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MINI-REVIEW

# Messengers for morphogenesis: inositol polyphosphate signaling and yeast pseudohyphal growth

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## Abstract

In response to various environmental stimuli and stressors, the budding yeast *Saccharomyces cerevisiae* can initiate a striking morphological transition from its classic growth mode as isolated single cells to a filamentous form in which elongated cells remain connected post-cytokinesis in multi-cellular pseudohyphae. The formation of pseudohyphal filaments is regulated through an expansive signaling network, encompassing well studied and highly conserved pathways enabling changes in cell polarity, budding, cytoskeletal organization, and cell adhesion; however, changes in metabolite levels underlying the pseudohyphal growth transition are less well understood. We have recently identified a function for second messenger inositol polyphosphates (InsPs) in regulating pseudohyphal growth. InsPs are formed through the cleavage of membrane-bound phosphatidylinositol 4,5-bisphosphate ($PIP_2$), and these soluble compounds are now being appreciated as important regulators of diverse processes, from phosphate homeostasis to cell migration. We find that kinases in the InsP pathway are required for wild-type pseudohyphal growth, and that InsP species exhibit characteristic profiles under conditions promoting filamentation. Ratios of the doubly phosphorylated $InsP_7$ isoforms 5PP-$InsP_5$ to 1PP-$InsP_5$ are elevated in mutants exhibiting exaggerated pseudohyphal growth. Interestingly, *S. cerevisiae* mutants deleted of the mitogen-activated protein kinases (MAPKs) Kss1p or Fus3p or the AMP-activated kinase (AMPK) family member Snf1p display mutant InsP profiles, suggesting that these signaling pathways may contribute to the regulatory mechanism controlling InsP levels. Consequently, analyses of yeast pseudohyphal growth may be informative in identifying mechanisms regulating InsPs, while indicating a new function for these conserved second messengers in modulating cell stress responses and morphogenesis.

**Keywords** Pseudohyphal growth · Yeast · *Saccharomyces cerevisiae* · Inositol polyphosphate · Inositol pyrophosphate · MAPK · AMPK

As is true of many fungi, the budding yeast *Saccharomyces cerevisiae* can exist in more than one morphological type, and the transition between these morphological states is accomplished through a controlled and precise interplay between hundreds of genes enabling dramatic changes in most aspects of cell function (Gimeno et al. 1992; Cullen and Sprague 2012). Under conditions of nitrogen or glucose limitation, certain strains of *S. cerevisiae* (e.g., Σ1278b) can undergo a transition from the typical budding yeast-like growth form to one in which cells elongate and remain connected following cell division, forming multicellular filaments termed pseudohyphae for their superficial resemblance to multinucleate hyphal tubes (Fig. 1) (Gimeno et al. 1992; Cullen and Sprague 2000). Pseudohyphal filaments can be seen spreading outward from a colony over a solid surface substrate and/or invading the surface below the colony. Pseudohyphal growth is thought to be a scavenging mechanism, enabling non-motile yeast to spread out over a greater surface area in search of regions with more readily available nutrients. Pseudohyphal filamentation has been studied extensively over the years, as the process closely resembles pseudohyphal and hyphal growth transitions that are required for virulence in the related opportunistic human fungal pathogen *Candida albicans* (Braun and Johnson

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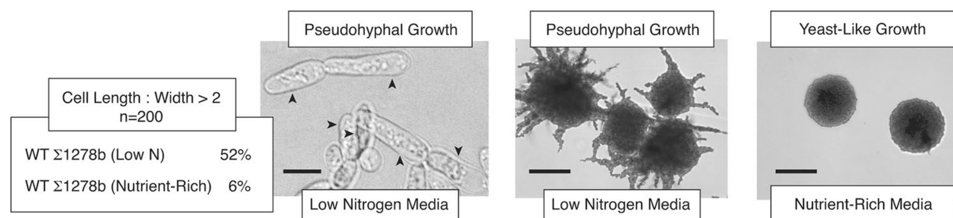


**Fig. 1** Morphological changes in *S. cerevisiae* pseudohyphal growth. Images of yeast cells and colonies grown in media with limited ammonium sulfate as a nitrogen source (low nitrogen media) or in media with normal nutrient availability. Quantification of cell elongation is indicated as the percentage of cells exhibiting a cell length:width ratio of greater than two. Arrowheads indicate elongated cells typical of pseudohyphal growth. Colony images are shown from a culture spread on an agar plate after 3 days growth. Scale bar for cells, 3 µm; scale bars for colony images are each 2 mm

1997; Lo et al. 1997; Mitchell 1998). Further, the signaling pathways that enable pseudohyphal growth in *S. cerevisiae* are conserved, with orthologous signaling systems regulating cell growth and responses to nutrient availability in metazoans (Cook et al. 1996; Erdman et al. 1998; Liu et al. 1993; Madhani and Fink 1997; Mosch et al. 1996; Pan and Heitman 1999).

Systematic screens for genes contributing to wild-type pseudohyphal growth have identified over 500 genes either required for pseudohyphal growth or that yield pseudohyphal growth phenotypes upon overexpression (Jin et al. 2008; Ryan et al. 2012; Shively et al. 2013). This collective gene set is broad, and the elucidation of pathways and mechanisms involving these genes warrants significant attention from the biological community, encompassing both ongoing and future studies. Much current and past research into yeast pseudohyphal growth has centered upon key and highly conserved signaling pathways required for wild-type filamentation. Pioneering research into the genetic basis of pseudohyphal growth identified the MAPK cascade of Ste11p, Ste7p, and the MAPK Kss1p (Cook et al. 1997; Liu et al. 1993; Madhani et al. 1997; Roberts and Fink 1994). Among its functions, phosphorylated Kss1p activates pseudohyphal growth through the transcription factors Ste12p and Tec1p, which form a complex that can recognize filamentation-responsive elements (FREs) in the promoters of target genes (Bardwell et al. 1998; Madhani and Fink 1997; Madhani et al. 1997). The mating pathway MAPK Fus3p inhibits pseudohyphal growth by phosphorylating Tec1p in response to pheromone, targeting Tec1p for degradation by the proteasome (Bao et al. 2004). The rat sarcoma (RAS)/protein kinase A pathway regulates pseudohyphal growth through several mechanisms, including phosphorylation of the pseudohyphal growth transcription factor Flo8p by Tpk2p, a catalytic subunit of protein kinase A (Pan and Heitman 1999; Robertson and Fink 1998; Iyer and Bhat 2017). The highly conserved nutrient-sensing target of rapamycin (TOR) pathway regulates pseudohyphal growth through the transcription factor Gcn4p, which in turn regulates expression of the flocculin Flo11p (Boeckstaens et al. 2008; Braus et al. 2003). The AMPK ortholog Snf1p functions in a glucose-sensing and regulatory pathway [reviewed in Simpson-Lavy and Kupiec (2018)], contributing to the control of filamentation by regulating the pseudohyphal growth repressors Nrg1p and Nrg2p at the *FLO11* promoter (Kuchin et al. 2002; Lo and Dranginis 1998; Vyas et al. 2003). The *FLO11* gene contains an unusually large promoter that is targeted by transcriptional regulators downstream of the MAPK, RAS/protein kinase A, TOR, and Snf1p signaling pathways (Rupp et al. 1999).

The brief overview above highlights a few critical pseudohyphal growth signaling pathways that contribute to broad and striking cellular changes in polarity, budding, cytoskeletal organization, cell cycle progression, and cell–cell adhesion. During pseudohyphal growth, yeast cells exhibit an increase in polarized apical growth occurring at the cell tip. Accordingly, the actin cytoskeleton in cells undergoing pseudohyphal growth is highly polarized, and the polarisome machinery, encompassing the formin Bni1p and the polarity control GTPase Cdc42p, is required for polarized growth during filamentation (Evangelista et al. 1997; Gladfelter et al. 2005). Under nutrient-limiting conditions that induce pseudohyphal growth, both haploid and diploid cells of filamentation-competent strains exhibit distal-unipolar budding, with buds forming predominantly at the distal pole (Gimeno et al. 1992). The distal marker Bud8p is required for unipolar budding during filamentation and is the preferential polar landmark over other positional cues in filamentous cells (Cullen and Sprague 2002; Harkins et al. 2001). Perspectives regarding budding and septin assembly in non-filamentous yeast are presented in Kang and Lew (2017). Cell elongation during pseudohyphal growth is also achieved through a delay in progression through G2/M, extending a period of directed apical growth, relative to uniform isotropic growth spread around the cell cortex (Kron et al. 1994). Filament formation requires enhanced adhesion between cells, and Flo11p is the principal expressed flocculin, with other *FLO* gene family members located subtelomerically in transcriptionally

repressed chromosomal regions (Guo et al. 2000; Lambrechts et al. 1996; Lo and Dranginis 1996). As suggested by its complex transcriptional regulation, alterations in Flo11p levels significantly impact cell adhesion (Fidalgo et al. 2006; Karunanithi et al. 2010). Interestingly, *FLO11* expression is regulated epigenetically and can be subject to rapid change, yielding yeast cell populations with heterogeneous adhesion properties (Halme et al. 2004; Verstrepen et al. 2005).

Although much still remains to be understood regarding the changes in cellular properties, signaling pathways, and proteins underlying the pseudohyphal growth transition, even less is known with respect to the changes in metabolites associated with filamentation. Short-chain alcohols, such as 1-butanol, can induce pseudohyphal growth, and these alcohols are now recognized as part of a quorum-sensing mechanism in *S. cerevisiae* (Chen and Fink 2006; Lorenz et al. 2000). Yeast cells secrete alcohol, such that corresponding alcohol levels roughly gauge cell density and population (Chen and Fink 2006). Tetrahydrofolate (vitamin B9) also induces pseudohyphal growth through uncharacterized mechanisms that impact *FLO11* expression levels (Guldener et al. 2004). The phytohormone indole-3-acetic acid is produced in yeasts and is known to induce pseudohyphal filamentation (Rao et al. 2010); its mechanism of action is unclear.

Our studies of pseudohyphal growth signaling pathways inadvertently led us to consider the role of another metabolite, inositol polyphosphate, in the yeast pseudohyphal growth transition. Using quantitative phosphoproteomics to profile changes in protein phosphorylation dependent upon a set of eight kinases required for wild-type pseudohyphal growth (Ste20p, Ste11p, Ste7p, Kss1p, Fus3p, Tpk2p, Snf1p, and Elm1p), we observed differences in the phosphorylation state of several kinases in the InsP biosynthetic pathway (Shively et al. 2015). InsPs are a ubiquitous class of second messengers with an increasingly recognized role in a diverse array of cellular processes.

Soluble InsPs are derived from membrane-bound phosphatidylinositol 4,5-bisphosphate ($PIP_2$) through the action of phospholipase C (Flick and Thorner 1993), which cleaves $InsP_3$ from $PIP_2$. The inositol polyphosphate $InsP_3$ is a 6-carbon cyclic alcohol with phosphate groups at the carbon-1, carbon-4, and carbon-5 positions. A variety of InsP species with additional phosphate groups are derived from $InsP_3$ through a sequentially acting set of InsP kinases and phosphatases [reviewed in Monserrate and York (2010)]. Arg82p, the *S. cerevisiae* ortholog of human IMPK, generates $InsP_5$ from $InsP_3$ through reactions that sequentially add phosphate groups to the carbon-6 and then carbon-3 positions of $InsP_3$ (Hatch and York 2010; Saiardi et al. 1999). The InsP kinase Ipk1p can convert $InsP_5$ to $InsP_6$ (York et al. 1999). Both $InsP_5$ and $InsP_6$ can be pyrophosphorylated, acquiring two phosphate groups at a single carbon position. In *S. cerevisiae*, the kinases Kcs1p and Vip1p are capable of pyrophosphorylating InsPs (Mulugu et al. 2007). Pyrophosphorylated isoforms of $InsP_7$ and $InsP_8$, as well as the kinases catalyzing the respective reactions, are summarized in Fig. 2. InsP phosphorylation is balanced by dephosphorylation through the phosphatases Siw14p, Ddp1p, and Vip1p, with the latter exhibiting both kinase and phosphatase activity (Pohlmann et al. 2014; Steidle et al. 2016; Wundenberg et al. 2014). The actions of these kinases and phosphatases generate dynamic changes in the cellular abundance and availability of different InsP isoforms, making them strong candidates to act as signal transducers in response to environmental perturbations.

InsPs have indeed come to be recognized as secondary messengers for cellular signal transduction. Perhaps most famously, $InsP_3$ binds to calcium channel receptors, regulating intracellular calcium release; however, this regulatory effect is not observed in *S. cerevisiae*, being restricted to higher eukaryotes (Michell et al. 1981). InsPs and inositol pyrophosphates have been shown to regulate a broad range of processes, including phosphate sensing, insulin secretion, viral particle release, glycolysis, ribosome synthesis, telomere length, cellular energy dynamics, dynein-driven transport, prion propagation, and amino acid signaling (Azevedo et al. 2009; Chakraborty et al. 2010; Chanduri

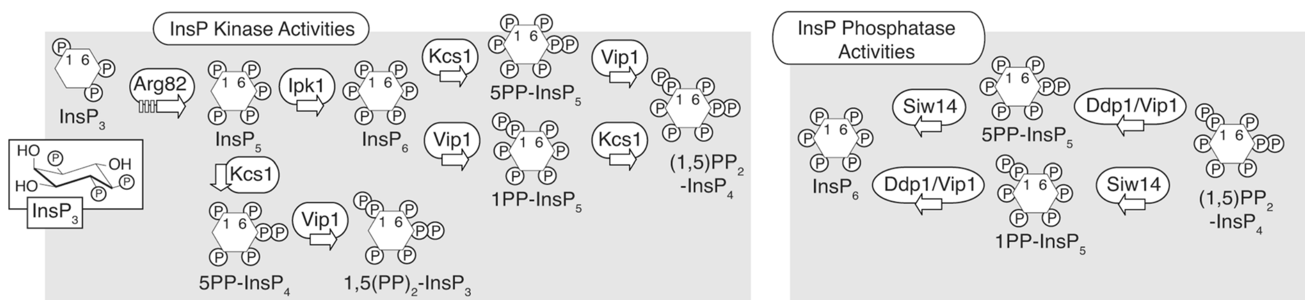


**Fig. 2** Diagram of the inositol polyphosphate biosynthetic pathway. The boat conformation for $InsP_3$ is indicated beneath a diagrammatic representation of inositol with the 1 and 6 positions indicated. InsP kinase and phosphatase activities are shown; respective proteins catalyzing each reaction are boxed

et al. 2016; Kim et al. 2011; Lee et al. 2007; Saiardi et al. 2005; Szijgyarto et al. 2011; Thota et al. 2015; Wickner et al. 2017, 2018; Wild et al. 2016). Although it has been suggested that $InsP_3$ is the only true second messenger among the InsP species (Shears et al. 2012), there is no doubt that inositol pyrophosphates are important for cellular signaling, particularly since they contain high-energy phosphate bonds (Bennett et al. 2006; Chakraborty et al. 2011). Two mechanisms have been proposed for the actions of pyrophosphates in cell signaling. First, inositol pyrophosphates may bind to proteins allosterically, changing their conformation, localization, and activity (Wu et al. 2016). Secondly, InsPs may regulate signaling through the transfer of a phosphate group to previously phosphorylated serine residues, generating pyrophosphorylated proteins (Bhandari et al. 2007; Saiardi 2016). In the pathogenic fungus *Cryptococcus neoformans*, $InsP_7$ was found to be crucial for metabolic adaptation to host environment and virulence (Lev et al. 2015). Asp1p, an ortholog of Vip1p in *S. pombe*, regulates polarized growth and the dimorphic switch (Pohlmann and Fleig 2010). Hence, like membrane constituent phospholipids (Rao et al. 2018), soluble InsPs can regulate physiological responses to various environmental stimuli in yeast.

Building on our observation that InsP pathway kinases are differentially phosphorylated in pseudohyphal growth kinase mutants, recent work from our laboratory indicates that InsP signaling regulates pseudohyphal growth (Norman et al. 2018). Genes encoding kinases and phosphatases in the InsP biosynthetic pathway are required for wild-type pseudohyphal growth under conditions of nitrogen limitation. Under these conditions, two isoforms of $InsP_7$, 5PP-$InsP_5$ and 1PP-$InsP_5$, can be distinguished, and elevated ratios of 5PP-$InsP_5$ to 1PP-$InsP_5$ are diagnostic of mutant strains exhibiting exaggerated pseudohyphal filamentation. Overexpression of *KCS1*, promoting elevated levels of the 5PP-$InsP_5$ isoform of $InsP_7$, is sufficient to drive pseudohyphal filamentation under otherwise non-inducing conditions.

The studies described above indicate a role for inositol polyphosphate signaling in pseudohyphal growth, but the findings also raise an open question as to the signaling pathways and networks that regulate inositol polyphosphate levels. Currently, relatively little is known regarding the regulation of inositol polyphosphate signaling in yeast or other eukaryotes. We find that InsP profiles are perturbed under conditions of nitrogen limitation in *S. cerevisiae* mutants of the filamentous Σ1278b background singly deleted of the pseudohyphal growth regulatory genes *KSS1, FUS3*, or *SNF1* (Norman et al. 2018). While we lack a regulatory mechanism by which these encoded kinases may modulate InsP kinase phosphorylation, the potential exists for control of the InsP biosynthetic pathway in yeast by the corresponding Kss1p and Fus3p MAPK cascades, as well as by the glucose-responsive AMPK Snf1p pathway. Prior to this work, Arg82p, Ksp1p, and Vip1p were independently identified in proteomic studies as phosphoproteins (Swaney et al. 2013), and *VIP1* may be subject to transcriptional regulation under conditions of heat stress by global regulators, such as Xbp1p, as assessed through large-scale chromatin immunoprecipitation analysis (Venters et al. 2011). It is certainly feasible, if not likely, that the InsP pathway is subject to significant regulatory control, both at the level of transcription and translation.

In sum, InsP signaling continues to emerge as a prominent second messenger system. The broad scope of processes impacted by InsP signaling likely reflects the diverse set of proteins bound by various InsP and pyrophosphate species, with the relative levels of particular species affecting substrate protein activity and cellular processes. Inositol pyrophosphates, in particular, may fill important regulatory roles, as their levels have been observed to increase in response to some conditions of stress and nutrient limitation (Dubois et al. 2002; Gibney et al. 2013; Worley et al. 2013). Additional work will be needed to identify and dissect the signaling pathways that act upstream of the InsP biosynthetic pathway. Given the diversity of cell processes affected by InsP signaling, it seems most likely that multiple signaling pathways act in parallel or in a convergent pattern to precisely modulate the respective activities of kinases and phosphatases in the InsP pathway. In that respect, the AMPK and MAPK nutrient/stress-responsive pathways may be part of a larger network affecting InsP levels in response to given cellular states and environmental conditions. On a broader level, analyses of InsP signaling highlight the need to consider the functions and changes associated with other cell metabolites, as ongoing and future metabolite profiling studies across single-cell eukaryotic and metazoan systems hold tremendous promise for the discovery of new and important biology.

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