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Egr-1 decreases adipocyte insulin sensitivity by tilting PI3K/Akt and MAPK signal balance in mice

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1st Editorial Decision 25 January 2011

Dear Dr. Li,

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office.

I have received a full set of comments (enclosed below) on your paper that outlines possible contributions of Egr-1 signaling to insulin sensitivity. As you will see, the referees appreciate this interesting and clinically relevant proposal. However, they also raise important points that would need to be experimentally addressed before we would be able to reach a final decision on suitability of your study for publication here.

Specifically, experiments demonstrating the proportion of adipocytes in injected epididymal fat pads that express the virus, and whether viral expression indeed originates from adipocytes or rather non-adipocytic cell types, such as macrophages. Further, more detailed in vivo studies on the metabolic phenotypes and in general better data quantification would be essential to corroborate the proposed Egr-1's function. As definitive molecular insight is a critical measure for consideration at The EMBO Journal, I urge you to take these remarks serious and invest the necessary time and experimental efforts to convince our referees from the significance of your findings. This should also avoid later disappointments. I like to add that we are able to grant additional time beyond our usual three-month deadline to facilitate requested experimentation upon authors request.

Finally, I do have to remind you that it is EMBO_J policy to allow a single round of major revisions and that the final decision on acceptance or rejection entirely depends on the content and strength of the final version of your manuscript. In case of further questions please do not hesitate to contact me, preferably via E-mail.

Looking forward to assess your revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

The manuscript by Yu et al elucidated the role of EGR1 in regulating insulin sensitivity through modulating PI3K/AKT and MAPK signaling pathways. The authors showed that EGR1 expression was elevated in diabetic rodents and patients. Overexpession of EGR1 or inhibiting EGR1 in adipocytes or adipose tissue in mice led to insulin resistance or enhanced insulin sensitivity, respectively. The findings are interesting and reveal a new role for EGR1 in regulating insulin sensitivity in adipose. The manuscript was well written. The experiments were carried out carefully and the data quality is adequate. Additional questions and comments for the authors are listed below.

1. The authors showed that EGR1 expression was elevated in adipose tissue from diabetic patients and db/db mice compared with their controls. It is important to find out whether EGR1 expression was elevated in insulin resistant, a prediabetic state, or only in diabetic state. This is can be done by using ob or diet-induced obese C57bl6 mice.
2. The information on patients' BMI has to be included in the supplemental Table 1. In addition, information on which adipose depot where biopsies were taken from patients needs to be include. It has been shown that the gene expression in each fat depot and their contribution to insulin resistant is quite different.

3. In fig3, 4 and 5 where the authors either overexpressed EGR1, dnEGR1 or siEGR1 in adipocytes, were these human adipocytes or 3T3-L1 adipocytes? It needs to be clarified in the text or fig legends. Does overexpression of EGR1 or blocking EGR1 activity with dnEGR1 or siEGR1 affect the differentiation of adipocytes? It it does, it will complicated the interpretation of the data since dedifferentiation of adipocytes will affect insulin sensitivity and glucose uptake. The authors should measure the expression of GLUT4, PPARg, C/EBPa and adipsin which are markers of adipocyte differentiation in these in vitro experiments.

Referee #2:

In this paper, the authors explore the concept that the hyperinsulinemia of chronic insulin resistant states can cause insulin resistance by signaling through Map kinase and JNK1 pathways leading to Egr-1 expression. This concept of mixed insulin resistance has received a great deal of attention over many years, and, in these studies, the authors add to the information base on this subject. They implicate insulin mediated Egr-1 expression as a culprit which can eventually cause insulin resistance. However, there are a number of questions that should be resolved in these experiments.

1. In all studies on this subject, there is the usual chicken and egg issue. If the hyperinsulinemia is necessary to cause insulin resistance in the author's model, then what causes the hyperinsulinemia in the first place? Usually, one thinks of insulin resistance as the cause of the hyperinsulinemia, not the other way around. Therefore, are the authors really proposing that there is some other cause of insulin resistance and that the resulting hyperinsulinemia then makes it worse through the Egr-1 mechanism?

2. In Figure 1 the loading levels are clearly unequal between normal and diabetics in Figure 1A. This clearly skews the results, and the data need to be accurately quantitated as a ratio of Egr1 or GGPPS to actin. The relationship between Figure 1 and Supplemental Figure 1 is not clear. Is the Supplemental figure the scanned results of the blots shown in Figure 1? How many replicates were

performed in Figure 1A, C, E, and G, and please be explicit as to the quantitation of these Western blots by scanning and statistics.

3. Supplemental Fig. 2. It appears that there are small differences in body weight in the db/db mouse and over 7 days this could be significant. There also appears to be changes in food intake in the wild types with adenovirus injection. Could these changes affect the in vivo metabolic data?

4. Adenoviral injection directly into tissues has been tried many times, and the key question is not whether or not the viral encoded genes are expressed in the injected tissue, but what percentage of the cells in the tissue express the gene. In other words, is Egr-1 expressed in 10% of the cells or 90% of the total adipocytes in the depot? Also, since the injections were into adipose tissue of obese mice, how much of the Egr-1 is being expressed in non-adipocyte cell types in the adipose tissue, such as immune cells?

5. Given the known effects of Egr-1, by what mechanism do the authors think IRS1 phosphorylation increases in Figure 2, and how are the effects transmitted to the liver?

6. In the signaling studies, the authors are hypothesizing that Egr-1 "tilts the balance" of PI3 kinase/Akt signaling. However, while Akt is measured, no measures of PI3 kinase are included. It is mandatory that direct measures of PI3 kinase activity be provided throughout these experiments.

7. In Figure 4, they provide nice evidence for the effect of Egr-1 mediated through PTEN and GGPPS. These studies again call for direct measurements of PI3 kinase. Given the targets of PTEN and GGPPS, how do the authors interpret the increased p-IRS-I data in Figure 2?

8. In Figures 5A&B, they conduct long term experiments with insulin treatment. However, it has been well described that chronic hyperinsulinemia in vitro leads to dramatic down regulation of the insulin receptor and this would be through a mechanism that has nothing to do with the Egr-1 story. In what way can they show that the effects they observed are due to the Egr-1 mechanism and not do to the well known insulin receptor down regulation? Furthermore, chronic hyperinsulinemia has been shown to increase IRS1 serine phosphorylation, which decreases downstream IRS1 signaling. Therefore, there are plenty of mechanisms already published, independent of Egr-1 which explains all of these results. In fact, wouldn't the dnEgr1 experiment indicate that the effects of insulin on receptor down regulation and IRS1 serine phosphorylation are blocked. This would be contrary to the authors' hypothesis, but measurements of insulin receptor surface content and IRS1 serine phosphorylation must be provided.

9. It is hard to see that the small differences in glucose levels in Figure 5E are meaningful. Given the large changes in 5D, it is hard to reconcile the small changes in 5E.

10. As a general comment, the measures of insulin sensitivity and glucose homeostasis are a bit superficial. This study would be greatly strengthened by more measurements of insulin secretion, circulating insulin levels, as well as glucose clamp studies in the various mouse models.

11. Figure 5A is interesting, but these are cytokines not adipokines and the authors do not know the cellular origin of the cytokines. This begs the question as to what is the effect on circulating concentrations of adiponectin and leptin. This should be provided.

12. In Figure 6D, the authors conclude that Egr-1 overexpression in fat blocked insulin induced Akt phosphorylation in the liver of BKs WT mice. However, there are two individual mice presented and pAkt looks very similar to GFP treatment in the first individual.

13. The co-culture experiments in Figure 6F don't make much sense. They are showing that the co-culture changes insulin stimulated glucose uptake in hepatocytes. However, insulin has no effect to stimulate glucose uptake in hepatocytes, unlike its well known effects in adipocytes and muscle cells. Therefore, how can they have insulin stimulated glucose uptake effects on this process with the various co-culture conditions measured, since it is not a part of insulin action in liver.

14. In general, the manipulations of PTEN and GGPPS are interesting, but throughout the paper the authors tend to conflate these two discrete effects, since they are both end points of Egr-1s

transcriptional effects. For example, the GGPPS adipocyte knockout mouse would be an interesting story all by itself, and the data presented in this manuscript on this interesting mouse model are very preliminary and superficial.

15. Egr-1 has been reported to have potent effects on adipocytes biology, independent of insulin signaling. In the discussion, the authors mention unpublished data on adipocyte size, etc. This should be presented in the manuscript, since changes in adipocyte size or adipogenesis could also explain the in vivo effects the authors observe.

16. What is the evidence that Map kinase signaling leads to IRS1 serine phosphorylation?

17. In the epididymal pad adenoviral injection studies, if the adenovirus was taken up into immune cells within the adipose tissue, would that give the expected effects that they observe?

18. Some references included in the text cannot be found in the bibliography (i.e. page 4, line 10, refers to Kaneto et al., 2004, Luan et al., 2009, manning & Davis 2003) - none of which are present in the bibliography.)

19. The fasting blood glucose values shown in figure 2E seem exceptionally high considering these mice were fasted for 16 hours.

20. Within the methods section please clarify the strains of mice/background used for the various components of the study and explain why they were selected.

21. This manuscript would also benefit from further proofreading. There are spelling mistakes, p4 line 15, i.e gen, should be gene. Supplemental Table 1, the body weight column appears to be out of alignment with the other columns. In methods section, companies should be quoted with state, i.e. Cell signaling Technology, MA)

Referee #3:

Review of EMBO article Yu et al
Yu et al. have elegantly presented a tremendous amount of work. They show that Egr-1 is up-regulated in diabetic humans and genetic or diet-induced obese mouse models, over-expression blunts insulin sensitivity in vivo and in vitro, dominant negative Egr-1 reverses insulin resistance in db/db mice and TNFa-induced insulin resistance. They show definitively that Egr-1 overexpression reciprocally regulates the PI3K/AKT (down) and ERK/MAPK (up) pathway through PTEN and GGPPS. Examination of cytokine expression in vivo and in vitro and co-culture studies nicely support mechanism of action. Overall, their work is very impressive in that they clearly investigate the role of Egr-1 in various models with complementary approaches. It is a novel and convincing manuscript. My concerns are overstatements and wrong word choice which can be corrected with very minor editing. Even the major comments are minor. I would change the title to be a bit more exciting and the current one does not represent the massive amount of work accomplished, especially since it says mice but they also have human data. "Adipose Egr-1 is upregulated in diabetic humans and animals and decreases insulin sensitivity by reciprocally regulating PI3K/AKT and MAPK pathways."

Major comments

1) Results Page 7 and used several times such as in figure legend for fig 1 on page 28: the authors say "constantly" elevated. However, they are only measuring 1 time point. Constantly implies elevations over several time points. Perhaps they mean consistently elevated between individuals? It may be easiest to just leave out a descriptor and say elevated.
2) Results Page 7 and throughout paper such as figure legend for fig 1 on page 28. Please write epi fat pad in text when referring to fat. Although it is in methods, I felt that it would be clearer if the authors stated in the results. Same for "adipocytes" later in manuscript. Please write 3T3L1 adipocytes so that the reader does not infer primary adipocytes.
3) Results Page 7- "Egr-1 exerts an effect" is not correct. You can't assume that it can exert an effect because you are not measuring that. Say "can be regulated" instead.

4) page 8 - sentence unclear. I do not understand what they mean at all. Ends with "epi fat pads that is visceral..."
5) page 8 - fig 2C conclusion overstated- it is not greatly impaired. Slightly is a more appropriate word.
6) page 12 - fig 5E conclusion. Say "slightly" or "minimally" more sensitive since there is only 1 time point different. Overall they are very very similar and you should be hesitant to say more sensitive. A clamp study would be able to definitively show increased insulin sensitivity in GGPPS-/-, but that is likely beyond the scope of this study.
7) page 15- I don't like the word "activating" as in "activating downstream genes involved in PI3K/AKT and MAPK signaling. Sustained "activating" Egr-1..." because activating implies activity such as a kinase activity or transcriptional activity. The authors should say "inducing the expression of downstream genes" or "sustained expression of Egr-1."
8) page 16- perhaps the authors can comments on the size of adipocytes where they mentioned data not shown. If the adipocytes are smaller, then they would be more insulin sensitive which would support their findings.
9) I hope that immunoblots shown are representative of larger human/animal studies. Please write in the figure legend that blots are representative.
10) figure legend 2C page 33- Egr-1 overexpression did not "greatly impair glucose tolerance". It slightly or minimally did. Please correct.
11) figure 1 image- page 34. In later figures authors denoted BKs or db/db under blots to help the reader. They should do the same for the first figure since there is so much data to get through.
12) sup fig S2 the authors show data for body weight and food intake when they injected Egr-1 and dnEgr-1. This should be mentioned in the text since it is relevant to systemic and hepatic insulin sensitivity. Also mention changes fat pad mass if obtained.

Minor comments:

It is impressive that they see effects in vivo after just 7 days of adenoviral injection into fat pads. I wonder if they would see stronger effects if they let the experiment go longer. (I am not asking for this since they have results, but perhaps results would be even stronger at 14 days for example).
Very well written but needs some clarity with English.
Some minor typos.
Page 6, spell out BKs
There could be exciting future studies in brown fat and/or macrophages, especially when using the FABP4 (aP2) driving CRE since aP2 is expressed in activated macrophages in adipose, atherosclerotic vessels, etc.

1st Revision - Authors' Response 03 June 2011

Referee #1:

1. The authors showed that EGR1 expression was elevated in adipose tissue from diabetic patients and db/db mice compared with their controls. It is important to find out whether EGR1 expression was elevated in insulin resistant, a prediabetic state, or only in diabetic state. This is can be done by using ob or diet-induced obese C57bl6 mice.

Yes, it is very suggestive. So we detected the Egr-1 protein level in the HFD-induced B6 mice for different times (Figure 1G). We can find that the protein level of Egr-1 and GGPPS increased after 3 weeks of HFD induction and increased more after 6 weeks of HFD induction.

2. The information on patients' BMI has to be included in the supplemental Table 1. In addition, information on which adipose depot where biopsies were taken from patients needs to be include. It has been shown that the gene expression in each fat depot and their contribution to insulin resistant is quite different.

We supplied BMI information in Supplemental Table1. The reviewer is right. Different fat contributes differently to insulin resistance. We have indicated in the text (Page 7 line 113-114) that

adipose depot from which we took was the abdominal fat of the patients, and the epididymal fat of the mice.

3. In fig3, 4 and 5 where the authors either overexpressed EGR1, dnEGR1 or siEGR1 in adipocytes, were these human adipocytes or 3T3-L1 adipocytes? It needs to be clarified in the text or fig legends.
Does overexpression of EGR1 or blocking EGR1 activity with dnEGR1 or siEGR1 affect the differentiation of adipocytes? It does, it will complicated the interpretation of the data since dedifferentiation of adipocytes will affect insulin sensitivity and glucose uptake. The authors should measure the expression of GLUT4, PPARg, C/EBPa and adipsin which are markers of adipocyte differentiation in these in vitro experiments.

It was 3T3-L1 adipocytes. We have added this clarification in the text and figure legends now. We have checked the expression of Glut4, PPAR gama, adiponectin and CEBPa and we found that there was no difference in control and experimental group. Thus overexpression of EGR1 or blocking EGR1 activity with dnEGR1 or siEGR1 had no effect on adipocytes differentiation. We added this result in the text (Page 10, line 185-187 and Supplemental Figure 3G.).

Referee #2:

1. In all studies on this subject, there is the usual chicken and egg issue. If the hyperinsulinemia is necessary to cause insulin resistance in the author's model, then what causes the hyperinsulinemia in the first place? Usually, one thinks of insulin resistance as the cause of the hyperinsulinemia, not the other way around. Therefore, are the authors really proposing that there is some other cause of insulin resistance and that the resulting hyperinsulinemia then makes it worse through the Egr-1 mechanism?

We agree that insulin resistance is usually the cause of the hyperinsulinism. But once circulation insulin level increases, hyperinsulinism will enhance insulin resistance and accelerate diabetes development. Here we try to figure out the molecular mechanism by which hyperinsulinism enhances insulin resistance. Our results suggest that hyperinsulinism can enhance insulin resistance through tilting the balance of two crucial signal pathways, in terms of PI3K/AKT and MAPK. Importantly, these two pathways are controlled by Egr-1. In our opinion, hyperinsulinism may not be the initial cause of insulin resistance, but make it worse under insulin resistant status.

2. In Figure 1 the loading levels are clearly unequal between normal and diabetics in Figure 1A. This clearly skews the results, and the data need to be accurately quantitated as a ratio of Egr1 or GGPPS to actin. The relationship between Figure 1 and Supplemental Figure 1 is not clear. Is the Supplemental figure the scanned results of the blots shown in Figure 1? How many replicates were performed in Figure 1A, C, E, and G, and please be explicit as to the quantitation of these Western blots by scanning and statistics.

Yes, indeed the loading levels in normal are higher than that in diabetics even though the densitometric analysis still shows a significant difference between them. So we have repeated this immunoblot and show it in Fig.1A after we carefully adjusted the loading levels. The densitometric analysis is shown in Supplemental Figure 1A.

Yes, Supplemental Figure 1 is the densitometric results of the blots shown in Figure 1. We have modified the description to make more clearly in the figure legend of Supplemental Figure 1. There are 10 samples of diabetic patients and 10 of normal people that we collected in Figure 1A and five replicates mice in each group in Figure 1C, E, and G, the light density of each blot was analyzed by the ImageQuant TL software from GE Healthcare Life Science and the statistics were supplied by the combination of all the scanning results. We have added this information in figure legend of Figure1 and Supplemental Figure 1.

3. Supplemental Fig. 2. It appears that there are small differences in body weight in the db/db mouse and over 7 days this could be significant. There also appears to be changes in food intake in the wild types with adenovirus injection. Could these changes affect the in vivo metabolic data?

The body weight data of mice we showed in Supplemental Fig. 2 was collected at 7 days after adenovirus injection but not at the first day of injection. We also monitored the injected mice for as long as 2 weeks, but there was no difference in body weight and food intake after injection, which we did not show in the manuscript.

4. Adenoviral injection directly into tissues has been tried many times, and the key question is not whether or not the viral encoded genes are expressed in the injected tissue, but what percentage of the cells in the tissue express the gene. In other words, is Egr-1 expressed in 10% of the cells or 90% of the total adipocytes in the depot? Also, since the injections were into adipose tissue of obese mice, how much of the Egr-1 is being expressed in non-adipocyte cell types in the adipose tissue, such as immune cells?

To make sure the virus can affect as many as adipocytes, we injected multiple sites in the fat tissue. Then we checked the expression efficiency with immunocytochemistry method against GFP since virus carried a separated GFP gene (Supplemental Figure 2C). The result shows that most of the fat cells are infected. But we can not find lymphocyte invasion in injected fat tissue. We also detect the expression of some mark genes of immune cells (Supplemental Figure 6B) and find that macrophage infiltration mark gene Lysozyme, macrophages polarization mark genes, IL-4 and IL-13 have no change after virus injection, which suggests that the immune cells may not be affected by the virus infection.

5. Given the known effects of Egr-1, by what mechanism do the authors think IRS1 phosphorylation increases in Figure 2, and how are the effects transmitted to the liver?

We have reported the mechanism by which IRS1 phosphorylation is affected by Egr-1 (Shen N, et, al., (2011) Journal of Biological Chemistry 286(16): 14508-14515). Egr-1 can promote ggpps transcription, which reactivate the Ras/MAPK/Erk1/2 pathway that can phosphorylate the IRS-1 on Serine 612 that is the inactivated site of IRS-1. The serine phosphorylation of IRS-1 impairs their interaction with the insulin receptor and consequently decreases their tyrosine phosphorylation by the insulin receptor (Paz K, Hemi R, LeRoith D, Karasik A, Elhanany E, Kanety H, Zick Y (1997); A molecular basis for insulin resistance. Journal of Biological Chemistry 272(47): 29911). Thus, disruption of Egr-1 function could inhibit the IRS-1 phosphorylation on Serine 612 and make tyrosine phosphorylation increase.

Additionally, as we mentioned in the Discussion that adipose tissue is the body's largest endocrine organ. Egr-1 can affect the synthesis and release of cytokines, such as TNF-a and IL-6, which can regulate liver insulin sensitivity.

6. In the signaling studies, the authors are hypothesizing that Egr-1 "tilts the balance" of PI3 kinase/Akt signaling. However, while Akt is measured, no measures of PI3 kinase are included. It is mandatory that direct measures of PI3 kinase activity be provided throughout these experiments.

We have measured the phosphorylation of PI3K under insulin treatment and embedded into Figure 3A, B, C, D.

7. In Figure 4, they provide nice evidence for the effect of Egr-1 mediated through PTEN and GGPPS. These studies again call for direct measurements of PI3 kinase. Given the targets of PTEN and GGPPS, how do the authors interpret the increased p-IRS-I data in Figure 2?

We have measured the phosphorylation of PI3K and embedded them in Figure 4D. The increase of p-IRS-1-Tyr in fat by over dnEgr-1 expression in Figure 2 is due to Egr-1 dependent GGPPS transcription (See our report in Shen N, et, al., (2011) Journal of Biological Chemistry 286(16): 14508-14515). Increased GGPPS in db/db mice augments Ras prenylation and then reactivates Ras/MAPK/Erk1/2 pathway that can phosphorylate the IRS-1 on Serine 612. The serine phosphorylation of IRS-1 impairs their interaction with the insulin receptor and consequently decreases their tyrosine phosphorylation by the insulin receptor (Paz K, Hemi R, LeRoith D, Karasik A, Elhanany E, Kanety H, Zick Y (1997); A molecular basis for insulin resistance. Journal of Biological Chemistry 272(47): 29911). Overexpression of dnEgr-1 in fat will decrease GGPPS

expression and then inhibit Serine 612 phosphorylation, thus in turn causes the increase of p-IRS-1-Tyr.

8. In Figures 5A&B, they conduct long term experiments with insulin treatment. However, it has been well described that chronic hyperinsulinemia in vitro leads to dramatic down regulation of the insulin receptor and this would be through a mechanism that has nothing to do with the Egr-1 story. In what way can they show that the effects they observed are due to the Egr-1 mechanism and not do to the well known insulin receptor down regulation? Furthermore, chronic hyperinsulinemia has been shown to increase IRS1 serine phosphorylation, which decreases downstream IRS1 signaling. Therefore, there are plenty of mechanisms already published, independent of Egr-1 which explains all of these results. In fact, wouldn't the dnEgr1 experiment indicate that the effects of insulin on receptor down regulation and IRS1 serine phosphorylation are blocked. This would be contrary to the authors' hypothesis, but measurements of insulin receptor surface content and IRS1 serine phosphorylation must be provided.

Yes, indeed, insulin receptor has been reported to decrease under hyperinsulinism and this has not been proved to do with the Egr-1. So we have detected the insulin receptor protein level and indeed the insulin receptor decreased along with the long term insulin treatment (Supplemental Figure 5B). However, in our study, the insulin sensitivity still could be improved when Egr-1 was disrupted though the decrease of insulin receptor was observed. Thus it looks like that the maintenance of insulin sensitivity may be a more complex process. IRS-1, the substrate of insulin receptor, has been reported to be inhibited by the serine phosphorylation which can also lead to insulin resistance under hyperinsulinism. As we previously reported (See our report in Shen N, et, al., (2011) Journal of Biological Chemistry 286(16): 14508-14515), Egr-1 dependant the serine 612 phosphorylation of IRS-1 was increased under long term insulin treatment. So both insulin receptor decrease and Egr-1dependant IRS-1 serine 612 phosphorylation contributes insulin resistance formation under hyperinsulinism stress. Disruption of Egr-1 could decrease serine 612 phosphorylation and increase the tyrosine phosphorylation of IRS-1 which enhances insulin signaling. Thus we think that the Egr-1-dependant IRS-1 activity also has effect on insulin signaling and the improved IRS-1 activity by disruption of Egr-1 may compensate the decrease of insulin receptor. We have discussed this in the text on Page 17 Line 330-335.

9. It is hard to see that the small differences in glucose levels in Figure 5E are meaningful. Given the large changes in 5D, it is hard to reconcile the small changes in 5E.
Actually, we collected the ITT and GTT data from both gender when we just have got the GGPPS deletion mice. When we have got enough mice, we analyzed ITT and GTT according their gender and turn out to be that male mice showed significantly difference between deletion mice and control mice. The female mice showed unstable hormone secretion and didn't supply more convincing and consistent data. We have replaced Fig.5E, F. with new male ones.

10. As a general comment, the measures of insulin sensitivity and glucose homeostasis are a bit superficial. This study would be greatly strengthened by more measurements of insulin secretion, circulating insulin levels, as well as glucose clamp studies in the various mouse models.

We agree with the reviewer's comment that more experiment should be done to study the function of Egr-1 on insulin resistance. We have followed the reviewer's suggestion by performing the glucose clamp studies to determine whether the whole-body insulin sensitivity has been improved after overexpression of dnEgr-1 in the epi fat tissue of db/db mice. The result has been shown in the Fig.5D.

11. Figure 5A is interesting, but these are cytokines not adipokines and the authors do not know the cellular origin of the cytokines. This begs the question as to what is the effect on circulating concentrations of adiponectin and leptin. This should be provided.

We have detected the adiponectin and leptin (Supplemental Figure 6) which shows no significant change when overexpression or disruption of Egr-1. We cultured 3T3-L1 adipocytes and overexpressed Egr-1 in them, we found that IL-6 and TNF-alpha were increased. Since the immune cells were not affected by Egr-1 overexpression, we think IL-6 and TNF-alpha were origin from Egr-1 overexpressed fat cells.

12. In Figure 6D, the authors conclude that Egr-1 overexpression in fat blocked insulin induced Akt phosphorylation in the liver of BKs WT mice. However, there are two individual mice presented and pAkt looks very similar to GFP treatment in the first individual.

We applied at least 5 mice to detect Akt phosphorylation in the liver of BKs WT and db/db mice and we randomly run the sample of every two mice and show the western blotting here. Since individual variance in animal; probably the basal level of Akt phosphorylation in the liver of each mouse was different. Therefore, we did densitometric Lanalysis of all five mice and data suggested that there was significant difference between Egr-1 overexpression group and control (Supplemental Figure 6C). Another reason is that there might be some differences in releasing and circulating cytokines after Egr-1 overexpression in fat and thus the response of liver is of course different.

13. The co-culture experiments in Figure 6F don't make much sense. They are showing that the co-culture changes insulin stimulated glucose uptake in hepatocytes. However, insulin has no effect to stimulate glucose uptake in hepatocytes, unlike its well known effects in adipocytes and muscle cells. Therefore, how can they have insulin stimulated glucose uptake effects on this process with the various co-culture conditions measured, since it is not a part of insulin action in liver.

Sorry that we make such a big mistake to detect glucose uptake in hepatocytes after insulin stimulation. We instead detect the glycogenesis in hepatocytes after co-cultured with adipocytes. Since insulin can increase glycogen synthase activity and glycogenesis in hepatocytes by triggering the PI3K/AKT and inactivating the GSK-3, then the free glucose in hepatocytes is decreased which indirectly causes glucose influx through Glut-2 in cell membrane. We added the glycogen synthesis result in the Figure 6F.

14. In general, the manipulations of PTEN and GGPPS are interesting, but throughout the paper the authors tend to conflate these two discrete effects, since they are both end points of Egr-1s transcriptional effects. For example, the GGPPS adipocyte knockout mouse would be an interesting story all by itself, and the data presented in this manuscript on this interesting mouse model are very preliminary and superficial.

Since both PTEN and GGPPS are both end points of Egr-1's transcriptional effects, it is difficult to distinguish their effect in wildtype mice. It has been reported that the PTEN adipocytes conditional knockout mice showed improved glucose tolerance which indicates that PTEN is crucial for insulin sensitivity of adipose tissue (Kurlawalla-Martinez C, Stiles B, Wang Y, Devaskar SU, Kahn BB, Wu H (2005) Insulin hypersensitivity and resistance to streptozotocin-induced diabetes in mice lacking PTEN in adipose tissue. Molecular and Cellular Biology 25(6): 2498). We generated GGPPS adipose knock out mouse and conducted some preliminary experiment to prove GGPPS, one of the Egr-1 target gene, is really involved in regulating insulin sensitivity in adipose tissue. Of course GGPPS adipocyte knockout mouse itself can make big story about adipocyte growth and differentiation, gender difference of insulin response, which we are examining and will report on other manuscript.

15. Egr-1 has been reported to have potent effects on adipocytes biology, independent of insulin signaling. In the discussion, the authors mention unpublished data on adipocyte size, etc. This should be presented in the manuscript, since changes in adipocyte size or adipogenesis could also explain the in vivo effects the authors observe.

We added this result in Supplemental Figure 2C. As shown in this figure, we did find a decrease in adipocyte size after disruption of Egr-1 function with dnEgr-1 in epididymal fat. This means that Egr-1 may regulate the insulin resistance through changing the situation of lipid metabolism of the adipocytes which is the second possible mechanism by which Egr-1 regulates the insulin resistance we mentioned in the Discussion.

16. What is the evidence that Map kinase signaling leads to IRS1 serine phosphorylation?

According to our study, Egr-1 plays an important role in decreasing adipocytes insulin sensitivity. So, as a major molecule marker of insulin sensitivity, the tyrosine phosphorylated IRS-1 will increase when the function of Egr-1 is inhibited. In detail, as we have reported (Shen N, et, al.,

(2011) Journal of Biological Chemistry 286(16): 14508-14515), Egr-1 can enhance the insulin resistance through promoting ggpps transcription. The GGPPS could reactivate the Ras/MAPK/Erk1/2 pathway through Ras prenylation that can phosphorylate the IRS-1 on Serine 612 which is the inactivated form of IRS-1. Thus, disruption of Egr-1 function could inhibit the IRS-1 phosphorylation on Serine 612 and make it tyrosine phosphorylation increase.

17. In the epididymal pad adenoviral injection studies, if the adenovirus was taken up into immune cells within the adipose tissue, would that give the expected effects that they observe?

As we provided the evidence in the Supplemental Figure 6, the virus injection did not affect the function of immune cells, such as infiltration and polarization. Thus, the immune cells in fat tissue would have no effect on insulin resistance.

18. Some references included in the text cannot be found in the bibliography (i.e. page 4, line 10, refers to Kaneto et al., 2004, Luan et al., 2009, manning & Davis 2003) - none of which are present in the bibliography.)

Sorry for the omission here and we have added these references into the text.

19. The fasting blood glucose values shown in figure 2E seem exceptionally high considering these mice were fasted for 16 hours.

The mice we used in Figure 2E were the diabetic mice, thus the fasting basal blood glucose would be at a quite high level.

20. Within the methods section please clarify the strains of mice/background used for the various components of the study and explain why they were selected.

We have clarified the background of BKs db/db mice on Page 6 Line 106, the GGPPS adipocytes conditional knockout mice on Page 24 Line 473-479. The db/db mice is a good model of insulin resistance which is the major subject in our study and the GGPPS adipocytes conditional knockout mice is used to determine whether GGPPS is involved in the regulation of adipocytes insulin sensitivity.

21. This manuscript would also benefit from further proofreading. There are spelling mistakes, p4 line 15, i.e gen, should be gene. Supplemental Table 1, the body weight column appears to be out of alignment with the other columns. In methods section, companies should be quoted with state, i.e. Cell signaling Technology, MA)

We have asked the nature editing group (http://languageediting.nature.com) to polish our manuscript and corrected the mistakes in the text.

Referee #3:

Major comments
1) Results Page 7 and used several times such as in figure legend for fig 1 on page 28: the authors say "constantly" elevated. However, they are only measuring 1 time point. Constantly implies elevations over several time points. Perhaps they mean consistently elevated between individuals? It may be easiest to just leave out a descriptor and say elevated.

We have leaved out the "constantly" in the text on Page 7 Line 119 and 124 and Page 32 Line 716.

2) Results Page 7 and throughout paper such as figure legend for fig 1 on page 28. Please write epi fat pad in text when referring to fat. Although it is in methods, I felt that it would be clearer if the authors stated in the results. Same for "adipocytes" later in manuscript. Please write 3T3L1 adipocytes so that the reader does not infer primary adipocytes.

We have changed this expression in the text.

3) Results Page 7- "Egr-1 exerts an effect" is not correct. You can't assume that it can exert an effect because you are not measuring that. Say "can be regulated" instead.

Yes, the reviewer is right. We have changed it into "can be regulated by insulin" in the text on Page 7 Line 129.

4) page 8 - sentence unclear. I do not understand what they mean at all. Ends with "epi fat pads that is visceral..."

Sorry for this mistake, what we really wanted to express is that the epi fat pad where we injected the adenovirus is the predominant fat tissue in mice. We have corrected this expression in the text on Page 8 Line 141.

5) page 8 - fig 2C conclusion overstated- it is not greatly impaired. Slightly is a more appropriate word.

Yes, we have corrected it in the text on Page 8 Line 154.

6) page 12 - fig 5E conclusion. Say "slightly" or "minimally" more sensitive since there is only 1 time point different. Overall they are very very similar and you should be hesitant to say more sensitive. A clamp study would be able to definitively show increased insulin sensitivity in GGPPS-/-, but that is likely beyond the scope of this study.

Yes, indeed. We have changed the expression in the text.

7) page 15- I don't like the word "activating" as in "activating downstream genes involved in PI3K/AKT and MAPK signaling. Sustained "activating" Egr-1..." because activating implies activity such as a kinase activity or transcriptional activity. The authors should say "inducing the expression of downstream genes" or "sustained expression of Egr-1."

We have changed the expression in the text on Page 16 Line 305 and 306.

8) page 16- perhaps the authors can comments on the size of adipocytes where they mentioned data not shown. If the adipocytes are smaller, then they would be more insulin sensitive which would support their findings.

Yes, the size of adipocytes is smaller and we added this data in the Supplemental Figure 2C

9) I hope that immunoblots shown are representative of larger human/animal studies. Please write in the figure legend that blots are representative.

The human samples we collected contain 6 man and 4 women in normal and diabetic groups and the animal study contains at least 5 mice in each group, this has been clarified in the figure legends. The immunoblots of the human or animal study shown are representative of all the replicates and we added this information in each figure legend.

10) figure legend 2C page 33- Egr-1 overexpression did not "greatly impair glucose tolerance". It slightly or minimally did. Please correct.

Yes, we overstated the situation and have changed this expression in the text on Page 33 Line 747.

11) figure 1 image- page 34. In later figures authors denoted BKs or db/db under blots to help the reader. They should do the same for the first figure since there is so much data to get through.

We denoted the background of mice in the figure 1.

12) sup fig S2 the authors show data for body weight and food intake when they injected Egr-1 and dnEgr-1. This should be mentioned in the text since it is relevant to systemic and hepatic insulin sensitivity. Also mention changes fat pad mass if obtained.

We have described this in the text on Page 8 Line 146-148.

Minor comments:
It is impressive that they see effects in vivo after just 7 days of adenoviral injection into fat pads. I wonder if they would see stronger effects if they let the experiment go longer. (I am not asking for this since they have results, but perhaps results would be even stronger at 14 days for example).

We have done the injection experiment and monitored for 2 weeks, however, they were no stronger results, probably because the efficiency of the adenovirus can't last for 2 weeks. For example, in the article that published in 2006, the level of UCP1 overexpression in Epi can hardly be detected at 7 days (Yamada T, Katagiri H, Ishigaki Y, Ogihara T, Imai J, Uno K, Hasegawa Y, Gao J, Ishihara H, Niijima A (2006) Signals from intra-abdominal fat modulate insulin and leptin sensitivity through different mechanisms: neuronal involvement in food-intake regulation. Cell Metabolism 3(3): 223-229).

Very well written but needs some clarity with English.
Some minor typos.
Page 6, spell out BKs

We have spelled out in the text on Page 6 Line 106.

There could be exciting future studies in brown fat and/or macrophages, especially when using the FABP4 (aP2) driving CRE since aP2 is expressed in activated macrophages in adipose, atherosclerotic vessels, etc.

Yes, indeed. GGPPS adipocyte and macrophages knockout mouse can make big story about adipocyte growth and differentiation, gender difference of insulin response and atherosclerotic vessels which we are examining and will report on other manuscript.

2nd Editorial Decision 20 June 2011

Dear Dr. Li,

Your revised manuscript has now been re-assessed by one of the original referees whose comments you will find enclosed. This scientist asks for two minor revisions. I kindly ask you to incorporate these and provide us with a final word-file of your paper to facilitate efficient acceptance.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORT:

Referee #2:

The authors have revised the manuscript well. I have just two minor revisions.
1. Please include the body weight data of the db/db mice referred to in the original supplemental figure 2.
2. The authors show that the GGPPS ITT data (Fig 5E &F) is more striking when they include only make mice and not females. This suggests there is a gender specific effect and this should be referred to in the text.

2nd Revision - Authors' Response 24 June 2011

Referee #2:

The authors have revised the manuscript well. I have just two minor revisions.
1. Please include the body weight data of the db/db mice referred to in the original supplemental fig 2.

Sorry for the insufficient information. We supplied the body weight result in the supplemental fig 2E and described it in detail in the legend of the supplemental figure 2. Also, we indicated it in the text (Line 148-150 and Line 479-480).

2. The authors show that the GGPPS ITT data (Fig 5E &F) is more striking when they include only make mice and not females. This suggests there is a gender specific effect and this should be referred to in the text.

Yes, it is very suggestive that there is a gender specific effect in our knockout mice according to the ITT data. So we indicated this in the text (Line 256-263)

