Multiple Phosphatases Regulate Carbon Source-Dependent Germination and Primary Metabolism in Aspergillus nidulans

Leandro José de Assis,* Laure Nicolas Annick Ries,* Marcela Savoldi,* Taisa Magnani Dinamarco,† Gustavo Henrique Goldman,*‡ and Neil Andrew Brown*,†

*Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Brazil CEP14040-903, †Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Brazil, CEP14040-901 and ‡Laboratório Nacional de Ciência e Tecnologia do Bioetanol, CTBE-CNPEM, Brazil CEP13083-970

ABSTRACT Aspergillus nidulans is an important mold and a model system for the study of fungal cell biology. In addition, invasive A. nidulans pulmonary infections are common in humans with chronic granulomatous disease. The morphological and biochemical transition from dormant conidia into active, growing, filamentous hyphae requires the coordination of numerous biosynthetic, developmental, and metabolic processes. The present study exhibited the diversity of roles performed by seven phosphatases in regulating cell cycle, development, and metabolism in response to glucose and alternative carbon sources. The identified phosphatases highlighted the importance of several signaling pathways regulating filamentous growth, the action of the pyruvate dehydrogenase complex as a metabolic switch controlling carbon usage, and the identification of the key function performed by the α-ketoglutarate dehydrogenase during germination. These novel insights into the fundamental roles of numerous phosphatases in germination and carbon sensing have provided new avenues of research into the identification of inhibitors of fungal germination, with implications for the food, feed, and pharmaceutical industries.

KEYWORDS phosphatase germination glucose metabolism cell cycle

Aspergillus nidulans is an important mold and a model system for the study of fungal cell biology. In addition, invasive A. nidulans pulmonary infections are common in humans with chronic granulomatous disease, resulting in a high rate of mortality (Henriet et al. 2012). Reproduction in A. nidulans yields asexual conidia and sexual ascospores that are essential for fungal dispersal and the survival of harsh environments. Spore dispersal and germination is a key factor in the spoilage of food and pathogenicity (Klich 2009). Dormant conidia can remain viable for years until the appropriate conditions for germination are detected, including the exogenous presence of water, salt, carbon, and nitrogen sources (Osherov and May 2000, 2001). Conidial germination is characterized by two morphological steps, isotropic growth (conidial swelling) and the establishment of polarization and germ-tube emergence (D’Enfert 1997).

Dormant A. nidulans conidia are arrested in the G1 of the cell cycle (Bergen and Morris 1983). With the detection of conditions suitable for germination, the cell cycle is reinitiated. Entry into the S phase occurs during isotropic growth and the first round of mitosis occurs before polarization and germ-tube emergence (Bergen and Morris 1983; Momany and Taylor 2000). Hence, germination involves the detection of external nutrients, which results in primary metabolism shifting toward energy-yielding reactions that sequentially use intracellular stores prior to extracellular sources, as well as the initiation of the cell cycle and the biosynthesis of cellular components, and the differentiation of growth morphology. The signaling networks regulating the aforementioned processes involved in germination are unclear. An enhanced understanding of these signaling pathways would have a wide ranging impact on fungal biology and subsequently represents the focus of this study.
A. nidulans can germinate and sustain growth on a diverse range of simple and complex carbon sources, including saccharides, alcohols, proteins, and lipids. This metabolic flexibility has endowed it with the ability to adjust metabolism and nutrient uptake to fit the encountered environment (MacCabe 2003). Glucose metabolism represents the greatest energetic gain and is the preferred carbon source for the majority of microbes. The ability to sense intracellular or extracellular nutrient sources enables the coordination of cellular metabolism and the preferential consumption of glucose prior to alternative carbon sources, referred to as carbon catabolite repression (CCR) (Brown et al. 2014). Primary glucose metabolism is well-characterized in fungi where the carbon locked within the glucose is directed toward energy-producing reactions such as its oxidation via glycolysis and the tricarboxylic acid (TCA) cycle, fermentation, and the production of ethanol, or alternatively the production of storage sugars such as trehalose and glycerol (Figure 1). During the isotropic growth phase of germination, internal trehalose and mannitol are metabolized (D’Enfert 1997; D’Enfert et al. 1999; Fillinger et al. 2002) prior to the uptake and metabolism of the external carbon source, which triggered the breaking of dormancy (MacCabe 2003). Therefore, during the short period of conidial germination, primary metabolism is shifted multiple times.

Nutrient sensing and the downstream signaling cascades have a profound impact on the regulation of biochemical metabolic pathways, biosynthetic processes, and developmental changes. Signaling cascade intermediates are subject to post-translational modifications that influence target protein activity, localization, and function, such as protein phosphorylation, mediated by the opposing function of protein kinases and phosphatases (Shi 2009). In Saccharomyces cerevisiae, the cAMP-dependent protein kinase A (PKA) pathway mediates glucose sensing, promoting glucose uptake and utilization while inhibiting the sucrose nonfermenting kinase (Snf1p)-mediated alternative carbon usage pathway and influencing cell cycle progression (Farkas et al. 1989; Dong et al. 1995; Pedruzzi et al. 2003; Barrett et al. 2012).

In filamentous fungi, such as A. nidulans, the G-protein-coupled receptor-mediated and Ras-mediated cAMP-PKA pathways regulate germination and trehalose metabolism, whereas the SchA kinase performs parallel functions (Fillinger et al. 2002; Lafon et al. 2005, 2006). Despite advancements in the understanding of the A. nidulans phosphotproteome and the roles played by several central protein kinases, such as PkaA, SchA, and SnfA (Fillinger et al. 2002; Brown et al. 2013; Ramsubramaniam et al. 2014), current knowledge of the functions performed by the opposing phosphatases in carbon metabolism and germination is scarce. The recent generation of an A. nidulans null mutant collection containing 103 nonessential protein kinases (NPKs) and 28 nonessential protein phosphatases (NPPs) has assisted in the dissection of the signaling pathways involved in fungal development and cell cycle (Son and Osmani 2009; De Souza et al. 2013). A subsequent screen of the A. nidulans NPK and NPP mutant collection identified NPKs and NPPs involved in the regulation of CCR and cellulose utilization (Brown et al. 2014). Additionally, seven NPPs were shown to be unable to utilize glucose as a sole carbon source. An enhanced understanding of how these seven NPPs influenced carbon sensing, metabolism, and germination in A. nidulans would be of great interest. Hence, the present study of A. nidulans characterized the different functions of these seven NPPs in carbon sensing. This investigation showed the essentiality of multiple NPPs in the regulation of shared and distinct processes that impacted on primary carbon metabolism, cell cycle, and, ultimately, germination.

**MATERIALS AND METHODS**

**Strains and culture conditions**

The two A. nidulans strains FGSC A4 (pyrG89; wA3; pyroA4; veA1) and TN02A3 (pyrG89; pyroA4; nkuA:argB) were used as reference for comparisons with the NPP mutant collection. The 28 NPP null mutants were created by in vitro recombination in S. cerevisiae followed by A. nidulans protoplast transformation (http://www.fgsc.net/Aspergillus/KO_Cassettes.htm) (Son and Osmani 2009; De Souza et al. 2013). All fungal strains were propagated on complete media (2% w/v glucose, 0.5% w/v yeast extract, trace elements) or minimal media (1% w/v glucose or hydrolyzed casein (CA), trace elements, supplemented with pyridoxine, riboflavin, and uracil/uridine, pH 6.5) plus or minus 2% w/v agar.

**Screening for growth defects and sensitivity to 2-DG**

For all radial growth experiments, the respective strains were point-inoculated onto minimal media agar plates (±1% glucose) supplemented with 1% alternative carbon source. For pre-incubation experiments, 1×10⁷ conidia were incubated in liquid minimal media supplemented with 1% CA for 4 hr prior to inoculating a 5-µl droplet, or point of inoculation, onto solid minimal media plus 1% glucose. Plates were incubated for 48 hr at 37ºC before examination. The toxic glucose analog, 2-deoxyglucose (2-DG; Sigma), was used to measure sensitivity to CCR via growing the strains in minimal media plus CA (1%) and increasing concentrations of 2-DG (25–100 µM) for 96 hr at 37ºC. In addition, to observe the post-germination influence of 2-DG, conidia were pre-inoculated in CA for 4 hr and then inoculated onto 1% CA plus 2-DG (5-60 µM) for 48 hr at 37ºC.

To map the block in carbon metabolism, all strains were inoculated onto media containing combinations of amino acids (50 mM each amino acid) that enter into the tricarboxylic acid (TCA) cycle at specific steps: pyruvate (alanine and glycine), acetyl-CoA (leucine and lysine), ketoglutarate (arginine, proline, histidine and glutamine), succinyl-CoA (isoleucine, valine, methionine, and threonine), fumarate (tyrosine and phenylalanine), and oxaloacetate (asparagine and aspartate) as a sole carbon source for 96 hr at 37ºC.

**Double reciprocal shift assays**

The reciprocal shift experiments used to determine the phase of the cell cycle at which the phosphatase mutants arrested were performed as described previously (Bergen et al. 1984). Briefly, conidia from the phosphatase mutants were inoculated on cover slips and incubated in minimal media plus 1% glucose for 7 hr at 37ºC. Untreated germlings were fixed (as a control), transferred to minimal media plus 1% glucose for 3 hr, or transferred to minimal media plus 1% CA with 25 mM hydroxyurea (HU) for 3 hr. For the reciprocal experiments, conidia were arrested in S-phase by incubation in minimal media plus 1% CA containing 25 mM HU for 7 hr at 37ºC. The cover slips were either fixed (as a control) or transferred to minimal media plus 1% CA without HU or shifted to minimal media plus 1% glucose without HU. Cover slips were stained with Hoechst (3 µg/ml) for 2 min and washed in phosphate-buffered saline (PBS; 1×, NaCl 8 g/liter, KCl, 0.2 g/liter, Na₂HPO₄, 1.44 g/liter, and KH₂PO₄, 0.24 g/liter, pH 7.4). Subsequently, the number of nuclei per germling was counted for 80 to 120 germlings.

**Glucose uptake**

Minimal media cultures (30 ml) containing 1% CA as a carbon source were inoculated with 3×10⁷ conidia and subsequently incubated for
Figure 1 Primary carbon metabolism in A. nidulans and the representative genes involved in each biochemical step of glycolysis, glycerol metabolism, fermentation, TCA cycle (represented in red), and gluconeogenesis (represented in blue). G6P = glucose 6 phosphate; F6P = fructose 6 phosphate; F1,6BP = fructose 1,6 biphosphate; GA3P = glyceraldehyde 3 phosphate; DHAP = dihydroxyacetone phosphate; G3P = glycerol 3 phosphate; 1,3diPG = 1,3 diphosphoglycerate; 3PG = 3 phosphoglycerate; 2PG = 2 phosphoglycerate; PEP = phosphoenolpyruvate; DH = dehydrogenase.
24 h at 37°C in a rotary shaker (180 rpm). The mycelia were then transferred to minimal medium plus 1% glucose as a carbon source for an additional 24 hr. The amount of free glucose remaining in the medium was measured using the Glucose GOD-PAP Liquid Stable Mono-reagent kit (LaborLab Laboratories Ltd. Guarulhos, São Paulo, Brazil), according to the manufacturer’s instructions. Glucose uptake was calculated via determining the difference in glucose present in the initial media and after 24-hr incubation.

**Oxygen uptake measurements**

Minimal media cultures (30 ml) containing 1% CA as a carbon source were inoculated with 3×10⁶ conidia and subsequently incubated for 24 hr at 37°C in a rotary shaker (180 rpm). The mycelia were then transferred to minimal medium plus 1% glucose as a carbon source for an additional 24 hr. The mycelia were centrifuged, and the oxygen consumption was measured by a Clark-type electrode (Hansatech Instruments Ltd Oxygen Electrode Measurement Systems) in 1 ml of minimal medium supplemented with 1% glucose. Measurements were normalized using mycelial dry weight (Dinamarco et al. 2010).

**RNA extraction and RT-qPCR**

Mycelia were harvested by vacuum filtration and immediately frozen in liquid nitrogen. Mycelia were ground into a fine powder in liquid nitrogen and the total RNA was extracted using TRIZOL (Invitrogen) before being treated with RNase-free DNase (Promega) and purified with the RNEasy Mini Kit (Qiagen). RNA integrity was confirmed using the Bioanalyser Nano Kit (Agilent Technologies) and the Agilent Bioanalyser 2100. The SuperScript III First Strand Synthesis system (Invitrogen) and oligo(dT) primers were used for cDNA synthesis according to the manufacturer’s instructions. All RT-qPCR reactions were performed using an ABI 7500 Fast Real-Time PCR System and Taq-Man Universal PCR Master Mix kit (Applied Biosystems). The tubulin gene tubC was used as an endogenous control. The primers used for the glucose transporter and metabolic enzyme encoding genes are listed in Support Information, Table S1.

**Pyrurate, ethanol, glycerol, and trehalose measurements in mycelia**

Mycelia of the respective strains were grown under the same experimental conditions as described for the glucose uptake investigation. Mycelia were ground in liquid nitrogen and immediately resuspended by inversion in extraction buffer [50 mM: Tris base pH 7.0, 50 mM NaF, 1 mM Na₃VO₄, 1 mM DTT, phosphatase inhibitor cocktail P0044 (Sigma), and an EDTA-free protease inhibitor cocktail (Roche)] prior to centrifugation for 5 min at 14,000g, and washed once using 1 ml of water. The conidia were broken using 0.3 g of glass beads (0.65 μm) and 1 ml of buffer extraction [50 mM Tris base pH 7.0, 50 mM NaF, 1 mM Na₃VO₄, 1 mM DTT, phosphatase inhibitor cocktail P0044 (Sigma) and an EDTA-free protease inhibitor cocktail (Roche)]. The trehalose content was measured using Trehalose Assay kit K-TREH 11/12 (Megazyme) according to the manufacturer’s instructions with additional standard curve (0–4 μg) of the trehalose dihydrate.

**Trehalose measurements in germinating conidia**

Trehalose was extracted from 5×10⁷ fresh conidia, plus conidia germinating in minimal media containing either glucose or CA as a single carbon source. Conidia were incubated for 150 min at 37°C in 1 ml of the respective media, collected by centrifuge for 5 min at 14,000g at 4°C, and washed once using 1 ml of water. The conidia were broken using 0.3 g of glass beads (0.65 μm) and 1 ml of buffer extraction [50 mM Tris base pH 7.0, 50 mM NaF, 1 mM Na₃VO₄, 1 mM DTT, phosphatase inhibitor cocktail P0044 (Sigma) and an EDTA-free protease inhibitor cocktail (Roche)]. The trehalose content was measured using Trehalose Assay kit K-TREH 11/12 (Megazyme) according to the manufacturer’s instructions with additional standard curve (0–4 μg) of the trehalose dihydrate.

**Alpha-ketoglutarate assay**

Total cell lysate was prepared as described in the previous section. Alpha-ketoglutarate activity was measured in 30 μg of the cell lysates (Chretien et al. 1995; Tretter and Adam-Vizi 2004; Hadelhah et al. 2004), with modifications to the reaction buffer (100 mM Tris-HCl pH 7.0, 5 mM 2-mercapto ethanol, 1 mM MgCl₂, 2 mM TPP, 5 mM alpha-ketoglutarate acid disodium salt, 1 mM NAD⁺ and 0.2 mM Coenzyme A). The generation of the reduced form of NADH was measured at 340 nm using a SpectraMax I3 spectrometer (Molecular Devices).

**ADP/ATP ratio measurement**

Mycelia of the respective strains were grown under the same experimental conditions as described for the glucose uptake investigation. Total cell lysate was prepared as described in the previous section and the ADP/ATP ratio was measured in 10 μg of the cell lysate using the ADP/ATP ratio assay kit MAK135 (Sigma) following the manufacturer’s instructions and was read using a SpectraMax I3 spectrometer (Molecular Devices).

**Isotrophic growth measurements**

Fresh conidia were harvested in ultrapure sterile water and immediately observed by microscopy, and the radial diameter was measured immediately (control) or after incubation for 2 hr in minimal media plus glucose as a carbon source (treatment). The radial diameter was used to calculate the volume of conidia before and after treatment, following the assumption that the conidia were spherical, with the following formula (V = 4/3πR³).

**Statistical analysis of biochemical assays**

Statistical analyses were performed on three biological replicates using a one-tailed t-test (Prism, GraphPad) with significance levels of *P < 0.05, **P < 0.01, and ***P < 0.001.

**Microarray analysis**

Initially 1×10⁷ conidia were incubated in minimal media containing 1% CA as a carbon source at 37°C in a rotatory shaker (180 rpm)
for 24 hr. Subsequently, mycelia were washed with sterile water and then incubated in minimal media plus 1% glucose at 37°F for an additional 4 hr. At each step the mycelia from three biological replicates were collected by vacuum filtration and immediately frozen in liquid nitrogen. Agilent custom-designed oligonucleotide arrays (Colabardini et al. 2014) were used to identify the transcriptional differences during growth on CA (Cy3 reference) and glucose (Cy5) for the wild-type and ΔptpB strains. Total RNA was extracted and RNA integrity was confirmed as described previously. Array hybridization and data analysis were performed according to Colabardini et al. (2014). The dataset was deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE61980). Genes were determined as differentially expressed between carbon sources by applying a t-test (P < 0.01) performed within the Mev software (Saeed et al. 2003). The differentially expressed genes were divided into those that were upregulated or downregulated and strain-specific transcriptional alterations were identified via Venn analysis. The functional profile and identification of overrepresented GO terms within the differentially expressed gene sets from each strain under the two nutritional conditions were identified using the FetGOat software (http://www.broadinstitute.org/fetgoat/index.html) and FunCat (http://pedant.gsf.de/pedant3htmlview/pedant3view?Method=analysis&Db=p3_p130_Aspx_nidul).}

**RESULTS**

**Identification of seven NPPs that play important roles in carbon source–dependent germination**

To identify NPPs involved in glucose sensing and CCR, a collection of 28 *A. nidulans* NPP null mutants was previously screened for an altered ability to grow on either glucose or cellulose as a sole carbon source. Seven NPP null mutants were shown to be unable to grow directly on glucose (Brown et al. 2013). BLASTp analyses of the seven NPP encoding genes identified the phosphatase subfamily and the orthologous genes in *S. cerevisiae* (Table 1) that have been shown to regulate the mitochondrial pyruvate dehydrogenase complex (paralogs PTC5 and PTC6), the pheromone and cell wall integrity MAPK pathways (paralogs MSG5 and SDP1), the Msn2p-mediated general stress response (PSR1), filamentation (PTP1), and cell cycle (PPS1). The *A. nidulans* phosphatases were named accordingly (Table 1).

The seven NPP mutants were also unable to grow directly on xylose, glycerol, ethanol, and acetate as sole carbon sources, but they were able to grow directly on hydrolyzed casein (CA) or tributyrin and media containing both CA and glucose (Figure 2). This suggested that the seven NPP mutants possessed defects in the sensing and/or metabolism of carbon sources that entered primary carbon metabolism prior to the TCA cycle but were able to grow on amino acids and lipids that entered primary carbon metabolism as TCA cycle intermediates.

To validate the identification of the identified NPPs in glucose signaling, metabolism, and CCR during germination, the seven NPPs were grown directly on CA plus an increasing concentration of 2-DG, a toxic glucose analog that mimics glucose signaling but cannot be metabolized (Figure S1). Compared to the wild-type strain, ΔpsrA showed similar 2-DG sensitivity, ΔppsA was moderately more resistant to 2-DG, whereas ΔmsgA, ΔptcD, and ΔptcE were significantly more resistant to 2-DG. This suggests that the latter three strains may possess impaired glucose sensing or glucose uptake pathways. Conversely, ΔptpB and ΔsdpA exhibited a significant and moderate increase in sensitivity to 2-DG, respectively. This implied that these two NPP mutants were unable to grow on glucose due to a deficiency in glucose metabolism and not a specific issue in glucose sensing or uptake.

A similar approach was used to evaluate the post-germination defects in glucose sensing and/or metabolism, where the wild-type and NPP mutants were pre-incubated in CA for 4 hr and transferred to CA plus increasing concentrations of 2-DG. All the NPP mutants showed increased resistance to 2-DG compared to the wild-type strain, especially ΔsdpA (Figure S2). This demonstrated that all strains possessed defects in glucose sensing and/or metabolism after germination.

In *A. nidulans*, conidial germination requires the sensing of the appropriate conditions to support growth resulting in the breaking of dormancy, as represented by isotropic growth, whereas the subsequent polarization and germ-tube emergence is an energy-consuming process (Fillinger et al. 2002; MacCabe 2003; Hayer et al. 2014). To assess if the NPP mutants were unable to grow on glucose due to a defect in germination, conidia were pre-incubated in liquid media containing CA as a carbon source for 4 hr prior to plating on solid media containing glucose as a sole carbon source. Pre-incubation in CA restored the ability of all seven NPP mutants to grow on glucose (Figure 2). However, the seven NPP mutants exhibited reduced growth on glucose after germination on CA compared to the wild-type strain (Table 2). This suggested that these NPP mutants possessed defects in carbon sensing and/or metabolism, mechanisms that were essential for germination, in addition to post-germination defects in carbon metabolism.

---

**Table 1** Seven nonessential *A. nidulans* phosphatases null mutants

| *A. nidulans* Genes | Familya | Subfamilyb | Class/Domainc | *S. cerevisiae* Gene | E Value | Identity (%) |
|---------------------|---------|------------|---------------|---------------------|---------|--------------|
| AN4544 (msgA)       | PTP     | Dual-specificity | DSP          | MSG5               | 1e-22   | 30           |
| AN0129 (ppsA)       | PTP     | Dual-specificity | DSP          | PPS1               | 1e-51   | 31           |
| AN10077 (psrA)      | S/T     | Asp-based   | HAD          | PSR1               | 3e-92   | 69           |
| AN0914 (ptcD)       | S/T     | PPM         | PP2C         | PTC6               | 1e-25   | 33           |
| AN5722 (ptcE)       | S/T     | PPM         | PP2C         | PTC6               | 5e-112  | 44           |
| AN4896 (ptpB)       | PTP     | Classical   | PTP          | PTP1               | 3e-39   | 33           |
| AN10138 (sdpA)      | PTP     | Dual-specificity | DSP          | SDP1               | 9e-05   | 33           |

Seven nonessential *A. nidulans* phosphatases null mutants that cannot germinate on glucose as a sole carbon source and the homologous functionally characterized *S. cerevisiae* phosphatases.

a Family abbreviations: S/T, serine/threonine; PTP, protein tyrosine phosphatase.

b Subfamily abbreviations: Asp-based, aspartate-based phosphatase.

c Class/domain abbreviations: PP2C, protein phosphatase 2C catalytic subunit; PPM, Mg2+ or Mn2+-dependent protein phosphatase; HAD, haloacid dehalogenase; PTP, protein tyrosine phosphatase catalytic subunit; DSPc, dual-specificity phosphatase catalytic subunit.
NPPs influence glucose-dependent breaking of conidial dormancy and reinitiation of cell cycle

To further evaluate the impact of the seven NPP mutants on germination, the ability to break dormancy and produce isotropic growth in glucose-containing media was assessed (Table 3). The ΔptcE mutant was unable to induce swelling, suggesting a major defect in the detection of glucose, whereas the ΔptcD, ΔpsrA, and ΔsdpA mutants also showed a significant reduction in conidial swelling. The ΔmsgA, ΔtpbB, and ΔppsA mutants showed a significant increase in conidial swelling (>75%) and were therefore unlikely to possess major defects in glucose detection.

Reciprocal shift assays were performed to determine at which stage of interphase the ΔmsgA, ΔtpbB, and ΔppsA mutants were arrested (Table 4). Wild-type A. nidulans dormant conidia predominantly arrest in G1 or prior to S-phase, whereas a small subpopulation, approximately 10%, contains more DNA and may be binucleate (Bergen and Morris 1983). When conidia from the three NPP mutants were incubated in glucose, only 4–8% of nuclei underwent mitotic division. However, after transfer to CA, 54% and 63% of the ΔtpbB and ΔppsA germlings had multiple nuclei, demonstrating the completion of at least a single turn of the cell cycle. Shifting the ΔtpbB and ΔppsA conidia from glucose to CA plus hydroxyurea (HU), which blocks cell cycle progression in S-phase, revealed that ΔtpbB was unable to perform mitosis, with nearly all conidia still possessing a single nucleus, whereas ΔppsA was able to complete the cell cycle with the majority of germlings (70%) containing multiple nuclei. Alternatively, transfer of the ΔmsgA conidia from glucose to CA resulted in a delayed reactivation of the cell cycle, whereas prolonged incubation in CA plus HU did not permit cell cycle progression and an increase in nuclei number.

As a control, the reciprocal experiment was performed in which conidia were primarily incubated in CA plus HU to synchronize the conidia in S phase and then shifted to glucose or CA without HU (Table 4). Releasing cell cycle in CA without HU resulted in 76–83% of germlings from the three mutants having multiple nuclei. Similarly, after shifting the three mutants to glucose, between 78% and 84% of germlings also underwent mitotic division. These findings suggest that the ΔtpbB and ΔmsgA conidia possess a defect that causes them to be unable to exit from G1-S phase during germination on glucose. In addition, ΔmsgA also showed a delay in the reactivation of cell cycle.

Figure 2. The growth of the seven NPP null mutants and the wild-type strain (FGSC A4) in the presence of various carbon sources. Fungal strains were grown directly on minimal media agar plates supplemented with 1% of the respective carbon source, except for the final column where conidia were pre-incubated in CA for 4 hr prior to plating on glucose. All cultures were incubated at 37° for 48 hr. CA, hydrolyzed casein; CA+Glu, hydrolyzed casein+glucose; and Pre-CA to Gluc, pre-grown in hydrolyzed casein and transferred to glucose.

NPPs influence trehalose levels in conidia and its breakdown on germination

Trehalose is stored within conidia and is metabolized during the isotropic growth phase at the onset of germination trehalose (D’Enfert 1997; D’Enfert et al. 1999, Fillinger et al. 2002) prior to the uptake and metabolism of the external carbon source, which triggered the breaking of dormancy (MacCabe 2003). Subsequently, trehalose levels within dormant conidia and after germination in glucose or CA were investigated (Figure 3). The ΔppsA and ΔptcE mutants showed higher trehalose levels under all conditions. Trehalose levels in the wild-type strain declined after germination in either glucose or CA. All seven NPP mutants also showed a decline in trehalose level during germination on glucose. However, excluding, ΔmsgA, the remaining six NPP mutants showed no significant decline in trehalose level during germination in CA. Therefore, it would appear that these six NPP mutants mobilized conidial trehalose stores to fuel germination on glucose, but downstream defects in saccharide metabolism impeded successful germination. Alternatively, during germination...
on CA, six NPP mutants did not mobilize conidial trehalose stores and possibly redirected metabolism toward the utilization of CA and successful germination.

**NPPs influence glucose and oxygen consumption**

A defect in the uptake and/or metabolism of saccharides may have contributed to the inability to germinate on such carbon sources. Subsequently, the capacity of the seven NPP mutants to consume glucose and oxygen was determined (Figure 4). The NPP mutants and the wild-type strain were pre-grown in media containing CA and then transferred to media containing glucose as a sole carbon source. After 24 hr, the amount of residual glucose in the media was measured. The wild-type strain plus the ΔptpB and ΔppsA mutants consumed all the available glucose, whereas the remaining five NPP mutants (ΔmsgA, ΔptcD, ΔptcE, ΔpsrA, and ΔsdpA) showed an approximate 60% reduction in glucose consumption (Figure 4A).

Simultaneously, the consumption of oxygen by the seven NPP mutants was determined. The ΔpsrA and ΔsdpA mutants consumed oxygen at a similar rate as the wild-type strain, whereas the ΔmsgA, ΔppsA, ΔptcD, ΔptcE, and ΔptpB showed a significant reduction in oxygen consumption (Figure 4B). Collectively, these data show that the NPP mutants that were more resistant to 2-DG (ΔmsgA, ΔptcB, and ΔptcE) had decreased consumption and/or uptake of glucose, coupled with a reduction in oxygen consumption, which conveyed 2-DG resistance. In accordance, a reduction in the expression of three glucose transporters, hxtB (AN1797), hxtC (AN10891), and hxE (AN6669), was observed in the ΔmsgA, ΔptcB, and ΔptcE strains after transfer from CA to glucose-rich media for 2 hr and 4 hr, suggesting that glucose uptake and signaling were impaired (Figure 5, A–C). Therefore, all seven NPP mutants were impaired in glucose uptake and/or respiration, suggesting that these NPPs perform additional roles in regulating metabolism beyond germination.

**Intracellular ethanol, glycerol, trehalose, and pyruvate levels imply shifts in primary metabolism in the NPP mutants**

The NPP mutants were unable to grow directly on glucose as a sole carbon source. However, when transferred from CA to glucose containing media, all strains were able to consume glucose and respire to an extent. Therefore, to assess where glucose metabolism had been redirected, the intracellular glycerol, trehalose, and pyruvate content were measured. The NPP mutants and the wild-type strain were grown on CA and then transferred to media containing glucose as a sole carbon source. Intracellular pyruvate levels in the ΔmsgA, ΔptcD, and ΔptcE mutants had significantly decreased whereas in the ΔpsrA, ΔptpB, and ΔsdpA mutants pyruvate levels had increased when compared with the wild-type strain (Figure 6A). No change was observed in the ΔppsA mutant (Figure 6A). Intracellular trehalose was reduced in the ΔmsgA, ΔptcE, ΔpsrA, ΔptpB, and ΔsdpA mutants, whereas ΔppsA and ΔptcD were similar to the wild-type strain (Figure 6B). The three NPP mutants with reduced pyruvate levels, ΔmsgA, ΔptcD, and ΔptcE, showed an approximate two-fold increase in intracellular glycerol levels compared with the wild-type strain (Figure 6C). These results suggest that glucose metabolism is directed toward glycerol production in the ΔmsgA, ΔptcD, and ΔptcE mutants, which seem unable to produce significant amounts of pyruvate. In contrast, the ΔptpB mutant had reduced glycerol levels, suggesting that glucose metabolism was redirected toward fermentation. Hence, ethanol production

---

**Table 2 Growth after germination (dry weight) of the seven NPP mutants and the wild-type strain on glucose**

| Trait | ΔpsrA | ΔmsgA | ΔptcD | ΔptcE | ΔptpB | ΔppsA | ΔsdpA | WT |
|-------|-------|-------|-------|-------|-------|-------|-------|----|
| Pre-CA for 4 hr prior to transfer to glucose | 0.002 ± 0.0002 | 0.032 ± 0.001 | 0.032 ± 0.004 | 0.033 ± 0.005 | 0.033 ± 0.006 | 0.033 ± 0.002 | 0.033 ± 0.001 | 0.056 ± 0.002 |
| Post-CA for 24 hr (dry weight in grams) | 0.033 ± 0.0002 | 0.033 ± 0.0003 | 0.035 ± 0.0002 | 0.035 ± 0.0002 | 0.035 ± 0.0002 | 0.035 ± 0.0002 | 0.035 ± 0.0002 | 0.035 ± 0.0002 |

1×10^7 conidia pre-germinated in CA minimal media for 4 hr prior to transfer to glucose minimal media for 24 hr. Level of significance compared to the wild-type strain: *P < 0.05*, **P < 0.01**, and ***P < 0.001***.
in the \(\Delta pt\) mutant was measured, revealing a three-fold increase in ethanol production \(0.09 \text{ g.L}^{-1}\) ethanol in the \(\Delta pt\) and wild-type strains, respectively. This result suggests that glucose metabolism is directed toward fermentation in the \(\Delta pt\) mutant.

**Mapping the block in glucose metabolism reveals TCA cycle defects in the NPP mutants**

The observation that the seven NPP mutants were unable to germinate on saccharide or alcohol carbon sources \((e.g., \text{glucose, xylose, glycerol, acetate, and ethanol; Figure 2})\) but could germinate and grow on CA or tributyrin suggested that these strains had defects in glycolysis, gluconeogenesis, or the TCA cycle. Hydrolyzed casein contains a mixture of amino acids that can be used by *A. nidulans* for carbon metabolism and growth. Individual amino acids enter carbon metabolism and growth. Individual amino acids enter

| Fungal Strain | Conidial Diameter (\(\mu m\)) | Conidial Volume (\(\mu m^3\)) | Swelling (%) | t test\(^a\) |
|---------------|--------------------------------|--------------------------------|-------------|-------------|
|               | Fresh (0 hr) Glucose (2 hr)   | Fresh (0 hr) Glucose (2 hr)   |             |             |
| WT            | 3.7 ± 0.04 5.4 ± 0.06         | 6.8 ± 7.16 81.1 ± 20.59      | 202.4 ± 15.23 | —           |
| \(\Delta msgA\) | 3.7 ± 0.03 4.5 ± 0.05         | 6.7 ± 6.50 46.7 ± 15.63      | 75.1 ± 18.99 | 0.0001      |
| \(\Delta ppsA\) | 4.1 ± 0.06 5.2 ± 0.08         | 36.6 ± 16.86 75.0 ± 29.94    | 105.0 ± 15.23 | 0.01        |
| \(\Delta psrA\) | 3.8 ± 0.02 4.1 ± 0.04         | 27.8 ± 5.88 35.0 ± 9.44      | 25.5 ± 4.70  | 0.0001      |
| \(\Delta ptcD\) | 3.8 ± 0.03 4.0 ± 0.05         | 27.5 ± 6.18 34.1 ± 10.66     | 24.2 ± 6.03  | 0.0001      |
| \(\Delta ptcE\) | 3.8 ± 0.03 3.5 ± 0.05         | 27.9 ± 7.30 23.1 ± 8.70      | 24.2 ± 6.03  | 0.0001      |
| \(\Delta ptpB\) | 3.7 ± 0.03 4.7 ± 0.04         | 26.9 ± 7.90 55.1 ± 14.78     | 105.0 ± 6.86 | 0.0001      |
| \(\Delta sdpA\) | 3.6 ± 0.03 4.2 ± 0.05         | 25.7 ± 6.68 37.4 ± 14.80     | 45.6 ± 8.68  | 0.0001      |

Presented are the conidial diameter and volume before and after incubation in glucose media for 2 hr plus the percentage change in conidial volume. \(^a\) t test wild-type vs. null mutant strain for swelling.

The NPP mutants were shown to have defects in the TCA cycle, inhibiting oxidative phosphorylation, which would therefore impede the production of ATP. Subsequently, the ADP/ATP ratio was measured under the same conditions as the glycerol, trehalose, and pyruvate measurements (Figure 8). The \(\Delta ppsA\) mutant had a similar ADP/ATP ratio to the wild-type strain, whereas the \(\Delta msgA\), \(\Delta psrA\), \(\Delta ptcD\), \(\Delta ptcE\), \(\Delta ptpB\), and \(\Delta sdpA\) mutants all had an increased ADP/ATP ratio. This showed that these six NPP mutants had reduced ATP level representative of a lower energetic state due to their defects in the TCA cycle.

Groups of amino acids were used to minimize the possible impact of defects in amino acid catabolism. In addition, the growth and germination of all the NPP deficient strains on minimal media plus CA were comparable to the wild-type, suggesting that there was no major defect in amino acid metabolism, whereas all NPP deficient strains also showed reduced growth rates on glucose (after germination). This strongly suggested that the metabolic defect(s) truly resided in carbon metabolism.

Collectively, these results demonstrate that multiple phosphatases influence primary carbon metabolism, whereas the deletion of these NPP-encoding genes impedes the production of energy and

**Table 4 Double reciprocal shift assay for the \(\Delta pt\), \(\Delta msg\), and \(\Delta pps\) A. nidulans phosphatase mutants**

| Fungal Strain | Initial Carbon Source (7 hr) | Shift Second Carbon Source | Germilngs with >1 Nucleus (%)\(^a\) |
|---------------|-----------------------------|----------------------------|----------------------------------|
| \(\Delta pt\)  | Glu No shift (0 hr)         | 7                          |                                  |
|                | CA (3 hr)                   | 54                         |                                  |
|                | Glu CA + HUb (3 hr)         | 6                          |                                  |
|                | CA + HUb CA                 | 8                          |                                  |
|                | CA + HUb Glu               | 76                         |                                  |
| \(\Delta msg\) | Glu No shift (0 hr)         | 4                          |                                  |
|                | CA (3 hr)                   | 3                          |                                  |
|                | Glu CA (5 hr)               | 78                         |                                  |
|                | Glu CA + HUb (3 hr)         | 1                          |                                  |
|                | Glu CA + HUb (5 hr)         | 3                          |                                  |
|                | CA + HUb CA                 | 7                          |                                  |
|                | CA + HUb Glu               | 78                         |                                  |
| \(\Delta pps\) | Glu No shift (0 hr)         | 8                          |                                  |
|                | CA (3 hr)                   | 63                         |                                  |
|                | Glu CA + HUb (3 hr)         | 70                         |                                  |
|                | CA + HUb Glu               | 2                          |                                  |
|                | CA + HUb CA                 | 83                         |                                  |
|                | CA + HUb Glu               | 84                         |                                  |

\(^a\) 100 germlings were counted for each experiment and the percentage having completing at least one nuclear division.

\(^b\) Germlings were incubated in the presence of 25 mM hydroxyurea (HU).
carbon compounds required for growth. In addition, these data implicated a crucial role for the α-KGDH in the regulation of conidial germination.

**NPPs influence alpha-ketoglutarate dehydrogenase activity**

In *S. cerevisiae*, two α-KGDH isoforms are encoded by the *KGD1* and *KGD2* genes (Repetto and Tzagoloff 1989, 1990). In *A. nidulans*, the genes *kgdA* (AN5571) and *kgdB* (AN3466) were identified with high identity to the two *S. cerevisiae* genes *KGD1* (e-value, 0.0; 65% identity; 79% similarity) and *KGD2* (e-value, 3.e-133; 60.2% identity; 74.1% similarity). To further support the hypothesis that the seven NPP mutants possessed defects in the ability to convert α-ketoglutarate to succinyl-CoA, α-KGDH activity was measured. The NPP mutants and the wild-type strain were grown in CA and then transferred to glucose media for an additional 24 hr. All seven of the NPP mutants had decreased α-KGDH activity when compared to the wild-type strain upregulated different genes in the conversion of α-ketoglutarate to succinyl-CoA and unable to grow on carbon sources prior to succinyl-CoA, due to a de-regulation of α-KGDH activity.

**Microarray analyses revealed that PtpB influences the transcriptional response to growth on glucose and alteration to primary metabolism**

In this study, the Δ*ptpB* strain was shown to be unable to grow on saccharides including glucose, to have a defect in cell cycle progression, and to have extreme sensitivity to 2-DG during germination, whereas mycelia were shown to have reduced respiration and increased resistance to 2-DG. To ensure that the Δ*ptpB* strain (containing Δ*ptpB::pyrGΔ* deletion cassette) did not possess additional mutations that impacted on carbon metabolism, it was sexually crossed with a strain containing the nonfunctional pyrG89 mutation, which is auxotrophic for uridine and uracil. The subsequent Δ*ptpB::pyrG89* progeny, which were uridine and uracil prototrophs, were selected. Phenotypic investigation of the progeny revealed Δ*ptpB::pyrGΔ* cassette co-segregated with an inability to germinate and grow on glucose (data not shown), strongly indicating that PtpB is responsible for the observed phenotypes.

As an initial step to characterize the effect of the phosphatase mutants in regulating glucose metabolism, we investigated the role of PtpB by using genome-wide microarray analyses. The main objective was to identify strain-specific transcriptional differences during the metabolism of CA or glucose as the sole carbon source. The wild-type and Δ*ptpB* strains were grown for 24 hr on CA and then transferred to glucose media for 4 hr. Genes that were differentially expressed between carbon sources in an individual strain were identified ($P < 0.01$) and subsequently divided into genes that were upregulated or downregulated after transfer to glucose. Strain-specific differences in the modulation of gene expression were identified via Venn analyses (Figure 10, A and B, Table S2, Table S3, Table S4, Table S5). An overview of the functional profile of the strain-specific gene sets was revealed via observing alterations in FunCat representation and the identification of over-represented gene ontologies (GO terms; $P < 0.05$) (Figure 10C, Table 5).

The two strains exhibited distinct transcriptional profiles as represented by the small proportion of genes that were modulated in both strains after transfer to glucose. Overall, the Δ*ptpB* mutant modulated the expression of a higher number of genes than the wild-type strain, particularly those involved in metabolism. Both strains showed an up-regulation of genes involved in the modulation of cell cycle and replication after transfer to glucose. The wild-type strain specifically showed an upregulation of processes related to protein translation, whereas the Δ*ptpB* strain upregulated different genes involved in ribosome biogenesis and downregulated alternative carbon usage when grown on glucose.

Subsequently, the transcriptional modulation of genes specifically involved in primary carbon metabolism, including glycolysis, gluconeogenesis, TCA cycle, glyoxylate cycle, alcohol fermentation, amino acid catabolism, lipid metabolism, and fatty acid beta-oxidation, were inspected (Figure 11). Several key steps in carbon metabolism were de-regulated in the Δ*ptpB* mutant when compared to the wild-type strain. These included the following: the *pfkA* phosphofructokinase, involved in glycolysis; the *acuG* fructose-1,6-bisphosphatase,
which is a key regulatory enzyme in gluconeogenesis; the acuK transcription factor that regulates gluconeogenesis; the \textit{FbaA} fructose-bisphosphate aldolase, which is important for growth on nonsugar carbon sources; subunits of the pyruvate dehydrogenase complex (3-methyl-2-oxobutanoate dehydrogenase, alpha-keto acid dehydrogenase E1 and E2; \textit{AN1726}, \textit{AN8559}, \textit{AN3639}) involved in amino acid catabolism; and the \textit{hadA} hydroxy-acyl-CoA dehydrogenase, the \textit{echA} enoyl-CoA hydratase, and \textit{mthA} ketoacyl-CoA thiolase, required for fatty acid beta-oxidation and amino acid catabolism. In addition, specifically in the \textit{DptpB} strain, an upregulation of the alcohol regulator \textit{alcR} and the alcohol dehydrogenase gene \textit{alcA} was observed.

Subsequent RT-qPCR analyses of genes encoding for metabolic enzymes involved in primary carbon metabolism (\textit{pfkA}, \textit{alcA}, \textit{pdhA}, \textit{carC} encoding 6-phosphofructokinase, alcohol dehydrogenase, alpha subunit of pyruvate dehydrogenase, and succinate dehydrogenase, respectively), which were differentially regulated in the \textit{DptpB} strain, were performed for the wild-type, \textit{DptpB}, \textit{DmsgA}, \textit{DptcD}, and \textit{DptcE} strains. These analyses confirmed the results observed in the microarray and demonstrated that primary carbon metabolism was similarly transcriptionally altered in the \textit{DmsgA}, \textit{DptcD}, and \textit{DptcE} strains (Figure 12, A–D). These results reveal how PtpB influences the transcriptional regulation of multiple key steps in primary carbon metabolism.

**DISCUSSION**

Conidial germination is an extremely complex biological process that requires the breaking of dormancy, morphogenesis, the reactivation of the cell cycle, the biosynthesis of new cell components, adaptations to stress, and the shifting of metabolism toward energy, yielding reactions. In \textit{A. nidulans}, conidia can germinate in

---

**Figure 5** The expression of three major glucose transporter encoding genes in three NPP mutants and the wild-type strain (FGSG A4). Mycelia were grown in minimal medium plus CA for 24 hr before being transferred to minimal medium plus glucose for 2 or 4 hr. Gene expression of (A) \textit{AN1797} (\textit{hxtB}), (B) \textit{AN10891} (\textit{hxtC}), and (C) \textit{AN6669} (\textit{hxtE}) was measured by RT-qPCR and values were normalized to the expression of \textit{tubC}. The values are the average of three independent experiments (± standard deviation). Glucose transporter encoding gene expression was significantly reduced in all the NPP mutants (\( P < 0.05 \)).

**Figure 6** Intracellular pyruvate (A), glycerol (B), and trehalose (C) levels in the mycelia of the NPP mutants and wild-type strain during growth on glucose as a sole carbon source. Conidia were incubated in minimal medium plus CA for 24 hr. Subsequently, the mycelia were transferred to minimal medium plus 1% glucose for 24 hr. The average concentrations were determined from three independent experiments (± standard deviation). The concentrations of glycerol, trehalose, and pyruvate are presented in \( \text{mol.mg}^{-1} \). Significance = *\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \).
the presence of water, salts, and a carbon source while being able to utilize a diverse array of simple and complex carbon sources. Hence, carbon sensing has a profound impact on *A. nidulans* germination, growth, and the aforementioned biological processes (Fillinger *et al.* 2002; Lafon *et al.* 2005, 2006). However, the signaling mechanisms that coordinate these processes are unclear. In the present study, seven phosphatases were identified to perform fundamental roles during conidial germination on saccharide and alcohol carbon sources, broadly influencing morphological adaptations, metabolism, and cell cycle (Table 6). This evidence represents the basis for the future dissection of carbon sensing and the signaling pathways involved in germination.

The immediate response of conidia to the detection of conditions suitable for growth is represented by isotropic growth or swelling, which occurs via the absorption of water, and the reactivation of cell cycle (Bergen and Morris 1983; D’Enfert 1997; Momany and Taylor 2000). After a suitable carbon source has been detected, metabolism is rapidly switched from the basal utilization of nonsaccharide carbon sources via gluconeogenesis and the glycoxylate cycle to the use of trehalose and mannitol via glycolysis, providing the energy and carbon compounds required for growth (D’Enfert 1997; Hayer *et al.* 2013, 2014). The absence of any swelling in the ΔptcE mutant suggests that it is unable to detect glucose. Interestingly, the ΔptcD and ΔpsrA mutants also showed substantially reduced swelling and may also possess defects in glucose sensing. However, conidial trehalose stores were mobilized during germination on glucose for all NPP mutants, as in the wild-type strain. Therefore, the reduced isotropic growth in response to glucose could also be the consequence of issues in trehalose metabolism, considering that all strains are unable to utilize any of the tested saccharides, a defect that was bypassed during germination on CA.

The homologs of the identified *A. nidulans* PtcD and PtcE phosphatases in *S. cerevisiae*, Ptc5p and Ptc6p, are mitochondrial...
phosphatases that regulate the pyruvate dehydrogenase complex (Gey et al. 2008). The pyruvate dehydrogenase complex (PDC) acts as a metabolic switch in mammalian cells that regulates the utilization of alternative carbon sources. The PDC generates NADPH and acetyl-CoA from the oxidative decarboxylation of pyruvate and facilitates uptake into the mitochondria. The phosphorylation state of the PDC controls the flux through this irreversible reaction, thus directing metabolism toward the consumption of glucose in respiration or the preservation of glucose for gluconeogenesis (Wu et al. 2001). Under carbon limitation, the pyruvate dehydrogenase kinase phosphorylates and inactivates the PDC, conserving glucose and promoting fatty acid utilization (Wu et al. 2001). Disruption of the A. nidulans pyruvate dehydrogenase kinase, pkpA, resulted in the loss of an ability to grow on recalcitrant cellulose, which represents an alternative carbon source that requires the utilization of fatty acids for the synthesis of cellulolytic enzymes (Brown et al. 2013). Conversely, the present study showed that the disruption of the opposing phosphatases, ptcD and ptcE, resulted in the loss of germination, but not growth, on glucose. Therefore, the PDC appears to perform a fundamental role in the regulation of carbon metabolism in A. nidulans, which during germination acts as a metabolic switch promoting the use of glycolytic carbon sources.

Dormant A. nidulans conidia reside in the G1 phase of the cell cycle. Prior to germ-tube emergence, at least one full cycle has been completed and the number of nuclei has increased (Bergen and Morris 1983; Momany and Taylor 2000). Here, several A. nidulans phosphatase mutants showed conidial swelling on glucose but did not establish polarity. Subsequently, these mutants were shown to be unable to complete the cell cycle. These A. nidulans phosphatases included the homologs of the S. cerevisiae phosphatases, Ptp1p, Pps1p, and Msg5p. These three S. cerevisiae phosphatases are involved in the regulation of pathways influencing cell cycle and filamentation. Overexpression of Pps1p resulted in synchronous growth arrest in S phase and an abolishment of DNA synthesis (Ernsting and Dixon 1997). A screen for suppressors, identified RAS2 as a multicopy suppressor, but not protein kinase A or the adenylate cyclase, suggesting that Pps1p influences a PKA-independent Ras2 pathway (Ernsting and Dixon 1997). Msg5p performs a role in the recovery from G1 arrest after exposure to the mating pheromone, suggesting that the kinase Fus3p, which must be dephosphorylated for recovery from G1 arrest, is a substrate for Msg5p (Doi et al. 1994). Ptp1p is a phosphotyrosine-specific protein phosphatase that dephosphorylates a broad range of substrates, including the Fpr3p nucleolar peptidyl-prolyl cis-trans isomerase that acts as a transcriptional repressor, whereas Ptp1p is proposed to be a negative regulator of filamentation (Wilson et al. 1995). In addition, Ptp1p can complement a mutation in an endogenous Schizosaccharomyces pombe phosphatase, Cdc25, which regulates a key cell cycle regulator, Cdc2 (Hannig et al.
In the reactivation of cell cycle progression, a block between the G1 and S phases in addition to showing a delay in germ-tube emergence (D’Enfert 1997), suggesting the Ptp1p also influences cell cycle. Here, the putative homologs in A. nidulans were shown to also possess defects in cell cycle progression during germination on glucose, with the ΔppsA strain possessing a block post S phase, possibly mitosis, and the ΔptpB strain possessing a block in the transition between the G1 and S phases. Interestingly, ΔmsgA also possessed a block between the G1 and S phases in addition to showing a delay in the reactivation of cell cycle progression, fitting with a putative role in the positive regulation of recovery from cell cycle arrest in G1.

The second developmental transition during germination in A. nidulans is represented by the establishment of polarity and germ-tube emergence (D’Enfert 1997). Filamentous growth in fungi is predominantly regulated by two mitogen-activated protein kinase (MAPK) cascades, termed the filamentous growth and cell wall integrity pathway (Rispail et al. 2009). In A. nidulans, the phosphatases regulating these MAPK cascades are unknown. In S. cerevisiae, two phosphatase paralogs, Msg5p and Sdp1p, regulate the phosphorylation state of the terminal kinase of the respective pseudohyphal growth/pheromone response and the CWI pathways, Fus3p/Kss1p and Stl2p (Palacios et al. 2011). Here, seven phosphatase mutants were unable to establish polarity and germ-tube emergence. Two of the identified A. nidulans phosphatases that could not germinate on saccharides were putative homologs of Msg5p and Sdp1p, suggesting these strains may have had defects in filamentous growth and CWI pathways, a hypothesis that is supported by the delayed germination of ΔmsgA on CA. The transition from conidia to hypha also exposes a germling to increased stress. This could contribute to the necessity for PsrA during A. nidulans germination on glucose, because the putative
**Figure 11** Heatmap displaying the transcriptional profiles of selected genes encoding for proteins involved in glycolysis, gluconeogenesis, fermentation, and the TCA cycle in the three independent replicates of the wild-type and ΔptpB strains.

*S. cerevisiae* homolog Psr1p is involved in the regulation of Msn2-mediated general stress response, which is activated by dephosphorylation (Kaida et al. 2002). The faster growth rate on glucose compared to CA may enhance the exposure to stress and increase the requirement for PsrA during *A. nidulans* germination on glucose.

The α-KGDH is a TCA cycle enzyme that catalyzes the conversion of α-ketoglutarate, coenzyme A, NAD+, succinyl-CoA, NADH, and CO₂ and requires thiamine pyrophosphate as a cofactor, which is inhibited by its end products (Tretter and Adam-Vizi 2004). A transcriptomic analysis of *A. niger* revealed that the α-KGDH is upregulated during germination (Novodvorska et al. 2013). The present study showed that the seven phosphatase mutants, which were all unable to germinate and grow on carbon sources prior to succinyl-CoA, also possessed reduced α-KGDH activity. The α-KGDH blockage in TCA cycle pushes down cell energy levels as the oxidative phosphorylation pathway requires NADH, produced by TCA cycle, to sustain ATP production. The ADP/ATP ratios were increased in all the NPP mutants but decreased in the wild-type strain during growth on glucose. This implicates the α-KGDH as a fundamental step in the regulation of conidial germination in *A. nidulans*.

In summary, the transition from dormant conidia into growing filamentous hyphae requires the activation of numerous processes. The present study showed the diversity of roles performed by multiple phosphatases in regulating cell cycle, development, and metabolism in response to glucose and alternative carbon sources. In addition, this study highlighted the importance of several signaling pathways regulating filamentous growth, the action of the PDC as a metabolic switch controlling carbon usage, and the identification of the key function performed by the α-KGDH during germination. The dispersal and germination of conidia has a profound impact on pathogenicity and the industrial application of *Aspergillus* species. The food and feed industry commonly use preservatives against molds such as propionate, which inhibits the PDC (Brock and Buckel 2004). Hence, these novel insights into the fundamental roles of numerous phosphatases in germination and carbon sensing will prompt the future dissection of the implicated pathways and mechanisms in the most highly conserved morphogenic program in fungi while providing new

**Figure 12** RT-qPCR analyses of genes encoding for metabolic enzymes involved in primary carbon metabolism in the wild-type, ΔptpB, ΔmsgA, ΔptcD, and ΔptcE strains after transfer from CA to glucose as a sole carbon source. (A) AN3223 (pfkA), (B) AN8979 (alcA), (C) AN1726 (pdhA), and (D) AN8793 (carC).
Table 6 Summary of the phenotypic characterization of the A. nidulans phosphatase mutants

| Trait | WT     | ∆msgA AN4544 | ∆ppsA AN0129 | ∆psrA AN10077 | ∆ptcD AN0914 | ∆ptcE AN5722 | ∆ptpB AN4896 | ∆sdpA AN10138 |
|-------|--------|--------------|--------------|---------------|--------------|--------------|--------------|--------------|
| Direct growth on glucose (Fig. 2) | +      | –            | –            | –             | –            | –            | –            | –            |
| After germination growth on glucose (Fig. 2) | +      | –            | –            | +             | –            | –            | –            | +            |
| Pre-CA and growth on glucose (Table 1) | 0.056  | 0.033**      | 0.032**      | 0.033**       | 0.033**      | 0.033**      | 0.030***     | 0.035***     |
| Isotropically growth on glucose (%)(Table 3) | 202    | 75***        | 105**        | 26****        | 24****       | 0****        | 105****      | 46****       |
| Cell cycle defect (Table 4) | N/A    | G1-S         | M            | N/A           | N/A          | N/A          | G1-S         | N/A          |
| Trehalose mobilization on Glucose (Fig. 3) | +      | +            | +            | +             | +            | +            | +            | +            |
| Fungal Strain | AN4544 | AN0129 | AN10077 | AN0914 | AN5722 | AN4896 | AN10138 |

Level of statistical significance: *P < 0.05, **P < 0.01, and ***P < 0.001. Level and direction of alteration in growth: +/-.

avenues of research into the identification of inhibitors for fungal germination.

ACKNOWLEDGMENTS
We thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for providing financial support. We also thank the two anonymous reviewers for their comments and suggestions.

LITERATURE CITED
Barrett, L., M. Orlova, M. Maziarz, and S. Kuchin, 2012 Protein kinase A contributes to the negative control of SufI protein kinase in Saccharomyces cerevisiae. Eukaryot. Cell 11: 119–128.
Bergen, L. G., and N. R. Morris, 1983 Kinetics of the nuclear division cycle of Aspergillus nidulans. J. Bacteriol. 156: 155–160.
Bergen, L. G., A. Upshall, and N. R. Morris, 1984 S-phase, G2, and nuclear division mutants of Aspergillus nidulans. J. Bacteriol. 159: 114–119.
Brock, M., and W. Buckel, 2004 On the mechanism of action of the antifungal agent propionate. Eur. J. Biochem. 271: 3227–3241.
Brown, N. A., P. F. de Gouvea, N. G. Krohn, M. Savoldi, and G. H. Goldman, 2013 Functional characterisation of the non-essential protein kinases and phosphatases regulating Aspergillus nidulans hydrolytic enzyme production. Biotechnol Biofuels 6: 91.
Brown, N. A., L. N. A. Ries, and G. H. Goldman, 2014 How nutritional status signalling coordinates metabolism and lignocellulosic enzyme secretion. Fungal Genet. Biol. 72: 48–63.
Chretien, D., M. Pourrier, T. Bourgeron, M. Séné, A. Rötig et al., 1995 An improved spectrophotometric assay of pyruvate dehydrogenase in lactate dehydrogenase contaminated mitochondrial preparations from human skeletal muscle. Clin. Chim. Acta 240: 129–136.
Colabardini, A. C., L. R. A. Nicolas, N. A. Brown, T. Fernanda, M. Savoldi et al., 2014 Functional characterization of a xylose transporter in Aspergillus nidulans. Biotechnol Biofuels 7: 46.

des Assis, L. J., R. B. Zingali, C. A. Masuda, S. P. Rodrigues, and M. Montero-Lomeli, 2013 Pyruvate decarboxylase activity is regulated by the Ser/Thr protein phosphatase Stp2 in the yeast Saccharomyces cerevisiae. FEMS Yeast Res. 13: 518–528.

D’Enfert, C., 1997 Fungal spore germination: Insights from the molecular genetics of Aspergillus nidulans and Neurospora crassa. Fungal Genet. Biol. 21: 163–172.

D’Enfert, C., B. M. Bonini, P. D. A. Zapella, T. Fontaline, A. M. Da Silva et al., 1999 Neutral trehalases catalyse intracellular trehalose breakdown in the filamentous fungi Aspergillus nidulans and Neurospora crassa. Mol. Microbiol. 32: 471–483.

Dinamarco T. M., Figueiredo Pimentel B. D. C., Savoldi M., Malavazi L., Soriani F. M. et al., G. H., 2010 The roles played by Aspergillus nidulans apoptosis-inducing factor (AIF)-like mitochondrial oxidoreductase (AiiA) and NADPH-ubiquinone oxidoreductases (NdeA-B and NdiA) in farnesol resistance. Fungal Genet. Biol. 47: 1055–1069.

Doi, K., A. Gartner, G. Ammerer, and B. Errede, 1994 MSG5, a novel protein phosphatase promotes adaptation to pheromone response in S. cerevisiae. EMBO J. 13: 61–70.

Dong, L., C. Chapline, B. Mousseau, L. Fowler, K. Ramsay et al., 1995 35H1, a sequence isolated as a protein kinase C binding protein, is a novel member of the Adducin family. J. Biol. Chem. 270: 25534–25540.

Ernsting, B. R., and J. E. Dixon, 1997 The PPS1 gene of Saccharomyces cerevisiae codes for a dual specificity protein phosphatase with a role in the DNA synthesis phase of the cell cycle. J. Biol. Chem. 272: 9332–9343.

Farkas, D. L., M. D. Wei, P. Febbroriello, J. H. Carson, and L. M. Loew, 1989 Simultaneous imaging of cell and mitochondrial membrane potentials. Biophys. J. 56: 1053–1069.

Fillingar, S., M.-K. Chaverroche, K. Shimizu, N. Keller, and C. D’Enfert, 2002 cAMP and ras signalling independently control spore germination in the filamentous fungus Aspergillus nidulans. Mol. Microbiol. 46: 1001–1016.

Gey, U., C. Czuapalla, B. Hofflack, G. Rödel, and U. Krause-Buchholz, 2008 Yeast pyruvate dehydrogenase complex is regulated by a concerted activity of two kinases and two phosphatases. J Biol Chem. 283(15): 9759–67.

Habelhah, H., A. Laine, H. Erdjument-Bromage, P. Tempst, M. E. Gershwin et al., 2011 Distinct docking mechanisms mediate interactions between the Msg5 phosphatase and mating or cell integrity mitogen-activated protein kinases (MAPKs) in Saccharomyces cerevisiae. J. Biol. Chem. 286: 42037–42050.

Pedruzzi, I., F. Duboulouz, E. Cameroni, V. Wanke, J. Roosen et al., 2003 TOR and PKA signaling pathways converge on the protein kinase Rim15 to control entry into G0. Mol. Cell. 12: 1607–1613.

Pohl, C. H., J. L. F. Kock, and V. S. Thibane, 2011 Antifungal free fatty acids: a review. Sci. Age Microb Path 1: 61–71.

Ramsubramaniam, N., S. D. Harris, and M. R. Marten, 2014 The phospho- proteome of Aspergillus nidulans reveals functional association with cellular processes involved in morphology and secretion. Proteomics 14: 2454–2459.

Repetto, B., and A. Tzagoloff, 1989 Structure and regulation of KGD2, the structural gene for yeast alpha-ketoglutarate dehydrogenase. Mol. Cell. Biol. 9: 2695–2705.

Repetto, B., and A. Tzagoloff, 1990 Structure and regulation of KGD2, the structural gene for yeast alpha-ketoglutarate dehydrogenase. Mol. Cell. Biol. 10: 4221–4232.

Risipul, N., D. M. Soanes, C. Ant, R. Czajkowski, A. Grünler et al., 2009 Comparative genomics of MAP kinase and calcium-calmodulin signalling components in plant and human pathogenic fungi. Fungal Genet. Biol. 46: 287–298.

Saeed, A. I., V. Sharov, J. White, J. Li, W. Liang et al., 2003 TM4: A free, open-source system for microarray data management and analysis. Bioinformatics 34: 374–378.

Shi, Y., 2009 Serine/threonine phosphatases: mechanism through structure. Cell 139: 468–484.

Son, S., and S. A. Osmani, 2009 Analysis of all protein phosphatase genes in Aspergillus nidulans identifies a new mitotic regulator, fcp1. Eukaryot. Cell 8: 573–585.

Souza, C. P., De, S. B. Hashmi, A. H. Osmani, P. Andrews, and C. S. Ringelberg et al., 2013 Functional analysis of the Aspergillus nidulans kinome. PLoS ONE 8: e58008.

Tretter, L., and V. Adam-Vizi, 2004 Generation of reactive oxygen species in the reaction catalyzed by alpha-ketoglutarate dehydrogenase. J. Neurosci. 24: 7771–7777.

Wilson, L. K., B. M. Benton, S. Zhou, J. Thorner, and G. S. Martin, 1995 The yeast immunophilin Fpr3 is a physiological substrate of the tyrosine-specific phosphoprotein phosphatase Ptp1. J. Biol. Chem. 270: 25185–25193.

Wu, P., J. M. Peters, and R. A. Harris, 2001 Adaptive increase in pyruvate dehydrogenase kinase 4 during starvation is mediated by peroxisome proliferator-activated receptor alpha. Biochem. Biophys. Res. Commun. 287: 391–396.

Communicating editor: M. S. Sachs