THE EMBO JOURNAL

Manuscript EMBO-2012-82507

Anchored Phosphatases Modulate Glucose Homeostasis

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Review timeline:

Submission date:	01 March 2012
Editorial Decision:	10 April 2012
Author Correspondence:	11 April 2012
Resubmission:	28 June 2012
Editorial Decision:	23 July 2012
Accepted:	23 July 2012

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 10 April 2012

Thanks so much for submitting your research paper addressing AKAP-150's physiological function in glucose homeostasis using mouse genetics for consideration to The EMBO Journal editorial office.

Having received two sets of consisting comments, I am in the position to reach a decision on the study. As you will recognize from the comments, both scientists agree on the technical quality and indeed potential interest of this paper. However, both also remark that further mechanistic analyses be provided to support the so far mostly genetic conclusions and significantly advance the actual molecular insights to the level they would have to demand for a more general title such as The EMBO Journal. Importantly, ref#2 indicates that altered systemic glucose homeostasis might compromise some of the conclusions and remarks that some further genetic experimentation would be needed to enable unambiguous discrimination of primary and secondary effects.
With these, as far as I can see mostly constructive comments, I am afraid I am not able to offer straightforward and timely very limited single revisions as I would essentially commit to the paper based on necessary experimentation with uncertain outcomes! Therefore, I do have to return the paper at this point to not only give you the opportunity to expense the necessary time and effort to develop it appropriately OR seek rapid publication presumably in a less demanding title.

I am fully aware that you would be in a strong position to address these concerns but I hope you understand the rationale of the current decision that is based on the preliminary, available dataset and leaves the decision how to pursue fully up to your discretion. We might indeed be able to reassess a strongly expanded version at a later time-point if you were to pursue publication at our title.

For the moment though, I am sorry that I cannot be more encouraging, but I hope that clear communication of our expectations might facilitate efficient further proceedings on this project.

Please do not hesitate to get in touch in case of necessary further clarifications (preferably via E-mail).

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

Hinke et al. describe the generation and analysis of ko mice lacking kinase/phosphatase anchoring protein AKAP150 or knock-in mice of AKAP150 mutants specifically deficient in either PKA or PP2A binding. They find that global deletion of AKAP 150 results in reduced insulin secretion. Yet, AKAP150-deficient mice display increased glucose tolerance due to enhanced insulin sensitivity of insulin-responsive tissues. Their experiments point to increased AKT signaling in skeletal muscle (but not in liver or adipocytes) as a potential source for the observed increased glucose tolerance. Physiologic, metabolic and biochemical analyses of knock-in mutant mice indicate that the observed effects in AKAP150 null mice are directly related to AKAP150's inability to recruit PP2A and not PKA. The knock-in mice expressing AKAP mutant species generated here provide some interesting and unexpected insight into AKAP150 function in glucose-stimulated insulin secretion. The analysis of these mice lack however depth with respect to mechanism.

Figure 1: In panel G the islet appears to be smaller. This may be just by coincidence. On the other hand if representative ,this could indicate effects on beta cell mass. The authors need to provide a statistical analysis of pancreatic sections including data on islet area/pancreatic area, islet mass and area as well as beta cell area and density (the amount of beta cells per area pancreas).

Figure 2: Are the analyses shown in panel A and B done with size-matched islets? How do the authors explain that first and second phase insulin secretion is hardly detectable in the islet perifusion experiment? AKAP150 is suggested to mediate voltage-gated Ca2+ channel phosphorylation. Hence, the attenuation of Ca2+ currents and perturbed glucose-stimulated Ca2+ flux associated with deletion of AKAP150 that the authors observe may relate to failure of PKA recruitment. If so beta cells from mice expressing a PKA deficient AKAP150 protein may demonstrate similar effects. Is this the case?

Figure 3: Skeletal muscle of AKAP150 ko mice display increased AKT phosphorylation on S473. This would be consistent with increased insulin signaling. The authors should complement their biochemical analysis of skeletal muscle by assessing the expression and phosphorylation state of other components of the insulin pathway including IRS. In addition, what happens to AMPK activation in response to insulin stimulation?

Figure 4 and 5: It is important to know whether there are any differences among islet area/pancreatic area, islet mass and area as well as beta cell area and density (the amount of beta cells per area pancreas) parameters in the different genotypes. Also insulin perifusion experiments in the background of these mutant protein expression should be assessed. This will provide insight whether one or the other mutant affects also beta cell mass as an alternative explanation of reduced insulin levels. From the densitometric scans in Figures 4 and 5, panels J, it appears that in each case there is about a 3-fold activation of AKT. Does this mean that the differential effects of the various AKAP mutants on glucose-stimulated insulin secretion in beta cells do not unfold in skeletal muscle tissue where neither PKA nor PP2A recruitment is important for AKT activation? As PKA and AMPK signaling demonstrate extensive interplay one wonders whether AMPK signaling is affected in the setting of these mutant proteins.

Referee #2:

In this manuscript Hinke et al. examined the effect of AKAP150 on glucose homeostasis using knockout and knockin mouse models, metabolic studies and ex-vivo analyses. Deletion of AKAP150 results in blunted insulin secretion from mouse islets and INS1 cells. However, the deletion also improves insulin sensitivity in muscle, resulting in improved glucose tolerance in whole-body AKAP150 KO mice. Notably, knock-in of a mutant AKAP150, which is unable to anchor PP2B, into mice results in very similar phenotype as seen in AKAP150 null mice, suggesting that PP2B plays an important role in systemic glucose homeostasis. These studies are well executed and the findings are timely and of general interest to the field.

Major comments:

1- Cell studies performed using gene silencing should include reconstitution experiments to determine if that will rescue the observed phenotype and rule any off target effects. Importantly, reconstitution of AKAP150 knockdown cells with the two mutants will be very informative not only in complementing findings from the different mouse models but also to demonstrate cell-autonomous effects. In Fig. 1D, the expression of transfected hGH should be presented. In addition, the authors need to also determine basal and glucose-stimulate insulin secretion in AKAP150 and 220 knockdown cells (including AKAP150 knockdown cells with AKAP150 reconstitution). In Fig. 1G a higher magnification of the AKAP150 expression in islets and well as its expression in alpha cells will be informative. In Fig. 1H the authors should also present the corresponding blood glucose levels (especially given the improved insulin sensitivity in the muscle of KO mice).

2- In addition to ex vivo GSIS data (Fig. 2A) the authors should present in vivo GSIS at 0, 2 and 15 minutes post glucose injection to determine both phases of insulin secretion in vivo. If WT and AKAP150 deficient cells produced similar levels of cAMP in response to forskolin (Fig 2K), why did forskolin have such a different effect on insulin release (Fig. 2B)? This should be discussed.

3- The authors should present insulin levels at 0, 10 and 20 minutes during GTT to determine the contribution of that to glucose levels as opposed to the effect of other peripheral-insulin responsive tissues. In addition to insulin levels in fed and fasted WT and KO mice the authors need to determine C-peptide levels to examine any alterations in clearance. Since glucagon levels are altered in the KO mice are there any alterations in gluconeogenesis. This could be easily measured by measuring expression of some genes (such as PEPCK, G6Pas etc.). Also the authors need to present islet mass/area in WT and KO mice.

Importantly, given that AKAP150 is deleted in other insulin-responsive tissues and insulin sensitivity of muscle is enhanced in KO mice the major issue with this study is that the altered systemic glucose homeostasis could be due, at least in large part, to increased insulin sensitivity in muscle and alterations in insulin levels are simply adaptations and secondary. To this reviewer the contribution of pancreatic AKAP150 deletion to systemic glucose homeostasis (without the compounding effects of its deletion in other tissues which have a profound effect on systemic homeostasis) could be convincingly achieved only by generating pancreas-specific KO mice. Since these are not available for these authors the authors should carefully restate their conclusions.

Author Correspondence 11 April 2012

Thanks for getting the comments back to us. Overall I agree with you that the reviewers comments were constructive. We can address these points, in some cases very easily, in the case of other points with a bit more work. Give us a couple of months and we will get a revised manuscript back to you.

John D. Scott FRS

Resubmission 28 June 2012

Referee #1:

1. Figure 1: In panel G the islet appears to be smaller. This may be just by coincidence. On the other hand if representative, this could indicate effects on beta cell mass. The authors need to provide a statistical analysis of pancreatic sections including data on islet area/pancreatic area, islet mass and area as well as beta cell area and density (the amount of beta cells per area pancreas).

Response: The referee makes a valid point, particularly since b-cell specific deletion of the regulatory subunit of calcineurin/PP2B reduces b-cell mass and has deleterious effects on glucose homeostasis [Heit et al, 2006 Nature 443:345-349]. Reviewer 2 also requested b-cell mass measurement.

Therefore we have performed a blinded morphometric analysis on 3 mice of each genotype (wildtype, AKAP150KO, AKAP150DPIX and AKAP150D36). Pancreata were dissected, fixed and paraffin embedded; 4 serial sections were taken at 200 mm intervals throughout each tissue block, and co-stained for insulin (blue), glucagon (green) and cadherin (red), or IgG controls. Immunofluorescence images were analyzed with NIS-Elements AR 3.2 software to measure islet area as a percentage of total pancreatic area. Calculation of b-cell mass using the wet weights of the dissected organs was performed. These new data are presented in Table I. No difference in islet area or b-cell mass were detected among the 4 lines of mice. Representative three-color images of WT and AKAP150KO sections have been added to Figure 1 (panels L & M).

2. Figure 2: Are the analyses shown in panel A and B done with size-matched islets? How do the authors explain that first and second phase insulin secretion is hardly detectable in the islet perifusion experiment? AKAP150 is suggested to mediate voltage-gated Ca2+ channel phosphorylation. Hence, the attenuation of Ca2+ currents and perturbed glucose-stimulated Ca2+ flux associated with deletion of AKAP150 that the authors observe may relate to failure of PKA recruitment. If so beta cells from mice expressing a PKA deficient AKAP150 protein may demonstrate similar effects. Is this the case?

Response: We have responded in three ways.

a) Analyses are done with size-matched islets (~70-300 mm in diameter), but normalized to total cell insulin content to account for any random bias. We apologize for not clarifying this point and have amended the materials and methods accordingly. [Page 18, Lines 18-19].

b) The reviewer was concerned about resolution of first and second phase insulin secretion during perifusion. Our studies monitored 100 islets/chamber at a buffer flow rate of 0.5mL/min using our previously published method [Hansotia et al, 2004, Diabetes 53: 1326-1335; Pamir et al, 2003 Am J Physiol 284: E931-939]. Fractions were collected at 1 min intervals and measured using ELISA. Similar parameters have been used to compare dynamic insulin secretion from rat and mouse islets, concluding that 1st and 2nd phases of insulin secretion cannot be resolved during perifusion of mouse islets [Zawalich et al, 2008 Metabolism 57:30-39]. Thus the inability to resolve 1st and 2nd phases of insulin secretion is a consequence of mouse models used for the analyses. This issue has now been mentioned in the text on page 19, lines 4-5.

c) The reviewer suggested that calcium flux in AKAP150D36 mice might be altered due to failure of PKA recruitment to the L-type calcium channel. This is a valid point. However, data presented in Figure 5 did not detect any measurable differences in insulin secretion from AKAP150D36 islets

(panels F & G). Likewise, we did not detect changes in circulating insulin or altered glucose homeostasis from AKAP150D36 animals (Figure 5D and H). Therefore it is unlikely that modulation of L-type calcium channels is altered in this genetic background. PKA regulation of calcium currents may be compensated by other anchoring proteins such as AKAP18 that also directly associate with this channel [Fraser et al, 1998, EMBO J 17:2261-2272]. This issue has been clarified in the text on page 13, lines 6-8.

3. Figure 3: Skeletal muscle of AKAP150KO mice display increased AKT phosphorylation on S473. This would be consistent with increased insulin signaling. The authors should complement their biochemical analysis of skeletal muscle by assessing the expression and phosphorylation state of other components of the insulin pathway including IRS. In addition, what happens to AMPK activation in response to insulin stimulation?

Response: This is an excellent point. New data added to supplemental Figure S3I & J shows that insulin responsive phosphorylation of Ser612 on IRS-1 is elevated in skeletal muscle from AKAP150 KO mice. Phosphorylation of this site is thought to modulate insulin action [Mothe and Van Obberghen, 1996 J. Biol. Chem. 271: 11222-11227].

The regulation of AMPK phosphorylation status by insulin has been extensively reported. Insulin stimulation attenuates AMPK activity, and is reflected in a diminished phosphorylation at Thr172 [Clark et al, 2004, Eur. J. Biochem 271:2215-2224; Horman et al, 2006, J. Biol. Chem. 281: 5335-5340; Yin et al, 2003, J. Biol. Chem 278: 43074-43080]. We have observed this negative feedback in our own experiments but this data is tangential focus of our article.

4. Figure 4 and 5: It is important to know whether there are any differences among islet area/pancreatic area, islet mass and area as well as beta cell area and density (the amount of beta cells per area pancreas) parameters in the different genotypes. Also insulin perifusion experiments in the background of these mutant protein expression should be assessed. This will provide insight whether one or the other mutant affects also beta cell mass as an alternative explanation of reduced insulin levels.

Response: There are two responses.

a) Morphometric analysis of the pancreas for AKAP150DPIX and AKAP150D36 sections have been performed as described above. As presented in Table I and Figures 5 and 6: no differences in b-cell mass, islet area or pancreatic insulin content were observed in any these genetically modified lines.

b) At the request of the reviewer we have performed additional dynamic insulin secretion measurements. The new insulin secretion data for the knock-in lines of mice can be found in Figures 5G and 6G. AKAP150DPIX islets display a secretory defect (Figure 6G), whereas islets from AKAP150D36 mice appear to have normal insulin secretion (Figure 5G), consistent with static insulin release experiments (Figures 5F and 6F).

5. From the densitometric scans in Figures 4 and 5, panels J, it appears that in each case there is about a 3-fold activation of AKT. Does this mean that the differential effects of the various AKAP mutants on glucose-stimulated insulin secretion in beta cells do not unfold in skeletal muscle tissue where neither PKA nor PP2B recruitment is important for AKT activation? As PKA and AMPK signaling demonstrate extensive interplay one wonders whether AMPK signaling is affected in the setting of these mutant proteins.

Response: We are unclear of the reviewers perspective on this point. We have included phospho-AMPK data for the AKAP150D36 and AKAP150DPIX mice as supplementary information (Figures S5F & G and S6F & G). In line with our data on the global AKAP150KO mice, we observed a trend towards enhanced (P)Thr172-AMPK in skeletal muscle extracts from AKAP150DPIX mice, but not from AKAP150D36 animals.

Referee #2:

1. (A) Cell studies performed using gene silencing should include reconstitution experiments to determine if that will rescue the observed phenotype and rule any off target effects. Importantly, reconstitution of AKAP150 knockdown cells with the two mutants will be very informative not only in complementing findings from the different mouse models but also to demonstrate cell-autonomous effects. (B) In Fig. 1D, the expression of transfected hGH should be presented. (C) In addition, the authors need to also determine basal and glucose-stimulate insulin secretion in AKAP150 and 220 knockdown cells (including AKAP150 knockdown cells with AKAP150 reconstitution). (D) In Fig. 1G a higher magnification of the AKAP150 expression in islets and well as its expression in alpha cells will be informative. (E) In Fig. 1H the authors should also present the corresponding blood glucose levels (especially given the improved insulin sensitivity in the muscle of KO mice).

Response: There are multiple responses.

(A) There are two responses to the first point. First, although siRNA is a useful tool to examine the acute depletion of a protein, this approach was simply used to screen for anchoring proteins that impact insulin secretion from a transformed tumor cell line. Once we had identified AKAP150 as a target much more extensive analyses were performed in four strains of genetically modified animals to define the role of this anchoring protein in glucose homeostasis. These include analysis of whole body AKAP150 deletion (Figures 1-3), conditional deletion of AKAP150 (new Figure 4) and metabolic profiling of mouse strains expressing AKAP150 forms unable to anchor PKA or PP2B (Figures 5 & 6).

Second, scaffolding proteins such as AKAP150, that function to bring together different combinations of proteins, are notoriously difficult to rescue. This is in part because low-level expression permits the organization of a few binding partners whereas high level or compartmentalized expression segregates the same pool of binding partners into partially formed complexes. As a result siRNA rescue experiments often are not technically feasible for AKAPs. A graphic example of this is presented in a paper we published in 2010 showing that low level expression of AKAP-Lbc enhanced signaling through the complex whereas higher level expression abolished signaling and gave the same result at siRNA knockdown (Smith et al., 2010, Nat. Cell Biol. 12:1242-1249). We obtained similar results when rescue experiments were attempted in INS-1(832/13) cells using the human AKAP79 otholog. Likewise, rescue of MyRIP, another AKAP that contributes to insulin secretion was not possible (Goehring et al, 2007, J. Biol. Chem. 282: 33155-67). Therefore, we have chosen not to include these data as the negative result does not add to our study.

(B) At the request of the referee the expression of the transfected hGH is now presented in Figure S1J as pg/1000 cells.

(C) We now include insulin secretion data from the INS-1(832/13) cells (Figure 1D and E). For this experiment, we had previously measured both insulin and hGH from the transfected INS-1(832/13) cells. Our insulin results from AKAP150i transfected cells is consistent with our results using the surrogate hGH secretory marker, Figure S1D & E. We observed no effect of AKAP220 siRNA when examining co-secreted hGH in our screen and thus have not continued this line of inquiry.

(D) We have incorporated images showing a higher magnification of the islet mantle in WT and AKAP150KO pancreata (Figure 1I). Because the immunofluorescent images presented in Figure 1H were not counterstained for glucagon, we performed immunoblots on a glucagon-secreting cell line (aTC-1-6). AKAP150 is expressed at only trace levels in aTC-1-6 cells. Thus AKAP150 negative cells of the islet mantle may include a cells. We hope that this new data, that has been included as supplemental information (Figure S1N), will satisfy the reviewer.

(E) The blood glucose data the referee requests is presented in Figure 3A. We apologize for any confusion we may have caused due to the layout and presentation of the original data set.

2. (A) In addition to ex vivo GSIS data (Fig. 2A) the authors should present in vivo GSIS at 0, 2 and 15 minutes post glucose injection to determine both phases of insulin secretion in vivo. (B) If WT and AKAP150 deficient cells produced similar levels of cAMP in response to forskolin (Fig 2K), why did forskolin have such a different effect on insulin release (Fig. 2B)? This should be discussed.

(A) In response to the referee's comment, we have performed early blood sampling time points during glucose tolerance testing. These are included in Figure S1O. Although our new findings are consistent with data from later time points, we wish to point out that the degree of time resolution *in vivo* that is necessary to resolve the phases of insulin secretion is not possible by this method. As discussed in response to Reviewer #1, mice lack a measurable 1st phase of insulin secretion [Zawalich et al, 2008 Metabolism 57:30-39]. Furthermore, additional variability may result from the stress of injection and the rate of glucose absorption from the IP cavity.

(B) We thank the reviewer for highlighting this point. We have amended our discussion with an added sentence to this effect, to address the concerns of the reviewer on page 13, lines 22-24.

3. (A) The authors should present insulin levels at 0, 10 and 20 minutes during GTT to determine the contribution of that to glucose levels as opposed to the effect of other peripheral-insulin responsive tissues. (B) In addition to insulin levels in fed and fasted WT and KO mice the authors need to determine C-peptide levels to examine any alterations in clearance. (C) Since glucagon levels are altered in the KO mice are there any alterations in gluconeogenesis. This could be easily measured by measuring expression of some genes (such as PEPCK, G6Pas etc.). (D) Also the authors need to present islet mass/area in WT and KO mice.

(A) As the reviewer also requested, we have added both a 2min and 10min measurements (Figure S1O), in addition to the 20min time point that was included in the initial submission.

(B) We have added the C-peptide measurements as requested by the reviewer in Figure S3C. The fed C-peptide concentrations between WT and AKAP150KO mice were not significantly different, suggesting that no overt differences in insulin clearance are responsible for the observed metabolic phenotype.

(C) We have performed real-time quantitative PCR using Taqman probes to address changes in gluconeogenesis in both liver and skeletal muscle (Figure S3G & H). In our initial submission, we also presented data from a pyruvate tolerance test (Figure 3I) that showed no difference between WT and KO animals in their ability to convert pyruvate into glucose by this test. We hope that this additional satisfies the reviewer.

(D) Reviewer #1 also requested islet mass for the global AKAP150KO mice, and the knock-in mouse lines. These data have been included in the revised manuscript (Figure 1L & M and Table I).

4. Importantly, given that AKAP150 is deleted in other insulin-responsive tissues and insulin sensitivity of muscle is enhanced in KO mice the major issue with this study is that the altered systemic glucose homeostasis could be due, at least in large part, to increased insulin sensitivity in muscle and alterations in insulin levels are simply adaptations and secondary. To this reviewer the contribution of pancreatic AKAP150 deletion to systemic glucose homeostasis (without the compounding effects of its deletion in other tissues which have a profound effect on systemic homeostasis) could be convincingly achieved only by generating pancreas-specific KO mice. Since these are not available for these authors the authors should carefully restate their conclusions.

Response: We thank the reviewer for this astute observation – one that we too had recognized. In this regard, we feel that the isolated tissue data does shed some light on this point. The blunted insulin secretion data we observe in isolated islets (Figure 2 A & B), where the homeostatic feedback loops are no longer present, indicates that the attenuated hormone release is not solely a consequence of enhanced insulin sensitivity.

We originally intended to publish a follow-up report on conditional deletion of the AKAP150 gene from pancreas, using the well established *Ins2-Cre* line [Postic et al, 1999, J. Biol. Chem. 274:305-15] with floxed AKAP150 mice. We have now decided to include data from conditional KO mice in Figure 4 in an effort to more directly respond to the reviewers concern. These animals demonstrate blunted circulating insulin levels in vivo, but display no difference in peripheral insulin sensitivity. We are also aware that any conclusion must be drawn with caution when using conditional knockout mice and emphasize this caveat in the discussion (Page 15, Lines 2-6).

2nd Editorial Decision 23 July 2012

Thank you very much for the thoroughly revised study that was assessed by one of the original referees being fully satisfied with the revisions provided.

Please allow me to congratulate to the study and point out that the editorial office will soon be in touch with necessary paperwork related to official acceptance.

Please also 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. This entails presentation of un-cropped/unprocessed scans for the KEY data of published work. We would be grateful for one PDF-file per figure combining this information. These will be linked online as supplementary "Source Data" files. Please do let me know if you have any questions regarding this initiative AND feel free to check the following URL for a recent example:
http://www.nature.com/emboj/journal/v30/n20/suppinfo/emboj2011298as1.html.

I am very much looking forward to efficient proceedings in this matter.

Yours sincerely,

Editor

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