THE EMBO JOURNAL

Manuscript EMBO-2013-86907

SIRT2 induces the checkpoint kinase BubR1 to increase lifespan

Brian J. North, Michael A. Rosenberg, Karthik B. Jeganathan, Angela V. Hafner, Shaday Michan, Jing Dai, Darren J. Baker, Yana Cen, Anthony A. Sauve , Jan M. van Deursen, Anthony Rosenzweig and David A. Sinclair

Corresponding author: David A. Sinclair, Harvard Medical School

Review timeline:

Submission date:	16 September 2013
Editorial Decision:	29 October 2013
Revision received:	22 January 2014
Accepted:	09 March 2014

Transaction Report:

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

Editor: Bernd Pulverer

1st Editorial Decision 29 October 2013

Thank you very much for submitting your manuscript for consideration by the EMBO Journal and for your patience in awaiting feedback on this reviewing process. I apologize for the slight delay. Your manuscript has been evaluated by two referees whose comments are shown below; we are still expecting a third report, but since we cannot justify any further delays, we are sending the decision at this time. If the report arrives in a reasonable time frame we will of course forward it.

Given referee2 and 3's positive recommendations, I would very much like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers.
Specifically,
1) both referee 2 and 3 request interaction data between SIRT2 and BubR1 at endogenous expression levels.
2) please experimentally investigate if the K668R and K668Q mutants of BubR1 exhibit differential protein stability (ref 3).
3) please expand the discussion of the BubR1 H/H / SIRT2 TG mouse phenotype to elaborate on the notion of a partial rescue and to speculate on molecular details underpinning these effects (ref 3).
4) please comment on the likelihood that another sirtuin may be involved in the rescue (ref 3).
5) please comment on referee 2's point regarding fig 3G.

If the report of referee 1 arrives in the near future, we will forward it and we would expect any essential experimental issues to be addressed as part of the revision. However, we will not hold up the publication process for the delayed report of referee 1.

Thank you for the opportunity to consider your work for publication. I look forward to your revision and we will ensure that the downstream editorial process will make up for the small delay at this stage.

REFEREE REPORTS

Referee 2

This is a remarkable report where authors identify an unexpected connection between two longevity proteins: BubR1 and Sirt2. Indeed, authors find that Sirt2, but not other sirtuins, stabilizes BubR1 through deacetylation of a particular residue (acetylation of this residue induces ubiquitylation and degradation of BubR1). Transgenic overexpression of Sirt1 increases the short lifespan of BubR1 hypmorphic mice. Finally, pharmacological activation of sirtuins with NAM restores de age-associated decline in BubR1 levels.

Together this is a very important advance for the aging field because it unifies under the same paradigm two previously unconnected longevity genes. It remains to be determined how BubR1 promotes longevity.

Technically, the paper is sound and convincing.

I only have minor criticisms:.
1. I miss demonstration of the SIRT2/BUBR1 interaction using endogenous proteins.
2. The ubiquitylation assay shown in Fig. 3G is poorly convincing.
3. As a marginal note: Sirt6 transgenic mice have been also reported to increase longevity selectively in males (and not in females).
...
Referee 3

In this manuscript, North and colleagues provide evidence to indicate that the mammalian sirtuin SIRT2 directly deacetylates the longevity factor BubR1 on lysine 668, increasing its stabilization by inhibiting proteosomal degradation. Further, overexpression of SIRT2 in mice rescues the shortened lifespan in BubR1 deficient mice. BubR1 has emerged in recent years as a critical modulator of lifespan, both in mice and humans. However, little is known on the mechanisms regulating this protein. As such, this study is quite novel and provides insight into the molecular mechanisms of BubR1 regulation, linking sirtuins to this modulation, and providing putative new ways to modulate lifespan through BubR1. Overall, the biochemistry is impeccable and the in vivo rescue compelling. Results in the manuscript are solid and clearly presented, supporting the authors' hypotheses. Surprisingly, the in vivo analysis falls short, given that the mice have been already generated, as indicated below.

Major comments:
- The authors claim that BubR1, SIRT2 and CBP interact, however this interaction is only measured with overexpressed proteins. Although it does not affect majorly the main conclusions of the paper, for the authors to state that these proteins interact, endogenous proteins should be measured.
- Although the ubiquitination data looks solid, do the BubR1 mutants (K668Q and K668R) exhibit differences in stability, as expected? In other words, expression of these proteins in cells leads to increased (K668R) and decreased (K668Q) levels of these mutants?
- The in vivo extension of lifespan in the BubR1 KO mice by overexpressing SIRT2 or treating with NMN is compelling. However, it is surprising how little is explored in the SIRT2 TG and NMN rescued mice. Why is lifespan extended? The authors mentioned briefly that it is likely through a senescence-independent mechanism (given the previous paper from the Van Deursen lab), and they also vaguely indicated in the Discussion that not all the phenotypes of the BubR1 mice were rescued, but no details are given or shown. For instance, are any of the hallmarks described in the BurB1 deficient mice (lordokyphosis, muscle atrophy, cataracts, lipolysis, etc.) rescued by SIRT2 or NMN? If so, can the authors at least speculate on the molecular reasons behind such rescue, in turn explaining what are the determinants of lifespan that are dependent on a SIRT2/BubR1 pathway?

- In Figure 5G, the authors claim that in SIRT2 KO MEFs, levels of BubR1 are not increased by NMN treatment, however in one of the two lines, there is a significant increase, indicating that possibly another sirtuin may influence the levels of BubR1 as well. It would be appropriate to at least refer to this point in the Discussion.

30 October 2013

Referee 1 comments

This is a great story, important and beautifully executed.
I have the only following reservation regarding the manuscript: the data looking at the effect of the SIRT2 transgenic crossed with the hypomorphic BubR1 mice is a bit light. For example, the authors could have looked at the level of BubR1 expression in these mice to confirm that BubR1 expression is indeed enhanced by SIRT2 overexpression. At the very least, I would ask them to tone down the conclusion of these experiments. For example, at the bottom of page 10, they state: "the results indicate that SIRT2 modulation of BuBR1 stability results in lifespan extension of male BuR1H/H mice". They clearly do not show this. I would ask them to delete this sentence and to replace by a statement to the effect that it is likely that SIRT2 has other targets beyond BubR1 and that the lifespan effect likely represent the integration of multiple targets that are regulated by SIRT2.
Finally, I noticed that most gels have very high background. In some cases, it makes the figures hard to look at (Fig. 1A,H, 2E,G, 3E, 5G). It should be easy to modify this in a revised manuscript.

1st Revision - authors' response 22 January 2014

We wish to thank the EMBO Journal editors and the reviewers for their encouraging and helpful comments regarding our prior submission. All three reviewers were positive about the initial submission and suggested additional experiments and changes to the text to improve the study. To quote from all three reviewers, the study:

• “is a great story, important and beautifully executed.”
• “is a remarkable report where authors identify an unexpected connection between two longevity proteins: BubR1 and Sirt2.”
• “is a very important advance for the aging field because it unifies under the same paradigm two previously unconnected longevity genes.”
• “is quite novel and provides insight into the molecular mechanisms of BubR1 regulation, linking sirtuins to this modulation, and providing putative new ways to modulate lifespan through BubR1. Overall, the biochemistry is impeccable and the *in vivo* rescue compelling.”

We agree that this novel connection between the longevity genes SIRT2 and BubR1 is exciting and provides a new approach for longevity-based therapeutics. Guided by suggestions from the editor and reviewers, we have performed numerous new experiments since our initial submission, including demonstrating interaction between BubR1 and SIRT2 at the endogenous level, showing the BubR1 acetylation mutants have altered stability, and performing new ubiquitination assays that demonstrate the effect of CBP and SIRT2 more clearly. Furthermore, we have assessed the levels of BubR1 in the hypomorphic mice in the presence and absence of SIRT2 overexpression. Finally, we have included data suggesting that the ability of SIRT2 to extend the lifespan of BubR1 hypomorphic mice is due to a reversal of cardiac conduction abnormalities caused by BubR1 depletion. All of this new data supports our original conclusions and provides a convincing case for the existence of an acetylation-based regulatory pathway of BubR1 controlled by SIRT2, a poorly characterized sirtuin. We hope that these new additional data will convince the reviewers that our study is now suitable for publication.

A detailed, point-by-point response is provided below:

Reviewer #1:
This is a great story, important and beautifully executed. I have the only following reservation regarding the manuscript: the data looking at the effect of the SIRT2 transgenic crossed with the hypomorphic BubR1 mice is a bit light. For example, the authors could have looked at the level of BubR1 expression in these mice to confirm that BubR1 expression is indeed enhanced by SIRT2 overexpression. At the very least, I would ask them to tone down the conclusion of these experiments. For example, at the bottom of page 10, they state: "the results indicate that SIRT2 modulation of BuBR1 stability results in lifespan extension of male BuR1H/H mice". They clearly do not show this. I would ask them to delete this sentence and to replace by a statement to the effect that it is likely that SIRT2 has other targets beyond BubR1 and that the lifespan effect likely represent the integration of multiple targets that are regulated by SIRT2.
Finally, I noticed that most gels have very high background. In some cases, it makes the figures hard to look at (Fig. 1A,H, 2E,G, 3E, 5G). It should be easy to modify this in a revised manuscript.

We thank the reviewer for their careful and constructive critique of our manuscript and for the view that "*This is a great story, important and beautifully executed*." We added further *in vivo* analysis of *BubR1H/H* mice with and without SIRT2 overexpression that have improved the technical rigor of our study and shed light on potential mechanisms by which SIRT2 extends lifespan of *BubR1H/H* mice.

1. The data looking at the effect of the SIRT2 transgenic crossed with the hypomorphic BubR1 mice is a bit light. For example, the authors could have looked at the level of BubR1 expression in these mice to confirm that BubR1 expression is indeed enhanced by SIRT2 overexpression.

As suggested we have extended our analysis of *BubR1H/H* mice with and without SIRT2 overexpression, including the assessment of BubR1 protein levels in various tissues (spleen, testes, and heart), which confirms that BubR1 protein levels are enhanced in response to SIRT2 overexpression. In addition, we have included data demonstrating that some aspects of cardiac electrophysiology that are altered in *BubR1H/H* mice are reversed by SIRT2 overexpression. This is particularly interesting in light of results from Jan van Deursen's lab (Baker *et al. Nature.* 2011) showing that the deletion of senescent cells in the *BubR1H/H* mice prevents many agerelated phenotypes but it has *no impact* on the lifespan of these animals. Their data indicate that this is due to altered cardiac electrophysiology. These mice tend to die in a sudden fashion, where one is unable to predict the lifespan of a particular animal based on the severity of its premature aging features. Here we show that overexpression of SIRT2 *extends the lifespan* of *BubR1H/H* mice with associated reversal of some cardiac abnormalities, a first for this model. We have added the new protein expression data from *BubR1H/H* and *SIRT2tg*/*BubR1H/H* mice to Figure 4 and the analysis of cardiac function in two new figures (Figure 5 and Supplemental Figure 5).

2. At the bottom of page 10, they state: "the results indicate that SIRT2 modulation of BuBR1 stability results in lifespan extension of male BuR1H/H mice". They clearly do not show this. I would ask them to delete this sentence and to replace by a statement to the effect that it is likely that SIRT2 has other targets beyond BubR1 and that the lifespan effect likely represent the integration of multiple targets that are regulated by SIRT2.

As suggested, we have toned-down the writing. Specifically, we have removed the statement "the results indicate that SIRT2 modulation of BubR1 stability results in lifespan extension of male *BubR1H/H* mice" and replaced it with "These results indicate that increasing SIRT2 activity can extend the lifespan of male *BubR1H/H* mice."
In addition, in the discussion section we have added the statement "Our results also indicate that inducing SIRT2 activity can counteract the aging effects caused by BubR1 depletion in the heart. Although our data suggest that SIRT2 mediated deacetylation and stabilization of BubR1 can reverse the effect of BubR1 depletion in *BubR1H/H* mice, there remains a strong possibility that SIRT2 has additional targets through which it might exert its lifespan extension effect under BubR1 depleted circumstances."

3. Finally, I noticed that most gels have very high background. In some cases, it makes the figures hard to look at (Fig. 1A,H, 2E,G, 3E, 5G).

We thank the reviewer for pointing this out. We will work with the editorial staff to make sure that all the gels are easy to view while making minimal adjustments to the original scans.

Reviewer #2:
This is a remarkable report where authors identify an unexpected connection between two longevity proteins: BubR1 and Sirt2. Indeed, authors find that Sirt2, but not other sirtuins, stabilizes BubR1 through deacetylation of a particular residue (acetylation of this residue induces ubiquitylation and degradation of BubR1). Transgenic overexpression of Sirt1 increases the short lifespan of BubR1 hypmorphic mice. Finally, pharmacological activation of sirtuins with NAM restores de age-associated decline in BubR1 levels.
Together this is a very important advance for the aging field because it unifies under the same paradigm two previously unconnected longevity genes. It remains to be determined how BubR1 promotes longevity.
Technically, the paper is sound and convincing.

We thank the reviewer for the positive comments and suggestions for improving the manuscript. We have addressed the concerns with further experimentation, which we hope convinces the reviewer the manuscript is now suitable for publication.

1. I miss demonstration of the SIRT2/BUBR1 interaction using endogenous proteins.

We agree with the reviewer that demonstrating interaction between two proteins is always more convincing if performed with endogenous proteins. We have now performed immunoprecipitations and are able to show interaction between SIRT2 and BubR1. Similar with our previous co-immunoprecipitation experiments, we find that the interaction is relatively weak, and may therefore be a transient interaction, which is often seen in enzyme-substrate interactions. This new experimental evidence is now shown in Figure 2E.

2. The ubiquitylation assay shown in Fig. 3G is poorly convincing.

We have carried out this experiment again, adjusting the duration of exposure to MG132 from 4 hours in the original experiment, to 16 hours. Using this longer duration, we now see a more substantial difference between the various conditions. We have replaced the original figure with this new data.

3. As a marginal note: Sirt6 transgenic mice have been also reported to increase longevity selectively in males (and not in females).

We thank the reviewer for reminding us of the gender bias in SIRT6 overexpression-mediated lifespan extension. We have discussed this in a new paragraph on Page 16.

Reviewer #3:
In this manuscript, North and colleagues provide evidence to indicate that the mammalian sirtuin SIRT2 directly deacetylates the longevity factor BubR1 on lysine 668, increasing its stabilization by inhibiting proteosomal degradation. Further, overexpression of SIRT2 in mice rescues the shortened lifespan in BubR1 deficient mice. BubR1 has emerged in recent years as a critical modulator of lifespan, both in mice and humans. However, little is known on the mechanisms regulating this protein. As such, this study is quite novel and provides insight into the molecular mechanisms of BubR1 regulation, linking sirtuins to this modulation, and providing putative new ways to modulate lifespan through BubR1. Overall, the biochemistry is impeccable and the in vivo rescue compelling. Results in the manuscript are solid and clearly presented, supporting the authors' hypotheses. Surprisingly, the in vivo analysis falls short, given that the mice have been already generated, as indicated below.

We thank the reviewer for their evaluation of our manuscript and for the positive comments and suggestions for improving the manuscript. We have addressed the reviewers concerns with further experimentation, and have added additional *in vivo* data from the mice as well as more detailed discussions related to the mouse phenotypes. We hope our added data and discussion convinces the reviewer the manuscript is now suitable for publication.

1. The authors claim that BubR1, SIRT2 and CBP interact, however this interaction is only measured with overexpressed proteins. Although it does not affect majorly the main conclusions of the paper, for the authors to state that these proteins interact, endogenous proteins should be measured.

We agree with the reviewer that it is ideal to assess interactions between proteins at the endogenous level. We have gone on to show interactions between BubR1 with SIRT2 at the endogenous level. We were unable to obtain clear interaction data at the endogenous level between BubR1 and CBP and have made sure we do not over-interpret our data in the text. The new data showing the endogenous BubR1-SIRT2 interaction is now represented in Figure 2E.

2. Although the ubiquitination data looks solid, do the BubR1 mutants (K668Q and K668R) exhibit differences in stability, as expected? In other words, expression of these proteins in cells leads to increased (K668R) and decreased (K668Q) levels of these mutants?

We thank the reviewer for the suggestion to assess the stability of BubR1 mutants. We went on to determine the stability of wild-type and the K668R and K668Q mutants utilizing cycloheximide time course experiments. Using this approach, we have confirmed that the K668Q mutant, which mimics acetylation and is ubiquitinated to a greater extent, has a shorter half-life than the wild-type protein. The K668R mutant has a half-life that is similar, or slightly longer, than the wild-type protein. These new data are included in Figure 3G-H.

3. The in vivo extension of lifespan in the BubR1 KO mice by overexpressing SIRT2 or treating with NMN is compelling. However, it is surprising how little is explored in the SIRT2 TG and NMN rescued mice. Why is lifespan extended? The authors mentioned briefly that it is likely through a senescence-independent mechanism (given the previous paper from the Van Deursen lab), and they also vaguely indicated in the Discussion that not all the phenotypes of the BubR1 mice were rescued, but no details are given or shown. For instance, are any of the hallmarks described in the BurB1 deficient mice (lordokyphosis, muscle atrophy, cataracts, lipolysis, etc.) rescued by SIRT2 or NMN? If so, can the authors at least speculate on the molecular reasons behind such rescue, in turn explaining what are the determinants of lifespan that are dependent on a SIRT2/BubR1 pathway?

We have expanded the discussion of the various phenotypes that are changed or unchanged in the BubR1 hypomorphic mice when crossed to SIRT2 overexpressing mice. Regarding why lifespan is extended by SIRT2, this is an excellent question that we have been addressing for the past few years and have included new data in the resubmission. What is surprising is that very few of the hallmarks of aging are significantly altered by SIRT2 overexpression, with longevity appearing to be the primary phenotype that is altered, along with cardiac improvements that we now include in the revised manuscript. This is quite interesting given a study from the van Duersen lab (Baker *et al. Nature* 2011) whereby they generated a *BubR1H/H* mouse in which they could delete senescent cells by overexpressing a caspase gene under a senescence-specific promoter. Mice with deleted senescent cells were more normal in size, exhibited reduced lordokyphosis, cataracts, etc. However, a striking point was that these *mice still had a short lifespan and deletion of senescence cells had no effect on lifespan of the BubR1H/H mice*. *BubR1H/H* mice tend to die in a sudden fashion, where one is unable to predict the lifespan of a particular animal based on severity of its premature aging features. There data indicated that lifespan is not extended because the treatment did not improve defective cardiac function, particularly cardiac electrophysiology.
We find that although SIRT2 overexpression *extends the lifespan* of *BubR1H/H* mice combined with reversal of some cardiac abnormalities. We appreciate that additional studies are necessary to further tease out this pathway, especially in the heart. However, we hope the reviewer concurs that this would fall outside the scope of the current study, and would constitute a subsequent study looking more specifically at SIRT2 and BubR1 function in the heart. In the new manuscript, we have extended our discussion of the phenotypes, including both the ones that are reversed and those that are not, and our thoughts on why this might be.
With regard to NMN, the current cost of NMN ($1600/g) precludes a long-term experiement. We are working to reduce this cost because this experiment will be very interesting. We suspect, as the reviewer also pointed out, that NMN treatment will likely lead to alterations in many

longevity pathways both dependent and independent of the SIRT2/BubR1 pathway.

4. In Figure 5G, the authors claim that in SIRT2 KO MEFs, levels of BubR1 are not increased by NMN treatment, however in one of the two lines, there is a significant increase, indicating that possibly another sirtuin may influence the levels of BubR1 as well. It would be appropriate to at least refer to this point in the Discussion.

We thank the reviewer suggesting that we further discuss these results and additional regulatory aspects of NMN on BubR1 protein abundance. Although we did not find that any other sirtuin has the ability to deacetylate BubR1, and our data indicates that SIRT2 is the primary mechanism of BubR1 deacetylation, we cannot rule out the possibility that NMN produces broad effects in the cell and there may be a number of other pathways that are modulated. We have expanded the discussion in the manuscript to include the possibility of additional regulatory mechanisms.

The authors should also directly show the effect of SIRT2 on BubR1 stability, not only on ubiquitination. They haven't really done adequate stability studies, and need a better examination of BubR1 half-life in absence and presence of SIRT2, in order to support their model.

This is a good suggestion, thank you. We have now determined the stability of wild-type and the K668R and K668Q mutants utilizing cycloheximide time course experiments. Using this approach, we have confirmed that the K668Q mutant, which mimics acetylation and is ubiquitinated to a greater extent, has a shorter half-life under in the presence of cycloheximide. The K668R mutant appears to have a half-life that is similar, or slightly longer, than the wildtype protein. These new data are included in Figures 3G-H.

2nd Editorial Decision 09 March 2014

I am very pleased indeed to inform you that your manuscript has been accepted for publication in the EMBO Journal.
I am sorry for the extensive delay in responding. We have obtained the positive re-evaluation by one key referee, which is shown below.

Please permit a couple of small suggestions regarding the title and abstract of your exciting study. The aim is to maximize the reach to the more general readership and the discoverability to search queries.
1) While the abstract is very clear and carefully worded, I suggest to mention that BubR1 is a mitotic spindle checkpoint kinase. You could also usefully describe the BubR1H/H model in a couple of words.
2) I suggest that the title could be a little more straightforward and to reflect the experimental data more clearly. How about: 'SIRT2 or NAD+ induce checkpoint kinase BubR1 to increase life span'

We will accelerate editorial production to ensure we make up for lost time in the review process.

Referee #3:

I find this revised version of the manuscript highly improved, in particular the new stability assays for BubR1, and the new in vivo data is particularly strong. The authors have adequately addressed most of the previous reviewers' concerns, and as such I find now the manuscript suitable for publication. The rescue of the BubR1 HH mice by SIRT2 overexpression is compelling, and I am sure these results will interest a broad audience.
