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Glycosylation of KEAP1 links nutrient sensing to redox stress signaling

Po-Han Chen, Timothy J. Smith, Jianli Wu, Priscila F. Siesser, Brittany J. Bisnett, Farhan Khan, Maxwell Hogue, Erik Soderblom, Flora Tang, Jeffrey R. Marks, Michael B. Major, Benjamin M. Swarts, Michael Boyce, and Jen-Tsan Chi

Corresponding authors: Michael Boyce and Jen-Tsan Chi, Duke University School of Medicine

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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.)

1st Editorial Decision 26 January 2017

Thank you for submitting your full manuscript on OGT-mediated Keap1 glycosylation in the regulation of Nrf2. We have now received feedback from three expert referees, whose reports are copied below for your information. As you will see, the referees consider your findings potentially interesting and important, but also list a number of issues in which the manuscript would need to be strengthened prior to publication. Should you be able to satisfactorily address these concerns, we would be happy to consider a revised version of the study further for The EMBO Journal.

Since it is our policy to allow only a single round of major revision, it will be important to carefully and comprehensively answer to all points raised at this stage. As it stands, the reviewers' concerns appear generally well-taken and relevant but also addressable in a straightforward manner. However, I would not consider a detailed investigation of Keap1-OGT interaction (ref 3) essential, and furthermore feel that dedicated in vivo follow-up studies as asked by referee 3 may also exceed the scope of a regular revision in this case - although any data you may already have to answer these points would certainly be beneficial. In any case, please do not hesitate to get back to me should you have any questions/comments regarding the referee reports or your revision work. We might further arrange for an extended revision period, during which time the publication of any competing work elsewhere would as usual have no negative impact on our final assessment of your own study.

REFEREE REPORTS

Referee #1:

Review of "Glycosylation of Keap1 links nutrient sensing to redox stress signaling"

The authors present convincing evidence that (1) modulating levels of O-GlcNAcylation in cells results in activation of Nrf2-dependent genes; (2) steady-state levels of Nrf2 are increased and ubiquitination of Nrf2 is decreased, when OGT is inhibited; (3) that the major regulator of Nrf2, the substrate adaptor Keap1, is modified by O-GlcNAcylation. The authors also identify 11 serine residues in Keap1 that are modified by O-GlcNAcylation and present functional data suggesting that one particular serine residue, Ser104 is a critical sensor for reduced glucose levels, leading to reduced O-GlcNAcylation of Keap1 and reduced assembly of a functional Keap1 ubiquitin ligase.

Many of the conclusions of the authors are supported by well-designed experiments, particularly points 1-3 above. The data in Figure 6, relating levels of glucose in the media to levels of Nrf2 are also important, although there is some concern that those experiments are not measuring changes in poly-ubiquitination of Nrf2 as claimed by the authors (see comment #2 below).

The work in this manuscript reveals an unanticipated and novel sensing mechanism by which regulation of Nrf2-dependent genes is linked to changes in glucose availability. This mechanism is likely to have a wide-spread role in maintaining cellular homeostasis, with important implications in cancer, immune regulation and neurodegenerative diseases. This manuscript will be of general interest to the audience of EMBO Journal.

However, the authors do not address a significant issue - the ubiquitous presence of a Ser or Thr residue, corresponding to Ser104 in Keap1, in all members of the KLHL family. The authors do not fully incorporate structural and functional data regarding the role of Ser104 and the likely consequences that modifying this residue with O-GlcNAcylation might have. Addressing this issue, which really has two points, would significantly strength the manuscript and increase the general interest in this manuscript.

Specific comments:

1: Ser 104 is nearly completely conserved across all members of the KLHL family (Keap1 is also known as KLHL19). One exception is KLHL11, which as a Thr in place of a Ser. Structural analysis of the BTB domains of several members of the family place this serine between two alpha helices. Furthermore, the corresponding Ser residue in gigaxonin (Ser52 in gigaxonin) has been suggested to be a disease-causing mutation in a patient with Giant Axonal Neuropathy.

Importantly, there is structural information on this region of Keap1 and several other KLHL proteins. The structural interface of KLHL11 and Cul3 (Canning et. al., JBC 288, 7803-7814, 2013) provides a detailed examination of how the BTB-BACK domains of the KLHL proteins interact with Cul3. Note that KLHL11 has a Thr residue (T119) in place of S104 in Keap1. In addition, there are several structures of the BTB domain of Keap1 in the Protein Data Base, along with a structure of the BTB-BACK domain of gigaxonin.

Thus, the data in this manuscript, if true, have broad implications for all members of the KLHL family. However, the author's interpretation of the role of S104 in the structure and function of Keap1 does not incorporate either the high degree of conservation of the corresponding Ser residue across the KLHL family or the structural information mentioned above.

Incorporating the existing structure data and determining if the central observation holds across other members of the KLHL family would strengthen the conclusions of this manuscript and broaden its relevance.

First, are other members of the KLHL family also regulated by O-linked glycosylation at the residue corresponding to Ser104? It would be relatively straightforward for the authors to demonstrate that other members of the KLHL family are modified by O-linked glycosylation, as the data reported in Figure 4D was obtained using MS analysis of recombinant Keap1 protein purified from HEK293T cells. The use of the GalNAz affinity reagent is another possible approach. If, for example, gigaxonin is also modified by O-linked glycosylation at Ser52, this would significantly extend the

importance of the central finding of this manuscript. A detailed functional analysis of such a modification, if found, would clearly be beyond the scope of the current manuscript, since substrates of gigaxonin-dependent ubiquitination have not clearly been established. But information relating to the generality of their observation, given that Ser104 is so highly conserved across all members of this important family of substrate adaptor proteins, is highly relevant. It may be the case that Keap1 is unique in terms of modifying this Ser residue. If so, that also would be important to know and substantiate the claim of the authors that this modification uniquely links regulation of Keap1 function to glucose availability.

A related issue to the data in Figure 4 is that it appears that only one of the 11 Ser residues in Keap1 that is modified by O-linked glycosylation is actually functionally relevant. This observation, at first glance, would suggest that many O-linked glycosylation events on many proteins in cells is irrelevant for their function. Can the authors comment on why there are so many O-linked glycosylation sites but only a few that appear to have functional consequences?

The second issue relates to the role of S104 in the structure of Keap1 and in terms of its interaction with different binding partners. The authors present Figure 4D as line-drawing of the Keap1 protein onto which the sites of O-linked glycosylation are mapped, along with regions of Keap1 that interact with known partners, including Cul3 and Nrf2 (The relevance of SQSTM1 interaction is not clear, since SQSTM1 binds to Keap1 but is not a ubiquitination substrate of the Keap1:Cul3 ubiquitin ligase complex). This line drawing does not adequately capture the structural data. I suggest that the authors provide a line drawing of the secondary structure elements of Keap1, onto which the interactions of Keap1 with its binding partners (Cul3 and Nrf2) could be mapped. This line diagram would clearly indicate the alpha helices that are likely to directly contact Cul3 (inferred from the KLHL11:Cul3 structure) and the so-called B-C loops in the Kelch domain that form the pocket of Keap1 for Nrf2. This line would more closely represent the structural information, in which alpha-helices at the N-terminal region of the BACK domain directly contact Cul3 which alpha-helices in the BTB domain are known to be involved in forming the anti-parallel Keap1 dimer. As a side note, the authors mention a controversy regarding the role of S104A in Keap1 dimerization and should discuss the structural information regarding dimer formation of KLHL proteins vs their interaction with Cul3.

The authors present functional data on the S104A mutant. The functional data on Nrf2 regulation could result from an altered conformation of the S104A dimer that changes the spatial orientation between the two Kelch domains and thereby perturbs efficient ubiquitination of the lysine residues in Nrf2. See the model in the Canning 2013 article and other structural and functional data (largely from the Yamamoto lab) in support of the two-site hinge and latch model for how Keap1 binds Nrf2 and presents lysine residues in Nrf2 for ubiquitin transfer mediated by the Rbx1 protein.

The authors demonstrate reduced association between Cul3 and the S104A mutant Keap1 protein. However, these experiments were performed with overexpressed protein and substantial (about 15-30%) of binding of Keap1 to Cul3 is still observed with the mutant protein. Again, the functional consequences of the S014A mutation are best interpreted in light of the two-site hinge and latch model rather than simply equating reduced Cul3 binding with reduced ubiquitination of Nrf2.

2: The multiple lines of evidence that inhibition of OGT results in reduced Nrf2 ubiquitination, increased steady-state levels of Nrf2 and increased mRNA levels of multiple Nrf2-dependent genes (Figures 1, 2 and 3) are strong. Those experiments are well designed with appropriate controls and the conclusions are solid. The demonstration that Keap1 is modified by GalNAz is also solid (Figure 4). However, the evidence for modulation of Nrf2 ubiquitination in the experiments reported in Figures 5 and 6 is not as strong as the data in, for example, Figure 3. The authors point to a minor form of Nrf2 that has slower migration in SDS-PAGE and claim that this is the ubiquitinated form of Nrf2. However, Nrf2 is often seen as a doublet (in the data presented by the authors and in many publications in the literature. When the authors are pointing out a form of Nrf2 that they claim is ubiquitinated, there is only a single band of Nrf2. See Figure 6B, 6C, 6D, for example. In contrast, in Figure 5A, the authors point to forms of Nrf2 that are much slower in migration, with a smear (not a single band) in lane 6 of Figure 5A. The data in Figures 6B, 6C and 6D are not convincing (at least to this reviewer) that the S104A protein changes the ubiquitination status of Nrf2, as it is probably the case that the single band that the authors point to in Figures 6B, C and D is NOT a poly-ubiquitinated forms of Nrf2. There are more conclusive ways of measuring ubiquitination of

Nrf2, particularly poly-ubiquitination, that should be performed for at least the key experiments in Figure 6 (See Figure 3B).

Referee #2:

The manuscript entitled, "Glycosylation of KEAP1 links nutrient sensing to redox stress Signaling", is a very interesting study that bring novel insights to the field. Here the authors have used an elegant methodologies starting from transcriptomic analysis and using these data to narrow down to deciphering molecular mechanism and provide some insight on the role of O-GlcNAcylation in cell physiology. This paper shows robust data supporting the claim that OGT targets and O-GlcNAcylates KEAP1, which in return leads the recruitment of CUL3 and the poly ubiquitination and degradation of NRF2. Based on there studies, the authors have suggested that glucose variation may interfere in the proper regulation of NRF2 and leads to tumor growth and chemotherapy resistance. Thus, overall, this is an important and interesting paper. However, some questions and concerns should be addressed:

1. The authors should provide the list of the up and down regulated gene identified in their microarray

2. Although the authors provide data supporting their model in different cell types, it is not clear why they are changing cell types throughout the manuscript. It would be clearest if they show the complete mechanism in one cell type and select some key experiments in other cell types showing the ubiquity of the mechanism?

3. O-GlcNAc antibodies, especially when used at high concentrations, may show some non-specific binding. Their use must be associated with controls such as a competition blots in presence of free GlcNAc or treatment of the samples with CpNagJ (or hexosamindase). This is particularly important for the IP blots.

4. The blot shown in Fig S3B doesn't really show an enrichment of NRF2 after IP. Can the authors provide a better experiment?

5. Metabolic labelling with the GalNAz is a powerful tool for O-GlcNAc protein identification, however this method doesn't allow a complete labeling due to competition with endogenous substrate, and the authors may miss a low O-GlcNAc stoichiometry on NRF2 and CUL3 for instance (Fig 4A). GalT labeling would provide a much higher labeling and can be done on endogenous protein without stressing the cell with the Ac5SGlcNAz treatment.

6. In figure 5E, the authors have used the 293T to show that O-GlcNAcylation of KEAP1 enhance its interaction with CUL3 with recombinant tagged constructs. Why have the authors have not used the H838 that doesn't express KEAP1 as they have done to point out the site specific function of the S104 residue?

7. In order to strengthened the role of the O-GlcNAcylation of the S104 on KEAP1 interaction with CUL3 and NRF2 activity, the authors could provide some experiments using high Glucose, Ac5SGlcNAc and/or TMG treatment and investigate the interaction/activity of KEAP1-S104A, CUL3 and NRF2?

Minor concerns:

1. A previous study has linked OGT/TET1 and NRF2 (Kyoung ah kang et al, oncotarget 2016). This study should be discussed.

2. References are not really up to date.

Referee #3:

This work revealed a novel mechanism that O-GlcNAcylation of KEAP1 is required to restrain the NRF2 pathway, and have important implications that nutrient status links to antioxidant signaling. Although it is an interesting finding, following points might be considered upon revision of this manuscript.

Minor points:

1. Although O-GlcNAcylation of KEAP1 is shown in this study, it remains to be seen whether interaction between OGT and KEAP1 is direct or indirect. To this end, interaction should be examined using recombinant proteins of KEAP1 and OGT.

2. In addition, responsible domain for interaction between domain KEAP1 and OGT should be determined. Although Figure S4C shows that BTB-deleted KEAP1 still interact with OGT, there is no mention in the text. The authors should explain this result and discuss how Keap1 binds to OGT.

3. Although it is a key finding that O-GlcNAcylation of KEAP1 is important for KEAP1-CUL3 interaction, Figures S5J,K showing that reduced interactions between Keap1 and CUL3 by 5SGlcNAc treatment are not convincing. The authors should improve these data and show statistical results.

4. It should be discussed why O-GlcNAcylation of KEAP1 S104 is structurally important for KEAP1-CUL3 interaction based on the previous report regarding structure of BTB domain (Cleasby et al, 2014; Huerta et al, 2016). Model analysis showing interaction between KEAP1 S104 O-GlcNAcylation with CUL3 would be nice.

5. All experiments in this study were conducted using culture cells. The authors should provide physiological evidence that nutrient status influence on KEAP1 O-GlcNAcylation and NRF2 activity in vivo. It would be great if hypoglycemia leads to activation of NRF2 pathway.

6. In Figure 3B, Myc-NRF2 protein was not accumulated by 5SGlcNAc treatment. As it seems inconsistent data, there should be some explanations.

1st Revision - authors' response 07 May 2017

We thank the referees for their positive comments on the overall convincing and significant nature of our results, and we are grateful for the opportunity to improve our manuscript further by responding to their suggestions and critiques. Below we provide point-by-point responses to each refereeís comments.

Referee 1: The authors present convincing evidence that (1) modulating levels of O-GlcNAcylation in cells results in activation of Nrf2-dependent genes; (2) steady-state levels of Nrf2 are increased and ubiquitination of Nrf2 is decreased, when OGT is inhibited; (3) that the major regulator of Nrf2, the substrate adaptor Keap1, is modified by O-GlcNAcylation. The authors also identify 11 serine residues in Keap1 that are modified by O-GlcNAcylation and present functional data suggesting that one particular serine residue, Ser104 is a critical sensor for reduced glucose levels, leading to reduced O-GlcNAcylation of Keap1 and reduced assembly of a functional Keap1 ubiquitin ligase.

Many of the conclusions of the authors are supported by well-designed experiments, particularly points 1-3 above. The data in Figure 6, relating levels of glucose in the media to levels of Nrf2 are also important, although there is some concern that those experiments are not measuring changes in poly-ubiquitination of Nrf2 as claimed by the authors (see comment #2 below).

The work in this manuscript reveals an unanticipated and novel sensing mechanism by which regulation of Nrf2-dependent genes is linked to changes in glucose availability. This mechanism is likely to have a wide-spread role in maintaining cellular homeostasis, with important implications in cancer, immune regulation and neurodegenerative diseases. This manuscript will be of general interest to the audience of EMBO Journal.

Reply: We are grateful for the refereeís positive assessment of our results, their significance, and their broad potential interest among EMBO readers. We agree that our findings on the regulation of KEAP1 by O-GlcNAcylation may have widespread implications for proteostasis. Indeed, we now provide new preliminary data indicating that other KLHL family members are O-GlcNAcylated (see below), suggesting that conserved, site-specific glycosylation may be a general mode of regulating the KLHL protein family. Although we believe a thorough testing of this hypothesis is beyond the scope of the current manuscript, we have revised our Discussion section (pages 2, 21-22) to propose this interesting possibility for future studies.

Referee 1: However, the authors do not address a significant issue - the ubiquitous presence of a Ser or Thr residue, corresponding to Ser104 in Keap1, in all members of the KLHL family. The authors do not fully incorporate structural and functional data regarding the role of Ser104 and the likely consequences that modifying this residue with O-GlcNAcylation might have. Addressing this issue, which really has two points, would significantly strength the manuscript and increase the general interest in this manuscript. Ser 104 is nearly completely conserved across all members of the KLHL family (Keap1 is also known as KLHL19). One exception is KLHL11, which as a Thr in place of a Ser. Structural analysis of the BTB domains of several members of the family place this serine between two alpha helices. Furthermore, the corresponding Ser residue in gigaxonin (Ser52 in gigaxonin) has been suggested to be a disease-causing mutation in a patient with Giant Axonal Neuropathy. Importantly, there is structural information on this region of Keap1 and several other KLHL proteins. The structural interface of KLHL11 and Cul3 (Canning et. al., JBC 288, 7803-7814, 2013) provides a detailed examination of how the BTB-BACK domains of the KLHL proteins interact with Cul3. Note that KLHL11 has a Thr residue (T119) in place of S104 in Keap1. In addition, there are several structures of the BTB domain of Keap1 in the Protein Data Base, along with a structure of the BTB-BACK domain of gigaxonin.

Thus, the data in this manuscript, if true, have broad implications for all members of the KLHL family. However, the author's interpretation of the role of S104 in the structure and function of Keap1 does not incorporate either the high degree of conservation of the corresponding Ser residue across the KLHL family or the structural information mentioned above.
Incorporating the existing structure data and determining if the central observation holds across other members of the KLHL family would strengthen the conclusions of this manuscript and broaden its relevance.

Reply: We thank the referee for his or her comments and we enthusiastically agree that the possibility of conserved, site-specific glycosylation of KLHL family members is an important implication of our work. In our revised manuscript, we have provided new figures (Figures EV5) to illustrate the conservation of the KEAP1 Ser104-cognate residue across evolution and among nearly all (37 of 42) human KLHL proteins, as the referee suggests. In particular, we have used modeling and the very recently reported structural data by Adamson et al. (PDB: 5NLB) to illustrate the 3D similarity between the BTB domains of KEAP1 and gigaxonin bound to CUL3, and their potentially conserved regulation through glycosylation at KEAP1 Ser104/gigaxonin Ser52 (Figures EV5D). Moreover, we now provide data indicating that gigaxonin itself is O-GlcNAcylated (Figure EV5E). Interestingly, as the referee notes, gigaxonin Ser52 is mutated in a subset of giant axonal neuropathy patients (Bomont et al, 2000; Mahammad et al, 2013), raising the possibility that dysregulated glycosylation of KLHL family members could contribute directly to human disease. We believe that a comprehensive test of this hypothesis is beyond the scope of our current manuscript, which focuses on the KEAP1/NRF2 axis. However, we have included a new discussion of this hypothesis in our revised manuscript (e.g., pages 2, 15 and 21-22), and have already initiated studies to test this hypothesis for future publications.

Referee 1: First, are other members of the KLHL family also regulated by O-linked glycosylation at the residue corresponding to Ser104? It would be relatively straightforward for the authors to demonstrate that other members of the KLHL family are modified by O-linked glycosylation, as the data reported in Figure 4D was obtained using MS analysis of recombinant Keap1 protein purified from HEK293T cells. The use of the GalNAz affinity reagent is another possible approach. If, for example, gigaxonin is also modified by O-linked glycosylation at Ser52, this would significantly extend the importance of the central finding of this manuscript. A detailed functional analysis of such a modification, if found, would clearly be beyond the scope of the current manuscript, since substrates of gigaxonin-dependent ubiquitination have not clearly been established. But information relating to the generality of their observation, given that Ser104 is so highly conserved across all members of this important family of substrate adaptor proteins, is highly relevant. It may be the case that Keap1 is unique in terms of modifying this Ser residue. If so, that also would be important to know and substantiate the claim of the authors that this modification uniquely links regulation of Keap1 function to glucose availability.

Reply: We agree with the referee that the potential glycosylation of other KLHL proteins, beyond KEAP1, is an important and relevant possibility. In our revised manuscript, we now provide new IP/Western data indicating that gigaxonin may indeed be O-GlcNAc-modified (Figure EV5E).

Experiments to determine the functional implications of gigaxonin Ser52 glycosylation are underway. We agree with the referee that these studies are beyond the scope of the current manuscript, but we have revised the manuscript in several places (e.g., pages 21-22) to highlight this possibility and its potential implications for the KLHL family of proteins.

Referee 1: A related issue to the data in Figure 4 is that it appears that only one of the 11 Ser residues in Keap1 that is modified by O-linked glycosylation is actually functionally relevant. This observation, at first glance, would suggest that many O-linked glycosylation events on many proteins in cells is irrelevant for their function. Can the authors comment on why there are so many O-linked glycosylation sites but only a few that appear to have functional consequences?

Reply: We agree with the referee that it is important to address why only Ser104, among the KEAP1 glycosylation sites we mapped, has a functional effect in our assays. Our glycosylation site-mapping experiment was performed via KEAP1 overexpression and pharmacological potentiation of global O-GlcNAcylation. This is a standard approach to mapping O-GlcNAc sites, because the modification is often sub-stoichiometric and is very labile under the peptide fragmentation methods used for mass spectrometry, making the site-mapping of O-GlcNAc moieties a significant technical challenge (Doll & Burlingame, 2015; Ma & Hart, 2017; Myers et al, 2013). It is possible that the other ten candidate O-GlcNAc sites on KEAP1 are glycosylated only in response to unknown stresses, signals or cellular states not tested in our work. Alternatively, some candidate sites may be rarely glycosylated under physiological conditions. Yet another possibility is that glycosylation at these sites is common but impacts other functions of KEAP1 that we have not yet tested, such as the regulation of substrates beyond NRF2. We plan to address these possibilities in future experiments. For the current work, we have revised our manuscript on pages 17-18 to discuss these potential reasons for the lack of functional effects in other KEAP1 glycosylation site point-mutants. Importantly, even if other glycosylation sites regulate KEAP1 under different pathophysiological conditions, we believe our data clearly demonstrate that O-GlcNAcylation of Ser104 is important for the O-GlcNAcylation-responsive regulation of the NRF2 pathway by KEAP1 (Figures 5, 6, EV3, EV4), identifying an important new regulatory connection between O-GlcNAc cycling and the antioxidant stress response.

Referee 1: The second issue relates to the role of S104 in the structure of Keap1 and in terms of its interaction with different binding partners. The authors present Figure 4D as line-drawing of the Keap1 protein onto which the sites of O-linked glycosylation are mapped, along with regions of Keap1 that interact with known partners, including Cul3 and Nrf2 (The relevance of SQSTM1 interaction is not clear, since SQSTM1 binds to Keap1 but is not a ubiquitination substrate of the Keap1:Cul3 ubiquitin ligase complex). This line drawing does not adequately capture the structural data. I suggest that the authors provide a line drawing of the secondary structure elements of Keap1, onto which the interactions of Keap1 with its binding partners (Cul3 and Nrf2) could be mapped. This line diagram would clearly indicate the alpha helices that are likely to directly contact Cul3 (inferred from the KLHL11:Cul3 structure) and the so-called B-C loops in the Kelch domain that form the pocket of Keap1 for Nrf2. This line would more closely represent the structural information, in which alpha-helices at the N-terminal region of the BACK domain directly contact Cul3 which alpha-helices in the BTB domain are known to be involved in forming the anti-parallel Keap1 dimer. As a side note, the authors mention a controversy regarding the role of S104A in Keap1 dimerization and should discuss the structural information regarding dimer formation of KLHL proteins vs their interaction with Cul3.

Reply: We agree with the referee that the secondary structural elements of KEAP1 are important to interpreting our results. We have added this information to Figure 4E, as requested, and have revised the manuscript text on pages 11 and 14-15 to better contextualize our results in the available structural data. In addition, we now discuss our results in light of the very recently available KEAP1 BTB-CUL3 complex structure (PDB: 5NLB) as the most relevant framework for our findings. Finally, we agree that prior structural studies on KEAP1 dimerization are germane for understanding the mechanism through which Ser104 glycosylation affects KEAP1 function. We have added a discussion of these structural studies to our revised manuscript (pages 14-15, 17-18 and Figure EV5A).

Referee 1: The authors present functional data on the S104A mutant. The functional data on Nrf2 regulation could result from an altered conformation of the S104A dimer that changes the spatial

orientation between the two Kelch domains and thereby perturbs efficient ubiquitination of the lysine residues in Nrf2. See the model in the Canning 2013 article and other structural and functional data (largely from the Yamamoto lab) in support of the two-site hinge and latch model for how Keap1 binds Nrf2 and presents lysine residues in Nrf2 for ubiquitin transfer mediated by the Rbx1 protein. The authors demonstrate reduced association between Cul3 and the S104A mutant Keap1 protein. However, these experiments were performed with overexpressed protein and substantial (about 15-30%) of binding of Keap1 to Cul3 is still observed with the mutant protein. Again, the functional consequences of the S014A mutation are best interpreted in light of the two-site hinge and latch model rather than simply equating reduced Cul3 binding with reduced ubiquitination of Nrf2.

Reply: We agree with the referee that Ser104 O-GlcNAcylation may induce a conformational change in KEAP1 to regulate the optimal positioning of NRF2 for ubiquitination, and that this conformational change may be the primary reason that reduced O-GlcNAc (e.g., upon OGT inhibition or glucose starvation) stabilizes NRF2. As the referee notes, the hinge and latch model is an important framework for considering this point. We have revised the manuscript text on pages 4, 14, 15 and 17 to highlight these points more clearly. In addition, we propose that the reduced KEAP1/CUL3 interaction upon loss of Ser104 O-GlcNAc may also contribute to the reduced ubiquitin-mediated destruction of NRF2 (Fig. 5E and F). Future biophysical and functional studies will be necessary to determine whether Ser104 glycosylation influences NRF2 ubiquitination through KEAP1 conformational changes, reduced CUL3 binding, both, or neither. We have revised the manuscript text on pages 17-18 to describe these considerations more fully.

Referee 1: The multiple lines of evidence that inhibition of OGT results in reduced Nrf2 ubiquitination, increased steady-state levels of Nrf2 and increased mRNA levels of multiple Nrf2-dependent genes (Figures 1, 2 and 3) are strong. Those experiments are well designed with appropriate controls and the conclusions are solid. The demonstration that Keap1 is modified by GalNAz is also solid (Figure 4). However, the evidence for modulation of Nrf2 ubiquitination in the experiments reported in Figures 5 and 6 is not as strong as the data in, for example, Figure 3. The authors point to a minor form of Nrf2 that has slower migration in SDS-PAGE and claim that this is the ubiquitinated form of Nrf2. However, Nrf2 is often seen as a doublet (in the data presented by the authors and in many publications in the literature. When the authors are pointing out a form of Nrf2 that they claim is ubiquitinated, there is only a single band of Nrf2. See Figure 6B, 6C, 6D, for example. In contrast, in Figure 5A, the authors point to forms of Nrf2 that are much slower in migration, with a smear (not a single band) in lane 6 of Figure 5A. The data in Figures 6B, 6C and 6D are not convincing (at least to this reviewer) that the S104A protein changes the ubiquitination status of Nrf2, as it is probably the case that the single band that the authors point to in Figures 6B, C and D is NOT a poly-ubiquitinated forms of Nrf2. There are more conclusive ways of measuring ubiquitination of Nrf2, particularly poly-ubiquitination, that should be performed for at least the key experiments in Figure 6 (See Figure 3B).

Reply: We believe the referee's comments resulted from our poor labeling of the original figures and we apologize for the confusion this caused. To clarify, we do not believe that any of the NRF2 bands depicted in Figures 6B-D represent ubiquitinated NRF2. The upper band (arrow) in the NRF2 blots in these figure panels is induced NRF2, not ubiquitinated NRF2. As noted by the referee, NRF2 often appears as a doublet around 100 kDa, consistent with our observations in multiple cell lines (e.g., Figure 2). However, the relative proportion and pattern of the NRF2 doublet bands are not always consistent among cell lines. For example, in Figure 2C, the induced NRF2 in MDA-MB-231 cells is likely the upper band, as indicated by its increased expression in both 5SGlcNAc- and tBHQ-treated cells. Consistent with our model (Figure 6G), treatment with GlcNAc, GlcN or an OGA inhibitor (Figures 6B-D) suppressed the induction of non-ubiquitinated NRF2 caused by glucose deprivation. We have improved the labeling of Figures 6B-D in the revised manuscript to rectify this ambiguity.

Referee 2: The manuscript entitled, "Glycosylation of KEAP1 links nutrient sensing to redox stress Signaling", is a very interesting study that bring novel insights to the field. Here the authors have used an elegant methodologies starting from transcriptomic analysis and using these data to narrow down to deciphering molecular mechanism and provide some insight on the role of O-GlcNAcylation in cell physiology. This paper shows robust data supporting the claim that OGT targets and O-GlcNAcylates KEAP1, which in return leads the recruitment of CUL3 and

the poly ubiquitination and degradation of NRF2. Based on there studies, the authors have suggested that glucose variation may interfere in the proper regulation of NRF2 and leads to tumor growth and chemotherapy resistance. Thus, overall, this is an important and interesting paper. However, some questions and concerns should be addressed:

1. The authors should provide the list of the up and down regulated gene identified in their microarray.

Reply: We thank the referee for his or her positive comments on the interest and importance of our work, the elegance of the methods and the robustness of the data. We have now included Excel files listing the up- and down-regulated genes as supplemental information in the revised manuscript, as requested (Appendix Table S1 and S2). In addition, our complete microarray datasets have been submitted to the NCBI (GEO: GSE81740), and the raw array files will be made freely available upon publication.

Referee 2: Although the authors provide data supporting their model in different cell types, it is not clear why they are changing cell types throughout the manuscript. It would be clearest if they show the complete mechanism in one cell type and select some key experiments in other cell types showing the ubiquity of the mechanism?

Reply: As the referee notes, we selected different cell types in some experiments to overcome specific technical obstacles. In most experiments, we used the MDA-MB-231 and MCF7 breast cancer lines, both of which express wild type KEAP1, because our initial microarray findings were obtained in MDA-MB-231 cells (Figure 1B). However, due to the low transfection efficiency in these cells, we used 293T instead for several protein purification and protein-protein interaction assays, a common practice in the field. To address the importance of each O-GlcNAc site on KEAP1, we used H838 and A549 cells, which harbor autochthonous KEAP1 mutations and are known to have dysregulated NRF2 activity (Singh et al, 2006). This KEAP1-defective background makes H838 and A549 cells appropriate choices for our functional assays involving wild type or mutant KEAP1 re-expression. We have revised the Results section of the manuscript (e.g., pages 11-12) to clarify the reasoning behind the choice of cell lines in several experiments. Importantly, we believe our results in multiple cell types indicate that site-specific KEAP1 O-GlcNAcylation may be a widespread mode of NRF2 pathway regulation.

Referee 2: O-GlcNAc antibodies, especially when used at high concentrations, may show some non-specific binding. Their use must be associated with controls such as a competition blots in presence of free GlcNAc or treatment of the samples with CpNagJ (or hexosamindase). This is particularly important for the IP blots.

Reply: We agree with the referee that additional specificity controls for anti-O-GlcNAc blots would be useful, especially for IP experiments. As requested, we IP-ed KEAP1 and blotted with anti-O-GlcNAc antibody in the presence or absence of free GlcNAc as a well-established specificity control (Sakabe et al, 2010). These IP-ed samples were blotted onto the same membrane with identical loading and chemiluminescence conditions. As indicated in Figure 4C, addition of free GlcNAc extinguished the ~75 kDa KEAP1 band, indicating that the observed signal is specific to KEAP1 O-GlcNAcylation. In addition, we note that 5SGlcNAc and Thiamet-G treatments invariably caused a decrease and increase, respectively, in anti-O-GlcNAc signal detected in input samples on our Western blots, as expected (Figures 2A, 2C, 2D, 3A, 3B, 6D, EV1A, EV2, EV4, S1B, and S3J). These observations reinforce the conclusion that the signal in our Western assays represents authentic O-GlcNAc, and not non-specific background.

Referee 2: The blot shown in Fig S3B doesn't really show an enrichment of NRF2 after IP. Can the authors provide a better experiment?

Reply: We have performed optimized NRF2 IPs with a different NRF2 antibody and have improved these results. Please find the new data in the revised Figure EV2B.

Referee 2: Metabolic labelling with the GalNAz is a powerful tool for O-GlcNAc protein identification, however this method doesn't allow a complete labeling due to competition with endogenous substrate, and the authors may miss a low O-GlcNAc stoichiometry on NRF2 and CUL3 for instance (Fig 4A). GalT labeling would provide a much higher labeling and can be done

on endogenous protein without stressing the cell with the Ac5SGlcNAz treatment.

Reply: We agree with the referee that our GalNAz result does not rule out the possibility that NRF2 and/or CUL3 is O-GlcNAcylated at very low levels or in response to stimuli or stresses that we have not yet examined. We have revised the manuscript text on page 19 to better acknowledge that fact. Importantly, however, we believe the conclusions from our current study would not be affected by future evidence of NRF2 or CUL3 O-GlcNAcylation, because we have demonstrated the requirement for KEAP1 Ser104 in the O-GlcNAc-mediated regulation of NRF2 (Figures 5, 6, EV3, EV4).

Referee 2: In figure 5E, the authors have used the 293T to show that O-GlcNAcylation of KEAP1 enhance its interaction with CUL3 with recombinant tagged constructs. Why have the authors have not used the H838 that doesn't express KEAP1 as they have done to point out the site specific function of the S104 residue?

Reply: We agree with the referee that repeating this experiment in KEAP1-deficient H838 cells would provide additional support for our conclusions. We performed the requested experiment in H838 cells (Figure 5E) and obtained a result similar to that from the 293T system (Figure 5F).

Referee 2: In order to strengthened the role of the O-GlcNAcylation of the S104 on KEAP1 interaction with CUL3 and NRF2 activity, the authors could provide some experiments using high Glucose, Ac5SGlcNAc and/or TMG treatment and investigate the interaction/activity of KEAP1-S104A, CUL3 and NRF2?

Reply: We agree with the referee that these proposed experiments would help reinforce our conclusions. In response to this request, we used IP/Western assays to test the KEAP1/CUL3 interaction under high and low glucose conditions. Consistent with our model (Figure 6G), glucose starvation diminished both KEAP1 O-GlcNAcylation (Figure 6E) and the KEAP1-CUL3 interaction (Figure 6F). This result phenocopies the effects of OGT inhibition (Figure EV4A, B) and the S104A mutation (Figures 5E and 5F) on the KEAP1-CUL3 interaction. We believe these data, taken together, strongly argue that nutrient-responsive KEAP1 Ser104 O-GlcNAcylation is required for optimal KEAP1-CUL3 interaction.

Referee 2: Minor concerns: A previous study has linked OGT/TET1 and NRF2 (Kyoung ah kang et al, oncotarget 2016). This study should be discussed.

Reply: We apologize for neglecting to cite the Kang et al. paper in our original submission. We have added discussion of this work to pages 20 of the revised manuscript. We note that Kang et al. reported a functional connection between OGT and the transcriptional control of the Nrf2 gene, but did not identify KEAP1 (or NRF2) as an OGT substrate, or identify any nutrient-responsive aspect of this connection, making our work complementary to theirs.

Referee 2: References are not really up to date.

Reply: We thank the referee for this suggestion and have carefully updated all references in our revised manuscript.

Referee 3: This work revealed a novel mechanism that O-GlcNAcylation of KEAP1 is required to restrain the NRF2 pathway, and have important implications that nutrient status links to antioxidant signaling. Although it is an interesting finding, following points might be considered upon revision of this manuscript.

Minor points:

Although O-GlcNAcylation of KEAP1 is shown in this study, it remains to be seen whether interaction between OGT and KEAP1 is direct or indirect. To this end, interaction should be examined using recombinant proteins of KEAP1 and OGT.

Reply: We thank the referee for his or her positive comments on the important implications of our work. Because OGT is the sole intracellular O-GlcNAc transferase in mammals, and because KEAP1 is O-GlcNAcylated (Fig. 4, EV3 and S2), we believe OGT and KEAP1 must interact directly

at least transiently. However, we agree with the referee that our co-IP data (Fig. 4D and EV3C (originally S4B)) do not strictly distinguish between direct and indirect interactions. In response to the refereeís comment, we have successfully expressed and purified soluble, recombinant GST-tagged KEAP1 and His-tagged OGT from E. coli. Although KEAP1 and OGT co-IP in human cell extracts (Figure 4D, EV3B, and EV3C), we did not detect any interaction between GST-KEAP1 and His-OGT in vitro (Figure EV3D). This result may indicate that additional proteins or post-translational modifications are required for the endogenous OGT-KEAP1 interaction, or that the GST or His tags interfere with direct binding. Further studies will be necessary to define the minimal components required for the KEAP1-OGT interaction. However, because OGT interacts with and modifies KEAP1 in human cells (Figures 4, EV3B, and EV3C), we believe the lack of evidence for direct interaction in vitro does not affect our overall conclusions. We have included these results in the revised manuscript (page 11) and acknowledged that the OGT-KEAP1 interaction in human cells may require other proteins or modifications.

Referee 3: In addition, responsible domain for interaction between domain KEAP1 and OGT should be determined. Although Figure S4C shows that BTB-deleted KEAP1 still interact with OGT, there is no mention in the text. The authors should explain this result and discuss how Keap1 binds to OGT.

Reply: We apologize for the insufficient description of Figure S4C in our original submission (now Figure EV3C). We have now added additional discussion about this experiment to the Results section (page 11). As the referee notes, the KEAP1 BTB domain is not required for its association with OGT in human cells (Figure EV3C). However, based on our newer in vitro data (Figure EV3D), the KEAP1-OGT interaction may require other proteins and/or post-translational modifications, which could confound the interpretation of additional experiments aimed at defining the interaction domains of KEAP1 and OGT in human cells. The biochemical reconstitution of the KEAP1-OGT interaction using minimal components will be an important goal for future studies, but we believe this substantial endeavor is beyond the scope of our current manuscript.

Referee 3. Although it is a key finding that O-GlcNAcylation of KEAP1 is important for KEAP1-CUL3 interaction, Figures S5J,K showing that reduced interactions between Keap1 and CUL3 by 5SGlcNAc treatment are not convincing. The authors should improve these data and show statistical results.

Reply: We agree with the referee that testing the effect of 5SGlcNAc on the KEAP1-CUL3 interaction is an important experiment. Therefore, we now show in two different cell lines that 5SGlcNAc treatment reduces the endogenous KEAP1-CUL3 interaction (Figures EV4A). Moreover, we have also used epitope-tagged KEAP1 and CUL3 to achieve the sensitivity required to quantify our Western blots, and demonstrated that 5SGlcNAc reduces the KEAP1-CUL3 interaction to a statistically significant extent in this context as well (Figure EV4B). Importantly, these results are in excellent agreement with our data demonstrating that the KEAP1 S104A mutation also reduces its interaction with CUL3 to a statistically significant extent (Figures 5E and 5F). We believe these experiments, taken together, strongly support our model that O-GlcNAcylation of KEAP1 S104 promotes its productive interaction with CUL3.

Referee 3: It should be discussed why O-GlcNAcylation of KEAP1 S104 is structurally important for KEAP1-CUL3 interaction based on the previous report regarding structure of BTB domain (Cleasby et al, 2014; Huerta et al, 2016). Model analysis showing interaction between KEAP1 S104 O-GlcNAcylation with CUL3 would be nice.

Reply: We agree with the referee that the available structural data on KEAP1 provide an important framework for interpreting our results. In our revised manuscript, we have provided new modeling figures based on the very recent structure of a KEAP1-CUL3 complex (PBD: 5NLB) (Figure EV5A and D) to illustrate the proposed biophysical basis for how Ser104 glycosylation might regulate KEAP1-mediated ubiquitination. In brief, we propose that O-GlcNAcylation of KEAP1 Ser104 induces a conformational change in the BTB domain that results in less efficient CUL3 binding and/or NRF2 ubiquitination. Our biochemical results are consistent with this proposed model (Figure 5, 6). We have also added a discussion of these structural considerations to the manuscript text on pages 14-15 and 21-22. In addition, as noted above, the KEAP1 Ser104 residue is conserved among nearly all (37 out of 42) KLHL proteins (Figures EV5B and C), and we now provide

evidence that other human KLHL family members beyond KEAP1 are O-GlcNAcylated (Figure EV5E). These observations suggest that site-specific O-GlcNAcylation may be a general mode of KLHL protein regulation, a possibility that we will test in future studies.

Referee 3: All experiments in this study were conducted using culture cells. The authors should provide physiological evidence that nutrient status influence on KEAP1 O-GlcNAcylation and NRF2 activity in vivo. It would be great if hypoglycemia leads to activation of NRF2 pathway.

Reply: We agree with the referee that in vivo studies of the relationship between nutrient status, KEAP1 O-GlcNAcylation and NRF2 activation should be a major focus of future work. In our current manuscript, we provide extensive evidence for the in vivo anti-correlation between the gene expression signatures of activated NRF2 and low OGT activity, querying six independent human tumor expression datasets (Figure 1C). These results suggest that our observations in cultured cells also hold true in human tumors in vivo. Directed experiments to characterize the functional connection between KEAP1 O-GlcNAcylation and NRF2 activation in various pathophysiological contexts (e.g., mouse models) will be an important priority for future studies. However, we believe such challenging experiments lie beyond the scope of the current manuscript.

Referee 3: In Figure 3B, Myc-NRF2 protein was not accumulated by 5SGlcNAc treatment. As it seems inconsistent data, there should be some explanations.

Reply: We thank the referee for noticing this important detail. In Figure 3B, 5SGlcNAc treatment does not increase Myc-NRF2 levels in the presence of MG132 (lanes 3 versus 4). This result mirrors the response we observe with endogenous NRF2 (e.g., Figure EV2A and EV2B). In this instance, it is likely that proteasome inhibition blocks all NRF2 degradation, and so OGT inhibition does not have an additional effect on NRF2 levels, consistent with our model (Figure 6G). Similarly, little accumulation of Myc-NRF2 is detectable in the absence of MG132 and presence of 5SGlcNAc (lanes 1 versus 2). It may be that the Myc-NRF2 levels are too low in these samples, relative to the MG132-treated samples, to be meaningfully compared in the same experiment, or that the Myc tag blunts the response of this construct to OGT inhibition. Importantly, we note that multiple lines of evidence (Figures 2A-G, 3A, 5A-C, 6A-D, EV2A, EV2B, EV4A, S1B, and S3G) demonstrate that site-specific O-GlcNAcylation of KEAP1 stabilizes endogenous NRF2 protein, consistent with our model (Figure 6G).

References:

Bomont P, Cavalier L, Blondeau F, Ben Hamida C, Belal S, Tazir M, Demir E, Topaloglu H, Korinthenberg R, Tuysuz B, Landrieu P, Hentati F, Koenig M (2000) The gene encoding gigaxonin, a new member of the cytoskeletal BTB/kelch repeat family, is mutated in giant axonal neuropathy. Nature genetics 26: 370-374

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Mahammad S, Murthy SN, Didonna A, Grin B, Israeli E, Perrot R, Bomont P, Julien JP, Kuczmarski E, Opal P, Goldman RD (2013) Giant axonal neuropathy-associated gigaxonin mutations impair intermediate filament protein degradation. The Journal of clinical investigation 123: 1964-1975

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Sakabe K, Wang Z, Hart GW (2010) -N-acetylglucosamine (O-GlcNAc) is part of the histone code. Proceedings of the National Academy of Sciences 107: 19915-19920

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Sidransky D, Gabrielson E, Brock MV, Biswal S (2006) Dysfunctional KEAP1ñNRF2 Interaction in Non-Small-Cell Lung Cancer. PLoS Med 3: e420

2nd Editorial Decision 22 May 2017

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees, whose comments are copied below. As you will see, both referees are satisfied with the improvements in this revision. We shall therefore be pleased to publish the study in The EMBO Journal, pending addressing of a number of remaining editorial issues. I am therefore returning the manuscript to you for an additional round of minor revision, to allow you to upload the accordingly modified files. Once we will have received this final version, we should hopefully be able to swiftly proceed with formal acceptance and production of the manuscript.

REFEREE REPORTS

Referee #1:

The work in this manuscript reveals a novel sensing mechanism by which regulation of Nrf2-dependent genes is linked to changes in glucose availability. This mechanism is likely to be widely utilized to maintain cellular homeostasis, with important implications in cancer, immune regulation and neurodegenerative diseases. This manuscript will be of general interest to the audience of EMBO Journal.

This manuscript presents convincing evidence that (1) modulating levels of O-GlcNAcylation in cells results in activation of Nrf2-dependent genes; (2) steady-state levels of Nrf2 are increased and ubiquitination of Nrf2 is decreased, when OGT is inhibited; (3) that the major regulator of Nrf2, the substrate adaptor Keap1, is modified by O-GlcNAcylation. The authors identify 11 serine residues in Keap1 that are modified by O-GlcNAcylation and present functional data suggesting that one particular serine residue, Ser104 is a critical sensor for reduced glucose levels, leading to reduced O-GlcNAcylation of Keap1 and reduced assembly of a functional Keap1 ubiquitin ligase. This residue is highly conserved across all members of the KLHL family and the mechanism propsed by the authors is likely to be broadly relevant for regulation of KLHL family proteins.

The authors have fully responded to the criticisms of the reviewers and improved the quality and readability of the manuscript. To this reviewer, the revised manuscript is fully acceptable for publication.

Referee #2:

The authors have done a nice job of responding to all of the prior issues. This is an important paper that is well suited to EMBO J.

2nd Revision - authors' response 29 May 2017

We are highly appreciative of the reviewersí comments and positive decision about our manuscript. We have made all the requested editorial changes and provided additional files as requested.

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A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

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- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
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  - are tests one-sided or two-sided?
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  - definition of 'center values' as median or average;
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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F- Data Accessibility

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