Sir2 histone deacetylase prevents programmed cell death caused by sustained activation of the Hog1 SAPK

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

1st Review 10 December 2010

REFEREE REPORTS:

Referee #1:

Overview & Summary: The studies described in this paper were provoked by trying to understand how prolonged stimulation of the HOG pathway leads to cell death. To chronically activate the Hog1 MAPK (a stress-activated MAPK is a SAPK), these investigators expressed at a very high level (under control of a GAL promoter) a constitutively-active allele of the immediate activator of Hog1, namely the Pbs2-DD mutant of the MAPKK Pbs2. However, it strikes this referee that this tactic was not very potent because even a full day after induction of Pbs2-DD there was not even one log of killing (the authors state that even 24 h after induction 24% of the cells still survived). What seems to have motivated this approach is that others had published that prolonged activation of the human ortholog of Hog1, p38/SAPK, reportedly causes apoptosis of animal cells. For this reason, these workers assessed whether "markers of apoptosis in yeast", including the appearance of single-strand DNA breaks (measured by TUNEL staining), an increase in cells with sub-G1 DNA content (via FACS), and an elevation in metacaspase activity (as judged by FLAM-FLICA assays) occurred in the culture after Pbs2-DD induction. Because others among the proponents of "yeast apoptosis" claim that an increase in reactive oxygen species (ROS) is a common cause of an apoptosis-like phenotype in yeast, these authors also measured ROS levels after Pbs2-DD induction and found it increased, and that the modest killing
caused by Pbs2-DD induction could be suppressed under anaerobic conditions. Somewhat, paradoxically, however, it is reported that O2 consumption was decreased after Pbs2-DD induction. Likewise, when Hog1 is activated in WT cells by shift to hyperosmotic (high salt) conditions, respiration rate (reduction of triphenyl tetrazolium) decreased in a Hog1-dependent manner. They then found that these phenotypes could be suppressed to some extent by mutations that affect the function of the essential ubiquitin ligase (E3) SCFCdc4. For example, a cdc4-1 mutation (a mutation in the F-box subunit of this E3) reduced the apoptosis-like killing caused by Pbs2-DD induction by ~50%. But, this is no surprise because initiation of DNA replication and cell cycle progression are compromised in such cells and damage to DNA was their primary assay. In any event, largely on this basis, and erroneously in the opinion of this referee the authors concluded that prolonged HOG pathway activation results in increased ROS, which is then the causative agent for the ensuing apoptosis-like phenotype, and that SCFCdc4 is necessary for the killing to occur. The authors then assumed, but did not demonstrate, that action of the SCFCdc4 E3 somehow contributes to ROS accumulation when Pbs2-DD is induced. Based on this assumption, they further explored the effects of the cdc4-1 mutation. Presence of this mutation did not change the dynamics of HOG pathway signaling; however, this mutant seemed slightly more osmoresistant than WT strains. Because SCFCdc4 has been shown by others to be responsible for the cell cycle-dependent degradation of many nuclear proteins (from the Far1 and Ste5 scaffold proteins to the Sic1 CDK inhibitor to several transcription factors), these authors looked at the induction of several HOG pathway genes during hyperosmotic shock in the cdc4-1 mutant. Genes known to be upregulated by the Msn2-Msn4 heterodimer (e.g., CTT1 and ALD3) seemed to be somewhat more highly induced in this mutant. Consistent with these results, as shown by others (Ref 20), SCFCdc4 is capable, at least in vitro, of ubiquitinating Msn2. These considerations lead them to examine whether Msn2 is degraded during hyperosmotic stress and after Pbs2-DD induction, and they found that it is and that the rate of this degradation is decreased in the cdc4-1 mutant. They then reasoned that the decrease in cell death upon Pbs2-DD induction in a cdc4-1 mutant might be due to maintenance of a higher level of Msn2. In agreement with this prediction, MSN2 overexpression ameliorates the cell death caused by Pbs2-DD induction and, conversely, a cdc4-1 mutation does not suppress Pbs2-DD-induced killing in cells lacking Msn2 and Msn4. Then, they examined the status of PNC1 expression because it has been reported previously that Msn2 and MSN4 have a role in yeast replicative lifespan, at least in part, because they are needed for expression of PNC1. PNC1 is among the many genes induced by hyperosmotic shock and encodes a nicotinamidase that degrades intracellular nicotinamide (and nicotinamide is a demonstrated competitive inhibitor of the Sir2 NAD+-dependent histone deacetylase). As expected due to slower rate of Msn2 degradation, the cdc4-1 mutant exhibited somewhat higher PNC1 induction that control cells. As expected if PNC1 is the primary target of Msn2 and Msn4 responsible for preventing Pbs2-DD-induced cell death, overexpression of PNC1 in otherwise WT cells prevented Pbs2-DD-induced killing and, conversely, a pnc1 mutation (like an msn2 msn4 double mutation) exacerbated cell killing in a cdc4-1 mutant upon Pbs2-DD induction. Since PNC1 induction stimulates Sir2, then a sir2 mutant should have the same phenotype as a pnc1 mutant; and, as expected, a sir2 mutation also prevented the ameliorating effect of cdc4-1 mutation upon Pbs2-DD induction. Likewise, treatment of WT cells with a drug (reversterol) that activates Sir2 decreased killing by Pbs2-DD induction. A net1 mutation, which is necessary for Sir2 localization at rDNA had the same effect as a sir2 mutation, whereas a sir4 mutation, which abrogates silencing at telomeres and at the cryptic mating-type loci, did not have this effect. On the basis of all of these data, the overall model proposed is that constitutive HOG pathway activation depresses respiration leading to an increase in ROS that causes an apoptosis-like phenotype. Compromising the function of SCFCdc4 prevents this death by sparing Msn2 from degradation, leading to a higher than normal induction of PNC1, which elevates Sir2 activity, which helps better maintain silenced rDNA, thus preventing formation of extra-chromosomal rDNA circles, which are thought to exacerbate the deleterious effects of ROS. The bottom line: This paper is totally inappropriate for the EMBO J. because: (a) it connects the dots between processes already known to be connected; (b) it provides no new insights at the molecular/mechanistic level; and, (c) the chronic overexpression of a MAPKK mutant from a GAL promoter is a totally artificial situation that would never be seen in nature. The strategy applied by the authors (overexpression of Pbs2-DD to chronically hyperactivate the HOG pathway) was simply a means to sensitize cells to the role that SCFCdc4 degradation is already known to play in controlling the level of Msn2. It is thus no surprise that a higher level of this transcription factor (Msn2) elicits greater expression of a gene (PNC1) already known to be under Msn2 control. It was already known that Pnc1 is an enzyme that removes an inhibitor of Sir2. It was already reported
quite some time ago that Sir2 action prevents the DNA damage caused by excision of rDNA circles. Thus, this very long "detective story" does not provide any new information and certainly does not tell us about the molecular basis / mechanistic cause of the cell death / "apoptosis-like phenomenon" responsible for the observed loss of viability.

Specific Concerns:

(1) Even if one accepted all of the evidence, conclusions, and claims made in this paper at face value about how artificially slowing down the rate of "aging" prevents cell death, these findings do NOT address how abnormally sustained Hog1 activation causes the death. What causes the purported increase in ROS? What is responsible for the reported inhibition of respiration? Therefore, no new biological insights arise from this work. Many additional experiments would need to be carried out to address the current deficiencies, such as:

(a) One of the observations made was that Msn2 is degraded during hyperosmotic shock and upon PBS2-DD induction. The observation that PBS2-DD induction causes Msn2 degradation strongly implies that this effect is dependent on Hog1; however, this should be proven (i.e. Fig. 3B should include a hog1 strain). To this reviewer's knowledge, no one has demonstrated direct phosphorylation of Msn2 by Hog1. Does Hog1 phosphorylate Msn2 to induce its degradation? Or, does Hog1 phosphorylate a component of the SCFCdc4 E3 to induce Msn2 degradation? Other kinases are known to phosphorylate Msn2; is it their phosphorylation that initiates the degradation? Basically, HOW, at the molecular / mechanistic level, activation of Hog1 is linked to destabilizing Msn2 is not elucidated in this work.

(b) Another observation made was that hyperosmotic stress leads to an inhibition of respiration. The study seems to imply that PBS2-DD-induced death is a combination of normal levels of aging, the inhibition of mitochondria and quite possibly other things. If Hog1 directly inhibits respiration, how is it doing this? The authors results predict that there must be other mutations that can suppress the cell death caused by prolonged Hog1 activation, namely ones that prevent respiration inhibition. There seems to have been no serious attempt to investigate that avenue in an unbiased manner If anything, the Sir2-dependent suppression is the least physiologically relevant since it only has an effect when Msn2 is artificially stabilized.

(2) The results of this paper involve several proteins and processes involved in NADH metabolism. The enzyme necessary for glycerol production during hyperosmotic stress, Gpd1, requires NADH as a cofactor for catalysis. There is no mention of that fact in the text, nor do the experiments performed reflect this interesting "coincidence." Hog1 may inhibit respiration simply by depleting the pool of NADH via glycerol synthesis. It would be appropriate to include a gpd1 strain and a strain overexpressing GPD1 in Fig. 2C to determine whether inhibition of respiration is simply a necessary and natural side effect of the consumption of NADH for glycerol production. Conversely, it is also possible that Hog1 increases glycerol production by preventing the oxidation of NADH in mitochondria, so more is available for Gpd1. Does respiration inhibition occur during PBS2-DD induction (Hog1 activity is necessary for respiration inhibition, but is it sufficient)? Would respiration inhibition occur during PBS2-DD induction in a gpd1 strain? The answers to these experiments would tell us whether the inhibition of respiration is a direct effect of Hog1, or a by-product of the adaptation process.

Referee #2:

In their manuscript, Vendrell and colleagues link stress signalling to yeast programmed cell death and sirtuins. Permanent activation of HOG1 (a stress activated protein kinase) triggers ROS mediated apoptosis that can be circumvented by activation of the cytoprotective MSN transcription factors, finally resulting in SIR2 activation.

Overall this is a very interesting and technically well done piece of work and I strongly recommend publication, given that the authors are willing to invest some more work:

- I think that:
"Sir2 histone deacetylase prevents programmed cell death in yeast by sustained activation of the Hog1 stress-activated protein kinase"

Would be a better title. EMBO is for non-specialized readers.

-While the pathway leading to and circumventing lethal stress is nicely characterized, the paper falls short in determining the corresponding downstream mechanisms. To this purpose, the authors should test the effects of sustained HOG1 activation in cells devoid of the yeast metacaspase YCA1, NUC1, Htra2/OMI as well as AIF1-deficient cells. These experiments would clarify, whether caspase-dependent or -independent cell death underlies this scenario.

- Msn2/Msn4 or Sir2 are important regulators in the physiological process of aging; in order to emphasize the physiological relevance of these results, experiments exploring the impact of Hog1 SAPK-mediated cell death on aging, which serves as an established model for both replicative and chronological aging (one model may be sufficient) would clearly strengthen the paper.

- The best way to proof that HOG1 activation inhibits mitochondrial respiration are plating assays or growth curve measurements on non-fermentable carbon sources. This is an easy and informative experiment.

Referee #3:

Vendrell et al. present a model that cell death caused by chronic hyperactivation of Hog1 is due to Msn2/4 mediated activation of Pnc1 and Sir2. This model is interesting, but the data presented here fall far short of providing convincing evidence that this is the case. The use of qualitative spot assays does not provide sufficient quantitative resolution and the authors fail to include important controls.

Major concerns:

1. In Fig 1., how do we know that the PBS2DD is expressed to similar levels in the mutant backgrounds compared to WT? Are these mutants slow growing? Are protein synthesis rates similar? The same question applies to the experiments in Figs 2 and 3. Does growth in low oxygen influence mRNA translation and protein synthesis? Growth rate? Such changes could easily influence the phenotypes being examined and must be controlled for.
2. Why aren't the survival values quantified by CFU counts for the cdc4-1 msn2 msn4 and cdc4-1 pnc1 experiments? Spot assays are not sensitive enough, especially when the effect of cdc4-1 on survival is relatively small.
3. The use of resveratrol in this study is tricky, since at least three different publications have suggested that resveratrol does not activate yeast Sir2 in vivo. It is important therefore to show that Sir2 is activated in vivo at the concentrations of resveratrol and conditions used here. Does overexpression of Sir2 show the expected phenotype?
4. The experiments with sir2 deletion need to be repeated with the HM locus deleted or in diploid cells.

Minor comments:

1. Deacetylase is misspelled as "deacetilase" on p. 4
2. "transcriptions factors" on p.8 should be "transcription factors"

1st Revision - authors' response 22 May 2011

Point by point Response to Reviewers

Referee #1. The reviewer found three aspects of this study not to be appropriated for its publication to The EMBO J. We discuss here the reviewer concerns point by point and we added new data that
addresses all the specific comments. The reviewer #1 feels that the paper was inappropriate for the EMBO J based mainly on three aspects: a) it connects the dots between processes already known to be connected; (b) it provides no new insights at the molecular/mechanistic level and; (c) the chronic overexpression of a MAPKK mutant from a GAL promoter is a totally artificial situation that would never be seen in nature. Then, based on these points, he/she feels that: this very long "detective story" does not provide any new information and certainly does not tell us about the molecular basis / mechanistic cause of the cell death / "apoptosis-like phenomenon" responsible for the observed loss of viability.

Here, our considerations to these three aspects:

a) It connects the dots between processes already known to be connected;
We would like to state that we go much beyond the connection between processes already known. It was not known that 1) activation of the Hog1 MAPK inhibited mitochondrial respiration, 2) activation of Hog1 induced oxidative stress, 3) sustained activation of Hog1 led to cell death dependent on the presence of O2 and correlated with apoptosis (it was known that cell growth was impaired upon Hog1 activation), 4) that the ubiquitin ligase Cdc4 targets Msn2 in vivo and this was important for gene expression upon osmostress and 5) that a pathway composed by Msn2, Pnc1 and Sir2 had a preventive role in MAPK-induced cell death. Taken together, we present solid evidence that Msn2 degradation by SCFCdc4 is a key factor to dictate the balance on cell survival due to SAPK-mediated ROS formation and the anti-apoptotic effect of the Sir2 pathway.

(b) It provides no new insights at the molecular/mechanistic level.
We absolutely disagree with the reviewer. At the cell biology level, the work presented here provides new insights on the regulation of the mitochondrial activity upon stress, the effect of a MAPK on ROS levels and its role on cell death. In addition, we provide the molecular mechanism by which Cdc4 and Msn2 counteract the cell death associated to activation of Hog1, which is the increase on Sir2 activity by the induction of Pnc1 transcription, a known activator of Sir2, due an increase on Msn2 levels. Therefore, the balance between cell lethality and the anti-apoptotic effect of the Sir2 pathway are clearly defined at the mechanistic level in the work presented here.

(c) The chronic overexpression of a MAPKK mutant from a GAL promoter is a totally artificial situation that would never be seen in nature.

We have made use of a genetic approach to unravel a connection that otherwise could have been difficult to decipher. However, we have demonstrated all along the manuscript that in response to osmostress the effect of activation of Hog1 upon osmostress is identical to the observed upon genetic activation. We have shown that upon osmostress respiration is blocked (dependent on Hog1; Figure 2D), that upon osmostress ROS levels increase in wild type and even more in a sir2 mutant (Figure 2B and S5). We also showed that upon osmostress, Msn2-responsive genes are induced more efficiently in a cdc4-1 mutant (Figure 3E) and that binding of Msn2 at promoters increases as well as its stability (Figures 3F and 3G). Moreover, we showed that Pnc1 transcription and protein levels increase upon stress and are affected in a cdc4-1 mutant (Figure 4B and 4C). In addition, even cell osmosensitivity increases when mutations in cdc4-1 are present (Figure 3C and 3D). Therefore, our study reports a very relevant scenario that occurs when the MAPK is activated upon osmostress.

In addition to the general considerations described above, Reviewer 1 also had specific comments that we have addressed experimentally.

1. The reviewer considered that additional experiments should be carried out to understand better how sustained Hog1 activation leads to cell death, suggesting two additional sets of experiments; a) The reviewer suggested to test whether degradation of Msn2 caused by Pbs2DD expression could be prevented in a hog1 strain. The reviewer also asked to test whether Msn2 could be directly phosphorylated by Hog1. We have performed both experiments and found that 1) degradation of Msn2 upon Pbs2DD induction is clearly reduced in a hog1 strain when compared to wild type and it is similar to cdc4-1 strain. This result supports our initial hypothesis and shows that degradation of Msn2 upon Pbs2DD depends on Hog1. These results have been included as Supplemental Figure 2A in the revised manuscript. We have also tested whether Hog1 is able to phosphorylate Msn2 directly in an in vitro kinase assay. Purified Msn2 from yeast was incubated in the presence of Hog1as (a modified form of Hog1 that uses PE-ATP as a substrate) in the presence or absence of an inhibitor for Hog1 (Macia et al., 2009). Only in the presence of active Hog1, Msn2 was phosphorylated. These data clearly show that Msn2 can be targeted, among other kinases such as PKA, by Hog1. The data have been included as a new figure 3H in the revised manuscript.

b) The reviewer asked to understand how Hog1 was able to inhibit mitochondrial respiration and
whether other mutations could prevent Hog1 lethality upon sustained activation. We agree with the reviewer that identifying the target for Hog1 in respiration would be of great interest. However, this goes beyond the scope of this work since this study is a complete story by itself. However, we provide strong evidence that inhibition of respiration occurs and that this inhibition is absolutely Hog1 dependent. The relevance of the target here is limited since our main focus is to describe that this inhibition of respiration leads to an increase on ROS levels that mediate cell death unless a survival pathway controlled by Sir2 prevents it.

2. The reviewer pointed out that the study provides evidence of the involvement of several proteins and processes related to NADH metabolism. He/She suggested to discuss this aspect on the manuscript and in addition proposed to test respiration in a gpd1 strain to assess whether the inhibition of respiration is a direct effect of Hog1, or a by-product of the adaptation process. We have done as suggested and found that inhibition of respiration (O2 consumption) in a gpd1 strain is similar to wild type. In contrast, the inhibition of respiration is completely abolished in a hog1 mutant. Therefore, these data supports our initial observations showing that inhibition of respiration it is not a by-product of the adaptation process. We have included the data as a new figure 2D.

Referee #2.
The reviewer found the manuscript very interesting, a technically well done piece of work and strongly recommend publication if we were able to invest some more work to support our observations. His/her comments were very helpful and we have included all the data requested in the revised version of the manuscript.

1. The reviewer suggested modifying the title to be more informative. We found his/her suggestion very adequate and modified the title accordingly to "Sir2 histone deacetylase prevents programmed cell death caused by sustained activation of the Hog1 SAPK"

2. The reviewer suggested to test cell lethality on a particular group of mutants to decipher whether cell death was caspase-dependent or independent. We have tested all the mutants suggested and found that whereas mutation of nuc1 was not able to rescue lethality observed by sustained activation of Hog1, deletion of yca1 had an intermediate effect and interestingly, deletion of nma11 (nuclear mediator of apoptosis) almost completely suppressed the lethality caused by Hog1 activation. Thus Nma11, a known regulator of apoptosis seems to mediate apoptosis by Hog1 (Walter D et al., 2006). This information has now been included in figures 1E and 1F in the revised manuscript.

3. The reviewer pointed out that defining better the Msn2/Msn4 or Sir2 are important regulators in the physiological process of aging; in order to emphasize the physiological relevance of these results, experiments exploring the impact of Hog1 SAPK-mediated cell death on aging, which serves as an established model for both replicative and chronological aging (one model may be sufficient) would clearly strengthen the paper. This is indeed an interesting point raised by the reviewer. Albeit, this falls far from the scope of this work, we have now referenced previous published data related to high osmolarity and life span (Kaeberlein et al., 2002).

4. The reviewer suggested to perform a plate assay or growth curve measurement on a non-fermentable carbon source. We have done as requested and found that in YPglycerol, cells are strongly sensitive to osmostress. We have included these results as a new supplemental figure S5B.

Referee #3
The reviewer found this study interesting and suggested to include quantitative data and additional controls to support our evidence.

1. The reviewer was concerned whether in all cells Pbs2DD was equally expressed as well as whether all mutant background had a similar growth under normal conditions. Following the reviewer suggestions, we have assessed cell growth and found no differences in any of the strains used in this study (we included this information in material and methods). Moreover, we have followed the levels of expression of Pbs2DD in several strains and found no difference of expression
in any strain tested. We have included these results as a new Supplemental Figure S4A. As for whether cells in low oxygen had similar rates of growth, mRNA translation or synthesis, we do not know. However, we included in all conditions the wild type strain as a reference and therefore, direct comparison can be made.

2. The reviewer asked to monitor CFU counts in addition to the dots presented for the experiments with msn2 msn4 and pnc1. As requested by the reviewer we measured CFU counts for those mutants and the results confirmed that the lack of pnc1 or msn2/msn4 abolished the preventive effect of cdc4-1.

3. The reviewer was concerned whether resveratrol activated Sir2 on the conditions tested. In figure 4D, it is shown that the effect of resveratrol in cell survival is abolished in a sir2 mutant. This is genetic evidence that links the suppressive effect of resveratrol with Sir2. To further confirm these results we followed the reviewer advice and overexpressed Sir2. As expected, overexpression of Sir2 suppressed the cell lethality observed by Pbs2DD in wild type cells. These results have been added to figure 4D.

4. The reviewer suggested repeating the experiments of cell survival with cells with deleted HM locus. We have done as suggested and found identical results to wild type or cdc4-1 cells. These results have been included as Supplemental Figure 4B.

5. The reviewer suggested to correct a few misspelled words in the manuscript. We have done as suggested.

2nd Review 17 June 2011

REFEREE REPORTS:

Referee #1:

Good revision. I have only one suggestion:
-the important result of cell death rescue by deletion of YCA1 and OMI should be mentioned in the abstract. This will justifye the term "PCD" in the title.

Referee #2:

Vendrell et al. present a model that cell death caused by chronic hyperactivation of Hog1 is due to Msn2/4 mediated activation of Pnc1 and Sir2. The manuscript has been improved in some respects and some interesting new data has been added, but still fails to provide convincing evidence for a coherent mechanism. I also have significant concerns about the quality of some of the data. The addition of quantitative rather than purely qualitative measures to some of the experiments (such as CFU measures in Fig 1) is a good start. Unfortunately, the authors chose not apply this level of rigor to other experiments and the CFU data raise additional concerns. The authors state that the error bars show standard deviation, but there is no indication for how this was calculated (replicate plates/replicate experiment; biological or technical replicates, etc.). Anyone who has done these types of assays would look at the very small error values reported and wonder about the way this was done. This raises an important additional point that the methods provided are woefully inadequate to allow for a careful evaluation of this work.
The resveratrol experiments are still problematic, first because the effect appears to be small in the wild type context and second because the epistasis with sir2 deletion is a negative and uninformative result. Since the sir2 mutant is hypersensitive, failure of resveratrol to protect in this context is meaningless. The bulk of published evidence now suggests that resveratrol does not activate yeast Sir2 in vivo. If the authors want to argue that this effect is via Sir2 activation, they need to demonstrate Sir2 activation in this experiment. Citing a single prior report that has been contradicted and is suspect is not sufficient. Again, there is no information regarding how this experiment was actually performed. How much drug was used? How long were the cells treated? Was there any effect of the drug on growth rate? Is there a direct effect on ROS (resveratrol is an anti-oxidant after all) or mitochondrial function? Etc.

The link to the rDNA function of Sir2 is very interesting, but also confusing in light of the authors’ model up to that point. Why would Sir2-dependent rDNA activity impinge on ROS and mitochondrial function? It's quite possible that two completely different mechanisms are interacting here and the authors have gotten fooled into thinking this is one pathway. An interesting experiment would be to ask whether deletion of Fob1 influences cell death in the context of Hog1 hyperactivation. If so, this would certainly argue that rDNA stability is the critical feature.

Response to Reviewers

Referee #1.
The reviewer suggested including in the abstract that deletion of YCA1 and OMI rescued the cell death caused by sustained activation of Hog1. We have included OMI (nma111) in the abstract since it is the mutant that displayed the strongest suppression.

Referee #2.
The reviewer found the manuscript improved over the initial submission and asked to include additional information on the quantitative analyses. We have modified figure legends and material and methods to include such information. Briefly, all error bars presented in our study indicate variations between at least three biological independent experiments.

The reviewer still considered that the experiments on resveratrol and its link to Sir2 were not sufficiently convincing based on previous reports. It is likely that the effect of resveratrol could be strain dependent and dose dependent. Cells were grown in the presence of resveratrol [5μM] in the plates. Based on reviewers concerns we have softened the conclusions about the use of resveratrol in the text. However, to conclude that Sir2 suppressed Hog1-mediated cell death we included additional evidence that support our findings such as that the deletion of SIR2 abolished the effect of resveratrol and the fact that overexpression of SIR2 suppressed cell death by Hog1. The reviewer was also interested on knowing the link on Sir2 in preventing cell lethality due to an increase on ROS levels and rDNA function. Indeed, this is an interesting aspect that arises from this study but we feel that goes beyond the scope of this manuscript. In the revised manuscript, we have highlighted this aspect on the discussion.