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RESEARCH ARTICLE

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Exploring the role of RRM domains and conserved aromatic residues in RGG motif of eIF4G-binding translation repressor protein Sbp1 [version 3; peer review: 4 approved]

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Abstract

Background: RNA binding proteins play crucial role in determining if a given mRNA will be translated, stored, or degraded. Sbp1 is an RGG-motif containing protein that is implicated in affecting mRNA decapping and translation. Sbp1 represses translation by binding eIF4G1 through its RGG-motif and activates decapping when overexpressed. In this report, we have assessed the genetic interaction of Sbp1 with decapping activators such as Dhh1, Pat1, and Scd6. We have further analyzed the importance of different domains and specific conserved residues of Sbp1 in its ability to cause over-expression mediated growth defect.
Method: Sequence alignment was performed to identify conserved aromatic residues to be mutated. Using site-directed mutagenesis several point mutations and domain deletions were created in Sbp1 expressed under a galactose-inducible promoter. The mutants were tested for their ability to cause growth defect upon over-expression. The ability of Sbp1 to affect over-expression mediated growth defect of other decapping activators was tested using growth assay. Live cell imaging was done to study localization of Sbp1 and its RRM-deletion mutants to RNA granules upon glucose starvation.
Results: Mutation of several aromatic residues in the RGG-motif and that of the phosphorylation sites in the RRM domain of Sbp1 did not affect the growth defect phenotype. Deletion of another eIF4G1-binding RGG-motif protein Scd6 does not affect the ability of Sbp1 to cause growth defect. Moreover, absence of Sbp1 did not affect the growth defect phenotypes observed upon overexpression of decapping activators Dhh1 and Pat1. Strikingly deletion of both the RRM domains (RRM1 and RRM2) and not the RNP motifs within them compromised the growth defect phenotype. Sbp1 mutant lacking both

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RRM1 and RRM2 was highly defective in localizing to RNA granules.

Conclusion: This study identifies an important role of RRM domains independent of the RNP motif in Sbp1 function.

Keywords

mRNA fate decisions, Translation control, RGG-motif, Sbp1, eIF4G, Translation repression

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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 regulates mRNA fate in the cytoplasm. RGG-motif containing proteins have recently emerged as an exciting class of RNA-binding proteins. A subset of RGG-motif proteins has recently been reported to repress translation by binding eIF4G1 (Rajyaguru *et al.*, 2012; Rajyaguru & Parker, 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 in response to glucose deprivation (Mitchell *et al.*, 2013). Sbp1 is a modular protein (Figure 2A) with two RNA Recognition Motifs (RRMs) sandwiching a central RGG-motif (Rajyaguru *et al.*, 2012). The RGG-motif is important for the translation repression activity of Sbp1. Interestingly the RGG-motif is interjected with aromatic residues (specifically phenylalanine and 'FRG' repeats) [Figure 1 and Figure 2A, the relevance of which is unclear.

RGG-motif of Sbp1 targets eIF4G1 to repress translation. 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 several RGG-motif proteins that bind eIF4G to repress translation indicated that the role of these proteins in translation initiation could be mRNP-specific. RNA-binding domains fused to the RGG-motif could orchestrate such specificity. The RRM domains could be performing similar function for Sbp1 however their contribution to Sbp1 repression activity has not been tested.

Interestingly, Sbp1 is phosphorylated and arginine methylated (Albuquerque *et al.*, 2008; Frankel & Clarke, 1999; Swaney *et al.*, 2013). The significance of arginine methylation has recently been reported in promoting translation repression activity of Sbp1 (Bhatter *et al.*, 2019). However the relevance of Sbp1 phosphorylation remains unclear. Interestingly the

RGG-motif of Sbp1 is interspersed with aromatic amino acid residues that are conserved (Figure 1). A similar pattern is observed in the RGG-motif of Scd6 (Roy & Rajyaguru, 2018). The importance of aromatic residues in RGG-motif has not been explored.

In this work, we have attempted to understand the following four aspects of Sbp1 function, a) the role of aromatic residues in the RGG-motif, b) the role of phosphorylation site, c) genetic interactions of SBP1 with decapping activators such as SCD6, PAT1 and DHH1, and d) the contribution of the two RRM domains towards Sbp1 function.

Results

Mutation in aromatic residues of RGG motif does not affect growth defect upon Sbp1 over expression

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-motif is also observed with another RGG-motif 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 RNP1 and RNP2 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 overexpression growth assay. Sbp1 overexpression leads to growth defect phenotype and mutants of Sbp1 defective in translation repression activity indicate compromised growth defect phenotype (Bhatter *et al.*, 2019). We mutated phenylalanine and tyrosine residues to alanine. We observe that mutating up to 5 phenylalanine and 1 tyrosine residue (Mut2) to alanine did not affect the ability of Sbp1 to cause a growth defect upon overexpression (Figure 2B). Based on the growth assay we conclude that mutated aromatic residues in RGG-motif perhaps do not play a very important role in Sbp1 over expression mediated growth defect.

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

Sbp1 gets phosphorylated at T91, T119, T242 and S244 (Figure 2A) (Albuquerque *et al.*, 2008; Holt *et al.*, 2009; Swaney *et al.*, 2013). Phosphorylation is a common post-translational modification that regulates protein function by altering protein-protein and/or protein-nucleic acid interaction. To test if phosphorylation of reported threonine and serine could alter Sbp1 function we created four different phospho-mimetic mutants (T91E, T119E, T242E and S244E). Amongst the known phosphorylation residues, T91 and T119 has been reported to get phosphorylated upon MMS treatment which causes DNA damage (Albuquerque *et al.*, 2008). We hypothesized that if
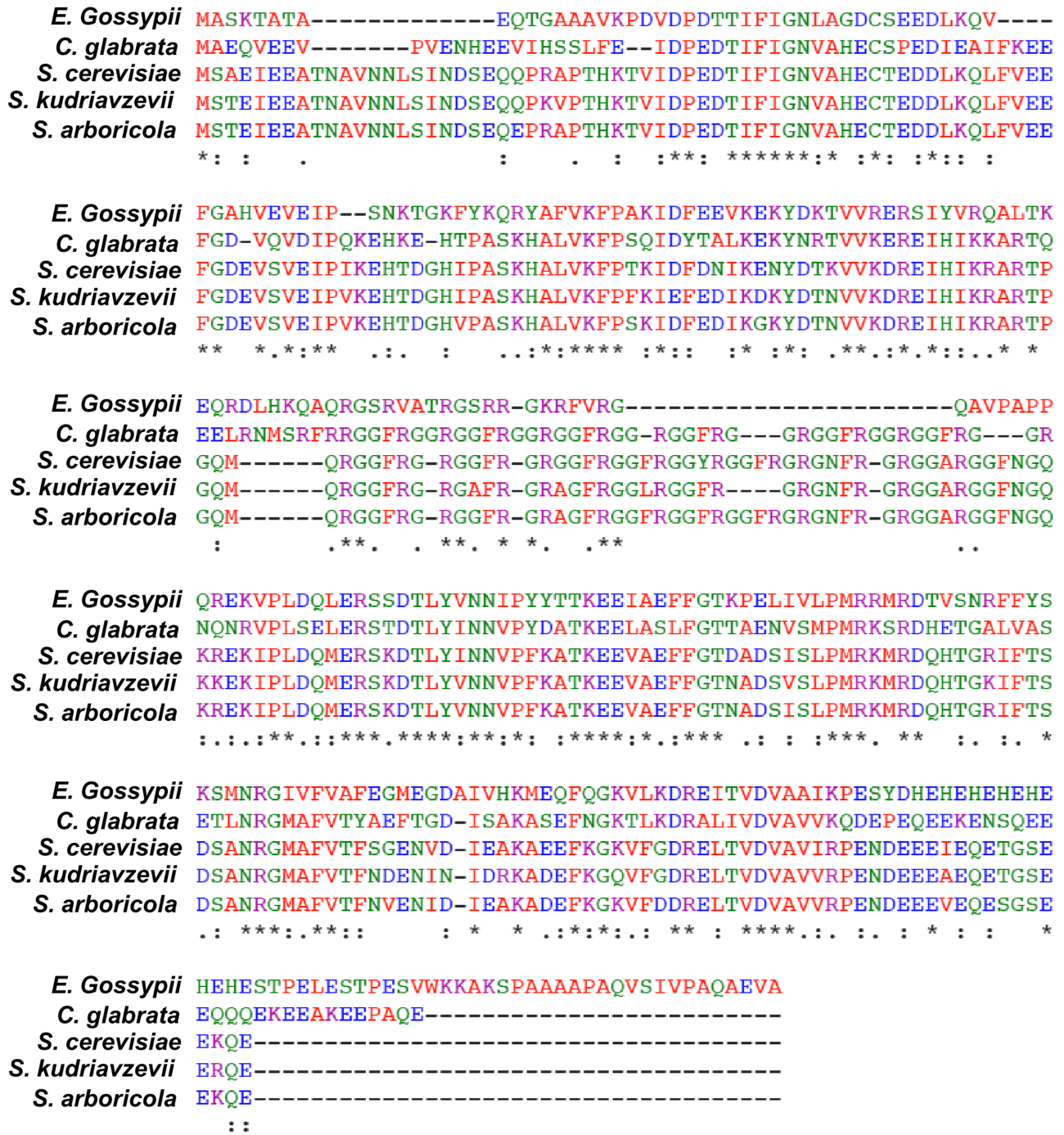
```
E. Gossypii     MASKTATA--------------EQTGAAAVKPDVDPDTTIFIGNLAGDCSEEDLKQV----
C. glabrata     MAEQVEEV-------PVENHEEVIHSSLFE--IDPEDTIFIGNVAHECSPEDIEAIFKEE
S. cerevisiae   MSAEIEEATNAVNNLSINDSEQQPRAPTHKTVIDPEDTIFIGNVAHECTEDDLKQLFVEE
S. kudriavzevii MSTEIEEATNAVNNLSINDSEQQPKVPTHKTVIDPEDTIFIGNVAHECTEDDLKQLFVEE
S. arboricola   MSTEIEEATNAVNNLSINDSEQEPRAPTHKTVIDPEDTIFIGNVAHECTEDDLKQLFVEE
                *: :     .           :       .   :   :**: ******:* :*: :*:: :

E. Gossypii     FGAHVEVEIP--SNKTGKFYKQRYAFVKFPAKIDFEEVKEKYDKTVVRERSIYVRQALTK
C. glabrata     FGD-VQVDIPQKEHKE-HTPASKHALVKFPSQIDYTALKEKYNRTVVKEREIHIKKARTQ
S. cerevisiae   FGDEVSVEIPIKEHTDGHIPASKHALVKFPTKIDFDNIKENYDTKVVKDREIHIKRARTP
S. kudriavzevii FGDEVSVEIPVKEHTDGHIPASKHALVKFPFKIEFEDIKDKYDTNVVKDREIHIKRARTP
S. arboricola   FGDEVSVEIPVKEHTDGHVPASKHALVKFPSKIDFEDIKGKYDTNVVKDREIHIKRARTP
                **   *.*:**   .:.  :    ..:*:**** :*::  :* :*: .**.:*.*::..* *

E. Gossypii     EQRDLHKQAQRGSRVATRGSRR-GKRFVRG-----------------------QAVPAPP
C. glabrata     EELRNMSRFRRGGFRGGRGGFRGGRGGFRGG-RGGFRG---GRGGFRGGRGGFRG---GR
S. cerevisiae   GQM------QRGGFRG-RGGFR-GRGGFRGGFRGGYRGGFRGRGNFR-GRGGARGGFNGQ
S. kudriavzevii GQM------QRGGFRG-RGAFR-GRAGFRGGLRGGFR----GRGNFR-GRGGARGGFNGQ
S. arboricola   GQM------QRGGFRG-RGGFR-GRAGFRGGFRGGFRGGFRGRGNFR-GRGGARGGFNGQ
                 :         .**.  . **. * *.  .**                        ..

E. Gossypii     QREKVPLDQLERSSDTLYVNNIPYYTTKEEIAEFFGTKPELIVLPMRRMRDTVSNRFFYS
C. glabrata     NQNRVPLSELERSTDTLYINNVPYDATKEELASLFGTTAENVSMPMRKSRDHETGALVAS
S. cerevisiae   KREKIPLDQMERSKDTLYINNVPFKATKEEVAEFFGTDADSISLPMRKMRDQHTGRIFTS
S. kudriavzevii KKEKIPLDQMERSKDTLYVNNVPFKATKEEVAEFFGTNADSVSLPMRKMRDQHTGKIFTS
S. arboricola   KREKIPLDQMERSKDTLYVNNVPFKATKEEVAEFFGTNADSISLPMRKMRDQHTGRIFTS
                :.:.:**.::***.****:**:*: :****:*.:*** .: : :***. **  :. :. *

E. Gossypii     KSMNRGIVFVAFEGMEGDAIVHKMEQFQGKVLKDREITVDVAAIKPESYDHEHEHEHEHE
C. glabrata     ETLNRGMAFVTYAEFTGD-ISAKASEFNGKTLKDRALIVDVAVKQDEPEQEEKENSQEE
S. cerevisiae   DSANRGMAFVTFSGENVD-IEAKAEEFKGKVFGDRELTVDVAVIRPENDEEEIEQETGSE
S. kudriavzevii DSANRGMAFVTFNDENIN-IDRKADEFKGQVFGDRELTVDVAVVRPENDEEEAEQETGSE
S. arboricola   DSANRGMAFVTFNVENID-IEAKADEFKGKVFDDRELTVDVAVVRPENDEEEVEQESGSE
                .: ***:.**::      : *  * .:*:*:.: ** : ****.:. :. : * : :    *

E. Gossypii     HEHESTPELESTPESVWKKAKSPAAAAPAQVSIVPAQAEVA
C. glabrata     EQQQEKEEAKEEPAQE-------------------------
S. cerevisiae   EKQE-------------------------------------
S. kudriavzevii ERQE-------------------------------------
S. arboricola   EKQE-------------------------------------
                ::
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Figure 1. Alignment of Sbp1 amino acid sequence from different genera of family Saccharomycetaceae using T-COFFEE multiple sequence alignment tool. Multiple sequence alignment of Sbp1 reveals conservation of aromatic residue in the RGG motif and RNP sequence in the RRM domain. Blue indicates negatively charged amino acids, purple indicate positively charged amino acids, red indicate hydrophobic amino acids and green indicate polar uncharged amino acid. Asterisks (*) indicate identical amino acid, colon (:) indicate similar amino acid and dots/periods (.) indicate weakly similar amino acid.

phosphorylation were required to activate the repression activity of Sbp1 then phospho-mimetic mutants could 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 2C). This indicated that phosphorylation of the specific residues that were tested did not affect the repression activity of Sbp1 based on the growth assay.
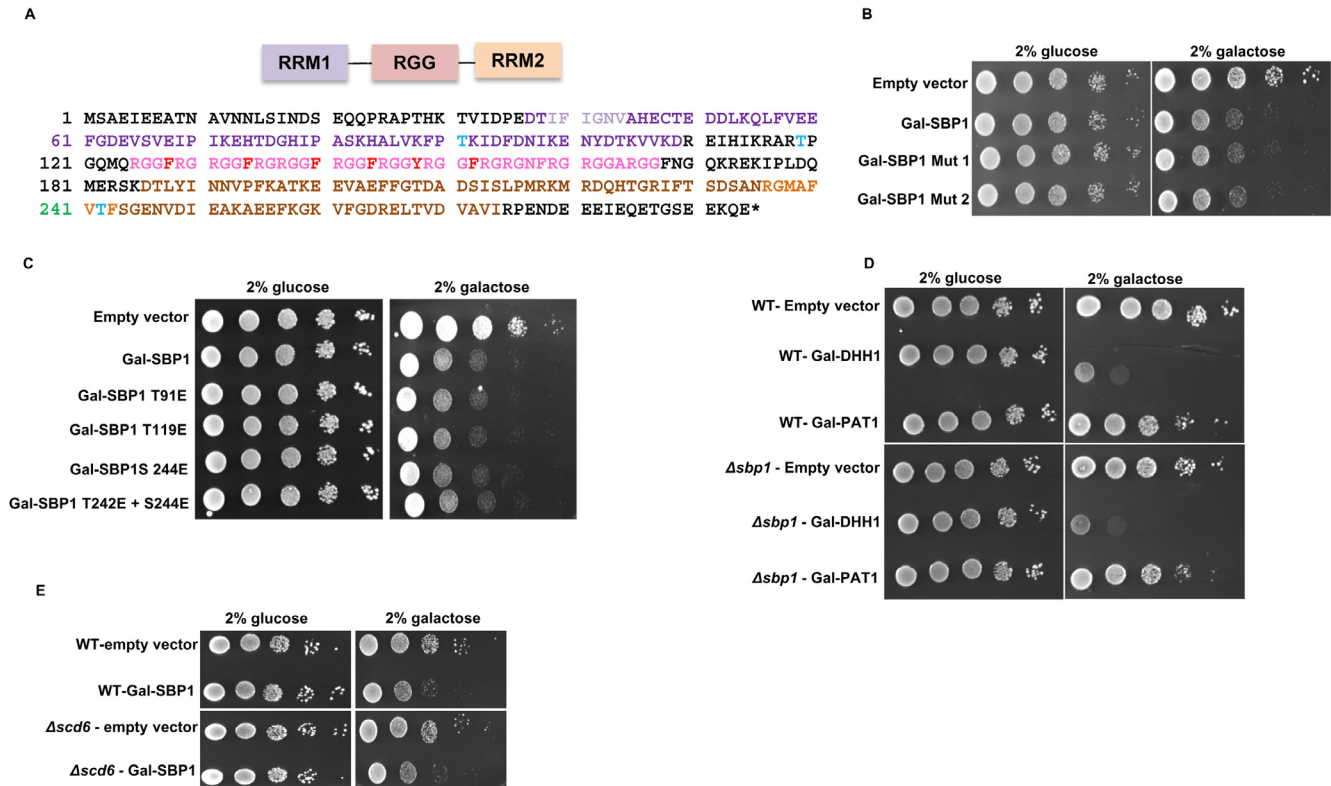
Figure 2. Effect of aromatic to alanine and phosphomimetic mutations in Sbp1 overexpression mediated growth defect and genetic interaction between Sbp1 and decapping activators such as Scd6, Dhh1 and Pat1. A. Amino acid sequence of Sbp1 showing different domains and motifs. Specific threonine and serine residues that were mutated to glutamic acid are marked blue. RGG motif (125 to 167 amino acids) is highlighted in pink, purple region marks RRM1 sequence (37 to 119 amino acid), light purple denotes RNP2 (39-44 amino acid) sequence, brown region denotes RRM2 sequence (186 to 274 amino acids) and orange denotes RNP1 (236 to 243 amino acid) sequence. Aromatic amino acid in RGG motif are marked red. Mut 1 has F128, F134 and F140 (marked red) mutated to alanine. Mut 2 has F144, Y148 and F152 (red) mutated to alanine along with the mutations present in Mut.1. **B**. Growth assay showing role of aromatic residues in the RGG motif on over expression mediated growth defect of SBP1. **C**. Growth assay images showing effect of constitutive phosphorylation on SBP1 over expression mediated growth defect. **D**. Growth assay showing effect of Sbp1 deletion on growth defect phenotype of translation repressors Pat1 and Dhh1. **E**. Growth assay showing effect of SCD6 deletion on SBP1 over–expression mediated growth defect. Top and bottom panels respectively in **D** and **E** are cropped from the same assay plate.

Scd6 does not affect the ability of Sbp1 to cause growth defect

Scd6, like Sbp1 is an RGG-motif containing translation repressor protein that binds eIF4G1 to repress translation (Roy & Rajyaguru, 2018). The significance of multiple RGG-motif containing translation repressor proteins binding eIF4G is unclear. It is possible that one RGG-motif containing repressor could affect the activity of another repressor. To address this, we decided to test the dependency of Sbp1 on Scd6 in causing growth defect upon over expression. We observed that absence of Scd6 did not alter the growth defect phenotype observed upon Sbp1 overexpression (Figure 2E). Based on these observations we conclude that Scd6 does not alter the ability of Sbp1 to cause growth defect. The reported role of Sbp1as 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 (Coller & Parker, 2005; Pilkington & Parker, 2008).

We overexpressed DHH1 and PAT1 in wild type and *Δsbp1* background. Absence of SBP1 did not affect the growth defect observed upon DHH1 and PAT1 overexpression (Figure 2D). This result indicates that Sbp1 does not alter overexpression growth defect of Dhh1 and Pat1.

Deletion of RRM domains of Sbp1 compromises overexpression mediated growth defect

RRM domains are highly conserved domains involved in binding both RNA and protein (Maris *et al.*, 2005). Two RRM domains that contain conserved RNP-motif sequence flank Sbp1 RGG motif. The RNP motifs contribute to the RNA binding activity in RRM domains. To test the role of RNP motifs we created deletions of RNP1, RNP2 and RNP1+RNP2. None of these mutations strongly affected the ability of Sbp1 to cause growth defect upon overexpression (Figure 3A). We next created deletion of RRM1, RRM2 and RRM1+2. Deletion of both the RRM domains led to partially compromised

growth defect phenotype (Figure 3B) whereas the deletion of individual RRM1 or RRM2 domains compromised the growth defect phenotype to a lesser extent than RRM1+2 (Figure 3B). Western blot analysis indicates that RRM1+2 mutant is expressed in manner comparable to wild type (Figure 3C). Based on these results we conclude that the RRM domains are important for causing growth phenotype. Since RNP1+2 deletion did not affect the growth defect phenotype, we interpret this result to suggest that the growth defect is independent of the RNP motifs.

Deletion of both the RRM domains impair the ability of Sbp1 to localize to granules upon glucose starvation

Sbp1 has been reported to localize to RNA granules upon glucose starvation which colocalizes with Edc3mcherry and Pub1mcherry (Mitchell et al., 2013; Segal et al., 2006). The compromised ability of RRM1+2 mutants in causing a growth defect could be due to its defective localization to RNA granules. We tested if Sbp1 RRM1+2 mutant localized to RNA granules in response to glucose starvation by using a construct where Sbp1 is under its own promoter (500 base-pairs upstream of start codon) and tagged to GFP (followed by ADH terminator sequence) at its C-terminus. We observed that Sbp1 localized to RNA granules upon glucose starvation stress as reported earlier but RRM 1+2 deletion mutant was impaired in its ability to localize to RNA granules (Figure 4A & B). Mutants with individual RRM domain deletion did not show a significant defect in localization to RNA granules upon glucose starvation compared to wild-type (Figure 4A & B). Localization of Edc3-mcherry in the cells expressing wild type or mutant Sbp1-GFP to granules was comparable suggesting that the inability of RRM 1+2 mutant to localize to RNA granules was not due to inadequate stress. Defective localization to RNA granules was not due to decreased protein expression since the RRM1+2 deletion mutant was expressed in manner comparable to the wild type protein (Figure 4C and D).

This result indicates that RRM domains are required for the localization of Sbp1 to RNA granules upon glucose starvation stress.
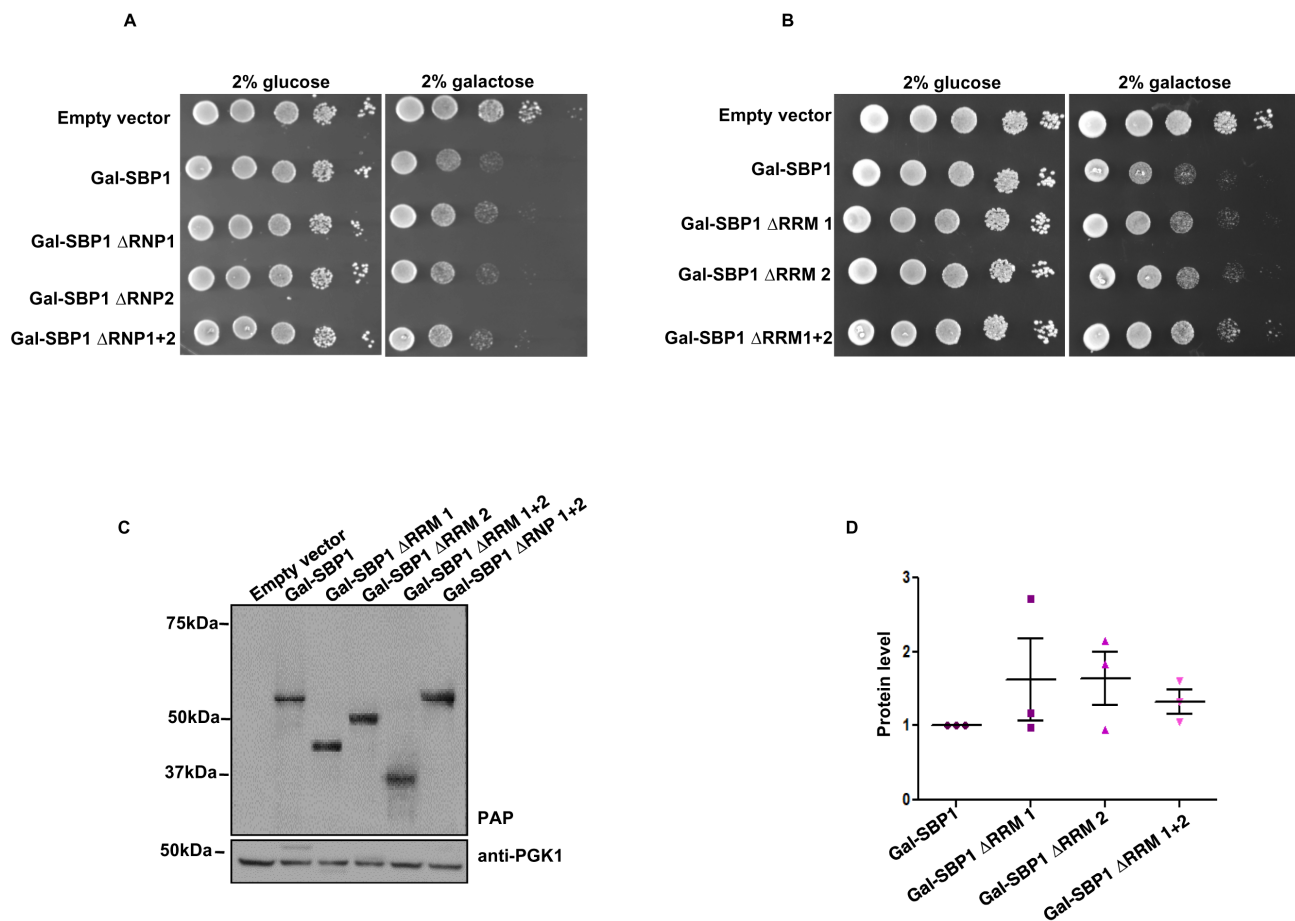

Figure 3. Effect of RRM domain and RNP-motif sequence deletion on SBP1 over expression mediated growth defect. A. Growth assay of Sbp1 RNP1, RNP2 and RNP 1+2 sequence deletion mutants (as indicated in Figure 2A). **B**. Growth assay with ΔRRM1, ΔRRM2 and ΔRRM 1+2 mutants of Sbp1. Images in both panels have been cropped from the same growth assay plate. **C**. Western blot using PAP antibody (Sigma) showing protein level of WT and indicated mutants of Sbp1. PAP will recognize the ZZ tag present in wild-type as well as Sbp1 mutants at the C-terminus. PGK1 served as loading control. Blot was first probed with PAP and then stripped followed by probing with anti-PGK1 antibody (Abcam). **D**. Graph showing quantitation of PAP bands observed in C after normalizing with protein loading control.
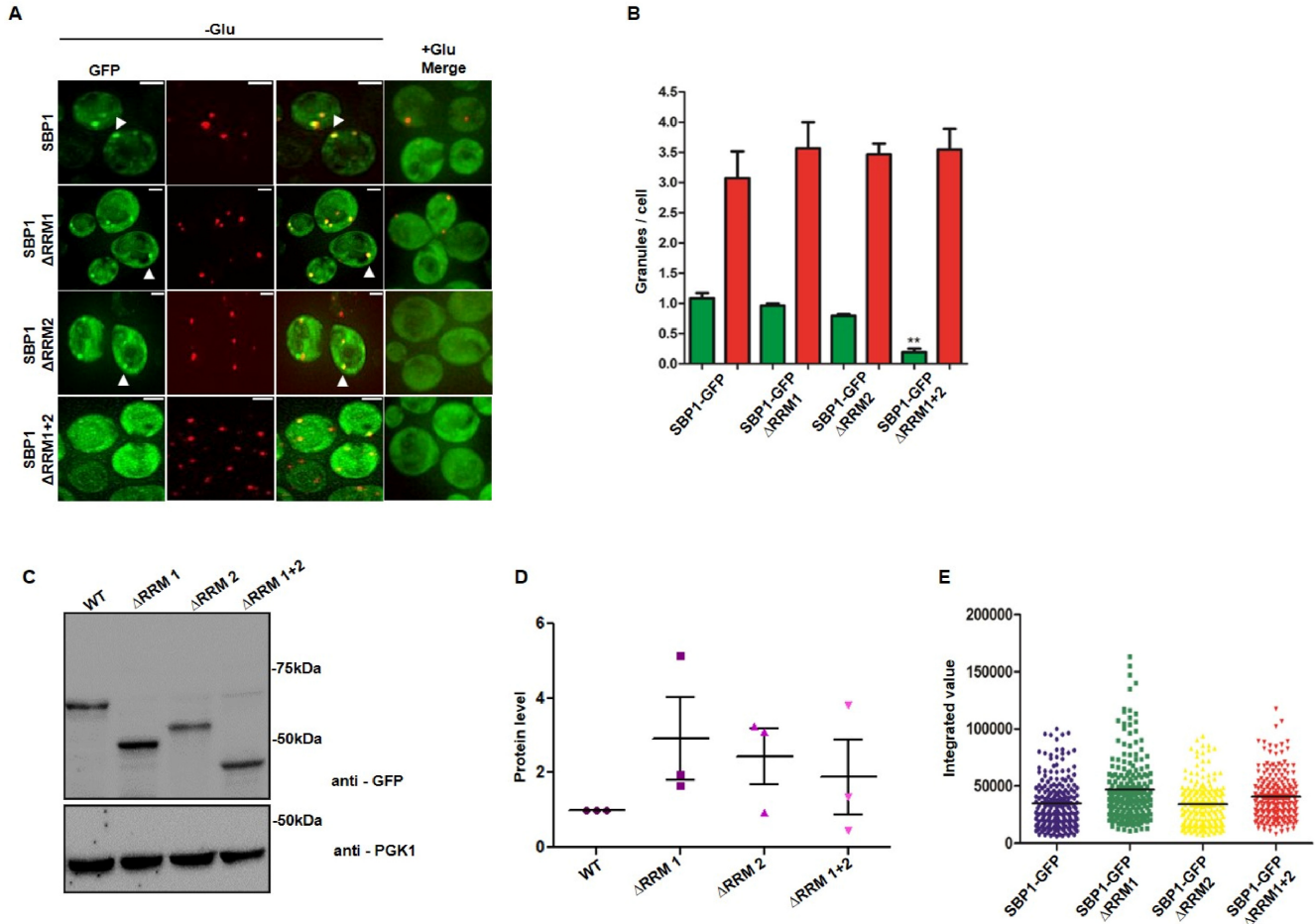
Figure 4. Effect of double deletion of RRM domains of Sbp1 on its ability to localize to RNA granules upon stress. A. Live cell imaging of WT and RRM domain deletion mutants of Sbp1 after 10 min of glucose starvation. Sbp1 was tagged to GFP and Edc3 was tagged to mCherry. Normally grown and glucose deprived cells were pelleted together at 14,100rpm for 12 s. For all experiments, glucose-starved cells were imaged first followed by cells grown with glucose. Scale denotes 2μm. **B**. Graph plotted for Sbp1-GFP and Edc3-mcherry granules per cell for n=3. p value for Sbp1-GFP wt and ΔRRM1+2 mutant is 0.0071. Asterisks denote statistical significance of the data plotted (** = p value> 0.01 and * = p value > 0.05). Red bar denotes Edc3-mCherry granule number and green bar denotes Sbp1-GFP granule number. **C**. Protein levels of WT Sbp1-GFP and its mutants treated with sodium azide. Blot was first probed with anti-GFP antibody followed by stripping and probing the same blot with anti-PGK antibody. **D**. Graph showing quantitation of protein bands obtained using anti-GFP antibody after normalizing with protein loading control. **E**. Intensity of GFP in cells expressing GFP tagged Sbp1 wt and mutants, quantitated from images of glucose starved cells (n=150).

Discussion

In this work, we provide evidence that a) aromatic (phenylalanine and tyrosine) residues interspersed in the RGG-motif and phosphorylation sites in the RRM domain of Sbp1 do not contribute to overexpression growth defect (Figure 2A-C) b) RGG-motif protein Scd6 does not affect the ability of Sbp1 to reduce growth upon over expression (Figure 2E) c) Sbp1 does not affect the ability of Dhh1 and Pat1 to cause growth defect (Figure 2D) d) Deletion of RRM domains compromises overexpression mediated growth defect (Figure 3A & B) e) RRM domains of Sbp1 are required for localization to RNA granules upon glucose starvation (Figure 4A & B).

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-motif 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 phenylalanine and tyrosine residues in the RGG-motif Sbp1 towards causing growth defect when over-expressed. Sbp1 mutant with 6 aromatic residues converted to alanine upon overexpression leads to a growth defect comparable to wild type Sbp1 (Figure 2B) indicating that the phenylalanine residues do not contribute to the growth defect.

The phosphorylation sites in Sbp1 are not in the RGG-motif, 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 one double phospho-mimetic mutant did not change the growth defect phenotype caused by over expression of Sbp1 (Figure 2C). It is possible that phospho-mimetic mutation of all sites simultaneously or creating phospho-dead mutants could provide further insight into the role of phosphorylation in Sbp1 function.

Even though both Scd6 and Sbp1 bind eIF4G to repress translation, Scd6 does not affect the ability of Sbp1 to repress translation, as deletion of Scd6 did not alter growth defect upon Sbp1 overexpression (Figure 2E). 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 RRMs are the RNA-binding domains of Sbp1. Comparing mRNA targets of Sbp1 and Scd6 would be an important future direction to understand the details of their repression activity.

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 2D). It must be noted that Pat1 overexpression has a very weak growth defect phenotype.

RNA recognition motif (RRM) is well known RNA binding domain present in proteins that are involved in RNA metabolism. This domain is often fused to RGG motif in proteins such as FUS, Nucleolin and TDP43 in mammals. Two RRM domains flank Sbp1 RGG motif and deletion of both the RRM domains of Sbp1 led to partial rescue of growth defect (Figure 3B) indicating that RRM domains along with the previously reported RGG-motif are important for growth defect phenotype and likely Sbp1 repression activity. The microscopy data provides further clear indication about the role of RRM domain in Sbp1 repression activity. Deletion of both the RRM domains renders the localization of Sbp1 to RNA granules defective upon glucose starvation (Figure 4A & B). Inability of the RRM deletion mutant to localize to RNA granules could be due to defective interaction with either mRNAs or a granule-resident protein that guides the localization of Sbp1 to granules or both. Surprisingly the deletion of consensus RNP motif sequences (RNP 1+2) did not affect the growth defect phenotype (Figure 3A). This indicates that contribution of RRM domains towards growth defect phenotype of Sbp1 is independent of the RNP motif. Identifying the mRNAs and/or proteins bound by the Sbp1 RRM domains will be an important future direction.

Overall, our growth-assay and live-cell imaging-based study provide insights into the role of RRM domains of Sbp1 in causing over-expression mediated growth defect and localize to cytoplasmic P bodies in response to nutrient starvation. It identifies a positive role of RRM domains in Sbp1 repression activity paving the way for addressing the mechanistic basis of the role of RRM domains in Sbp1 function.

Methods

Yeast strains and plasmids

All strains, plasmids and oligos used in this study are listed in Supplementary Table 1, 2 and 3 respectively. Please see 'Data availability' section below for more details regarding these tables. Yeast strains used in this study are BY4741 (wild type), *Δsbp1*(YSC1053, Dharmacon) 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. pPIR6 is BG1805 empty vector, a kind gift from Roy Parker lab (Nissan *et al.*, 2010).

Site-directed mutagenesis

For creating point mutations in construct expressing galactose-inducible Sbp1, primers were designed using Quick change 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 (Figure S3) (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 (L.B) 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 (6133000907, 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.

Live cell imaging

For glucose starvation stress with SBP1-GFP construct, yeast cultures were grown to OD_{600} of 0.5–0.6 in SD-Leu-ura + 2% glucose media at 30°C. Glucose starvation was done as described previously (Bhatter *et al.*, 2019). Briefly, after reaching desired O.D, cells were split into two equal volume followed by pelleting at 4200rpm for 10s at room temperature in eppendorf centrifuge. This was followed by washing cells with respective media (-glu pellet with SD URA- without glucose and +glu pellet with SD URA- media with glucose media). Final resuspended cells were allowed to grow for 10min in shaker incubator. This was followed by pelleting cells at 14200 rpm for 12s and spotting them on coverslip to observe under microscope at room temperature. All images were acquired using Deltavision Elite microscope (GE Healthcare) system running softWoRx 3.5.1 software (Applied Precision, LLC), using an Olympus 100×, oil-immersion 1.4 NA objective. Exposure time and transmittance for Green Fluorescent Protein (GFP) channel was 0.2 seconds and 32% respectively. Exposure time and transmittance for mCherry channel were 0.3 seconds and 32% respectively. Images were collected as 512×512 pixel files with a CoolSnapHQ camera (Photometrics) using 1×1 binning for yeast. All yeast images were deconvolved using standard softWoRx deconvolution algorithms. ImageJ was used to adjust all images to equal contrast ranges according to the experiment conducted or protein examined. For Sbp1-GFP experiment on an average, minimum of 100 cells was counted per experiment. Data from three independent experiments was used for quantitation and statistical significance was calculated using two-tailed paired *t*-test. Quantitation of intensity for glucose starved GFP cells were done as described previously (Parbin *et al.*, 2020).

Western blotting

To look at the protein level of wild type and mutants of Sbp1 in BG1805 construct, cells were first grown in SD ura-minimal media with glucose till 0.45–0.5 OD600 this was followed by pelleting and growing in SD ura- 2% galactose overnight. Cells were broken open using acid wash glass beads and 20microgram of total protein was loaded in 8% SDS polyacrylamide gel. The gel was transferred onto a nitrocellulose membrane using Bio-Rad wet transfer apparatus. Post transfer, the membrane was stained with Ponceau S to know the total protein present in each lane. The blot was washed and blocked using skimmed milk. PAP (1:5000, Sigma Aldrich cat# P1291) was used to detect over expressed Sbp1 and mutant proteins. Sbp1-GFP and its mutants protein level was looked at the same way as gal inducible Sbp1 except the use of anti-GFP anti body (1: 1000, BioLegend cat# 902602). For loading control, blot was stripped and put in anti - PGK1 antibody (1:1000, Abcam cat# AB113687).

Data availability

Underlying data

OSF: Characterizing mutations in and genetic interactions of RGG-motif translation repressor Sbp1.

https://doi.org/10.17605/OSF.IO/FNDY3 (Rajyaguru *et al.*, 2018)

This project contains the following underlying data:

- Growth assays

- Oligos used in this study

- Sequencing files

Extended data

Figshare: Exploring the role of RRM domains and conserved residues of eIF4G-binding translation repressor protein Sbp1 (supplementary data). https://doi.org/10.6084/m9.figshare.11733786.v1 (Bhatter *et al.*, 2020).

This project contains the following extended data:

- Supplementary Table 1: List of strains.

- Supplementary Table 2: List of plasmids.

- Supplementary Table 3: List of oligonucleotides.

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Open Peer Review

Current Peer Review Status: ✓ ✓ ✓ ✓

Reviewer Report 21 September 2021

https://doi.org/10.21956/wellcomeopenres.18850.r45934

✓ **Jeffrey Wilusz**
Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO, USA

I find the revised version to be improved and largely responsive to the points that I raised in the initial round of critiques. I have no further comments to make.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: mRNA stability; RNA virus host interactions; RNA biology.

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.

Reviewer Report 04 March 2020

https://doi.org/10.21956/wellcomeopenres.17163.r37997

✓ **Sarah F. Mitchell**
Department of Chemistry and Biochemistry, Loyola Marymount University, Los Angeles, CA, USA

This work investigates the growth defect caused by over expression of the translational repressor Sbp1. The roles of the RRM domains, individual amino acids in the RGG domain, and

phosphorylated amino acids are investigated. Though most of the results are negative, deletion of both RRM domains leads to a subtle reduction in the over-expression growth defect and a reduction in the presence of Sbp1 in RNA granules. These findings are minor, but will be of interest to researchers in the field. Some of the findings could be more strongly supported by a clearer presentation of several figures. I have several specific, predominantly minor, suggestions listed below.

1. In Figure 1, it would be helpful to the reader if the location of the RRM and RGG domains could be indicated.

2. In Figure 2A, the numbering in the sequence is not consistent with the numbering of the mutants described in the figure legend. The numbering for F133, F139, F143, Y147 and F151 appears to be shifted by one amino acid such that in the sequence the numbers appear to be higher (F134, F140, etc.). In addition, F151 is not colored as indicated in the legend.

3. In Figure 3B, the reduced growth defect is subtle. As mentioned by other reviewers, a growth curve would strengthen the evidence for this change. Another way to make the difference appear more convincing may be to present images that are more consistent. In this figure, the background for the 2% galactose is much darker than other figures in the manuscript. This makes a direct comparison more difficult.

4. Figure 3C shows a reduced amount of Gal-SBP1deltaRRM2 relative to other constructs. This does not alter the primary conclusion that deletion of both RRMs reduces the growth defect, but may alter the more minor conclusion that there is a lesser reduction in the growth defect for the individual RRM deletions. It should be mentioned in the manuscript.

5. In Figure 4A, the Edc3-mCherry panels do not align well with the GFP panels. This makes the results more difficult to interpret.

6. The authors write that the western blot in Figure 4C shows comparable expression of WT and deltaRRM1+2 proteins. As the control bands (Pgk1) appear different in these two samples, quantification of this blot would make this statement more convincing.

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?
Yes

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?
Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: mRNA-protein interactions, translation, post-transcriptional regulation of gene expression

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.

Author Response 03 Aug 2021

Purusharth Rajyaguru, Indian Institute of Science, Bangalore, India

Sarah F. Mitchell
Department of Chemistry and Biochemistry, Loyola Marymount University, Los Angeles, CA, USA
This work investigates the growth defect caused by over expression of the translational repressor Sbp1. The roles of the RRM domains, individual amino acids in the RGG domain, and phosphorylated amino acids are investigated. Though most of the results are negative, deletion of both RRM domains leads to a subtle reduction in the over-expression growth defect and a reduction in the presence of Sbp1 in RNA granules. These findings are minor, but will be of interest to researchers in the field. Some of the findings could be more strongly supported by a clearer presentation of several figures. I have several specific, predominantly minor, suggestions listed below.

Author's comment: The authors thank the reviewer for all the comments.

1. In Figure 1, it would be helpful to the reader if the location of the RRM and RGG domains could be indicated.

Author's comment: The details of RRM and RGG domain with amino acid number and location has been indicated in figure 2A cartoon. Further explanations are provided in the figure legends.

2. In Figure 2A, the numbering in the sequence is not consistent with the numbering of the mutants described in the figure legend. The numbering for F133, F139, F143, Y147 and F151 appears to be shifted by one amino acid such that in the sequence the numbers appear to be higher (F134, F140, etc.). In addition, F151 is not colored as indicated in the legend.

Author's comment: We have incorporated all the changes in Figure 2. The correct amino acid mutation for Mut1 is F128, F134 and F140 (marked red) which were mutated to alanine. Mut 2 has F144, Y148 and F152 in addition to mutations present in Mut1. All the corrected amino acid number are mentioned in the legend.

3. In Figure 3B, the reduced growth defect is subtle. As mentioned by other reviewers, a growth curve would strengthen the evidence for this change. Another way to make the difference appear more convincing may be to present images that are more consistent. In

this figure, the background for the 2% galactose is much darker than other figures in the manuscript. This makes a direct comparison more difficult.

Author's comment: We have repeated growth assay and replaced the older image with new one in Figure 3B. △RRM1+2 mutant of SBP1 has consistently shown partial rescue form over-expression mediated growth defect in spotting assays.

4. Figure 3C shows a reduced amount of Gal-SBP1deltaRRM2 relative to other constructs. This does not alter the primary conclusion that deletion of both RRMs reduces the growth defect, but may alter the more minor conclusion that there is a lesser reduction in the growth defect for the individual RRM deletions. It should be mentioned in the manuscript.

Author's comment: Reduced protein level of △RRM2 in previous pap blot in Figure 3C, was because of less protein loaded as indicated by anti-PGK1 blot. We repeated the protein level western blot result and found protein level of all the mutants to be comparable with wild-type. The previous Figure 3C is replaced by a new western blot figure and the PAP bands have been quantitated in figure 3C for n=3 experiments and plotted in Figure 3D.

5. In Figure 4A, the Edc3-mCherry panels do not align well with the GFP panels. This makes the results more difficult to interpret. The authors write that the western blot in Figure 4C shows comparable expression of WT and deltaRRM1+2 proteins. As the control bands (Pgk1) appear different in these two samples, quantification of this blot would make this statement more convincing.

Author's comment: We have incorporated the changes suggested by the Reviewer in figure 4A. We have repeated western blot of Sbp1-GFP in wild-type and mutant background and replaced 4C with new image where total protein level in each lane is more comparable. In figure 4D, we have plotted graphical quantitation of bands obtained by anti-GFP antibody in each lane and normalized with anti-PGK1 antibody (protein loading control). RRM deletion mutants of Sbp1 seem to express comparable to wild-type.

Competing Interests: No competing interests were disclosed.

Reviewer Report 03 March 2020

✔ **David Tollervey** (iD)

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

The authors have suitably addressed my initial comments and I am happy to approve the revised

manuscript.

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?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

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

Are the conclusions drawn adequately supported by the results?
Yes

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.

Reviewer Report 02 March 2020

https://doi.org/10.21956/wellcomeopenres.17163.r37998

?   **Jeffrey Wilusz**
Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO, USA

This is the first time that I've had the privilege to review this study involving mutational analyses of the Sbp1 translational regulator on cell growth and granule formation. Overall I feel that the study makes a measurable, albeit somewhat incremental, contribution to our understanding of the role of the RRM domains of this factor in yeast biology. I have several major issues as well as some minor points that should be addressed to make the study as compelling as possible.

Major Points:
1. Fig. 2C: The growth defect observed with the over expression of Sbp1 in this panel is not as dramatic as other panels – and thus the conclusion of no effect of the mutated phosphorylation sites is not as compelling as other studies in the figure. Thus I would recommend that the authors improve the quality of these data to better support their conclusion. Perhaps the original suggestion of including growth curves should be reconsidered.

2. Fig. 3C: The low expression of the delta RRM2 mutant does not allow clear conclusions to be made from this construct relative to the other constructs. This represents a fundamental flaw in the experiment that must be addressed.

3. Fig. 4C: The western blot clearly demonstrates that the RRM delta 1/2 protein is expressed much less than the WT protein in the experiment. This could be affecting the granule localization results and must to be addressed so that the conclusions drawn are fully convincing. As with the previous point, this represents a fundamental flaw in the experiment.

Minor Points:
1. It is important for authors to strive to polish the English language usage in their manuscripts to avoid unconscious bias by the reader that could affect the impact of their study. This is a potential issue with this manuscript in my opinion. There are numerous places in the manuscript, for example, where subject-verb agreement is lost which makes reading difficult. For example, the legend of Fig. 1. should be edited to read as follows (changes are in italics):
"Multiple sequence alignment of Sbp1 reveals conservation of aromatic residue in the RGG motif and RNP sequence in the RRM domain. Blue indicate*s* negatively charged amino acids, purple indicate*s* positively charged amino acids, red indicate*s* hydrophobic amino acids, and green indicate*s* polar uncharged amino acid*s*."

2. There are two other problems with Fig. 1 legend that need to be addressed. First, 'genera' not genuses, is the plural of genus. Second, the legend needs to indicate what the code of asterisks, colons and periods means to allow the reader to fully digest the figure.

3. Fig. 4B: The authors need to directly state what the red and green bars refer to in the legend to allow the graph to be readily interpreted.

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?
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?
Yes

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: mRNA stability; RNA virus host interactions; RNA biology.

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 03 Aug 2021
Purusharth Rajyaguru, Indian Institute of Science, Bangalore, India

Jeffrey Wilusz
Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO, USA
This is the first time that I've had the privilege to review this study involving mutational analyses of the Sbp1 translational regulator on cell growth and granule formation. Overall I feel that the study makes a measurable, albeit somewhat incremental, contribution to our understanding of the role of the RRM domains of this factor in yeast biology. I have several major issues as well as some minor points that should be addressed to make the study as compelling as possible.

Author's comment: We thank the reviewer for all the comments.

Major Points:
1.Fig. 2C: The growth defect observed with the over expression of Sbp1 in this panel is not as dramatic as other panels – and thus the conclusion of no effect of the mutated phosphorylation sites is not as compelling as other studies in the figure. Thus I would recommend that the authors improve the quality of these data to better support their conclusion. Perhaps the original suggestion of including growth curves should be reconsidered.

Author's comment: We have repeated growth assay of phosphomimetic mutants of Gal-SBP1 construct and replaced the older growth assay image with new one in Figure 2C. We agree with the limitation of spotting assay however within these limitations, we observe that the phosphomimetic mutants do not seem to affect the growth defect phenotype. Whether these mutations affect other phenotypes related to Sbp1 remains to be addressed.

2. Fig. 3C: The low expression of the delta RRM2 mutant does not allow clear conclusions to be made from this construct relative to the other constructs. This represents a fundamental flaw in the experiment that must be addressed.

Author's comment: We think reduced level of △RRM2 was because of less total protein as indicated by PGK1. We have looked at protein level of △RRM2 in our previous study as well (Bhatter *et al.*, 2019) and did not find it to be compromised. However, we have repeated measuring levels of △RRM1+2 mutant in this study. Figure 3D shows the quantitation of PAP intensity as observed in Figure 3C after normalizing with Ponceau stained blot. Together, the western blot (Figure 3C) and its graphical quantitation (Figure 3D) suggests that the protein level of the RRM mutants of Sbp1 is not compromised.

Fig. 4C: The western blot clearly demonstrates that the RRM delta 1/2 protein is expressed much less than the WT protein in the experiment. This could be affecting the granule localization results and must to be addressed so that the conclusions drawn are fully convincing. As with the previous point, this represents a fundamental flaw in the experiment.

Author's comment: We have repeated protein level analysis of Sbp1-GFP △RRM1+2 and quantitated the band intensity with wild-type for n=3 experiments (Figure 4D). We do not observe protein level of △RRM1+2 mutant to be compromised as compared to wild-type.

Minor Points:
It is important for authors to strive to polish the English language usage in their manuscripts to avoid unconscious bias by the reader that could affect the impact of their study. This is a potential issue with this manuscript in my opinion. There are numerous places in the manuscript, for example, where subject-verb agreement is lost which makes reading difficult. For example, the legend of Fig. 1. should be edited to read as follows (changes are in italics):
"Multiple sequence alignment of Sbp1 reveals conservation of aromatic residue in the RGG motif and RNP sequence in the RRM domain. Blue indicates negatively charged amino acids, purple indicates positively charged amino acids, red indicates hydrophobic amino acids, and green indicates polar uncharged amino acids."

There are two other problems with Fig. 1 legend that need to be addressed. First, 'genera' not genuses, is the plural of genus. Second, the legend needs to indicate what the code of asterisks, colons and periods means to allow the reader to fully digest the figure.

Author's comment: We have done the changes in the text as suggested by the reviewer. We thank the reviewer for thorough analysis of the text.

Fig. 4B: The authors need to directly state what the red and green bars refer to in the legend to allow the graph to be readily interpreted.

Author's comment: We have incorporated the graph details in the legends.

Competing Interests: No competing interests were disclosed.

Reviewer Report 24 February 2020

https://doi.org/10.21956/wellcomeopenres.17163.r37832

✔ **Tracy Nissan** (iD)

University of Sussex, Brighton, UK

The revised manuscript examines the translational repressor Sbp1. It dissects the domains and motifs region repressors required to inhibit growth upon overexpression. It further examines its effect in different mutant backgrounds. The authors have satisfied my earlier comments. I have a few minor comments that can be addressed by alterations of the texts and figures.

1. Some statements remain stating that the results demonstrate translational repression, when the results are instead consistent with a translation repression effect. For example:
   - In the abstract: "We have further analyzed the importance of different domains and specific conserved residues of Sbp1 in translation repression activity".

   - In the discussion: "This indicates that the site(s) required for Sbp1 interaction with mRNA and/or protein for translation repression function is not in the RNP motifs.
   Without examining translation directly (for example in a cell extract), these are suggested by the results. The text should be re-written to make this clearer.

2. The authors should indicate the FRG and RGG repeats in Figure 1 with boxes or shading. It is difficult to determine the frequency of these repeats in this figure.

3. Pat1 overexpression did not kill the cells in Figure 2D. This is interesting, since it has been reported by Pilkington and Parker, MCB 2008[1] and Sopko *et al*., Genes Dev 2006[2] among others. Since Pat1 overexpression does not cause cell death as previously reported, I am not sure it informative to keep this experiment in the manuscript. If they keep this data, they should indicate that Pat1 overexpression has been previously reported to be moderately lethal.

4. The "growth phenotype" and "overexpression phenotype" are used interchangeably in the manuscript. It would be clearer, if "growth phenotype" was changed to growth defect or similar language.

5. The authors should indicate where the Sbp1 granules are in Figure 4A. It is not clear how many foci are present in the selected cells and where they overlap with Edc3 foci.

6. It would be helpful to add a small additional panel to Figure 2 to show the Sbp1 protein as a rectangle and the RRM and RNP regions indicated by shading and labelling.

7. Supplementary tables are not accessible.

8. The methods section has some text that should have been removed in editing.

References

1. Pilkington GR, Parker R: Pat1 contains distinct functional domains that promote P-body assembly and activation of decapping.*Mol Cell Biol*. 2008; **28** (4): 1298-312 PubMed Abstract | Publisher Full Text

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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?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

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

Partly

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.

Author Response 03 Aug 2021

Purusharth Rajyaguru, Indian Institute of Science, Bangalore, India

Tracy Nissan
University of Sussex, Brighton, UK
The revised manuscript examines the translational repressor Sbp1. It dissects the domains and motifs region repressors required to inhibit growth upon overexpression. It further examines its effect in different mutant backgrounds. The authors have satisfied my earlier comments. I have a few minor comments that can be addressed by alterations of the texts and figures.
1. Some statements remain stating that the results demonstrate translational repression, when the results are instead consistent with a translation repression effect. For example:
- In the abstract: "We have further analyzed the importance of different domains and specific conserved residues of Sbp1 in translation repression activity".
- In the discussion: "This indicates that the site(s) required for Sbp1 interaction with mRNA and/or protein for translation repression function is not in the RNP motifs.

Author's comment: We agree with the reviewers and have incorporated the changes in the manuscript.

2. Without examining translation directly (for example in a cell extract), these are suggested by the results. The text should be re-written to make this clearer. The authors should indicate the FRG and RGG repeats in Figure 1 with boxes or shading. It is difficult to determine the frequency of these repeats in this figure.

Author's comment: We have removed the translation repression activity term from the text. The FRG and RGG repeats are highlighted in figure 2A.

3. Pat1 overexpression did not kill the cells in Figure 2D. This is interesting, since it has been reported by Pilkington and Parker, MCB 2008 and Sopko et al., Genes Dev 2006 among others. Since Pat1 overexpression does not cause cell death as previously reported, I am not sure it informative to keep this experiment in the manuscript. If they keep this data, they should indicate that Pat1 overexpression has been previously reported to be moderately lethal.

Author's comment: We agree with the reviewer. According to the report of Pilkington and Parker, 2008, the fifth dilution of cell after spotting grows slow when Pat1 is over-expressed (Figure 6B). In our report in figure 2D, the fifth dilution of Gal-Pat1 transformed cell seems to grow slow as compared to empty-vector, as reported in literature.

4. The "growth phenotype" and "overexpression phenotype" are used interchangeably in the manuscript. It would be clearer, if "growth phenotype" was changed to growth defect or similar language.

Author's comment: We have incorporated the changes in the manuscript text as suggested by the reviewer.

5. The authors should indicate where the Sbp1 granules are in Figure 4A. It is not clear how many foci are present in the selected cells and where they overlap with Edc3 foci. It would be helpful to add a small additional panel to Figure 2 to show the Sbp1 protein as a rectangle and the RRM and RNP regions indicated by shading and labelling.

Author's comment: We have indicated Sbp1 granules with an arrow in Figure 4A. A cartoon with different domains of Sbp1 is incorporated in Figure 2A along with the sequence.

6. Supplementary tables are not accessible.

Author's comment: We have made it accessible

7. The methods section has some text that should have been removed in editing.

Author's comment: It has been removed

- -

Version 1

Reviewer Report 10 September 2018

? **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

? **Tracy Nissan** (iD)

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 atthe 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