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Hexokinase II Knockdown Results in Exaggerated Cardiac Hypertrophy via Increased ROS Production

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(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 09 September 2011

Thank you for the submission of your manuscript "Hexokinase II knockdown in the mouse heart results in exaggerated hypertrophy via increased ROS production" to EMBO Molecular Medicine. You will see that the reviewers find the topic of your manuscript potentially interesting. However, they also raise significant concerns on the study, which should be addressed in a major revision of the manuscript.

Importantly, Reviewer #3 highlights that glucose and fatty acid oxidation should be investigated while Reviewer #1 highlights that a differentiation between ROS production and accumulation should be included. In addition, both Reviewers #1 and # point out that the data in Fig 3A should be improved.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the time constraints outlined below.

However, we realize that addressing all of the referees' criticisms might require a lot of additional time and effort and be technically challenging. I am uncertain whether you will be able (or willing) to return a revised manuscript within the 3 months deadline and I would also understand your decision if you chose to rather seek rapid publication elsewhere at this stage.

Should you decide to embark on such a revision, revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions.

Should you find that the requested revisions are not feasible within the constraints outlined here and choose, therefore, to submit your paper elsewhere, we would welcome a message to this effect.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1:

In the presented study Wu and colleagues evaluate the in vitro and in vivo effects a knockdown of Hexokinase on cardiac plasticity. Pressure overload experiments demonstrate exaggerated hypertrophic responses in heterozygous HK-II-knockout mice, si-RNA-mediated knockdown of HK-II also shows aggravated hypertrophy in Ang-II stimulated NVCM. Increased levels of reactive oxygen species (ROS) are argued to be the mediator of these hypertrophic effects. A lack or mitochondrial dissociation of HK-II is demonstrated to be associated with an increase in mitochondrial permeability and a reduction of cellular anti-oxidant activity.
Wu et coworkers conclude that the reduction of cellular HK-II levels aggravates hypertrophic responses to pathologic hypertrophic stimuli (TAC and Ang-II) by increasing cellular ROS production. These elevated ROS levels are attributed to increased mitochondrial permeability and reduced anti-oxidant activity.

The presented study addresses an interesting aspect of myocardial responses to hypertrophic stimuli and the potential crosstalk and multifacetted roles of proteins in the heart. The in vitro and in vivo experiments attempt to further elucidate these mechanisms of cardiac plasticity.

Specific comments:
1. The merely descriptive nature of the study is noted. More mechanistic and deeper analysis of the interesting findings presented in the study would definitely strengthen the impact of the data. Generally, associations detected in the study are often advocated to be of causative nature; however, the data does not mandate this causative statement. Surprisingly, a differentiation between production of ROS and the mere accumulation of ROS is not made or attempted. Instead the title of the paper suggests clear evidence of hypertrophy due to increased ROS production.

2. The abstract requires improvements in structure and language. The key findings could be stated more clearly.

3. There are problems with the presentation of immunostainings. The scaling bars are potentially misleading, although the conclusions and interpretation might still be applicable.
Concerning the numerous immunostainings (3B, 4D, 4F, 5A, 6C, 6E, 7A, 7C) it seems mandatory to the reviewer that all the images in one (sub)figure are presented in equal size. Scaling bars should not only indicate the same metric size but should be presented in equal size to visualise the effect of a treatment (e.g. treatment with Ang-II) to the reader. Some images lack scaling completely (1C, 2A).

4. Western blots do not show molecular weight marker.

5. Figure 1A first bar has no illustration of a SEM which seems odd, similarly 1E.

6. The M-Mode echo-images (fig. 1D) do not match and illustrate the reduction in Fractional Shortening demonstrated in Fig. 2E.

7. The Kaplan blot in 2D should have the y-axis start at 0%, also the red line should be denoted as non-continous in the legend as well.

8. Fig. 3A lacks explanation: antibodies used, ladder, etc. If - based on the incomplete explanations

provided - interpreted correctly by the reviewer the following is noted: Lane 2, 7, 8 look the same. Only lane 1 exhibits less protein. The conclusion derived from the data seems questionable on the basis of the presented Western blot, one cannot use this WB for densitometry. How often was the blot performed, maybe a more convincing blot can be shown.

9. Cell size analyses are presented throughout the study. How many times were these repeated? From the description the reviewer understands that a number of 75 cells per condition was counted once. This would not seem sufficient; the experiment should be repeated at least three times. In light of the above, the SEM (of the analysis of 75 cells) appears surprisingly narrow to the reviewer.

10. The densitometric analysis in 4C does not seem to match the WB results shown in 4B. Would an induction of aDNP be expected in Ang-II stimulated cells? The normalization by Ponceau exhibits marked differences.

11. Concerning Fig 4D-G the reviewer wonders whether a positive control for ROS induction might strengthen the demonstrated data. Is Ang-II to be expected to induce ROS 3-4 times in controls?

12. Fig 5B: Would you expect NAC to attenuate/reduce Ang-II mediated hypertrophy by 30%? Generally, a control (baseline data without Ang-II treatment) would validate the results presented in Fig. 5 A-E.

13. Fig 6 F-H lack the ctrl scram + NAC.

14. Fig. 7 A: A prohypertrophic effect of Angiotensin in the si-Ctrl panel as well and maybe more importantly in the siHK-II panel cannot be detected in the presented data.

15. Fig. 8 How does the (lack of) effect of Ang-II in regard to anti-oxidant activity relate to the current literature and the observed induction of ROS (compare Fig. 4).

16. Unfortunately there is no explanation how statistics/ statistical significance were calculated (t-test, ANOVA, program used, etc.).

Referee #2:

Wu et al. have examined the effects of generalised hemizygotic deletion of one allele of the hexokinase II (HKII) gene on the propensity of mice to develop cardiac hypertrophy. HKII is the isoform of HK that binds to the outer mitochondrial membrane. The rationale underlying the investigation is that hypertrophied hearts are supposedly more reliant on carbohydrate metabolism and less reliant on lipid metabolism than 'normal' hearts (though whether this is a consequence of reduced cardiac reserve and subliminal cellular hypoxia is not very clear, as far as I am concerned). Some investigators have extended this idea to proposing that the switch in fuel metabolism is an initiating factor in the development of cardiac hypertrophy. Personally, I have never been convinced by this hypothesis because I have always believed that the 'metabolic fuel switch' is a response, rather than a cause (see also this MS, page 10 lines 1-2). Be that as it may, if the 'metabolic fuel switch' hypothesis has any substance, it could be argued that reduction in carbohydrate utilisation should reduce the hypertrophic response to pro-hypertrophic interventions such as transverse aortic constriction (TAC). This was the starting point for this investigation. In fact contrary to the investigators' predictions, the hypertrophic response in the HKII+/- heterozygotes in response to TAC was exacerbated compared with the wild-type (WT) littermate controls. The investigators then examined the possible causes for the exacerbated hypertrophy. They suggest that one factor may be increased oxidative stress because of a decrease in NADPH reducing equivalents possibly provided by the pentose phosphate pathway (PPP), in which HKII-catalysed production of glucose-6-phosphate is supposed to play a role. A second cause may be that a reduction in HKII and hence in HKII-binding to mitochondria leads to opening of the mitochondrial permeability transition pore and an increase in oxidative stress. Oxidative stress does activate some of the intracellular signalling pathways that are thought to be involved in the development of the 'adaptive phase' of cardiac hypertrophy, though personally I think that oxidative stress is likelier to be involved in maladaptation and heart failure.

SUBSTANTIVE POINTS

1. Fig. 1C. It would be useful to show the morphological results for the sham mice here, if they are readily available.

2. Fig. 1D. It appears to me from the echocardiographic traces that the 4-week TAC/HKII+/- hearts are hypercontractile compared with the 4 w TAC/WT. Is this correct? However, Fig. 2E shows a lack of difference in fractional shortening.

3. Fig. 3A. What do the two sets of blots show? I presume that the upper blot is HKII and the lower blot is a reference protein, but which reference protein was used? I must admit that I was not entirely convinced by the blots and I was not sure that it was really necessary to include this information. In any case, the blots do not show an increase in 'total HKII expression' (page 6 line 1), they show an increase in HKII expression relative to some undefined protein.

4. Page 8 last paragraph. I think that the authors are being excessively speculative in implicating the PPP alone in provision of NADPH. I would not mention the PPP in this context because the authors have not been any direct measurements of its activity. It could be argued that the changes in NADPH/total NADP and GSSG/total glutathione are a response to activation of the MPTP. This point could be mentioned speculatively in the Discussion, though the authors even over-speculate even in the current Discussion (page 9 line 19).

5. Page 10, discussion on AMPK. I am not convinced that this paragraph is relevant. The glycogen super-compensation hypothesis may be relevant to skeletal muscle glycogen loading (as seen in trained endurance athletes), but I fail to see its relevance to TAC as there is no removal of the stress condition. If the authors wish to introduce this speculation into their discussion, they should have measured glycogen levels.

By the way, although Drug Discov Today Dis Mech 2005 is not cited in PubMed, the reference should be to Ashrafian & WATKINS.

6. Page 10 line 20. The use of 'glucose uptake' is not appropriate here. The GLUTs are largely responsible for glucose uptake. HKs are responsible for phosphorylation. Use 'glucose uptake and/or phosphorylation'.

7. The authors return to the PPP on page 11. Any speculation on the PPP in the Discussion should be contiguous.

8. I assume that the statistical test used would have been an unpaired t test, but I cannot see that this has been specifically stated

LESS IMPORTANT POINTS

1. Page 3 lines 27-28, lower glucose and insulin levels than what (presumably WT)? In fact, the glucose tolerance test responses in WT and HKII+/- are similar and it is only at 60 min that they show any slight divergence (Heikkinen et al. 1999, Fig. 5B). I suggest that the authors delete this. It is not really relevant and is somewhat counterintuitive. It is also somewhat contradicts the work on exercise tolerance and fat feeding where skeletal muscle glucose uptake in the HKII+/- is less than in the WT.

2. Page 8 line 4. Are the data on cell death with N-terminal HKII peptide shown? I cannot see them. If they are shown, mention where they are shown.

3. Page 15 lines 1-2. Did the authors measure 'total NADPH/NADP levels' or NADPH/total NADP (see Fig. 8A), i.e. NADPH/NADPH plus NADP+? The same point arises with the measurement of glutathione (see a few lines below). Total glutathione is GSSG + GSH, GSH:GSSG is the ratio between reduced and oxidised glutathione. This type of problem re-emerges in the legend to Fig. 8. Apart from the total NADP/NADPH type of problem (in which the numerator and denominator now appear to have been inverted, page 24 line 3), total NAD(P)H means NADPH + NADH.

4. Definition of abbreviations could be improved. In some cases, they are defined repeatedly (e.g. TAC), in others (e.g. FS for fractional shortening), they are not defined at all.

MINOR POINTS

In some places, phraseology and use of English could be improved. By way of example, an (incomplete) list includes the following :

Page 3 lines 11-12, when referring to HKII, it would be better to refer to independently-active catalytic domains than 'two similar halves'.
Page 3 line 25, 'homozygotic', not 'homozygote'; line 27, 'heterzygotic'.
Page 4 line 8 and elsewhere, 'dissociation' would be better than 'dislocation'.
Page 5 line 3, it is the HK enzymes that are 'rate-limiting', rather than HKII specifically. This point should be made in the Introduction, rather than in the Results section.
Page 4 line 5 and elsewhere: 'compared with', not 'compared to'. 'Compare to' is used to point out a similarity as in 'Shall I compare thee to a summer's day?'
Fig. 2G. Labelling of the ordinate, LW/BW is a ratio. Should it not be unitless, rather than 'arbitrary units'?
Page 6 last line, 'reduced the values of these variables following HKII knockdown to' would be better (current phrasing is clumsy and, in general usage, parameter means boundary or limit).
Page 7 line 7, the infinitive (to study) should not be split by the adverb (specifically).
Page 7 last line, cite Fig. 7A after 'after 24 h', rather than at the end of the next sentence.
Fig. 8A. The ordinate in Fig. 8A should be labelled 'NADPH/total NADP', should it not?
Page 10 line 16, 'major player' is not really appropriate scientific terminology.
Page 14 line 10, 'Zeiss'.

Referee #3:

SUMMARY

Wu et. al. use a gain/loss of function approach to look at the effects of reduced hexokinase-II (HKII) during hypertrophy. They found that the HKII +/- mouse hearts were more dysfunctional and hypertrophic than WT mice following TAC. According to siRNA experiments in NRCMs, reduction of HKII expression was sufficient to induce hypertrophy. According to experiments in which they administered NAC to NRCMs during hypertrophy, the authors suggest ROS production as the likely culprit in the generation of HKII-deficient cardiomyocyte hypertrophy. The authors also blocked HKII from binding to the mitochondria and showed that such blockade exacerbated ROS production and hypertrophy. The authors also show that MPT formation and reduction in NADPH and reduced glutathione levels are associated with HKII knockdown.

COMMENTS
Major:
1. The central question seems to be: "Are glucose oxidation and fatty acid oxidation altered in the HKII +/- hearts during TAC compared to WT TAC?". These data should be shown. All of the other changes reported by the authors could be secondary to these changes.
2. The authors report LVEDD and %FS as indicators of functional changes in the heart during TAC. Considering the equipment the authors have (Vevo), it is quite accessible to generate ventricular volume data, which is superior to simple one-dimensional changes in diameter (i.e. %FS). This concern is re-enforced by the apparent hyper-effectiveness of the HET hearts at 4wk TAC in Figure 1D.
3. Are the Ang II-induced changes (e.g. Fig 8) due to NADPH oxidase stimulated ROS? Or, is this an effect of hypertrophic changes?
4. Figures 6D, 7, & 8: Are these effects reversed by NAC (or other ROS scavengers/antioxidants)?
5. The method for MPT formation is not particularly clear. Please clarify what, exactly, was measured. Overall fluorescence will usually not decrease if there is a simple redistribution within the cell. In addition, more details regarding the imaging setup and conditions should be supplied (light source/power/exposure/objectives/etc).
6. The conclusions regarding the PPP are possible, but certainly not unequivocal based on the data

shown. The authors should consider mollifying their tone regarding alterations in the PPP, at least based on the data presently available.
7. Do the authors have data on the pressure gradient of the constriction? While the mice undergo the same surgery, there is variance in any surgical model. The authors do have in vitro data to support their claims, but it would benefit the manuscript if the authors showed that their control and KO mice are undergoing the same amount of pressure overload. This would assuage any doubt about the consistency in vivo model. After all, the in vivo data are potentially the most compelling component of the study.

Minor:
1) Figure 2D: no asterisk for significance.
2) Where were ANOVA and t-test used? For ANOVA, which post hoc analysis was used?

Referee #4 (Comments on Novelty/Model System):

Overall I very much like this study. The role of HKII in hypertrophy has never been studied before, and the paper provides considerable mechanistic insight into how a HKII may act in this context. The data are clear, thorough, and, for the most part, support the authors' conclusions. However, some additional experiments are required before the study is acceptable for publication. The authors need to test the effects of the n-HKII peptide on the thiols. They also need to actually examine the PPP is they are going to conclude that changes in this pathway are responsible for the changes in thiols. I would also like to see the effects of NAC treatment on the MPT response. For these reasons I recommend the paper be published if revised approprately..

Referee #4 (Other Remarks):

The study by Wu et al examines the effects of HKII depletion on the myocardial hypertrophic response. They demonstrate that mice heterozygous for the HKII gene exhibit an exacerbated hypertrophic response and cardiomyopathy following TAC, compared to wildtype controls. Knockdown of HKII or displacement of HKII from the mitochondria recapitulated this data in a cultured myocyte model. In both the in vivo and in vitro cases the enhanced hypertrophy was associated with enhanced ROS production, and treatment with an antioxidant attenuated the hypertrophy in vitro. Moreover, HKII depletion/dissociation induced mitochondrial permeability transition and a reduction in NADPH and GSH levels. The authors conclude that mitochondrial binding of HKII exerts an anti-hypertrophic effect by diminishing ROS production and maintaining anti-oxidant defenses.

Major Comments

1. The authors only examine the effects of the HKII siRNA on NADPH and GSH levels. They also need to test the effect of the n-HKII peptide on these thiols.

2. The authors imply that the alterations in the reduced thiol levels are due to changes in the pentose phosphate pathway. While this could certainly be the case, it could be equally possible that the enhanced ROS production is responsible for the decreases in NAPDH and GSH. To back up their statements, the authors should assess the activities of key enzymes in the PPP such as G6PD and/or 6PGD to see if HKII depletion does indeed affect the PPP.

3. The authors should also test whether NAC also attenuates the enhanced MPT response in the siHKII and n-HKII treated myocytes.

4. On a related note, the authors need to discuss why they think the robust MPT response seen in the HKII-depleted myocytes did not induce cell death (which would normally be expected).

1st Revision - Authors' Response 08 February 2012

We would like to thank the editor and the reviewers for their objective and constructive review of our manuscript *Hexokinase II knockdown in the mouse heart results in exaggerated hypertrophy via increased ROS production,* submitted by Wu, *et al.* The reviewers have raised several valid concerns and offered insightful suggestions that we have addressed and incorporated into the revised manuscript. We feel these changes have strengthened and expanded upon the original findings, creating a more cohesive and complete narrative. In the response below, we first address the specific concerns raised by the editor and then respond to the critiques from each individual reviewer. The original comments are highlighted in bold, followed by our response.

Response to the Editor

Reviewer #3 highlights that glucose and fatty acid oxidation should be investigated.

Response: We agree with the reviewer's suggestion. To address this concern, we generated additional $HKII^{+/-}$ mice and performed metabolic analysis on adult cardiomyocytes isolated from wildtype (WT) and $HKII^{+/-}$ mice exposed to either sham operation or transverse aortic constriction (TAC). Adult cardiomyocytes isolated from WT and $HKII^{+/-}$ mice demonstrate comparable levels of glucose metabolism when subjected to sham operation and increased glycolysis after TAC. $HKII^{+/-}$ cardiomyocytes displayed decreased glucose utilization compared to WT after TAC; however, this difference did not reach statistical significance. Rates of fatty acid oxidation were similar between the two groups after TAC. These results suggest the exaggerated hypertrophy observed with a reduction in HKII is not exclusively mediated by compensated changes in glucose metabolism, and implicate an alternative mechanism for the exaggerated response. The metabolic data are presented in Supplemental Figure S3. The procedures for adult cardiomyocyte isolation and the use of the Seahorse XF system to perform metabolic analysis are described in the supplemental methods section.

Reviewer #1 highlights that a differentiation between ROS production and accumulation should be included.

Response: The reviewer raises a valid point, and we acknowledge the important distinction between ROS production and ROS accumulation. To address this concern, we modified the manuscript to more accurately describe our results. We have also expanded on our original findings, and performed additional studies to better evaluate the sources of increased ROS. First, we agree that the data presented in figure 4A-D do not differentiate between ROS production and accumulation, and now refer to these findings as evidence of increased oxidative stress (4A and B) and ROS levels (4C and D). Mitochondrial permeability transition (MPT) is a source of ROS production under conditions of stress, resulting in both a transient ROS burst, as well as the potential induction of ROS in adjacent mitochondria (ROS induced ROS release - RIRR) (1). In the original manuscript we demonstrated increased MPT after AngII treatment with HKII knockdown, and show that dissociation of HKII from the mitochondria resulted in MPT. We now demonstrate that treatment with the antioxidant N-*Acetyl*-L-*Cysteine* (NAC) prevented the increased MPT observed with HKII knockdown or dissociation from the mitochondria, further implicating MPT as a source of ROS production (Figure 7). In addition, we examined the effects of HKII reduction on antioxidant expression and activity. We did not observe significant changes in Catalase, Superoxide Dismutase 1 (SOD1), and SOD2 protein levels after hypertrophic stimulation. More importantly, there were no significant differences between control groups and those with reduced HKII expression for any treatment condition. *In vitro* studies also showed that there was no significant change in total antioxidant activity with either HKII knockdown or dissociation. These data are now presented in Supplemental Figure S8 and S9. Taken together, the data implicate increased ROS production and not a decrease in antioxidant defense as the primary source of oxidative stress observed with reduced HKII expression or dissociation from the mitochondria. Overall, the data implicate a role for hexokinase and its mitochondrial binding in the attenuation of ROS production during the development of hypertrophy that is partially mediated through the effects of HKII on MPT.

In addition, both Reviewers #1 and # point out that the data in Fig 3A should be improved.

Response: We agree with this assessment and have repeated the experiment. The new data agree with the original observation; and the updated blot presented in Figure 3A more clearly demonstrates the increased total HKII expression observed after AngII treatment (12h, 250nM). The blot is now clearly labeled, and we regret the oversight in our original submission.

Referee #1:

The merely descriptive nature of the study is noted. More mechanistic and deeper analysis of the interesting findings presented in the study would definitely strengthen the impact of the data. Generally, associations detected in the study are often advocated to be of causative nature; however, the data does not mandate this causative statement. Surprisingly, a differentiation between production of ROS and the mere accumulation of ROS is not made or attempted. Instead the title of the paper suggests clear evidence of hypertrophy due to increased ROS production.

Response: We appreciate the reviewer's critique. After demonstrating that a reduction in HKII resulted in an exaggerated hypertrophy, we investigated possible mechanisms underlying this phenotype. Initial evidence indicated that a reduction of HKII increased oxidative stress levels and ROS accumulation after TAC or AngII treatment (Figure 4A-D), and we investigated increased ROS accumulation as a possible mechanism for the exaggerated hypertrophic response. First we demonstrated the involvement of ROS, as antioxidant treatment abrogated the exaggerated hypertrophy observed with HKII knockdown (Figure 5A-D). We then examined possible sources of increased ROS accumulation. Recent reports have implicated mitochondrial ROS in the development of hypertrophy, and increased HK expression and mitochondrial binding of hexokinase are known to reduce ROS levels (2-3). Thus, we focused on the mitochondria as a potential source of the increased ROS in our system, and showed that that the dissociation of HKII from the mitochondrial fraction led to increased superoxide production and *de novo* hypertrophy (Figure 6C-F) which was also attenuated by antioxidant treatment (Figure 6D-F). We then investigated MPT as a potential source of increased ROS in the setting of reduced HKII expression or dissociation from the mitochondria (Figure 7). In the updated manuscript, we demonstrate that treatment with NAC attenuates the increased ROS levels observed with HKII knockdown or dissociation from the mitochondria (Supplemental Figure S5). We also show that treatment with NAC prevented the increased MPT observed with HKII knockdown or dissociation (Figure 7). In addition, we now show that reduction of HKII or dissociation from the mitochondria did not result in a significant change in antioxidant expression or activity (Supplemental Figure S8 and 9).

The abstract requires improvements in structure and language. The key findings could be stated more clearly.

Response: We appreciate the reviewer's suggestion and have amended the abstract to more clearly describe the findings.

There are problems with the presentation of immunostainings. The scaling bars are potentially misleading, although the conclusions and interpretation might still be applicable. Concerning the numerous immunostainings (3B, 4D, 4F, 5A, 6C, 6E, 7A, 7C) it seems mandatory to the reviewer that all the images in one (sub)figure are presented in equal size. Scaling bars should not only indicate the same metric size but should be presented in equal size to visualise the effect of a treatment (e.g. treatment with Ang-II) to the reader. Some images lack scaling completely (1C, 2A).

Response: We agree with the reviewer's recommendation, and all images within each subfigure are now presented with equivalent scaling for proper visualization. Scale bars are now included in Figure 1C and 2A, and we regret the omission.

Western blots do not show molecular weight marker.

Response: The molecular weight marker is now included.

Figure 1A first bar has no illustration of a SEM which seems odd, similarly 1E.

Response: We have modified the layout of the figures so the error bars are now visible.

The M-Mode echo-images (fig. 1D) do not match and illustrate the reduction in Fractional Shortening demonstrated in Fig. 2E. The Kaplan blot in 2D should have the y-axis start at 0%, also the red line should be denoted as non-continuous in the legend as well.

Response: We thank the reviewer for their comment. We have replaced Figure 1D with a better representative echo-image. The y-axis for the Kaplan-Meier survival curve now starts at 0%, and the red line is denoted as non-continuous in the legend as suggested.

Fig. 3A lacks explanation: antibodies used, ladder, etc. If - based on the incomplete explanations provided - interpreted correctly by the reviewer the following is noted: Lane 2, 7, 8 look the same. Only lane 1 exhibits less protein. The conclusion derived from the data seems questionable on the

basis of the presented Western blot, one cannot use this WB for densitometry. How often was the blot performed, maybe a more convincing blot can be shown.

Response: We agree, please see our response to the editor in reference to this critique.

Cell size analyses are presented throughout the study. How many times were these repeated? From the description the reviewer understands that a number of 75 cells per condition was counted once. This would not seem sufficient; the experiment should be repeated at least three times. In light of the above, the SEM (of the analysis of 75 cells) appears surprisingly narrow to the reviewer.

Response: We regret the lack of clarity with regard to the *in vitro* cell size analysis. Briefly, total cell size was measured from a minimum of 5 separate fields taken from 3 independent experiments. The average (cell size/number of cells) was determined for each field, and a minimum of 75 cells total was evaluated for experimental condition. This is now more clearly described in the methods section. It is also important to note that cell size was not the only measure used to assess *in vitro* hypertrophy, and the data are corroborated by 2 additional markers (fetal gene expression and protein synthesis).

The densitometric analysis in 4C does not seem to match the WB results shown in 4B. Would an induction of aDNP be expected in Ang-II stimulated cells? The normalization by Ponceau exhibits marked differences.

Response: In our study AngII is associated with increased oxidative stress by the 24h time point, and this finding is corroborated by the increased ROS levels presented in Figure 4C-D. Dai, *et al.* have also shown increased protein carbonylation in hearts after AngII treatment, as well as in hypertrophied hearts from mice overexpressing Gaq (4). In their *in vitro* studies, changes in oxidative stress were observed at an earlier time point; however, a higher dose of AngII (1mM) was used. The differences in loading are reflected in graph, as the aDNP measurements were normalized to the Ponceau loading control.

Concerning Fig 4D-G the reviewer wonders whether a positive control for ROS induction might strengthen the demonstrated data. Is Ang-II to be expected to induce ROS 3-4 times in controls?

Response: The use of fluorescent probes to demonstrate increased ROS after treatment with hypertrophic agonists has been reported; however, the relative fold change may differ depending on the duration and concentration of the insult (4-5). Exogenous H_2O_2 or antimycin A were used as positive controls for these assays. However, they cause a significant increase in ROS levels at earlier timepoints, and ultimately resulted in increased cell death. As such, they were not analyzed for our hypertrophy studies.

Would you expect NAC to attenuate/reduce Ang-II mediated hypertrophy by 30%? Generally, a control (baseline data without Ang-II treatment) would validate the results presented in Fig. 5 A-E.

Response: Attenuation of AngII induced hypertrophy with NAC has previously been reported; however, the degree of attenuation may vary depending on the dose and time-course of the given treatments (6). Baseline data (without AngII) was acquired to verify the efficacy of AngII, and these data are now included in Figure 5A-D.

Fig 6 F-H lack the ctrl scram + NAC.

Response: These data are now included in Figure 6, and show that there was no difference between the scrambled peptide control (scram) and scram+NAC for any of the hypertrophy markers investigated.

Fig. 7 A: A prohypertrophic effect of Angiotensin in the si-Ctrl panel as well and maybe more importantly in the siHK-II panel cannot be detected in the presented data.

Response: We agree with this assessment, and have updated the images to better demonstrate the increased hypertrophic effect (i.e. calcein release) with HKII knockdown + AngII and HKII dissociation (Figure 7). We have also included a mitochondrial marker (Mitotracker Red) to better demonstrate changes in calcein distribution within the cell.

Fig. 8 How does the (lack of) effect of Ang-II in regard to anti-oxidant activity relate to the current literature and the observed induction of ROS (compare Fig. 4).

Response: These changes could reflect increased ROS production rather than a change in the antioxidant defenses, as other reviewers have suggested. We now demonstrate that there is no

significant difference in antioxidant expression or activity with reduced HKII expression when compared with control under any condition (Supplemental Figures S8 and S9). Collectively, these studies suggest that increased ROS production is driving the exaggerated response observed in our studies. Changes in NADPH and glutathione levels are now described in the context of oxidative stress, and presented in Supplemental Figure S10.

Unfortunately there is no explanation how statistics/ statistical significance were calculated (t-test, ANOVA, program used, etc.).

Response: We sincerely regret this oversight, and have included this information in the methods section.

Referee #2:

Fig. 1C. It would be useful to show the morphological results for the sham mice here, if they are readily available.

Response: The morphological results are now presented in Supplemental Figure S2.

Fig. 1D. It appears to me from the echocardiographic traces that the 4-week TAC/HKII+/- hearts are hypercontractile compared with the 4 w TAC/WT. Is this correct? However, Fig. 2E shows a lack of difference in fractional shortening.

Response: $HKII^{+/-}$ hearts are not hypercontractile compared with WT after TAC (4w). A more representative image is displayed in Figure 1D.

Fig. 3A. What do the two sets of blots show? I presume that the upper blot is HKII and the lower blot is a reference protein, but which reference protein was used? I must admit that I was not entirely convinced by the blots and I was not sure that it was really necessary to include this information. In any case, the blots do not show an increase in 'total HKII expression' (page 6 line 1), they show an increase in HKII expression relative to some undefined protein.

Response: We agree, and have updated this blot. Please see our response to the editor's concerns.

Page 8 last paragraph. I think that the authors are being excessively speculative in implicating the PPP alone in provision of NADPH. I would not mention the PPP in this context because the authors have not been any direct measurements of its activity. It could be argued that the changes in NADPH/total NADP and GSSG/total glutathione are a response to activation of the MPTP. This point could be mentioned speculatively in the Discussion, though the authors even over-speculate even in the current Discussion (page 9 line 19).

Response: We concede that the role of the Pentose Phosphate Pathway in the exaggerated hypertrophic response may be overstated based on our results. The central role of G6P, and thus HKII, in the PPP pathway seems to reasonably explain both the observed reduction in NADPH levels and the subsequent increase in oxidized glutathione; however, the role of the PPP in cardiomyocyte metabolism and the response to stress is still unclear. Furthermore, the reviewer makes the salient point that this result could be caused by increased MPT or perhaps dysregulation of some other intermediate in this pathway. A complete evaluation of the PPP in the hypertrophic response, along with all enzymes involved in both NADPH and GSH cycling would be required to sufficiently address this question, and goes beyond the scope of the current study. As such, we have incorporated the data into our results on sources of oxidative stress. In agreement with this position, we have significantly mollified our claims on the role of PPP in these findings and only comment on its potential involvement in the discussion.

Page 10, discussion on AMPK. I am not convinced that this paragraph is relevant. The glycogen super-compensation hypothesis may be relevant to skeletal muscle glycogen loading (as seen in trained endurance athletes), but I fail to see its relevance to TAC as there is no removal of the stress condition. If the authors wish to introduce this speculation into their discussion, they should have measured glycogen levels. By the way, although Drug Discov Today Dis Mech 2005 is not cited in PubMed, the reference should be to Ashrafian & WATKINS.

Response: We appreciate the reviewer's comments, and agree with the assessment. The paragraph has been removed, and the proper citation will be noted in the future.

Page 10 line 20. The use of 'glucose uptake' is not appropriate here. The GLUTs are largely responsible for glucose uptake. HKs are responsible for phosphorylation. Use 'glucose uptake and/or phosphorylation'.

Response: We thank the reviewer for highlighting this important distinction and have made the appropriate changes to the text.

The authors return to the PPP on page 11. Any speculation on the PPP in the Discussion should be contiguous.

Response: We have modified our results and discussion on the involvement of the PPP as described.

I assume that the statistical test used would have been an unpaired t test, but I cannot see that this has been specifically stated.

Response: We sincerely regret this oversight, and have included this information in the methods section.

LESS IMPORTANT POINTS

Page 3 lines 27-28, lower glucose and insulin levels than what (presumably WT)? In fact, the glucose tolerance test responses in WT and HKII+/- are similar and it is only at 60 min that they show any slight divergence (Heikkinen et al. 1999, Fig. 5B). I suggest that the authors delete this. It is not really relevant and is somewhat counterintuitive. It is also somewhat contradicts the work on exercise tolerance and fat feeding where skeletal muscle glucose uptake in the HKII+/- is less than in the WT.

Response: We regret any confusion with the presentation of the data and have amended the introduction to improve clarity.

Page 8 line 4. Are the data on cell death with N-terminal HKII peptide shown? I cannot see them. If they are shown, mention where they are shown.

Response: These data were reported in our previous study (7); however, evaluation of cell death after peptide treatment using both DAPI exclusion and Annexin V is now presented in Supplementary Figure S10.

Page 15 lines 1-2. Did the authors measure 'total NADPH/NADP levels' or NADPH/total NADP (see Fig. 8A), i.e. NADPH/NADPH plus NADP+? The same point arises with the measurement of glutathione (see a few lines below). Total glutathione is GSSG + GSH, GSH:GSSG is the ratio between reduced and oxidised glutathione. This type of problem re-emerges in the legend to Fig. 8. Apart from the total NADP/NADPH type of problem (in which the numerator and denominator now appear to have been inverted, page 24 line 3), total NAD(P)H means NADPH + NADH.

Response: We thank the reviewer for their suggestion, and regret the confusion. The axes are now clearly defined in the figures, legends, and the text.

Definition of abbreviations could be improved. In some cases, they are defined repeatedly (e.g. TAC), in others (e.g. FS for fractional shortening), they are not defined at all.

Response: We have made the suggested adjustments in our use of abbreviations.

MINOR POINTS – We thank the reviewer for the comments and have made changes to the manuscript.

Page 3 lines 11-12, when referring to HKII, it would be better to refer to independently-active catalytic domains *than 'two similar halves'.*

Page 3 line 25, 'homozygotic', not 'homozygote'; line 27, 'heterzygotic'.

Page 4 line 8 and elsewhere, 'dissociation' would be better than 'dislocation'.

Page 5 line 3, it is the HK enzymes that are 'rate-limiting', rather than HKII specifically. This point should be made in the Introduction, rather than in the Results section.

Page 4 line 5 and elsewhere: 'compared with', not 'compared to'. 'Compare to' is used to point out a similarity as in 'Shall I compare thee to a summer's day?'

Fig. 2G. Labelling of the ordinate, LW/BW is a ratio. Should it not be unitless, rather than 'arbitrary units'?

Page 6 last line, 'reduced the values of these variables following HKII knockdown to' would be better (current phrasing is clumsy and, in general usage, parameter means boundary or limit).

Page 7 line 7, the infinitive (to study) should not be split by the adverb (specifically).

Page 7 last line, cite Fig. 7A after 'after 24 h', rather than at the end of the next sentence.

Fig. 8A. The ordinate in Fig. 8A should be labelled 'NADPH/total NADP', should it not?

Page 10 line 16, 'major player' is not really appropriate scientific terminology.

Page 14 line 10, 'Zeiss'.

Referee #3:

The central question seems to be: "Are glucose oxidation and fatty acid oxidation altered in the HKII +/- hearts during TAC compared to WT TAC?". These data should be shown. All of the other changes reported by the authors could be secondary to these changes.

Response: We agree with the reviewer's critique, and evaluation of the metabolic phenotype is described in our response to the editor. These results are presented in Supplemental Figure S3 and offer further evidence that an alternative mechanism (ROS) plays a major role in the exaggerated hypertrophic response.

The authors report LVEDD and %FS as indicators of functional changes in the heart during TAC. Considering the equipment the authors have (Vevo), it is quite accessible to generate ventricular volume data, which is superior to simple one-dimensional changes in diameter (i.e. %FS). This concern is re-enforced by the apparent hyper-effectiveness of the HET hearts at 4wk TAC in Figure 1D.

Response: We appreciate the reviewer's suggestion, and now present the ejection fraction (%EF) for these studies (obtained from B mode) in figure 1D to better understand the functional changes in HKII+/- hearts during TAC.

Are the Ang II-induced changes (e.g. Fig 8) due to NADPH oxidase stimulated ROS? Or, is this an effect of hypertrophic changes?

Response: The exact mechanisms of AngII induced increased in ROS are still unclear. We would argue that AngII has both Nox dependent and independent effects on hypertrophy. How Nox specific ROS generation might influence ROS production and the MPT is an interesting question that is likely to involve multiple Nox isoforms and intermediate mechanisms that are beyond the scope of this study.

Figures 6D, 7, & 8: Are these effects reversed by NAC (or other ROS scavengers/antioxidants)?

Response: In the updated manuscript we have shown that NAC decreased mitochondrial superoxide levels after HKII dissociation (Figure 6C). We also show that NAC attenuates the increased MPT observed with HKII knockdown + AngII, as well as the MPT resulting from HKII mitochondrial dissociation (Figures 7).

The method for MPT formation is not particularly clear. Please clarify what, exactly, was measured. Overall fluorescence will usually not decrease if there is a simple redistribution within the cell. In addition, more details regarding the imaging setup and conditions should be supplied (light source/power/exposure/objectives/etc).

Response: We regret the lack of clarity with regard to our study of MPT, and have expanded the methods section to better explain our technique and imaging setup. In these studies we evaluated calcein redistribution from the mitochondria, and have updated the images and included a mitochondrial marker to better demonstrate this redistribution.

The conclusions regarding the PPP are possible, but certainly not unequivocal based on the data shown. The authors should consider mollifying their tone regarding alterations in the PPP, at least based on the data presently available.

Response: We agree, please see our response to reviewer 2 with regard to the PPP.

Do the authors have data on the pressure gradient of the constriction? While the mice undergo the same surgery, there is variance in any surgical model. The authors do have in vitro data to support their claims, but it would benefit the manuscript if the authors showed that their control and KO mice are undergoing the same amount of pressure overload. This would assuage any doubt about

the consistency in vivo model. After all, the in vivo data are potentially the most compelling component of the study.

Response: The pressure gradient was assessed in our initial studies, but not reported. The data are now presented in Supplementary Figure S4.

Minor – we appreciate the reviewer's suggestions and have made the changes accordingly.

1) Figure 2D: no asterisk for significance.

2) Where were ANOVA and t-test used? For ANOVA, which post hoc analysis was used?

Referee #4:

The authors only examine the effects of the HKII siRNA on NADPH and GSH levels. They also need to test the effect of the n-HKII peptide on these thiols.

Response: We appreciate the reviewer's suggestion. HKII dissociation from the mitochondria did not lead to a significant change in the thiol levels. The data are presented in Supplemental Figure S10.

The authors imply that the alterations in the reduced thiol levels are due to changes in the pentose phosphate pathway. While this could certainly be the case, it could be equally possible that the enhanced ROS production is responsible for the decreases in NAPDH and GSH. To back up their statements, the authors should assess the activities of key enzymes in the PPP such as G6PD and/or 6PGD to see if HKII depletion does indeed affect the PPP.

Response: We have amended our speculation on the involvement of the PPP in these studies, as described in our response to critiques from other reviewers.

The authors should also test whether NAC also attenuates the enhanced MPT response in the siHKII and n-HKII treated myocytes.

Response: We appreciate the reviewer's suggestion. The original data has been updated, clearly demonstrating increased calcein release (i.e. MPT) with HKII knockdown and dissociation, and now abrogation of this effect after treatment with NAC.

On a related note, the authors need to discuss why they think the robust MPT response seen in the HKII-depleted myocytes did not induce cell death (which would normally be expected).

We appreciate the suggestion, and understand the need to discuss these findings given the literature describing the prominent role of MPT in cell death. An expanded description and interpretation of our results, along with supporting literature citations are presented in the updated discussion section.

1. Zorov DB, Filburn CR, Klotz LO, Zweier JL, & Sollott SJ (2000) Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. (Translated from eng) *The Journal of experimental medicine* 192(7):1001-1014 (in eng).

2. da-Silva WS*, et al.* (2004) Mitochondrial bound hexokinase activity as a preventive antioxidant defense: steady-state ADP formation as a regulatory mechanism of membrane potential and reactive oxygen species generation in mitochondria. *J Biol Chem* 279(38):39846-39855.

3. Sun L, Shukair S, Naik TJ, Moazed F, & Ardehali H (2008) Glucose phosphorylation and mitochondrial binding are required for the protective effects of hexokinases I and II. *Mol Cell Biol* 28(3):1007-1017.

4. Dai DF*, et al.* (2011) Mitochondrial oxidative stress mediates angiotensin II-induced cardiac hypertrophy and Galphaq overexpression-induced heart failure. (Translated from eng) *Circ Res* 108(7):837-846 (in eng).

5. Sundaresan NR*, et al.* (2009) Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. (Translated from eng) *J Clin Invest* 119(9):2758-2771 (in eng).

6. Nakagami H, Takemoto M, & Liao JK (2003) NADPH oxidase-derived superoxide anion mediates angiotensin II-induced cardiac hypertrophy. (Translated from eng) *J Mol Cell Cardiol* 35(7):851-859 (in eng).

7. Wu R*, et al.* (2011) Reduction in hexokinase II levels results in decreased cardiac function and altered remodeling after ischemia/reperfusion injury. (Translated from eng) *Circ Res* 108(1):60-69 (in eng).

2nd Editorial Decision 29 February 2012

Thank you for the submission of your revised manuscript "Hexokinase II knockdown results in exaggerated cardiac hypertrophy via increased ROS production" to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it.

As you will see, the reviewers are now globally supportive and we are happy to inform you that we can proceed with the official acceptance of your manuscript once the points below have been convincingly addressed.

- Please see below for an excerpt from our Guidelines on Statistical analysis and mention the actual p value in each case.
- Please include a Table of Contents as the first page of the Supplementary Material
- In addition, please note that it is our standard procedure to request the original files from which the cropped figures were obtained for increased transparency during the editorial process. I would thus like to ask you whether you could provide us with the original western blots (uncropped, unmodified) used in your manuscript (specifically Fig 6B) when you return a revised version of it.

Statistical analysis:
The description of all reported data that includes statistical testing must state the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the actual P value for each test (not merely 'significant' or 'P < 0.05').

Revised manuscripts should be submitted within one month of a request for revision; they will otherwise be treated as new submissions, unless arranged otherwise with the editor.

We also must alert you to a very important impending change at EMM that will affect your article: The journal will convert to full open access in March 2012. We are sending you this notification ahead of a formal announcement, as your paper would be subject to the 'Article Processing Charge' (APC) of 3,000 Euros if it is accepted for publication after the open access conversion date. The APC replaces the current page charges, and there are no submission fees and no other additional costs. As with page charges, the APC may be waived in exceptional circumstance for authors who provide evidence that they are unable to pay the fees.

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Please contact Bernd Pulverer, Head of Scientific Publications, EMBO, with any questions or comments on the open access conversion of the journal (direct line: +4962218891501; bernd.pulverer@embo.org), or myself if the question regards the manuscript.

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

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1:

Well done

Referee #2:

I thought that this MS was an interesting contribution when I first reviewed it and rated it 'suitable for publication after minor revision'. The authors have attended to all of the points that I raised in my first review (which were relatively minor). Clarity has been improved. I think that the MS could now be accepted.

Referee #3:

Suitable for publication.

2nd Revision 15 March 2012

Note: As requested by the editor the authors have included the actual P value for each test.