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ORIGINAL ARTICLE

Trehalose biosynthetic pathway regulates filamentation response in *Saccharomyces cerevisiae*

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Abstract

Background Diploid cells of *Saccharomyces cerevisiae* undergo either pseudohyphal differentiation or sporulation in response to depletion of carbon and nitrogen sources. Distinct signaling pathways regulate filamentation and sporulation in response to nutrient limitation. How these pathways are coordinated for implementing distinct cell fate decisions in response to similar nutritional cues is an enigma. Although the role of trehalose pathway in sporulation has been extensively studied, it's possible role in pseudohyphal differentiation has been unexplored.

Methods and results Briefly, *tps1* and *tps2* mutants were tested for their ability to form pseudohyphae independently as well as in the background of *GPR1* and *RAS2* mutations. Here, we demonstrate that disruption of *TPS1* but not *TPS2* inhibits pseudohyphae formation. Interestingly, deletion of *GPR1* suppresses the above defect. Further genetic analysis revealed that *TPS1* and *TPS2* exert opposing effects in triggering filamentation.

Conclusion We provide new insights into the role of an otherwise well-known pathway of trehalose biosynthesis in pseudohyphal differentiation. Based on additional data we propose that downstream signaling, mediated by cAMP may be modulated by nutrient mediated differential regulation of *RAS2* by *TPS1* and *TPS2*.

Keywords *TPS1* · *TPS2* · *RAS2* · *GPR1* · Pseudohyphae · Trehalose

Introduction

Organisms have evolved a plethora of developmental and differentiation mechanisms to overcome nutritional deprivation. This involves extensive rewiring of signaling mechanisms to be able to meet the changing metabolic requirements of the cell. Yeast, *Saccharomyces cerevisiae*, undergoes both metabolic as well as morphologic adaptation in order to overcome nutrient deprivation [1, 2]. Thus, diploid cells of *S. cerevisiae* have the potential to either sporulate or achieve pseudohyphal differentiation in response to carbon and/or nitrogen depletion [3–6]. Despite intense investigations, it is still unclear as to how these two developmental processes of pseudohyphae formation and sporulation emerge in response to the common trigger of low nitrogen and low glucose [4, 7, 8]. Interestingly, the cAMP-PKA pathway is involved in both acetate mediated spore formation [9, 10] as well as glucose mediated pseudohyphal differentiation [1, 11].

Signaling for pseudohyphal transition in response to low ammonium occurs via *MEP2*, an ammonium transporter [12]. It was demonstrated that *MEP2* is a transceptor i.e. in addition to signaling, the transport function of Mep2p was necessary for filamentation response [13]. *NPR1*, a *TORC1* effector kinase, positively regulates *MEP2* under conditions of poor nitrogen availability to trigger pseudohyphae formation [14, 15]. Although it has been demonstrated that *MEP2* signals via cAMP [12], the underlying mechanisms have not been fully elucidated. [13]. Of relevance here is the observation that the filamentation defect of a *mep2* mutant is overcome upon addition of cAMP or by constitutively active alleles of *GPA2* or *RAS2* [12].

Pseudohyphal differentiation was originally thought to occur only in response to low ammonium and abundant glucose [5]. In contrast, Iyer et al. [4] demonstrated that in addition to signaling from low ammonium, low glucose signaling was also essential for filamentation response. Glucose mediated signaling for filamentation occurs

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through *GPR1-GPA2* axis [7, 16]. *GPA2* relays the signal to cAMP via adenylate cyclase [7] as does *RAS2* [17]. The activation of either *RAS2* or *GPA2* can elicit the transcriptional changes required for glucose mediated increase of cAMP [2]. It was previously reported that *KRH1/2* interfere with *GPR1-GPA2* coupling thereby inhibiting downstream signaling via cAMP [18]. Iyer and Bhat [19] demonstrated that *KRH1* and *KRH2* are non-redundant and uncovered distinct roles for these two kelch proteins in inducing pseudohyphae by using low glucose. Thus, this study highlighted the significance of glucose limiting condition in filamentation. This was consistent with the observation that *FLO11*, a key gene regulating pseudohyphal differentiation [20], is glucose repressed [21]. Further, *SNF1*, a gene that is required to alleviate glucose repression [22], is also necessary for formation of pseudohyphae [23]. Thus, it is evident that glucose limitation is also a key component of pseudohyphal differentiation process just as it is for spore formation. This compelled us to revisit the possibility of new players in regulating pseudohyphal differentiation in response to depleting ammonium when glucose is limiting.

It has been observed that components of the glucose regulated trehalose biosynthetic pathway [24] are essential for sporulation [25]. The trehalose pathway involves two enzymes namely *TPS1* (trehalose phosphate synthase) and *TPS2* (trehalose phosphate phosphatase). *TPS1* catalyzes the formation of trehalose-6-phosphate (Tre6P) from UDP-Glucose and glucose-6-phosphate [26]. Observations in *Candida albicans* had indicated that disruption of *TPS1* resulted in a decrease in virulence of the strain as well as an inability to form hyphae [27]. Disruption of *TPS2* caused a reduction in virulence without affecting hyphae formation [28]. Further, it was demonstrated that *TPS2* and *GPR1* functioned synergistically in trehalose metabolism as well as virulence [29]. The *TPS* genes have been shown not only to regulate differentiation in *C. albicans* but also to regulate several processes in plants ranging from cell morphology to architecture of inflorescence and other developmental processes [30].

Based on the above and the observation that low glucose is pivotal in pseudohyphae formation as well as in restoring the glycolytic imbalance in a *tps1* mutant, we hypothesized that the trehalose biosynthetic pathway could be involved in filamentation response. Here, we show that *tps1* but not *tps2* mutant is defective in pseudohyphae formation. The use of SLALD (Synthetic low ammonium low dextrose) medium [4] in addition to SLAD (Synthetic low ammonium dextrose) medium [12] enabled the dissection of the independent roles of *TPS1* and *TPS2*. Our results demonstrate that *TPS1* and *TPS2* may regulate *RAS2* differentially depending upon the availability of nutrients, to signal filamentation via cAMP/PKA pathway.

Materials and methods

Media and strains

The strains used in this study are isogenic derivatives of ∑1278b strain (as listed in Online Resource 1). The strains were constructed using standard methods [31]. Genes were disrupted (primers listed in Online Resource 2) using marker based polymerase chain reaction (PCR) methods [32]. For all mutants used in this study, the entire coding region was replaced by the disruption cassette. Double disruptants were generated by mating the individual mutants followed by sporulation and segregation of haploids. The haploid strains were diploidized using mating type switching induced by HO plasmid.

Pseudohyphal growth assay

Synthetic low ammonium dextrose (SLAD) medium (50 µM ammonium sulphate and 2% glucose) or Synthetic low ammonium low dextrose (SLALD) medium (50 µM ammonium sulphate and 0.05% glucose) were used to score pseudohyphal growth [3, 4, 33]. Cells were spread for single colonies and incubated for 6 days at 30 °C unless mentioned otherwise. Images of colonies were captured at × 10 magnification using a Nikon Coolpix 8400 camera attached to a Nikon TS 100 microscope. Three independent colony images which are representative of at least three experimental repetitions are shown.

Spotting assay

The glucose growth phenotype was determined using the spotting assay. Cells were grown to 0.5 OD_{600}, washed twice with sterile distilled water and five-fold serial dilutions were spotted on yeast extract peptone dextrose (YPD) agar (1% yeast extract, 2% peptone and 2% glucose) or yeast extract peptone galactose (YPGal) agar (1% yeast extract, 2% peptone and 2% galactose). YPGal medium served as a control. Images were captured after 2 days of incubation.

Western blot analysis

Crude cell extracts were prepared as described by [34]. Briefly, cells were grown to OD_{600} of 3–4 in YPD medium and then harvested. For analysis in SLALD medium, cells grown to 3–4 OD_{600} were collected by centrifugation, washed twice with sterile distilled water, re-suspended in SLALD and transferred to SLALD (one-fifth volume) and incubated for an additional 8 h before being harvested. 200 mg cells were lysed with 0.2 g of glass beads after

adding lysis buffer as described [34]. Centrifugation at 8000 rpm for 5 min yielded the crude protein extract used for western blot analysis. 20 µg protein was loaded. The blots were developed with antibodies against yeast Ras2p from Santacruz Biotechnology Inc. For the loading control, Glucose 6-phosphate dehydrogenase (G6PDH), antibodies from Sigma-Aldrich were used. Both the anti-mouse pAb (for Ras2p) as well as the anti-rabbit pAb (for G6PDH) were alkaline phosphatase conjugates obtained from Sigma-Aldrich. Quantification of western blot data was carried out using ImageJ analysis [35]. The final normalized values i.e. the ratio of the net bands (after background subtraction) namely the net protein band to the net loading control band are represented in the graph. The data represented is an average of three experimental repetitions with the error bars indicating the standard deviation. Variation in protein loaded is less than 10% based on quantification of G6PDH band intensity (see Online Resource 3).

Results

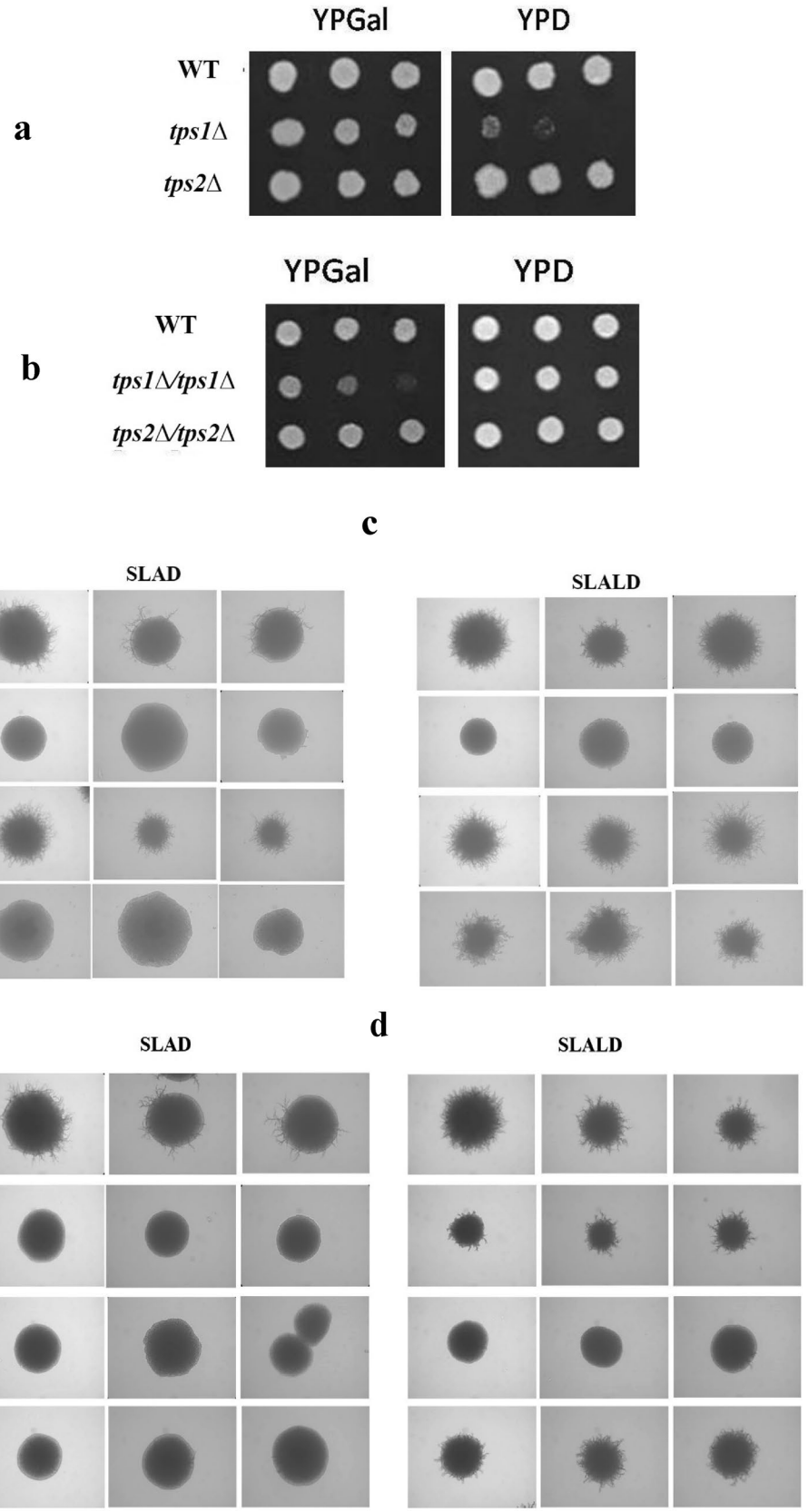
Glucose growth defect of the *tps1* mutant is both strain as well as ploidy dependent

It is well established that mutation in *TPS1* causes a growth defect on fermentable carbon sources [34, 36]. This phenotype was observed in a haploid i.e. *tps1::TRP1* of the W303 strain background [34]. In the study by van Heerden et al. [36], the diploid strain of S288c i.e. *tps1::G418/tps1::G418* was used (personal communication). Suzuki et al. [37], reported that transposon insertion mutation at the *TPS1* locus i.e. *tps1::mTn3* was lethal in the ∑1278 strain background. Contrary to this finding, we observed that the *tps1* mutant in the ∑1278 background i.e. *tps1::G418* was viable. In an attempt to determine the glucose growth phenotype of the ∑1278 *tps::G418* mutants, we analyzed growth on glucose as well as galactose media. Galactose medium was used as a control condition. We observed that the *tps2* mutant did not exhibit any growth defect. Although the *tps1Δ* haploid strain in the ∑1278 background had a growth defect (Fig. 1a), the diploid strain was able to grow on glucose (Fig. 1b). Thus it is evident that glucose growth phenotype of the *tps1* mutant strains varies with different lineages as well as the ploidy status of the cell. Surprisingly, we observed that the diploid *tps1* mutant exhibited a growth defect on galactose. While it is difficult to explain this observation, we surmise that this may be a deleterious effect caused because glucose-6-phosphate formed from galactose accumulates above a threshold in the cell, in the absence of *TPS1*.

TPS1 is required for filamentation response

Trehalose synthesis in yeast occurs in response to adverse environmental conditions, including nutritional stress [38, 39]. Since filamentation occurs in response to nutrient limitation, we hypothesized that *TPS1* and/or *TPS2* may be involved in regulating this response. Therefore, independent colonies of *tps1* and *tps2* as well as the *tps1tps2* double mutant were analyzed for filamentation in SLAD as well as SLALD media (Fig. 1c). As expected, *tps1* was defective in pseudohyphae formation. In contrast, the *tps2* mutant had no filamentation defect indicating that *TPS1* but not *TPS2* is required for this response. However, the *tps1tps2* double mutant formed pseudohyphae only in SLALD but not SLAD medium. A possible explanation for this would be that *TPS2* is required to alleviate glucose mediated repression of filamentation. In SLALD medium, this requirement is of no consequence as the glucose concentration is such that repression does not occur. Further, strains heterozygous for the *TPS1* as well as the *TPS2* loci exhibit different phenotypes as compared to the strains homozygous for the same loci (Fig. 1d), indicating that the effective concentrations of intermediates of the trehalose synthesis pathway may play a role.

The next step was to determine the pathway through which *TPS1* acts. Studies in *C. albicans* have demonstrated that *TPS* enzymatic activity was higher in the *gpr1*Δ strain [40]. To determine if there was any genetic interaction between *GPR1* and *TPS1* or *TPS2*, in *S. cerevisiae*, pseudohyphal growth of *gpr1tps1* and *gpr1tps2* mutants was monitored. Both the haploid as well as the diploid strains of the *gpr1tps1* double mutant exhibited a growth defect on high glucose (data not shown). It is possible that in the absence of both *TPS1* as well as *GPR1*, in an ammonium deficient environment, the cell experiences severe effects of perceived absence of glucose, resulting in the growth defect. In SLALD medium, however, the filamentation defect of *tps1* mutant was overcome upon disruption of *GPR1* (Fig. 2a, right panel and Fig. 2b), indicating that mutation in *GPR1* was epistatic over mutation in *TPS1*, in signaling for pseudohyphae. This effect is probably mediated via the cAMP-PKA pathway as extraneous addition of cAMP overcomes the filamentation defect of the *tps1* mutant in SLALD medium (Fig. 3, left panel). Further, our observations indicate that mutation in *TPS2* is epistatic over mutation in *GPR1* in SLAD medium (Fig. 2a, compare top and bottom panels). This is in contrast to that observed in *C. albicans* where mutation in *TPS2* and *GPR1* result in a synergistic effect in suppression of filamentation [29]. The only plausible explanation for this phenotype is that accumulation of Tre6P probably triggers filamentation through some unknown mechanism. However, this is counter-intuitive given that pseudohyphae


a
YPGal
YPD
WT
tps1Δ
tps2Δ
b
YPGal
YPD
WT
tps1Δ/tps1Δ
tps2Δ/tps2Δ
c
SLAD
SLALD
WT
tps1Δ/tps1Δ
tps2Δ/tps2Δ
tps1Δ/tps1Δ
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d
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tps2Δ/TPS2
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◂**Fig. 1** Effect of carbon source on *tps* mutants. Glucose growth phenotype of **a** haploid and **b** diploid *TPS* mutants. Three dilutions (five-fold) were spotted. Effect of zygosity on pseudohyphal growth phenotype. Colony images of **c** homozygous and **d** heterozygous *TPS* mutants are shown

formation is favoured by low glucose conditions [4] while Tre6P promotes glucose repression [41].

TPS1 and *TPS2* have a differential role in regulating *RAS2* depending upon nutrient availability

It has been observed that *tps1* mutation results in activation of *RAS2* [34]. However, the effect of *tps2* mutation on *RAS2* is not known. In order to determine whether the effect on *RAS2* is limited only to *TPS1* or does *TPS2* also have a role and whether this effect led to regulation of pseudohyphal growth, filamentation was monitored in both *ras2tps1* as well as *ras2tps2* double mutants. Interestingly, disruption of *tps2* but not *tps1* restored pseudohyphae formation in the *ras2* mutant (Fig. 3b). Further, pseudohyphae formation in the *ras2tps2* double mutant was enhanced in SLALD as compared to SLAD medium (Fig. 3b, compare left and right panels). Therefore, expression of Ras2p was monitored under nutrient complete (YPD medium) as well as nutrient limiting (SLALD medium) conditions in the diploid strains of *tps1*Δ and *tps2*Δ. Diploid strains were used for this experiment to ensure that observations are a more accurate reflection of the role played by *TPS* genes, as pseudohyphal differentiation is exhibited only by diploid cells. Contrary to earlier reports in the haploid *tps1* mutant where *RAS2* is activated in glucose causing the growth defect [34], we observed that Ras2p expression was reduced in the diploid *tps1* mutant in YPD medium (Fig. 4a and c). This meant one of two possibilities; either that regulation of Ras2p by *TPS1* resulted in opposite effects in the haploid versus the diploid cell or that Ras2p is upregulated upon *TPS1* disruption in W303 strain [34] while it is downregulated in the ∑1278 strain background (this study). Whatever the reason, our observations indicate that this mutant is unable to form pseudohyphae. Since, Ras2p expression is reduced in high glucose in a diploid *tps1* mutant, the defect in pseudohyphae formation is probably due to lower levels of Ras2p. Further, our data indicates that in YPD medium, Ras2p expression is upregulated in a *tps2* mutant. This suggests that opposing effects exerted by *TPS* and *TPS2* may be co-ordinated to effect signaling mediated through *RAS2*. In SLALD medium, however, Ras2p expression is decreased in *tps2* but not *tps1* mutant (Fig. 4b and c). In a *tps1* mutant, even though Ras2p is expressed in SLALD medium, pseudohyphae formation is inhibited suggesting that *TPS1* also regulates pseudohyphal growth in a *RAS2* independent manner. To summarize, our observations indicate that *TPS1* and *TPS2* possibly regulate *RAS2* differentially under conditions of nutrient abundance or depletion, to trigger pseudohyphal differentiation.

To determine whether exogenous cAMP could rescue the defect caused by reduction in Ras2p levels mediated through *TPS1*, effect of extraneous addition of cAMP was monitored in the *tps1*, *tps1ras2* as well as the *ras2* mutant as a control (Fig. 3a). As discussed in the earlier section, the pseudohyphal defect of the *tps1* mutant was rescued on addition of cAMP in SLALD but not SLAD medium (Fig. 3a, left panel). The filamentation defect of the *ras2* mutant was restored on exogenous cAMP addition in SLAD medium only (Fig. 3a, middle panel) while the *ras2tps1* double mutant remained defective (Fig. 3a, Right panel). Surprisingly, addition of cAMP did not rescue the filamentation defect of either the *ras2* mutant or the *ras2tps1* double mutant on SLALD medium (Fig. 3a, middle and right panels). These results indicate the possibility that signaling from glucose is necessary in addition to that from cAMP in the absence of *RAS2*.

Discussion

TPS1/2 regulate dimorphic transition and thereby virulence in *Candida albicans* [28] as well as *Magnaporthe grisea* [42]. There is evidence to show that *TPS* genes regulate multiple processes involved in growth and development in plants as well [30, 43]. In *S. cerevisiae*, although there is a large body of data available on the deleterious effects of *TPS1* or *TPS2* mutations [36, 44–46], how these effects are generated is unclear. Gibney et al. [47] demonstrated that phenotypes of the *tps1* mutant could not be reversed by simply increasing intracellular concentration of trehalose. This meant that the phenotypes were not due to the depletion of intracellular trehalose concentration per se. It is possible that the enzymes of trehalose pathway exert a more complex metabolic effect on the physiology of the cell [47, 48].

Our observations imply that components of the trehalose biosynthetic pathway may determine whether the cell goes into pseudohyphal differentiation or sporulation in response to nutritional stress by regulation of cAMP and thereby downstream signaling. This is in accordance with an earlier observation that intracellular concentration of trehalose correlated with pseudohyphae formation [4]. Based on our results, we propose a model (Fig. 5) wherein, the trehalose biosynthetic pathway regulates pseudohyphal differentiation in multiple ways. We propose that *TPS1* functions in two ways. On one hand it acts by activation of *RAS2*. On the other hand, it positively regulates cAMP in a *RAS2* independent manner. This is supported by our observation that the *tps1* mutant is unable to form pseudohyphae in SLALD medium (Fig. 1c) although expression of *RAS2* is not reduced in SLALD medium in this strain (Fig. 4). This

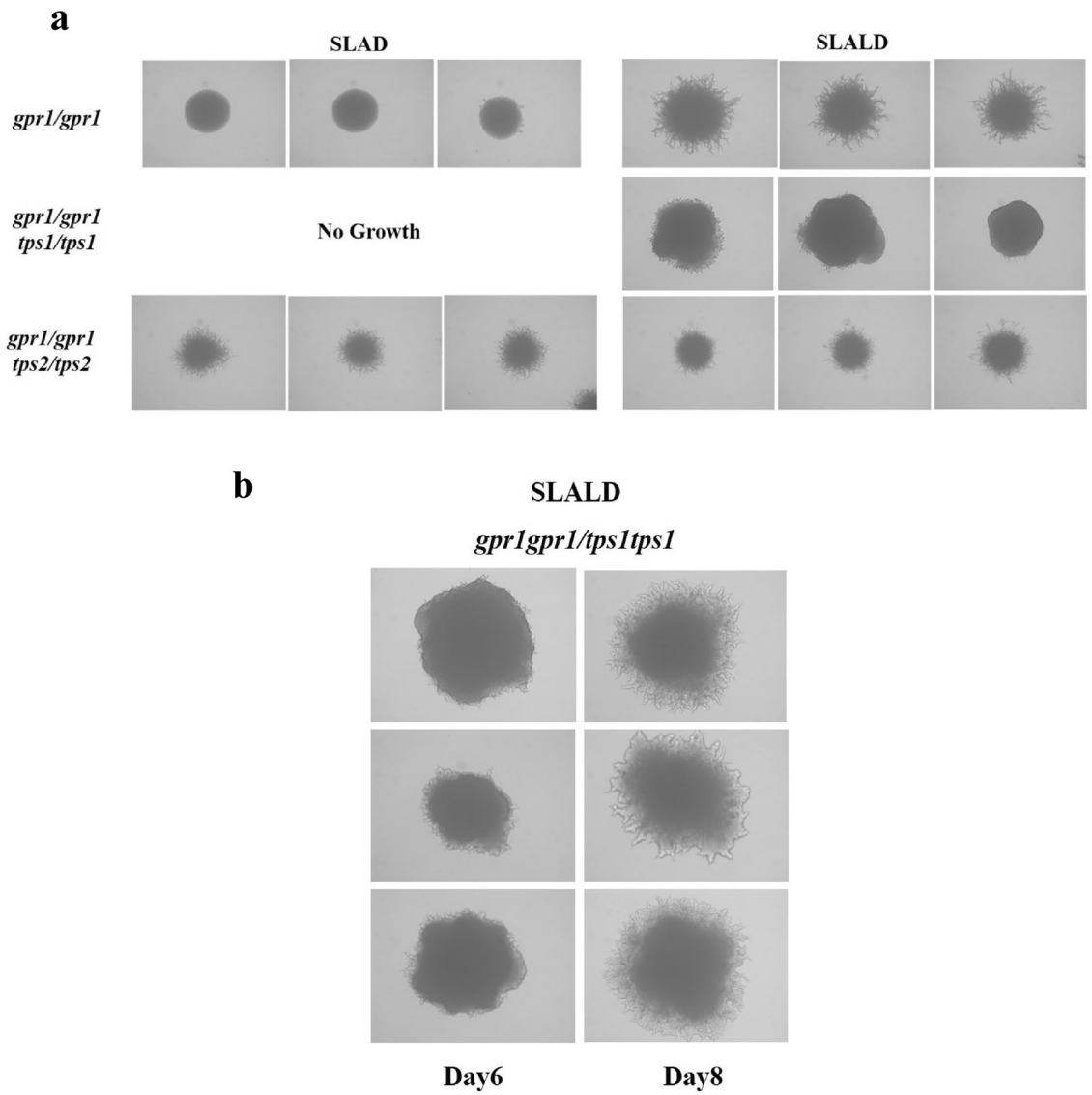

Fig. 2 Effect of *GPR1* disruption on filamentation in the background of *TPS* mutations. **a** Pseudohyphae formation at the end of 6 days of incubation. **b** Filamentation response of the *gpr1tps1* double mutant at the end of 6 or 8 days of incubation on SLALD medium. The photographs at the end of 8 days are not of the same colonies as that shown at the end of 6 days

RAS2 independent effect of *TPS1* could be mediated via *GPR1* or through a hitherto unidentified mechanism.

TPS2 however, appears to play a predominant and more complicated role. While our observations suggest that *TPS2* is not involved in pseudohyphae formation, mutation in *tps2* overcomes the filamentation defect of *tps1* mutant in SLALD (Fig. 1c, bottom right), that of *gpr1* mutant in SLAD (Fig. 2a, bottom left) and that of *ras2* mutant in SLAD as well as SLALD media (Fig. 3b). According to our model, it is likely that *TPS2* exerts a regulatory effect by suppressing *RAS2* in addition to exerting a metabolic or regulatory effect mediated through Tre6P. It has been reported that Tre6P promotes glucose repression by suppressing genes required for gluconeogenesis [41]. We propose that this is the basis for the filamentation response of the *tps1tps2* mutant (Fig. 1c, compare bottom left and right panels). In this double mutant, it is possible that Tre6P accumulates because of *tps2* mutation and prevents pseudohyphae formation in SLAD medium. However, in SLALD medium, the glucose concentration is below that required for glucose repression and the *tps1tps2* double mutant is able to overcome the filamentation defect by virtue of the general effect of alleviation of glucose repression. Our observation that filamentation defect of the *gpr1* mutant is overcome upon *tps2* mutation (Fig. 2a, compare top and bottom of left panel) supports the idea that Tre6P exerts a positive regulatory effect on pseudohyphae formation. However, this argument is counter-intuitive as Tre6P is known to promote

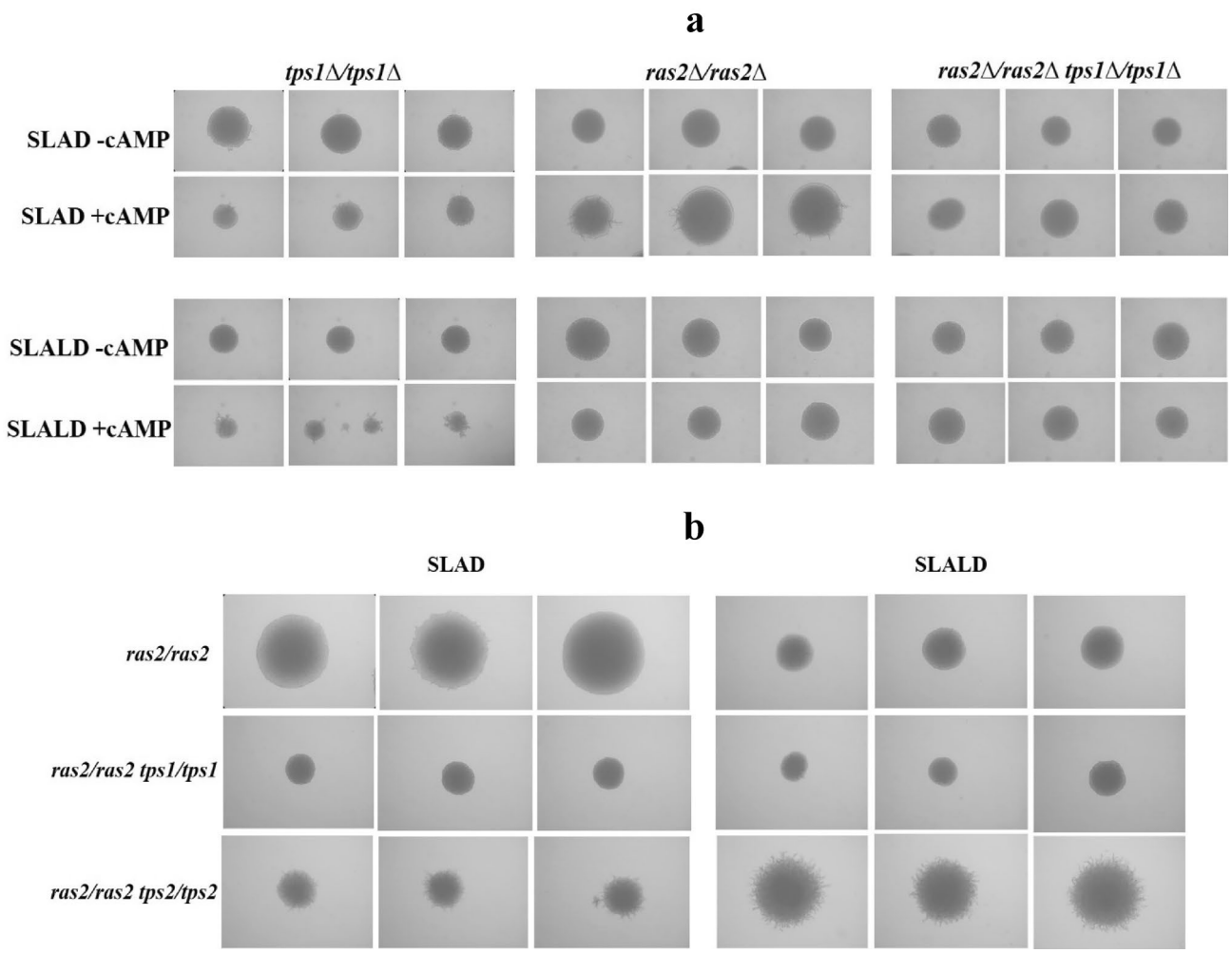

Fig. 3 **a** Effect of extraneous addition of 1 mM cAMP on pseudohyphal differentiation of *ras2* disruption in the background of *tps* mutations. **b** Effect of *RAS2* disruption on pseudohyphae formation in the background of *TPS* mutations

glucose repression which is a condition that inhibits filamentation and probably not likely to occur. Thus, the role of Tre6P if any, is not clear. The positive effect of *tps2* mutation in the background of *ras2* mutations is possibly due to fall in trehalose levels. This is based on our earlier observation that lower trehalose level in the cell correlates with pseudohyphae formation [4]. Thus, our data suggests that both Tps1p and Tps2p could be bi-functional proteins exerting both a regulatory as well as a metabolic effect.

Based on the results of western blot, we further hypothesize that *TPS1* and *TPS2* coordinate to regulate the expression of Ras2p based on glucose availability (Fig. 5) and thereby affect the downstream concentration of cAMP, to trigger filamentation. That glucose is a key nutrient in this signaling is strengthened by our observations on SLALD medium supplemented with cAMP (Fig. 3a, middle panel), where exogenous cAMP addition is able to rescue filamentation in SLAD but not SLALD medium. This data suggests that signaling from glucose is essential in addition to that from cAMP in the absence of *RAS2*, to trigger pseudohyphae formation. It is possible that this signal is transmitted through *TPS1* either via *GPR1-GPA2* axis or directly via glucose. All things considered, our data clearly implies that *RAS2* signaling in the context of pseudohyphal differentiation needs further evaluation. This idea is supported by the existence of conflicting reports on the filamentation response of the *ras2* mutant [49–51]. Monitoring the effect of perturbations in ammonium and glucose sensing pathways on *RAS2* expression in the *tps* mutants could shed some light on the possible mechanism mediated through the trehalose biosynthetic pathway.

To summarize, the results of this study shed light on the metabolic basis of cellular differentiation effected via *TPS1/2*. However, it is still unclear whether the observed effects are mediated through trehalose, the metabolic intermediates or through the *TPS1/2* encoded proteins per se or

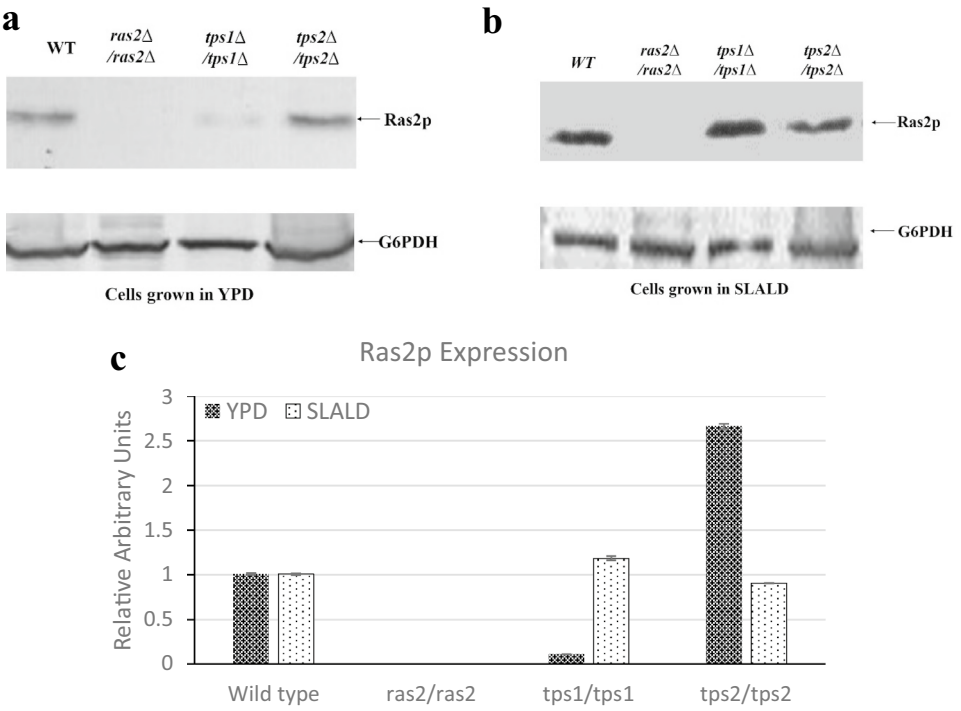

Fig. 4 Expression of Ras2p in the *TPS* mutants. **a** In nutrient rich YPD medium. **b** In nutrient deficient SLALD medium. **c** Western blot quantification data. The bars represent the ratio of the normalized values i.e. background-subtracted Ras2p to background-subtracted G6PDH, error bars show standard deviation (n = 3)

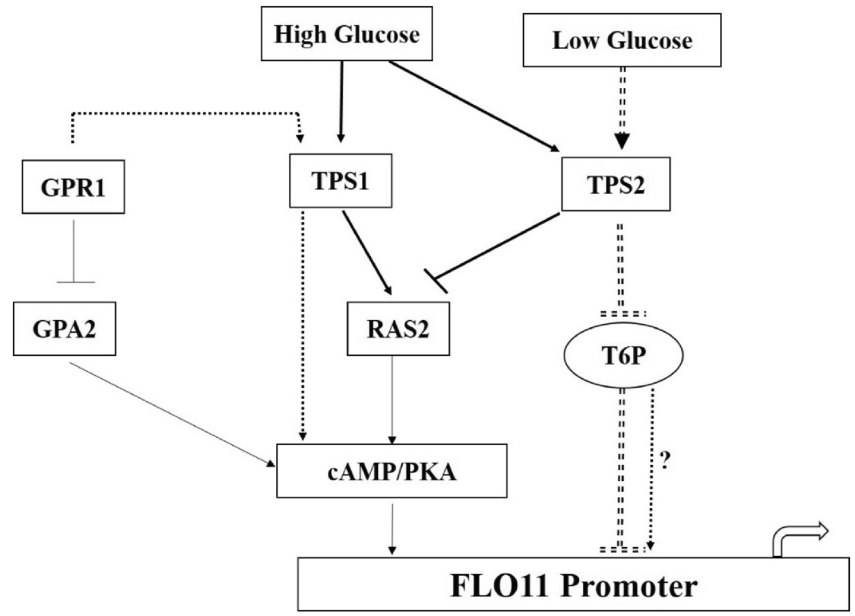

Fig. 5 Schematic illustration of signaling and possible metabolic effect mediated by the trehalose biosynthetic pathway components. Extracellular glucose availability determines regulation of *RAS2* by *TPS1* and *TPS2* which in turn regulates cAMP effects. In addition, *TPS1* exerts an independent effect probably mediated through *GPR1* while *TPS2* possibly alleviates glucose repression by directing the metabolic flux from Tre6P to trehalose. Bold lines represent interactions based on our observations, dotted lines represent proposed interactions, compound dotted lines represent proposed glucose mediated signaling via *TPS2*. Key: → Activation ⊣ Inhibition

a combination thereof. One way of addressing this would be to isolate missense mutations in *TPS1* that specifically knock off the enzyme function without affecting pseudohyphae formation. Understanding the mechanistic basis of this metabolic effect is fundamental for elucidating the mechanism of fungal virulence. The wider and more significant implication of the study is in understanding the metabolic basis of differentiation in response to nutrient limitation as the nutrient dependent *TOR*, *SNF1* and PKA mediated signalling pathways are highly conserved amongst eukaryotes.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-022-07792-5.

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Author contributions Both authors contributed to the study conception and design. Material preparation, data collection and analysis were

performed by Dr. RI. The first draft of the manuscript was written by Dr. RI. Both authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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