THE EMBO JOURNAL

Manuscript EMBO-2010-73801

Cytosolic pH is a second messenger for glucose and regulates the PKA pathway through V-ATPase

Reinhard Dechant, Matteo Binda, Sung Sik Lee, Serge Pelet, Joris Winderickx, and Matthias Peter

Corresponding authors: Reinhard Dechant, ETH Zurich; Matthias Peter, ETH Zurich

Review timeline:

Submission date:	28 January 2010
1st Editorial Decision:	24 February 2010
1st Revision received:	10 May 2010
2nd Editorial Decision:	26 May 2010
2nd Revision received:	28 May 2010
Accepted:	28 May 2010

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 24 February 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see while all three referees consider the study as very interesting and potentially important they all feel that the causal link between cytoplasmic pH/V-ATPase and PKA needs to be strengthened considerably before they can support publication of the paper here. In addition they also mention a few other points, which in their view should be addressed. Taking together all issues raised we should be able to consider a revised manuscript in which the referees' concerns are addressed in an adequate manner and to their satisfaction. I would like to point out that I do not think that point 3 of referee 1 should be followed in a way that evidence is removed. Still some "standardisation" of read-outs should be done nevertheless. Also, I would like to urge you to consider re-writing the manuscript text in a clearer way that makes it easier to the non-specialist reader to follow. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript as well as on the final assessment by the referees.

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the conceptual advance provided by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

When preparing your letter of response to the referees' comments, please bear in mind that this will

form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This manuscript describes evidence that, in yeast, decreased glucose leads to decreased cytosolic pH which in turn leads to V-ATPase disassembly, decreased adenylate cyclase activity and decreased PKA activity. A glucose dependent change in the pH of the insulin-containing compartment in Min6 cells and a role for V-ATPase activity in glucose-dependent insulin secretion and PKA activation in these cells is also reported. While the results are potentially important, the conclusions drawn by the authors are not fully justified by the data presented. Acceptance of this manuscript for publication would be contingent upon the authors adequately addressing the issues raised below.

1. The authors suggest that V-ATPase assembly represents a key link between the observed changes in cytoplasmic pH with glucose and changes in PKA activity. Unfortunately, this linkage rests primarily on the experiments shown in Fig.2 employing dinitrophenol (DNP) to equilibrate the cytoplasmic and extracellular pH. The authors interpret the results in parts A and B to indicate that acidification of the cytosol leads to V-ATPase dissociation. However, because DNP is an uncoupler that, minimally, depletes cells of ATP and changes pH gradients across both intracellular and surface membranes, it is not possible to draw this conclusion. It also appears from the legend that DNP is not included in parts C and D of this figure. If this is the case, how can the authors make statements about the effect of cytoplasmic pH on dissociation from the data in Figs 2C and D. If DNP is included in parts C and D, it would appear that even under low pH conditions, glucose still induces dissociation of the V-ATPase (compare Fig2B and D), suggesting changes in pH are not the primary means of regulating V-ATPase assembly.

2. Many of the results in this manuscript are complicated by the fact that glucose is able to alter adenylate cyclase and PKA activity in yeast by two parallel pathways. One of these involves the G-protein coupled receptor Gpr1 and the G-alpha subunit Gpa2 while the other, the authors suggest, involves changes in cytoplasmic pH. In one experiment (Fig.4D) the authors attempt to separate these pathways by looking at a double mutant disrupted in both pathways. They show that Hsp12 expression (as an indirect readout of PKA activation) is additive upon disruption of gpa2 and vma5. The authors need to do other experiments in the gpa2 disrupted strain where complications of the effect of glucose on PKA via this route do not exist. Thus, for example, the experiments shown in parts A and B of Fig.4 should be repeated in the double deletion strain. In this case, the authors model predicts that Msn2 should not undergo translocation to the nucleus upon glucose depletion and cAMP should not increase in response to glucose addition.

3. This manuscript is unnecessarily lengthened and complicated by the multitude of methods used to mimic glucose depletion (addition of deoxyglucose, switch of carbon source from glucose to galactose in a strain disrupted in Pgm2, disruption of pyruvate kinase) and in the indirect readouts used as a measure of PKA activity (Msn2 localization, Hsp12 expression). The authors should employ a single method (for example glucose depletion) and a single readout (for example Msn2 localization) throughout the manuscript. The authors should also make it clear that they are using only indirect measures of PKA activity and that their results therefore need to be interpreted with caution.

4. The authors use GFP-tagged Vma5p to monitor in vivo assembly, but they do not show that the remainder of the V1 domain dissociates upon glucose removal in vivo. Experiments following fluorescence localization of another V1 subunit (for example subunit A or B) should also be included to document that true dissociation of the V-ATPase complex is being followed as opposed to simply dissociation of subunit C. It is also important to document that the GFP tagged subunit C behaves the same way as the native subunit C with respect to glucose-dependent dissociation, assembly and activity. This can be done by using the more conventional IP/Western method to evaluate glucose-dependent dissociation and comparing the behavior of complexes in strains expressing the GFP-tagged and untagged protein.

5. In Fig.S3, the change in Hsp12 expression in a vma5 disrupted strain following addition of cAMP is quite small and unconvincing. The authors also do not discuss how addition of cAMP (20 mM) to the outside of yeast is resulting in increased cAMP inside the cells or document that such a change in cytoplasmic cAMP actually occurs.

6. The effect of the D329N mutant of Vph1p on activity and assembly should be shown and the authors should explain why dissociation is delayed by more than 5 min (Fig.2F) and yet nuclear localization of Msn2 is delayed by only approximately 1 min.

7. The authors should explain why their model predicts a constitutively high activity for Ras even in the absence of glucose for the vma minus strain, since this was not really clear from either the Results or Discussion. The authors should also include in the Discussion a more complete description of the role of Ira proteins in activating Ras in response to elevated glucose, since this is a well documented pathway.

8. In Fig.5B the authors report a reduction of insulin in the media after treatment of Min6 cells with elevated glucose if the V-ATPase has been inhibited by concanamycin A. It is unclear, however, whether this is due to reduced insulin processing, interference in glucose-dependent signaling or due to a direct defect in fusion of insulin containing vesicles with the plasma membrane.

9. In their discussion of previous results documenting the role of the Ras/cAMP/PKA pathway in control of V-ATPase assembly in yeast, they suggest that this result is not reflective of the in vivo situtation but rather a "positive feedback loop from PKA to V-ATPase assembly which can be detected in vitro but which is too weak to influence V-ATPase assembly in vivo". This ignores the observation, however, that in the YPH500 strain disrupted in either Ira1 or Ira2, the cells retain the red pigment indicative of assembled and functional V-ATPase complexes in vivo. The authors also need to include a discussion of the considerable evidence for PKA regulating V-ATPase assembly in insect cells.

10. In general this manuscript needs to be re-written in much clearer style.

Referee #2 (Remarks to the Author):

The manuscript Dechant et al., "Cytosolic pH is a second messenger for glucose and regulates the PKA pathway via V-ATPase" describes the identification of a novel connection between basic carbon metabolism and intracellular pH using mainly the yeast S. cerevisiae as a model system. The authors further provide evidence for a connection between vacuolar V-ATPase function and activity of protein kinase A - a central metabolic regulatory pathway.
The data presented are of highest technical quality and nicely demonstrate how high-end techniques using the Saccharomyces cerevisiae system contribute to a quantitative understanding of signalling processes in vivo. The paper is clearly written and the referencing is fair and seems up-to date.
The involvement of V-ATPase in signalling intracellular pH to other signalling pathways is an exciting proposal. The authors convincingly show a functional connection between V-ATPase, glycolytic flux and intracellular pH. They further prove a direct function of V-ATPase disassembly for metabolic signalling by using a novel point mutated allele of the Vph1 V-ATPase subunit. Additional sections demonstrate the partial dependence of PKA on the intracellular pH and establish the V-ATPase as a mediator. In the last part a role of the V-ATPase for glucose-regulated insulin secretion of a beta cell line is demonstrated and thus the extend their idea of V-ATPase as a

mediator of a intracellular metabolic signal to higher eukaryotes. Together, these results and ideas will spark novel thinking and thus novel insights for old and "textbooked" metabolic pathways.

Points:
(1) It is absolutely clear from the data shown that PKA is somehow differently wired in the V-ATPase mutant and thus certain responses are delayed. However, the connection of V-ATPase to PKA could be demonstrated more carefully. The genetic connection between the PKA pathway and the V-ATPase (also Figure S4B) can now be more quantitatively analyzed using the available datasets of Constanzo et al., (Science 327, 425). This could strengthen the proposed mechanisms. Additionally, a more global assessment of the influence of V-ATPase on the expression level of other PKA target genes might provide additional support since the HSP12 transcript level is also regulated by several other cues. Finally, the connection of V-ATPase to the PKA pathway might be further substantiated by determination of cAMP levels and PKA activity in mutants combining the V-ATPase and the GPCR glucose sensing complex including Gpa2 (e.g. vma2 gpa2).

More specific points:
2) P8 L10: "...the mutation did not affect V-ATPase assembly or function ..." How was V-ATPase function tested in the Vph1-D329N mutant version?
3) P8 L12: Which data are not shown?
4) P8 L14: "efficiently uncoupled" is perhaps overstated.
5) P9 L1: Please give a reference for "reduced glycolytic flux"

Figures:
6) Figure 1: Treatment times should be indicated in the figures
7) Figure 2: E and F: D329N should be replaced with Vph1-D329N in the figure
8) Figure 2: Does the Vph1-D329N mutant allele cause changes in morphology?
9) Figure 4C: The method for Ras-GTP measurement not described sufficiently in the materials and methods. The legend says Ras2-GTP and the figure Ras-GTP.
10) D: What are the units for HSP12 expression?
11) E: D329N should be replaced with Vph1-D329N.
12) Figures S1 and especially the genetic data of S4 are of more general interest and should be presented in the paper.
13) For Figure S5 only a microscopic picture is shown, however, quantification as shown in other figures (Figure 2) for V-ATPase assembly is missing.
14) Figure 6 and S6: It is no clear why two schemata are presented. One general should be enough. Figure S6: The red hexagons representing glucose - are not optimal from a chemist´s point of view.
15) Table S4: The quantitative contribution of the ATP/ADP ratio to H+ load of the cytosol is perhaps negligible. In this respect the title of table S4 is misleading. It is not the "ATP demand" but the ratio of amount of Pi or the ratio of ATP/ADP which contributes to cytosolic H+. The quantitative contribution of these should be discussed.

Methods:
16) The scoring method for the assembly of V-ATPase in cells is not described sufficiently. The Ras-GTP measurement should also be mentioned here - at least as a reference.

Discussion:
17) The authors might also consider a model including direct regulation of PKA such as factors downstream of Gpa2 via the kelch repeat proteins Krh1 and 2, which are negative regulators of PKA activity.

18) P16L11
I guess should it read
"... glucose sensing via INactivation of ATP-dependent K+-channels ..."
based on the reference given: McDonald et al., 2000.
"...ATP can inhibit K+ATP channels at the Kir6.2 subunit ... (Tucker et al. 1997; John et al. 1998; Mikhailov et al. 1998)"
19) Reference (McDonald et al., 2000) should be marked as review and/or original literature be cited.

20) P16 L13

The conclusion " ... further suggesting ... similar principles in yeast and mammalian cells" is not clear based on the arguments mentioned in the paragraph. ATP inhibits mammalian ß-cell K+Channels and activates yeast H+ATPase. Please clarify the connection.

21) P16 L18
" ... V-ATPase is required for cAMP production" Figure 4B shows that vma2 and vma5 deletion mutant produce less cAMP - V-ATPase is therefore not required.

22) P16 L21
This sentence needs rephrasing.

23) P17 L3
Since PKA is INactivated by starvation it might be more clearer to state that the activity of PKA induces Glucose-dependent ASSEMBLY of the vacuolar (H+)-ATPase instead of the starvation induced disassembly.

24) P17 L9
The here postulated feedback loop is difficult to understand. It is also not included in the schemata (Fig 6 and S6). Please explain the idea more clearly.

Referee #3 (Remarks to the Author):

This manuscript presents evidence that cytosolic pH and the vacuolar ATPase play important roles in glucose sensing and signal transduction. For many years, people have tried to identify the exact nature of the glucose signal. In this paper, the authors build a very strong correlation between glycolytic flux and cytosolic pH. Could the glucose signal be the concentration of protons? This idea is enticing in its simplicity.

1. The main weakness in this paper is the lack of connection between the V-ATPase and PKA. Both molecules show an effect of cytosolic pH. The V-ATPase dissociates in low pH. PKA is activated in high pH. Is there a causative connection here or are they parallel recipients of the pH signal? The authors offer a couple experiments to show a connection but none are direct. Does the Msn2-NLS-GFP construct contain the Ser582 site? Do mutations of the PKA site (Ser582Ala or Ser582Glu) eliminate the effect of the VPH1-D329N mutation?

2. One strength of this manuscript is that it brings together two distinct fields of study. Because of this, the authors are obligated to provide sufficient background information for readers from both fields to understand the experimental design. The introduction does not provide sufficient background for those coming from the glucose sensing field. The intro needs to include more basic information about the V-ATPase - namely that it is a large macromolecular complex that is conserved between yeast and mammals; that it is composed of two functional domains, V0 which is embedded in the vacuole membrane and V1 which reversibly associates with V0. This information is critical for the reader to interpret data in Figures 1 and 2. The authors use Vma5-GFP to monitor V-ATPase disassembly and they use Vph1-GFP as a control. This only makes sense if the reader knows that Vph1 is part of the V0 complex and that Vma5 is part of the V1 complex.

3. The authors often state the pH is a regulator of PKA. However, the data in Figure 4B and 4C suggest that pH is a regulator of cAMP levels, and not PKA directly. Is there a way to determine whether cytosolic pH is affecting adenylate cyclase or phosphodiesterase? In any case, the authors need to be more careful with their words and should say that pH regulates the PKA pathway rather than PKA itself.

4. Is it possible to perform a direct assay of PKA activity in cells with high and low cytosolic pH? They have shown that they can manipulate cytosolic pH by use of 2,4-DNP. Does this treatment affect PKA activity? Either PKA activity in a kinase pull-down assay or a western detecting the phosphorylation of a known PKA site (Ser 582 of Msn2) would greatly support the idea that pH regulates PKA.

5. Experimental protocol with the microfluidics chamber is not explained or referred to unless you read the Materials and Methods section. My first examination of Figure 1 B,C and D left me wondering how the cells were switched between media so rapidly. The authors use a very sophisticated method and it should be mentioned in results section and figure legend.

6. What are the units on the Y axis for the nuclear localization of MSN2 (Fig 4A) and the relative assembly of the V-ATPase (Fig 1B, 2F)? In some figures they use % assembled? Is there some reason they have to use arbitrary units instead of % assembled?

1st Revision - authors' response 10 May 2010

Referee #1 (Remarks to the Author):

1. "The authors suggest that V-ATPase assembly represents a key link between the observed changes in cytoplasmic pH with glucose and changes in PKA activity. Unfortunately, this linkage rests primarily on the experiments shown in Fig.2 employing dinitrophenol (DNP) to equilibrate the cytoplasmic and extracellular pH. The authors interpret the results in parts A and B to indicate that acidification of the cytosol leads to V-ATPase dissociation. However, because DNP is an uncoupler that, minimally, depletes cells of ATP and changes pH gradients across both intracellular and surface membranes, it is not possible to draw this conclusion. It also appears from the legend that DNP is not included in parts C and D of this figure. If this is the case, how can the authors make statements about the effect of cytoplasmic pH on dissociation from the data in Figs 2C and D. If DNP is included in parts C and D, it would appear that even under low pH conditions, glucose still induces dissociation of the V-ATPase (compare Fig2B and D), suggesting changes in pH are not the primary means of regulating V-ATPase assembly.

Our conclusion that V-ATPase is regulated by cytosolic pH is supported by multiple lines of evidence. First, intracellular acidification of cells by adding 2,4-DNP leads to the disassembly of V-ATPase as judged by Vma5p-GFP localization. We now provide automated quantifications of these experiments (revised Figure 2B). Second, we have directly addressed if 2,4-DNP might affect V-ATPase assembly by acting as an uncoupler of mitochondrial activity. As shown in the new Figure 2B, V-ATPase assembly is unaffected by treatment with Antimycin A, which inhibits mitochondrial respiration. Third, we repeated the starvation experiments with medium adjusted to high or low pH. As before, starvation of cells in medium of high pH impaired V-ATPase disassembly. In the new experiments, we have now simultaneously measured cytosolic pH under these conditions in time-lapse experiments and demonstrate that such treatment prevents cytosolic acidification. These data were indeed performed in the absence of any protonophores and demonstrate that reduction of cytosolic pH upon starvation is caused by influx of protons from the medium into the cells. Finally, direct regulation of V-ATPase assembly is further supported by the described pH-resistant *vph1-D329N* mutant and our observations that cytosolic pH and the assembly state of V-ATPase are co-regulated under all conditions tested (see for example new Figure 3D and E). Yet, inactivation of V-ATPase does not alter pH regulation, demonstrating that cytosolic pH is not a direct consequence of V-ATPase regulation. In the revised manuscript we have included more data demonstrating this under various metabolic perturbations (revised Figure 4), and rephrased the paragraphs describing Figure 2. We believe that these changes and additional data strongly support our conclusion on pH regulation of V-ATPase.

2. "Many of the results in this manuscript are complicated by the fact that glucose is able to alter adenylate cyclase and PKA activity in yeast by two parallel pathways. One of these involves the G-protein coupled receptor Gpr1 and the G-alpha subunit Gpa2 while the other, the authors suggest, involves changes in cytoplasmic pH. In one experiment (Fig.4D) the authors attempt to separate these pathways by looking at a double mutant disrupted in both pathways. They show that Hsp12 expression (as an indirect readout of PKA activation) is additive upon disruption of gpa2 and vma5. The authors need to do other experiments in the gpa2 disrupted strain where complications of the effect of glucose on PKA via this route do not exist. Thus, for example, the experiments shown in parts A and B of Fig.4 should be repeated in the double deletion strain. In this case, the authors model predicts that Msn2 should not undergo translocation to the nucleus upon glucose depletion

and cAMP should not increase in response to glucose addition."

As suggested by the reviewer, we have included cAMP measurements (in vma5Δ; gpa2Δ and vma2Δ;gpa2Δ) and Msn2p relocalization data upon readdition of starved cells in *vma5*Δ single and *vma5*Δ*;gpa2*Δ double mutants. As expected, the double mutants lead to a strongly enhanced defect in PKA pathway activity upon glucose addition to starved cells (revised Figures 5B and 5D).

3. "This manuscript is unnecessarily lengthened and complicated by the multitude of methods used to mimic glucose depletion (addition of deoxyglucose, switch of carbon source from glucose to galactose in a strain disrupted in Pgm2, disruption of pyruvate kinase) and in the indirect readouts used as a measure of PKA activity (Msn2 localization, Hsp12 expression). The authors should employ a single method (for example glucose depletion) and a single readout (for example Msn2 localization) throughout the manuscript. The authors should also make it clear that they are using only indirect measures of PKA activity and that their results therefore need to be interpreted with caution."

In the original manuscript we have included experiments that independently mimic glucose starvation using both genetic means and with the glucose analog 2-DOG to address the regulation of cytosolic pH, V-ATPase assembly and PKA pathway activity. We believe that these experiments are complementing each other rather than simply showing the same effects with multiple means. Specifically, we designed those experiments to carefully address the metabolic requirements of the regulation of pH and PKA pathway regulation. Glucose starvation leads to the inactivation of all glucose sensors, including the Gpr1p/Gpa2p module regulating PKA in response to extracellular glucose levels. In contrast, addition of excess 2-DOG, while keeping glucose concentration constant, allows us to specifically inhibit intracellular signals activating PKA. Thus, this experiment increases the specificity of the treatment and allows for additional conclusions. We therefore prefer to include these data in the revised version, but have rewritten these paragraphs to clarify these issues. Moreover, we have reorganized the figures to minimize complications due to the different assays used to monitor PKA pathway activity. In particular, we present all the experiments using the pHSP12qV reporter now in a single supplementary Figure (new Figure S5), and use only PKA-pathway specific readouts (cAMP measurements and Ras2p-GTP loading) together with Msn2p localizations in the main Figure (new Figure 5). Where necessary, we now clearly indicate the indirect nature of the PKA activity assays.

4. "The authors use GFP-tagged Vma5p to monitor in vivo assembly, but they do not show that the remainder of the V1 domain dissociates upon glucose removal in vivo. Experiments following fluorescence localization of another V1 subunit (for example subunit A or B) should also be included to document that true dissociation of the V-ATPase complex is being followed as opposed to simply dissociation of subunit C. It is also important to document that the GFP tagged subunit C behaves the same way as the native subunit C with respect to glucose-dependent dissociation, assembly and activity. This can be done by using the more conventional IP/Western method to evaluate glucose-dependent dissociation and comparing the behavior of complexes in strains expressing the GFP-tagged and untagged protein."

In the initial characterization of the *in vivo* regulation of V-ATPase, we have performed experiments with Vma2p-GFP and Vma5p-GFP, both being functional fusions and reporting on V-ATPase disassembly. Both GFP-fusions have previously been used as *in vivo* markers for V-ATPase assembly ((Sambade et al, 2005; Seol et al, 2001; Smardon & Kane, 2007)). As requested by the reviewer, we included an additional panel following V-ATPase assembly using Vma2p-GFP in the Supplementary material (revised Figure S1). In addition, we have analyzed strains containing a marker for Vph1p (V0 sector) (revised Figure 1), and tested its functionality of the GFP fusion (revised Figure S2). We hope that inclusion of these additional data further establishes these markers for an *in vivo* assessment of V-ATPase assembly. As briefly discussed in the revised discussion of the manuscript, given the sensitivity of V-ATPase assembly to cytosolic pH, we believe that this *in vivo* assay more accurately reflects V-ATPase assembly compared to immunoprecipitation experiments performed under constant pH conditions.

5. "In Fig.S3, the change in Hsp12 expression in a vma5 disrupted strain following addition of cAMP is quite small and unconvincing. The authors also do not discuss how addition of cAMP (20 mM) to the outside of yeast is resulting in increased cAMP inside the cells or document that such a

change in cytoplasmic cAMP actually occurs."

We agree that the change appears small with only the histograms from the FACS analysis presented, yet is statistically significant and reproducible. To demonstrate this, we now also include an additional panel (new Supplementary Figure S5) presenting the mean and standard deviation of 5 independent experiments to better demonstrate the observed changes. We decided to present these data in the Supplementary material, together with all measurements of pHSP12 activity, as HSP12 expression, as also discussed in the response to the other reviewers, only indirectly reflects PKA pathway activity.

cAMP is often used to activate the PKA pathway by addition to the growth medium, as it effectively permeates yeast cells. Indeed, addition of cAMP to the growth medium is sufficient to support growth in a strain deficient for adenylate cyclase, demonstrating its ability to enter cells. However, once cAMP enters the cells it is rapidly degraded through phosphodiesterases. Addition of cAMP is therefore more effective in cells deleted for the phosphodiesterase Pde2p. As this deletion also leads to hyperactivation of the PKA pathway, we decided to rather add high concentrations of cAMP, without further genetic interference with PKA activity.

6. "The effect of the D329N mutant of Vph1p on activity and assembly should be shown and the authors should explain why dissociation is delayed by more than 5 min (Fig.2F) and yet nuclear localization of Msn2p is delayed by only approximately 1 min."

As requested by the reviewer, experiments assessing the activity of the *vph1-D329N* mutation have been included as supplementary data (Figure S2). The discrepancy of the time delays for V-ATPase disassembly and Msn2p relocalization is probably caused by the redundancy in the activation of PKA by glucose. In this experiment, Msn2p is regulated by both V-ATPase and Gpa2p, yet only the V-ATPase input is affected by the D329N allele.

7. " The authors should explain why their model predicts a constitutively high activity for Ras even in the absence of glucose for the vma minus strain, since this was not really clear from either the Results or Discussion. The authors should also include in the Discussion a more complete description of the role of Ira proteins in activating Ras in response to elevated glucose, since this is a well documented pathway."

We have revised this section to account for the rather counterintuitive phenomenon. We initially omitted a further discussion of the role of the regulation of Ras by either the Ira proteins and/or Cdc25p, as our genetic data suggest that V-ATPase regulates the PKA pathway in parallel (or downstream of Ras2p). Nevertheless, for the sake of completeness, we have included a note on this regulation with appropriate references in the revised version of the manuscript.

8. "In Fig.5B the authors report a reduction of insulin in the media after treatment of Min6 cells with elevated glucose if the V-ATPase has been inhibited by concanamycin A. It is unclear, however, whether this is due to reduced insulin processing, interference in glucose-dependent signaling or due to a direct defect in fusion of insulin containing vesicles with the plasma membrane."

As referenced in the manuscript, the effect of V-ATPase inhibitors on insulin secretion has been reported previously. It was described that this phenotype cannot be explained by inappropriate processing of the insulin molecule, but could be caused by an effect on vesicle fusion (Sun-Wada et al, 2006). Our experiments now open a novel possibility, namely that V-ATPase contributes to glucose signaling and thus controls insulin secretion. We agree that the measurement of secreted insulin represents a rather indirect indication of defects in glucose signaling, and have thus also used CREB phosphorylation to follow PKA activity. In the revised manuscript we rephrased this section for clarification, and interpret the results with caution.

9. "In their discussion of previous results documenting the role of the Ras/cAMP/PKA pathway in control of V-ATPase assembly in yeast, they suggest that this result is not reflective of the in vivo situation but rather a positive feedback loop from PKA to V-ATPase assembly which can be detected in vitro but which is too weak to influence V-ATPase assembly in vivo. This ignores the observation, however, that in the YPH500 strain disrupted in either Ira1 or Ira2, the cells retain the red pigment indicative of assembled and functional V-ATPase complexes in vivo. The authors also need

to include a discussion of the considerable evidence for PKA regulating V-ATPase assembly in insect cells."

As the reviewer correctly states, there is indeed significant evidence for a regulation of V-ATPase by PKA in insect cells, but also mammalian cells. We have included a paragraph on this regulation in the discussion of the revised manuscript. These data from other systems might be reflecting a similar mechanism as the proposed feedback loop from PKA to V-ATPase activity. We proposed this feed back loop to explain conflicting results reported on a regulation of V-ATPase by PKA. It has been reported that disruption of *IRA2* is sufficient to retain V-ATPase assembly upon starvation *in vitro*, based on a biochemical pull-down assay, and promotes the formation of red pigment, indicative of increased V-ATPase activity on Raffinose media. In contrast, we present evidence that *in vivo*, deletion of *IRA2* does not affect V-ATPase disassembly. We note that the formation of red pigment is only an indirect readout of V-ATPase assembly and has been scored under different experimental conditions (raffinose medium rather that glucose starvation), which may allow detecting the effect of the negative feed back loop. However, as expression of genes required for Adenine metabolism is also strongly dependent on the environmental conditions, it cannot be excluded that transcriptional regulation of *ADE*-genes contributes to the formation of the red pigment in *ira2*Δ cells.

10. "In general this manuscript needs to be re-written in much clearer style."

We have rewritten a substantial amount of the manuscript. In particular, we expanded the introduction and discussion and have rephrased the result section to be more explicit and clear.

Referee #2 (Remarks to the Author):

1. "It is absolutely clear from the data shown that PKA is somehow differently wired in the V-ATPase mutant and thus certain responses are delayed. However, the connection of V-ATPase to PKA could be demonstrated more carefully. The genetic connection between the PKA pathway and the V-ATPase (also Figure S3B) can now be more quantitatively analyzed using the available datasets of Constanzo et al., (Science 327, 425). This could strengthen the proposed mechanisms. Additionally, a more global assessment of the influence of V-ATPase on the expression level of other PKA target genes might provide additional support since the HSP12 transcript level is also regulated by several other cues. Finally, the connection of V-ATPase to the PKA pathway might be further substantiated by determination of cAMP levels and PKA activity in mutants combining the V-ATPase and the GPCR glucose sensing complex including Gpa2 (e.g. vma2 gpa2)."

As suggested, we now included an assessment of the published genetic interactions between components of the Ras/PKA pathway and genes involved in V-ATPase assembly and activity. As also discussed in the response to the other reviewers, we have included a careful analysis of double mutants, defective in V-ATPase activity and the GPCR complex, assaying both cAMP and Msn2p relocalization in the revised Figure 5.

2. *"P8 L10: "...the mutation did not affect V-ATPase assembly or function ..." How was V-ATPase function tested in the Vph1-D329N mutant version? P8 L12: Which data are not shown?"*

We compared vacuolar accumulation of Quinacrine and Ca^{2+} sensitivity and growth on glycerol and high pH media of Vph1-D329N mutants, wild-type and *vph1*Δ strains, and did not observe any difference. We now included these data in Supplementary Figure S2. We also rephrased the results section to be more explicit.

3. P8 L14: "efficiently uncoupled" is perhaps overstated."

We agree that this phrase somewhat exaggerating and have changed the respective sentence. It now reads: "…this mutation allows partial uncoupling of V-ATPase assembly from glucose levels …"

4. *"P9 L1: Please give a reference for "reduced glycolytic flux"*

A reference has been inserted as requested.

5. "Figure 1: Treatment times should be indicated in the figures"

Treatment times have been included in the Figure legends and Figures as requested.

6. "Figure 2: E and F: D329N should be replaced with Vph1-D329N in the figure"

This has been changed accordingly.

7. "Figure 2: Does the Vph1-D329N mutant allele cause changes in morphology?"

We did realize that the Vph1p-D329N mutation does seem to contain more vacuoles that are of somewhat smaller size, but this difference is hard to quantify and we therefore did not comment on this.

8. "Figure 4C: The method for Ras-GTP measurement not described sufficiently in the materials and methods. The legend says Ras2-GTP and the figure Ras-GTP."

As also requested by other reviewers, we now added a method description in the revised Method section. As western blotting was performed with a Ras2p-specific antibody, it should indeed read Ras2p-GTP throughout the manuscript. We apologize for this inaccuracy.

9. "D: What are the units for HSP12 expression?"

The units are fluorescence intensities measured by FACS and as such are arbitrary units. For better readability, we have reformatted the quantifications to now represent relative increase in fluorescence by normalizing the values to 1 for the wild-type control in each panel (Figure S5).

10. "E: D329N should be replaced with Vph1-D329N."

This has been adjusted accordingly.

11. "Figures S1 and especially the genetic data of S4 are of more general interest and should be presented in the paper."

As suggested, we included one panel of Figure S1 into the main document. However, with the incorporation of additional data into Figure 4 we fear that providing the genetic data of Supplementary Figure S4 in the main text would lead to a data overload. Moreover, while the genetic interaction between *gpa2*Δ and mutations in components of V-ATPase is very consistent with a role of V-ATPase regulating the PKA pathway, these data on their own are not conclusive. Thus, we prefer to present more specific data (cAMP measurements, Ras2p-GTP and Msn2p localization) in the main text and keep the genetic data in the supplement. However, we included some double mutants described to display synthetic sickness in Figure S4 in the new panels of Figure 5. As discussed in the response to reviewer 1, we also show a meta-analysis of genetic interaction data from the Boone lab in the revised Supplementary Figure S5.

12. "For Figure S5 only a microscopic picture is shown, however, quantification as shown in other figures (Figure 2) for V-ATPase assembly is missing."

As requested, we have included an appropriate quantification.

13. "Figure 6 and S6: It is no clear why two schemata are presented. One general should be enough."

We separated these models to both convey the basic message of the manuscript in a simplified and easy to understand manner and also to be able to include a more detailed view, which hopefully allows the reader to understand the model presented in the discussion more easily. As suggested by reviewer 3 we have even expanded the supplementary model to include the feedback loops discussed. We therefore feel that this separation is still justified and hopefully contributes to the readability of the manuscript, yet at the cost of an additional Supplementary figure.

14. "Figure S6: The red hexagons representing glucose - are not optimal from a chemist's point of view."

We greatly appreciate the detailed and thoughtful comments of the reviewer. However, since glucose is a hexose, containing 6 C-atoms and in solution exists predominantly as the cyclic form (pyranose), with a ring structure containing 5 C-atoms and one O, we feel that it is justified to use a simple hexagon for the representation of glucose. We also note that the use of a hexagon for glucose is not unprecedented in the literature (Gancedo, 2008; Moriya & Johnston, 2004).

15. "Table S4: The quantitative contribution of the ATP/ADP ratio to H+ load of the cytosol is perhaps negligible. In this respect the title of table S4 is misleading. It is not the "ATP demand" but the ratio of amount of Pi or the ratio of ATP/ADP, which contributes to cytosolic H+. The quantitative contribution of these should be discussed."

We agree and have rephrased this section in the revised manuscript.

16. "The scoring method for the assembly of V-ATPase in cells is not described sufficiently. The Ras-GTP measurement should also be mentioned here - at least as a reference."

As also discussed in the response to the other reviewers, a more detailed description of the scoring method has been provided, including an additional panel in Supplementary Figure 1. We apologize for the omission of a better description of the Ras2p-GTP measurements. We have now included this in the Methods section and provide the appropriate reference.

17. "The authors might also consider a model including direct regulation of PKA such as factors downstream of Gpa2 via the kelch repeat proteins Krh1 and 2, which are negative regulators of PKA activity."

We have tried to genetically characterize how the V-ATPase dependent signal integrates with the major known inputs into the Ras/PKA pathway (Gpa2p and Ras2p). This analysis suggests that V-ATPase acts in parallel to Gpa2p and in parallel or downstream of Ras2p. We have not directly tested if the regulation of the PKA pathway by V-ATPase requires Gpb1p/2p (Krh1p/2p). However, Gpb1p/2p directly interact with Gpa2p and Ira1p/2p, and regulate Ira1/2 stability. Thus, Gpb1p/2p genetically act upstream of Ras. Moreover, phenotypes of *gpb1,2*ΔΔ cells are at least partially suppressed by deletion of *GPA2*. Thus, both observations rather suggest that V-ATPase does not act through regulation of Gpb1p/2p. Considering the already rather lengthy discussion of our manuscript, we prefer not to discuss this rather unlikely possibility, although admittedly at the cost of overall completeness.

18. "P16L11: I guess should it read "... glucose sensing via INactivation of ATP-dependent K+-channels ..." based on the reference given: McDonald et al., 2000.
"...ATP can inhibit K+ATP channels at the Kir6.2 subunit ... (Tucker et al. 1997; John et al. 1998; Mikhailov et al. 1998)"

We apologize for this typographical and unfortunate mistake, and have corrected the text accordingly.

19. "Reference (McDonald et al., 2000) should be marked as review and/or original literature be cited."

This has been changed accordingly.

20. "P16 L13: The conclusion " ... further suggesting ... similar principles in yeast and mammalian cells" is not clear based on the arguments mentioned in the paragraph. ATP inhibits mammalian ß-cell K+Channels and activates yeast H+ATPase. Please clarify the connection."

The analogy between yeast and mammalian cells in this context is based on the existence of an ATP sensitive ion-channel / ion-pump, which modulates the membrane potential in response to glucose in both systems. We agree with the reviewer that the analogy is somewhat reduced as ATP

activates P-ATPase, but closes the K+-channels in mammalian cells. We have rephrased this section to more explicitly mention the proposed similarity, while mentioning the evolutionary differences.

21. "P16 L18 " ... V-ATPase is required for cAMP production" Figure 4B shows that vma2 and vma5 deletion mutant produce less cAMP - V-ATPase is therefore not required."

We agree with the reviewer and have changed this sentence as follows: "V-ATPase is required for maximal stimulation of cAMP production upon glucose addition".

22. "P16 L21: This sentence needs rephrasing."

This revised sentence now reads: "Similarly, inhibition of V-ATPase activity using Concanamycin A impaired glucose stimulated CREB1 phosphorylation at a PKA specific site (S133), and reduced cAMP accumulation."

23. "P17 L3: Since PKA is INactivated by starvation it might be more clearer to state that the activity of PKA induces Glucose-dependent ASSEMBLY of the vacuolar (H+)-ATPase instead of the starvation induced disassembly."

We have used this admittedly somewhat cumbersome phrasing to appropriately report on the published data, but have rewritten this section for clarity. In fact, to our best knowledge, no evidence is reported in the literature that PKA activity is indeed required for V-ATPase assembly in *S. cerevisiae*. Rather, V-ATPase disassembly is studied under starvation conditions and found to be impaired in strains with elevated PKA activity. Our own unpublished data indicate that cytosolic pH is unaffected upon inactivation of Cdc25p, a critical activator of the PKA pathway. We therefore postulate the existence of a PKA-dependent feedback loop that regulates V-ATPase as described in the discussion (see also below).

24. "P17 L9: The here postulated feedback loop is difficult to understand. It is also not included in the schemata (Fig 6 and S6). Please explain the idea more clearly."

As suggested, we included this feedback loop in the revised Supplementary model (Figure S8). In addition, we have rephrased this section in the revised discussion.

Referee #3 (Remarks to the Author):

1. "The main weakness in this paper is the lack of connection between the V-ATPase and PKA. Both molecules show an effect of cytosolic pH. The V-ATPase dissociates in low pH. PKA is activated in high pH. Is there a causative connection here or are they parallel recipients of the pH signal? The authors offer a couple experiments to show a connection but none are direct. Does the Msn2-NLS-GFP construct contain the Ser582 site? Do mutations of the PKA site (Ser582Ala or Ser582Glu) eliminate the effect of the VPH1-D329N mutation?"

We have revised the manuscript to include more information about the Msn2p-NLS construct used for this experiment. S582 is indeed part of the Msn2p-NLS construct. In addition, this fragment contains more PKA-dependent sites, and simultaneous substitution of these sites to Ala does abolish glucose regulation of this fragment. Since the characterization of this mutation is published, and all glucose-dependent regulation is abolished, we feel that the analysis of this mutation does not add additional information for the present study. As discussed in the response to the other reviewers, we have thus included additional data on the influence of *VMA* deletion on PKA pathway activity and rephrased this section to better describe the data that lead us to conclude a causal link between V-ATPase and the PKA pathway.

2. "One strength of this manuscript is that it brings together two distinct fields of study. Because of this, the authors are obligated to provide sufficient background information for readers from both fields to understand the experimental design. The introduction does not provide sufficient background for those coming from the glucose sensing field. The intro needs to include more basic information about the V-ATPase - namely that it is a large macromolecular complex that is conserved between yeast and mammals; that it is composed of two functional domains, V0 which is embedded in the vacuole membrane and V1 which reversibly associates with V0. This information is

critical for the reader to interpret data in Figures 1 and 2. The authors use Vma5-GFP to monitor V-ATPase disassembly and they use Vph1-GFP as a control. This only makes sense if the reader knows that Vph1 is part of the V0 complex and that Vma5 is part of the V1 complex."

We have changed the introduction to provide more background information on V-ATPase regulation, and included a schematic drawing of V-ATPase in Figure 1 to improve understanding for non-specialists.

3. "The authors often state the pH is a regulator of PKA. However, the data in Figure 4B and 4C suggest that pH is a regulator of cAMP levels, and not PKA directly. Is there a way to determine whether cytosolic pH is affecting adenylate cyclase or phosphodiesterase? In any case, the authors need to be more careful with their words and should say that pH regulates the PKA pathway rather than PKA itself."

As also suggested by the other reviewers, we have revised the manuscript and refer to the "PKA pathway" instead of PKA, where only indirect evidence for PKA activity is presented. Currently, the data do not allow us to unambiguously discriminate between a regulation of adenylate cyclase or phosphodiesterase by V-ATPase. Two lines of evidence however suggest a regulation of adenylate cyclase rather than phosphodiesterases. First, the cAMP measurements in the Min6 cell line were performed in the presence of IBMX, an inhibitor of phosphodiesterase, yet we demonstrate a reduced cAMP content in the presence of the V-ATPase inhibitor, suggesting reduced adenylate cyclase activity. Second, we observe similar kinetics, but reduced amplitude of cAMP accumulation, suggesting that the feedback loops responsible for the largely transient nature of cAMP accumulation are unaffected. In contrast, overexpression of PDE1 leads to reduced but sustained accumulation of cAMP (Ma *et al*. Mol.Biol.Cell, 1998). Accordingly, we discuss this alternative possibility in the discussion and have adjusted the model in Supplementary Figure 6.

4. "Is it possible to perform a direct assay of PKA activity in cells with high and low cytosolic pH? They have shown that they can manipulate cytosolic pH by use of 2,4-DNP. Does this treatment affect PKA activity? Either PKA activity in a kinase pull-down assay or a western detecting the phosphorylation of a known PKA site (Ser 582 of Msn2) would greatly support the idea that pH regulates PKA."

Unfortunately, it is not possible to perform kinase assays from pull-down experiments with PKA that accurately report on *in vivo* PKA activity. This is caused by the fact that during cell-lysis, cAMP levels are reduced leading to reassociation of catalytic subunits of PKA and its regulatory subunit, Bcy1p.

However, we have measured PKA activity using the phosphospecific antibody recognizing S582 of Msn2p by western blotting in *pgm2*Δ cells (Figure 3). In this mutant, cytosolic pH is specifically reduced on galactose, which correlates with reduced PKA activity and can be suppressed by addition of exogenous cAMP. Furthermore, we analyzed the Msn2p-NLS reporter under several conditions reducing cytosolic pH and observed robust nuclear localization due to reduced PKA activity.

It should also be noted that multiple studies suggest that intracellular acidification using several ionophores including 2,4-DNP, leads to the activation of the PKA pathway (Colombo et al, 1998; Trevillyan & Pall, 1979). However, *in vivo*, glucose triggers a significant increase of cytosolic pH rather than acidification (Figure 1, (Ma et al, 1997; Orij et al, 2009), while clearly activating PKA. Moreover, 2,4-DNP could trigger cAMP production also in a strain deleted for the RasGEFs, Cdc25p and Sdc25p (Colombo et al, 1998), which are essential for PKA activation by glucose (Folch-Mallol et al, 2004; Gross et al, 1992; Van Aelst et al, 1990). In contrast, addition of 2-DOG effectively reduces cytoplasmic pH, but fails to trigger a cAMP response (Joëlle Rosseels and JW, unpublished data). Thus, activation of the PKA pathway by 2,4-DNP does not seem to reflect activation of PKA by glucose under physiological conditions. Rather, it appears likely that activation of Ras/PKA activity might have been caused by indirect effects of 2,4-DNP.

5. "Experimental protocol with the microfluidics chamber is not explained or referred to unless you read the Materials and Methods section. My first examination of Figure 1 B,C and D left me wondering how the cells were switched between media so rapidly. The authors use a very sophisticated method and it should be mentioned in results section and figure legend."

As suggested, we have included an additional panel in Supplementary Figure 1 to illustrate the technique and have more clearly referred to this method in the revised text and figure legend.

6. "What are the units on the Y axis for the nuclear localization of MSN2 (Fig 4A) and the relative assembly of the V-ATPase (Fig 1B, 2F)? In some figures they use % assembled? Is there some reason they have to use arbitrary units instead of % assembled?"

The discrepancy in the two different scoring methods stems from the fact that only time course experiments performed in the microfluidic chambers were automatically quantified using MATLAB based programs. In the original Figures 2B and 2D, cells were manually scored for V-ATPase assembly, while in Figures 1B, 2F, 4A, and 4F, V-ATPase assembly and Msn2p-localization were scored automatically. To apply a single quantification method throughout the paper we have repeated the experiments in Figures 2B and 2D and now also applied automated scoring. For V-ATPase the Coefficient of Variation of the pixel intensity of the total cellular fluorescence was calculated, while the Coefficient of Variation of the pixel intensity of the 500 brightest pixels was used to quantify Msn2p-GFP localization (a region approximately twice the size of the nucleus). To better illustrate the scoring method, we have included an additional panel in Supplementary Figure S1.

References:

Colombo S, Ma P, Cauwenberg L, Winderickx J, Crauwels M, Teunissen A, Nauwelaers D, de Winde JH, Gorwa MF, Colavizza D, Thevelein JM (1998) Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signalling in the yeast Saccharomyces cerevisiae. *EMBO J* **17**(12)**:** 3326-3341

Folch-Mallol JL, Martinez LM, Casas SJ, Yang R, Martinez-Anaya C, Lopez L, Hernandez A, Nieto-Sotelo J (2004) New roles for CDC25 in growth control, galactose regulation and cellular differentiation in Saccharomyces cerevisiae. *Microbiology* **150**(Pt 9)**:** 2865-2879

Gancedo JM (2008) The early steps of glucose signalling in yeast. *FEMS Microbiol Rev* **32**(4)**:** 673-704

Gross E, Goldberg D, Levitzki A (1992) Phosphorylation of the S. cerevisiae Cdc25 in response to glucose results in its dissociation from Ras. *Nature* **360**(6406)**:** 762-765

Ma P, Goncalves T, Maretzek A, Dias MC, Thevelein JM (1997) The lag phase rather than the exponential-growth phase on glucose is associated with a higher cAMP level in wild-type and cAPK-attenuated strains of the yeast Saccharomyces cerevisiae. *Microbiology* **143 (Pt 11):** 3451-3459

Moriya H, Johnston M (2004) Glucose sensing and signaling in Saccharomyces cerevisiae through the Rgt2 glucose sensor and casein kinase I. *Proc Natl Acad Sci U S A* **101**(6)**:** 1572-1577

Orij R, Postmus J, Ter Beek A, Brul S, Smits GJ (2009) In vivo measurement of cytosolic and mitochondrial pH using a pH-sensitive GFP derivative in Saccharomyces cerevisiae reveals a relation between intracellular pH and growth. *Microbiology* **155**(Pt 1)**:** 268-278

Sambade M, Alba M, Smardon AM, West RW, Kane PM (2005) A genomic screen for yeast vacuolar membrane ATPase mutants. *Genetics* **170**(4)**:** 1539-1551

Seol JH, Shevchenko A, Deshaies RJ (2001) Skp1 forms multiple protein complexes, including RAVE, a regulator of V-ATPase assembly. *Nat Cell Biol* **3**(4)**:** 384-391

Smardon AM, Kane PM (2007) RAVE is essential for the efficient assembly of the C subunit with the vacuolar H(+)-ATPase. *J Biol Chem* **282**(36)**:** 26185-26194

Sun-Wada GH, Toyomura T, Murata Y, Yamamoto A, Futai M, Wada Y (2006) The a3 isoform of V-ATPase regulates insulin secretion from pancreatic beta-cells. *J Cell Sci* **119**(Pt 21)**:** 4531-4540

Trevillyan JM, Pall ML (1979) Control of cyclic adenosine 3',5'-monophosphate levels by depolarizing agents in fungi. *J Bacteriol* **138**(2)**:** 397-403

Van Aelst L, Boy-Marcotte E, Camonis JH, Thevelein JM, Jacquet M (1990) The C-terminal part of the CDC25 gene product plays a key role in signal transduction in the glucose-induced modulation of cAMP level in Saccharomyces cerevisiae. *Eur J Biochem* **193**(3)**:** 675-680

2nd Editorial Decision 26 May 2010

Thank you for sending us your revised manuscript. Our original referees have now seen it again, and you will be pleased to learn that in their view you have addressed the criticisms in a satisfactory manner, and that the paper will therefore be publishable in The EMBO Journal.

Before this will happen, however, I was wondering whether you would like to consider addressing the minor issues suggested by referee 2 (see below). Please let us have a suitably amended manuscript as soon as possible. I will then formally accept the manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2 (Remarks to the Author):

The authors have addressed the points and have improved the manuscript. A broader analysis of transcription of PKA responsive genes other than HSP12 (as suggested in my previous comment) might have been supportive, however as a whole, the body of evidence is convincing. I have only a few minor remarks.

Some editorial changes might still improve the manuscript:
1) Introduction P5 L3: the statement that ...PKA is the major glucose sensing pathway ... does not give credit to the essential role of AMPK. PKA is one of the two major glucose sensing pathways. In fact, PKA can be eliminated and cells still respond to glucose.
2) Figure 5: To point (8) of the previous review: despite the claim in the rebuttal letter „Ras-GTP" is in the figure but not Ras2-GTP.
3) Figure S5: The added e-map of genetic interactions would profit from the inclusion of a colour bar to get an idea of the quantitative aspect of the genetic interactions.
4) Figure S7: A final comment to the discussion about graphics: as a teacher witnessing decline of chemical understanding of students, I have the opinion that a hexagon is not an appropriate symbol for glucose (even if other people have used this): the 6 corners of glucose are not equal, the molecule is not planar and one carbon is not part of the ring. The authors might consider either using a symbol that is not cyclohexan or a graph that is more like glucose.

Referee #3 (Remarks to the Author):

The authors have revised the manuscript and have addressed all the concerns raised by this reviewer.

2nd Revision - authors' response 28 May 2010

Thank you very much for your mail and your decision on the review process of our manuscript EMBOJ-2010-73801R, entitled *"Cytosolic pH is a second messenger for glucose and regulates the PKA pathway via V-ATPase"*. Following the suggestions of the reviewer, we have now prepared a modified version of the manuscript to address the remaining issues. Once again, we would like to thank you and the reviewers for all the efforts in the review process.

