 during treatments with cAMP? Of fasting/refeeding?

A2. In response to this comment, we investigated the effects of forskolin or 8-Br-AMP on the subcellular localization of FCoR. These data are described in the revised manuscript (page 10, line 1-page 12, line 2 and Figure 4).

Q3. Does inhibition of Foxo1 transcriptional activity affect UCP1 and Adrb3 mRNA levels, as well as PGC1a levels (as seen in Nakae, 2008).

A3. As Referee #3 points out, *aP2-FLAG-D*256 mice, in which transactivation-defective Foxo1 (D256) was expressed in both WAT and BAT, showed increased oxygen consumption accompanied by increased expression of PGC1a, uncoupling protein (UCP)-1, UCP-2, and b3-adrenergic receptor (b3-AR). We describe this inconsistency in the Discussion section of the revised manuscript (page 21, line 9-page 22, line 2).

Q4. In Figure 5, authors should use another control for total RNA levels, since actin mimics differences in levels of P13 in the two different lines.

A4. In response to this suggestion, we changed the control RNA panel from b-actin to 28S rRNA in Figure 6B in the revised manuscript.

2nd Editorial Decision 27 January 2012

Thank you very much for submission of your revised paper that has now been seen by one of the original referees. I would be grateful for addressing these comments and some further experimentation as you find feasible before submitting a final version of your study. I am sure that these could be performed within a rather moderate timeframe and thus look forward to your final amendments.

Please also notice that The EMBO Journal encourages the publication of source data along with accepted articles, particularly for electrophoretic gels and blots, with the aim of making primary data

more accessible and transparent to the reader. This is a voluntary policy to present un-cropped/unprocessed scans at least for the key data of published work. If you agree to this initiative, we would be grateful for files that each combine the un-cropped blots presented in the individual figure(s). These will be linked online as a supplementary "Source Data" file for the figures you provide the information.
Please let me know if you have any questions about this AND please check the below URL for a recent example:
http://www.nature.com/emboj/journal/v30/n20/suppinfo/emboj2011298as1.html

A I am very much looking forward to your final responses and your opinion on the source data to enable efficient proceedings.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #2:

The manuscript by Nakae and colleagues is very much improved with the removal of the original weaker data that P13 (now better named FCor) regulates CRTC2 in addition to FOXO. Many aspects of the original manuscript are enhanced, not the least of which is the generation and inclusion here of whole-body KO for FCor. The authors new model is that following forksolin, PKA induces phosphorylation and nuclear translocation of FCor, which acts as an acetylase for FOXO, resulting in FOXO inhibition, as its acetylation has been shown to do in many cellular contexts. While greatly improved, there are a limited number of minor issues the authors need to consider prior to the manuscript being suitable for publication.

Minor comments:
1. How does the PKA phosphorylation and presumed activation of FCor fit with the observed decrease in FCor mRNA and protein by fasting and increase in FCor mRNA and protein by feeding? Why would there be a dramatic increase in FCor levels in the fed state if it is not active in that state? More needs to be done with the author's Phospho-FCor antibody to ensure that the regulation of this site in response to fasting and feeding is as expected. It may be that the active form is unstable or turned over rapidly but the authors need to address that in the discussion then.

2. The authors should comment on how the regulation of FOXO in response to forskolin they are studying here is completely opposite to the recently reported nuclear translocation of a deacetylase for FOXO in HDAC4 and HDAC5 following forskolin or glucagon in hepatocytes (Mihaylova et al Cell 2011, Wang et al. Cell 2011). Does this suggest that FOXO is acutely acetylated by FCor following forskolin in adipocytes yet in liver after forkolin, FOXO is deacetylated? It is well-established that in hepatocytes, FOXO is actively promoting transcription of its target genes after forkolin, glucagon, or cAMP treatment, so if FCor is expressed in liver the predicted acetylation and inhibition of FOXO run counter to the known activation of FOXO by forkolin/ cAMP agonists.

3. Considering the interaction of Foxo and FCoR will be serum dependent and later show to be so (Fig2B, 4L), the authors need to specify if the immunoprecipitations are done under serum free conditions or normal media in Figure 1.

4. The authors perhaps need to do qPCR analysis for the Foxo family members in WAT and BAT, rather than using antibodies (in Figure S2), especially given that not a single ab is able to detect all family members.

5. In figure 1C, it is important to show the total levels of Foxo and FCoR in the endogenous IPs as a control.

6. It seems that in Fig 1K there is more FCoR in the WAT and not the BAT of the "control mice" which is the opposite of what is reported in figure 1E and 1I. Can the authors comment on this discrepancy?

7. It would have been nice to see the forskolin induced nuclear accumulation in a more physiologically relevant cell type such as the 3T3-F442 cells, rather than in 293 cells.

8. In figure S3, where the cells are treated with cAMP it seems that myc FCoR is still cytoplasmic here. Based on their model of PKA mediated phosphoryation and nuclear localization, the majority of FCoR should be nuclear.

9. Pg 8 - Recently the Class IIa HDACs and Class I HDAC3 have also been shown to regulate Foxo1 deacetylation. The authers here pre-treat cells with TSA, which will inhibit Class I and Class II HDACs as well.

10. Authors should have included known HATs for Foxo1, such as p300 as a control and comparison in the in vitro acetylation assay in Fig 3E. These are commercially available and a necessary control for comparison to determine the relative activity of FCor as an acetyltransferase.

11. In Figure 3E authors say that the truncated GST Foxo1 is aa251-409 and later go on to claim that this truncated version includes K219, K242 and K245 which would be absent according in the 251-409 Foxo1 version they use.

12. How does FCoR mediated suppression of PGC1a relate to the proposed physiological function of FCoR in adipose tissue? Why did the authors not look at PGC1a levels and regulation on the WAT FCoR over-expressing mice? Why are PGC1a mRNA levels not included in figure 8F?

13. Better explanation of characterization of transgenic mice in Fig 6 is needed. How does the adipocyte size relate to the function of FCoR in adipocyte differentiation?

14. The authors must give a better introduction of the known physiological role of Foxo proteins in adipose tissues as that is not clearly addressed in the introduction.

2nd Revision - Authors' Response 07 March 2012

Response to Referee #2

We would like to thank Referee #2 for the insightful and constructive comments. These comments helped us strengthen and clarify our report.

We changed introduction and discussion in this revised manuscript in response to Referee #2's comments. We added the description about the physiological roles of Foxo1 in adipose tissues and adipocytes in Introduction, the deacetylation and activation of Foxo1 by class IIa and I HDACs, and explanation of characterization of transgenic mice in Discussion of the revised manuscript.

We also have done several experiments, including real-time PCR of Foxo familymembers in WAT and BAT (Supplementary figure S2), immunofluorescence of endogenous FCoR in 3T3-F442A cells during differentiation (Figure 4C), real-time PCR of Ppargc1a in WAT of WFCoR transgenic mice and Fcor KO mice (Figure 6K and 8f), and in vitro acetylation assay using p300 as a positive control (Figure 3F and 3G).

Finally, we revised several figures and made some changes in the manuscript in response to the suggestions from Referee #2, as noted below.

Q1. How does the PKA phosphorylation and presumed activation of FCor fit with the observed decrease in FCor mRNA and protein by fasting and increase in FCor mRNA and protein by feeding? Why would there be a dramatic increase in FCor levels in the fed state if it is not active in that state? More needs to be done with the author's Phospho-FCor antibody to ensure that the

regulation of this site in response to fasting and feeding is as expected. It may be that the active form is unstable or turned over rapidly but the authors need to address that in the discussion then.

A1. We agree with this comment. In response to this comment, we performed western blotting of phosphorylated endogenous FCoR in WAT and BAT at fasting and fed states. However, we could not detect bands of phosphorylated endogenous FCoR in these tissues at both states. We also perform immunoprecipitation using anti-FCoR antibody and blotted with anti-phospho-FCoR antibody. However, we could not detect any band of phosphorylated FCoR. Therefore, we just described the speculation that, at fasting state, FCoR is phosphorylated in WAT and BAT but is maybe unstable and turn over rapidly, leading to low expression levels at fasting state in Discussion of the revised manuscript (Page 20, lines 15-18 in the revised manuscript) .

Q2. The authors should comment on how the regulation of FOXO in response to forskolin they are studying here is completely opposite to the recently reported nuclear translocation of a deacetylase for FOXO in HDAC4 and HDAC5 following forskolin or glucagon in hepatocytes (Mihaylova et al Cell 2011, Wang et al. Cell 2011). Does this suggest that FOXO is acutely acetylated by FCor following forskolin in adipocytes yet in liver after forkolin, FOXO is deacetylated? It is well-established that in hepatocytes, FOXO is actively promoting transcription of its target genes after forkolin, glucagon, or cAMP treatment, so if FCor is expressed in liver the predicted acetylation and inhibition of FOXO run counter to the known activation of FOXO by forkolin/ cAMP agonists.

A2. In response to this comment, we described about Foxo and HDACs and cited new references (Mihaylova et al Cell 2011, Wang et al. Cell 2011) in Discussion of the revised manuscript. Furthermore, we described the possibility that FCoR may run counter to deacetylation and activation of Foxo in liver if FCoR is expressed in liver (Page 21, lines 6-17 in the revised manuscript). Furthermore, although we didn't describe at this time, overexpressed FCoR by adenoviral infection encoding FCoR decreased fasting blood glucose and expression levels of Ppargc1a, G6pc, and Pck1 (Figure 7 in the 1st manuscript). In the following future experiments, we will try to see acetylation-status of Foxo1 in liver after deletion or overexpression of FCoR.

Q3. Considering the interaction of Foxo and FCoR will be serum dependent and later show to be so (Fig2B, 4L), the authors need to specify if the immunoprecipitations are done under serum free conditions or normal media in Figure 1.

A3. We agree with this comment. We performed coimmunoptrcipitation studies in Figure 1B in the presence of serum. We described this condition in the revised manuscript and figure legend (Page 6, line 10 and Page 44, line 14 in the revised manuscript).

Q4. The authors perhaps need to do qPCR analysis for the Foxo family members in WAT and BAT, rather than using antibodies (in Figure S2), especially given that not a single ab is able to detect all family members.

A4. We agree with this comment. We performed real-time PCR of Foxo1, 3a, and 4 using RNA from WAT and BAT. We made new supplementary figure 2 and replaced it with the previous supplementary figure 2. We also added primer sequences of Foxo1, Foxo3a, and Foxo4 in Supplementary Table 1.

Q5. In figure 1C, it is important to show the total levels of Foxo and FCoR in the endogenous IPs as a control.

A5. In response to this suggestion, we added western blot of endogenous protein using anti-FCoR and anti-FOXO1 antibodies and made a new Figure 1C in the revised manuscript.

Q6. It seems that in Fig 1K there is more FCoR in the WAT and not the BAT of the "control mice" which is the opposite of what is reported in figure 1E and 1I. Can the authors comment on this discrepancy?

A6. We agree with this comment. In the original manuscript, it is difficult to see the differences of RNA amount of lane 1 and 3 (b-actin) due to darkness. We reduced darkness of the original picture and replaced it with the original panel of b-actin. We think that the amount of total RNA of lane 1

was much more than of lane 3. Therefore, it looked like more expression level of Fcor in lane 1. We measured density of Fcor and b-actin of lanes 1 and 3 and calculated relative expression level of Fcor using this Figure. We calculated that the expression level of Fcor in lane 1 was decreased by 40% compared with lane 3 (Figure 1K in the revised manuscript).

Q7. It would have been nice to see the forskolin induced nuclear accumulation in a more physiologically relevant cell type such as the 3T3-F442 cells, rather than in 293 cells.

A7. We agree with this opinion. In order to clarify physiological significance of FCoR, we investigated subcellular localization of endogenous FCoR in 3T3-F442A cells during differentiation. At this time, we used IBMX for 48 hours after induction of differentiation. These studies demonstrated that endogenous FCoR was in nucleus at day 2 and, thereafter, in cytosol at day 6. We think that these data supported the observation that PKA induced nuclear localization of endogenous FCoR. We described them in the revised manuscript and added a new Figure 4C (Page 11, lines 5-11, page 51, lines 8-11, and Figure 4C in the revised manuscript).

Q8. In figure S3, where the cells are treated with cAMP it seems that myc FCoR is still cytoplasmic here. Based on their model of PKA mediated phosphoryation and nuclear localization, the majority of FCoR should be nuclear.

A8. We agree with this comment. We darkened the original Figure S3 by Photoshop. We think that most of Myc-FCoR are in cytosol. We made a new Figure S3 in the revised manuscript.

Q9. Pg 8 - Recently the Class IIa HDACs and Class I HDAC3 have also been shown to regulate Foxo1 deacetylation. The authers here pre-treat cells with TSA, which will inhibit Class I and Class II HDACs as well.

A9. In response to this comment, we described the interaction between Foxo and class IIa and I HDACs in Discussion in the revised manuscript (Page 21, lines 6-17 in the revised manuscript)

Q10. Authors should have included known HATs for Foxo1, such as p300 as a control and comparison in the in vitro acetylation assay in Fig 3E. These are commercially available and a necessary control for comparison to determine the relative activity of FCor as an acetyltransferase.

A10. We agree with this comment. In response to this comment, we performed in vitro acetylation assay using GST-FCoR (4mg) and recombinant p300 (0.5 mg from ACTIVE MOTIF) and demonstrated that relative activity of FCoR was approximately 10% of p300. We described them and made new figures 3F and 3G in the revised manuscript (Page 10, lines 10-11, page 31, line 10, page 50, lines 3-12, and Figures 3F and 3G in the revised manuscript).

Q11. In Figure 3E authors say that the truncated GST Foxo1 is aa251-409 and later go on to claim that this truncated version includes K219, K242 and K245 which would be absent according in the 251-409 Foxo1 version they use.

A11. We agree with this comment. We made a mistake for description of this point in the original manuscript. Therefore, we deleted K219, K242, and K245 in the revised manuscript (Page 10, line 8 in the revised manuscript).

Q12. How does FCoR mediated suppression of PGC1a relate to the proposed physiological function of FCoR in adipose tissue? Why did the authors not look at PGC1a levels and regulation on the WAT FCoR over-expressing mice? Why are PGC1a mRNA levels not included in figure 8F?

A12. In response to this comment, we performed real-time PCR of Ppargc1a using total RNA of WAT from WFCoR and FcorKO mice. Expression level of Ppargc1a in WAT of WFCoR or FcorKO mice was similar to control mice. We added these data in new Figure 6K and 8F in the revised manuscript.

Q13. Better explanation of characterization of transgenic mice in Fig 6 is needed. How does the adipocyte size relate to the function of FCoR in adipocyte differentiation?

A13. In response to this comment, we changed a part of Discussion in the revised manuscript (Page 22, line 19-Page 23, line 11). Foxo1 is involved in the early stage of adipose conversion and may inhibit it. Therefore, inhibition of Foxo1 leads to increased conversion of adipocytes and to smaller adipocytes. The mechanism how inhibition of Foxo1 leads to smaller adipocytes is not still clarified. However, some of them are related to regulation of PPARg activity or of *Pparg* gene expression. We think that FCoR regulates adipose conversion through inhibition of Foxo1 activity and increased expression of *Pparg*. Accordingly, we changed Discussion in the revised manuscript.

Q14. The authors must give a better introduction of the known physiological role of Foxo proteins in adipose tissues as that is not clearly addressed in the introduction.

A14. In response to this comment, we described the physiological roles of Foxo1 in adipose tissues in Introduction of the revised manuscript (Page 3, line 13-page 4, line 9 in the revised manuscript).

Editorial Correspondence 15 March 2012

Thank you very much for your further revisions that I assessed in detail and are thus happy to convey that we would in principle be happy to formerly accept the paper.

Checking through the figures though, I noticed that the blot for myc-tagged FCoR appears rather blurred, and I was thus wondering whether a more informative example for this experiment could be provided.

Furthermore, please notice that The EMBO Journal encourages the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would thus be grateful for one PDF-file per figure you decide to provide this information for. These will be linked online as supplementary "Source Data" files.

Please let me know if you have any questions about this AND check the URL below for a recent example: http://www.nature.com/emboj/journal/v30/n20/suppinfo/emboj2011298as1.html

I am very much looking forward to your timely response in this matter that will determine efficient further steps towards publication of your study.

Yours sincerely,

Editor
The EMBO Journal