A pharmaco-epistasis strategy reveals a new cell size controlling pathway in yeast

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1st Editorial Decision 16 September 2013

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from two of the three referees whom we asked to evaluate your manuscript. Since their recommendations are very similar, I prefer to make a decision now rather than further delaying the process. As you will see from the reports below, the reviewers acknowledge that your study presents potentially interesting findings regarding cell size control and ageing. However, they list a number of issues that should be addressed and make suggestions for modifications, which we would ask you to carefully address in a revision of the manuscript.

Without repeating all the points listed below, both reviewers point out that the findings should be cautiously interpreted and correctly presented in the context of the existing knowledge, in order to avoid overstatements. Moreover, they refer to the need to provide additional explanations and clarifications concerning the analysis and interpretation of the data. Finally, reviewer #3 mentions that additional evidence needs to be presented to convincingly support the proposed role of NAD+ as a regulator of cell size and includes suggestions in this regard.

Thank you for submitting this paper to Molecular Systems Biology.

REFEEEREE REPORTS:

Reviewer #1:

The article by Daignan-Fornier et al. entitled "A pharmaco-epistasis strategy reveals a new cell size controlling pathway in yeast" is well written manuscript detailing a number of significant insights into the mechanisms of cell size control. The investigators use the particularly innovative approach
of pharmacogenomics to yield a number of intriguing and thought provoking results with respect to the genetics of size control. By taking advantage of the ability of treatment with nicotinamide, to mimic a sir2 deletion, the authors have conducted a large scale epistasis analysis of the potential role of Sir2 in size control. A second pharmaco-epistasis strategy using DAB yields equally important and fascinating results. The manuscript has significant experimental support and yields noteworthy and captivating results that are bound to advance both the fields of cell size control and aging. While the manuscript is, in general, well-designed and logically presented, there are areas that could use clarification/correction in order to improve the overall quality of the manuscript.

Points for consideration:
1. There are several areas where the authors seem to unaware of the fact that their observation(s) has already been published. First, Yang et al 2011, clearly report that sir2 deletions increase cell size and discuss the implications of this result. This fact needs to be taken into account in the presentation of the authors' sir2/NAM results. In addition, the statement of pg 11 that "our results established the Sir2/60S pathway as a new way to modulate cell size" once again negates the fact that this was a major observation reported in the Yang et al 2011 paper. Second, the labs of Futcher, Tyers, and Alberghina have spent parts of their entire careers elucidating at the molecular level the mechanism whereby nutrients control cell size. As such, the statement on pg 8 that such an understanding was first worked out in B. subtilis in 2007 is not accurate. Third, the authors seem to unaware that the Polymenis lab (Hoose et al 2012) has already published nearly the exact same data that is presented in Fig 3 A-B. Finally, the Bilinski lab has published a number of articles showing that enlargement of yeast using alpha factor does indeed shorten lifespan. These observations are now regarded as more likely to reflect physiological reality as opposed to the 1994 Guarente paper that is cited. Furthermore, in a significant number of places a less than ideal reference is provided or no reference is given at all. Examples of this are:
   a. pg 3: "In fact, cells grown in poor media are significantly smaller than those grown in rich media." A reference should be cited.
   b. pg 4 "Cln3p/Cdc28 stimulates the transcriptional activation of more (sic) hundred genes involved in the transition from G1 to S phase" should reference Spellman et al 1998.
   c. References to Polymenis lab work regarding the complexity of cell size control, e.g. Hoose et al are missing in several places.
   d. The use of Turner et al. as a reference for Coulter Counter methods is less than ideal. Many better references exist.
   e. The statement that critical cell size is "constant" in a culture is not accurate. Critical size increases for each cell each generation.
2. While the pharmacogenomics approach is very appealing and innovative, many of the observed results are potentially over-interpreted. Nicotinamide is a drug/vitamin that could have many off target effects. Thus, while it is really cool to use an inhibitor of Sir2 to mimic a sir2 deletion, failure to take into account any additional non-Sir2 based effects of nicotinamide can lead to misinterpretation of the data. In fact, additional non-Sir2 dependent phenotypes might partially explain some of the perplexing aspects of the results report in this work. As a case in point, Kaeberlein et al. has shown that nicotinamide clearly has important phenotypes in cells lacking SIR2 (e.g. shortened lifespan). This is a critical point that needs to be addressed in this work. Moreover, this same point applies to the DAB and nicotinic acid experiments.
3. The types of pharmaco-epistatic experiments present here are very innovative, but need to be interpreted with care. Nam phenocopies but is not the same as a sir2- deletion as discussed above. While combining opposite phenotypes can clearly demonstrate epistasis, it does not necessarily translate to gene order in pathways. For example, since we don't know how sir2- makes cells large or how rpl35b- or rpa49a make cells small, it is difficult to state if they function upstream or downstream from one another. Thus sir2- might make cells larger thru the accumulation of ERCs while rpl35b could result in less Whi5 being produced. The result is that ERCs may not increase the size of cells lacking whi5 but this says little about the pathway order of these genes biochemically.
4. On page 9, the authors state that "the large and small subunits, at least in part, act independently
on cell size homeostasis." Given the complexity of cell size control, it is probably an over generalization to make this statement based on two genetic crosses.

5. The concentrations of DAB and cycloheximide used in this work need to be better justified. In each case, they are ~25-30 lower than used in the Steffen et al 2008 work. How can the authors be sure that they are working with a physiologically relevant concentration? Comparisons between published data are very difficult when such different levels of drugs are used.

6. The statement on page 10 that orange dots reflect false positives is unsubstantiated. How do the authors justify this statement?

7. In figure 2A, a significant portion of the "whi" mutants are shown larger than WT cells. Why is this? This needs to be addressed! The green line needs to originate from the origin not above it. On page 7, it is stated that since 74 "whi" mutants respond similar to wild type cells after NAM treatment (i.e. they get bigger), they probably function through independent means to affect cell size. Couldn't the NAM also be functioning downstream of the "whi" mutation but in the same pathway?

8. Page 14 - Creating actual combinations with sir2 deletions is far from impossible, just more difficult.

9. Page 16 - Analyzing genetic networks in yeast haploid cells is relatively simple. However, one must keep in mind the detrimental effects that suppressor mutations could have on genetic combinations when working with haploids and not diploid or higher ploidy cells.

10. The final paragraph needs to address the complex issue surrounding a potential role for cell size in lifespan determination more thoroughly. The authors appropriately conclude that no simple connection between these two phenomena can be drawn, but they only point out evidence opposing a causal relationship between cell size and lifespan when they have equally compelling data in favor of this conclusion. For example, the apparently central role of Sir2/60S in both size and lifespan is intriguing. The authors' own work shows that nicotinic acid reduces cell size presumably by activating Sir2 which is known to extend lifespan. Finally, the authors' use of their fob1 data to supposedly refute a relationship between size and lifespan is somewhat ironic as Fig 7C-D shows no effect of the deletion of FOB1 on either size or lifespan.

All in all, the work presented here is innovative, thought-provoking, and highly important to the field. However, a number of issues need to be addressed to significantly improve the impact of this manuscript.

Reviewer #3:

The manuscript by Moretto et al describes the discovery of a new size modulating pathway in yeast using a chemical - genetic approach. This manuscript provides two new insights which are of significant importance to the community: 1. Sir2 modulates cell size in a manner which is at least partially independent of previously described pathways. 2. The small and large ribosomal subunit proteins seem to affect growth and division in fundamentally different ways. Though none of their findings are backed up biochemically, the genetic evidence presented seems solid and carefully done. However, I think several issues need to be addressed
1. In the introduction and discussion the authors only briefly touch upon current models of cell size control (such as the threshold model) and do not discuss their findings in this context (figure 6D is based mainly on findings from the Tyers lab). Cell size is a result of the balance of growth and division, and it would thus be useful if the authors could place their findings in this theme rather than using the abstract concept of "cell size pathway". Growth rates/doubling times are only mentioned in fig 3C. However, Jorgensen (2002) and others have demonstrated how important growth rates are for the interpretation of cell size data. Therefore all cell size phenotypes which are important for the overall result should be controlled for growth rates.
2. The interpretation of the Swi4/6 phenotypes is ambiguous. These mutants tend to arrest permanently at the G1/S transition.
3. If the authors are measuring NAD+ by metabolomics anyway, it would be useful to report the concentrations of NADH and other precursors as well, since these compounds must have been
measured in the same run. Do other metabolites respond to NA and correlate with cell size?

4. The proposed role of NAD+ as an upstream regulator of cell size is interesting and provocative. However, the presented evidence for this is rather weak. Can a correlation of NAD+ concentrations and cell size be constructed from literature data?

Minor points:
1. The chosen cut-offs for "hits" seem very arbitrary, please explain.
2. Figure 2B and C show different WT and sir2 size distributions
3. The authors could speculate how Sir2's role in size control fits with its well characterized function as a deacetylase.

Reviewer #1 (Remarks to the Author):

Points for consideration:
1. There are several areas where the authors seem to unaware of the fact that their observation(s) has already been published. First, Yang et al 2011, clearly report that sir2 deletions increase cell size and discuss the implications of this result. This fact needs to be taken into account in the presentation of the authors' sir2/NAM results.

The report by Yang et al. is now cited and discussed page 6 line 5. The discussion on sir2 effects on cell size, in the Yang et al paper, are centered on the role of ERC accumulation in ageing and cell size control. Our experiments on the sir2 fob1 double mutant presented in figure 7 do not favor a role of ERC in cell size homeostasis.

In addition, the statement of pg 11 that "our results established the Sir2/60S pathway as a new way to modulate cell size" once again negates the fact that this was a major observation reported in the Yang et al 2011 paper.

We agree that data in the Yang et al paper document the effect of Sir2 on cell size. However, the connection between Sir2 and the 60S ribosomal subunit was not established by these authors and therefore the Sir2/60S signaling pathway disclosed by our large scale epistasis is a “new pathway”. Our approach based on utilization of a set of pre-identified mutants is not aimed at identifying new players but at elucidating the functional relationship between known players using epistasis i.e. identifying new signaling pathways. The sentence (page 11 first sentence of second paragraph) has been changed to make that point clearer.

Second, the labs of Futcher, Tyers, and Alberghina have spent parts of their entire careers elucidating at the molecular level the mechanism whereby nutrients control cell size. As such, the statement on pg 8 that such an understanding was first worked out in B. subtilis in 2007 is not accurate.

Sorry! Our written English is clearly not good enough to apprehend all language subtleties. In that particular case, our purpose was obviously not to ignore or deny the work of the people of the yeast field. What we intended to say was just that the full understanding of the intimate molecular mechanisms connecting nutrient to cell size was reported for B. subtilis by Weart et al. in 2007. This sentence has been changed page 3 beginning of paragraph 2.

Third, the authors seem to unaware that the Polymenis lab (Hoose et al 2012) has already published nearly the exact same data that is presented in Fig 3 A-B.

The results shown in Hoose et al. 2012 are now cited and discussed page 9 (end of first paragraph).
coworkers (compiled by Hoose et al) were obtained in growth conditions very different from ours. Please see also point 7 below.

Finally, the Bilinski lab has published a number of articles showing that enlargement of yeast using alpha factor does indeed shorten lifespan. These observations are now regarded as more likely to reflect physiological reality as opposed to the 1994 Guarente paper that is cited.

The disagreement between Guarente and Bilinski results is now presented (page 18 line 5).

Furthermore, in a significant number of places a less than ideal reference is provided or no reference is given at all. Examples of this are:

a. pg 3: "In fact, cells grown in poor media are significantly smaller than those grown in rich media." A reference should be cited.

A reference has been added page 3 line 11

b. pg 4 "Chn3p/Cdc28 stimulates the transcriptional activation of more than (sic) hundred genes involved in the transition from G1 to S phase" should reference Spellman et al 1998.

This reference has been added page 4 line 17

c. References to Polymenis lab work regarding the complexity of cell size control, e.g. Hoose et al are missing in several places.

This work is now cited page 5 first line and page 9 line 8.

d. The use of Turner et al. as a reference for Coulter Counter methods is less than ideal. Many better references exist

We agree that this reference is obviously not the first paper that uses Coulter to measure yeast cell volume. However, we used this reference because the author provides a critical comparison of currently used methods to measure yeast cell size. The sentence has been changed to make it clear page 20 line 8-10

e. The statement that critical cell size is "constant" in a culture is not accurate. Critical size increases for each cell each generation.

We meant that the averaged critical size is constant (in the population). In fact, here what we use for comparison is the median cell volume. This has been changed (page 4 line 3).

2. While the pharmacogenomics approach is very appealing and innovative, many of the observed results are potentially over-interpreted. Nicotinamide is a drug/vitamin that could have many off target effects. Thus, while it is really cool to use an inhibitor of Sir2 to mimic a sir2 deletion, failure to take into account any additional non-Sir2 based effects of nicotinamide can lead to misinterpretation of the data. In fact, additional non-Sir2 dependent phenotypes might partially explain some of the perplexing aspects of the results report in this work. As a case in point, Kaeberlein et al. has shown that nicotinamide clearly has important phenotypes in cells lacking SIR2 (e.g. shortened lifespan). This is a critical point that needs to be addressed in this work. Moreover, this same point applies to the DAB and nicotinic acid experiments.

We agree that this is an important issue. Off target effects cannot be surely eliminated but we have done our best to reduce them and to validate our results by independent means. This was done by using as low as possible concentrations of inhibitors without abolishing the cell size effect (0.1mM for NAM compared to 5mM in Kaeberlein et al experiments; 0.5 mg/L DAB compared to 15 mg/L in Steffen et al). In addition, we carefully confirmed the pharmacoc-epistasis results by classical genetics. This was done with several mutants in the case of NAM and for all of them in the case of DAB. We think that this classical genetic reconstitution is very strong evidence that the effects we observed are not the result of off-target effects. This point is now discussed page 14 line 12 of discussion.
3. The types of pharma-co-epistatic experiments present here are very innovative, but need to be interpreted with care. Nam phenocopies but is not the same as a sir2- deletion as discussed above. While combining opposite phenotypes can clearly demonstrate epistasis, it does not necessarily translate to gene order in pathways. For example, since we don’t know how sir2- makes cells large or how rpl33b- or rpa49a make cells small, it is difficult to state if they function upstream or downstream from one another. Thus sir2- might make cells larger thru the accumulation of ERCs while rpl33b could result in less Whi5 being produced. The result is that ERCs may not increase the size of cells lacking whi5 but this says little about the pathway order of these genes biochemically.

Although epistasis is widely used and largely accepted as a tool to order genes into linear pathways, we agree that it is not a formal and definitive demonstration but it is a very strong indication. This is especially true when (as we do here) epistasis is used in its strict (Bateson) sense i.e.: “…when one mutation (or drug mimicking the mutation) masks the effects of another mutation”. We first concentrated on these situations because they are relatively easy to interpret and allow positioning genes into linear regulatory pathways. (Discussion page 14).

As suggested by the reviewer, the conclusions of epistasis analyses have been attenuated page 7 line 5 from the bottom, page 9 fourth line starting from the bottom and page 11 end of first paragraph.

4. On page 9, the authors state that "the large and small subunits, at least in part, act independently on cell size homeostasis.” Given the complexity of cell size control, it is probably an over generalization to make this statement based on two genetic crosses.

We agree with the reviewer. The statement has been tempered (page 9 line 5 of second paragraph)

5. The concentrations of DAB and cycloheximide used in this work need to be better justified. In each case, they are ~25-30 lower than used in the Steffen et al 2008 work. How can the authors be sure that they are working with a physiologically relevant concentration? Comparisons between published data are very difficult when such different levels of drugs are used.

In the experiment presented in Fig. 3C, DAB and cycloheximide (CHX) were used at a low concentration affecting only slightly and similarly generation time. The rationale here was to use drug concentrations that are effective (as attested by the slight increase in generation times) but do not arrest cells. We do not believe that using higher drug concentration for which cells hardly proliferate are physiologically relevant conditions to measure an effect on cell size. Of note, in the work of Steffen et al (Fig. 3C) the concentration of CHX used (25ng/mL) is not so much higher than the one we used (10ng/mL) in Fig. 3C.

6. The statement on page 10 that orange dots reflect false positives is unsubstantiated. How do the authors justify this statement?

The two mutants that could not be confirmed by classical genetics were considered to respond to DAB in a way that could not be mimicked by the rpa49 mutation and were therefore named “false positive” since they are positive for the drug response but not confirmed by classical genetics. The sentence (page 10 line 13) has been changed to make it more clear.

7. In figure 2A, a significant portion of the "whi" mutants are shown larger than WT cells. Why is this? This needs to be addressed!

Indeed, we observed several discrepancies with the data from Jorgensen et al. As mentioned by the reviewer, several whi mutants were not found whi in our hands. This is most likely due to differences in culture conditions. As discussed page 20 line 10-15 growth conditions are critical and we paid a lot of attention to keep them as constant as possible. In particular maintaining the cells in exponential growth for at least 24 h was very important for reproducibility. For example Jorgensen et al. did not identify sir2 as a large mutant and we found the exact same result when using their growth conditions (data not shown) while sir2 was clearly large under our growth conditions. This point is now addressed page 7 line 3 of second paragraph

The green line needs to originate from the origin not above it.
For the sake of visibility, the origin of the graph has been set at 25fl (not zero). This has been noted more clearly on Fig. 2A and is now specifically mentioned in the figure legend.

On page 7, it is stated that since 74 "whi" mutants respond similar to wild type cells after NAM treatment (i.e. they get bigger), they probably function through independent means to affect cell size. Couldn't the NAM also be functioning downstream of the "whi" mutation but in the same pathway?

If this was the case, NAM should be fully “epistatic” on these mutants and consequently they should reach the size of a wild-type strain treated with NAM (same size as a sir2 mutant). This is clearly not the case for most of them, for which NAM seems to rather act additively (not epistatically) to their Whi phenotype. Additivity is interpreted here as an indication of action through independent means.

8. Page 14 - Creating actual combinations with sir2 deletions is far from impossible, just more difficult.

Yes and we indeed constructed a few of them. This has been changed (Page 14 line 10 of discussion).

9. Page 16 - Analyzing genetic networks in yeast haploid cells is relatively simple. However, one must keep in mind the detrimental effects that suppressor mutations could have on genetic combinations when working with haploids and not diploid or higher ploidy cells.

We fully agree and therefore segregated a large number of mutants through meiosis to ensure that their size phenotype segregated correctly (2:2) in the meiotic progeny. Occasionally we observed suppressor mutations or aberrant segregation most likely due to aneuploidy (which could also occur in diploids).

10. The final paragraph needs to address the complex issue surrounding a potential role for cell size in lifespan determination more thoroughly. The authors appropriately conclude that no simple connection between these two phenomena can be drawn, but they only point out evidence opposing a causal relationship between cell size and lifespan when they have equally compelling data in favor of this conclusion. For example, the apparently central role of Sir2/60S in both size and lifespan is intriguing. The authors' own work shows that nicotinic acid reduces cell size presumably by activating Sir2 which is known to extend lifespan. Finally, the authors' use of their fob1 data to supposedly refute a relationship between size and lifespan is somewhat ironic as Fig 7C-D shows no effect of the deletion of FOB1 on either size or lifespan.

We think that a couple of counter-examples are a stronger indication than a correlation but we agree that this is largely a question of interpretation. A sentence has been added in the discussion section (page 18 end of the paragraph).

All in all, the work presented here is innovative, thought-provoking, and highly important to the field. However, a number of issues need to be addressed to significantly improve the impact of this manuscript.

Reviewer #3 (Remarks to the Author):

The manuscript by Moretto et al describes the discovery of a new size modulating pathway in yeast using a chemical - genetic approach. This manuscript provides two new insights which are of significant importance to the community: 1. Sir2 modulates cell size in a manner which is at least partially independent of previously described pathways. 2. The small and large ribosomal subunit proteins seem to affect growth and division in fundamentally different ways.

Though none of their findings are backed up biochemically, the genetic evidence presented seems solid and carefully done. However, I think several issues need to be addressed

1. In the introduction and discussion the authors only briefly touch upon current models of cell size
control (such as the threshold model) and do not discuss their findings in this context (figure 6D is based mainly on findings from the Tyers lab). Cell size is a result of the balance of growth and division, and it would thus be useful if the authors could place their findings in this theme rather than using the abstract concept of "cell size pathway". Growth rates/doubling times are only mentioned in fig 3C. However, Jørgensen (2002) and others have demonstrated how important growth rates are for the interpretation of cell size data. Therefore all cell size phenotypes which are important for the overall result should be controlled for growth rates.

We agree that doubling time is an important parameter and doubling times of the various strains have now been added in the text (added page 20 line 4). In this work we observed that the doubling time of the sir2 large mutant is the same as the wild-type doubling time (100 to 105 minutes) and therefore the larger size of sir2 cells cannot be simply attributed to a slower progression in the cell cycle. The whi mutants rpl31a, rpl33b and rpa49 have longer doubling times (120, 130 and 135 minutes, respectively). These results illustrate the fact that a longer generation time is not necessarily associated to a large phenotype and can even be found for whi mutants. These observations are consistent with those reported by Polymenis and coworkers showing that many mutations disturbing cell cycle progression do not necessarily affect cell size (Hoose et al 2012) (see added sentence in the Introduction section page 4 last line).

2. The interpretation of the Swi4/6 phenotypes is ambiguous. These mutants tend to arrest permanently at the G1/S transition.

Our goal in this work was not to determine why the various mutants are large or whi but rather to organize the mutants into genetic networks through epistasis relationships. We are aware that the swi4 and swi6 mutants are altered for cell cycle progression and consequently have longer doubling times (around 165 minutes) (added page 20 line 4). The phenotypes of swi4 and swi6 are interpreted in terms of epistasis by comparison with mbp1 which clearly behaved differently in the epistasis analysis (page 11 end of first paragraph). As suggested by reviewer 1 interpretation of epistasis results are now discussed more cautiously than in the initial manuscript (please see also the answer to point 3 from reviewer 1).

3. If the authors are measuring NAD+ by metabolomics anyway, it would be useful to report the concentrations of NADH and other precursors as well, since these compounds must have been measured in the same run. Do other metabolites respond to NA and correlate with cell size?

We have not done a thorough metabolomics analysis. However, several other metabolites (almost all purine and pyrimidine nucleotides, nucleosides and bases) have been measured in the same run with NAD+. The conclusion is that none of those correlates with cell size variations. Unfortunately, in our running conditions, NAM and NMR (the precursors of NAD(H)) are detected in the same elution peak, preventing any concentration calculation for these molecules. Concerning NADH it was found correlated to NAD+ as previously reported by others. Formally NADH could thus be the signal but we favor the hypothesis of NAD+ being the signal for the following reasons. First NAD+ is several orders of magnitude more abundant than free NADH (see discussion page 17 line 8). Second, NAD+ is an activator of Sir2 while NADH is rather an inhibitor of Sir2 (Lin et al 2004). Since nicotinic acid addition increases both NADH and NAD+ concentrations and decreases cell size in a way that is Sir2-dependent, i.e.: through Sir2 activation (Fig. 5B), we favor the idea that NAD+ is the signal but we have not excluded that a derivative could also play a role (see Introduction page 5 last paragraph and Results page 11 last line). We also have attenuated the statement concerning NAD+ in the abstract.

4. The proposed role of NAD+ as an upstream regulator of cell size is interesting and provocative. However, the presented evidence for this is rather weak. Can a correlation of NAD+ concentrations and cell size be constructed from literature data?

This sounds like an attractive possibility. however, as shown and discussed in several places in our manuscript, cell size measurements and quantitative metabolomics being exquisitely sensitive to growth conditions, we think that it would be very perilous to compare sets of data obtained in different conditions.
Minor points:
1. The chosen cut-offs for "hits" seem very arbitrary, please explain.

The cut-off at + or - 5% was chosen on the basis of our routine standard error around 5% (page 15 line 9).

2. Figure 2B and C show different WT and sir2 size distributions

These are two totally independent experiments. Size distributions are never strictly superimposable although median cell sizes are very close to each other.

3. The authors could speculate how Sir2's role in size control fits with its well characterized function as a deacetylase.

A sentence has been added to the discussion section page 15 line 4 of second paragraph.