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Manuscript EMBO-2013-87224

Regulation of G6PD acetylation by KAT9/SIRT2 modulates NADPH homeostasis and cell survival during oxidative stress

Yi-Ping Wang, Li-Sha Zhou, Yu-Zheng Zhao, Shi-Wen Wang, Lei-Lei Chen, Li-Xia Liu, Zhi-Qiang Ling Fu-Jun Hu, Yi-Ping Sun, Jing-Ye Zhang, Chen Yang, Yi Yang, Yue Xiong, Kun-Liang Guan, and Dan Ye

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Anne Nielsen

1st Editorial Decision 13 December 2013

Thank you for submitting your manuscript for consideration by The EMBO Journal and my apologies for the unusually long duration of the review period in this case. Your study has now been seen by two referees whose comments are shown below. As you will see, while the referees do express interest in the work and topic in principle, they do not offer strong support for publication in The EMBO Journal.

I will not repeat all their individual points of criticism here, but it becomes clear that ref #1 finds the depth of analysis, conclusiveness, and physiological significance of the reported model to be too limited, while ref #2 raises strong conceptual concerns in light of the existing literature on sirtuins acting in redox homeostasis and metabolic regulation. Clearly, an substantial amount of further-reaching experimentation would be required to address these issues and to bring the study to the level of insight and significance required for its publication here.

Given these negative opinions I am afraid we have little choice but to return the manuscript to you at the current stage with the message that we are unable to offer further steps towards publication in The EMBO Journal.

Thank you in any case for the opportunity to consider this manuscript and my apologies again for the delay in communicating our decision here. I am sorry that we cannot be more positive on this occasion, but we do hope that you will nevertheless find our referees' comments helpful.

REFEREE REPORTS:

Referee #1:

EMBO J Wang et al.

One of the major NADPH-producing enzymes is glucose-6-phosphate dehydrogenase (G6PD), the only NADPH-producing enzyme that is activated. It is an important enzyme to understand because it controls the rate-limiting and primary control step of the NADPH-generating portion in the pentose phosphate pathway and G6PD deficiency is the most common human enzyme defect, being present in more than 400 million people worldwide, possibly because it provides protection against malaria. Sirtuins (SIRT1-7) are NAD+-dependent deacetylases that are continuing to attract attention with almost 100 papers per year. Sirt2, the cytoplasmic sirtuin, is the least understood sirtuin with only a handful of papers.

This study by Wang et al. is directed towards understanding the molecular mechanism by which acetylation regulates G6PD activity to control NADPH homeostasis and oxidative stress response. They present evidence indicating that SIRT2-mediated decacetylation of G6PD controls its dimerization an activity.

The study is comprehensive in its analysis and is mostly convincing. It uses innovative techniques (e.g. cytosolic redox-sensitive green fluorescent protein 1 (HEK293roGFP1) and the data is well presented and of high quality. The use of KO animals further raises the level of this study and its relevance to the in vivo situation. Statistical analyses performed with a two-tailed unpaired Student's t-test are appropriate with a cutoff of 0.05.
There are some overstatements and missing key experiments. With some additional experiments it will be greatly improved. The study should be of interest to a wide audience interested in metabolism, oxidative stress, acetylation, malaria resistance, and aging.

Major Points

1. In figure 1A, the authors test NAM + TSA but not TSA. Does TSA alone affect acetylation, which would indicate that another HDAC is involved.

2. It is unclear why the authors would test cobB, a bacterial relative of a sirtuin, not a mammalian sirtuin from the outset. Was this simply more available?

3. In figure 1C-E, the authors state that "K403 is the major, if not the sole, regulatory acetylation site which plays a critical role in controlling G6PD activity." This is based on mutation and anti-Ac antibodies. The issue is that the conclusion can not be drawn. Anti-Ac antibodies are notorious for their lack of generality. A pan-Ac antibody is not "pan". Moreover, the authors don't show whether the other sites that affect activity also affect the ability of the Ab to detect the site. For example, is K171 or K386 K or Q detectable by the Ab and to what extent? Were these avoided because it would be a lot more work to study them? For this to be done well, the authors would need to use mass spectroscopy, which is less biased. Solution: either do mass spec, or show the effect of the K171 and K386 mutations on acetylation. As it is, the authors can not be so definitive and there as a

gaping hole.

4. Where do the other mutations that affect activity (K171, K386) lie within the G6PD structure? Can they authors explain why they lost activity.

5. The authors found that the substitution of K403Q, but not K403R, disrupted the interaction between G6PD subunits. Couldn't K171 or K386 K substitutions, which affect activity but presumably not dimerization, serve as negative controls?

6. In figure S3, the production of recombinant mutant G6PD is presented. The authors need to test if the protein has folded correctly, because this could also explain lack of dimerization and catalytic activity. This can be tested using a variety of methods including melting temperature and fluorescence polarization compared to WT.

7. The authors observed that G6PD "directly interacted with SIRT2, but not SIRT1 (Figure 3A and 3B)." But this is using overexpressed proteins. The endogenous interaction needs to be shown or doubts could be raised about its physiological significance.

8. The SIRT2-specific inhibitor AGK2 is good but compounds always have off targets. The authors need to test a SIRT2 knockdown/KO in the biochem/acetylation experiments to be sure the effect is specific. Ideally, with reconstitution mutant constructs. Similarly, in figure 5, inhibition of SIRT2 by AGK2 in the presence of menadione on ROS production is not sufficient. The G6PD knock-down in HEK293T and cells rescued by WT G6PD or the K403Q mutant (Supp. Figure 5) could be useful in this regard.

9. The authors should discuss how altering NAD+ or SIRT2 levels/activity could be therapeutic, against malaria for example, (see Shin Imai's work) and what impact the decline in NAD levels during aging could have. See Ding et al., 2013 "G6PD (Xq28)-were associated (P {less than or equal to} 1 ◊ 10(-6)) with RBC traits in the discovery cohort."

10. There is no information on Sirtuins in the introduction. What is known about Sirt2? The manuscript is too focused on G6PD and misses the opportunity to present the big picture and implications, linking sirtuins to glucose metabolism to oxidative stress resistance and diseases/aging.

11. There is new work indicating that GPPD deficiency confers an advantage against the development of cancer, reduces the risk of coronary diseases and has a beneficial effect in terms of longevity." See Manganelli et al., 2013. PMID: 23534950. Again, the authors miss an opportunity to discuss the wider implications.

Minor Points

12. Make sure the methods are complete. e.g. Where are the KO cells from? Whose mouse? What's the deletion?

13. The authors use HEK293 cells but this is not mentioned early in the results section, leaving the reader guessing if it's human, mouse, or other.

14. Nicotinamide was discovered as an inhibitor by Bitterman, 2002, JBC.

15. "triplicated results with standard deviation" should be "triplicate experiments" or similar.

16. P20, 21 and possibly other places. "37 ∫C", should have no space.

Referee #2:

The study by Wang et al describes regulation of G6PD after oxidative stress through deacetylation of G6PD by SIRT and consequent increased G6PD activity. Being a key enzyme for the pentose phosphate shunt this regulation results in an increased NADPH and reduced glutathione. As the authors show this is (yet again) a mechanism that contributes to redox homeostasis.

This study represents a concise and clear set of data that by and large support the conclusions drawn. Thus a well executed study with solid conclusions. However, previous proteomic studies albeit limited in quality already indicated that acetylation occurs rather common on metabolic enzymes including G6PD. Also the combination acetylation and metabolism results in a sort of default implication of SIRT in deacetylating these events. A mindset that is quite common in this field of research. Whether or not this mindset is correct applied to this study it results in yet another critical role of SIRT etc. amongst the many other critical and essential roles of SIRTs.

Minor comments:

It is shown that SIRT2 deacetylates K403 and that oxidative stress results in deacetylation of K403, but these two observations are not connected experimentally i.e does oxidative stress enhances the interaction between G6PD and SIRT2 in order to increase deacetylation. Otherwise G6PD under basal conditions already shows substantial interaction with SIRT2 why is K403 deacetylation apparently so inefficient under basal conditions? The apparent lack of robustness of regulation by SIRT is also reflected by the rather mariginal increase in acetylation in SIRT-/- MEFs

Given that the role of SIRTs is sort of to be expected one wonders what the HAT involved would be. It is relatively easy to test HAT involvement by employing a targeted siRNA library.

Appeal 20 December 2013

Thank you for your email of December 14th. We appreciate the efforts by you and the reviewers for evaluating our work (EMBOJ-2013 -87224). We are pleased to see that both reviewers expressed positive view of our study. Reviewer 1 has commented that "the study is comprehensive in its analysis and is mostly convincing. It uses innovative techniques...and the data is well presented and of high quality. The use of KO animals further raises the level of this study and its relevance to the in vivo situation." Reviewer 2 commented that "This study represents a concise and clear set of data that by and large support the conclusions drawn. Thus a well executed study with solid conclusions."

A major concern raised by Reviewer 2, as you noted, is the conceptual advancement of the current study to the 'the existing literature on sirtuins acting in redox homeostasis and metabolic regulation'. Specifically, Reviewer 2 commented that "previous proteomic studies albeit limited in quality already indicated that acetylation occurs rather common on metabolic enzymes including G6PD. Also the combination acetylation and metabolism results in a sort of default implication of SIRT in deacetylating these events. A mindset that is quite common in this field of research. Whether or not this mindset is correct applied to this study it results in yet another critical role of SIRT etc. amongst the many other critical and essential roles of SIRTs."

We respectfully disagree with this assertion. This is like to say that abundant studies from past decades have already demonstrated the critical and essential roles of protein kinases and ubiquitin ligases in the regulation of various cellular pathways. As such, any study on the identification and functional characterization of new kinase or E3 ligase substrates has only 'a sort of default

implication'. The mindset that previous reported roles of SIRTs (total 7 in human cells) would make the current (and any future) study on a new SIRT substrate being 'quite common' is like to say that the value of discovering another target gene of p53 (e.g. Bax, Puma, Mdm2 etc.) is low after the discovery of p21 as a p53 target gene in 1993. Would the reviewer also suggest that any study on the crystal structure of GPCR (e.g. 2AR by Brian Kobilka in 2011) is not significant after the first report of Rhodopin structure by Masashi Miyano in 2000?

More to the point, our recent studies have revealed that individual SIRT enzyme can regulate multiple metabolic enzymes from different metabolic pathways by distinct mechanisms. For example, by deacetylating a specific lysine (K5) in lactate dehydrogenase A (LDHA), SIRT2 blocks chaperone-mediated autophagy, leading to increased LDHA protein level and activity and thereby supporting the metabolic reprogramming in cancer cells (Zhao et al., *Cancer Cell* 2013, 23: 464-476; PMID:23523103). The present study reveals a yet another novel function of SIRT2 in a different metabolic pathway by a different mechanism: deacetylating K403 of G6PD to facilitate the formation of active dimmer, and a new physiological regulation of G6PD by acetylation in mediating cellular response to oxidative stress. It is worth noting that G6PD is the rate-limiting enzyme in the pentose phosphate shunt pathway, which is critically important for ribose synthesis and NADPH production.

Reviewer 1 has raised various specific concerns, and we felt that we can address this reviewers' concerns by further experimentation as described in the attached point-to-point response plan. Therefore, I would like to ask your reconsideration of our manuscript and offer us an opportunity for submitting a revision.

2nd Editorial Decision 23 January 2014

Thank you for your email requesting us to change our decision concerning your manuscript and my apologies for the unusual delay in my reply to you. I have now had the chance to go through both your manuscript and the referee reports once more in light of your comments.

I appreciate your efforts to outline the experiments that can be included to address the criticisms raised by the referees and we would be willing to consider an extensively revised manuscript, addressing all concerns as described in your outline. However, given the significant level of revision involved and the still uncertain outcome and timeline for several of the suggested experiments, we may want to re-assess the revised manuscript editorially at the time of resubmission. In light of the slight discrepancy in the assessment of your work by the two referees - especially with regards to the strong conceptual concerns raised by ref #2 - we may also want to involve a third arbitrating referee at a later stage.

If you are able to address all concerns raised by the referees, we would therefore invite you submit such an extensively revised manuscript using the link provided below.

Thank you again for submitting your work to The EMBO Journal and for your patience in this matter. I look forward to seeing your revised manuscript.

1st Revision - authors' response 25 January 2014

RE: Wang et al. EMBOJ-2013-87224

Dear Dr. Nielsen,

Thank you for your efforts in handling our paper and for offering us an opportunity to revise our paper.

As described in my letter which was sent to you right before we received your email, while waiting for your decision, we have been continuing the study and carried out extensive experimentations to address the specific concerns raised by both reviewers. As you can see from the attached point-by-point response, I believe that we have satisfactorily addressed all concerns raised by the reviewers. These include three major additions.

1. We have carried out siRNA screen against 19 lysine acetyltransferases (KATs) and identified KAT9/ELP3 as the one responsible for G6PD K403 acetylation and enzyme activity regulation (new Fig 3).
2. We have further explored how cells sense oxidative stress to regulate G6PD acetylation. We found that either H_2O_2 or menadione did not change the transcriptional expression of *SIRT2* gene, but did significantly activate SIRT2 enzyme activity in vitro (new Figure 5C), and importantly, profoundly enhanced G6PD-SIRT2 interaction (new Figure 5D).
3. Of 7 lysine residues found to be acetylated by proteomic studies, we have previously shown that substitution with an acetylation mimetic glutamine of three (K171, K386 and K403) substantially reduced G6PD activity (previous Fig. 1D). We now demonstrated that only K403R mutation, but not K171R and K386R mutations, significantly reduced overall G6PD acetylation (new Fig 1E). We also illustrated that substitution of only K403, but neither K171 nor K386, disrupted G6PD dimerization (new Figure 2B). These new results provide further evidence supporting the importance of K403 acetylation in the control of G6PD activity.

A major concern raised by Reviewer 2, as you noted, is whether our study contains sufficient conceptual advancement or has already been indicated by the previous proteomic studies that 'acetylation occurs rather common on metabolic enzymes including G6PD', or falls in 'a sort of default implication of SIRT in deacetylating these events' that is quite common in this field of research'. We respectfully disagree. This is like to say that abundant studies in past decades have already demonstrated the critical and essential roles for protein kinases and ubiquitin ligases. As such, any study on a new substrate has only 'a sort of default implication'. The mindset that previous reported roles of SIRTs (total 7 in human cells) would make the current (and any future) study on

a new SIRT substrate being ‘quite common’ (presumably not novel) is like to say that the value of discovering another target gene of p53 (e.g. Bax, Puma, Mdm2 etc.) is low after the discovery of its first target, p21 CDK inhibitor, in 1993. As the reviewer 1 noted, ‘G6PD deficiency is the most common human enzyme defect, being present in more than 400 million people worldwide’ and ‘confers advantage against the development of cancer’. As more acetylation substrates are being characterized, one would expect to see the function of Sirts to be continuously expanded not only in the metabolic regulation, but also multiple additional pathways and in various organisms.

With these extensive experimentation and clarification, the quality of the manuscript is significantly improved. I hope you and the reviewers will now be satisfied.

If you have any question, please feel free to contact me. I am looking forwards to hearing from you soon.

Kind regards,

Dan

Dan Ye, Ph.D.
Associate Professor
Institute of Biomedical Sciences
Shanghai College of Medicine
Fudan University

Referee #1:

One of the major NADPH-producing enzymes is glucose-6-phosphate dehydrogenase (G6PD), the only NADPH-producing enzyme that is activated. It is an important enzyme to understand because it controls the rate-limiting and primary control step of the NADPH-generating portion in the pentose phosphate pathway and G6PD deficiency is the most common human enzyme defect, being present in more than 400 million people worldwide, possibly because it provides protection against malaria. Sirtuins (SIRT1-7) are NAD+-dependent deacetylases that are continuing to attract attention with almost 100 papers per year. Sirt2, the cytoplasmic sirtuin, is the least understood sirtuin with only a handful of papers.

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The study is comprehensive in its analysis and is mostly convincing. It uses innovative techniques (e.g. cytosolic redox-sensitive green fluorescent protein 1 (HEK293roGFP1) and the data is well presented and of high quality. The use of KO animals further raises the level of this study and its relevance to the in vivo situation. Statistical analyses performed with a two-tailed unpaired Student's t-test are appropriate with a cutoff of 0.05.

We thank the reviewer for the positive comments regarding the quality of presented data and the importance of this manuscript.

There are some overstatements and missing key experiments. With some additional experiments it will be greatly improved. The study should be of interest to a wide audience interested in metabolism, oxidative stress, acetylation, malaria resistance, and aging.

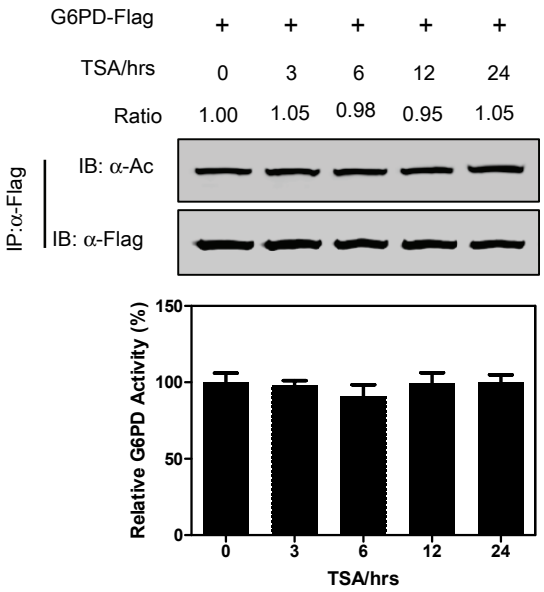
We thank the reviewer for the constructive comments. Please find our point-to-point response below.

Major Points

1. In figure 1A, the authors test NAM + TSA but not TSA. Does TSA alone affect acetylation, which would indicate that another HDAC is involved?

Response: Following reviewer's suggestion, we have determined whether TSA alone would affect the acetylation of G6PD acetylation, and found that treatment with TSA did NOT affect G6PD acetylation and activity. This data is now present in new supplementary Figure 2.

New supplementary Figure 2



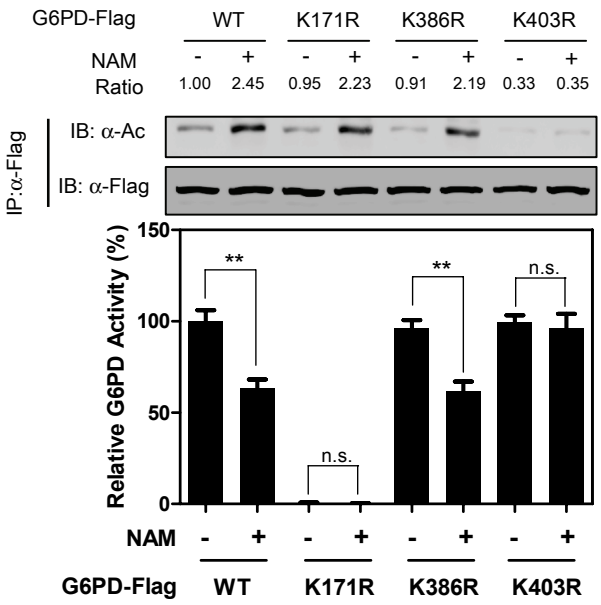
2. It is unclear why the authors would test cobB, a bacterial relative of a sirtuin, not a mammalian sirtuin from the outset. Was this simply more available?

Response: CobB, a known Sir2-like bacterial lysine deacetylase, can be readily expressed and purified from bacteria. It efficiently removes the acetyl group from acetylated lysine. The purpose of this experiment is to show that in vitro deacetylation of G6PD via cobB can activate G6PD activity to support the notion that acetylation negatively regulates G6PD activity. This is like to use bacterial λ-phosphatase to demonstrate the function of phosphorylation.

3. In figure 1C-E, the authors state that "K403 is the major, if not the sole, regulatory acetylation site which plays a critical role in controlling G6PD activity." This is based on mutation and anti-Ac antibodies. The issue is that the conclusion cannot be drawn. Anti-Ac antibodies are notorious for their lack of generality. A pan-Ac antibody is not "pan". Moreover, the authors don't show whether the other sites that affect activity also affect the ability of the Ab to detect the site. For example, is K171 or K386 K or Q detectable by the Ab and to what extent? Were these avoided because it would be a lot more work to study them? For this to be done well, the authors would need to use mass spectroscopy, which is less biased. Solution: either do mass spec, or show the effect of the K171 and K386 mutations on acetylation. As it is, the authors cannot be so definitive and there as a gaping hole.

Response: We agree with the reviewer's comment and have adjusted our original statement. Furthermore, we have determined the effect of K171R and K386R mutations on G6PD acetylation and activity in cells treated with or without NAM. Our data clearly suggest that K403 is an important regulatory acetylation site which controls G6PD activity. This data is now present in new Figure 1E.

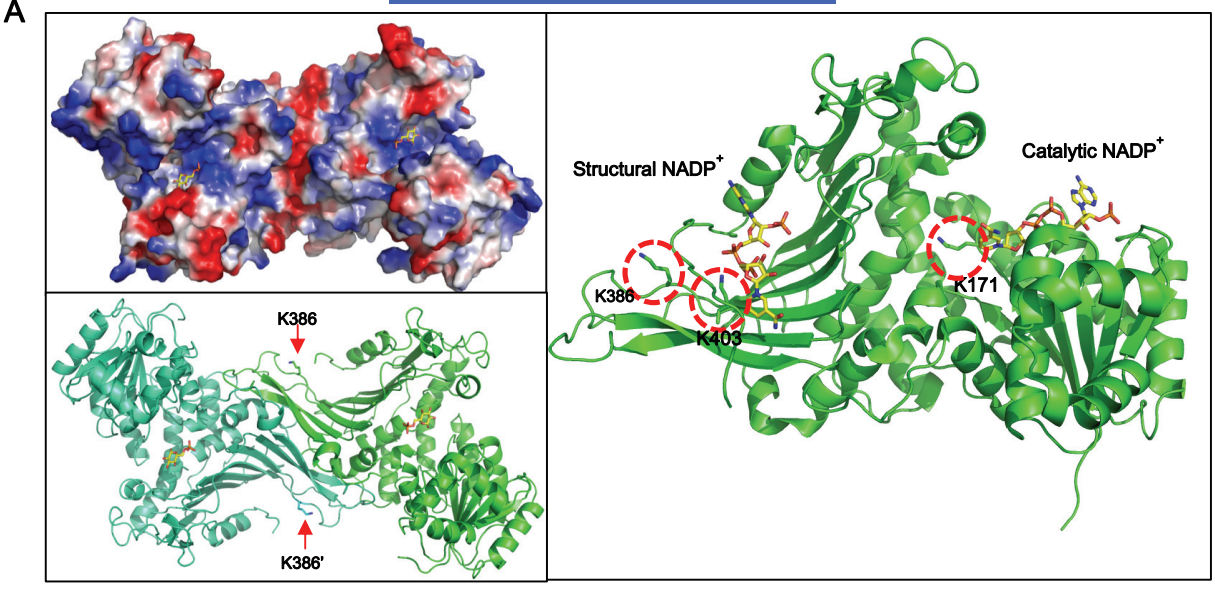
New Figure 1E

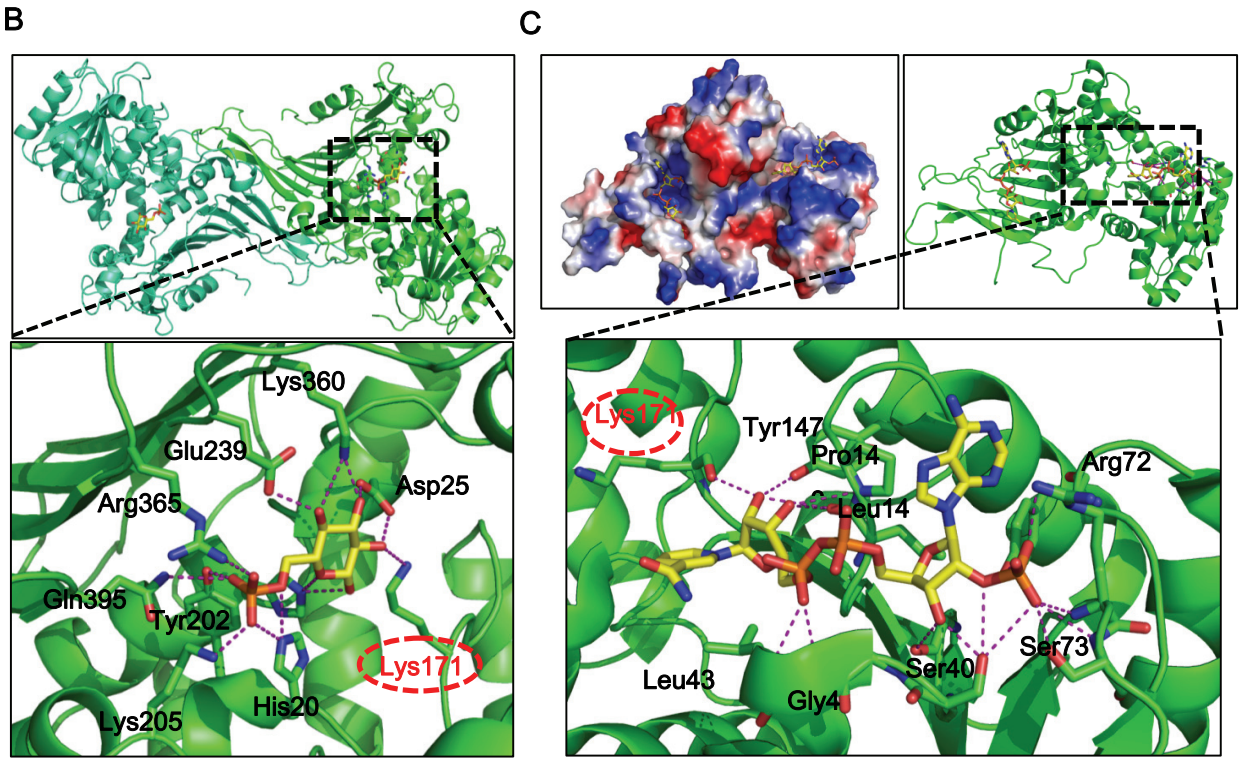


4. Where do the other mutations that affect activity (K171, K386) lie within the G6PD structure? Can they authors explain why they lost activity.

Response: The K171 site lies in the catalysis pocket of G6PD, thereby directly interacting with substrates, $NADP^+$ and G6P. The K386 site locates in the dimer interface, and therefore, K386 mutation would be expected to affect the dimer formation of G6PD. To further clarify this, we have made a structural illustration for K403, K171, and K386 within the G6PD protein, which will be helpful for better understanding why these site mutations would affect G6PD activity (new supplementary Figure 4).

New Supplementary Figure 4

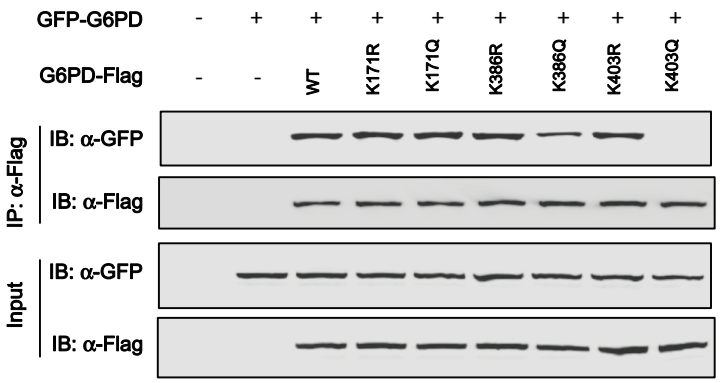




5. The authors found that the substitution of K403Q, but not K403R, disrupted the interaction between G6PD subunits. Couldn't K171 or K386 K substitutions, which affect activity but presumably not dimerization, serve as negative controls?

Response: Following reviewer's suggestion, we have determined the effect of K171 or K386 substitutions on the interaction between G6PD subunits, and found that substitutions of K171R/Q did not affect the interaction between G6PD subunits, while substitution of K386Q, but not K386R, slightly reduced the interaction between G6PD subunits. Note that in the same experiment, substitution of K403Q, but not K403R, completely disrupted the interaction between G6PD subunits, indicating that K403 acetylation largely hinders the interaction between G6PD subunits (new Figure 2B).

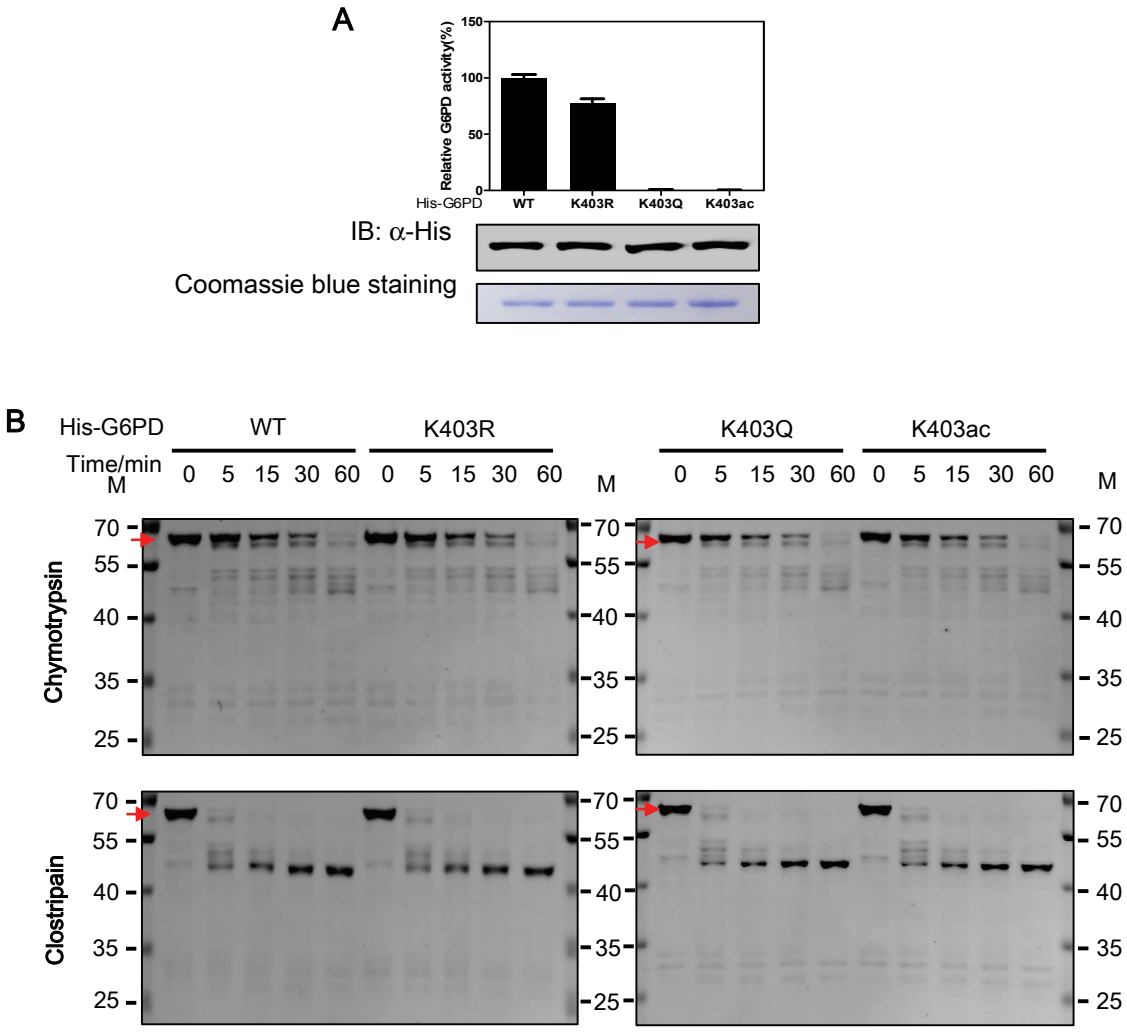
New Figure 2B



6. In figure S3, the production of recombinant mutant G6PD is presented. The authors need to test if the protein has folded correctly, because this could also explain lack of dimerization and catalytic activity. This can be tested using a variety of methods including melting temperature and fluorescence polarization compared to WT.

Response: To address this question, we performed limited proteolysis to test whether the recombinant mutant G6PD (G6PD^{K403ac} protein) can be folded correctly or not. We found that as compared to wild-type and K403R/Q mutants of G6PD, the recombinant G6PD^{K403ac} protein displayed the identical pattern of proteolytic cleavage after treatments with proteases, chymotrypsin and clostripain. Moreover, the G6PD^{K403ac} protein exhibited normal thermodynamic stability when compared to wild-type and K403R/Q mutants of G6PD (new supplementary Figure 8).

New Supplementary Figure 8



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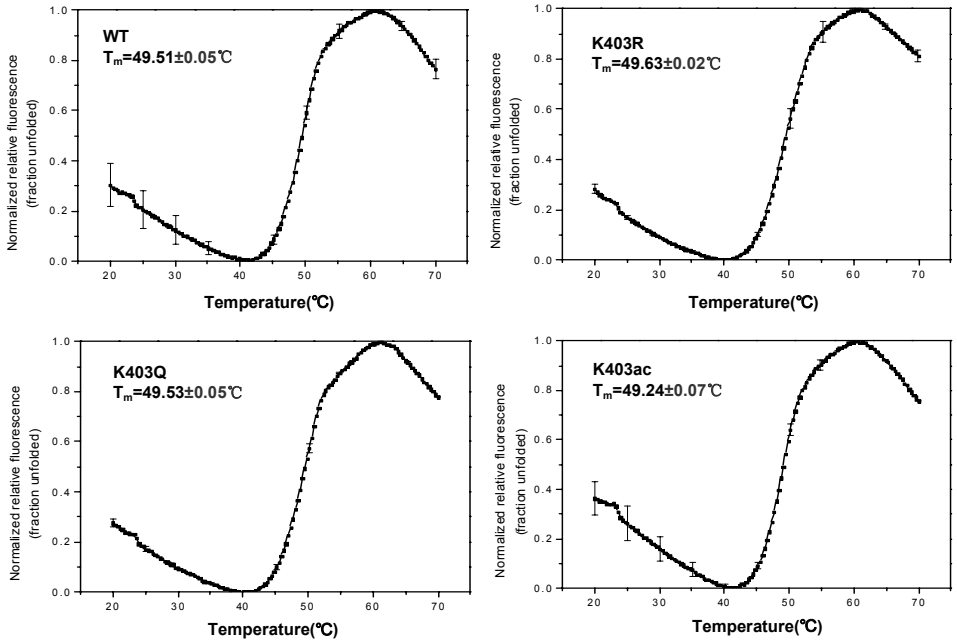
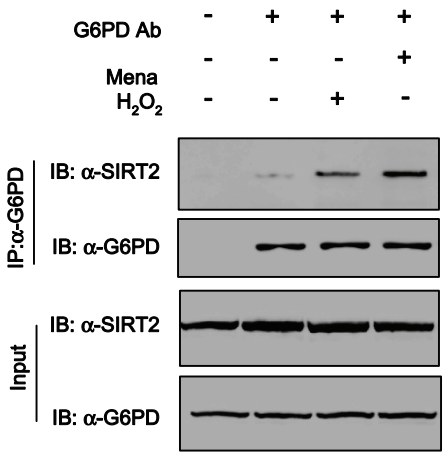


7. The authors observed that G6PD "directly interacted with SIRT2, but not SIRT1 (Figure 3A and 3B)." But this is using overexpressed proteins. The endogenous interaction needs to be shown or doubts could be raised about its physiological significance.

Response: To address this question, we have determined the interaction between endogenous G6PD and SIRT2 in cells under non-stressed or oxidative stress condition. We found that the protein interaction between endogenous SIRT2 and G6PD was weak in cells under non-stressed condition, and this interaction was profoundly enhanced by treatment with either H_2O_2 or menadione (new Figure 6D). This result provides further evidence supporting a major conclusion of the paper that SIRT2-mediated G6PD deacetylation and activation to counteract oxidative damage.

New Figure 6D

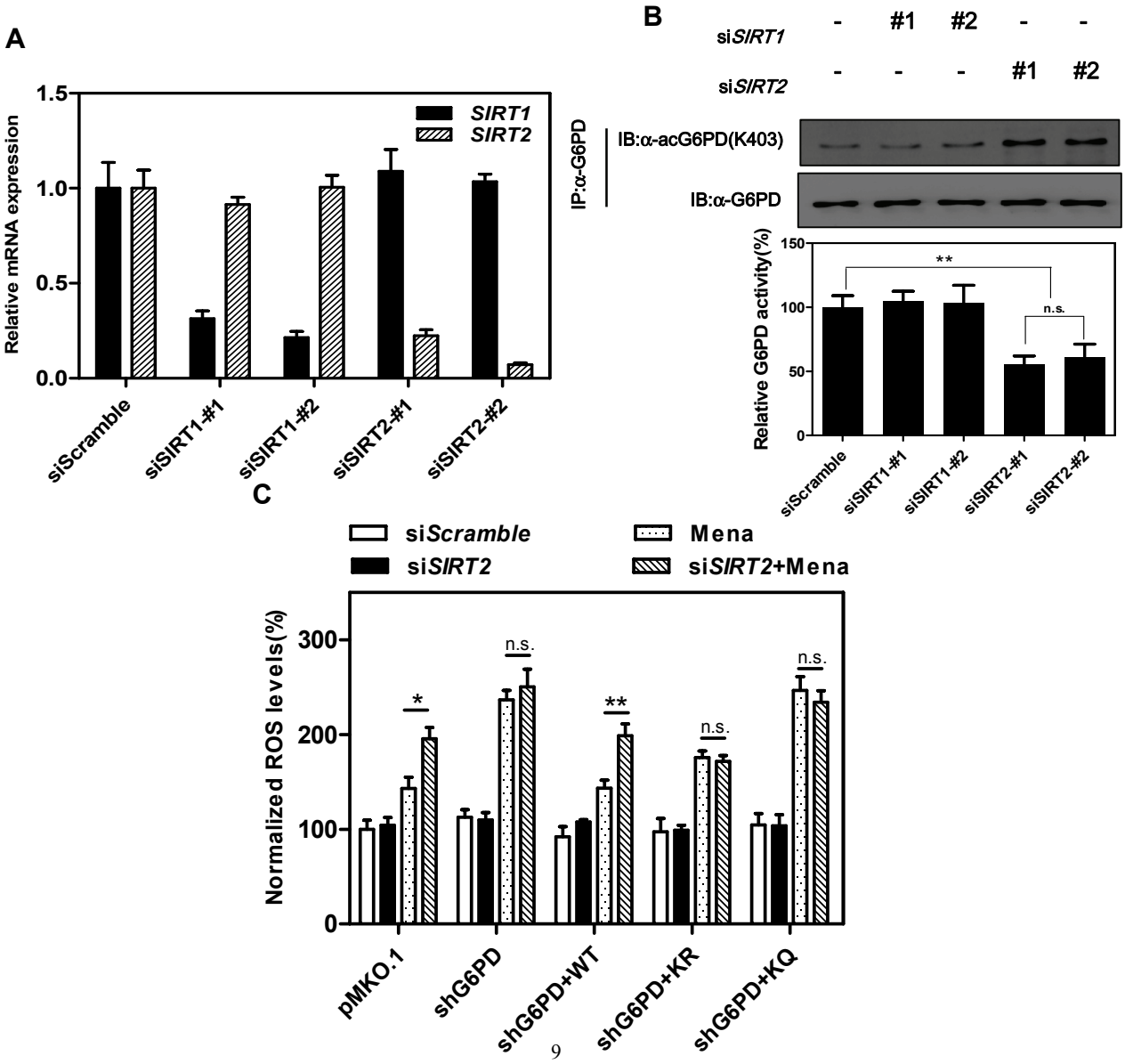


8. The SIRT2-specific inhibitor AGK2 is good but compounds always have off targets. The

authors need to test a SIRT2 knockdown/KO in the biochem/acetylation experiments to be sure the effect is specific. Ideally, with reconstitution mutant constructs. Similarly, in figure 5, inhibition of SIRT2 by AGK2 in the presence of menadione on ROS production is not sufficient. The G6PD knock-down in HEK293T and cells rescued by WT G6PD or the K403Q mutant (Supp. Figure 5) could be useful in this regard.

Response: To address the reviewer's concern, we have repeated the experiments in Figure 5 by knocking-down *SIRT2* in HEK293 cells. We found that transient knocking-down *SIRT2*, but not *SIRT1*, increased the K403 acetylation level of endogenous G6PD, and enhanced the effect of menadione on ROS production and cell death in both the control and G6PD rescued cells, but not in the G6PD knockdown or K403R/K403Q rescued cells (new supplementary Figure 12). These data support that the observed effects of the SIRT2 inhibitor AGK2 on ROS production and cell viability are mediated by G6PD K403 acetylation.

New Supplementary Figure 12



9. The authors should discuss how altering NAD+ or SIRT2 levels/activity could be therapeutic, against malaria for example, (see Shin Imai's work) and what impact the decline in NAD+ levels during aging could have. See Ding et al., 2013 "G6PD (Xq28)-were associated with RBC traits in the discovery cohort."

Response: We appreciate the reviewer's suggestion to further point out the significance of our study. We have discussed the therapeutic implications of altering SIRT2 levels/activity for regulating G6PD activity for treating related diseases, such as malaria, hemolysis, diabetes, aldosterone-induced endothelial dysfunction.

10. There is no information on Sirtuins in the introduction. What is known about Sirt2? The manuscript is too focused on G6PD and misses the opportunity to present the big picture and implications, linking sirtuins to glucose metabolism to oxidative stress resistance and diseases/aging.

Response: Following reviewer's suggestion, we have discussed lysine acetyltransferases (KATs) and deacetylases (KDACs) in the introduction. Moreover, we have added more discussion about the known and speculated biological functions of sirtuins (SIRTs), in particular their roles in metabolism regulation and cellular energy response. Finally, we have discussed the therapeutic implications of altering SIRT2 levels/activity for regulating G6PD activity for treating related diseases, such as hemolysis, diabetes, aldosterone-induced endothelial dysfunction.

11. There is new work indicating that GPPD deficiency confers an advantage against the development of cancer, reduces the risk of coronary diseases and has a beneficial effect in terms of longevity." See Manganelli et al., 2013. PMID: 23534950. Again, the authors miss an opportunity to discuss the wider implications.

Response: We appreciate for the reviewer's suggestion to further point out the significance of our study. As described above, we have added more discussion in order to strengthen the clinical implications of our study with regards to the correlation between G6PD dysfunction and human diseases.

Minor Points

12. Make sure the methods are complete. e.g. Where are the KO cells from? Whose mouse? What's the deletion?

Response: We have added more details on the source of these reagents.

13. The authors use HEK293 cells but this is not mentioned early in the results section, leaving the reader guessing if it's human, mouse, or other.

Response: We thank the reviewer for pointing this out and have added this information in the revised manuscript.

14. Nicotinamide was discovered as an inhibitor by Bitterman, 2002, JBC.

Response: We have cited this reference in the revised manuscript.

15. "triplicated results with standard deviation" should be "triplicate experiments" or similar.

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Major Points

1. This study represents a concise and clear set of data that by and large support the conclusions drawn. Thus a well-executed study with solid conclusions. However, previous proteomic studies albeit limited in quality already indicated that acetylation occurs rather common on metabolic enzymes including G6PD.

Response: We respectfully disagree with the Reviewer's comment about the significance of our study. Previous proteomic studies by us and others have shown that lysine acetylation occurs rather commonly in more than 4,500 proteins, including many metabolic enzymes as well as multiple additional cellular pathways, such as histone modification, in various organisms (e.g. Choudhary et al *Science* 2009, PMID: 19608861, Wang et al. *Science* 2010, PMID: 20167787, Weinert et al. *Science Signaling*, 2011, PMID: 21791702, Lundby et al. *Cell Reports* 2012, PMID: 22902405). These proteomic studies opened the door for studying cellular regulation by another major post-translational modification (PTM), but did not address the function and regulation of acetylation in individual protein that participates in specific biological processes.

In this study, the proteomic study has led us to investigate the mechanism for how acetylation regulates the activity of G6PD, the rate-limiting enzyme of the pentose phosphate shunt pathway and a major contributor for cellular NADPH pools. At present, very little is known about how extracellular oxidative stimuli affect NADPH homeostasis and thus cellular redox status through G6PD. To date, the only reported PTM regulation of G6PD is phosphorylation (Pan et al. *Arterioscler Thromb Vasc Biol*. 2009, PMID: 19359662). In this study, we demonstrate that up to 50% of G6PD protein is acetylated in vivo at K403 and that the SIRT2-mediated K403 deacetylation plays a critical role in vivo to protect cells from oxidative damage. Moreover, we illustrated that acetylation at the site of K403 negatively controls the activity of G6PD via impeding its dimerization. To the best of our knowledge, this is the first report on the regulation of G6PD by acetylation, the only PTM regulation of G6PD besides phosphorylation, and the first report on the PTM regulation of G6PD dimerization.

2. Also the combination acetylation and metabolism results in a sort of default implication of SIRT in deacetylating these events. A mindset that is quite common in this field of research. Whether or not this mindset is correct applied to this study it results in yet another critical role of SIRT etc. amongst the many other critical and essential roles of SIRTs.

Response: This comment raised by the reviewer is quite broad and puzzling. If we

understand it correctly, it is like to say that abundant studies in past decades have already demonstrated the critical and essential roles for protein kinases and ubiquitin ligases in the regulation of various cellular pathways. As such, any study on the identification and functional characterization of a new substrate has only 'a sort of default implication'. The mindset that previous reported roles of SIRTs (total 7 in human cells) would make the current (and any future) study on a new SIRT substrate being 'quite common' (presumably not novel) is like to say that the value of discovering another target gene of p53 (e.g. Bax, Puma, Mdm2 etc.) is low after the discovery of its first target, p21 CDK inhibitor, in 1993.

There are 7 sirtuins and 11 HDACs in humans, each of which presumably carries distinct functions. Our recent studies have revealed that Sirt2 alone can regulate multiple metabolic enzymes from different metabolic pathways by distinct mechanisms. For example, by deacetylating a specific lysine (K5) in lactate dehydrogenase A (LDHA), Sirt2 blocks chaperone-mediated autophagy, leading to increased LDHA protein level and activity and thereby promoting glycolysis and cell proliferation (Zhao et al., *Cancer Cell* 2013, 23: 464-476; PMID:23523103). The current study reveals a yet another novel function of Sirt2 in a different metabolic pathway (oxidative stress response) by a different mechanism (regulating the formation of active dimmer). As pointed out by the reviewer #1, G6PD is the rating-limiting enzyme in the pentose phosphate shunt pathway, which is important for not only ribose synthesis but also NADPH production. Elucidating the molecular mechanism of G6PD regulation, as shown by acetylation in this study, would not only advance our knowledge of oxidative stress response, but also that of G6PD deficiency. As the reviewer 1 noted, 'G6PD deficiency is the most common human enzyme defect, being present in more than 400 million people worldwide' and 'confers advantage against the development of cancer'. As more acetylation substrates are being characterized, one would expect to see the function of Sirts to be continuously expanded not only in the metabolic regulation, but also multiple additional pathways and in various organisms.

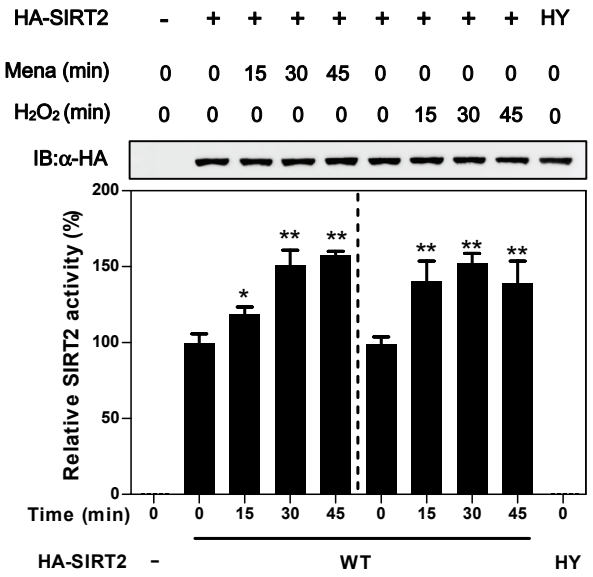
Minor comments:

1. It is shown that SIRT2 deacetylates K403 and that oxidative stress results in deacetylation of K403, but these two observations are not connected experimentally i.e does oxidative stress enhances the interaction between G6PD and SIRT2 in order to increase deacetylation. Otherwise G6PD under basal conditions already shows substantial interaction with SIRT2 why is K403 deacetylation apparently so inefficient under basal conditions?

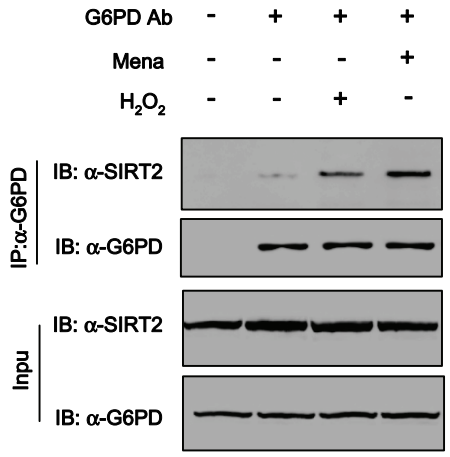
Response: To address this issue, we have tested whether oxidative stress would affect SIRT2-G6PD interaction, and transcriptional expression and enzyme activity of SIRT2. We found that either H_2O_2 or menadione did not change the transcriptional expression of *SIRT2* gene, but did significantly activate SIRT2 enzyme activity in vitro (new Figure 6C). Moreover, we found that the protein interaction between endogenous SIRT2 and G6PD was weak in cells under non-stressed condition, and this interaction was profoundly enhanced by treatment with either H_2O_2 or menadione (new Figure 6D). These data

have addressed this reviewer's question as to how oxidative stress results in SIRT2-mediated deacetylation on K403 of G6PD.

New Figure 6C



New Figure 6D



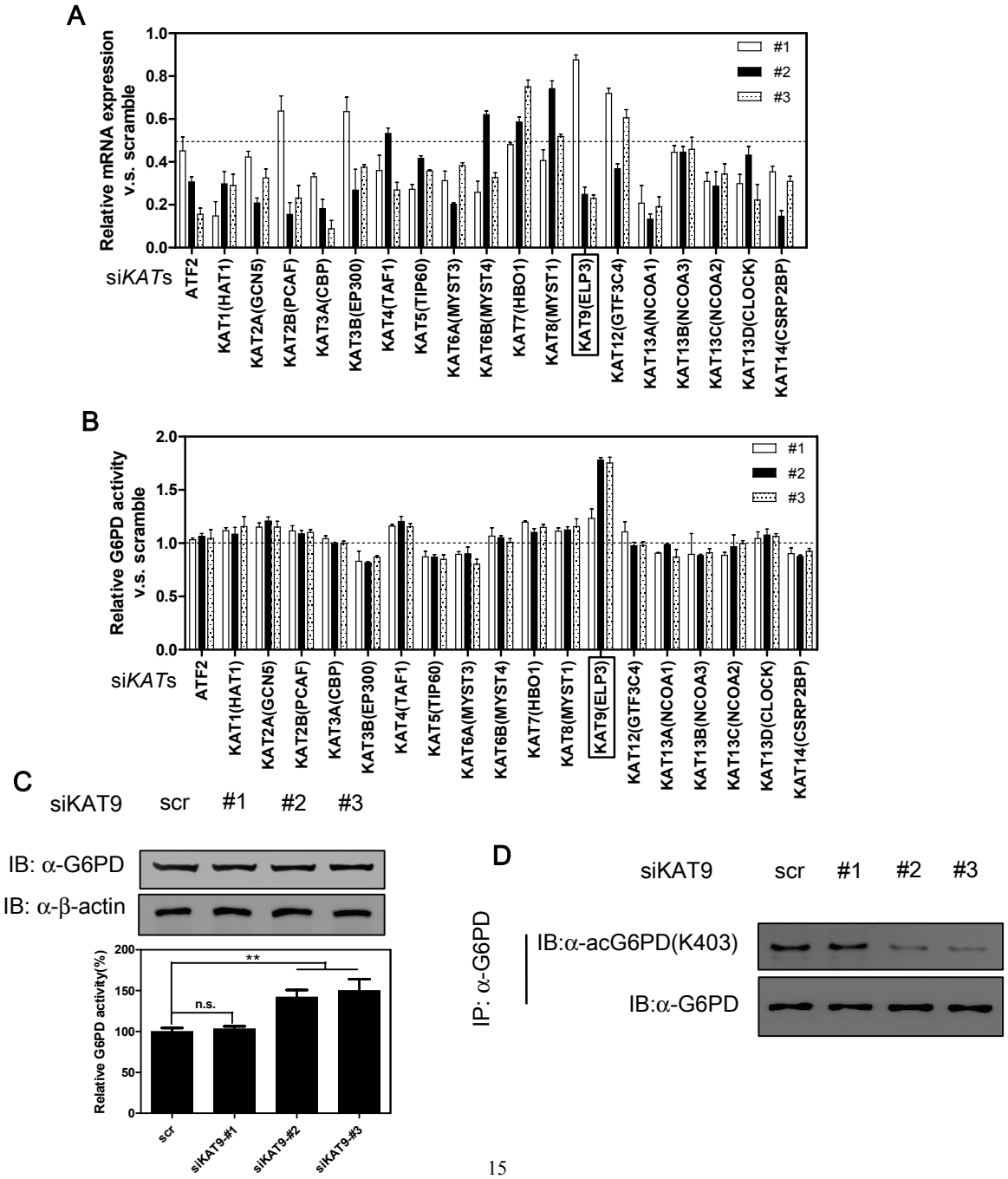
2. The apparent lack of robustness of regulation by SIRT is also reflected by the rather mariginal increase in acetylation in SIRT-/- MEFs. Given that the role of SIRTs is sort of to be expected one wonders what the HAT involved would be. It is relatively easy to test HAT involvement by employing a targeted siRNA library.

Response: We respectfully disagree with the Reviewer's comment about "rather marginal increase in acetylation in SIRT-/- MEFs". As shown earlier in Figure 7D, sirt2 deficiency leads to a remarkable, NOT marginal, increase (by >1.8-fold) in the G6pd K403 acetylation level in mouse MEFs. As a result, G6pd activity was significantly reduced in SIRT2-/- MEFs under both non-stressed and oxidative stress conditions.

To address the reviewer's concern about the involvement of HATs in acetylating G6PD,

we have established a siRNA library against 19 known *HAT* genes, and then carried out an unbiased screen to search for potential HAT(s) which are involved in the regulation of G6PD acetylation and activity. We found that knocking-down of most *HAT* genes did not substantially affect the enzyme activity of endogenous G6PD. With one exception, knocking-down *KAT9* (also known as *ELP3*) significantly stimulated the activity of endogenous G6PD. Moreover, transient knockdown of *KAT9* decreased the K403 acetylation levels of endogenous G6PD without changing its protein expression, further supporting the notion that KAT9 is the potential acetyltransferase of G6PD. All these data are now presented in new Figure 3.

New Figure 3



3rd Editorial Decision 18 March 2014

Thank you for submitting your revised manuscript to The EMBO Journal and please accept my sincere apologies for the unusual delay in the review process in this case, brought on by extensive travelling on my side.

Your study has now been seen by the two original referees (comments enclosed below) and as you will see they both find that all original criticisms have been adequately addressed in this revised version. I am therefore happy to inform you that your manuscript has been accepted for publication in The EMBO Journal.

REFEREE REPORTS:

Referee #1:

The revised version of the manuscript by Wang et al. comprehensively addresses previous concerns. All the suggested experiments and improvements are now in the manuscript, to a degree rarely seen in revised manuscripts. For example, in testing whether the recombinant proteins are folded correctly, they not only performed denaturation experiments but went to the additional effort to do a limited proteolysis experiment. When they were asked about HAT involvement, they tested all of them in a screen. The authors are to be commended.

Prompted by the reviewer comments, I recommend adapting the title and the abstract to increase the readership and do the work justice. For example, they could change it from

"G6PD activity is regulated by acetylation to modulate NADPH homeostasis during oxidative stress" to

"Control of G6PD acetylation by KAT9/SIRT2 regulates NADPH homeostasis and cell survival" or something to that effect.

and the abstract could end by saying that this work points to new therapies to treat G6PD deficiency. This is a big deal given that G6PD deficiency is the most common human enzyme defect, up to 1 in 10 african Americans.

Referee #2:

The authors have responded to my concerns/questions and although I disagree with some of their opinions/answers I do not think this disagreement should preclude acceptance of this manuscript.