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Research paper

Low complexity RGG-motif containing proteins Scd6 and Psp2 act as suppressors of clathrin heavy chain deficiency

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ABSTRACT

Clathrin, made up of the heavy- and light-chains, constitutes one of the most abundant proteins involved in intracellular protein trafficking and endocytosis. *YPR129W*, which encodes RGG-motif containing translation repressor was identified as a part of the multi-gene construct (SCD6) that suppressed clathrin deficiency. However, the contribution of *YPR129W* alone in suppressing clathrin deficiency has not been documented. This study identifies *YPR129W* as a necessary and sufficient gene in a multi-gene construct SCD6 that suppresses clathrin deficiency. Importantly, we also identify cytoplasmic RGG-motif protein encoding gene *PSP2* as another novel suppressor of clathrin deficiency. Detailed domain analysis of the two suppressors reveals that the RGG-motif of both Scd6 and Psp2 is important for suppressing clathrin deficiency. Interestingly, the endocytosis function of clathrin heavy chain assayed by internalization of GFP-Snc1 and α-factor secretion activity are not complemented by either Scd6 or Psp2. We further observe that inhibition of TORC1 compromises the suppression activity of both *SCD6* and *PSP2* to different extent, suggesting that two suppressors are differentially regulated. Scd6 granules increased based on its RGG-motif upon Chc1 depletion. Strikingly, Psp2 overexpression increased the abundance of ubiquitin-conjugated proteins in Chc1 depleted cells in its RGG-motif dependent manner and also decreased the accumulation of GFP-Atg8 foci. Overall based on our results using *SCD6* and *PSP2*, we identify a novel role of RGG-motif containing proteins in suppressing clathrin deficiency. Since both the suppressors are RNA-binding proteins, this study opens an exciting avenue for exploring the connection between clathrin function and post-transcriptional gene control processes.

1. Introduction

Clathrin is a protein of vital importance in cells. Clathrin-coated vesicles (CCVs) play a key role in endocytosis and various intracellular trafficking. The triskelion, which is made up of the clathrin light chain and heavy chain monomers, forms a typical polygonal surface lattice on CCVs. Clathrin-mediated endocytosis, which is highly conserved from yeast to humans, is the major pathway for the internalization of external and membrane molecules into the cell. There are clathrin-independent pathways, however in the absence of clathrin-mediated endocytosis, uptake is greatly reduced [1–4]. CCVs are also involved in the intracellular trafficking pathway. Deficiency of clathrin in unicellular organisms like yeast, amoeba and protozoa are associated with defects in intracellular trafficking and receptor-mediated endocytosis [5,6].

In certain yeast strains, the deletion of the clathrin heavy chain-1 gene (*CHC1*) renders cells inviable, whereas in others, cells are viable. This is owing to the presence of an independently segregating gene – suppressor of clathrin deficiency 1 (*SCD1*). Presence of the *scd1-i* allele leads to lethality in *chc1* deficient cells, whereas the *scd1-v* allele allows growth, albeit poorly. Along with slow growth, *Δchc1 scd1-v* cells have abnormal morphology, genetic instability, reduced endocytosis and mis-localization of endoproteases from the *trans*-Golgi network to the cell membrane [7–12]. *SCD1* was recently identified to encode *PAL2*, a protein localized to cortical patches with other endocytic factors, thereby hypothesized to facilitate endocytosis. It was found that *scd1-i* allele has a premature stop codon which results in a truncated version of wild type *PAL2*, encoded by the *scd1-v* allele [13].

In 1993, Nelson and Lemmon identified six multi-gene and multi-copy plasmids (YEpSCD2–7) in a genetic screen that suppressed the growth defect associated with Chc1 depletion [14]. YEpSCD6 was one of those suppressors that contains a total of 8 genes along with *YPR129W*. A personal communication on the *Saccharomyces* genome database indicated *YPR129W* as the underlying multicopy suppressor of clathrin depletion from YEpSCD6 plasmid (Gelperin et al. 1995, personal

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communication to SGD). *YPR129W* encodes an RGG-motif containing translation repressor protein (Scd6) that targets translation initiation factor eIF4G1 [15].

RGG-motif proteins contain single or multiple repeats (Table 1) of RGG/RGX ('X' being any residue). This motif is known to be involved in protein-nucleic acids and protein-protein interactions [16]. Such proteins are known to function in processes like transcription, cell cycle, apoptosis and synaptic plasticity and can localize to both nucleus and cytoplasm. The RGG-motif constitutes the second most common class of domains/motifs that contribute to RNA-binding [17]. A subset of yeast RGG-motif proteins (such as Scd6, Sbp1, Npl3 and Ded1) have been implicated in translation control [15,18] as these proteins bind conserved translation initiation factor eIF4G through their RGG-motif to repress translation. Scd6 and Sbp1 are translation repressors and decapping activator proteins. Consistent with their role, both Scd6 and Sbp1 localize to RNA granules that are sites of translation repression and mRNA decay [15,19,20]. *PSP2* encodes a cytosolic RGG-motif containing protein (Table 1) originally identified as a suppressor of intron-splicing defects of a mutation in *MRS2* and subsequently as a suppressor of conditional mutation in DNA POL I in yeast [21,22]. Psp2 localizes to processing bodies during glucose deprivation [19]. *DED1* encodes a DEAD-box RNA helicase, an RNA granule resident protein that can both promote and inhibit translation initiation in yeast [23,24]. *GBP2* encodes a nucleo-cytoplasmic shuttling protein involved in telomere length modification [25], mRNA export [26–28] and mRNA quality control [29]. Despite being a nuclear protein, Gbp2 associates with polysomes and is a component of stress granules [30,31]. Recent reports implicate Gbp2 as a translation repressor protein thereby explaining its role in the cytoplasm [32]. *DHH1* encodes a DEAD-box RNA helicase that acts as a translation repressor and decapping activator [33]. It localizes to RNA granules and regulates P-body assembly in an ATP-dependent manner [34]. In this work, we report *YPR129W* and *PSP2* as bonafide suppressors of clathrin deficiency. The RGG-motif of both Scd6 and Psp2 is important for the suppression of clathrin deficiency. These results establish an unprecedented link between clathrin heavy chain function and RNA-binding RGG-motif proteins.

2. Results

2.1. YPR129W is necessary and sufficient to suppress clathrin deficiency

YEpSCD6 (pPIR144), a high copy number multi-gene plasmid was reported to be capable of rescuing inviability of clathrin heavy chain-deficient yeast strain expressing endogenous *CHC1* under a galactose-inducible promoter [14]. This multi-gene construct contained 7 other genes along with *YPR129W*. Based on this study and the personal communication (Gelperin et al. 1995, personal communication to SGD), we were intrigued by the possible role of an RNA-binding translation repressor protein in suppressing clathrin heavy chain requirement and therefore wanted to indeed confirm the role of *YPR129W* in clathrin function. To test in our hands the necessity and sufficiency of *YPR129W*, we created YEpΔ*scd6* (lacking *YPR129W*, pPIR159) and YEponly*SCD6* (YEpo*SCD6*; expressing only *YPR129W*, pPIR158) by site-directed mutagenesis. These plasmids (Table 3) were transformed into *GAL1:CHC1* strain (yPIR28) and the transformants were assayed for growth on glucose and galactose. We observed that YEpΔ*scd6* transformant grew as poorly as the empty vector control on the glucose plate (Fig. 1A), indicating that in the absence of *SCD6*, none of the other 7 genes could suppress the clathrin deficiency growth defect. Thus, *SCD6* was necessary in the multi-gene construct to suppress clathrin deficiency mediated growth defect. YEpo*SCD6* transformed cells grew in a manner comparable to YEp*SCD6* transformed cells suggesting that *SCD6* was sufficient to suppress the clathrin deficiency mediated growth defect (Fig. 1A). In order to quantify the extent of rescue, a plating assay was carried out on YEP media, wherein after depleting Chc1, cells were plated on galactose and glucose followed by counting the colonies. Consistent with the growth assay, cells expressing YEp*SCD6* and YEpo*SCD6* but not YEpΔ*scd6* could suppress clathrin deficiency (Fig. 1B). To further confirm its role, we cloned *YPR129W* in a 2μ plasmid pGP564 with a C-terminal His-tag (pPIR133) and checked its ability to suppress clathrin deficiency. We observed that this plasmid encoding *SCD6* suppressed the clathrin deficiency-mediated growth defect in a manner comparable to YEp*SCD6* (Fig. 1C). Based on these results we confirmed and established that *YPR129W* is a suppressor of clathrin deficiency.

Table 1
Number of RGG/RGX (X = any amino acid) in different proteins tested in this study.

Protein (# of amino acids)	# of RGG-/RGX-	Location of RGG-/RGX- motifs
Scd6 (349)	1/7	C-terminal
Psp2 (593)	4/10	C-terminal
Sbp1 (294)	8/5	Central
Ded1 (604)	4/4	Dispersed
Gbp2 (427)	4/6	Predominantly towards N-terminal

2.2. PSP2, but not other RGG-motif protein-encoding genes, can suppress clathrin deficiency defect

SCD6 encodes RGG-motif containing protein which acts as a translation repressor. We wanted to check whether other genes encoding RGG-motif proteins and/or translation repressors can suppress clathrin deficiency. We, therefore, tested some cytoplasmic RGG-motif containing proteins including D*ed1*, Sbp1, Dhh1, and Psp2 for their suppression activity. Sbp1, Ded1 and Dhh1 can act as translation repressor proteins. Sbp1 and Ded1 (but not Dhh1) contain RGG/RGX motifs which vary in the number of repeats and location in the protein sequence (Table 1). Psp2 is an RGG-motif containing protein that localizes to RNA granules and has recently been reported to modulate the translation of specific mRNAs involved in autophagy [35]. Apart from these proteins, we also tested one nuclear RGG-motif containing protein that has recently been found out to be a translation repressor, Gbp2 [32]. Interestingly *DED1*, *SBP1*, *DHH1* and *GBP2* did not suppress the clathrin deficiency mediated growth defect (Fig. 2A and B). Plating assay further confirmed the growth assay results (Fig. 2C). Strikingly, *PSP2* suppressed clathrin deficiency mediated growth defect (Fig. 2D). Plating analysis followed by colony count confirmed the growth assay results (Fig. 2E). The percentage rescue by *PSP2* improved when the plating assay was performed on selective synthetic media plates, likely due to increased retention of the plasmid (Supplementary Fig. 1). We conclude that *PSP2* is a novel suppressor of clathrin deficiency based on these observations. These results also indicate that the genes encoding RGG-motif containing or translation repressor proteins, in general, do not suppress clathrin deficiency, highlighting the specificity of suppression by *SCD6* and *PSP2*.

2.3. RGG-motif plays a vital role in suppressing clathrin-deficiency

Both *PSP2* and *SCD6* contain RGG-motif rich C-terminal region (Figs. 3A and 4A). *SCD6* also contains an LSm domain at its N-terminus and a central FDF domain. *PSP2* does not have any other canonical domain/motif other than the RGG-motif. We investigated which domain of these proteins was responsible for the observed suppression of growth defect. To this end, we generated domain deletion constructs and assayed them for suppression of clathrin deficiency mediated growth defect. An RGG-deletion mutant of *PSP2* was created (pPIR227) and tested for its ability to suppress clathrin deficiency. We observed that the RGG-motif deletion mutant of *PSP2* was defective in suppressing clathrin deficiency (Fig. 3B). The mutant was not compromised in its expression but interestingly was significantly more than that of wild type protein (Fig. 3C and D). Plating assays followed by colony counting further confirmed that the RGG-motif deletion mutant of *PSP2* was

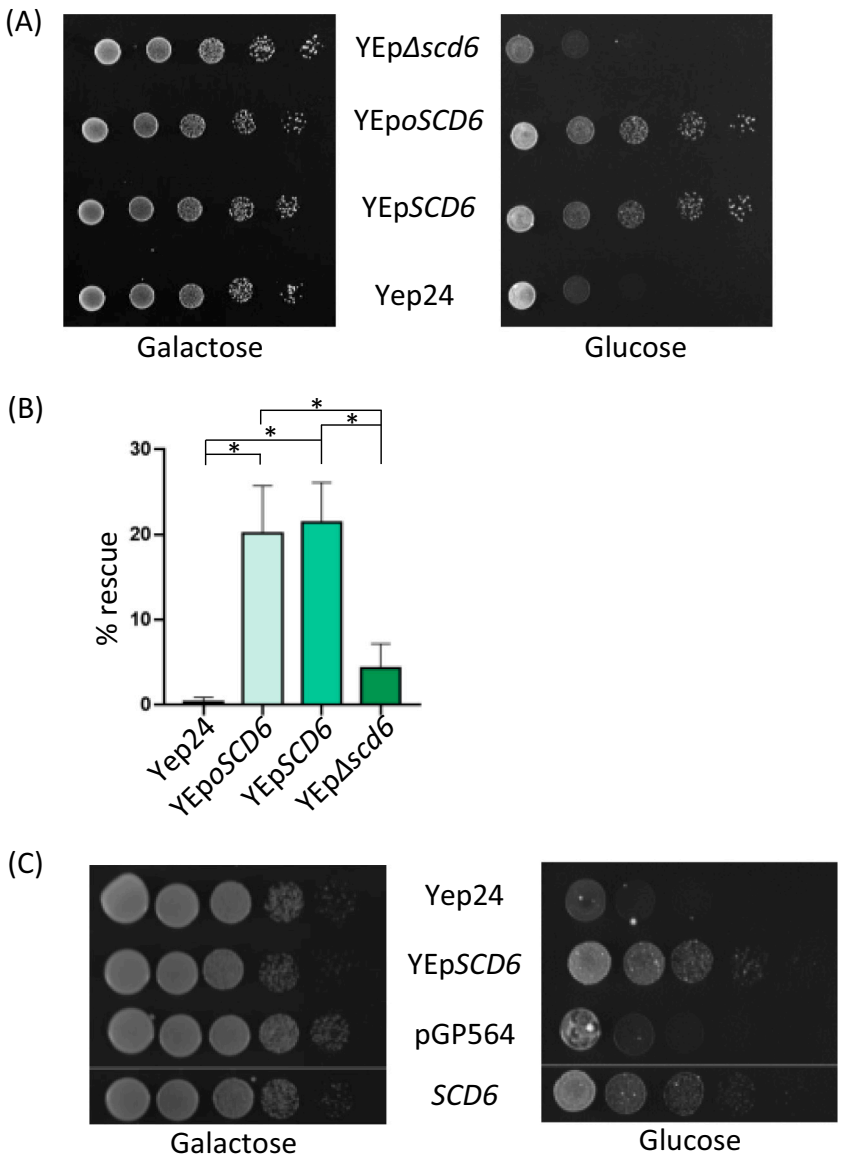

Fig. 1. *YPR129W* is necessary and sufficient to rescue clathrin deficiency. (A) Growth assay of *GAL1:CHC1* cells (endogenous *CHC1* is under galactose-inducible promoter, yPIR28) transformed with YEp24 (pPIR142), YEp*SCD6* (pPIR144), YEp*Δscd6* (lacking *YPR129W*, pPIR159) and YEp*oSCD6* (YEponly*SCD6* expressing only *YPR129W*, pPIR158) plasmids. 10 OD_{600} cells were serially diluted and spotted on glucose or galactose containing selective media plates. These plates were incubated at 30 °C for 2 days (galactose media) or 4–5 days (glucose media) before imaging. (B) Quantitation of the percentage rescue of *GAL1:CHC1* transformants upon clathrin deficiency. Percentage rescue was calculated as (number of colonies on glucose / number of colonies on galactose) * 100. A two-tailed paired student *t*-test was used to calculate the p-values (* indicates $p < 0.05$) and error bars represent mean ± SEM. (C) Growth assay of *GAL1:CHC1* transformants. *SCD6* panel refers to the transformant expressing *SCD6* (pPIR133) from pGP564 vector with C-terminal His-tag (pPIR130). *SCD6* expressed from pGP564 vector was spotted on the same plate as the rest of the three spottings. Image was spliced to remove other spottings not relevant to this figure. (Note: Because of the difference between the selection marker between Yep and pGP564, this assay was done on YEP plates.) Plates were incubated at 30 °C for 2 days (galactose media) or 4–5 days (glucose plate) before imaging.

defective in rescuing growth on the glucose media (Fig. 3E and Supplementary Fig. 1). We conclude based on these results that the RGG-motif of *PSP2* plays an important role in suppressing clathrin deficiency.

Like *PSP2*, the RGG-motif deletion construct of *SCD6* (pPIR169) was highly defective in suppressing clathrin deficiency (Fig. 4B). Like *psp2ΔRGG*, *scd6ΔRGG* protein levels were also higher than that of the full-length protein (Fig. 4C and D). Further, the other domains/motifs were also assayed for their role in the suppression phenotype (Supplementary Fig. 2A). The LSm domain deletion construct (pPIR135) was also defective in this phenotype, albeit slightly lesser than the RGG-motif deletion construct. Deletion of the FDF motif (pPIR137) weakly affected the ability of the mutant to suppress clathrin deficiency. The RGG-motif (282–348 residues) of *SCD6* as annotated in literature [36] comprises of RGG-rich sequences (282–310 residues) and QN-rich sequences (311–348 residues). Deletion of just the RGG-rich sequences (282–310) (*scd6RGG′*, pPIR141) resulted in a defect that was comparable to the deletion of 282–348 residues (pPIR139) indicating that the RGG-rich sequence was indeed important for the rescue of clathrin deficiency (Supplementary Fig. 2B). Based on these results, we conclude that all three domains affect the ability of *SCD6* to suppress clathrin deficiency. RGG-motif is the least and FDF-domain is the most dispensable domain for suppressing clathrin deficiency. Plating experiments followed by colony counting confirmed the growth assay results (Supplementary Fig. 2C). Interestingly, the FDF domain deletion mutant was also defective in suppressing clathrin deficiency in the plating assay, which was not evident as much in the growth assay (Supplementary Fig. 2B and C). The differential behavior of this mutant in two different assays is interesting however the basis for this is unclear. Some of the arginine residues in the RGG-motif of Scd6 are methylated and this modification promotes the repression activity [37]. We tested the role of arginine residues of the RGG-motif in suppressing clathrin deficiency. We observed that the arginine methylation defective mutant (AMD; with 9 R to A substitutions, pPIR170) failed to suppress the clathrin deficiency both in growth and plating assay (Fig. 4E and F). It is possible that arginine methylation of *SCD6* RGG-motif play a role in the suppression of clathrin deficiency. Endogenous Scd6 is expressed in low copy number [1280 molecules/cell [38]] and we believe that its expression is highly regulated. Our several attempts to raise anti-Scd6 antibody using full-length protein and peptides as antigens have not been successful. Even though the GFP-tagged version of the proteins were detected, visualization of Scd6 and its mutants expressed from pGP564 (Fig. 4E and Supplementary Fig. 2) using both poly- and monoclonal anti-His

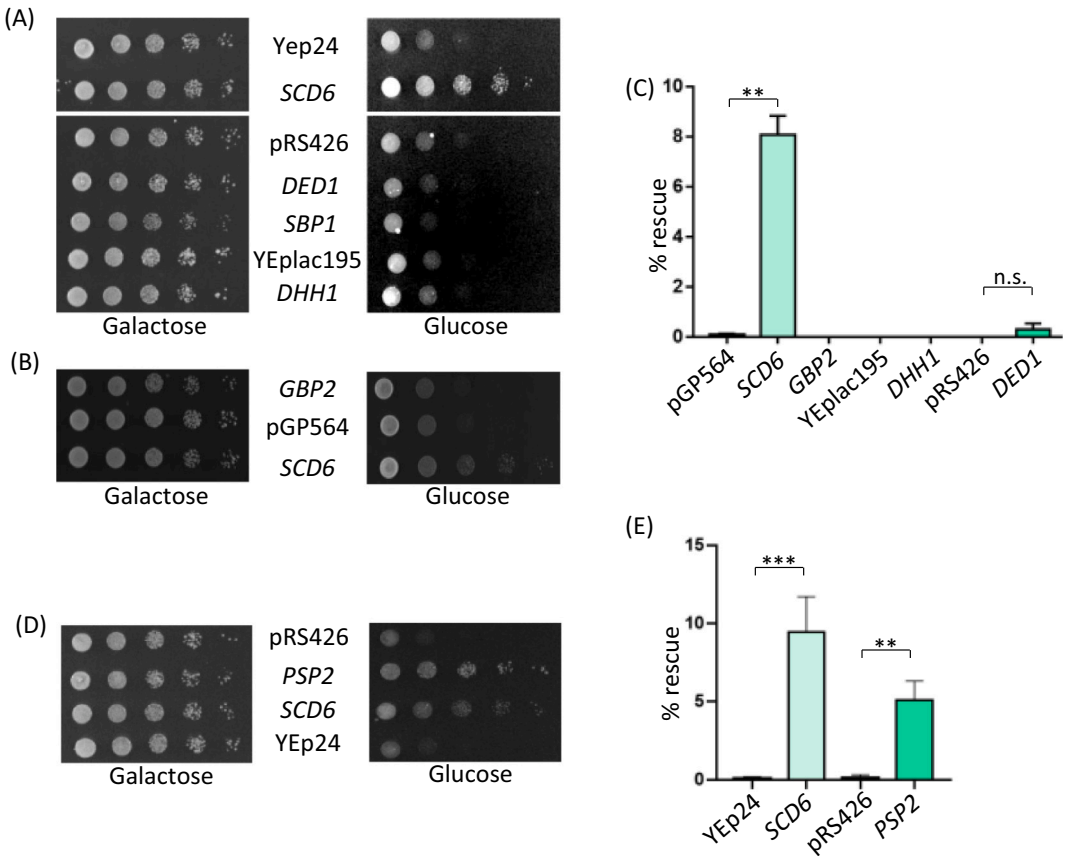

Fig. 2. *PSP2* is a novel suppressor of clathrin deficiency. (A and B) Growth assay of *GAL1:CHC1* cells transformed with different plasmids as labelled. 10 OD_{600} cells were serially diluted and spotted on glucose or galactose containing selective media plates. These plates were incubated at 30 °C for 2 days (galactose media) or 4–5 days (glucose media) before imaging. YEp24 and *SCD6* transformed cells were spotted on the same plate as the rest of the spottings. (C) Quantitation of the percentage rescue of *GAL1:CHC1* transformants upon clathrin deficiency. Percentage rescue was calculated as (number of colonies on glucose / number of colonies on galactose) * 100. (D) Growth assay of *GAL1:CHC1* cells transformed with pRS426 (empty vector, pPIR150) or pRS426-*PSP2* (pPIR153) as in A. (E) Quantitation of the percentage rescue by *PSP2* upon clathrin deficiency using plating assay. Percentage rescue was calculated as the number of colonies on glucose plate / number of colonies on galactose plate * 100. A two-tailed paired student *t*-test was used to calculate the p-values (** and *** indicates $p < 0.01$ and $p < 0.001$, respectively) and error bars represent mean ± SEM.

antibodies has been unsuccessful. We, however, know that expression of AMD mutant of Scd6 is not compromised when expressed under galactose-inducible promoter from a 2μ plasmid (BG1805 vector) and detected using PAP reagent that detects the C-terminal ZZ-tag [37]. We also know that LSm domain deletion mutant expression is not compromised when expressed in the BG1805 vector [39]. Overall, these results indicate that the Scd6 RGG-motif and arginines in RGG-motif are important for the suppression of clathrin deficiency. Interestingly, the LSm and the FDF (to a certain extent) domains also contribute to the suppression of clathrin deficiency by Scd6.

2.4. Depletion of Chc1 in the absence of PSP2 leads to a growth defect

Since Scd6 and Psp2 overexpression suppressed the growth defect, we asked if the deletion of these proteins had any effect on Chc1 depleted cells. To this end, we created Δ*psp2* (yPIR92) or Δ*scd6* (yPIR91) in *GAL:CHC1 scd1-v* (yPIR29) strain and analyzed the growth in glucose. Δ*scd6* grew comparable to its wild-type counterpart yPIR29, but there was a significant growth defect in the Δ*psp2* background (Fig. 5A). Deletion of *PSP2* did not have any growth defect of its own (Supplementary Fig. 3). With this, we conclude that *PSP2* deletion affects the basal growth of *GAL:CHC1 scd1-v* strain. Notably, such phenotype has also been reported for some of the other suppressors identified earlier, like *SCD3 (BMH2)* [40]. The difference between Scd6 and Psp2 in this context points towards the difference between the characteristics of the two suppressor proteins.

To check if the degree of overexpression could have any effect on the suppression activity of Scd6 and Psp2, we cloned *SCD6* (pPIR262) and *PSP2* (pPIR292) in a CEN plasmid pRS315 (pPIR260) and assessed for the growth rescue phenotype. Both the proteins were unable to rescue the growth defect when overexpressed from a low copy CEN plasmid compared to a high-copy 2μ plasmid (Fig. 5B). Based on this observation, we conclude that a robust overexpression is required for the suppression activity of Scd6 and Psp2. Overall, Scd6 and Psp2 are high-copy suppressors and deletion of *PSP2*, not *SCD6*, leads to a growth defect when combined with depletion of *CHC1*.

2.5. SCD6 and PSP2 suppression activity does not depend on SCD1

SCD1 has previously been identified as one of the suppressors of growth defect associated with Chc1 depletion. It encodes for Pal2 protein that localizes to the cortical patches indicating its direct relation to the endocytosis [13]. To assess if Scd6 and Psp2 suppression activity were dependent on Scd1, we evaluated the growth of *GAL:CHC1 scd1-i* (yPIR28) and *GAL:CHC1 scd1-v* (yPIR29) upon overexpression of either Scd6 or Psp2 (Fig. 6A). *GAL:CHC1 scd1-v* strain grows better than *GAL:CHC1 scd1-i* upon depletion of *CHC1* as reported earlier. Overexpression of Scd6 and Psp2 suppressed the growth defect of both the strains thus, indicating that the suppression activity is independent of the status of the *SCD1* allele. Interestingly, even though the growth rescue phenotype

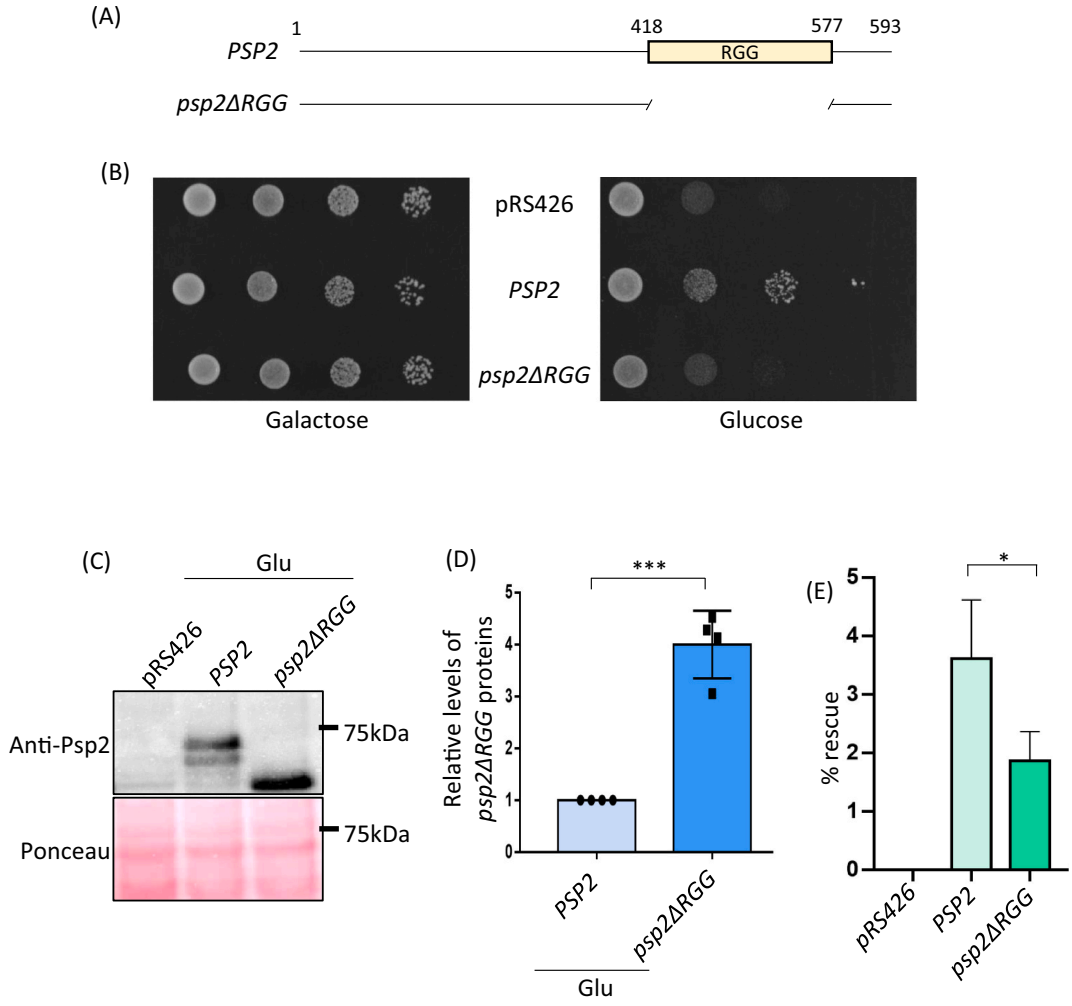

Fig. 3. Deletion of RGG-motif compromises the ability of Psp2 to suppress clathrin deficiency mediated growth defect. (A) Schematic showing the RGG deletion mutant of Psp2 constructed. Numbers denote the amino acid residue demarcating the RGG-motif of the full-length protein. (B) Growth assay of *GAL1:CHC1* cells transformed with wild type (pPIR153) and RGG-motif deletion mutants of Psp2 (pPIR227). 10 OD_{600} cells were serially diluted and spotted on glucose or galactose containing selective media plates. These plates were incubated at 30 °C for 2 days (galactose media) or 4–5 days (glucose media) before imaging. (C) Western analysis for *psp2ΔRGG* from the respective growth assay spots as performed in B. Ponceau serves as the loading control. (D) Quantitation of *psp2ΔRGG* levels from four independent experiments (n = 4) that were performed as in C. (E) Quantitation of the percentage rescue of *GAL1:CHC1* transformants with wild type or RGG-motif deletion mutant of *PSP2* upon clathrin deficiency. Percentage rescue was calculated as the number of colonies on glucose plate / number of colonies on galactose plate * 100. A two-tailed paired student *t*-test was used to calculate the p-values (* and *** indicates $p < 0.05$ and $p < 0.001$, respectively) and error bars represent mean ± SEM.

was relatively the same, there was a differential overexpression of Psp2 in *GAL:CHC1 scd1-i* and *GAL:CHC1 scd1-v* (Fig. 6B and C). This observation points towards a possible role of Scd1 in regulating Psp2 expression.

2.6. *SCD6 and PSP2 do not complement Snc1 endocytosis and trans-Golgi network sorting defect of Chc1 depleted cells*

We next tested the ability of *SCD6* and *PSP2* to complement specific functions of Chc1. First, we checked for endocytosis by assessing the distribution of GFP-Snc1, as Chc1 is known to play a key role in Snc1 endocytosis [41]. We tested whether overexpression of either Scd6 or Psp2 in Chc1-depleted conditions rescued the Snc1 endocytosis defect. Upon live-cell imaging analysis, there was a significant defect in the uptake and localization of GFP-Snc1 to the bud cell in the absence of Chc1 (Fig. 7A and B). Overexpression of Scd6 did not affect the defective localization of GFP-Snc1 (Fig. 7A). Surprisingly, there was a considerable reduction in GFP-Snc1 internalization when Psp2 was overexpressed (Fig. 7B). Along a similar line, there was a mild yet significant increase in the localization of GFP-Snc1 to the bud cell in Δ*psp2*, but not in Δ*scd6*, as compared to the wild-type cells (Fig. 7C). Based on this, we conclude that Scd6 and Psp2 do not complement the function of Chc1 in Snc1 endocytosis. Our result also points towards an antagonistic effect of Psp2 on Snc1 internalization.

Second, we assessed the ability of Scd6 and Psp2 to rescue the trans-Golgi network (TGN) sorting defects of *CHC1* depleted cells. MATα cells are defective in the secretion of the mature form of the mating pheromone α-factor in the absence of clathrin. This is due to the mislocalization of α-factor processing enzymes from the trans-Golgi network to the plasma membrane [9]. As a result, MATα cells with defective clathrin function do not inhibit the growth of MATa cells, otherwise evident by a zone of growth inhibition (halo) in the halo assay. Since *SCD6* and *PSP2* rescue the growth defect phenotypes upon depletion of Chc1, we decided to test their contribution to α-factor secretion. *GAL1:CHC1* MATα cells (yPIR33) overexpressing plasmid encoding *CHC1*, *SCD6*, *SBP1*, *PSP2*, *GBP2*, or *DED1* were depleted of Chc1 by growing on glucose and spotted on a lawn of MATa cells (yPIR32) expressing the *sst1–2* allele. *GAL1:CHC1* MATα (yPIR28) cells

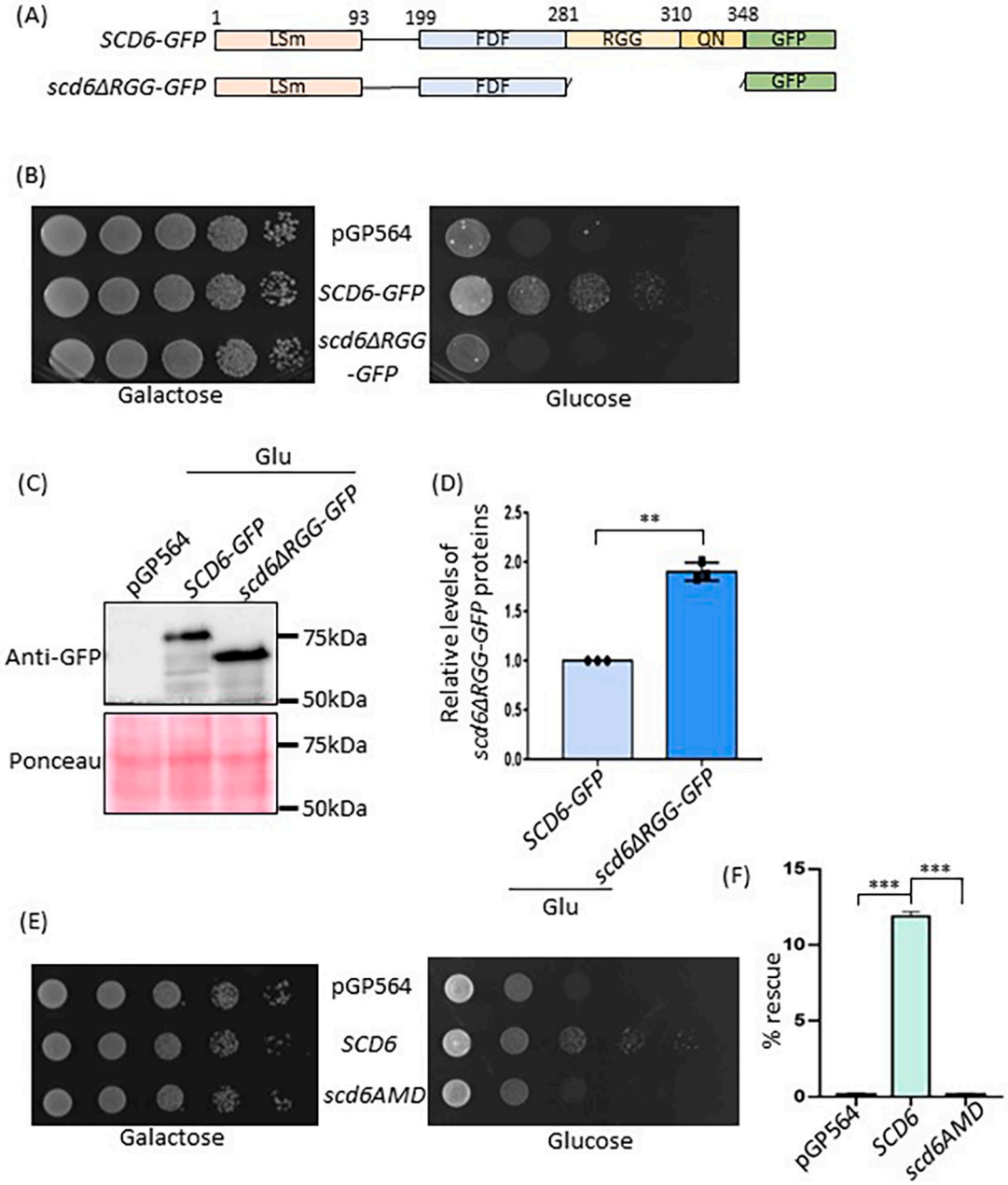

Fig. 4. RGG-motif is important for suppression of clathrin deficiency growth defect by Scd6. (A) Schematic showing the deletion mutant of Scd6 as used in B. Numbers denote the amino acid residue demarcating the different domains/motifs of the full-length protein. (B) Growth assay of *GAL1:CHC1* cells transformed with plasmids expressing wild type and RGG-motif deletion mutant of Scd6-GFP. 10 OD_{600} cells were serially diluted and spotted on glucose or galactose containing selective media plates. These plates were incubated at 30 °C for 2 days (galactose media) or 5 days (glucose media) before imaging. (C) Western analysis for *scd6ΔRGG* from the respective growth assay spots as performed in B. Ponceau serves as the loading control. (D) Quantitation of *scd6ΔRGG* levels from three independent experiments (n = 3) that were performed as in C. (E) Growth assay to test the ability of arginine methylation defective (AMD) mutant of *SCD6* (pPIR170) in suppressing clathrin deficiency. (F) Quantitation of the percentage rescue of *GAL1:CHC1* transformants by the AMD mutant of *SCD6* upon clathrin deficiency. Percentage rescue was calculated as the number of colonies on glucose plate / number of colonies on galactose plate * 100. A two-tailed paired student *t*-test was used to calculate the p-values (** and *** indicates $p < 0.01$ and $p < 0.001$, respectively) and error bars represent mean ± SEM.

overexpressing *CHC1* from a plasmid (pPIR145) form a clear zone of growth inhibition (halo) however, such a zone of inhibition was absent for cells overexpressing any of the tested genes including *SCD6* and *PSP2* (Fig. 8). We interpret this result to suggest that the suppression of clathrin deficiency growth defect by *SCD6* and *PSP2* does not involve complementation of the TGN function. Taken together, these results indicate that Scd6 and Psp2 do not complement GFP-Snc1 endocytosis and TGN sorting functions of Chc1.

2.7. Suppression activity of SCD6 and PSP2 is dependent on TORC1 activity

We next hypothesized if suppression activity of Scd6 and Psp2 was mediated by clearance of cargo proteins of clathrin-mediated endocytosis. Psp2 has recently been identified as a regulator of autophagy. It can positively regulate the translation of *ATG8* and *ATG13* mRNAs in nitrogen starvation conditions to induce autophagy [35]. Considering this, we aimed to check if the suppression activity of Psp2 is due to the activation of autophagy which could clear the cargo proteins thereby

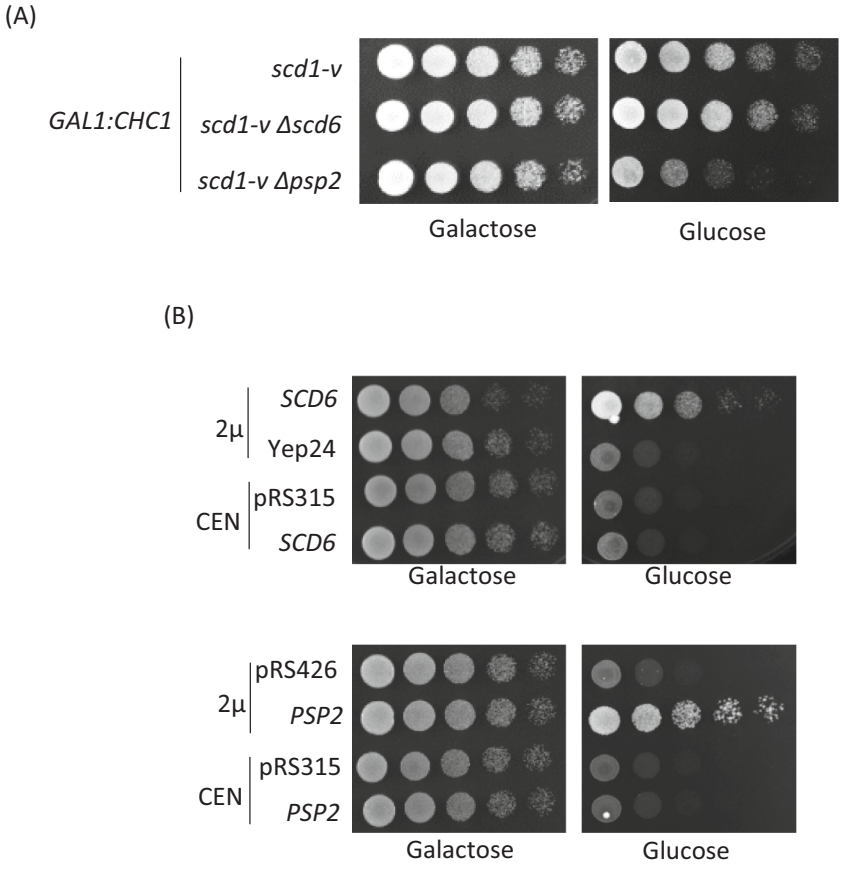

Fig. 5. Deletion of *PSP2*, but not *SCD6*, leads to growth defect upon depletion of *CHC1*. (A) Growth assay of *GAL1:CHC1 scd1-v* cells (yPIR29) with deletion of *SCD6* (yPIR91) or *PSP2* (yPIR92). Cells were first depleted of Chc1 by growing in glucose for 15 h. Thereafter, 10 OD_{600} cells were serially diluted and spotted on glucose or galactose containing YEP plates. These plates were incubated at 30 °C for 2 days (galactose media) or 3 days (glucose media) before imaging. (B) Growth assay of *GAL1:CHC1* cells transformed with high copy 2u (Yep24, pPIR142 and pRS426, pPIR150) and low copy CEN (pRS315, pPIR260) plasmids expressing the respective gene as labelled. 10 OD_{600} cells were serially diluted and spotted on glucose or galactose containing YEP media plates. Plates were incubated for 2 days (galactose media) or 4–5 days (glucose media) before imaging at 30 °C temperature.

reducing the load on clathrin machinery. To this end, first, we studied the localization of GFP-Atg8 (expressed from a plasmid, pPIR290) in Chc1 depleted cells in control and Psp2 overexpression. Under Chc1-repleted condition, GFP-Atg8 accumulated at a perivacuolar site known as the phagophore assembly site (PAS), as previously reported [42]. We found an increase in the number of GFP-Atg8 granules upon depletion of Chc1 in empty vector transformed cells (Fig. 9 A and B, galactose vs glucose of pRS426 transformed cells). Accumulation of such granules reflect an induction in autophagy, but a defect in the fusion of autophagosome to the vacuole like observed in deletion of *VPS18* [43,44]. Interestingly, upon Psp2 overexpression the number of these granules reduced significantly (Fig. 9A and B) depicting a possible role of Psp2 in regulating autophagy induction in this condition.

Further, we did growth assays on plates containing 1 ng/ml of rapamycin, an inhibitor of TORC1, and an activator of autophagy [45]. Surprisingly, upon treatment with rapamycin, the suppression activity of Scd6 and Psp2 was compromised (Fig. 9C). The impact of rapamycin treatment on Psp2 was weaker than in the case of Scd6 as the suppression activity of Psp2 was still evident. Treatment with rapamycin also increased the expression of Psp2 in Chc1 depleted conditions (Fig. 9D and E). Interestingly, there was no corresponding increase in the growth rescue by the increased protein expression in rapamycin treated cells. Since Scd6 could not be detected in the cells used for growth assay, we used a different strain and checked the effect of rapamycin on Scd6 levels. We did not find any change in Scd6 levels (Supplementary Fig. 4). These results indicate that an active TORC1 is important for the suppression activity of Scd6 and Psp2, to a certain extent.

2.8. Localization of Scd6 to puncta increases upon Chc1 depletion

Since rapamycin can have impact on global translation as well, we next tested if global translation repression can suppress the growth defect associated with Chc1 depletion by reducing the load on endocytic machinery by using cycloheximide. Growth assays with 0.1 μg/ml CHX showed complete loss of the growth rescue by the target suppressor protein (Supplementary Fig. 5). This indicated that active protein translation is required for the suppression activity of Scd6 and Psp2.

Considering the known role of Scd6 in translation repression and related localization to RNA granules upon stress, we tested the localization of Scd6 upon depletion of Chc1. Scd6 granules were found to be increased in Chc1 depletion (Fig. 10A). Even though RGG-motif deletion mutant was defective in granule formation, the mutant protein was also found in granules more upon depletion of Chc1 albeit to a lesser extent than full length protein (Fig. 10A and B). This phenotype suggests a possible role of Scd6 granules in suppression of growth defect of Chc1 depleted cells that can be explored further.

2.9. Psp2 increases the abundance of ubiquitin conjugates in cells depleted of Chc1

Accumulation of the ubiquitin-conjugated proteins has been reported earlier in Chc1 depleted cells [14]. We therefore checked if there is any change in the abundance of ubiquitin-conjugated proteins in Scd6 and Psp2 overexpression by western blotting. We saw an increase in the ubiquitin-conjugated proteins upon depletion of Chc1 as reported earlier (Supplementary Fig. 6). Strikingly, there was an accumulation of higher molecular weight proteins tagged with ubiquitin upon overexpression of Psp2 in conjunction with Chc1 depletion (Fig. 11B). Such an increase was not observed upon Scd6 overexpression (Fig. 11A). Further, the overexpression of an RGG-motif deletion mutant of Psp2 was significantly defective in increasing Ub-conjugates (Fig. 11B). To understand if increase in ubiquitin-conjugates was due to an increase in the ubiquitin protein, free ubiquitin levels were also compared. We did not find any significant change in free ubiquitin levels upon Psp2 overexpression as

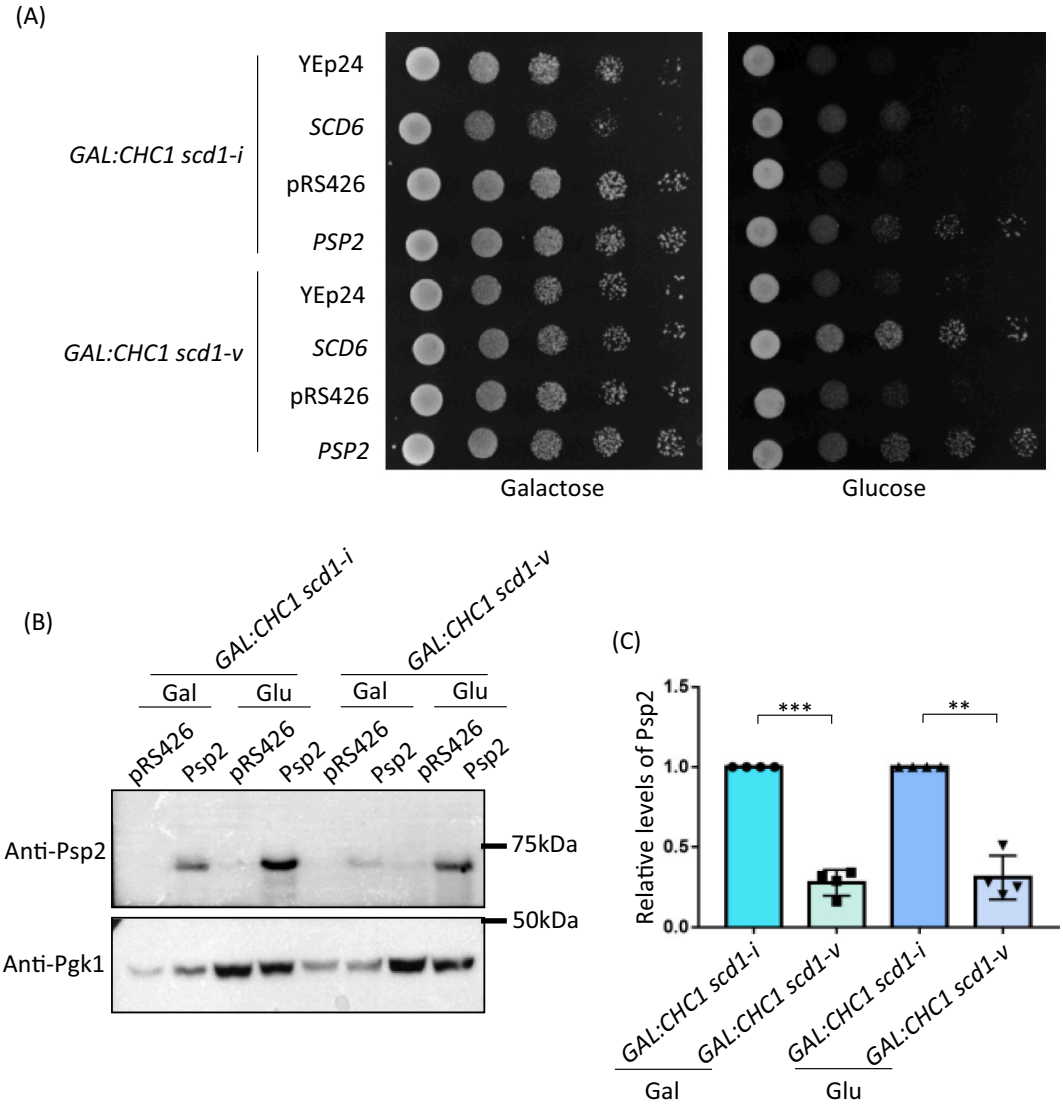

Fig. 6. Suppression activity of Scd6 and Psp2 is independent of the presence of Scd1. (A) Growth assay of *GAL1:CHC1 scd1-i* (yPIR28) and *GAL1:CHC1 scd1-v* (yPIR29) cells transformed with different plasmids as labelled. 10 OD_{600} cells were serially diluted and spotted on glucose or galactose containing selective media plates. These plates were incubated at 30 °C for 2 days (galactose media) or 4–5 days (glucose media) before imaging. (B) Western analysis for Psp2 from the respective growth assay spots as performed in A. Pgk1 serves as the loading control. (C) Quantitation of Psp2 levels from four independent experiments (n = 4) that were performed as in B. A two-tailed paired student *t*-test was used to calculate the p-values (** and *** indicates $p < 0.01$ and $p < 0.001$, respectively) and error bars represent mean ± SEM.

compared to the control (Supplementary Fig. 7). Based on these observations, we conclude that Psp2 might be increasing the basal levels of ubiquitin-conjugated proteins in Chc1 depleted cells in an RGG-motif dependent manner without affecting the free ubiquitin pool.

3. Discussion

We provide evidence suggesting that two genes encoding RGG-motif proteins, function as suppressors of clathrin deficiency. This will be the first peer-reviewed report implicating RGG-motif proteins in clathrin function to the best of our knowledge. Our conclusion is based on the following observations, a) *SCD6* and *PSP2* act as a suppressor of clathrin deficiency (Figs. 1 & 2), b) RGG-motifs of Psp2 and Scd6 are required for suppressing clathrin deficiency (Figs. 3 and 4), c) Arginine residues of the Scd6 RGG-motif play an important role in suppressing clathrin deficiency (Fig. 4E and F), d) Scd6 granules increased upon depletion of Chc1 (Fig. 10) and, e) Psp2 overexpression increased the levels of ubiquitin-conjugated proteins upon depletion of Chc1 in RGG-motif dependent manner (Fig. 11B).

SCD6 was identified as one of the genes in a multi-gene construct that suppressed clathrin heavy chain deficiency; however the specific contribution of *SCD6* remained to be confirmed [14]. Our results prove that in the reported multi-gene construct, *SCD6* is both necessary and sufficient to suppress clathrin deficiency mediated growth defect. This, along with the observation that *SCD6* cloned in a different vector also suppresses clathrin deficiency, indicates that it is a genuine suppressor of clathrin deficiency. Reported roles of *SCD6* orthologs point towards a role of this family of proteins in endocytosis and/or trafficking. *CAR-1* (worm ortholog) plays a role in maintaining ER organization as upon *CAR-1* knockdown, ER is disorganized into large patches and thick strands [46]. Trailer hitch (*Drosophila* ortholog) localizes to ER-exit sites, which persist as large foci upon Tral knockdown [47].

Suppression of clathrin deficiency by *PSP2* is a striking result. Psp2 is known to localize to RNA granules [19] and upon overexpression, can rescue P-body formation in *edc3Δlsm4Δc* strain [48]. Scd6 binds *TIF4631* (eIF4G1) to repress translation and interestingly Psp2 is reported to

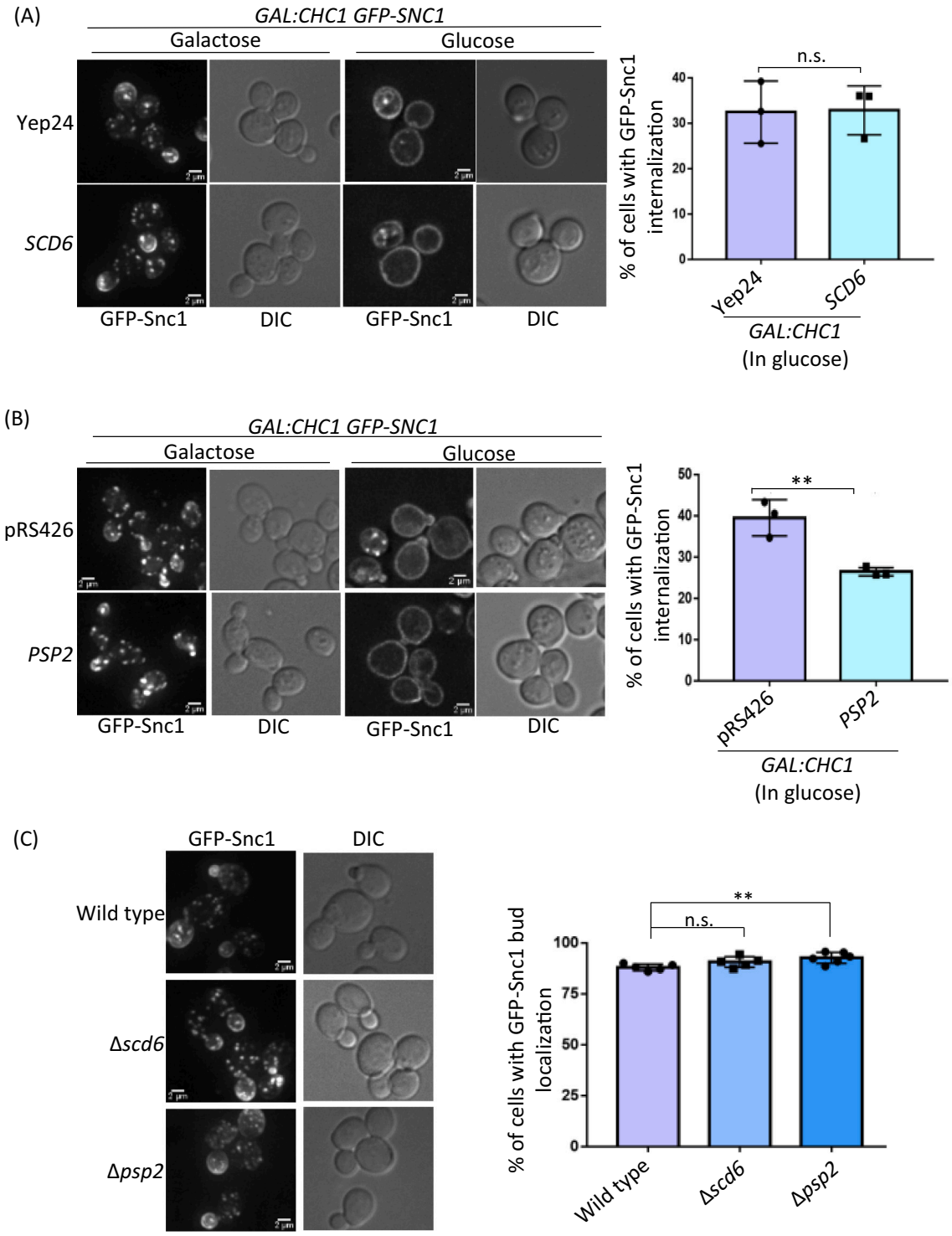

Fig. 7. *SCD6* and *PSP2* fail to complement endocytosis in the absence of Chc1. (A and B) Microscopy analysis showing the localization of GFP-Snc1 in *GAL1:CHC1* cells transformed with (A) Yep24 (pPIR142) and *SCD6* (pPIR158) and (B) pRS426 (pPIR150) and *PSP2* (pPIR153) expressing plasmids with quantitation of three independent experiments (n = 3) on the right. (C) Microscopy analysis showing the localization of GFP-Snc1 in wild type (yPIR1), Δ*scd6* (yPIR24) and Δ*psp2* (yPIR94) cells. Graph on the right represents quantitation of five independent experiments (n = 5) that were performed as in C. A two-tailed paired student t-test was used to calculate the p-values (** indicates $p < 0.01$) and error bars represent mean ± SEM.

physically interact with its paralog *TIF4632* [49]. A recent report has indeed implicated Psp2-TIF4632 interaction in modulating the translation of autophagy genes [35]. Whether Psp2 can act as a translation repressor of specific mRNAs has not been explored.

Lack of suppression by other RGG-motif proteins and translation repressors (Sbp1, Ded1, Dhh1 and Gbp2) highlights the specificity of Scd6 and Psp2 in clathrin function. It is unlikely that the contribution of Scd6 and Psp2 in clathrin function is dependent on the number of RGG/RGX repeats as Sbp1 contains more repeats than Scd6 (Table 1) but fails to suppress. Remarkably both Scd6 and Psp2 harbor RGG-motifs at their C-terminal but none of the other tested proteins, including Sbp1, have RGG-motifs at their C-terminus. It is noteworthy that although the Scd6

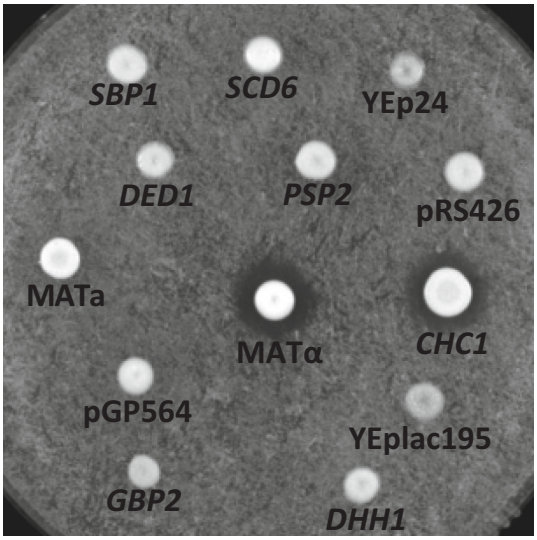

Fig. 8. *SCD6* and *PSP2* do not complement trans-Golgi network sorting defect. MATα strain expressing endogenous *CHC1* under galactose-inducible (pPIR28) promoter was transformed with plasmids expressing *SCD6*, *SBP1*, *DED1*, *PSP2*, *GBP2*, *DHH1* or *CHC1*. Respective empty vectors were used as control. Transformants were spotted on a lawn of MATa (yPIR31) cells carrying the *sst1–2* allele and incubated on YEP glucose plate until (2–3 days) the appearance of a zone of clearance (halo). YEp24 is the empty vector control for *SCD6* and *CHC1*. pRS426 is the empty vector control for *DED1*, *SBP1* and *PSP2*. pGP564 is the empty vector control for *GBP2* and YEplac195 is the empty vector control for *DHH1*. Wild type MATa (yPIR32) and MATα (yPIR33) strains act as negative and positive controls, respectively.

and Psp2 RGG-deletion mutants failed to act as suppressors, their protein levels were significantly more than that of the wild-type protein. This observation might point towards an interplay between RGG-motif and protein degradation which will be explored in future studies.

Suppressors of clathrin deficiency might likely fall into one of the following two categories: a) Suppressors that directly contribute to clathrin function in vesicular transport/endocytosis and, b) Suppressors that indirectly increase tolerance to clathrin deficiency. *SCD4* [50] and *SCD5* [11] belong to the former category that contributes directly to vesicular transport. *SCD2*, on the other hand, belongs to the latter category of suppressors. It was identified as *UBI4* [14], which likely suppresses clathrin deficiency defect by accelerating clearance of mislocalized proteins.

Our results do not appear to support a role of Scd6 and Psp2 in directly contributing to the Chc1 function. Scd6 fails to complement both the trans-Golgi network sorting and Snc1 endocytosis function of clathrin heavy chain (Figs. 7A and 8). We also did not observe any effect on Snc1 internalization upon deletion of Scd6 (Fig. 7C). Like Scd6, Psp2 fails to complement the trans-Golgi network sorting function of Chc1 (Fig. 8) and uptake of lucifer yellow upon clathrin depletion (data not shown). However, overexpression of *PSP2* upon depletion of *CHC1* has an antagonistic effect on the internalization of GFP-Snc1 (Fig. 7B). This observation is unexpected and intriguing. In clathrin-depleted cells, the residual endocytosis largely depends on clathrin-independent pathways. A clathrin-independent pathway, like Tor2-Rho1 dependent endocytosis [4,51], could contribute to the basal uptake of Snc1 in the absence of Chc1. Based on our data, we propose that Psp2 might be a negative regulator of a clathrin-independent pathway and overexpression of Psp2 in the absence of Chc1 could lead to decreased uptake of Snc1, thereby reducing the intracellular trafficking load leading to the observed growth rescue.

In light of the lack of complementation of endocytic functions of Chc1 by Scd6 and Psp2, we hypothesized that these proteins could rescue clathrin deficiency by increasing the tolerance of cells to clathrin deficiency. Previously, another suppressor, Ubi4, was suggested to work indirectly by accelerating the clearance of mislocalized proteins [14]. Global translation repression, as achieved by cycloheximide treatment, abrogated the growth rescue phenotype of both Scd6 and Psp2 (Supplementary Fig. 5). This indicated that active translation was required for suppression by Scd6 and Psp2. We also tested the role of Psp2 and Scd6 in clearing the mislocalized cargo proteins in the absence of Chc1 through autophagy. A selective autophagy pathway has been observed to degrade endocytic macromolecular complexes [52]. It is also reported that Psp2 is a positive regulator of the autophagy pathway [35]. We observe an increase in the number of Atg8 granules upon depletion of Chc1 (Fig. 9A and B). This increase was not surprising as membrane trafficking had been found to be an integral part of autophagy [53]. Clathrin is found to take part not only in autophagosome formation but also in its fusion to lysosome in mammalian cells [54,55]. An increase in Atg8 granules in our system point towards autophagy induction but a defect in the fusion of autophagosome to the vacuole and hence defect in the completion of autophagy [43,44]. Further, we saw that Psp2 overexpression accumulated less GFP-Atg8 puncta compared to the empty vector transformed cells, which possibly point towards an involvement of Psp2 in negatively affecting autophagosome formation. Further, rapamycin treatment (an inducer of autophagy) also did not have any impact on the growth of empty vector transformed cells (Fig. 9C) but strikingly Scd6 suppression activity is compromised upon treatment with TORC1 inhibitor rapamycin (Fig. 9C; glucose panel), whereas Psp2 suppression activity is mildly compromised (Fig. 9C; glucose panel). These results point towards the involvement of a major signaling complex TORC1 in the suppression activity of Scd6 and Psp2.

Along with autophagy, ubiquitination is another major signal that can target a protein for degradation. Such proteins can be targeted either to proteasomal machinery or to vacuole for their clearance [56,57]. Our data indicates an increase in the accumulation of ubiquitin conjugates in Psp2 overexpression that was dependent on its RGG-motif without affecting the free ubiquitin pool (Fig. 11B and Supplementary Fig. 7). Increased ubiquitination and hence degradation of mislocalized proteins could be a possible explanation for Psp2 dependent growth rescue in Chc1-depleted cells. Whether ubiquitination is increasing in a global manner or is specific to certain proteins still need to be analyzed. It might be possible that overexpression of Psp2 is diverting the protein degradation traffic from autophagy to proteasomal machinery. This would explain the observations of decrease in Atg8 granules, decreased growth rescue in the presence of rapamycin and increase ubiquitin-conjugates in Psp2 overexpression. This hypothesis will be experimentally validated and addressed in more details in subsequent studies.

Several observations point towards the importance of translation related function in the suppression activity: (a) RGG-motif, which is required for the translation repression activity of *SCD6* [15,37,58] is also required to suppress clathrin deficiency defect by *SCD6*, (b) *SCD6* AMD mutant is defective in repressing translation as well as in suppressing clathrin deficiency and, (c) LSm domain is required for the translation repression activity of Scd6 [39] and also for suppressing clathrin deficiency. Further, an active translation and an active TORC1 are also required for the suppression activity of Scd6 (Supplementary Fig. 5 and Fig. 9C). It is noteworthy that the inhibition of TORC1 is known to compromise Hmt1 methyltransferase activity [59]. In such a scenario, methylation of Scd6 could be defective, leading to reduced translation repression activity as methylation is known to promote Scd6 repression activity [37]. This might explain the loss in growth rescue with rapamycin treatment as seen with the AMD mutant (Fig. 4E). Checking the methylation status of Scd6 upon perturbation of TORC1 will be an important future direction.

It is also possible that translation control of specific mRNAs contributes to the suppression of clathrin deficiency. We have looked for the possible role of Scd6 in the stabilization of *CHC1* mRNA upon shift to glucose but failed to observe any significant change (Supplementary Fig. 8). Mild yet consistent decrease in endocytosis of Snc1 upon Psp2

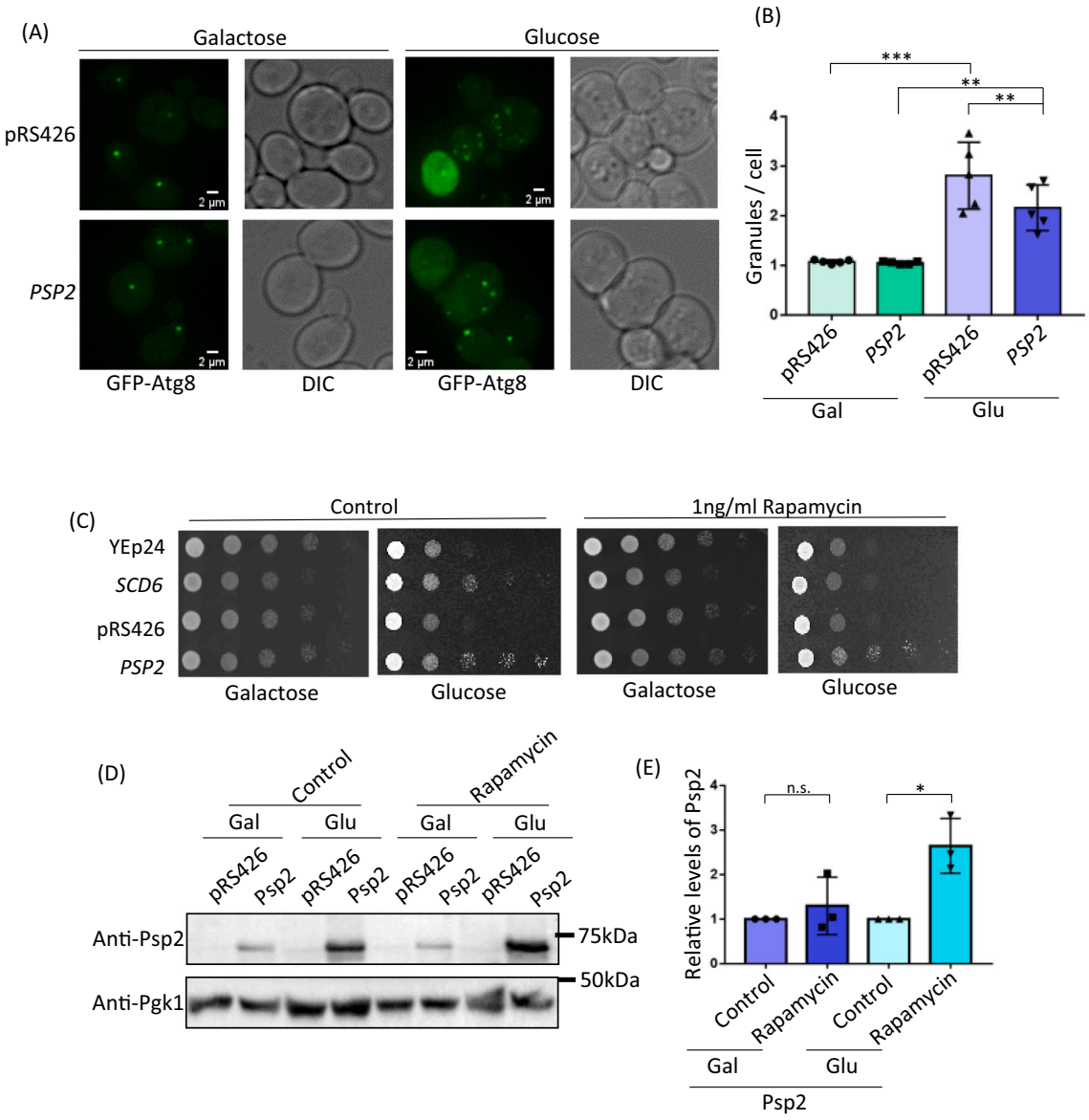

Fig. 9. Suppression activity of Scd6 and Psp2 is dependent on TORC1. (A) Microscopy analysis showing the localization of GFP-Atg8 in *GAL1:CHC1* cells transformed with respective plasmid. Cells were grown either in galactose (Chc1 repleted) or glucose (chc1 depleted). (B) Quantitation of the granules per cells of five independent experiments (n = 5) as performed in A. (C) Growth assay of *GAL1:CHC1* cells transformed with different plasmids as labelled. 10 OD_{600} cells were serially diluted and spotted on glucose or galactose containing selective media plates supplemented with or without 1 ng/ml of Rapamycin. These plates were incubated at 30 °C for 2 days (galactose media) or 4–5 days (glucose media) before imaging. (D) Western analysis for Psp2 from the respective growth assay spots as performed in C. Pgk1 serves as the loading control. (E) Quantitation of Psp2 levels from three independent experiments (n = 3) that were performed as in D. A two-tailed paired student *t*-test was used to calculate the p-values (* indicates $p < 0.05$) and error bars represent mean ± SEM.

overexpression could be mediated by post-transcriptional regulation of specific transcript(s) involved in clathrin independent pathway. Similarly the increased ubiquitin conjugates upon Psp2 overexpression also could be due to specific regulation of transcript(s) involved in the ubiquitin pathway. Identifying specific mRNA targets of Scd6 and Psp2 will provide important insights in this regard. Since Scd6 and Psp2 localizes to RNA granules which are sites of translation repression and mRNA decay, we also tested the localization of Scd6 upon clathrin depletion. We observed a significant increase in the localization of Scd6 to foci (Fig. 10), suggesting that suppression is likely mediated by changes in localization to foci. The role of these puncta in rescuing the growth defect in Chc1-depleted cells remains to be addressed. It is possible that these puncta could regulate fate of transcripts encoding ancillary proteins involved in clathrin related functions. The prediction would be that such transcripts are stabilized by Scd6 thereby leading to increased expression and rescue of clathrin deficiency. LSM14 is the human ortholog of Scd6 [36,60]. The role of Scd6 identified in mRNA fate determination in yeast is conserved in humans. It is possible that LSM14 could contribute to clathrin function in a manner comparable to described in this report for Scd6. This exciting possibility will be tested in future.

Overall, our results identify bonafide suppressors of clathrin heavy chain deficiency. It establishes a new link between RGG-motif containing RNA-binding proteins and clathrin function. Analyzing further the details of this link could lead to the unraveling of the role of mRNA metabolism in clathrin-mediated endocytosis. It will also be pertinent to test if the players contributing to the endocytic/trafficking pathway including Chc1 are modulating cytoplasmic mRNA fate through Scd6 and Psp2. Our current work thus highlights several research questions, and addressing these questions will be an exciting future endeavor.

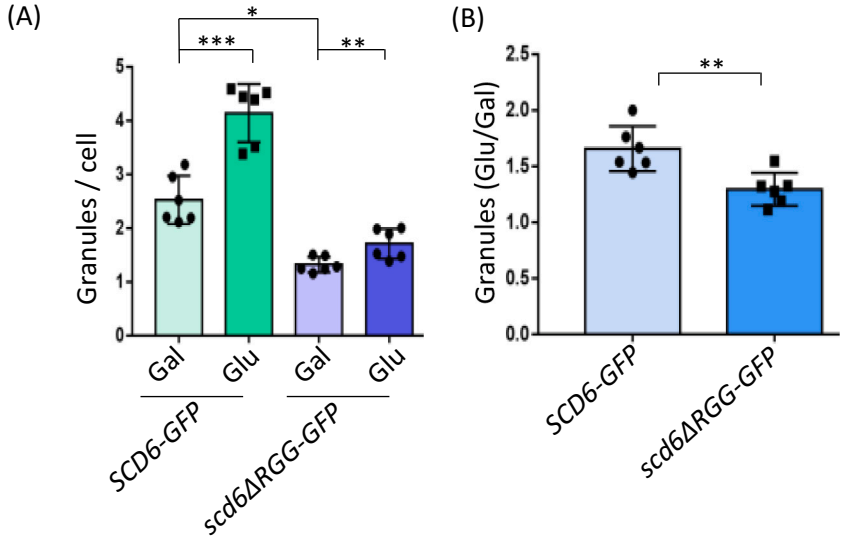

Fig. 10. Scd6 granules increase upon depletion of Chc1. (A) Quantitation of the granules per cells of six independent experiments (n = 6) of Scd6-GFP and *scd6ΔRGG-GFP* in *GAL1:CHC1* cells transformed with respective plasmid. Cells were grown either in galactose (Chc1 repleted) or glucose (Chc1 depleted). A two-tailed paired student *t*-test was used to calculate the p-values (*, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) and error bars represent mean ± SEM. (B) Ratio of granules in glucose to that of galactose grown cells as done in A. A two-tailed unpaired student t-test was used to calculate the p-values (** indicates $p < 0.01$) and error bars represent mean ± SEM.

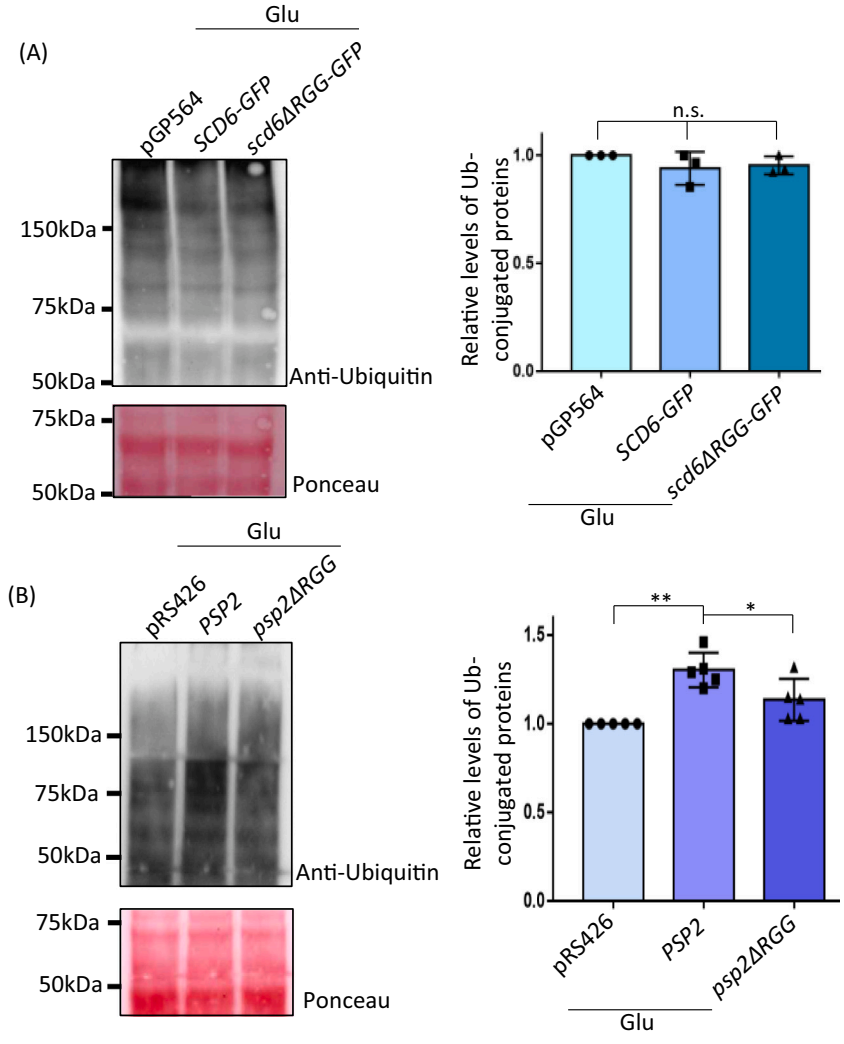

Fig. 11. Overexpression of Psp2 in Chc1 depleted condition increases the abundance of Ub-conjugated proteins. (A and B) Western analysis for Ub-conjugated proteins in Chc1 depleted (Glu) cells upon overexpression of Scd6 (A) or Psp2 (B) and their respective RGG-motif deletion mutant. Ponceau image serves as the loading control. A two-tailed paired student t-test was used to calculate the p-values (* and ** indicate $p < 0.05$ and $p < 0.01$, respectively) and error bars represent mean ± SEM.

4. Materials and methods

4.1. Strains and plasmids

Strains and plasmids used in this study are enlisted in Tables 2 and 3, respectively.

4.2. Growth assay

Growth assays were performed to assess the ability of plasmids expressing desired genes to rescue clathrin deficiency mediated growth defect of the *GAL:CHC1* strain on glucose media. In *GAL:CHC1* strain, *CHC1* is under a galactose inducible promoter [14] which results in a growth defect on glucose media. Such growth defect will not be observed for wild type (yPIR1; BY4741) strain used in Supplementary Fig. 3. Transformants grown on galactose containing media (10 OD_{600}) were serially diluted and spotted on the respective minimal media containing glucose and galactose plates. All the plates were incubated at 30 °C and imaged after 2 days (galactose media) or 4–5 days (glucose

Table 2
List of strains used in this study.

Name	Genotype	Description	Source
yPIR28	*MATα GAL1:CHC1 scdl-i leu2 ura3-52 trpl hisl GAL2*	Wild type *Saccharomyces cerevisiae* with the inviable allele of *SCD1* and *CHC1* under galactose inducible promoter	[14]
yPIR93	*MATα GAL1:CHC1 scdl-i leu2 ura3-52 trpl hisl GAL2* GFP-*SNC1-SUC2*	Wild type *Saccharomyces cerevisiae* with the inviable allele of *SCD1*, *CHC1* under galactose inducible promoter and genomic integration of pHB4 (Addgene: 53462)	This study
yPIR31	*MATa sst1-2 ade2-1 his6 met1 cyh2 rme1 ura1 can1*	*Saccharomyces cerevisiae* with defective adaptation to alpha-factor response pathway	[61]
yPIR32	*MATa leu2 ura3-52 trp1 his3-200*	Wild type *Saccharomyces cerevisiae* with 'a' mating type	[61]
yPIR33	*MATα leu2 ura3-52 trp1 his3-200*	Wild type *Saccharomyces cerevisiae* with 'alpha' mating type	[61]
yPIR29	*MATα GAL1:CHC1 scdl-v leu2 ura3-52 trpl hisl GAL2*	Wild type *Saccharomyces cerevisiae* with the viable allele of *SCD1* and *CHC1* under galactose inducible promoter	[14]
yPIR91	*MATα GAL1:CHC1 scdl-v leu2 ura3-52 trpl hisl GAL2 Δscd6*	Wild type *Saccharomyces cerevisiae* with the viable allele of *SCD1*, *CHC1* under galactose inducible promoter and *SCD6* deletion	This study
yPIR92	*MATα GAL1:CHC1 scdl-v leu2 ura3-52 trpl hisl GAL2 Δpsp2*	Wild type *Saccharomyces cerevisiae* with the viable allele of *SCD1*, *CHC1* under galactose inducible promoter and *PSP2* deletion	This study
yPIR1	*MATa his3D1 leu2D0 met15D0 ura3D0 ('BY4741')*	Wild type *Saccharomyces cerevisiae*	[37]
yPIR24	*MATa his3D1 leu2 ura3 his3 met15 scd6Δ::KanMX (Δscd6)*	Wild type *Saccharomyces cerevisiae* with *SCD6* deletion	[15]
yPIR94	*MATa his3D1 leu2 ura3 his3 met15 psp2Δ::KanMX (Δpsp2)*	Wild type *Saccharomyces cerevisiae* with *PSP2* deletion	Saccharomyces Genome Deletion project library

Table 3
List of plasmids used in this study.

Name	Description	Vector	Source
pPIR142	YEp24 library empty vector, multicopy 2μ plasmid with *URA3* and ampicillin resistance genes	YEp24	[14]
pPIR144	YEp24 cloned with a fragment from digested yeast genome containing *YPR129W* along with other flanking genes	YEp24	[14]
pPIR158	Plasmid with only *SCD6* gene from YEp*SCD6*, other flanking genes deleted	YEp24	This study
pPIR159	Plasmids retaining all genes except *SCD6* from YEp*SCD6*	YEp24	This study
pPIR145	Plasmid expressing *CHC1* ORF from a CEN plasmid YCp50	YCp50	[14]
pPIR130	Yeast shuttle vector, multicopy 2μ plasmid with *LEU2* and kanamycin resistance genes	pGP564	YGTC library, Lab Stock
pPIR133	pGP564 containing *SCD6* under its own promoter. Along with the ORF 550 nucleotides upstream and 225 nucleotides downstream are present. A 6X-His tag is present before the stop codon of *SCD6*.	pGP564	This study
pPIR135	pGP *SCD6* with LSm domain deletion: amino acids deleted: 2–93; *scd6*ΔLSm	pGP564	This study
pPIR137	pGP *SCD6* with FDF domain deletion; amino acids deleted: 199–281; *scd6*ΔFDF	pGP564	This study
pPIR139	pGP *SCD6* with RGG domain (including QN rich part) deletion; amino acids deleted: 282–348; *scd6*ΔRGG	pGP564	This study
pPIR141	pGP *SCD6* with RGG domain (excluding QN rich part) deletion, amino acids deletion: 282–310; *scd6*ΔRGG′	pGP564	This study
pPIR170	pGP *SCD6* with R288A, R292A, R294A, R298A, R301A, R304A, R306A, R310A, R316A; *scd6*AMD	pGP564	This study
pPIR150	Yeast shuttle vector, multicopy 2μ plasmid with *URA3* and ampicillin resistance genes	pRS426	[20]
pPIR153	pRS426 expressing *PSP2* under its own promoter	pRS426	[48]
pPIR227	pRS426 *PSP2* with RGG domain deletion; amino acids deleted: 418–577	pRS426	This study
pPIR152	pRS426 expressing *DED1* under its own promoter	pRS426	[18]
pPIR151	pRS426 expressing *SBP1* under its own promoter	pRS426	[20]
pPIR154	Yeast shuttle vector, multicopy 2μ plasmid with *URA3* and ampicillin resistance genes	YEplac195	[62]
pPIR155	YEplac195 expressing *DHH1* under its own promoter	YEplac195	[62]
pPIR121	pPS1372 expressing *GBP2-GFP* under its own promoter, multicopy 2μ plasmid with *LEU* and ampicillin resistance genes	pPS1372	[29]
pPIR291	Addgene: 53462; ADHpr driven chimeric protein with SNC1, N-terminal GFP and C-terminal SUC2 tagged	pRS316	Addgene
pPIR260	Yeast shuttle vector with mCheery ORF, low copy CEN plasmid with *LEU2* and ampicillin resistance genes	pRS315	
pPIR262	pRS315 expressing *SCD6* under its own promoter and C-terminally tagged with Flag-mCherry tag.	pRS315	[32]
pPIR292	pRS315 expressing *PSP2* under its own promoter and C-terminally tagged with Flag-mCherry tag.	pRS315	This study
pPIR168	pGP564 containing *SCD6* under its own promoter. Along with the ORF 550 nucleotides upstream and 225 nucleotides downstream are present. A GFP tag is present before the stop codon of *SCD6*.	pGP564	This study

(*continued on next page*)

Table 3 (*continued*)

Name	Description	Vector	Source
pPIR169	pGP *SCD6*-GFP with RGG domain (including QN rich part) deletion; amino acids deleted: 282–348; *scd6*ΔRGG-GFP	pGP564	This study
pPIR290	Addgene ID: 49424; pRS414 expressing GFP-Atg8; low copy CEN plasmid with *TRP1* and ampicillin resistance genes	pRS414	Addgene

media) unless mentioned otherwise. Growth assays were performed at least three times with comparable results.

4.3. Plating assay

After growing cells in YEP-glucose for >16–20 h, different *GAL:CHC1* transformants were diluted to 0.001 OD_{600}. Three different volumes (50 μl, 100 μl and 200 μl) were plated on YEP-galactose and glucose plates. The galactose containing plates were incubated for 4 days and glucose plates for 7 days before imaging. Colonies were counted and plotted as percentage rescue which was calculated as [(no. of colonies on glucose / no. of colonies on galactose) * 100]. Plating assays were performed at least three times with comparable results which were quantitated.

4.4. Halo assay

The background tester MATa strain (BJ3556) carrying the *sst1–2* allele and the different *GAL:CHC1* transformants were grown on YEP-glucose for 10–12 h and 16–20 h, respectively. The tester strain was diluted to 0.1 OD_{600} and spread onto YEP-glucose plate. Subsequently different *GAL:CHC1* transformant cultures were spotted onto the plate which were incubated for 2–3 days before imaging. MATa and MATα strains labelled in Fig. 8 are wild type strain controls.

4.5. Endocytosis assay

GFP-Snc1 was used either in the plasmid form (pGSS; Addgene 53462) or integrated into the genome as described in Dalton et al. [63]. *GAL:CHC1* cells were grown in 2 % glucose or galactose containing media for 14 h in the form of a primary culture (for the assay in Fig. 7C, cells were grown only in 2 % glucose). A secondary culture was set up in the respective carbon source at 0.1 OD_{600} and the cultures were allowed to grow till 0.4–0.5 OD_{600}. A fraction of cells were pelleted and taken for microscopy analysis.

4.6. Atg8/Scd6 localisation assay

For Atg8 or Scd6 localisation assay, *GAL:CHC1* cells were transformed with respective plasmid pPIR290/pPIR168/pPIR169. Cells were either depleted of Chc1 by using glucose as the carbon source or not by maintaining galactose as the carbon source. A secondary culture was set up in the respective carbon source at 0.1 OD_{600} and the cultures were allowed to grow till 0.4–0.5 OD_{600}. Cells were pelleted and taken for imaging.

4.7. Microscopy analysis

Cells were centrifuged at 14,000 rpm for 15 s and pellets were resuspended in 10 μl of supernatant media. A total of 5 μl of the cell suspensions was spotted on coverslips for live cell imaging. Deltavision Elite microscope system was used to acquire all the images. The system was equipped with softWoRx 3.5.1 software (Applied Precision, LLC) and an Olympus 100×, oil-immersion 1.4 NA objective. Exposure time and transmittance settings for the Green Fluorescent Protein (GFP) channel were 0.25 s and 32 %, respectively. Images were captured as 512 × 512-pixel files with a CoolSnapHQ camera (Photometrics) using 1 × 1 binning for yeast. All the images were deconvolved using standard softWoRx deconvolution algorithms. ImageJ was used to adjust all images to equalize contrast ranges.

Granules were counted using 'Find Maxima' tool from Fiji-ImageJ software. Briefly, the images were converted to 8-bit and the plugin was run. The prominence was set to 30 and the number of granules and cells were counted.

4.8. Rapamycin treatment

For growth assays, the plates contained rapamycin to a final concentration of 1 ng/ml in their respective media. For Supplementary Fig. 4, cells were grown till mid-log phase (0.5–0.6 OD_{600}) and then treated with 100 nM rapamycin for 45 min. The cells were collected and taken ahead for western analysis.

4.9. Western analysis

Cells from the growth assay plate were scrapped and resuspended into 1× SDS running buffer containing 1× PIC and 1 mM PMSF. After addition of 1× SDS-PAGE loading dye, the cells were heated at 100 °C for 5 min. The debris was pelleted using a mini-spin and the supernatant was considered as the whole cell lysate. Western analysis was performed as per the standard protocol. The antibodies used in this study are as follows: Anti-Psp2 (GenScript, SC1195), anti-Pgk1 (Abcam, Ab113687), anti-mCh (Abcam, Ab167453), anti-Ub (BioLegend, 838703), anti-GFP (BioLegend, 902602) anti-mouse (Jackson ImmunoResearch Laboratories, 115-035-003) and anti-rabbit (Jackson ImmunoResearch Laboratories, 111-035-003).

CRediT authorship contribution statement

Mani Garg: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing – review & editing. **Debadrita Roy:** Conceptualization, Methodology, Investigation, Formal analysis. **Purusharth I. Rajyaguru:** Conceptualization, Methodology, Project administration, Resources, Supervision, Writing – original draft, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Purusharth I Rajyaguru reports financial support was provided by DBT-IISc. Purusharth I Rajyaguru reports financial support was provided by Department of Science and Technology. Purusharth I Rajyaguru reports financial support was provided by Wellcome Trust DBT India Alliance. Mani Garg reports a relationship with University Grants Commission that includes: funding grants. Debadrita Roy reports a relationship with DST-INSPIRE that includes: funding grants. The authors declare no competing interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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