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ORIGINAL ARTICLE

KRH1 and *KRH2* are functionally non-redundant in signaling for pseudohyphal differentiation in *Saccharomyces cerevisiae*

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Abstract Diploid cells of *Saccharomyces cerevisiae* undergo pseudohyphal differentiation in response to nutrient depletion. Although this dimorphic transition occurs due to signals originating from carbon and nitrogen limitation, how these signals are coordinated and integrated is not understood. Results of this study indicate that the pseudohyphal defect of the *mep2*Δ mutant is overcome upon disruption of *KRH2*/*GPB1* but not *KRH1*/*GPB2*. Further, the agar invasion defect observed in a *mep2* mutant strain is suppressed only by deleting *KRH2* and not *KRH1*. Thus, the results presented indicate that *MEP2* functions by inhibiting *KRH2* to trigger filamentation response when glucose becomes limiting. Biochemical data and phenotypic response to glucose replenishment reveal that *KRH1* and *KRH2* are differentially regulated by glucose and ammonium to induce pseudohyphae formation via the cAMP-PKA pathway. In contrast to the current view, this study clearly demonstrates that, *KRH1* and *KRH2* are not functionally redundant.

Keywords *KRH1* · *KRH2* · *MEP2* · Pseudohyphae

Introduction

Evolutionary considerations predict that fermentation, an energy generating pathway in which ATP yield is low but operates at a high rate, has a selective advantage when organisms compete for shared energy sources. On the other hand, respiration, a pathway that offers high ATP yield at a lower rate, lends itself for co-operative utilization of energy resources and may allow the cells to spatially organize into defined structures (Pfeiffer et al. 2001). A classic example that seems to follow this dictum is the fermentative mode of growth exhibited by the yeast *Saccharomyces cerevisiae* (Pfeiffer and Schuster 2005) in presence of abundant glucose. It switches over to respiration as glucose concentration decreases (van Dijken et al. 1993). In addition to this metabolic switch, it has been demonstrated that diploid cells of *Saccharomyces cerevisiae* exhibit a morphologic switch when exposed to abundant glucose but low ammonium, forming a spatially defined structure called pseudohypha at the end of 6 days of incubation (Gimeno et al. 1992; Lorenz and Heitman 1997). Recent evidence, however, has indicated that pseudohyphal differentiation occurs only when the glucose concentration is reduced to a level that results in a switch to the respiratory mode of growth (Iyer et al. 2008). This is consistent with the evolutionary considerations discussed above. That the respiratory pathway of energy production favors pseudohyphal differentiation is supported by other independent observations. For example, petite cells that are incapable of respiration are defective in pseudohyphal differentiation (Kang and Jiang 2005; Jin et al. 2008). *SNF1*, required to de-repress mitochondrial function as the glucose concentration decreases is necessary for pseudohyphal transition as well (Kuchin et al. 2002). Thus, the fundamental question is how does *S. cerevisiae* signal the shift from fermentative to respiratory mode of growth to the downstream elements that induce the pseudohyphal differentiation?

PKA mediated signaling is pivotal in regulating growth, filamentation and stress response depending upon nutrient

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availability (reviewed in Rubio-Texeira et al. 2009; Leadsham and Gourlay 2010; Papp et al. 2016). For example, rapid cell division in response to abundant glucose is mediated through the cAMP-PKA pathway (Smets et al. 2010) which also activates the expression of *FLO11*, an essential and the primeval target for filamentation response in *S. cerevisiae* (Rupp et al. 1999). However, *FLO11* expression is repressed under high glucose through the well known glucose repression signaling mechanism (Gagiano et al. 1999; Kuchin et al. 2002). Thus, *FLO11* is under positive regulation mediated by PKA pathway and negative control exerted by excess glucose. The paradox, however, is how does the alleviation of glucose repression of *FLO11* which occurs as the glucose concentration decreases, co-ordinate with the signaling machinery that sustains the PKA mediated positive signal for filamentation. In other words, upon glucose depletion, what are the signaling mechanisms that trigger pseudohyphal differentiation?

MEP2, an ammonium transceptor, was shown to be essential for nitrogen mediated signaling of pseudohyphal differentiation (Lorenz and Heitman 1998). The same authors showed that a constitutive allele of *GPA2* overcomes the filamentation defect of a *mep2* mutant indicating that *MEP2* triggers pseudophyphal differentiation by activating the cAMP-PKA pathway (Lorenz and Heitman 1998). A large body of data is available on *MEP2* mediated signaling (Boeckstaens et al. 2007, 2014; Rutherford et al. 2008) including a recent study where the crystal structure of Mep2p has been reported (van den Berg et al. 2016). Nonetheless, how *MEP2* signals the PKA pathway in a *GPA2* dependent fashion in response to low glucose and low ammonium remains unclear.

GPR1, a glucose sensing GPCR protein, which was identified in a two-hybrid screen using the Gα protein *GPA2* as bait (Yun et al. 1997; Xue et al. 1998; Kraakman et al. 1999) was also shown to trigger filamentation via the cAMP-PKA pathway (Lorenz et al. 2000). Subsequently, it was proposed that the redundant kelch repeat proteins *KRH1* (*GPB1*) and *KRH2* (*GPB2*) prevent pseudohyphal differentiation through negative regulation of PKA signaling by inhibiting *GPA2* function (Harashima and Heitman 2002; Battle et al. 2003). These proteins, thought to be Gβ mimics (Harashima and Heitman 2002), were shown to inhibit *RAS* signaling (Harashima et al. 2006). Krh/Gpb proteins seem to interconnect signaling from *GPR1* and *RAS* to regulate the cAMP-PKA pathway by way of their interaction with *GPA2* as well as *IRA1*/2 (reviewed in Cullen and Spargue 2012). However, there are opposing reports on the interaction between *KRH1*/2 and *IRA1*/2 where on one hand evidence supports the possibility of *IRA* being stabilized by *KRH* (Harashima et al. 2006) while on the other hand it appears that *KRH* facilitates degradation of *IRA* (Phan et al. 2010). Deletion of either *KRH1*/*GPB2* or *KRH2*/*GPB1* resulted in enhanced filamentation response. It was demonstrated that *KRH1*/2 negatively regulated filamentation response by inhibiting *GPA2-GPR1* coupling (Harashima and Heitman 2005). In contrast, another study (Peeters et al. 2006) showed that *KRH1*/2 increased the interaction between the catalytic and regulatory subunit of PKA which was later shown to be achieved through the inhibition of PKA mediated phosphorylation of *BCY1* by *KRH1*/2 (Budhwar et al. 2010). A subsequent study showed that *KRH1*/2 mediated effect on *BCY1* is an indirect consequence of their effect on PKA (Budhwar et al. 2011). Peeters et al. (2006) also showed that the deletion of PKA catalytic subunits abolished the down regulation of glycogen and trehalose accumulation observed upon *KRH1*/2 deletion, suggesting that *KRH1*/2 inhibit PKA. Based on these observations, and contrary to the previous report (Harashima and Heitman 2005), it was proposed (Peeters et al. 2006) that *GPA2* activates PKA by inhibiting *KRH1*/2. Although there is evidence to indicate that *KRH1*/2 function is independent of cAMP (Lu and Hirsch 2005), an earlier report showed that *KRH1*/2 deletion resulted in elevation of intracellular cAMP concentrations (Harashima and Heitman 2002). Counter to the above studies which considered *KRH1*/2 to be functionally redundant in pseudohyphal differentiation, the only study that demonstrated distinct functions for *KRH1* and *KRH2* was with regard to their role in *IRA2* mediated *RAS* signaling (Phan et al. 2010).

It has been suggested that the promiscuous interaction of *KRH1*/2 may facilitate the coupling of nutrient sensing to cAMP production to execute diverse responses (Harashima and Heitman 2005). It was also proposed that in addition to *GPA2*, the activity of *KRH1*/2 may be controlled by other factors, and thus *KRH1*/2 may play a role in integrating the diverse signals that impinge on PKA (Peeters et al. 2006). Based on our previous observation that cells committed to pseudohyphal differentiation become refractory to glucose mediated repression of pseudohyphae formation (Iyer et al. 2008) and on the ability of *KRH1*/2 to interact with *GPR1, GPA2* as well as PKA, we hypothesized that *KRH1* and *KRH2* could play distinct roles. We surmised that such a differential role could be uncovered only on the induction of pseudohyphae in synthetic low ammonium and low dextrose (SLALD) medium (Iyer et al. 2008) but not in synthetic low ammonium dextrose (SLAD) medium (Gimeno et al. 1992). Our results clearly demonstrate that *KRH1* and *KRH2* are non-redundant with respect to their function in filamentation response. Further, our observations indicate that *MEP2* probably activates the cAMP-PKA pathway through suppression of *KRH2* but not *KRH1*. We suggest that these distinct regulatory mechanisms of *KRH1* and *KRH2* play an important role in processing and conveying the signal to the downstream elements in response to

glucose as well as ammonium depletion to elicit the filamentation response.

Materials and methods

Yeast strains and plasmids

Strains used in this study are isogenic derivatives of Σ1278 strain and listed in Table 1. Gene deletions were carried out using marker based polymerase chain reaction (PCR) methods as described (Wach et al. 1994). Plasmid pRI3 (for *KRH1* over-expression) was constructed by cloning 6his-tagged *KRH1* as a SmaI-PstI fragment under GPD promoter in p426GPD vector. Plasmid pRI4 (for *KRH2* over-expression) was constructed using recombination mediated in vivo ligation in yeast as described earlier (Oldenburg et al. 1997) to clone 6his-tagged *KRH2* under GPD promoter in p426GPD vector.

Assay for filamentation response

Standard methods were used to score pseudohyphal growth (Gimeno et al. 1992; Lorenz and Heitman 1997). Briefly, diploid yeast cells pre-grown in YPD or synthetic uracil drop-out media were washed and then spread on Synthetic low ammonium dextrose (SLAD) or synthetic low ammonium low dextrose (SLALD) media as indicated in the figures. Representative colonies were photographed at the end of 6 days of incubation at 30 °C. Glucose replenishment assay was carried out as described earlier (Iyer et al. 2008). In the current study, however, the wild-type or mutant strains transformed with the vector and exposed to glucose replenishment served as the corresponding experimental controls. Images of the colonies were captured at the end of 4 days of incubation at 30 °C after glucose addition. All experiments were repeated at least three times before representative colonies were photographed. Colonies were photographed at 10× magnification using a Nikon Coolpix camera attached to a Nikon microscope. For the invasive growth assay, haploid strains were patched onto Yeast extract Peptone Dextrose (YPD) agar and the plates were photographed before as well as after washing the agar surface as described earlier (Kuchin et al. 2002).

β galactosidase assay

FLO11 activity was measured using a P_{FLO11}-*lacZ* construct with the full-length promoter sequence as reported earlier (Rupp et al. 1999). β galactosidase assay using chlorophenyl β-D-galactopyranoside as a substrate was employed as described (Som et al. 1988). Assays were done in triplicates and repeated at least five times. Statistical significance of the result was determined using the student's *t* test.

Western blot analysis

Cells from confluent 20 h old cultures in synthetic uracil drop-out medium were washed twice, transferred to SLAD or SLALD medium as required and grown for 12–14 h. The 6his-tagged Krh1/2p present in the cell free extract of the above cultures was allowed to bind to Ni–NTA agarose beads from QIAGEN. The bound fraction was eluted and subjected to western blot analysis using anti-His antibody from Santa Cruz Biotechnology Inc., as per the manufacturer's recommendations. The enrichment of Krh1/2p was required as we were unable to detect either Krh1p or Krh2p

Table 1 List of strains used in this study

Strains	Genotype	Reference
MLY40α (WT)	ura3-52 MATα	Lorenz and Heitman (1997)
MLY61 (WT)	ura3-52/ura3-52 MATa/α	Lorenz and Heitman (1997)
MLY108	Δmep2::LEU2/Δmep2::LEU2 ura3-52/ura3-52 Δleu2::hisG/Δleu2::hisG MATa/α	Lorenz and Heitman (1998)
MLY108α	Δmep2::LEU2 ura3-52 Δleu2::hisG MATα	Lorenz and Heitman (1998)
THY204	Δkrh1::G418/Δkrh1::G418 ura3-52/ura3-52 MATa/α	Harashima and Heitman (2002)
THY204α	Δkrh1::G418 ura3-52 MATα	Harashima and Heitman (2002)
THY206	Δkrh2::hph/Δkrh2::hph ura3-52/ura3-52 MATa/α	Harashima and Heitman (2002)
THY206α	Δkrh2::hph ura3-52 MATα	Harashima and Heitman (2002)
THY212	Δkrh1::G418/Δkrh1::G418 Δkrh2::hph/Δkrh2::hph ura3-52/ura3-52 MATa/α	Harashima and Heitman (2002)
RIY116	Δmep2::LEU2/Δmep2::LEU2 Δkrh1::G418/Δkrh1::G418 ura3-52/ura3-52 MATa/α	This study
RIY016α	Δmep2::LEU2 Δkrh1::G418 ura3-52 MATα	This study
RIY117	Δmep2::G418/Δmep2::G418 Δkrh2::hph/Δkrh2::hph ura3-52/ura3-52 MATa/α	This study
RIY017α	Δmep2::G418 Δkrh2::hph ura3-52 MATα	This study

Strains are isogenic derivatives of ∑1278b

Table 2 Relative expression of *FLO11* in different strains

A					B	
Strains	WT	mep2Δ	mep2Δkrh1Δ	mep2Δkrh2Δ	mep2Δkrh2Δ Vs mep2Δkrh1Δ	mep2Δkrh2Δ Vs WT
Fold increase*	1.5	Nil	Nil	3.1	6.7	0.7

Part A – The numbers indicate ratios of the specific activity of β galactosidase in SLALD medium relative to that in SLAD medium. Part B – The numbers denote ratios of the specific activity in the *mep2Δkrh2Δ* mutant relative to that of the *mep2Δkrh1Δ* mutant or WT as indicated in the table, in SLALD medium

*Significant at $p < 0.05$

in whole cell extracts obtained from cells grown on synthetic uracil drop-out medium. The western blot analysis was performed multiple times and a representative result is presented.

Results

MEP2 signals for pseudohyphal differentiation by inhibiting *KRH2*

Iyer et al. demonstrated that pseudohyphae formation is a response to ammonium depletion only when glucose is limiting (Iyer et al. 2008). The authors further showed that *GPR1* suppressed pseudohyphae and that the filamentation defect of a *mep2* mutant is overcome upon disruption of *GPR1* in low glucose. Although experimental evidence indicates that *MEP2* functions upstream of *GPA2* to trigger pseudohyphal response through the cAMP-PKA pathway (Lorenz and Heitman 1998), no signaling partner of *MEP2* has been identified till date. Based on the above and the observation that *KRH1/2* inhibit pseudohyphae through *GPR1*-*GPA2*-cAMP signaling (Battle et al. 2003; Harashima and Heitman 2002, 2005; Peeters et al. 2006), we surmised that *MEP2* may regulate filamentation response through *KRH1/2*.

To test this hypothesis, *KRH1* and *KRH2* were independently disrupted in a *mep2Δ* mutant strain to monitor pseudohyphae formation. Both the *mep2Δkrh1Δ* as well as the *mep2Δkrh2Δ* mutants were unable to form pseudohyphae in SLAD (Synthetic low ammonium dextrose) medium (Fig. 1). In SLALD (Synthetic low ammonium low dextrose), however, the inability of the *mep2Δ* mutant to put forth pseudohyphae was overcome only by *KRH2* disruption, although the pseudohyphae formed were fewer and shorter. On the contrary, the *mep2Δkrh1Δ* double mutant was unable to filament indicating that *KRH2* but not *KRH1* suppresses filamentation in the absence of *MEP2* in low glucose (Fig. 1). This result was further supported by the invasive growth assay where the *mep2Δkrh2Δ* strain exhibited invasive growth while the *mep2Δkrh1Δ* strain did not (Fig. 2). For biochemical corroboration of the above

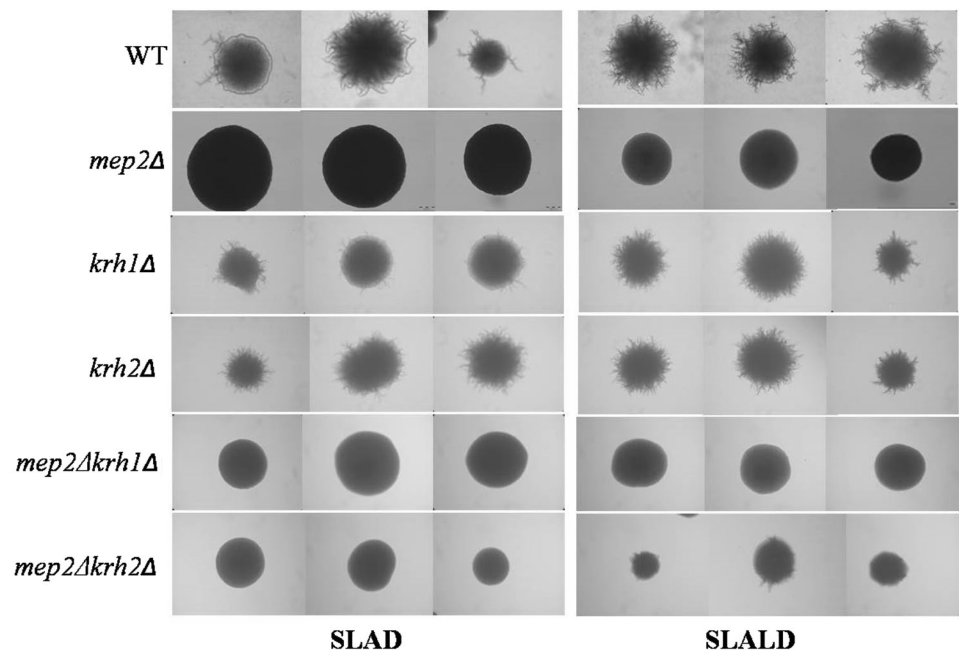

Fig. 1 Pseudohyphal growth phenotype of mutant strains Images of colonies on SLAD as well as SLALD media as indicated in the figure. Three representative colonies are shown for each mutant

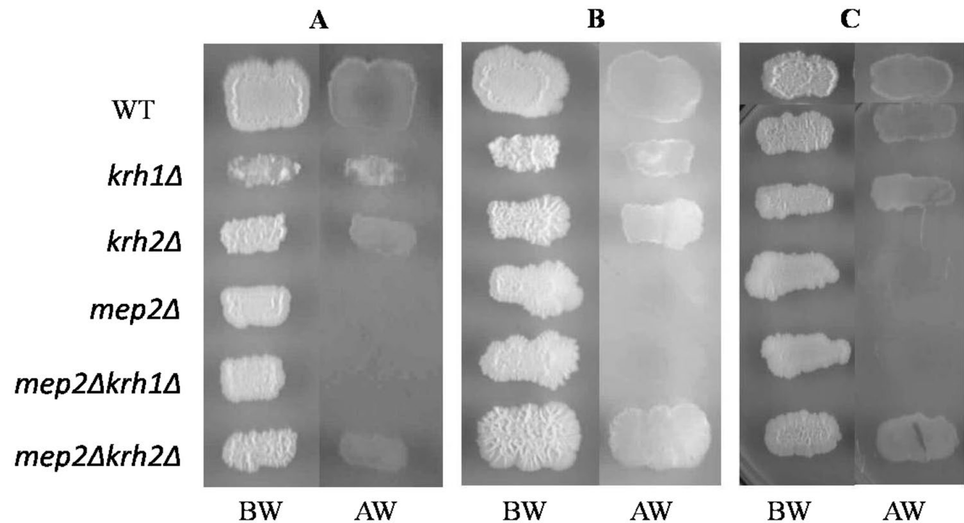

Fig. 2 Haploid invasive growth phenotype of mutant strains Images of colony patches on YPD medium; *BW* Before wash, *AW* After wash. **a, b** and **c** represent three independent experiments

observation, *FLO11* expression was determined as a function of β-galactosidase activity in SLAD as well as SLALD medium using a P_{FLO11}-*lacZ* reporter plasmid (Table 2). As expected, in SLALD medium, the *mep2Δkrh2Δ* mutant exhibited higher β-galactosidase activity as compared to that in SLAD medium. Moreover, *FLO11* expression was significantly higher in *mep2Δkrh2Δ* strain as compared to the *mep2Δkrh1Δ* strain, consistent with the filamentation response of the two strains. The diminished filamentation exhibited by the *mep2Δkrh2Δ* strain in SLALD (see Fig. 1) is probably due to lower *FLO11* expression when compared to that of the wild-type strain. The above observations indicate that *MEP2* exerts an inhibitory effect on *KRH2* thereby activating filamentation response. Thus, the results presented here provide the first genetic evidence of a possible interacting partner for *MEP2*. The genetic interaction of *MEP2* with *KRH2* but not *KRH1* indicates that *KRH1* and *KRH2* may be functionally non-redundant.

KRH1 and *KRH2* are functionally non-redundant

It is clear from the above results that the disparate behaviour of *KRH1* and *KRH2* could be ascertained only in SLALD but not SLAD medium. To further test whether extracellular low glucose concentration modulates the function of *KRH1* and *KRH2* differentially, pseudohyphae formation in response to over-expression of *KRH1*/*2* under GPD promoter was monitored on SLAD as well as SLALD media. We deliberately used a heterologous promoter to ensure that expression is independent of the growth conditions which can otherwise confound our interpretation. As reported earlier (Battle et al. 2003; Harashima and Heitman 2002), over-expression of either *KRH1* or *KRH2* suppressed pseudohyphae on SLAD medium (Fig. 3). However, filamentation was suppressed upon *KRH2* but not *KRH1* over-expression in SLALD medium (Fig. 4, compare middle and bottom – panels a, b and c). Only in the *krh2*Δ strain, we observed a mild retardation in pseudohyphae formation upon *KRH1* overexpression when compared with the corresponding vector control. It is possible that in the absence of *KRH2*, overexpression of *KRH1* has a mild, non-specific negative effect by virtue of being a paralogue of *KRH2*. Nonetheless, the pseudohyphae formed were more pronounced as compared to the wild-type strain under the same conditions. It is clear from the above data that *KRH1* and *KRH2* may have different roles only when both glucose as well as ammonium are limiting. Surprisingly, western blot analysis revealed that although *KRH1*

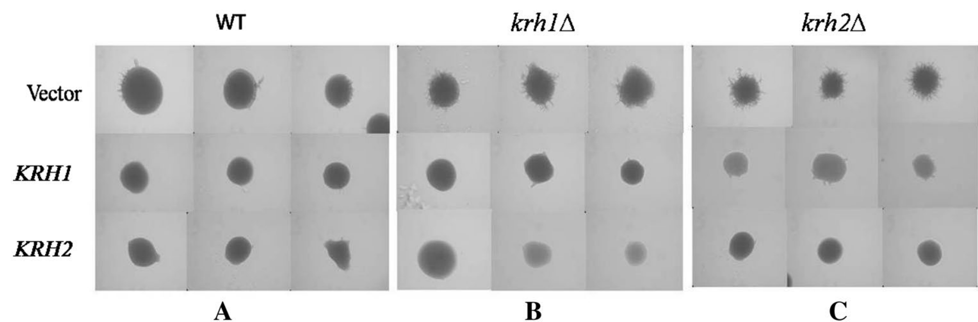

Fig. 3 Effect of over-expression of *KRH1*/*2* in SLAD medium Over-expression of *KRH1* and *KRH2* independently in the wild-type (*WT*), *krh1Δ* as well as *krh2Δ* strains. When over-expressed both *KRH1* and *KRH2* inhibit pseudohyphae formation in SLAD medium. Images of three representative colonies are shown for each experimental condition

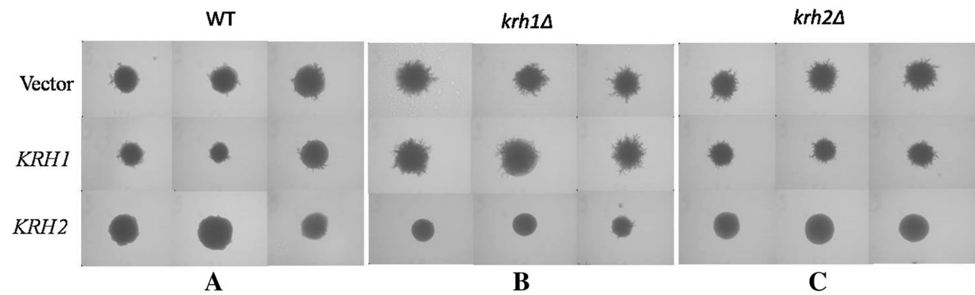

Fig. 4 Effect of over-expression of *KRH1*/*2* in SLALD medium *KRH1* and *KRH2* are separately over-expressed in the WT, *krh1Δ* as well as *krh2Δ* strains. In SLALD medium, upon over-expression *KRH2* but not *KRH1* suppresses filamentation. Three representative colonies are shown for each experimental condition

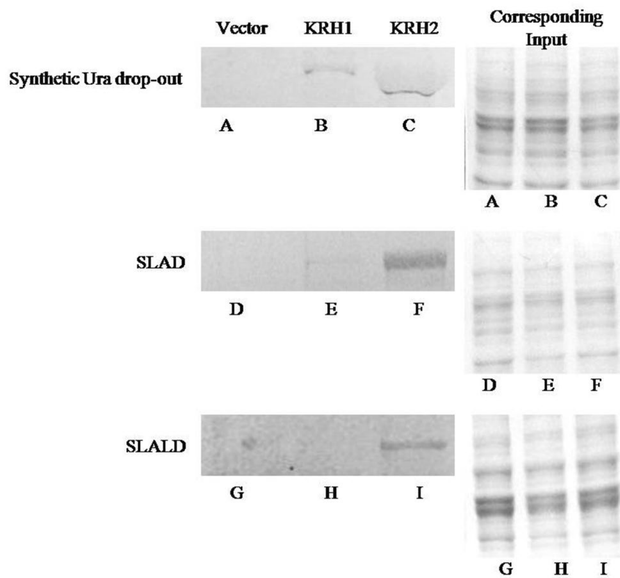

Fig. 5 *KRH1*/*2* expression under different nutrient conditions *KRH1* is inactivated in low ammonium concentration. His-tagged *KRH1* as well as *KRH2* were purified using Ni–NTA agarose column and probed using anti-His antibodies. Equal protein was loaded onto the Ni–NTA column as represented by the coommassie blue stained, corresponding input fraction which represents 0.1% of the total protein used for binding

as well as *KRH2* were expressed in synthetic uracil drop-out medium (with abundant glucose and ammonium), only *KRH2* was expressed in SLAD or SLALD medium (Fig. 5) indicating that *KRH1* may be suppressed when ammonium levels are low. In other words, when ammonium limitation occurs simultaneously with glucose limitation, *MEP2* is probably required to suppress only *KRH2* but not *KRH1* to trigger filamentation response. To examine further whether glucose too has a role in the regulation of *KRH1*/*2* we employed our earlier observation that in a wild-type strain, glucose replenishment was unable to inhibit filamentation once the cells are committed to differentiation. That is, glucose replenishment suppressed pseudohyphae formation only in SLAD but not in SLALD medium (Iyer et al. 2008). To determine whether *KRH1*/2 have any role in this glucose dependent commitment to pseudohyphae formation, the effect of glucose replenishment was monitored under SLAD as well as SLALD conditions in strains wherein *KRH1*/2 is either deleted or over-expressed. Upon glucose replenishment in SLAD medium with abundant glucose, pseudohyphae were suppressed in a *krh1Δ* but not *krh2Δ* mutant (Fig. 6, compare b and c of left panel) demonstrating that *KRH1* and *KRH2* are functionally non-redundant. In addition, under the same condition of growth, filamentation in the *krh2Δ* mutant was suppressed upon *KRH2* over-expression (Fig. 6, compare left and right panels of c), indicating that *KRH2* function is not suppressed by glucose in SLAD medium. However, in SLALD medium, upon glucose replenishment, pseudohyphae were formed even when *KRH2* was over-expressed (Fig. 6, compare c and g of right panel) indicating that glucose suppressed the function of *KRH2*. Thus, it is evident from the difference in the phenotypes observed in SLAD as compared to SLALD medium that glucose mediated suppression of *KRH2* function is dependent on initial glucose concentration.

Based on the results presented above, we surmised that *KRH2* may function to prevent filamentation response when glucose is abundant while *KRH1* may not be required for this function. To determine whether the above role of *KRH2* is in any way modulated by *KRH1*, the effect of glucose replenishment on *KRH2* over expression was monitored separately in the mutants defective for *KRH1* and *KRH2*. Interestingly, glucose suppressed *KRH2* more strongly in the *krh2Δ* strain resulting in increased pseudohyphae formation in a *krh2Δ* mutant compared to a *krh1Δ* strain (Fig. 6, compare f and g of right panel). Further, pseudohyphae formed in the *krh2Δ* mutant upon glucose replenishment when *KRH2* was over-expressed were abolished in a *krh1Δkrh2Δ* double mutant (Fig. 6, compare g and h of right panel) indicating that filamentation occurring as a result of glucose mediated suppression of *KRH2* is dependent on *KRH1*.

Discussion

There is a large body of evidence to show that transcriptional factors responsive to glucose deprivation regulate

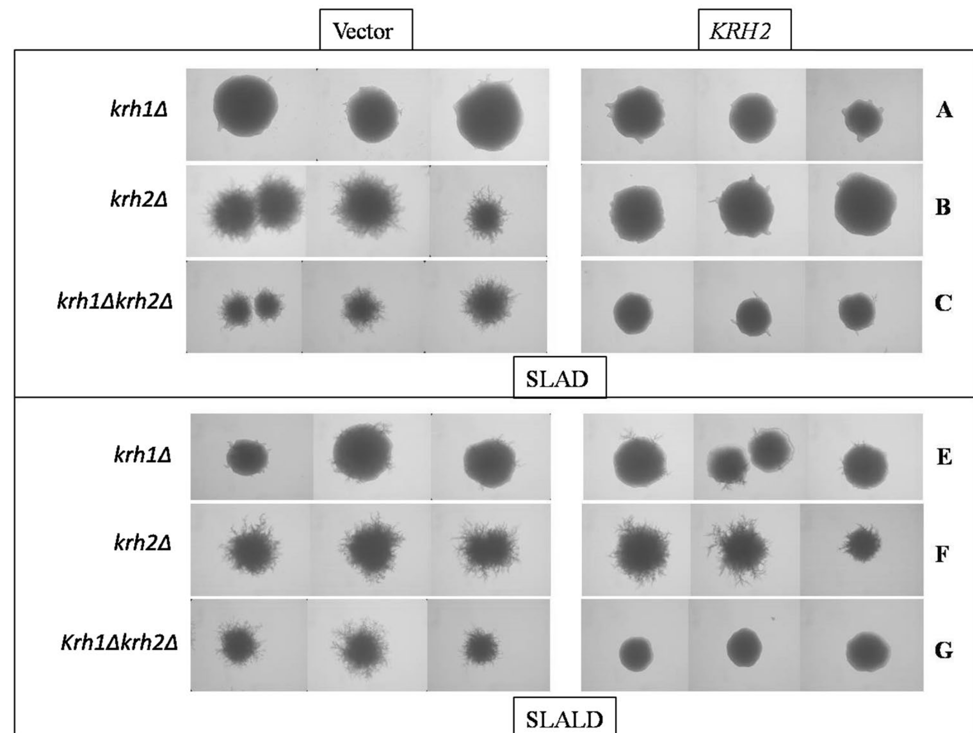

Fig. 6 Effect of glucose replenishment Over-expression of *KRH2* in the WT, *krh1Δ, krh2Δ* and *krh1Δkrh2Δ* strains respectively in SLAD (**a, b, c, d**) and SLALD (**e, f, g, h**) medium as indicated in the figure. Images represent colonies (in triplicates) after exposure to glucose addition

metabolism in *S. cerevisiae* (Soontorngun 2016). The fundamental question, however, is what would be the metabolic basis of pseudohyphal differentiation in response to decreasing concentration of glucose when ammonium concentration is low to begin with. While the biochemical interactions occurring during this process have been studied in great detail (Broach 2012; Cullen and Sprague 2012), the mechanism of how such interactions give rise to a specific phenotype depending upon the availability of glucose as well as ammonium is not clear. Here, to investigate the role of *KRH1* and *KRH2* in the context of depleting glucose as well as ammonium concentrations, we undertook a detailed study of different mutant strains under varying growth conditions.

Based on our previous data and the results presented in this study, we consider the following possibility (refer to Fig. 7) when a wild-type cell is allowed to grow in SLAD medium (i.e. when glucose is abundant to start with). *GPR1* senses abundant glucose to activate *GPA2* on one hand and inhibit *KRH2* on the other. Simultaneous activation of *GPA2* and inhibition of *KRH2* triggers the cAMP pathway favouring the vegetative mode of growth. As the glucose concentration diminishes, glucose mediated inhibition of *KRH2* is alleviated thereby allowing inhibition of *GPA2* and PKA by *KRH2*. When ammonium concentration decreases, repression by *MEP2* prevents *KRH2* from inhibiting *GPA2* and PKA. It appears that *MEP2* mediated inhibition of *KRH2* occurs only when glucose dependent inhibition of *KRH2* is alleviated in response to decreasing glucose concentration. Under these conditions, *KRH2* can no longer inhibit *GPA2* and PKA, thereby triggering pseudohyphae formation. We had previously reported that *gpr1*Δ mutant exhibits profuse pseudohyphae in SLALD but not in SLAD medium (Iyer et al. 2008). Further, deletion of *GPR1* suppressed the inability of the *MEP2* mutant to put forth pseudohyphae in SLALD but not in SLAD medium. In the absence of *MEP2* as well as *GPR1*, although *KRH2* is free to suppress PKA signaling, it is possible that pseudohyphae are facilitated by alleviation of glucose mediated repression of *FLO11* in SLALD medium. Thus, the above mechanism seems to operate by co-ordinating the alleviation of glucose repression of *FLO11*, with that of *MEP2* dependent activation of PKA, through *GPR1-GPA2* axis. Keeping *FLO11* under tight glucose repression, probably allows the cells to grow rapidly even under low ammonium until glucose repression is alleviated. Once the glucose levels decrease pseudohyphal differentiation may be triggered through *MEP2*. Thus, we suggest that the cAMP-PKA pathway favours vegetative growth only as long as glucose is in abundance. However, as nutrients get depleted, signals from *MEP2* as well as from low glucose impinge on the cAMP-PKA pathway so that it favours filamentation response.

Our observations indicate that *KRH2* is inhibited by *MEP2* in addition to being modulated by glucose signaling. Overall, the results of this study suggest that *KRH2* receives and integrates signals from both glucose as well as ammonium. On the other hand, our observation that *KRH1*

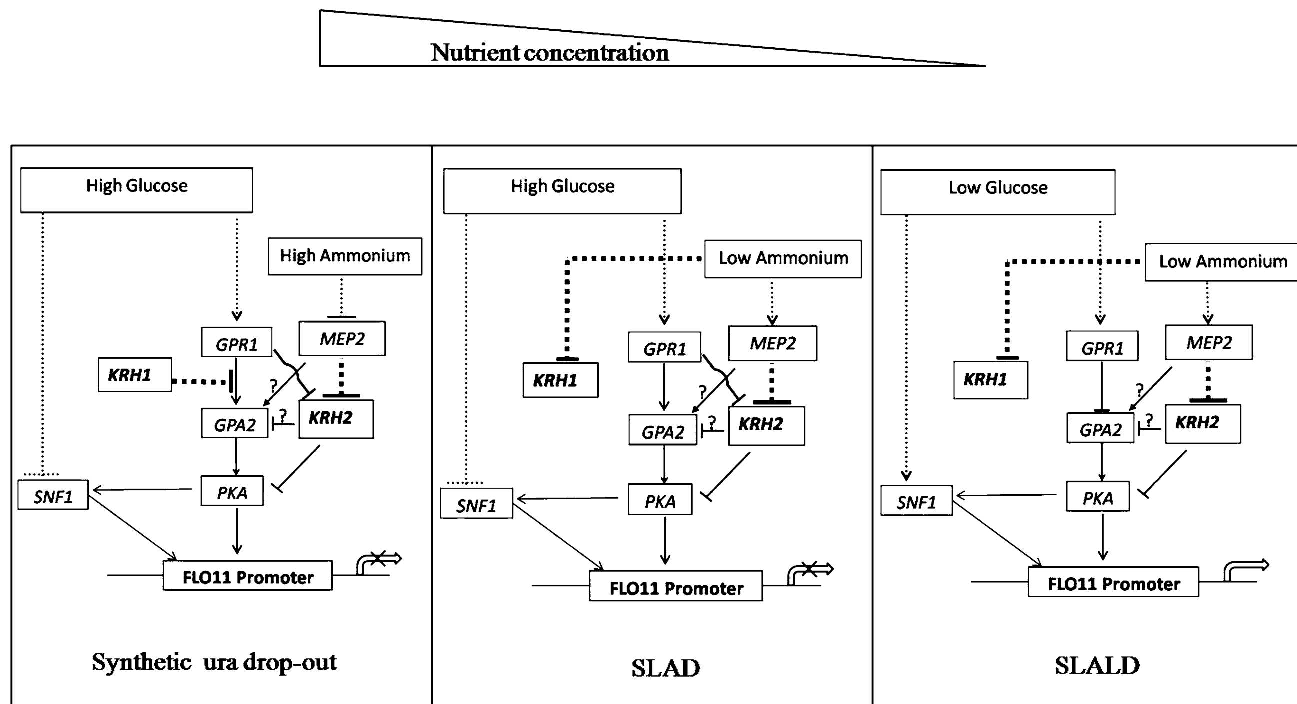

Fig. 7 Model depicting the shift in signaling as glucose and ammonium concentrations decrease Under condition of high glucose concentration, filamentation is suppressed by *KRH1* mediated down-regulation of *GPR1*-*GPA2* signaling as well as by glucose mediated activation of *KRH2* through *GPR1*. In response to depleting ammonium *KRH1* is down-regulated thereby alleviating *KRH1* mediated suppression of PKA. Simultaneously, suppression of *KRH2* mediated by *MEP2* occurs. As glucose is utilized by the cells, the concentration decreases and pseudohyphae formation is triggered by alleviation of glucose mediated repression of *FLO11*

is inactivated by low ammonium suggests that *KRH1* may be involved only in cAMP-PKA signaling in response to glucose and may not play a role in pseudohyphal differentiation. In contrast to the current understanding that *KRH1* and *KRH2* are functionally equivalent or that they are redundant (reviewed in Peeters et al. 2007), we propose that *KRH1* and *KRH2* are non-redundant with respect to their function in filamentation response. Our earlier results suggested a paradigm shift in that low glucose-low ammonium is a physiologically relevant signal for dimorphic transition in *S. cerevisiae* (Iyer et al. 2008). It is only in this changed paradigm, that we are able to dissect out the differential role of *KRH1* and *KRH2*. Thus, genetic and biochemical evidence presented in this study suggests that cells constantly monitor the level of glucose to decide when they should quit from normal proliferation and switch over to pseudohyphal mode of growth. Our data imply that given an opportunity, the cell would prefer to grow vegetatively rather than put-forth pseudohyphae as long as sufficient glucose is available.

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