THE EMBO JOURNAL

Manuscript EMBO-2009-70764

The Snf1 kinase and proteasome-associated Rad23 regulate UV-responsive gene expression

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Review timeline:

Submission date:	03 March 2009
Editorial Decision:	30 March 2009
Revision received:	29 June 2009
Editorial Decision:	02 July 2009
Revision received:	14 July 2009
Accepted:	16 July 2009

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 30 March 2009

Dear Dr. Auble,

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see while referee 2 is not in favour of publication of the paper here the other referees are very positive in principle and would support publication of the paper here after appropriate revision. Taking together all issues raised we have come to the conclusion that we would be able to consider a revised manuscript in which you will need to address the referees' concerns (including the more specific points raised by referee 2) in an adequate manner. In particular it would be important to address the differential requirement of Mig3 versus Mig1 downstream of different stimuli, but involving the same kinase (Snf1) in some more depth as put forward by referees 1 and 3. I should also point out that as also mentioned by referee 1 the microarray data need to be deposited in one of the public databases (for details, please see "author instructions" on our webpage) and the accession details need to be made available at the time the revised manuscript is submitted.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This paper describes an interesting role for the Snf1 kinase and the proteasome-associated Rad23 protein in UV-responsive gene expression and UV resistance in yeast. Snf1 is the yeast homolog of the mammalian AMP-activated kinase. This important kinase family has been highly studied with regards to the cellular response to nutrient starvation. Although Snf1 has also been implicated in the response to several other stresses, little is known of its roles in these responses. The authors show here that it is required for a normal transcriptional response to UV, although the snf1 mutant is not UV sensitive. Rather surprisingly, this is apparently due to some functional redundancy with Rad23, a proteasome-associated protein that is implicated in the NER pathway. The snf1 rad23 double mutant is significantly sensitive to UV and shows greater transcriptional defects than either single mutant. Previous work had implicated the putative transcriptional repressor Mig3 as a target of Snf1 in response to hydroxyurea, but its gene targets were unknown. The authors show that deletion of MIG3 suppresses the UV sensitivity of the snf1 rad23 mutant. They further show that Mig3 binds the HUG1 gene and this binding is inhibited after UV irradiation in a manner dependant on Snf1 and Rad23. Finally, the authors show that the effect of a rad23 mutant on the UV transcriptome is similar to that of 19S proteasome mutants, thereby suggesting that the role of the 19S proteasome complex may be mediated by Rad23. The general interet of this paper is the demonstration of an important functionally overlapping role for the Snf1 kinase and the Rad23-19S complex in UV-induced transcription and UV resistance. The suppression of the UV sensitivity of snf1 rad23 by deletion of MIG3, and the identification of HUG1 as a target gene, are also important results because it pinpoints a specific transcriptional repressor and gene target in this pathway. These are novel and interesting results.

Major Comments:

1. Some major conclusions regarding the importance of Snf1 and Rad23 in the transcriptomic response to UV irradiation are based on microarray data that are summarized in the form of heat maps, Venn diagrams, and tables with GO classifications. I assume that the microarray data is or will be deposited in public databases. Nevertheless, I was surprised that Excel tables with lists of genes showing differential expression, and the levels of differential expression, were not made available in Supplementary material for at least the most affected genes. I'm not a microarray expert, so I don't know whether this is typical or not. For the HUG1, RAD51, and HMS1 genes, there is convincing confirmatory data provided.

2. The authors claim that Snf1 or Rad23 are required for over half of all UV-responsive gene expression. Does this include the so-called general "environmental stress response" genes?

3. There are some surprising aspects to the UV activation of Snf1. Activating phosphorylation of Snf1 or the mammalian AMPK requires very high doses and is very transient. Mig3 appears to be mainly phosphorylated in a Snf1-dependent manner even before UV treatment. This result is remarkable because Snf1 is thought to have little activity under these conditions. This suggests that the basal activity of Snf1 may be sufficient for UV responsiveness and Mig3 is a privileged substrate. It's not clear that the transient activating phosphorylation of Snf1 is important in the UV response. The microarray data of the authors apparently also suggest a significant effect of Snf1 on the transcriptome of yeast cells under conditions in which Snf1 has often been considered to be inactive or weakly active.

4. The authors provide genetic data suggesting the importance of Mig3 inactivation on the UV resistance of cells, but the critical target genes are not identified. HUG1 is clearly a candidate. Have the authors tried to ectopically express HUG1 in the snf1 rad23 to see if it could suppress UV sensitivity? A negative result wouldn't mean much, but a positive result would be telling.

5. The authors partially examine the issue of crosstalk between the Mig1 and Mig3 repressors (Mig2 is not considered). Mig1 is mainly implicated in the glucose starvation response of Snf1 whereas Mig3 has been implicated in response to HU and now UV. The Mig proteins have similar DNA binding specificity in vitro, but they clearly have distinct functions in vivo. The authors confirm that Mig3 is degraded upon glucose starvation, which can explain why Mig3 does not interfere with depression of Mig1-target genes under these conditions. However, what happens to the UV resistance of cells when they are grown in the absence of glucose and Mig3 is turned over? Is the UV resistance of rad23 changed under these conditions? The authors show that Mig1 and Mig3 are both apparently bound to the HUG1 promoter, but only Mig3 is dissociated upon UV irradiation. Presumably, the Mig1 that remains bound to HUG1 is not active as a repressor for unknown reasons, or perhaps is bound only part of the time or in a fraction of cells.

6. Fig. S4-A seems to show that a fraction of snf1 bar1 cells don't recover from alpha factor arrest. This should be pointed out. Fig. S4-D shows differences as well in the recovery of the asynchronous snf1 and rad23 mutants after UV irradiation compared to the WT. It's striking that the cells do not accumulate in G2/M at 180 minutes as for the WT. This may suggest that the mutant G1 cells do not recover well after UV irradiation. This may be an important clue concerning the function of Snf1 and Rad23 in response to UV and I am surprised that it was not studied in more detail by the authors. Since snf1 does not appear to recover normally from alpha factor treatment, it might be useful to try elutration or nocodazole synchronization to better study the cell cycle progression of the snf1 mutant after UV irradiation.

Minor Comments :

1. You should consider adding Snf1 and proteasome-associated Rad23 to the title, for example : The Snf1 kinase and the proteasome-associated Rad23 regulate UV-responsive gene expression.

2. p. 9: HUG1 and RAD51 are involved in cell cycle arrest?

3. Fig. S1-A: it looks to me like the snf1 rad23 double mutant may be growing a bit worse on raffinose compared to the snf1 single mutant.

4. Fig. 3E and p.17: WT levels are never reached in snf1 rad23. Add: "during the 120 minute time course." It's possible that the induction is just further delayed relative to the single mutants.

Referee #2 (Remarks to the Author):

The manuscript by Wade et al investigated the role of Snf1 and Rad23 in cellular responses to UV-induced DNA damage. They reported certain phenotypes in the absence of these genes such as increased loss of viability and alternative gene expression profiles. Metabolic signaling (including AMPK/Snf1) is clearly recognized as an important pathway for cell to respond to stress conditions such as DNA damage. So the connection between UV and Snf1 is not very novel or surprising. Overall, the data presented are too preliminary, and the observed effect of snf1 and rad23 mutations could be very indirect. The study fails to provide any significant new mechanistic insight. Moreover, many of the results are inconsistent and unconvincing.

Specific comments:

(1) Figures 1, 3, 5: Numerous DNA microarray studies have been performed over the years with a myriad of stress conditions, and snf1 mutants. So the gene expression profiles are not very informative.

(2) Figure 2, the mutant cells are clearly sensitive to low UV irradiation. Why did Snf1 T210 phosphorylation only respond to high UV dosage (100-300 J/m2)? Is T210 phosphorylation truly correlative to Snf1 kinase activity? The loadings are not equal in 2A and 2B.

(3) Figure 3E and 3F, the effect of snf1 and rad23 mutations on expression of HUG1 and RAD51 is

very small. Error bars need to be included to ensure that they are not experimental variations.

(4) Figure 4. 2D gels are not very quantitative. The tiny difference in Mig3 phosphorylation (4D) could be easily due to loading errors.

(5) Figure 5C, changes in Mig3 promoter occupancy in different mutants do not correlate well with the change in HUG3 expression (3E). The ChIP assay with snf1Δ rad23Δ mutant needs to be included. Expression data are missing for HMS1, SUC2 and ACT1 in various mutants under different conditions.

Referee #3 (Remarks to the Author):

This manuscript examines the transcriptional response to UV irradiation. The novel finding is that the nutrient sensing kinase Snf1 joins Rad23 to regulate the transcription of UV response genes. Neither protein would have been predicted to play roles in the transcriptional response to UV damage. A second important point is the idea that two distinct stimuli can converge on one kinase which can then promote different responses. Both glucose starvation and UV irradiation cause activation of Snf1 as judged by the phosphorylation of the Snf1 activation loop. Yet, the responses diverge. When UV is the stimulus, Mig3 is phosphorylated and removed from the UV responsive HUG1 promoter. When glucose starvation is the stimulus, Mig1 is phosphorylated and removed from glucose responsive SUC2 promoter. The authors use ChIP analysis in Figure 5 to demonstrate this. These data left me wondering if glucose starvation led to Mig3 phosphorylation and if UV irradiation led to Mig1 phosphorylation. In other words, two stimuli converge on Snf1. Where do the responses diverge? During the process of substrate selection? Or, after. Some the data needed to answer these questions are in this paper, some is in the earlier Dubacq 2004 paper and some is not provided. The reason it matters where the two pathways diverge is that it could explain why Rad23 is needed. UV response genes need not only active Snf1 (leading to phosphorylated Mig3) but also Rad23. Activation of UV responsive genes does not occur when glucose activates Snf1 because the UV-induced Rad23 activity is lacking.

This paper would be strengthened if this issue were more clearly resolved. Does UV irradiation lead to Mig1 phosphorylation? This should be easy to answer. Does glucose starvation lead to Mig3 phosphorylation? Actually the answer to this is yes and documented in the Supplemental data and the Dubacq 2004 paper. The authors here need only to discuss this more fully.

Minor issues:

1. (Page 7, middle) "including a protein which associates with the Snf1 kinase complex" should say "including Elc1, a protein which associates with a regulatory subunit of the Snf1 kinase complex" .

2. (Page 10, top) The authors discuss the role of Snf1 as a transcriptional regulator and highlight its role phosphorylating histone H3 serine 10. I'm not sure I'd want to state that. That result comes from Shelly Berger's lab and has not been reproduced in other labs (several have tried). Furthermore, Shelly and her postdoc have now reported in two separate meetings that they now believe the Snf1 target is Threonine 39 in histone H2B (unpublished). Therefore, it might be better to discuss Snf1 as a histone kinase without actually mentioning the H3 serine 10 site.

3. (Page 17, bottom) "Mig1 and Mig3 are highly related transcriptional repressors". Actually, they are only highly related in their DNA binding domains, a 60 amino acid stretch in their N-termini. The Snf1 responsive domain in Mig1 is not conserved at all in Mig2 or Mig3.

4. (page 18, first line). The authors mention that Mig3 is phosphorylated and degraded in response to glucose starvation. They cite Figure 5F, S8 and Dubacq 2004. Figure S8 and the Dubacq paper address this point directly but Figure 5F is a ChIP analysis of Mig1.

5. Figure 1. The authors use two-dimensional hierarchical clustering. A reference for this method should be added to the methods section.

1st Revision - authors' response 29 June 2009

This letter accompanies a revised manuscript entitled "The Snf1 Kinase and Proteasome-Associated Rad23 Regulate UV-Responsive Gene Expression". The title has been modified in response to a comment from reviewer 1. Two reviewers of the original submission were "very positive in principle and would support publication of the paper… after appropriate revision". We were urged to revise and resubmit the manuscript in accordance with their comments, as well as the specific comments raised by the other reviewer who was not supportive of the original submission. In accordance with your suggestion, we have addressed the specific criticisms of all three reviewers. The general perception of reviewer 2 regarding the overall impact of our work is straightforward to rebut. One need only appreciate that the importance of the relationship between metabolic regulation and diseases of genomic instability such as cancer is currently a very hot area (e.g. Jones and Thompson, Genes & Dev 23:537, 2009; Vaughn and Deshmukh, Nat. Cell Biol. 10:1477, 2008; Vander Heiden et al, Science 324:1029, 2009; in addition to papers cited in the manuscript). Our manuscript provides a link- the first clear and mechanistically detailed link- between metabolic stress responses and damage signaling in yeast, not to mention new global transcriptional roles for Rad23 and the proteasome. I would be happy to provide a more detailed response if it would be useful.

In this revision we have meaningfully addressed every specific point raised by the previous reviewers, and all but one of the new experimental results is included in the revision. (One result is provided below for the reviewer's evaluation.) Owing to space constraints, a number of the new experimental results are relegated to the Supplement, which now consists of 14 figures and 7 tables. The revised manuscript is slightly above the character limit. This is entirely a consequence of the incorporation of new results obtained in response to reviewers' concerns. None of the new results affect the main conclusions of the original submission. As requested by reviewer 1, we also include an excel file with lists of affected genes, and all of the microarray data have been deposited to GEO and are available for reviewer scrutiny (link below).

These are the main changes (detailed responses follow):

·We provide estimates of the error associated with the relative expression levels of the various genes we analyzed by real-time PCR or Northern blotting. We also report associated errors for all ChIP data.

·To address the question of whether Snf1 activation or just "basal" kinase activity is involved in the UV response, we now report expression levels of Snf1-dependent genes in Snf1-T210A strains. In new results, we also genetically analyzed *snf4*Δ cells, which are impaired in activated Snf1 activity, and we do a better job of explaining how all of the data- ours and others- provide a consistent picture of the role of Snf1 basal and activated kinase activity in the UV response.

·We clarify the distinct roles of Mig1 and Mig3 by presenting new data for the behavior of each in response to both glucose limitation and UV irradiation. The experiments involved genetic analyses as well as analyses of the protein via western blotting.

·We clarify the relationships of Snf1 and Rad23 to damage-induced cell cycle arrest by providing extensive new flow cytometry data using a nocodazole arrest, followed by release into alpha factor. We show that *snf1*Δ *rad23*Δ cells delay re-entry to the cell cycle, supporting the reviewer's hunch that these factors may indeed participate in this process, perhaps by regulating expression of cell cycle regulation genes as we originally noticed from the microarray data.

We are grateful to the reviewers for their comments, and with these changes, we feel that the manuscript is markedly improved. As we have now addressed all of the reviewers' specific concerns, we hope that you will find this manuscript acceptable for publication in *EMBO Journal.* We look forward to your reply.

Referee #1:

Major Comments:

1. Some major conclusions regarding the importance of Snf1 and Rad23 in the transcriptomic response to UV irradiation are based on microarray data that are summarized in the form of heat maps, Venn diagrams, and tables with GO classifications. I assume that the microarray data is or will be deposited in public databases. Nevertheless, I was surprised that Excel tables with lists of genes showing differential expression, and the levels of differential expression, were not made available in Supplementary material for at least the most affected genes. I'm not a microarray expert, so I don't know whether this is typical or not. For the HUG1, RAD51, and HMS1 genes, there is convincing confirmatory data provided.

All microarray data have now been deposited to the Gene Expression Omnibus repository. The data can be accessed via the following reviewer link:

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=jluvhaygaqsoqfq&acc=GSE16799

When this work is published, all microarray data will become publicly available via the GEO link. In addition, an Excel spreadsheet listing all UV responsive genes has been included as Table S1.

2. The authors claim that Snf1 or Rad23 are required for over half of all UV-responsive gene expression. Does this include the so-called general "environmental stress response" genes?

We have compared genes whose UV-response is either Snf1 or Rad23-dependent to the "environmental stress response" (ESR) genes identified in Gasch et al (2000). This comparison is provided in new Figure S4 and shows that about 40% of ESR genes are either Snf1- or Rad23-dependent for proper UV response and about 20% of all ESR genes require both Snf1 and Rad23 for appropriate UV-mediated regulation.
A sentence has been added on page 10 as follows:

"This gene set includes about 40% of the general environmental stress response genes identified by Gasch et al (2000; Figure S4)."

3. There are some surprising aspects to the UV activation of Snf1. Activating phosphorylation of Snf1 or the mammalian AMPK requires very high doses and is very transient. Mig3 appears to be mainly phosphorylated in a Snf1-dependent manner even before UV treatment. This result is remarkable because Snf1 is thought to have little activity under these conditions. This suggests that the basal activity of Snf1 may be sufficient for UV responsiveness and Mig3 is a privileged substrate. It's not clear that the transient activating phosphorylation of Snf1 is important in the UV response. The microarray data of the authors apparently also suggest a significant effect of Snf1 on the transcriptome of yeast cells under conditions in which Snf1 has often been considered to be inactive or weakly active.

This is an interesting comment. The reviewer makes a couple of important points here. First, is the transient activation of Snf1 important? Our original genetic analysis (Figures 3B and C in the revision) showed that the inability to activate Snf1 by phosphorylation was less of a problem for damaged cells than complete loss of kinase activity, at least in terms of cell survival. In the revision we provide new information that leads us to conclude that Snf1 activation plays some role in the UV response, but it is not apparently essential. This conclusion is in excellent agreement with the work of Dubacq et al, 2004, who likewise reached the conclusion that while Snf1 kinase activity is important for the response of cells to hydroxyurea, kinase activation is not essential. In new results we measured the expression of three genes in WT versus *snf1*Δ, *snf1-K84R*, and *snf1-T210A* cells (Figures 3D-E), and consistent with the genetics, we find that while a catalytically-dead kinase mutant (K84R) behaved equivalently to *snf1*Δ, the inability to activate Snf1 (T210A) had less of an effect (or in some cases, was of no detectable importance). We conclude that Snf1 activation apparently plays a less important and gene-specific role. We also show in new data (Figure S2) that loss of Snf4 (a Snf1 kinase complex subunit involved in kinase activation) does not have the same effect on UV survival as loss of Snf1 kinase activity. Again, this is what one would expect if kinase activation is not absolutely required for the Snf1-mediated UV response. However, it is interesting

that a more severe genetic interaction was observed when all three upstream Snf1 kinases were deleted (Figure S2), which we speculate could mean that these kinases target other substrates in addition to Snf1. The new results are presented beginning on the top of page 8 and extend to the top of page 9, where we also make plainer the consistency of the results presented here with those of Dubacq et al.

The reviewer also points out that the data support a role for Snf1 in the absence of cellular stress and kinase activation. We quite agree that this is striking, and is an apparently overlooked point. In our opinion, this is highly relevant for the preceding discussion, because under these conditions the generally accepted view would be that the kinase is inactive. In retrospect, I suppose we should have anticipated a role for Snf1 in nutrient replete conditions for the simple reason that *snf1*Δ cells grow slowly even in rich media.

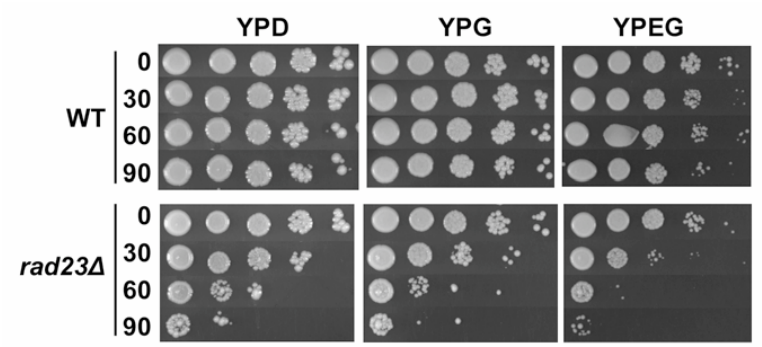
4. The authors provide genetic data suggesting the importance of Mig3 inactivation on the UV resistance of cells, but the critical target genes are not identified. HUG1 is clearly a candidate. Have the authors tried to ectopically express HUG1 in the snf1 rad23 to see if it could suppress UV sensitivity? A negative result wouldn't mean much, but a positive result would be telling.

Given the robust UV-mediated regulation of *HUG1*, this was a reasonable suggestion. We did the experiment using a high-copy plasmid carrying *HUG1*, and alas, increasing *HUG1* dosage did not suppress the UV sensitivity of snf1Δ rad23Δ cells. The results are shown in Figure S8 and presented on page 11:

"Given the roles of Snf1 and Rad23 in *HUG1* expression (Figure 4E), and evidence implicating *HUG1* in the *MEC1* damage response pathway (Basrai et al, 1999), we tested the possibility that a defect in *HUG1* expression was responsible for reduced cell survival in response to irradiation. However, high-copy *HUG1* did not rescue the UV phenotype of *snf1*Δ *rad23*Δ cells (Figure S8). "

5. The authors partially examine the issue of crosstalk between the Mig1 and Mig3 repressors (Mig2 is not considered). Mig1 is mainly implicated in the glucose starvation response of Snf1 whereas Mig3 has been implicated in response to HU and now UV. The Mig proteins have similar DNA binding specificity in vitro, but they clearly have distinct functions in vivo. The authors confirm that Mig3 is degraded upon glucose starvation, which can explain why Mig3 does not interfere with depression of Mig1-target genes under these conditions. However, what happens to the UV resistance of cells when they are grown in the absence of glucose and Mig3 is turned over? Is the UV resistance of rad23 changed under these conditions?

Since Mig3 generally functions as a repressor, perhaps the reviewer was considering the possibility that loss of *MIG3* would suppress (or partially suppress) the UV defect in *rad23*Δ cells. However, one has to remember that Rad23 plays important roles in both repair and transcription, and the phenotypic effect of Rad23 on transcription was only uncovered in *snf1*Δ cells. Thus, we would not anticipate a genetic interaction between *rad23*Δ and *mig3*Δ as suggested. Prior to the previous submission, we had already tested *rad23*Δ *mig3*Δ cells and found that they are indistinguishable from *rad23*Δ cells in terms of their UV sensitivity. (The reviewer had no way of knowing this since it wasn't in the paper.) Even so, something else could in principle be going on, or perhaps we have misinterpreted the reviewer's suggestion, so we performed the experiment suggested by the reviewer and include the result below. The UV resistance of *rad23*Δ cells was not affected by growth on galactose. There was a slight increase in UV sensitivity of *rad23*Δ cells on YPEG (2% ethanol, 2% glycerol). However, because Mig3 is degraded upon a switch to galactose as well, this cannot be interpreted as an effect of Mig3 degradation. Since (to us at least) the result is not very illuminating, and because it would require significant text to explain the rationale and the result, we provide the result here but not in the revision.



WT *or rad23*Δ cells were spotted in 10-fold serial dilutions onto agar plates containing YPD (rich media, 2% glucose), YPG (2% galactose) or YPEG (2% ethanol, 2% glycerol). Each row of cells was then subjected to the indicated dose of UV light indicated on the left (J/m2).

The authors show that Mig1 and Mig3 are both apparently bound to the HUG1 promoter, but only Mig3 is dissociated upon UV irradiation. Presumably, the Mig1 that remains bound to HUG1 is not active as a repressor for unknown reasons, or perhaps is bound only part of the time or in a fraction of cells.

We hadn't tested directly whether Mig1 participates in *HUG1* regulation, so we performed this experiment. Interestingly, new expression data shows that Mig1 can indeed repress *HUG1*, however not to the extent of Mig3 (Table S5). This is consistent with the idea that the Mig1 we detect by ChIP at the *HUG1* promoter is functionally significant. If that is the case, why then do we not detect its dissociation following UV irradiation? There are many possible explanations, including the inherent limitations of the ChIP assay. (As the reviewer points out, Mig1 could be very transiently associated with the *HUG1* promoter or only bound in a fraction of cells.) Alternatively, we may be misled by the Mig1 ChIP results, and perhaps the effect of Mig1 on *HUG1* expression is indirect. Although we have done more work to clarify this issue, we still can't provide a complete mechanistic explanation for the differential transcriptional effects of Mig1 and Mig3. Overall, we feel that this manuscript provides a reasonably complete first step in dissecting the functional roles of these related proteins and that the more detailed exploration of Mig1 versus Mig3 function is beyond the scope of this study.

We address all of the results regarding Mig1 and Mig3 beginning on page 19 about mid-page.

6. Fig. S4-A seems to show that a fraction of snf1 bar1 cells don't recover from alpha factor arrest. This should be pointed out. Fig. S4-D shows differences as well in the recovery of the asynchronous snf1 and rad23 mutants after UV irradiation compared to the WT. It's striking that the cells do not accumulate in G2/M at 180 minutes as for the WT. This may suggest that the mutant G1 cells do not recover well after UV irradiation. This may be an important clue concerning the function of Snf1 and Rad23 in response to UV and I am surprised that it was not studied in more detail by the authors. Since snf1 does not appear to recover normally from alpha factor treatment, it might be useful to try elutration or nocodazole synchronization to better study the cell cycle progression of the snf1 mutant after UV irradiation.

Many thanks to the reviewer for this astute observation. We repeated the cell cycle analysis using a nocodazole block, followed by release into alpha factor. We released the cells into alpha factor to ensure that cells in the G2 peak following release were cells delayed there and not cells that had traversed the cell cycle and re-entered G2. The experiment was not trivial due to the longer cycling time of *snf1*Δ cells, which necessitated an arrest with nocodazole twice as long as with WT or *rad23*Δ cells. The prolonged arrest caused some cell death (as evidenced by the increased sub-G1 population) and over-replication of mitochondrial DNA (observable as a broadening of the G2 peak). Nonetheless, as shown in Figure S7, compared to WT or single mutants, *snf1*Δ *rad23*Δ cells did have a significant delay in re-entering the cell cycle following nocodazole arrest. This delay is consistent with the roles for Snf1 and Rad23 in expression of cell cycle regulatory genes uncovered in the microarray data, and provides a new biological consequence of Snf1 and Rad23 action for

future study. These new results replace the previous alpha factor arrest experiment and in combination with other results led us to write (page 11):

"Taken together, the results suggest that the synthetic UV phenotype observed in *snf1*Δ *rad23*Δ cells is due to the additive effect of gene expression changes that influence cell cycle arrest pathways and possibly other DNA damage response mechanisms."

Minor Comments:

1. You should consider adding Snf1 and proteasome-associated Rad23 to the title, for example: The Snf1 kinase and the proteasome-associated Rad23 regulate UV-responsive gene expression.

Good idea. Done.

2. p. 9: HUG1 and RAD51 are involved in cell cycle arrest?

HUG1 has been implicated in cell cycle arrest via the *MEC1* pathway whereas *RAD51* is involved in repair but to our knowledge has not been directly implicated in cell cycle arrest. We have clarified this point in the revision by discussing the genes separately (page 11).

3. Fig. S1-A: it looks to me like the snf1 rad23 double mutant may be growing a bit worse on raffinose compared to the snf1 single mutant.

While there appears to be a slight difference in the growth between these two strains on raffinose plates, we do not believe this to be significant as it is a very small difference and was not reproducible.

4. Fig. 3E and p.17: WT levels are never reached in snf1 rad23. Add: "during the 120 minute time course." It's possible that the induction is just further delayed relative to the single mutants.

The wording of this section has been changed as suggested.

Referee #2:

Specific comments:

(1) Figures 1, 3, 5: Numerous DNA microarray studies have been performed over the years with a myriad of stress conditions, and snf1 mutants. So the gene expression profiles are not very informative.

While microarray data is available for many different types of stress conditions, and *snf1* cells have also been analyzed previously, the data presented here adds considerably to our understanding of Snf1 function, and we use these data to uncover new functions for other factors, including Rad23, which had no previously known role in transcription. In contrast to the reviewer's comment, there was no solid evidence linking Snf1 to the UV damage response, let alone mechanistic insight into how that might work. We made the discovery via the genetic connection we report here between Snf1 and Rad23, which uncovered a UV phenotype. Considering that the cellular response to damage is one of the most intensely studied responses, we find it striking and of considerable importance that Snf1 and Rad23 are required for most damage induced gene expression, and yet prior to this study neither one had been implicated in this response. The prior work of Carl Mann and colleagues (Dubacq et al, 2004) regarding the role of Snf1 in the response to HU was intriguing, but the study did not directly link a phenotype to gene expression. As pointed out by the first reviewer, a large-scale role for Snf1 in undamaged cells grown in rich medium is novel since the kinase has been thought to be inactive under these conditions. Thus, this study provides new insight into Snf1 function, even considering an area in which Snf1 function was thought to be well understood. We are confident that this work will be of broad interest, notably among individuals who work in the Snf1/AMPK field.

A related point is that the reviewer's comment dismisses or ignores other major aspects of this manuscript. We used the expression analyses documenting the UV response and the role of Snf1 to

understand functional roles for Rad23, Mig3, the proteasome, and Rad4 in gene expression, as well as some of their combinatorial effects. Of course microarray data alone tend in general to provide limited insight, which is why we combined expression analyses with genetic analyses, ChIP, and analyses of protein modification state. High quality damage response and *snf1*Δ datasets were required for comparison with other congenic strains; the results alluded to by the reviewer aren't the point of the study; rather they were just the beginning.

(2) Figure 2, the mutant cells are clearly sensitive to low UV irradiation. Why did Snf1 T210 phosphorylation only respond to high UV dosage (100-300 J/m2)? Is T210 phosphorylation truly correlative to Snf1 kinase activity? The loadings are not equal in 2A and 2B.

The question of whether Snf1 activation is functionally important for the UV response is a good one. Please see the response to reviewer 1, point 3, for a description of new data that address this issue. We now provide analyses of the effects of abrogating Snf1 activation on Snf1- and UV-dependent gene expression, as well as new genetic results using *snf4*Δ cells. Taken together, the results presented here as well as the results of Dubacq et al, 2004, provide a reasonably coherent picture in which Snf1 activation contributes to, but is not essential for, the Snf1-mediated damage response. In addition, one should keep in mind a technical issue: the ability to detect low-level Snf1 activation may be limited by the antibody and western blotting assay. The data do not rule out the possibility of low-level Snf1 activation at lower doses of irradiation.

The reviewer points out unequal loading in Figures 2A and 2B, however this does not affect the interpretation of the results. Loading between comparable samples (glucose vs galactose, unirradiated vs irradiated) is consistent. Precipitation of more total Snf1-HA in lanes 7 and 8 of Figure 2B indicates that the phosphorylation levels seen following damage are higher that those seen in cells in galactose-containing media and the text has been amended (page 6) to make this point:

"In proportion to the total level of Snf1, levels of activation in response to 300 J/m2 UV irradiation were somewhat higher than those in cells switched to galactose for one hour (Figure 2B)."

(3) Figure 3E and 3F, the effect of snf1 and rad23 mutations on expression of HUG1 and RAD51 is very small. Error bars need to be included to ensure that they are not experimental variations.

Error bars have been added in what is now Figure 4E, 4F and S5. The error bars do not change the original conclusions.

(4) Figure 4. 2D gels are not very quantitative. The tiny difference in Mig3 phosphorylation (4D) could be easily due to loading errors.

The differences that we report are modest, and vary in magnitude from experiment to experiment. However, we feel these are comparable to other published results obtained from 2D Western blots to detect relatively non-abundant proteins. These are difficult experiments. We have included additional replicates of this experiment in the supplement (Figure S11) which illustrate the reproducibility of a 15-20% increase in phosphorylated Mig3-myc following damage. We disagree with the reviewer that the differences can be attributable to loading errors. A hypothetical loading error might affect the overall signal on the blot but could not in any obvious way give rise to a change in the proportions of the phosphorylated and unphosphorylated forms in a homogeneous sample.

*(5) Figure 5C, changes in Mig3 promoter occupancy in different mutants do not correlate well with the change in HUG3 expression (3E). The ChIP assay with snf1*Δ *rad23*Δ *mutant needs to be included. Expression data are missing for HMS1, SUC2 and ACT1 in various mutants under different conditions.*

Changes in Mig3 promoter occupancy *do* correlate with *HUG1* expression. This is explained on page 14 of the revision:

"A ten-fold enrichment in ChIP signal was seen at the *HUG1* promoter for Mig3-myc, whereas no such localization was observed at the *ACT1* promoter (Figures 6C and D). In addition, Mig3 was released from the *HUG1* promoter after UV irradiation, and this release was Snf1-dependent (Figure

6C). Most interestingly, the release of Mig3 from the *HUG1* promoter was also dependent on Rad23 (Figure 6C). Displacement of Mig3 from the promoter preceded the accumulation of *HUG1* mRNA, which peaked at 60 min (Figures 4E and S5), as expected if the events were mechanistically coupled. The requirement for both Snf1 and Rad23 for release of Mig3 from the *HUG1* promoter offers a molecular explanation for the contributions of these two factors to UV-dependent *HUG1* expression."

(The relevant results are also reviewed on pages 18 and 19 of the Discussion.)

ChIP results using *snf1*Δ *rad23*Δ cells are now shown in Figure 6C. In this strain, Mig3 occupied the promoter in undamaged cells and failed to vacate the promoter following UV. This is fully consistent with the model we present, data from the single mutants, and all of the other results.

Expression data are included in the revision for all genes used in the ChIP assays, and in all strains examined. In addition to data presented in multiple figures in the body of the paper and in the Supplement, Table S5 summarizes all of the results.

Referee #3:

This manuscript examines the transcriptional response to UV irradiation. The novel finding is that the nutrient sensing kinase Snf1 joins Rad23 to regulate the transcription of UV response genes. Neither protein would have been predicted to play roles in the transcriptional response to UV damage. A second important point is the idea that two distinct stimuli can converge on one kinase which can then promote different responses. Both glucose starvation and UV irradiation cause activation of Snf1 as judged by the phosphorylation of the Snf1 activation loop. Yet, the responses diverge. When UV is the stimulus, Mig3 is phosphorylated and removed from the UV responsive HUG1 promoter. When glucose starvation is the stimulus, Mig1 is phosphorylated and removed from glucose responsive SUC2 promoter. The authors use ChIP analysis in Figure 5 to demonstrate this. These data left me wondering if glucose starvation led to Mig3 phosphorylation and if UV irradiation led to Mig1 phosphorylation.

Mig3 phosphorylation increased following UV irradiation and it was known to be degraded upon glucose starvation. Degradation of Mig3 is now recapitulated in the revision in Figure S12 and the results are in good agreement with the results of Dubacq et al, 2004. Thus, Mig3 is a target of both UV and starvation responses, however the function of Mig3 in gene expression is dependent on the stimulus, at least to a first approximation. We also show in Figure S12 that Mig1, on the other hand, is phosphorylated upon starvation but not following UV irradiation- even at very high UV doses. This suggests that Mig1 is a specific target of the starvation response. These results are discussed on page 19 of the revision:

"Genetic results presented here show that there is a fairly clean delineation in the response of Mig1 or Mig3 to a particular stimulus (Figures 5A-B and S10). In addition, Mig1 is phosphorylated and vacates promoters in response to glucose limitation but not irradiation and Mig3 vacates only UV-responsive promoters upon irradiation (Figures 6C and S12). On the other hand, despite the lack of evidence for a functional role for Mig3 in the starvation response, it is modified and degraded in response to glucose deprivation (Figure S12; Dubacq et al, 2004) and there is overlap in the DNA binding site preferences of Mig1 and Mig3 (Figure 6C-F and S12; Lutfiyya et al, 1998). These results imply that Mig1 and Mig3 are necessary but insufficient for dictating the observed transcriptional regulatory patterns, and that other co-activators/co-repressors likely delimit how and where Mig1 and Mig3 act."

In other words, two stimuli converge on Snf1. Where do the responses diverge? During the process of substrate selection? Or, after. Some the data needed to answer these questions are in this paper, some is in the earlier Dubacq 2004 paper and some is not provided. The reason it matters where the two pathways diverge is that it could explain why Rad23 is needed. UV response genes need not only active Snf1 (leading to phosphorylated Mig3) but also Rad23. Activation of UV responsive genes does not occur when glucose activates Snf1 because the UV-induced Rad23 activity is lacking. This paper would be strengthened if this issue were more clearly resolved. Does UV irradiation lead to Mig1 phosphorylation? This should be easy to answer. Does glucose starvation lead to Mig3 phosphorylation? Actually the answer to this is yes and documented in the Supplemental data

and the Dubacq 2004 paper. The authors here need only to discuss this more fully.

We appreciate the reviewer's thoughtful comments on this issue. As this is an elaboration on the points raised above, our thoughts on this are mostly captured in the preceding response. As described above, new results show that Mig1 is phosphorylated in response to glucose limitation but not irradiation. Yes, glucose starvation led to modification of Mig3, and then its degradation. The modification of Mig3 under these conditions is Snf1-dependent but the shift in mobility is quite large and may reflect coupled phosphorylation and ubiquitylation. To our knowledge, the precise modification state of Mig3 in starvation, prior to destruction, has not been nailed down. In any event, the data we present in Figure S12 is in good agreement with Dubacq et al, 2004.

Minor issues:

1. (Page 7, middle) "including a protein which associates with the Snf1 kinase complex" should say "including Elc1, a protein which associates with a regulatory subunit of the Snf1 kinase complex" .

The sentence has been changed as suggested.

2. (Page 10, top) The authors discuss the role of Snf1 as a transcriptional regulator and highlight its role phosphorylating histone H3 serine 10. I'm not sure I'd want to state that. That result comes from Shelly Berger's lab and has not been reproduced in other labs (several have tried). Furthermore, Shelly and her postdoc have now reported in two separate meetings that they now believe the Snf1 target is Threonine 39 in histone H2B (unpublished). Therefore, it might be better to discuss Snf1 as a histone kinase without actually mentioning the H3 serine 10 site.

With all due respect, we disagree. The permanent scientific record is the published record, and to date we are aware of no clarification about this in the published record. We are among those who have failed to reproduce some of those results, and we believe others would benefit from knowing our results as they might inform their own work. Indeed, had we known at the time that H3 S10 was in doubt as a substrate, we may not have spent the effort to test it. We also feel that the broader scientific community- those who haven't had the opportunity to hear Shelley speak- ought to be aware that there are other observations that have been made regarding the possible roles of H3 S10 modification by Snf1. Finally, strictly speaking, our results don't challenge anything previously published; after all, one point of this manuscript is that Snf1 targets different substrates in response to different stimuli.

3. (Page 17, bottom) "Mig1 and Mig3 are highly related transcriptional repressors". Actually, they are only highly related in their DNA binding domains, a 60 amino acid stretch in their N-termini. The Snf1 responsive domain in Mig1 is not conserved at all in Mig2 or Mig3.

Thanks for pointing this out. The sentence has been edited as follows (page 19):

"Mig1 and Mig3 are homologous transcriptional repressors with highly related DNA binding domains, yet they have distinct functional specificities *in vivo* (Lutfiyya et al, 1998; Treitel et al, 1998; Figures 5, 6 and S12)."

4. (page 18, first line). The authors mention that Mig3 is phosphorylated and degraded in response to glucose starvation. They cite Figure 5F, S8 and Dubacq 2004. Figure S8 and the Dubacq paper address this point directly but Figure 5F is a ChIP analysis of Mig1.

This has been corrected.

5. Figure 1. The authors use two-dimensional hierarchical clustering. A reference for this method should be added to the methods section.

This is now explained in the Materials and Methods section (page 26) as follows:

"In order to identify genes with similar expression profiles across our time course, clustering analysis was performed using the clustergram package from the Bioinformatics toolbox in MATLAB (Bar-Joseph et al, 2001; Eisen et al, 1998). This package creates a dendogram and

heatmap of the gene expression data and the samples for two way clustering. The Spearman rank correlation was used as a measure of gene expression profile similarity to generate the hierarchical tree, and the clustering was performed on both genes and on strains (i.e., two way clustering)."

2nd Editorial Decision 02 July 2009

Thank you for sending us your revised manuscript. Our original referees 1 and 3 have now seen it again, and you will be pleased to learn that in their view you have addressed their criticisms in a satisfactory manner, and that the paper will therefore be publishable in The EMBO Journal.

Before this will happen, however, I was wondering whether you would like to consider addressing the minor issue suggested by referee 1 (see below). Furthermore, I need to ask you to submit your microarray data to one of the relevant public databases and to include the accession details into the final version of the manuscript according to our policies (please follow "author instructions" on our web page).

Please let us have a suitably amended manuscript as soon as possible. I will then formally accept the manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors have addressed all my concerns and I feel that their paper is of high quality and novelty and should be of great interest to all those working on the Snf1/AMP-activated kinases or cellular responses to genotoxic stress. My only technical comment concerning the revision has to with the new cell cycle analyses. The authors provide interesting new data showing an inhibition of mitotic exit for the snf1 rad23 double mutant after UV-irradiation. Their original data suggested that there might also be a defect in G1/S recovery after UV and a defect in alpha factor recovery of the snf1 mutant that could have been retained as well in the revised manuscript.

Referee #3 (Remarks to the Author):

The authors have responded to all the points raised by me and the other reviewers.

2nd Revision - authors' response 14 July 2009

This letter accompanies a second revision of the manuscript entitled "The Snf1 Kinase and Proteasome-Associated Rad23 Regulate UV-Responsive Gene Expression". Two issues were raised regarding our first revision. Reviewer 1 requested that we include the original flow cytometry data. In the first revision we replaced these results with new flow cytometry results from experiments performed in a different way. Here we include both sets of results as requested (Figures S7 and S15). In addition, you requested that we submit our microarray data to a public database and provide an

accession number as stipulated by The EMBO Journal author guidelines. All microarray data have been deposited to GEO and we state in the Materials and Methods section (page 26):

"Microarray data has been deposited to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/): accession number GSE16799."

