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SLR-2 and JMJC-1 regulate an evolutionarily conserved stress-response network

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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 11 July 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the exceptionally long delay in getting back to you with a decision. Unfortunately, we experienced difficulties in finding suitable and willing referees for this manuscript. In addition, two of the referees were not able to get back to us with their reports as quickly as initially expected.

Your manuscript has now finally been seen by three referees whose comments to the authors are shown below. As you will see all three referees consider the study as interesting in principle. Referee 1 is very positive, but the other two referees feel that some more work is required before they can support publication of the study here. One major issue refers to the role of NO66 in mammalian cells. Both referees feel that knockdown experiments should be performed for NO66 in mammalian cells to causally link NO66 to the mammalian stress response. Furthermore, both referees feel that you should compare you dataset to other/more published datasets on daf-16. Also, referee 3 feels that the functional significance of the ESREs should be tested more directly. All in all we should thus be able to consider a revised manuscript if you can address the issues put forward by the referees in an adequate manner and to their satisfaction.

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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This is an outstanding paper; the authors are to be commended.

I have only a few minor comments.

1) Although I am convinced that DAF-16 has little relevance to control by SLR-2/JMJC-1; data for the converse are not as clear. This should be corrected or the statements about cross-talk/co-regulation/independence modified somewhat.

2) Put SLR-2 into the title: Perhaps "SLR-2: a master-regulator of a new evolutionarily conserved stress-response pathway"

3) There certainly could (and probably should) be an additional paragraph that frames these results in the framework of the "regulation of aging". This compound term is misused in the aging field because it is underlying stress-response pathways that are being regulated and differential longevity is the outcome.

Referee #2 (Remarks to the Author):

The Kirienko et al. manuscript describes two new players important for general stress responses in C. elegans. SLR-2 is a zinc finger protein previously known to affect nutrient utilization in C. elegans. In this paper, the authors demonstrated that the expression of slr-2 is induced upon a variety of stress stimuli, including heat, hypertonic, ethanol, and oxidative stress. Based on data from previous microarray studies, the authors identified a set of genes carrying a consensus ESRE motif, and their expression is stress-induced and subjected to regulation by SLR-2. Consistent with its role in stress response, a slr-2 mutant is sensitive to a wide-variety of stress stimuli, whereas overexpression of slr-2 confers greater resistance to ethanol stress. The authors also provided evidence that slr-2 regulates the expression of a downstream effector jmjc-1. jmjc-1 expression is also induced upon stress and is required for stress-induced upregulation of ESRE-containing genes. jmjc-1 is evolutionarily conserved; its inactivation in C. elegans and Drosophila lead to reduced survival upon stress, and its expression is induced by stress in mammals. Overall, this is an interesting paper and will be of general interest to a wide range of readers. In general, the experiments are well-done, and the data presented appear solid. I have a few comments, which should help to strengthen the conclusion of the paper.

Specific Comments:

1) For all the qRT-PCR experiments, the authors should indicate how many independent trials were carried out, and whether the SEM represents errors among different trials.

2) Both the slr-2 and jmjc-1 mutants are sensitive to a variety of stress stimuli. The authors showed that overexpression of slr-2 confers resistance to ethanol. Does it confer resistance to other stresses as well?

What about overexpression of jmjc-1?

3) It seems that Figure 4D and Fig. S8 B are showing results from different trials of the same experiment? If so, then showing it once is probably sufficient.

4) For Fig. S8, the lifespan data (survival curves) under normal culturing condition should be shown.

5) For Fig. 6C, to be consistent with the other figures and to more rigorously compare the data, the fold change should compare the expression under stressed and unstressed condition for each of the genotype. This is important, as some of the genes may show lower basal expression in the mutant genotype, and their expression upon stress may be lower than that in wild-type, but the fold induction can be similar.

6) Likewise, for Fig S6, fold change should be compared the basal expression of each gene in the respective genotype.

7) For comparison with df-16 microarray data, the authors should consider including the data from Murphy et al., 2003. This dataset is quite different from the McElwee dataset and will be interesting to see if ESRE motif may be detected in the Murphy dataset.

8) Have the authors tested whether hsf-1 is required for slr-2 induction upon stress?

9) Have the authors tested whether knockdown of NO66 in mammalian cells will confer sensitivity to various stress stimuli?

Referee #3 (Remarks to the Author):

COMMENTS TO AUTHORS:
The paper is interesting and makes important observations. However, there are a number of concerns that need to be addressed. Importantly, some conclusions overstate the data.

1. There are problems with literature citations. Page 3, bottom: This sentence implies that NRF2 is regulated by heat. There is no evidence in Kell et al for this. Sentence also implies that PDHK-2 etc. are important for the heat-induced translocation of SKN-1. Again, there is no evidence for this in Kell et al.

Page 4, top: Lamitina and Strange did not show that DAF-16 is activated by hypertonic stress. They showed that DAF-16 activation by mutations conferred hypertonic stress resistance.

2. Statistical analysis of gene expression data needs to be presented throughout. qRT-PCR data should be presented in Supplemental materials.

3. Motif analysis is interesting, but it does not provide compelling evidence that the motif has a regulatory role related to slr-2 or jmjc-1. Molecular studies are required before the motif can be termed an ESRE.

4. Page 8, second paragraph: The so-called "kinetic data" do not match slr-2 expression as claimed. Slr-2 shows little if any upregulation until between 4-12 h post heat stress. Many of the genes examined show striking upregulation within 4 h.

5. Page 9: Text should refer to Figure S5 not S4. I am not at all convinced that comparing slr-2 mutants to unstressed wild type is a fair comparison.

6. Page 10, top: Recovery rates were not measured. Text should refer to Figure S8 not S6.

7. Page 10, bottom: There are several daf-16 expression studies. Unfortunately, there is not a lot of consistency between them. How does comparison to other data sets impact your conclusions? Data sets should also be analyzed for DAF-16 associated element (DAE) identified by Murphy et al and Oh et al.

8. Page 12, middle: "Abrogated" is not used correctly. There still seems to be significant upregulation of jmjc-1 in slr-2 mutants. Is expression merely slowed or reduced (it is not eliminated)?

9. The labels in Figure 4 and S8 are not consistent with standard nomenclature and make it difficult to understand the genotype being examined.

10. Figure 14, top: What is a "biological repeat"?

11. Page 15: Knockdown studies should be conducted in human cells to determine whether NO66 is important for survival under stress conditions.

12. Page 16: No evidence is presented demonstrating that the ESRE plays any role in stress response. It is merely present in many stress regulated genes. No data are presented demonstrating that jmjc-1 plays a role in stress responses in mammalian cells. Concluding that it is a master stress pathway in distantly related taxa is overstated.

13. Page 17, bottom: The slr-2 pathway is not conserved.

1st Revision - authors' response 29 October 2009

We very much appreciate the referees positive comments and constructive suggestions. We have made strong efforts to address all of the reviewer's points both in writing as well as additional experimentation and data analysis.

Referee #1 (Remarks to the Author):

1) Although I am convinced that DAF-16 has little relevance to control by SLR-2/JMJC-1; data for the converse are not as clear. This should be corrected or the statements about cross-talk/coregulation/independence modified somewhat.

We appreciate the reviewer's point. To address this concern, we significantly expanded this section (pages 12-13) through further analysis of our microarray data along with published data on DAF-16. We observed that only 1 of the 244 genes upregulated in the daf-16 microarray paper by Murphy, et al. (2003) was also upregulated by in slr-2 mutants. Genes downregulated in both studies did in fact show a greater overlap, but most of these genes also had an ESRE motif that would allow for DAF-16-independent regulation, which is further consistent with our findings that ESRE genes don't show a difference in expression between wild-type and daf-16(mu86) mutants. We also note that at least two bona fide daf-16 targets, mtl-1 and sod-3 were not differentially expressed in slr-2 mutants whereas the daf-16 targets hsp-16.1 and hsp-16.49, which do contain ESRE sites, were downregulated in slr-2 mutants. Finally, we also observed that canonical DAF-16 targets were not overrepresented in our slr-2 microarray data. Combined, these data strengthen and support our arguments that DAF-16 and SLR-2 function independently in the response to stress.

2) Put SLR-2 into the title: Perhaps "SLR-2: a master-regulator of a new evolutionarily conserved stress-response pathway"

Although SLR-2 obviously plays a central role in this paper, slr-2 does not have any clear orthologs outside of nematode species. Thus, we feel that adding slr-2 to the title could be misleading. That said, the ESRE network and jmjc-1 are both conserved, however we prefer to leave the title more general if possible.

3) There certainly could (and probably should) be an additional paragraph that frames these results in the framework of the "regulation of aging". This compound term is misused in the aging field because it is underlying stress-response pathways that are being regulated and differential longevity is the outcome.

This is a valid point and we have now added the following paragraph addressing this issue to the Discussion section (page 21).

> "A significant body of data have intimated a close connection between the molecular mechanisms regulating stress resistance and longevity (Chen et al., 2007; Johnson et al., 2001; Johnson et al., 1996; Lithgow et al., 1995; Murphy et al., 2003; Oh et al., 2005; Oliveira et al., 2009; Samuelson et al., 2007) Moreover, according to the stress response hypothesis proposed by Johnson and colleagues, much of the increased longevity observed in gerontogene mutants (e.g. age-1, daf-2, etc.) is due to their greater resistance to exogenous and endogenous stresses (Johnson et al., 2001). This hypothesis is supported by findings that overexpression of several stress-response factors, including SKN-1, PHA-4, ABU-11, and DAF-16 increases lifespan (Haskins et al., 2008; Henderson and Johnson, 2001; Lin et al., 2001; Panowski et al., 2007; Tullet et al., 2008; Viswanathan et al., 2005). Consistent with these findings, we observed a modest, though statistically significant, lifespan extension in strains that carried multiple copies of slr-2."

Referee #2 (Remarks to the Author):

Specific Comments:

1) For all the qRT-PCR experiments, the authors should indicate how many independent trials were carried out, and whether the SEM represents errors among different trials.

We have now added information regarding this issue to the qRT-PCR Materials and Methods section (page 25).

2) Both the slr-2 and jmjc-1 mutants are sensitive to a variety of stress stimuli. The authors showed that overexpression of slr-2 confers resistance to ethanol. Does it confer resistance to other stresses as well?

This is a good question. We have addressed this by describing the increased resistance to heat, oxidative stress, and hypertonic stress that are conferred by overexpression of slr-2 (pages 15-16). We have also included additional figures that show these data (Figure 6C and Supplementary Figure S9A-C).

What about overexpression of jmjc-1?

We have addressed this in the text (pages 15-16) and have now shown that, like slr-2, overexpression of jmjc-1 confers increased resistance to heat shock and ethanol, oxidative, and hypertonic stresses. Data for these findings are shown in Figure 6BC and Supplementary Figure S9A-C.

3) It seems that Figure 4D and Fig. S8B are showing results from different trials of the same experiment? If so, then showing it once is probably sufficient.

In fact, these were slightly different conditions, representing different concentrations of ethanol. Nevertheless, we agree with the reviewer that these data were overly redundant, and have replaced the supplementary figure with data regarding different modes of stress (e.g., Figure 6B and Supplementary Figure S9A-C).

4) For Fig. S8, the lifespan data (survival curves) under normal culturing condition should be shown.

We appreciate the reviewer bringing this to our attention. To clarify this issue, we have performed longevity assays at both 20°C, Figure 6D, and 16°C, Supplementary Figure S9D. Interestingly, we do now see a statistically significant increase in the longevity strains that

overexpress slr-2 (primarily in the latter portion of the time course) that our previous analysis had missed.

5) For Fig. 6C, to be consistent with the other figures and to more rigorously compare the data, the fold change should compare the expression under stressed and unstressed condition for each of the genotype. This is important, as some of the genes may show lower basal expression in the mutant genotype, and their expression upon stress may be lower than that in wild-type, but the fold induction can be similar.

This is also a very good point. To clarify this matter, we have modified all figures containing qRT-PCR data (Figure 2B-D, Figure 7C, Figure 8C, and Supplementary Figure S6B-C) to now show normalization to both genotypic and wild-type cohorts. We feel that showing both comparisons provides biologically relevant information and more accurately highlights some of the important differences observed between strains and conditions.

6) Likewise, for Fig S6, fold change should be compared the basal expression of each gene in the respective genotype.

We have made the change requested, as described in our response to point 5 above.

7) For comparison with daf-16 microarray data, the authors should consider including the data from Murphy et al., 2003. This dataset is quite different from the McElwee dataset and will be interesting to see if ESRE motif may be detected in the Murphy dataset.

We agree that analysis of data from the Murphy, et al., 2003 microarray study would be informative to our discussion regarding the independence of SLR-2 and DAF-16 activities. To that end, we have analyzed these data and added our findings to pages 12-13. This analysis has further strengthened our conclusion that DAF-16 does not regulate genes through the ESRE motif and that DAF-16 and SLR-2 have largely non-overlapping sets of targets.

8) Have the authors tested whether hsf-1 is required for slr-2 induction upon stress?

To address this question, we tested baseline and fold-induction of ESRE genes in hsf-1 mutants, but observed no apparent difference between these mutants and wild type. Given that this is a negative result, we have not included this data in the current manuscript.

9) Have the authors tested whether knockdown of NO66 in mammalian cells will confer sensitivity to various stress stimuli?

To more directly address the function of NO66 in mammalian cells, we have now tested the effects of treating mammalian cells with an siRNA construct targeting NO66. Consistent with JMJC-1 orthologs in worms and flies, NO66 is required for the maintenance of basal ESRE gene expression levels and for induction of ESRE genes following stress. We have considerably reworked this section of the paper and our new findings are described on pages 17-18 and in Figure 8.

Referee #3 (Remarks to the Author):

COMMENTS TO AUTHORS:

1. There are problems with literature citations. Page 3, bottom: This sentence implies that NRF2 is regulated by heat. There is no evidence in Kell et al for this. Sentence also implies that PDHK-2 etc. are important for the heat-induced translocation of SKN-1. Again, there is no evidence for this in Kell et al.

Page 4, top: Lamitina and Strange did not show that DAF-16 is activated by hypertonic stress. They showed that DAF-16 activation by mutations conferred hypertonic stress resistance.

We are grateful to the reviewer for bringing these issues to our attention and have corrected the relevant statement and literature citations as suggested (pages 3-4).

2. Statistical analysis of gene expression data needs to be presented throughout. qRT-PCR data should be presented in Supplemental materials.

For all qRT-PCR experiments, we have shown statistical analysis in the form of standard error of the mean, which was performed independently for each gene measured. This treatment is quite standard for data of this nature. We also didn't feel comfortable with adding P-values to these data, given that such experiments are based on three biological replicates (as well as 3 trials for each replicate). Also, these graphs are already quite busy, and we feel that adding additional information would render the figures more difficult to parse. We do maintain that the figures, in their present form (with S.E.M. shown), effectively convey the biological relevance of our findings. We also note that additional qRTPCR data are presented in supplementary figures, for example Supplementary Figure S6.

3. Motif analysis is interesting, but it does not provide compelling evidence that the motif has a regulatory role related to slr-2 or jmjc-1. Molecular studies are required before the motif can be termed an ESRE.

This is a very valid point. As described on pages 8-10 and 24, we constructed a 3X-ESRE::GFP reporter and observed induction of fluorescence by four different forms of stress (heat shock, ethanol stress, oxidative stress, and hypertonic stress; see Figure 3 and Supplementary Figure S5). Further, induction of this construct was reduced in slr-2 and jmjc-1 mutants. Thus, we can conclude that the ESRE motif can be sufficient for stress-induced gene expression and that robust activation is SLR-2 and JMJC-1 dependent.

Regarding the term ESRE, this motif was given this name by Kwon and colleagues, in a paper published in Genomics in 2004. They had determined that the transcriptome response to ethanol stress showed enrichment of genes with this regulatory motif, and therefore called it an ethanol and stress response element (ESRE). As this element was previously named ESRE, we retained the name to prevent confusion in the literature.

4. Page 8, second paragraph: The so-called "kinetic data" do not match slr-2 expression as claimed. Slr-2 shows little if any upregulation until between 4-12 h post heat stress. Many of the genes examined show striking upregulation within 4 h.

We understand the reviewer's concern regarding our use of the term "kinetic" and have modified our statements on pages 11-12. Regarding the pattern of slr-2 and ESRE gene induction, by four hours following induction, expression of slr-2 shows a ~1.6-2.0-fold increase, and by 12 hours after stress, expression of slr-2 is upregulated from ~2.5-4.7-fold, depending on the nature of the stress (Figure 4). This general pattern of induction, if not the precise magnitude of upregulation, is in fact consistent with what is observed for most ESRE genes at these time points (Figure 2 and S6). At earlier times after induction, such as 1 hour, we do not see robust upregulation of either slr-2 or ESRE genes (Figure 4 and data not shown).

5. Page 9: Text should refer to Figure S5 not S4. I am not at all convinced that comparing slr-2 mutants to unstressed wild type is a fair comparison.

We thank the reviewer for bringing the misreferenced figure to our attention, and we have corrected it. With respect to comparing unstressed slr-2 mutants to unstressed wild-type controls, this practice is certainly standard for our field and has been proven to yield relevant biological insights. With respect to other types of comparisons, such as those integral to our qRT-PCR data, we have addressed this issue in our response to reviewer #2, point #5.

6. Page 10, top: Recovery rates were not measured. Text should refer to Figure S8 not S6.

The reviewer is correct and we have amended our text to more accurately reflect the specific nature of the test performed (page 12), which was to expose worms to a brief, acute stress, followed by a thirty-minute window of recovery time, after which individuals were scored. We have also corrected the misreferenced figure.

7. Page 10, bottom: There are several daf-16 expression studies. Unfortunately, there is not a lot of consistency between them. How does comparison to other data sets impact your conclusions? Data sets should also be analyzed for DAF-16 associated element (DAE) identified by Murphy et al and Oh et al.

We appreciate the reviewer bringing this matter to our attention as this partially overlaps with a comment from reviewer #2 (point #7). In response, we have analyzed additional daf-16 expression data (Murphy, et al., 2003) and our conclusions are virtually the same (pages 12-13).

With respect to the DAE, (identified by Murphy, et al., 2003 and Oh, et al., 2006), this element has at its core a GATA submotif that is likely recognized by ELT-3. Budovskaya and colleagues (2008) demonstrated that ELT-3 is required in part for the regulation of a number of GATA genes associated with life-extension in daf-2 mutants. We have also recently shown (Kirienko et al., 2008) that many genes containing a GATA motif are differentially regulated in slr-2 mutants. In our case, however, these GATA genes highly overlap with previously published ELT-2 targets (McGhee, et al., 2007), and are thought to be involved in intestinal development and function and not life-span control. Furthermore, SLR-2 GATA genes did not significantly overlap with DAF-16-ELT-3 GATA targets. We thus feel that given the divergence in processes regulated by the ELT-2 and ELT-3 GATA-binding proteins, drawing attention to GATA sight enrichment in slr-2 mutants would likely be misleading in the context of the role of SLR-2 in stress response.

8. Page 12, middle: "Abrogated" is not used correctly. There still seems to be significant upregulation of jmjc-1 in slr-2 mutants. Is expression merely slowed or reduced (it is not eliminated)?

The text has been changed to "reduced" (page 15).

9. The labels in Figure 4 and S8 are not consistent with standard nomenclature and make it difficult to understand the genotype being examined.

These labels have been changed, as suggested (Figure 6 and S9).

10. Figure 14, top: What is a "biological repeat"?

A definition for this term was added to the qRT-PCR portion of the Materials and Methods section on page 25.

11. Page 15: Knockdown studies should be conducted in human cells to determine whether NO66 is important for survival under stress conditions.

A similar point was raised by reviewer #2 and was addressed in point #9.

12. Page 16: No evidence is presented demonstrating that the ESRE plays any role in stress response. It is merely present in many stress regulated genes. No data are presented demonstrating that jmjc-1 plays a role in stress responses in mammalian cells. Concluding that it is a master stress pathway in distantly related taxa is overstated.

These issues have been addressed: see point #3 (above) and reviewer #2 point #9.

13. Page 17, bottom: The slr-2 pathway is not conserved.

We have corrected portions of the text that explicitly imply conservation of SLR-2 function (e.g., see page 22). We note that although SLR-2 has no apparent mammalian ortholog, JMJC-1 is conserved in both Drosophila and mammals. Furthermore, JMJC-1, as well as the ESRE motif, have conserved roles in the regulation of stress-responsive genes in multiple species tested.

2nd Editorial Decision 23 November 2009

Thank you for sending us your revised manuscript. Our original referees 2 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 would like to ask you to address/respond to the minor issues still suggested by the referees (see below).

Please let us have a suitably amended manuscript as soon as possible.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2 (Remarks to the Author):

The authors have done a nice job in addressing my previous comments.
I believe the title can be changed to more specifically discuss slr-2 & jmjc-1. The current title is quite vague and general.

Referee #3 (Remarks to the Author):

The analysis of qRT-PCR data is still a concern. We do extensive qRT-PCR and always use statistical analyses to determine if differences observed are significant. It is not adequate to simply report means and errors and conclude that differences observed are meaningful. Statistics need to be performed. If the sample size is not adequate, additional experiments should be carried out.

2nd Revision - authors' response 28 November 2009

We have changed the title as suggested by Reviewer #2 to include JMJC-1 and SLR-2: "SLR-2 and JMJC-1 regulate an evolutionarily conserved stress-response network".

We have also added all the statistical information requested by Reviewer #3. This includes changes to Figures 4 and 6 and to figure legends 2, 4, 6, 7, 8, and S6. Most importantly, we have added Supplemental Table 1, which provides statistical analysis and support for data contained in Figures 2, 7, 8, and S6. We would underscore that this analysis, which included the derivation of ~500 p values, completely supports all of our previous and current stated conclusions.

