PtdIns(4,5) P_2 and phospholipase C-independent Ins(1,4,5) P_3 signals induced by a nitrogen source in nitrogen-starved yeast cells

Jan C. T. BERGSMA*, Nael Nadif KASRI*, Monica C. V. DONATON*, Veerle DE WEVER*, Renata TISI†, Johannes H. DE WINDE*¹, Enzo MARTEGANI†, Johan M. THEVELEIN* and Stefaan WERA*²

*Laboratorium voor Moleculaire Celbiologie, Katholieke Universiteit Leuven, Kasteelpark Arenberg 31, B-3001 Leuven-Heverlee, Flanders, Belgium, and †Dipartimento di Biotecnologie e Bioscienze, Università degli Studi di Milano-Bicocca, Piazza delle Scienze 2, I-20126 Milan, Italy

Addition of ammonium sulphate to nitrogen-depleted yeast cells resulted in a transient increase in Ins(1,4,5) P_3 , with a maximum concentration reached after 7–8 min, as determined by radioligand assay and confirmed by chromatography. Surprisingly, the transient increase in Ins(1,4,5) P_3 did not trigger an increase in the concentration of intracellular calcium, as determined *in vivo* using the aequorin method. Similar Ins(1,4,5) P_3 signals were also observed in wild-type cells treated with the phospholipase C inhibitor 3-nitrocoumarin and in cells deleted for the only phospholipase C-encoding gene in yeast, *PLC1*. This showed clearly that Ins(1,4,5) P_3 was not generated by phospholipase C-dependent cleavage of PtdIns(4,5) P_2 . Apart from a transient increase in Ins(1,4,5) P_3 , we observed a transient increase in

PtdIns(4,5) P_2 after the addition of a nitrogen source to nitrogen-starved glucose-repressed cells. Inhibition by wortmannin of the phosphatidylinositol 4-kinase, Stt4, which is involved in PtdIns(4,5) P_2 formation, did not affect the Ins(1,4,5) P_3 signal, but significantly delayed the PtdIns(4,5) P_2 signal. Moreover, wortmannin addition inhibited the nitrogen-induced activation of trehalase and the subsequent mobilization of trehalose, suggesting a role for PtdIns(4,5) P_2 in nitrogen activation of the fermentable-growth-medium-induced signalling pathway.

Key words: inositol 1,4,5-trisphosphate, nitrogen sensing, phosphoinositides, trehalase.

INTRODUCTION

Yeast cells growing in batch culture rapidly exhaust the growth medium of essential nutrients, leading to growth arrest and entry into the G_0 phase of the cell cycle. In many instances, such as during wine fermentation, nitrogen becomes the limiting factor for yeast growth and division. Upon re-addition of the lacking nutrients to the growth medium yeast cells activate several signal-transduction pathways to quickly adapt to the improved conditions. Re-addition of a nitrogen source, such as aspartate or ammonium sulphate, to yeast cells depleted of nitrogen in the presence of sufficient amounts of glucose (or another fermentable sugar) activates the fermentable-growth-medium-induced (FGM) pathway. Sustained activation of the FGM pathway requires both a fermentable carbon source and a complete growth medium, hence the name. FGM signalling results in the activation of trehalase, the repression of STRE (stress-regulated element)-controlled genes and the induction of ribosomal protein genes (reviewed in [1]).

The correlation between FGM and other nutrient-induced pathways such as the target of rapamycin (TOR) pathway or the cAMP/protein kinase A pathway is not clear. Nevertheless, it was shown that the cAMP concentration remains constant during FGM signalling [2] and that the Sch9 protein kinase is essential for FGM signalling [3]. The catalytic domain and C-terminus of Sch9 are highly similar to mammalian protein kinase B (PKB). Overexpression of PKB can partially restore the signalling defect

of an *sch9* deletion strain [4]. Since PKB was shown to phosphorylate mammalian TOR (mTOR) in mammalian cells [5], a possible correlation between the FGM and TOR pathways in yeast cannot be excluded.

Little is known about the factors acting upstream of Sch9 in the FGM pathway. To tackle this problem, we re-investigated the earlier observation by Schomerus and Kuntzel [6] that addition of ammonium sulphate to starved yeast cells led to a 3–4-fold increase in Ins(1,4,5) P_3 (which will be referred to throughout the paper as Ins P_3). These authors favoured the idea that nitrogen-induced signalling is dependent on Plc1 (the gene product of *PLC1*, a single gene encoding phospholipase C in yeast) and the GDP/GTP-exchange factor Cdc25. The recent discovery of an interaction between the G-protein-coupled receptor Gpr1 and Plc1 [7] is also suggestive of a role for Plc1 in nutrient-induced signal transduction in yeast. We confirm here to a certain extent the data obtained by Schomerus and Kuntzel [6], by showing an increase in Ins P_3 upon ammonium sulphate addition to nitrogen-depleted yeast cells. However, we also show that this Ins P_3 signal is transient, not dependent on Plc1 and does not lead to an increase in intracellular calcium.

Following the classical pathway, mainly established in mammalian cells, Ins P_3 is generated by cleavage of PtdIns(4,5) P_2 into diacylglycerol and Ins P_3 in a reaction catalysed by phospholipase C [8]. Recently, however, an alternative mechanism for the generation of Ins P_3 in eukaryotic cells was discovered in *Dictyostelium*. This mechanism involves dephosphorylation of

Abbreviations used: groPIns(4,5) P_2 , glycerophosphoinositol 4,5-bisphosphate; FGM, fermentable-growth-medium-induced; PKB, protein kinase B; TOR, target of rapamycin.

¹ Present address: DSM Bakery Ingredients, Technology Cluster, Beijerinck Laboratory, P.O. Box 1, 2600 MA Delft, The Netherlands.

² To whom correspondence should be addressed (e-mail stefaan.wera@bio.kuleuven.ac.be).

Ins(1,3,4,5,6) P_3 , Ins(1,3,4,5) P_4 and Ins(1,4,5,6) P_4 to Ins P_3 , by an inositol polyphosphatase [9,10]. Our results suggest that such a mechanism might also be operative in yeast.

MATERIALS AND METHODS

Yeast strains and media

The following strains were used: wild type (WPY260), *Matx ura3-52 trp1-Δ901 ade2-101*; and *plc1*-deletion (WPY300), *Mata ura3-52 trp1-Δ901 ade2-101 plc1Δ::URA3*. Cells were grown to exponential phase on 1% (w/v) yeast extract/2% (w/v) peptone containing 2% (w/v) glucose (YPD) and starved of nitrogen on 1.67% (w/v) Yeast Nitrogen Base without amino acids and without ammonium sulphate (Difco) containing 4% (w/v) glucose (nitrogen-starvation medium).

Determination of Ins P_3 and PtdIns(4,5) P_2

Nitrogen-starved cells were pre-incubated at 30 °C at a density of 37.5 mg (wet weight)/ml in nitrogen-starvation medium containing 4% glucose for 20 min with or without inhibitors. At time point zero, ammonium sulphate was added at a final concentration of 5 or 10 mM. Samples containing 75 mg of cells were quenched in 10 ml of 60% methanol at -40 °C and subsequently harvested by centrifugation. Ice-cold perchloric acid (1 ml) and 1.5 g of glass beads (0.5 mm diameter) were added and the cells were broken by vigorous shaking at 4 °C. The cell homogenates were centrifuged at high speed, after which the soluble fraction (extract) was used to determine Ins P_3 and the pellet (insoluble fraction) to determine PtdIns(4,5) P_2 levels.

Ins P_3 was measured in neutralized cell extracts using an Ins P_3 bovine adrenal-binding-protein-based radioligand assay (Amersham Pharmacia Biotech). PtdIns(4,5) P_2 was converted into Ins P_3 under alkaline conditions as described by Chilvers et al. [11] and Ins P_3 was measured with the binding protein assay described above. Illustrated data represent typical experiments which were performed at least three times. Basal Ins P_3 concentrations measured were 0.08 ± 0.02 pmol/mg of cells (wet weight). Basal PtdIns(4,5) P_2 concentrations, assuming a 100% recovery from the PtdIns(4,5) P_2 membrane-extraction procedure, amounted to 0.04 ± 0.01 pmol/mg of cells (wet weight).

3-Nitrocoumarin was synthesized according to the procedure of Perrella et al. [12]. The product was crystallized twice and purity was greater than 98%. The product was dissolved at 10 mg/ml in DMSO.

Purification of Ins P_3

Cell extracts were prepared and neutralized using the same method as for the assay of Ins P_3 . Samples were chromatographed on a MonoQ (HR10/10) equilibrated in 0.2 mM HCl and developed with a HCl gradient to 0.4 M following the method described in [13]. This method allows the separation of Ins P_3 from higher and lower inositol phosphates and from other trisphosphorylated compounds such as ATP. Column fractions (1 ml) were neutralized using KOH and assayed for Ins P_3 . Radioactively labelled [3 H]inositol phosphates (2500–7000 c.p.m.; NEN and Amersham Pharmacia Biotech) were loaded on to the column, which was developed in the same way. Fractions were counted in the scintillation counter. [3 H]Glycerophosphoinositol 4,5-bisphosphate {[3 H]groPIns(4,5) P_2 } was prepared by deacylation of [3 H]PtdIns(4,5) P_2 (NEN) as described in [14] and subsequently purified on a Bio-Rad Ag 1 ($\times 8$, 200–400 mesh) formate-form resin [15].

Monitoring of intracellular calcium

Intracellular calcium was monitored following the method described by Nakajima-Shimada et al. [16] with some modifications. Aequorin-expressing cells were obtained by transforming wild-type yeast with the pYX012-aequorin vector and selection on kanamycin-containing medium. pYX012-aequorin was prepared by inserting the haemagglutinin-tagged aequorin-encoding *EcoRI* fragment from the cytAEQ/pcDNAI vector (Molecular Probes) into the *EcoRI* sites of the integrative yeast plasmid pYX012 (R&D systems). Finally, the Kan^R gene was inserted into the *SmaI/EcoRV* sites of the resulting construct.

Prior to the experiment, aequorin-expressing cells were pre-incubated in nitrogen-starvation medium containing 4% (w/v) glucose and 50 μ M coelenterazin (Molecular Probes). Cells were washed and resuspended to a density of 15 mg of cells (wet weight)/ml in nitrogen-starvation medium containing 4% (w/v) glucose. Ammonium sulphate was added at a final concentration of 5 mM to 3 mg of cells. Luminescence was measured in a Lumicount (Packard) luminometer. For salicylate-induction experiments, 5 mM ammonium sulphate was replaced by 5 mM sodium salicylate.

For hypotonic shock experiments, cells were grown in synthetic medium containing 2% (w/v) glucose. Hypotonic shock was performed by adding to the cells one volume of diluted (6.7%) medium.

Determination of trehalase activity

Trehalase activity and trehalose content were determined as described previously [3]. Wortmannin from *Penicillium funiculosum* was purchased from Fluka and dissolved (50 mg/ml) in DMSO.

Figure 1 Nitrogen-induced Ins P_3 signal in nitrogen-starved cells

Yeast cells were starved of nitrogen in the presence of 4% glucose for 16 h. At the indicated time point (arrow) ammonium sulphate was added at a final concentration of 5 mM. Before and at the indicated time points after ammonium sulphate addition samples were taken to assay for Ins P_3 . Means $\pm$ S.E.M. from three independent experiments are shown.

RESULTS AND DISCUSSION

Addition of a nitrogen source to nitrogen-depleted, glucose-repressed cells results in an InsP_3 signal

Wild-type yeast cells were grown in YPD to an attenuation (D_{600}) of 1.0 and subsequently incubated for 16 h in nitrogen-starvation medium containing 4% glucose. As shown in Figure 1, addition of 5 mM ammonium sulphate resulted in a transient increase in the concentration of InsP_3 . A maximal concentration was reached around 7 min after nitrogen-source addition and concentrations returned to basal levels within minutes. Maximal InsP_3 concentrations reached about 2–3-fold of the basal level.

Since the InsP_3 assay was performed with a radioligand assay using a highly specific binding protein, it is unlikely that metabolites or components other than InsP_3 could have interfered with our measurements. However, to check the possible interference of other components with the assay, we purified the yeast extracts by ion-exchange chromatography prior to the assay. We first demonstrated that ion exchange allowed the separation of InsP_3 from other inositol phosphates, since those components are most likely to interfere with the assay. From Figure 2(A) it is clear that a complete separation of radioactive isotopes of InsP , InsP_2 , InsP_3 and InsP_4 is obtained, with InsP_3 eluting in fractions 21–25. We subsequently checked whether $\text{groPIns}(4,5)\text{P}_2$, the deacylation product of $\text{PtdIns}(4,5)\text{P}_2$, might co-elute with InsP_3 under the conditions used. This was especially important since $\text{groPIns}(4,5)\text{P}_2$ is chemically related to InsP_3 and was found to be produced by yeast cells under conditions of refeeding glucose to starved cells [15]. As can be seen in Figure 2(A), however, radioactive $\text{groPIns}(4,5)\text{P}_2$ is separated from InsP_3 during chromatography. Moreover, $\text{groPIns}(4,5)\text{P}_2$ did not significantly interact with the InsP_3 radioligand assay used (results not shown). We then applied a yeast extract to the column and assayed InsP_3 in all fractions using the radioligand assay (Figure 2B). We could only measure InsP_3 in fractions 21–25, exactly where radioactive InsP_3 eluted, offering further support for the specificity of the assay used. Finally, we applied the extracts, prepared at different time points after the administration of ammonium sulphate to starved, glucose-repressed yeast cells (from Figure 1), to the ion exchanger, and assayed fractions 21–25 of each time point for InsP_3 . The total InsP_3 content of fractions 21–25 for each time point was calculated. The results (Figure 2C) show clearly that the InsP_3 concentration displayed a similar transient increase in these purified samples (not containing any higher- or lower-order inositol phosphates) as in total extracts. Although we cannot fully exclude that a compound different from InsP_3 , but interacting with the InsP_3 -binding protein and co-migrating with InsP_3 during chromatography, is responsible for the observed effects, this is highly unlikely given the high specificity of the InsP_3 -binding protein used for the assay and given the efficient separation of InsP_3 from related

Figure 2 Specificity of the InsP_3 assay

(A) $[\text{H}] \text{Ins}1\text{P}$ ($\square$), $[\text{H}] \text{Ins}(1,4)\text{P}_2$ ($\diamond$), $[\text{H}] \text{InsP}_3$ ($\blacklozenge$), $[\text{H}] \text{Ins}(1,3,4,5)\text{P}_4$ ($\blacktriangle$) and $[\text{H}] \text{groPIns}(4,5)\text{P}_2$ ($+$) were separated on a MonoQ column and detected by scintillation counting. (B) A yeast extract was separated on a MonoQ column under the same conditions as in (A) and fractions were assayed for InsP_3 using the radioligand assay method. (C) Yeast cells were starved of nitrogen in the presence of 4% glucose during 16 h. At the indicated time point (arrow) ammonium sulphate was added at a final concentration of 5 mM. Before and at the indicated time points after ammonium sulphate addition samples were taken, separated on MonoQ and fractions 21–25 were assayed for InsP_3 . The total InsP_3 content of fractions 21–25 was calculated for each time point. Means $\pm$ S.E.M. from three independent experiments are shown.

compounds during the chromatography prior to the assay (Figure 2A).

Addition of a nitrogen source to nitrogen-depleted, glucose-repressed cells does not result in a Ca^{2+} signal

To follow the intracellular Ca^{2+} concentration, we transformed the yeast cells with an integrative aequorin expression vector. In the presence of coelenterazine, a Ca^{2+} signal in such cells results in luminescence at 470 nm. To check whether a Ca^{2+} signal could be detected at all in the transformed cells, we either treated exponential-phase cells with 5 mM sodium salicylate or subjected the cells to hypotonic shock. Both conditions are known [17,18] to induce an intracellular Ca^{2+} increase. Under sets of both conditions we clearly observed an increase in luminescence, reaching a maximum at 4 min after salicylate addition (results not shown) or 12 s after hypotonic shock (Figure 3A), showing the reliability of the method in our conditions. As shown in Figure 3(B), salicylate also induced a Ca^{2+} increase in nitrogen-starved, glucose-repressed cells, indicating that in these cells the aequorin-coelenterazine Ca^{2+} assay also works efficiently and that cells under these conditions are able to generate a Ca^{2+} signal.

We then checked whether addition of ammonium sulphate to nitrogen-depleted yeast cells results in a Ca^{2+} increase. Unexpectedly, however, no increase in luminescence was observed

Figure 4 Similar InsP_3 signals in wild-type versus $\text{plc1}\Delta$ cells

Wild-type ($\blacklozenge$) and $\text{plc1}\Delta$ ($\diamond$) cells were starved of nitrogen in the presence of 4% glucose for 16 h. At the indicated time point (arrow) ammonium sulphate was added at a final concentration of 5 mM. Before and at the indicated time points after ammonium sulphate addition samples were taken for the assay of InsP_3 . Means $\pm$ S.E.M. from three independent experiments are shown.

Figure 3 Addition of a nitrogen source to nitrogen-starved, glucose-repressed cells does not result in a Ca^{2+} signal

(A) Apo-aequorin-expressing cells submitted to hypotonic shock (arrow). (B) Apo-aequorin-expressing cells were incubated in nitrogen-starvation medium containing 4% glucose for 16 h. At the indicated time point (arrow) sodium salicylate was added at a final concentration of 5 mM. (C) Apo-aequorin-expressing cells were incubated in nitrogen-starvation medium containing 4% glucose for 16 h. At the indicated time point (arrow) ammonium sulphate was added at a final concentration of 5 mM. In all cases, luminescence at 470 nm was monitored; rlu, relative luminescence units.

during the first 25 min following ammonium sulphate addition (Figure 3C). In a parallel experiment, using the same batch of cells, we measured the InsP_3 concentration and clearly observed a peak at about 8 min after ammonium sulphate addition (results not shown). In conclusion, our results show that addition of ammonium sulphate to nitrogen-starved yeast cells results in a transient InsP_3 increase but not in a Ca^{2+} signal. Possibly the InsP_3 signal we observed was too short-lived to generate a calcium signal. On the other hand, as far as we know no studies have directly demonstrated an InsP_3 -dependent calcium release in yeast *in vivo* [19]. The elegant experiments by Belde et al. [20] demonstrate an InsP_3 -dependent calcium release from isolated yeast vacuoles and indicate the presence of an InsP_3 -specific Ca^{2+} channel in yeast. Half-maximal release of calcium was observed at $0.4 \mu\text{M}$ InsP_3 , close to the estimated maximal InsP_3 concentration reached under our conditions. Although our data do not rule out that such an InsP_3 -dependent calcium release occurs *in vivo* under other conditions, they do open the possibility that in yeast cells InsP_3 may have an additional calcium-independent signalling function.

The InsP_3 signal is not dependent on phospholipase C

Our observation that re-addition of a nitrogen source to nitrogen-starved cells induces a transient increase in InsP_3 confirmed to a certain extent the earlier work by Schomerus and Kuntzel [6], which indicated an InsP_3 increase after nitrogen addition to starved cells. These authors, however, observed a 3-fold increase, which was stable for about 1 h. In contrast the InsP_3 concentration in our experiments increased 2–3-fold and dropped back within minutes to control values (Figure 1). About 12 min after nitrogen-source addition InsP_3 levels had returned to basal levels in all our experiments.

Schomerus and Kuntzel [6] suggested the involvement of phospholipase C in the InsP_3 increase. Yeast phospholipase C is encoded by a single gene [21–23], called *PLC1*. We ruled out the involvement of the *Plc1* gene product in three independent ways. First, addition of $15 \mu\text{g/ml}$ 3-nitrocoumarin, a potent inhibitor of phospholipase C [12,24], did not affect the nitrogen-induced InsP_3 signal in wild-type cells (results not shown). Secondly, similar nitrogen-induced InsP_3 signals were observed in wild-type versus *plc1*-deleted cells (Figure 4), clearly ruling out the involvement of *Plc1* in this process. Further support for the *Plc1* and $\text{PtdIns}(4,5)\text{P}_2$ -independent formation of InsP_3 comes from the observation that addition of $10 \mu\text{g/ml}$ wortmannin did not affect the InsP_3 signal (results not shown). The primary target of wortmannin in yeast is the phosphatidylinositol 4-kinase, *Stt4* [25]. *Stt4* catalyses the formation of $\text{PtdIns}4\text{P}$ [26], a precursor of $\text{PtdIns}(4,5)\text{P}_2$, and inhibition of *Stt4* is hence expected to block *Plc1*-dependent InsP_3 formation. Taken together, our data indicate that the InsP_3 signal observed occurs independently of *Plc1*. Possibly InsP_3 is generated under these conditions from dephosphorylation of inositol polyphosphates, as was shown to occur in *Dictyostelium* [9,10]. The generation of InsP_3 in the absence of *Plc1* is also in agreement with results from *in vivo*

Figure 5 Nitrogen-induced $\text{PtdIns}(4,5)\text{P}_2$ signal

Yeast cells were starved of nitrogen in the presence of 4% glucose for 16 h. At the indicated time point (arrow) ammonium sulphate was added at a final concentration of 5 mM. Before and at the indicated time points after ammonium sulphate addition samples were taken for the assay of $\text{PtdIns}(4,5)\text{P}_2$ (A), trehalase activity (B) or trehalose (C). Closed symbols represent controls, open symbols indicate the presence of $1 \mu\text{g/ml}$ wortmannin.

labelling experiments with [^3H]inositol, which demonstrated the presence of InsP_3 in a *plc1/inp51* double-deletion strain [27]. *INP51* encodes a lipid phosphatase which *in vitro* dephosphorylates the 5-position of the inositol ring of $\text{PtdIns}(4,5)\text{P}_2$, but not InsP_3 . However, the *plc1* single deletion could not be labelled efficiently due to a growth defect on synthetic medium.

Addition of a nitrogen source to nitrogen-depleted, glucose-repressed cells results in a transient increase in $\text{PtdIns}(4,5)\text{P}_2$

Wild-type yeast cells were grown in YPD to an attenuation (D_{600}) of 1 and subsequently incubated for 16 h in nitrogen-starvation medium containing 4% glucose. As shown in Figure 5(A), addition of 10 mM ammonium sulphate resulted in a transient increase in the concentration of $\text{PtdIns}(4,5)\text{P}_2$. A maximal concentration was reached around 9 min after nitrogen-source addition and concentrations returned to basal levels within 25 min. Maximal $\text{PtdIns}(4,5)\text{P}_2$ concentrations reached about 5-fold the basal level. Addition of 1 $\mu\text{g}/\text{ml}$ wortmannin, a concentration known to inhibit the PtdIns 4-kinase *Stt4* [25], delayed the $\text{PtdIns}(4,5)\text{P}_2$ increase from 9 to 15 min after nitrogen-source addition (Figure 5A). This suggests that the $\text{PtdIns}(4,5)\text{P}_2$ signal is generated following the classical phosphoinositide pathway involving *Stt4* and *Mss4*. Neither addition of higher wortmannin concentrations (10 $\mu\text{g}/\text{ml}$) nor re-addition of wortmannin during the incubation led to a complete disappearance of the $\text{PtdIns}(4,5)\text{P}_2$ signal. Both sets of conditions resulted in a similar delay of the $\text{PtdIns}(4,5)\text{P}_2$ increase from 9 to 15 min after nitrogen-source addition (results not shown).

Addition of wortmannin at a concentration of 1 $\mu\text{g}/\text{ml}$ (Figure 5B) inhibited the activation of trehalase and the mobilization of trehalose (Figure 5C), which occurs in response to the addition of a nitrogen source to nitrogen-depleted glucose-repressed cells. Since nitrogen-induced trehalase activation occurred in a similar way in wild-type and *plc1*Δ mutants (results not shown), we could also rule out the involvement of *Plc1*. Activation of trehalase is a rapid effect of the nitrogen-induced activation of the FGM pathway. The fact that this activation is sensitive to the *Stt4* inhibitor wortmannin, together with the observation that wortmannin delays the nitrogen-induced $\text{PtdIns}(4,5)\text{P}_2$ signal, indicates a possible role for *Stt4* and $\text{PtdIns}(4,5)\text{P}_2$ in FGM signalling.

Conclusions

Our data indicate that both InsP_3 and $\text{PtdIns}(4,5)\text{P}_2$ may have a signalling function in the FGM pathway. It is noteworthy that the protein kinase *Sch9*, which occupies a key role in FGM signalling [3], contains a C2 domain. C2 domains in other proteins are known to bind negatively charged lipids such as $\text{PtdIns}(4,5)\text{P}_2$, often in a calcium-dependent manner [28]. However, until now we were unable to find any evidence for an interaction between either InsP_3 or $\text{PtdIns}(4,5)\text{P}_2$ and *Sch9* (J. C. T. Bergsma, J. M. Thevelein and S. Wera, unpublished work). Apart from the involvement of other second messengers, one explanation might be the presence of additional components of the FGM signalling cascade between $\text{InsP}_3/\text{PtdIns}(4,5)\text{P}_2$ and *Sch9*. Good candidates might be the protein kinases *Pkh1* and *Pkh2*, which are the yeast homologues of the mammalian PKB-activating protein kinase *PDK1* [29].

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