RESEARCH ARTICLE

Characterizing mutations in and genetic interactions of RGG-motif translation repressor Sbp1 [version 1; peer review: 2 approved with reservations]

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Abstract

Background: Mechanisms of mRNA fate decisions play an important role in determining if a given mRNA will be translated, stored or degraded upon arrival to cytoplasm. Sbp1 is an important RGG-motif containing protein that is implicated in mRNA fate decisions since it can affect mRNA decapping and translation. Sbp1 represses translation by binding eIF4G1 through its RGG-motif and activates decapping when overexpressed. In order to understand the amino acids important for translation repression activity of Sbp1 we performed mutational analysis of Sbp1 combined with assessing its genetic interaction with another RGG-motif protein Scd6. We created two classes of point mutations a) in aromatic residues of the RGG-motif and b) in residues reported to be phosphorylated.

Method: Sequence alignment was performed to identify aromatic residues to be mutated based on conservation. Site-directed mutagenesis approach was used to create several point mutations in Sbp1 expressed under galactose-inducible promoter. The mutants were tested for their ability to cause growth defect upon overexpression. The ability of Sbp1 to affect repression activity of other decapping activators was tested using the same growth assay.

Results: Mutation of several aromatic residues in the RGG-motif of Sbp1 led to a weak rescue phenotype. However the phospho-mimetic mutants of Sbp1 did not lead to any kind of growth defect rescue. Deletion of another eIF4G1-binding RGG-motif protein Scd6 does not affect ability of Sbp1 to cause growth defect. On the other hand absence of Sbp1 does not affect ability of Dhh1 and Pat1 to repress translation.

Conclusion: Based on our growth assay analysis we conclude that mutated aromatic residues contribute marginally to repression activity of Sbp1 whereas phospho-mimetic mutants do not alter ability

Open Peer Review

Reviewer Status

Invited Reviewers

1. Tracy Nissan, University of Sussex, Brighton, UK
2. David Tollervey, University of Edinburgh, Edinburgh, UK
3. Jeffrey Wilusz, Colorado State University, Fort Collins, USA
4. Sarah F. Mitchell, Loyola Marymount University, Los Angeles, USA

Any reports and responses or comments on the article can be found at the end of the article.
of Sbp1 to cause growth defect. Interestingly Scd6 does not affect ability of Sbp1 to repress translation, which in turn does not affect Dhh1 and Pat1.

**Keywords**
mRNA fate decisions, Translation control, RGG-domain, Sbp1, eIF4G, Translation repression

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**Author roles:** Bhatter N: Investigation, Validation, Writing – Review & Editing; Iyyappan R: Investigation; Rajyaguru PI: Conceptualization, Formal Analysis, Funding Acquisition, Methodology, Project Administration, Resources, Supervision, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

**Competing interests:** No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Introduction

Regulation of mRNA stability and translation plays a key role in cellular processes. RNA binding proteins orchestrate such regulatory processes. Translation repressors are an important class of RNA binding proteins that regulated mRNA fate in the cytoplasm. RGG-domain containing proteins have recently emerged as an exciting class of RNA-binding proteins. A subset of RGG-domain proteins has recently been reported to repress translation by binding eIF4G1 (Rajyaguru et al., 2012).

Sbp1 was identified as a single stranded nucleic acid binding protein (Jong & Campbell, 1986). It can act as a decapping activator and translation repressor (Segal et al., 2006). Consistent with its role in translation and mRNA decay it can bind mRNA and localizes to RNA granules such as P-bodies and stress granules (Mitchell et al., 2013). Sbp1 is a modular protein with two RNA Recognition Motifs (RRMs) sandwiching a central RGG-domain (Rajyaguru et al., 2012). The RGG-domain is important for the translation repression activity of Sbp1. Interestingly the RGG-domain is interjected with aromatic residues (specifically phenylalanine and ‘FRG’ repeats) in Sbp1, the relevance of which is unclear.

RGG-domain of Sbp1 targets eIF4G1. During translation initiation, eIF4G plays an important role as a scaffolding initiation factor that recruits other initiation factors such as eIF4E, eIF4A and Pab1 (Merrick, 2015) to orchestrate formation of the cap-binding complex. Identification of RGG-domain proteins that bind eIF4G to repress translation indicated that the role of this protein in translation initiation could be mRNP-specific depending on the proteins recruited by it (Rajyaguru & Parker, 2012). The impact on global translation of several RGG-domain proteins targeting eIF4G remains unclear. It is possible that one RGG-domain protein could affect the repression activity of another through co-operativity or competition.

Interestingly, Sbp1 has been reported to be phosphorylated in vivo and arginine methylated in vitro (Albuquerque et al., 2008; Frankel & Clarke, 1999; Swaney et al., 2013) the significance of which is not reported. Both phosphorylation and arginine methylation are important posttranslational modifications that regulate protein functions by acting as an on/off switch and/or a fine tuner.

In this work we have addressed three aspects of Sbp1’s role in repression. We have attempted to understand a) the role of aromatic residues in the RGG-domain, b) the role of phosphorylation sites and c) genetic interactions of Sbp1 with decapping activators such as Scd6, Pat1 and Dhh1.

Mutations in aromatic residues of Sbp1 marginally rescue growth defect upon overexpression

Sbp1 contains 8 aromatic residues (7 phenylalanine and 1 tyrosine) in the RGG-domain (125-165). Out of these, 6 phenylalanine occur as ‘FRG’ repeats interspersed with ‘RGG’ repeats. Alignment of the Sbp1 protein sequence revealed that the ‘FRG’ repeats are fairly conserved in other Saccharomyces species as well as in Candida glabrata (Figure 1). Abundance of aromatic residues in RGG-domain is also observed with another RGG-domain protein Scd6. Aromatic amino acids have been fairly well characterized in RNA binding proteins and reported to contribute to RNA-binding through base stacking interactions (Moras & Poterszman, 1995; Rahman et al., 2015). Specifically aromatic residues surrounded by charged residues (Figure 2A) have been implicated in RNA binding for example in the case of the RNPI and RNPS sequence motifs present in RNA Recognition Motifs (RRMs) (Maris et al., 2005). This led us to hypothesize that the conserved aromatic residues in the RGG-motif of Sbp1 could contribute to the repression activity of Sbp1 presumably through binding RNA. We decided to test the importance of these residues using a simple but elegant growth assay. Overexpression of translation repressors in general leads to a growth defect (Coller & Parker, 2005; Hilliker et al., 2011; Nissan et al., 2010) due to global translation repression and mutants defective in translation repression rescue the growth defect. Overexpression of Sbp1 also leads to growth defects. The RGG-domain deletion mutant that is defective in repressing translation is also defective in causing the growth defect (Unpublished study, Bhatter, Roy, Shah, Shastri, Iyyappan, Kankaria and Rajyaguru, communicated). We mutated phenylalanine and tyrosine residues (Figure 2B) to alanine. We observe that mutating up to 5 phenylalanine and 1 tyrosine residue to alanine marginally affect the ability of Sbp1 to cause a growth defect upon overexpression. Based on the growth assay we conclude that mutated aromatic residues in RGG-domain perhaps do not play a very important role in Sbp1 repression activity (Figure 2C).

Phospho-mimetic mutants of Sbp1 do not alter growth defect phenotype upon overexpression

Sbp1 gets phosphorylated at T91, T119 and T230 (Figure 3A) (Albuquerque et al., 2008; Chi et al., 2007; Holt et al., 2009; Swaney et al., 2013). Phosphorylation is a common posttranslational modification that regulates protein function by altering protein-protein and/or protein-nucleic acid interaction. To test if phosphorylation of reported threonines and serine could alter Sbp1 repression activity we created four different phospho-mimetic mutants (T91E, T119E, T242E and S244E). We hypothesized that if phosphorylation were required to activate the repression activity of Sbp1 then phosphor-mimetic mutants would lead to a stronger growth defect phenotype. We observed that none of the mutations affected the ability of Sbp1 to cause growth defects upon overexpression (Figure 3B). This indicated that constitutive phosphorylation did not affect the repression activity of Sbp1 based on growth assay.

Scd6 does not affect the ability of Sbp1 to repress translation

Scd6, like Sbp1 is an RGG-motif containing translation repressor protein that binds eIF4G1 to repress translation (Roy & Rajyaguru, 2018). Recent studies indicate that eIF4G, considered to be a bona fide translation initiation factor, can also aid in mRNA translation repression by acting as a scaffold to recruit negative regulators of translation such as the RGG-motif proteins (Rajyaguru et al., 2012). The significance of multiple RGG-motif containing translation repressor proteins binding eIF4G is unclear. Such interactions could lead to mRNA subset specific repression or stronger repression (Rajyaguru & Parker, 2012). To address this, we decided to test the dependency of Sbp1 on Scd6 in repressing translation using a growth assay. We compared the
Figure 1. Sequence alignment of Sbp1 motif between various groups of fungi shows conservation of aromatic residues and phosphorylation sites.

ability of Sbp1 to repress translation in the Δscd6 strain. We observed that absence of Scd6 did not alter the growth defect phenotype observed upon Sbp1 overexpression (Figure 4A). Based on these observations we conclude that Scd6 does alter the ability of Sbp1 to repress translation as observed in the growth assay.

Sbp1 does not affect the ability of Dhh1 and Pat1 to repress translation

The reported role of Sbp1 as a decapping activator (Segal et al., 2006) prompted us to test if Sbp1 could modulate the repression activity of other decapping activators and translation repressors such as Dhh1 and Pat1. Ded1 is an interesting DEAD-box RNA helicase, which can a) promote the translation initiation process (Chuang et al., 1997) (b) repress initiation (Hilliker et al., 2011) and c) activate decapping (Coller & Parker, 2005). Pat1 on the other hand can act as a translation repressor and stimulator of decapping activity (Nissan et al., 2010; Pilkington & Parker, 2008).

We overexpressed Dhh1 and Pat1 in wild type and Δsdp1 background. Absence of Sbp1 did not affect the growth defect observed upon Dhh1 and Pat1 overexpression (Figure 4B). This result indicates a lack of genetic interaction between Sbp1
Figure 2. A. Protein sequence of Sbp1 showing presence of seven phenylalanine residues in the RGG motif B. Phenylalanine residues in the RGG motif were mutated sequentially to alanine. Mut.3 has first 3 Phe converted to Ala, Mut.4 has first 5 Phe converted to Ala and Mut.5 has all the 7 converted to Ala. C. Sbp1 upon over expression causes growth defect. However, upon mutation of Phe residues to Ala in the RGG motif, there is no effect on the growth defect phenotype.

Figure 3. A. Sequence of Sbp1 showing specific threonine and serine residues that were mutated to glutamic acid. B. Growth assay images showing phosphomimetic mutations in Sbp1 has almost no affect on its growth defect phenotype.
Aromatic residues have been implicated in RNA-binding through base stacking interactions. Sbp1 binds a subset of mRNA in yeast (Mitchell et al., 2013). Sbp1 has two RRM domains, which are likely to be involved in RNA binding. RGG-domains have also been reported to bind RNA, specifically G-rich structures such as G-quadruplex (Fay et al., 2017). Whether Sbp1 binds RNA with G-quadruplex structure is not known however we decided to test the role of RGG-domain phenylalanine in Sbp1 repression activity through growth assays. Sbp1 mutant with 6 aromatic residues converted to alanine upon overexpression leads to a growth defect comparable to wild type Sbp1 (Figure 2) indicating that the mutated aromatic residues do not contribute to the repression activity.

and Dhh1/Pat1 based on the overexpression mediated growth defect assay.

**Discussion**

In this work, based on growth assay results we provide evidence that a) Aromatic (phenylalanine and tyrosine) residues interspersed in the RGG-domain of Sbp1 marginally affect its ability to repress translation (Figure 2) b) Phospho-mimetic mutations do not alter the ability of Sbp1 to repress translation (Figure 3) c) Another RGG-domain protein Scd6 does not affect the ability of Sbp1 to repress translation (Figure 4A) d) Sbp1 does not affect the ability of Dhh1 and Pat1 to repress translation (Figure 4B).
The phosphorylation sites in Sbp1 are not in the RGG-domain, which is important for Sbp1 repression activity. The significance of Sbp1 phosphorylation is not known. We tested the contribution of phosphorylation sites in Sbp1 and observed that three single, and a one double phospho-mimetic mutant did not change the growth defect phenotype caused by overexpression of Sbp1 (Figure 3). It is possible that mutation of all sites simultaneously, and/or creating phospho-dead mutants could provide further insight into the role of phosphorylation in Sbp1 function.

Even though both Scd6 and Sbp1 act through binding eIF4G, it seems that Scd6 does not affect the ability of Sbp1 to repress translation, as deletion of Scd6 did not alter growth defect upon Sbp1 overexpression (Figure 4A). This result points to the idea that despite targeting the same initiation factor both Scd6 and Sbp1 might have non-overlapping mRNA targets. Scd6 contains Lsm and FDF domains as RNA-binding domains, whereas RRM are the RNA-binding domains of Sbp1. Comparing mRNA targets of Sbp1 and Scd6 would be an important future direction.

We also tested if Sbp1 could affect the role of other translation repressors and decapping activators. It was recently demonstrated that Sbp1, Dhh1 and Pat1 bind to common mRNA subsets suggesting a cumulative role of these factors in affecting translation and/or stability of target mRNAs (Mitchell et al., 2013). We observe that absence of Sbp1 did not affect overexpression growth defect phenotype of Dhh1 and Pat1 (Figure 4B). It must be noted that Pat1 overexpression has a very weak growth defect phenotype.

Overall our growth-assay based study provides a systematic and hypothesis-driven analysis of several residues in Sbp1 and decapping factors in context of Sbp1 repression activity.

Methods

Yeast strains and plasmids
All plasmids used in this study are listed in Supplementary Table 1. Yeast strains used in this study are BY4741 (wild type), Δsbp1 (YSC1053, Dhharmacon) and Δscd6. Strains were grown on synthetic medium (SC) supplemented without uracil and 2% glucose (51758, Sisco Research Laboratories) or galactose (G0750, Sigma Aldrich). All strains were grown at 30°C.

Site-directed mutagenesis
For creating point mutations in construct expressing galactose-inducible Sbp1, primers were designed using Quickchange primer design tool from Agilent. The oligos were procured from Bioserve Biotechnologies. Phusion taq polymerase (FNZ520S, Thermo Fisher) was used for PCR. 4 cycles of PCR was done with forward and reverse primers (sequences are listed in Supplementary Table 2) (Bioserve Biotechnologies) in different vial along with PCR reaction mixture using thermal cycler (6331000017, Eppendorf). The conditions of PCR were as follows: initial denaturation at 98°C for 10 minutes followed by cycles of denaturation at 98°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 4 minutes 30 seconds and final extension at 72°C for 10 minutes. This step allows amplification of single strand of the plasmid with mutation in desirable position as present in the primer. Before starting the next 21 PCR cycles (cycling conditions used were same as above), the contents of the two tubes were mixed and put in same vial. After PCR, the reaction mixture was subjected to treatment with Dpn1 (ER1701, Thermo Scientific) restriction enzyme (before adding the enzyme, 1/5th volume of the reaction was taken out to be used as control). Both treated and untreated PCR reaction mixture were then transformed in E.coli XL1BLUE strain (a kind gift from Parker lab) and selected in Luria Bertini (LB) agar plates (‘Molecular Cloning: A Laboratory Protocol, CSHL press) supplemented with 100ug/ul of ampicillin (61314, Sisco Research Laboratories). Components for media were procured from Himedia labs (Tryptone – RM014, Peptone-RM001, Yeast extract-RM027, Agar-RM301 and GRM026, Sodium Chloride- 33205). Colonies obtained by transforming Dpn1-treated PCR-mix were screened for mutation and confirmed by colony PCR (wherever applicable) and Sanger sequencing (Medauxin, Bangalore).

Growth assays
All strains were patched on synthetic medium without uracil and allowed to grow overnight. Next day cells from patches were re-suspended and Optical density of culture was measured at 600 nm wavelength using water as blank with the help of spectrophotometer (613300907, Eppendorf). The following dilutions were prepared 10, 1, 0.1, 0.01 and 0.001 in 96-well plates. In all the growth assays 5 µl of diluted culture was spotted on both SD-URA plates with 2% glucose and 2% galactose. Glucose and galactose plates were imaged at 36–48 h and 60–72 h timeframe respectively using gel documentation system (Image Quanta LAS 4000, GE Healthcare). The settings of camera for imaging were tray position 2, precision setting, 1/30 seconds and brightness at 6.

Data availability
The data underlying this study is available from Open Science Framework. Dataset 1: Characterizing mutations in and genetic interactions of RGG-motif translation repressor Sbp1
https://doi.org/10.17605/OSF.IO/ZVRH7 (Rajyaguru, 2018)

This dataset is available under a CC by 4.0 license

Grant information
This study was supported by the Wellcome Trust through a Wellcome Trust/DBT India Alliance Intermediate fellowship to PIR (IA/I/12/2/500625). NB had financial support from a Council of Scientific and Industrial Research, Senior Research Fellowship. RI was supported by a Science and Engineering Research Board National Post-Doctoral Fellowship (PDF/2015/000143) from Department of Science and Technology.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Supplementary material

Supplementary Table 1: List of strains and plasmids
Click here to access the data.

Supplementary Table 2: List of primers used
Click here to access the data.

References

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[Image 269x671 to 287x689]
[Image 303x671 to 321x689]
[Image 29x649 to 580x650]
[Image 171x483 to 183x495]
[Image 58x479 to 76x497]

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David Tollervey
Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh, UK

The authors report a mutational analysis of the RGG region of the protein Spb1. As an assay they use the dominant negative growth phenotype that was previously reported to be caused by over expression of Spb1. The chief problem in interpreting the results is that the basis of the growth phenotype remains unclear and none of the mutations tested clearly affected growth in the plate tests shown.

Overall, while the results of the study are certainly worth recording as information for the field, this does not constitute a body of work that should be included in PubMed. I regret to write that I could not recommend acceptance for indexing without considerable additional work.

Specific comments:

1. It might be expected that the aromatic residues within the RGG domain contribute to RNA interactions. However, the effects are not very marked at the level of growth. The enhanced growth for Mut1 and Mut2 relative to Spb1 is not readily visible in Figure 2C. Given that this alteration in growth rate is the only positive result reported in the MS, this conclusion should be more robustly supported.

   This should include growth curves with error bars indicating the reproducibility of the effects. This would also reveal whether the alteration affects maximal growth rate or some other feature of the growth curve.

   For plate tests it would also be better to inoculate with cultures grown to defined OD, rather than patches of cells grown overnight, which might be different between strains.

2. The authors test phosphomimetic mutants and conclude that these do not affect growth. Is there reason to believe that these sites are ever phosphorylated?

   Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
No source data required

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Gene expression, RNA processing, RNA-protein interactions, ribosome synthesis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 17 Jan 2020

Purusharth Rajyaguru, Indian Institute of Science, Bangalore, India

Author's response
We thank the reviewer for the comments on this manuscript.

Specific comments:
1. It might be expected that the aromatic residues within the RGG domain contribute to RNA interactions. However, the effects are not very marked at the level of growth. The enhanced growth for Mut1 and Mut2 relative to Spb1 is not readily visible in Figure 2C. Given that this alteration in growth rate is the only positive result reported in the MS, this conclusion should be more robustly supported. This should include growth curves with error bars indicating the reproducibility of the effects. This would also reveal whether the alteration affects maximal growth rate or some other feature of the growth curve. For plate tests it would also be better to inoculate with cultures grown to defined OD, rather than patches of cells grown overnight, which might be different between strains.

Author's response
We agree with the reviewer and have changed the conclusion after repeating growth assay for Mut 1 and 2. The growth alteration for Mut1 and Mut2 are not very notable.
To bolster the impact of the work we have now incorporated two results (Figure 3 & 4) that demonstrate the positive role of RRM domains in Sbp1 function, which has not been reported earlier. We provide evidence that the RRM domains are required for Sbp1 overexpression mediated growth defect and for localization of Sbp1 to granules. We think these results significantly elevate the importance of the manuscript.

2. The authors test phosphomimetic mutants and conclude that these do not affect growth. Is there reason to believe that these sites are ever phosphorylated?

**Author's response**

We have incorporated the reference that reported Sbp1 phosphorylation at the specific residue that we mutated in the manuscript.

**Competing Interests:** None

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**Reviewer Report 23 August 2018**

https://doi.org/10.21956/wellcomeopenres.16019.r33744

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Tracy Nissan

University of Sussex, Brighton, UK

This manuscript addresses the ability of the translational repressors Sbp1, Dhh1 and Pat1 to inhibit growth and therefore likely repress translation. The contribution of this work is to demonstrate that mutated residues in Sbp1's RGG motif in contrast to other RGG proteins are apparently not important for its function. In addition, the manuscript addresses the importance of Scd6 for growth inhibition of Sbp1 and whether the absence of Sbp1 would affect the growth inhibition caused by overexpression of Dhh1 or Pat1. This work provides a further step for understanding the residues important for Sbp1 translational repression as assessed by growth inhibition as well as providing more insight into the genetic relations between translational repressors. Suggestions for addressing my reservations are indicated below:

1. Since the ability to repress translation was not directly tested, the authors should alter the statement at the end of the Conclusion on p1: “Interestingly Scd6 does not affect ability of Sbp1 to repress translation, which in turn does not affect Dhh1 and Pat1.” to indicate that it likely does not affect the ability to repress translation or similar statement.

2. It would be helpful to indicate under what conditions is Sbp1 phosphorylated in previous studies (p3) to assist in interpretation of the results of the phosphomimetic experiment (Fig 3B).
3. I cannot see the difference between the growth inhibition of Sbp1 with the phosphomimetic mutants in Figure 3B. It would be helpful to indicate where the growth was marginally less inhibited. In addition, a statement of how many times the experiment was performed should be indicated.

4. A reference should be added for the plasmid pIR6 in the materials section. In addition the regions cloned into that plasmid from the SBP1 gene should be indicated.

5. There is a mistake on p4, where Ded1 is discussed instead of Dhh1.

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**
Not applicable

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Partly

**Competing Interests:** No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 17 Jan 2020

**Purusharth Rajyaguru,** Indian Institute of Science, Bangalore, India

**Author’s response**

The authors thank the reviewer for comments on this manuscript.

We would like to bring to notice of the reviewer that we have now incorporated two results (Figure 3 & 4) that demonstrate the role of RRM domains in Sbp1 function. This has not been reported earlier. We provide evidence that the RRM domains are required for Sbp1 overexpression mediated growth defect and for localization of Sbp1 to granules. We think these results significantly elevate the importance of the manuscript.

The manuscript has been revised and rewritten to interpret the new data and to address
reviewer's concerns.

**Suggestions for addressing my reservations are indicated below:**
1. Since the ability to repress translation was not directly tested, the authors should alter the statement at the end of the Conclusion on p1: “Interestingly Scd6 does not affect ability of Sbp1 to repress translation, which in turn does not affect Dhh1 and Pat1.” to indicate that it likely does not affect the ability to repress translation or similar statement.

**Author’s response**
We have done the changes in the text.

2. It would be helpful to indicate under what conditions is Sbp1 phosphorylated in previous studies (p3) to assist in interpretation of the results of the phosphomimetic experiment (Fig 3B).

**Author’s response**
We have included the information on the text. (line 133).

3. I cannot see the difference between the growth inhibition of Sbp1 with the phosphomimetic mutants in Figure 3B. It would be helpful to indicate where the growth was marginally less inhibited. In addition, a statement of how many times the experiment was performed should be indicated.

**Author’s response**
We have not observed any impact of the phosphomimetic mutants on the Sbp1 overexpression mediated growth defect phenotype and concluded accordingly.

4. A reference should be added for the plasmid pIR6 in the materials section. In addition the regions cloned into that plasmid from the SBP1 gene should be indicated.

**Author's response**
We have incorporated the details in the text (line 278)

5. There is a mistake on p4, where Ded1 is discussed instead of Dhh1.

**Author's response**
We have incorporated the changes.

**Competing Interests:** None