

A Mep2-dependent Transcriptional Profile Links Permease Function to Gene Expression during Pseudohyphal Growth in *Saccharomyces cerevisiae*

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The ammonium permease Mep2 is required for the induction of pseudohyphal growth, a process in *Saccharomyces cerevisiae* that occurs in response to nutrient limitation. Mep2 has both a transport and a regulatory function, supporting models in which Mep2 acts as a sensor of ammonium availability. Potentially similar ammonium permease-dependent regulatory cascades operate in other fungi, and they may also function in animals via the homologous Rh proteins; however, little is known about the molecular mechanisms that mediate ammonium sensing. We show that Mep2 is localized to the cell surface during pseudohyphal growth, and it is required for both filamentous and invasive growth. Analysis of site-directed Mep2 mutants in residues lining the ammonia-conducting channel reveal separation of function alleles (transport and signaling defective; transport-proficient/signaling defective), indicating transport is necessary but not sufficient to sense ammonia. Furthermore, Mep2 overexpression enhances differentiation under normally repressive conditions and induces a transcriptional profile that is consistent with activation of the mitogen-activated protein (MAP) kinase pathway. This finding is supported by epistasis analysis establishing that the known role of the MAP kinase pathway in pseudohyphal growth is linked to Mep2 function. Together, these data strengthen the model that Mep2-like proteins are nutrient sensing transceptors that govern cellular differentiation.

INTRODUCTION

Ammonia is an important nutrient for many microorganisms and plants, and in animals the catabolism of amino acids produces ammonia as a by-product that must be excreted from the body to prevent its toxic accumulation. The Amt/Mep/Rh proteins form an evolutionary conserved family of permeases that mediate ammonium transport across cell membranes (reviewed in Andrade and Einsle, 2007). Structural and biochemical studies of bacterial members of this transporter family reveal that the Amt/Mep/Rh proteins form a trimeric complex that facilitates passive diffusion of ammonia gas (Khademi *et al.*, 2004; Zheng *et al.*, 2004; Andrade *et al.*, 2005; Ishikita and Knapp, 2007). Conserved key residues required for ammonium translocation have been identified by site-directed mutagenesis and provide evidence that the mechanism of ammonium transport is evolutionarily conserved (Javelle, *et al.*, 2006; Marini *et al.*, 2006).

Previous studies support the model that certain members of the Amt/Mep/Rh family play a direct role in fungal

development as ammonium receptors. The high-affinity ammonium permeases of *Saccharomyces cerevisiae*, *Candida albicans*, and *Ustilago maydis* are required for filamentous growth in response to low ammonium conditions (Lorenz and Heitman, 1998a; Smith *et al.*, 2003; Biswas and Morschhäuser, 2005). The fission yeast *Schizosaccharomyces pombe* undergoes haploid invasive growth during ammonium limitation, and this is dependent on the ammonium permease Amt1, and, to a lesser extent, its paralogue, Amt2 (Mitsu-zawa, 2006). Haploid invasive growth and mating by the basidiomycetous fungus *Cryptococcus neoformans* is induced by ammonium limitation and requires the high-affinity ammonium permease, Amt2 (Rutherford *et al.*, 2008). However, the mechanisms that link ammonium transport to development are not understood. One hypothesis is that these permeases act as ammonium sensors, or transceptors, that regulate downstream effector molecules (Lorenz and Heitman, 1998a). This mechanism couples the physical process of nutrient transport to nutrient sensing and is distinct from a regulatory mechanism that responds to the changing metabolized levels of a transported nutrient (Holsbeeks *et al.*, 2004).

Current evidence supports the hypothesis that fungal high-affinity permeases function as ammonium sensors. *S. cerevisiae* cells lacking the high-affinity permease Mep2 do not undergo pseudohyphal growth or exhibit any change in the activity of nitrogen metabolic enzymes (Lorenz and Heitman, 1998a). The C-terminal cytoplasmic domain of the *C. albicans* Mep2 protein is essential for the induction of filamentous growth but dispensable for transport activity

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(Biswas and Morschhäuser, 2005). Therefore, the roles of Mep2 in ammonium transport and induction of filamentous growth are separable. Independently of its role in pseudohyphal growth, Mep2 acts as a transceptor in the cAMP-independent activation of the protein kinase A (PKA) pathway after ammonium addition to starved cells (Van Nuland *et al.*, 2006). This rapid response is not dependent on ammonium metabolism and can be induced by the transport of the nonmetabolizable ammonium analogue methylamine, which establishes the paradigm of Mep2 as a transceptor. It is not clear whether Mep2 has an equivalent transceptor function during pseudohyphal growth or whether it interacts with any of the established signal transduction pathways that are essential for this process. Epistasis analysis is consistent with the RAS-cAMP being a possible downstream target of Mep2 (Lorenz and Heitman, 1998a); however, a direct interaction between Mep2 and any signaling pathway has not been demonstrated in relation to pseudohyphal growth.

In this report, we present evidence that Mep2 forms a multimeric complex localized to the cell membrane during pseudohyphal growth. Similar to *S. pombe* and *C. neoformans*, haploid *S. cerevisiae* cells undergo invasive growth in response to ammonium limitation and exhibit morphological changes analogous to those observed in pseudohyphal cells. We find that invasive growth requires Mep2, the Npr1 kinase, and elements of the PKA and mitogen-activated protein (MAP) kinase pathways. Furthermore, Mep2-dependent pseudohyphal and invasive growth requires two conserved histidine residues within the hydrophobic channel of Mep2 that have been predicted to be essential for ammonium translocation through the permease. Remarkably, constitutive Mep2 expression induces pseudohyphal growth on nitrogen-replete medium. Under these conditions, Mep2 induces a haploid- and diploid-specific transcriptional profile that includes genes known or predicted to be differentially regulated during pseudohyphal growth. Included within this group of genes are those controlled by the MAP kinase-regulated transcription factor Ste12. Consistent with a functional link between Mep2 and Ste12, constitutive *MEP2* overexpression restores pseudohyphal growth in cells lacking PKA pathway elements but not in cells lacking Ste12. In accord with this finding, overexpression of Ste12, or of the MADS box transcription factor Mcm1, restores pseudohyphal growth in cells lacking Mep2 under ammonium limiting conditions. Therefore, the established role of the MAP kinase pathway in pseudohyphal growth may, in part, be due to its role as a downstream effector of the ammonium receptor function of Mep2. Collectively, these data further support a role for the Mep2 family of permeases as sensors of ammonium availability.

EXPERIMENTAL PROCEDURES

Strains and Growth Media

The *S. cerevisiae* strains used in this study are listed in Table 1. *S. cerevisiae* cells were grown in synthetic minimal medium with either 2% glucose, raffinose, or galactose as the carbon source. To induce pseudohyphal growth, cells were grown with 50 μ M ammonium sulfate as the nitrogen source. Otherwise, cells were grown with the standard concentration of ammonium sulfate in yeast nitrogen base (5 g/l). Yeast cells were transformed as described previously (Schiestl and Gietz, 1989).

Plasmids

The plasmids used in this study are listed in Table 2. All manipulations of DNA fragments and plasmids were carried out using standard procedures and the *Escherichia coli* strain DH5 α (Sambrook *et al.*, 1989). All DNA sequences encoding Mep2, epitope-tagged Mep2, and mutant derivatives of these were generated and inserted into the vector pRS316 (*URA3*) by homologous recombination (Ma *et al.*, 1987).

Table 1. Yeast strains used in this study

Strain	Reference
Congenic with Σ 1278b	
HLY352 MATa/ α <i>ste12::LEU2/ste12::LEU2</i>	Liu <i>et al.</i> (1993)
MLY41 MATa <i>ura3-52</i>	Lorenz and Heitman (1997)
MLY54a MATa <i>ura3-52</i> <i>npr1::LEU2 leu2::hisG</i>	Lorenz and Heitman (1998a)
MLY54a/ α MATa/ α <i>ura3-52/ura3-52</i> <i>npr1::LEU2/npr1::LEU2</i> <i>leu2::hisG/leu2::hisG</i>	Lorenz and Heitman (1998a)
MLY61a/ α MATa/ α <i>ura3-52/ura3-52</i>	Lorenz and Heitman (1997)
MLY104a MATa <i>ura3-52</i> <i>mep1::LEU2 leu2::hisG</i>	Lorenz and Heitman (1998a)
MLY108a MATa <i>ura3-52</i> <i>mep2::G418</i>	Lorenz and Heitman (1998a)
MLY128a MATa <i>ura3-52</i> <i>mep3::G418</i>	Lorenz and Heitman (1998a)
MLY132a MATa <i>ura3-52</i> <i>gpa2::G418</i>	Lorenz and Heitman (1997)
MLY132a/ α MATa/ α <i>ura3-52/ura3-52</i> <i>gpa2::G418/gpa2::G418</i>	Lorenz and Heitman (1997)
MLY140a/ α MATa/ α <i>ura3-52/ura3-52</i> <i>ure2::G418/ure2::G418</i>	Lorenz and Heitman (1998a)
MLY183a MATa <i>ura3-52</i> <i>tec1::G418</i>	Lorenz and Heitman (1998b)
MLY186a MATa <i>ura3-52</i> <i>ras1::G418</i>	Lorenz unpublished
MLY187a MATa <i>ura3-52</i> <i>ras2::G418</i>	Lorenz <i>et al.</i> (2000a)
MLY187a/ α MATa/ α <i>ura3-52/ura3-52</i> <i>ras2::G418/ras2::G418</i>	Lorenz <i>et al.</i> (2000a)
MLY216a MATa <i>ura3-52</i> <i>ste12::G418</i>	Lorenz unpublished
MLY232a MATa <i>ura3-52</i> <i>gpr1::G418</i>	Lorenz <i>et al.</i> (2000a)
MLY232a/ α MATa/ α <i>ura3-52/ura3-52</i> <i>gpr1::G418/gpr1::G418</i>	Lorenz <i>et al.</i> (2000a)
XPY5-1a MATa <i>ura3-52</i> <i>tpk2::G418</i>	Pan and Heitman (1999)
XP5a/ α MATa/ α <i>ura3-52/ura3-52</i> <i>tpk2::G418/tpk2::G418</i>	Pan and Heitman (1999)
XPY14-1a MATa <i>ura3-52</i> <i>tpk1::G418 tpk3::G418</i>	Pan and Heitman, (1999)
XPY207a MATa <i>ura3-52</i> <i>she2::G418</i>	Pan and Heitman (2000)
Other strains	
SY1 MATa <i>ura3-52 leu2-3</i> <i>his4-619 GAL sec6-4</i>	Novick <i>et al.</i> (1980)
Mep2-GFP MAT α <i>his3Δ1</i> <i>leu2Δ0 lys2Δ0 ura3Δ0</i> <i>MEP2::GFP-HIS3</i>	Huh <i>et al.</i> (2003)

ogous recombination (Ma *et al.*, 1987). In each case, the *MEP2* gene included its own promoter (1 kb), and, except in the Mep2-green fluorescent protein (GFP) fusion encoding plasmid and its mutant derivatives, the *MEP2* terminator. The Mep2-GFP encoding sequences were amplified from the relevant strain of the yeast GFP clone collection to include the *ADHI*-terminating sequences (Huh *et al.*, 2003). All Mep2-FLAG fusions contain the N4Q mutation to prevent glycosylation of Mep2 (Marini and André, 2000). *MCMI1*, including its own promoter and terminator, was inserted into the vector pRS426 (*URA3*) by homologous recombination. All newly generated plasmid inserts were verified by DNA sequencing. The plasmid pYes2.1-Mep2, a kind

Table 2. Plasmids used in this study

Plasmid	Function	Reference
pJRH1	Mep2-GFP <i>CEN</i>	This study
pJRH2	Mep2-D186A-GFP <i>CEN</i>	This study
pJRH3	Mep2-H194A-GFP <i>CEN</i>	This study
pJRH4	Mep2-H348A-GFP <i>CEN</i>	This study
pJRH7	Mep2-N4Q-FLAG <i>CEN</i>	This study
pJRH8	Mep2-N4Q-H194A-FLAG <i>CEN</i>	This study
pJRH9	Mep2-N4Q-H348A-FLAG <i>CEN</i>	This study
pJRH10	Mep2-N4Q-D186A-FLAG <i>CEN</i>	This study
pJRH11	Mep2-N4Q-T288A-FLAG <i>CEN</i>	This study
pJRH17	<i>MCM1</i> 2 μ	This study
pML151	<i>MEP2</i> 2 μ	Lorenz and Heitman (1998a)
pYES2.1-MEP2	<i>GAL-MEP2</i> 2 μ	Smith <i>et al.</i> (2003)
pNC252	<i>GAL-STE12</i> <i>CEN</i>	Liu <i>et al.</i> (1993)
pSC4	<i>STE12</i> <i>CEN</i>	Fields and Herskowitz (1987)
pSL1509	<i>STE11-4</i> <i>CEN</i>	Stevenson <i>et al.</i> (1992)
pMW2	<i>RAS2^{Val19}</i> <i>CEN</i>	Ward <i>et al.</i> (1995)

All novel plasmids were generated using pRS316 (*URA3*) except pJRH17 (pRS426-*URA3*).

gift from Mike Perlin (University of Louisville, Louisville, KY), was used to express *MEP2* to high levels from the galactose-inducible promoter (Smith *et al.*, 2003).

Membrane Preparation, Immunoprecipitation, and Western Immunoblotting

The preparation of cell membranes was carried out as described previously (Galan *et al.*, 1996). Briefly, yeast cells in the exponential growth phase were harvested by centrifugation, washed in distilled water, and suspended in lysis buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 5 mM EDTA) and a mixture of proteinase inhibitors (cocktail IV; Calbiochem, San Diego, CA) and 0.5 mM phenylmethylsulfonyl fluoride. Glass beads (0.45 μ m in diameter) were added, and the cells were lysed by vigorous vortex mixing for 3 min. The resulting homogenate was diluted threefold with lysis buffer and centrifuged at 3000 rpm for 3 min at 4°C. The plasma membrane-enriched fraction was collected by centrifugation for 45 min at 12,000 rpm at 4°C and then suspended in lysis buffer and trichloroacetic acid (10%). For Western analysis, the precipitates were neutralized and dissolved in 20 μ l of 1 M Tris base plus 80 μ l of sample buffer (100 mM Tris-HCl, pH 6.8, 4 mM EDTA, 4% SDS, 20% glycerol, and 0.02% bromophenol blue) containing 2% 2-mercaptoethanol and heated at 37°C for 15 min. For immunoprecipitation, 2-mercaptoethanol was omitted from the samples, which were diluted with 0.6 ml of TNET buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100) plus the proteinase inhibitors described. Western analysis was carried out using antibodies, either monoclonal ANTI-FLAG M2 (Sigma-Aldrich, St. Louis, MO), monoclonal anti-GFP (Roche Diagnostics, Indianapolis, IN), or anti-Pma1 (Cardenas *et al.*, 1990), and the relevant horseradish peroxidase-conjugated anti-IgG second antibody, followed by ECL chemiluminescence (GE Healthcare, Chalfont St. Giles, United Kingdom). In the experiments using the *npr1* Δ strain, relative abundance of Mep2-FLAG was quantified using Quantity One version 4.6.2. (Bio-Rad, Hemel Hempstead, United Kingdom). Immunoprecipitation was carried out as described previously (Harashima and Heitman, 2002) by using EZview Red ANTI-FLAG M2 affinity gel (Sigma-Aldrich). Native Mep2 was resolved by suspending membrane samples in 1 \times Tris-glycine native sample buffer and separating on 6% NOVEX Tris-glycine gels with Tris-glycine native running buffer (Invitrogen).

Protein Localization

Cells containing plasmid expressed Mep2-GFP were grown in synthetic minimal media and examined for protein localization under a fluorescent microscope (Axioskop2 Plus; Carl Zeiss, Thornwood, NY).

Pseudohyphal and Invasive Growth

Cells undergoing pseudohyphal and invasive growth assays were grown for 6 d on low-ammonium SLAD medium (50 μ M ammonium sulfate) and investigated as described previously (Harashima *et al.*, 2006).

Microarray Analysis

S288C haploid and Σ 1278b diploid wild-type strains containing a 2 μ plasmid consisting of a *GAL1/10*-driven *MEP2* gene were grown concurrently with isogenic strains containing an empty vector to mid-log phase in selective liquid medium plus 2% raffinose. *MEP2* gene expression was induced for 3 h in 2% galactose. Comparison of the S288C and Σ 1278b diploid wild-type strains was carried out in synthetic complete medium with 2% glucose. RNA preparation, hybridization, image acquisition, and data processing for microarrays were performed as described previously (Grigull *et al.*, 2004). Each microarray experiment was replicated in dye reverse.

RESULTS

Mep2 Forms a Multimeric Complex In Vivo

To facilitate studies of the role of Mep2 in the initiation of pseudohyphal growth, a plasmid was generated expressing Mep2 fused to a C-terminal FLAG epitope (Mep2-N4Q-FLAG) from the *MEP2* endogenous promoter. In this allele, the fourth asparagine residue of Mep2 was substituted with glutamine, which prevents glycosylation but does not significantly influence the ability of Mep2 to transport ammonium or induce pseudohyphal growth (Marini and André, 2000). The Mep2-N4Q-FLAG protein is functional; transformation of cells lacking endogenous Mep2 with the Mep2-N4Q-FLAG plasmid restored pseudohyphal growth and growth under ammonium limiting conditions (Figure 5).

The bacterial Amt ammonium permeases form trimeric membrane protein complexes (Conroy *et al.*, 2004; Zheng *et al.*, 2004). Potential multimeric forms of *S. cerevisiae* Mep2 have also been detected following SDS electrophoresis (Marini and André, 2000). We observed that Mep2-N4Q-FLAG migrated at a size consistent with that of a trimeric complex under native electrophoresis conditions (Figure 1A). To confirm that Mep2 monomers interact in vivo, Mep2-N4Q-FLAG was coexpressed with Mep2 fused to

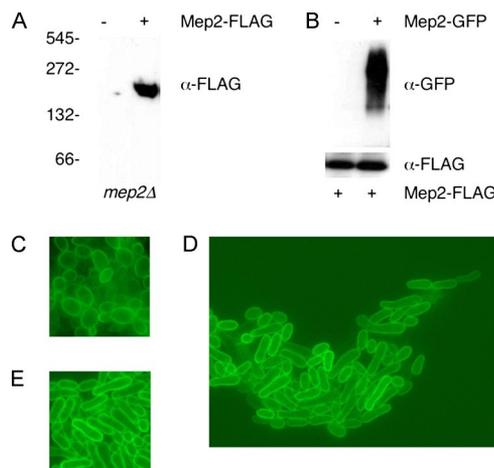


Figure 1. Mep2 forms a multimeric complex and is membrane localized in cells undergoing pseudohyphal growth. (A) Membrane fractions from haploid *mep2* Δ cells containing a plasmid expressing Mep-N4Q-FLAG or a control vector that had been grown in low-ammonium medium (SLAD) were resolved under native conditions, and the FLAG epitope was detected by Western analysis. (B) Mep2-FLAG was immunoprecipitated from control cells and cells expressing an Mep2-GFP fusion protein that had been grown in SLAD medium. Diploid MLY108a/ α cells (*mep2* Δ /*mep2* Δ) expressing Mep2-GFP were grown on low-ammonium medium (SLAD) plates for 6 d. The Mep2 fusion protein was localized by direct epifluorescence microscopy in cells at the center of the colony (C), within cells that had formed pseudohyphae (D), and at the edge of the colony (E).

green fluorescent protein. Immunoprecipitation of Mep2-N4Q-FLAG resulted in coimmunoprecipitation of Mep2-GFP, confirming the two epitope-tagged Mep2 derivatives interact (Figure 1B).

Mep2 Localization on the Cell Membrane Requires the Exocyst Complex

Diploid *S. cerevisiae* cells undergo polarized growth during pseudohyphal growth that is, in some respects, similar to polarized growth of haploid cells during mating. In response to mating pheromone, cells develop a mating projection, or shmoo, that grows toward the mating partner to enable cell-cell contact (Jackson and Hartwell, 1990). Proteins important for polarized growth during mating are localized to the shmoo (Bagnat and Simons, 2002). Because pseudohyphal growth involves polarized growth, the extent to which Mep2 is localized to the growing tip of a filament during pseudohyphal growth was examined.

Cells expressing the Mep2-GFP fusion protein were grown on solid agar under pseudohyphal conditions. Within the center of the colony, cells were oval in shape (Figure 1C), whereas cells at the colony edge exhibited the elongated morphology characteristic of pseudohyphal growth (Figure 1E). In both cases, Mep2-GFP was expressed and localized throughout the cell membrane. In addition, Mep2-GFP was localized throughout the cell membrane of cells that had grown away from the center of the colony (Figure 1D). Unlike the signaling components important for mating, Mep2-GFP does not exhibit any preferential localization to any discrete area in the cell membrane or at the growing tip of the cell during pseudohyphal growth.

We next examined whether Mep2 might be transiently localized during its initial expression after the transition from high- to low-ammonium media (Marini *et al.*, 1997) (Figure 2A). Under these conditions, Mep2-GFP localizes to the entire cell membrane within 2 h (Figure 2A). In budding cells, however, Mep2-GFP is initially (within 30 min) localized to small daughter cells, and in larger daughter cells, to the bud neck (Figure 2B). Longer incubation periods resulted in Mep2-GFP becoming gradually localized throughout more of the cell membrane, until Mep2-GFP covered the entire cell membrane by 2 h. The polarized transport of vesicles to the mother-bud neck or the bud tip is dependent on the actin cytoskeleton and the Myo2 myosin motor protein (Pruyne *et al.*, 1998; Karpova *et al.*, 2000). An alternative mechanism to ensure daughter-cell-specific protein expression is myosin-dependent mRNA transport from mother to daughter cell (Takizawa *et al.*, 1997). To distinguish between these two mechanisms, the localization of Mep2-GFP was

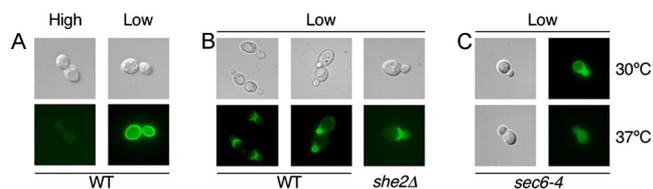


Figure 2. Mep2 is localized to the cell membrane via the exocyst complex. Direct epifluorescence microscopy of cells expressing Mep2-GFP was performed with wild-type cells that were grown in high-ammonium medium, pelleted, washed and then grown in high- or low (50 μ M)-ammonium sulfate medium for 2 h (A), wild-type and *she2* Δ cells that were transferred from growth in high-ammonium to low-ammonium medium and grown for 20 min (B), and *sec6-4* cells that were grown at the permissive and nonpermissive temperature in low-ammonium medium (C).

analyzed in cells lacking the She2 protein and cells that contain the temperature-sensitive *sec6-4* allele. She2 is an RNA binding protein essential for daughter-cell-specific mRNA transport (Long *et al.*, 2000), whereas Sec6 is an integral component of the exocyst complex that mediates polarized targeting of secretory vesicles to sites of active exocytosis (TerBush *et al.*, 1996; TerBush and Novick, 1995). Mep2-GFP exhibited a wild-type pattern of localization in a *she2* Δ mutant strain; however, this pattern of localization was absent in *sec6-4* cells grown at the restrictive temperature (Figure 2C). Thus, Mep2 distribution on the cell membrane involves the exocyst complex.

Mep2 Is Correctly Localized in Mutants Lacking Npr1 and Ure2 and Is Required for Pseudohyphal Growth on Nitrogen Sources Other Than Ammonium

To further analyze the link between Mep2 localization and pseudohyphal signaling, the localization of Mep2 was analyzed in mutants lacking Ure2 and Npr1 that do not form pseudohyphae (Lorenz and Heitman, 1998a). Ure2 is a regulatory protein that binds to the Gln3 transcription factor and thereby represses transcription of nitrogen catabolite-regulated genes (Blinder *et al.*, 1996). The Npr1 kinase regulates sorting and localization of the general amino acid permease Gap1 (De Craene *et al.*, 2001). We observed that Mep2-GFP is normally localized to the plasma membrane in both *ure2* and *npr1* mutant strains when grown under high and low levels of ammonium (Figure 3A). Therefore, the loss of either Ure2 or Npr1 results in the derepression of *MEP2* gene expression, and the lack of pseudohyphal growth of these mutants cannot be attributed to the lack of Mep2 localization to the cell membrane. In addition, the GFP fluorescence observed in *npr1* Δ cells expressing Mep2-GFP was greater than in wild-type cells, and Mep2-N4Q-FLAG ex-

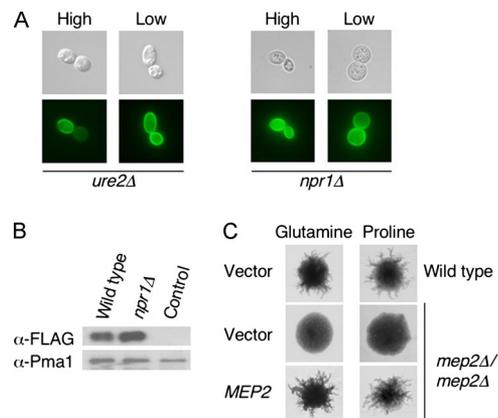


Figure 3. Mep2 is correctly localized in mutants lacking Npr1 and Ure2, and it is required for pseudohyphal growth on nitrogen sources other than ammonium. (A) Epifluorescence microscopy of *ure2* Δ and *npr1* Δ cells containing Mep2-GFP that had been grown in high-ammonium medium, pelleted, washed, and then grown in high- or low-ammonium sulfate medium for 2 h. (B) The levels of Mep2-N4Q-FLAG within the membrane fraction of wild-type and *npr1* Δ cells grown in low (50 μ M) ammonium sulfate medium were quantified by Western analysis. Levels of the plasma membrane H^+ -ATPase Pma1 served as a loading control. Cells not expressing Mep2-N4Q-FLAG served as a negative control. (C) Diploid wild-type and *mep2* Δ cells containing either a control plasmid or low copy plasmid expressing *MEP2* from its own promoter were grown with either glutamine (100 μ M) or proline (100 μ M) as the sole nitrogen source for 6 d.

pression was also ~30% higher in these cells as detected by Western blotting (Figure 3B).

Our previous studies reported that Mep2 induced pseudohyphal growth in response to growth on ammonium alone and not when cells were grown on alternative sources of nitrogen (Lorenz and Heitman, 1998a). This suggested that ammonium transport through Mep2 is required for the initiation of pseudohyphal growth. Conversely, studies in *C. albicans* have found that Mep2 is required for the induction of filamentous growth in response to nitrogen sources other than ammonium (Biswas and Morschhäuser, 2005). We re-analyzed the growth of diploid *mep2Δ* mutants on agar media containing either glutamine or proline as a nitrogen source. Under these conditions, wild-type cells produced pseudohyphae, whereas the diploid *mep2Δ* mutant strain did not (see *Discussion*). Expression of the *MEP2* gene in the diploid *mep2Δ* strain from a centromeric plasmid restored the ability of the mutant strain to undergo pseudohyphal growth (Figure 3C). Therefore, consistent with what has been described in *C. albicans*, Mep2 is required for the initiation of pseudohyphal growth on nitrogen sources other than ammonium.

Mep2 Induces Haploid Invasive Growth in Response to Ammonium Limiting Conditions

Dimorphic growth by *S. cerevisiae* in response to ammonium limitation has traditionally been investigated using diploid cells. Haploid *S. cerevisiae* cells undergo invasive growth when grown in rich medium or in response to fusel alcohols (Roberts and Fink, 1994; Lorenz *et al.*, 2000b), but their response to ammonium levels has not been extensively studied. The finding that the basidiomycetous fungus *C. neoformans* and the fission yeast *S. pombe* undergo haploid invasive growth in response to ammonium limitation suggests that this pattern of growth may be conserved within fungi (Mitsuzawa, 2006; Rutherford *et al.*, 2008). Growth of wild-type cells on low-ammonium media resulted in invasive growth and was dependent on the presence of Mep2 but not on the presence of the Mep1 or Mep3 ammonium permeases (Figure 4A). Complementation of the *mep2Δ* mutation with *MEP2* regulated by its native promoter from a low copy number centromeric plasmid restored invasive growth (Figure 5D). Microscopic examination of the areas of invasive growth revealed formation of chains of elongated cells (Figure 4B). Consistent with the morphology of cells undergoing pseudohyphal growth, some haploid cells exhibited an elongated cell shape when grown under ammonium limiting conditions (Figure 4C).

The extent of invasive growth under low ammonium conditions was then analyzed in mutant strains lacking genes involved in diploid pseudohyphal growth. Tpk1, Tpk2, and Tpk3 are the PKA catalytic subunits, and Ste12 and Tec1 are transcription factors regulated by the MAP kinase pathway. Mutants lacking Npr1, Tpk2, Ste12, or Tec1 exhibited reduced invasive growth under ammonium limiting conditions. Interestingly, a *tpk1 tpk3* double mutant exhibited increased invasive growth under the same conditions. The link between ammonium-responsive invasive growth is consistent with the finding that cAMP can restore invasive growth in a *S. pombe* mutant that lacks *amt1Δ* (Mitsuzawa, 2006). The Gpr1 G protein-coupled receptor and the G α subunit Gpa2 activate the cAMP-PKA pathway and are required for pseudohyphal growth. Ras2 is a guanosine triphosphate-binding protein that activates both the cAMP-PKA and the MAP kinase pathways (reviewed in Pan *et al.*, 2000). Mutants lacking Gpr1, Gpa2, Ras1, or Ras2 exhibited

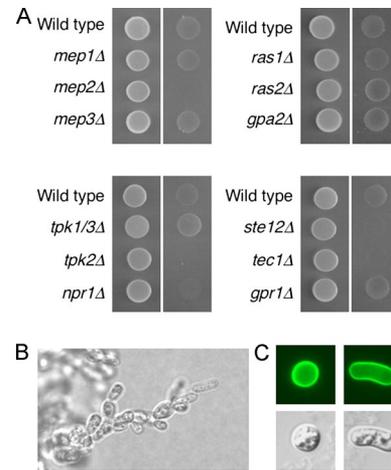


Figure 4. Mep2 is required for haploid invasive growth under nitrogen limiting conditions. (A) Haploid wild-type and mutant cells containing a control plasmid were spotted onto low ammonium sulfate (50 μ M) solid medium lacking uracil and grown for 6 d. Panels show cells before (left) and after (right) cells have been washed off the surface of the plate to reveal the extent of invasive growth. (B) The morphology of invading cells was visualized with a Zeiss Axiophot 2 Plus fluorescence microscope revealing, in some areas, filaments of pseudohyphal-like chains of cells. (C) Wild-type cells expressing Mep2-GFP that had undergone haploid invasive growth exhibit either morphology consistent with cell cycle arrest or pseudohyphal-like elongation.

invasive growth equivalent to wild-type haploid cells under ammonium limiting conditions (Figure 4A).

Ammonium Transport Is Linked to Pseudohyphal and Invasive Growth

Transceptors bind and/or transport nutrients as part of their sensing mechanism. If Mep2 functions as a transceptor, then mutations that affect transport would be predicted to play a role in pseudohyphal differentiation. In contrast, if Mep2 signals in the absence of its ligand, then mutations that prevent Mep2 from binding or transporting ammonia/ammonium might mimic the ligand free receptor state and activate pseudohyphal growth. These opposing models were tested by identifying and mutating residues involved in Mep2 transport function. An aspartate residue in AmtB that is conserved throughout the Mep/Amt/Rh family is required for ammonium transport (Javelle *et al.*, 2004). In addition, two conserved histidine residues that line the hydrophobic channel of the ammonium permease family are essential for ammonium transport by AmtB (Javelle *et al.*, 2006). These residues are equivalent to D186, H194, and H348 in Mep2 of *S. cerevisiae*.

Individual alanine substitutions of residues D186, H194, and H348 were generated in the Mep2-N4Q-FLAG and Mep2-GFP fusion proteins. The H194A and H348A Mep2 proteins were stably expressed based on Western blot analysis, and they were also localized to the surface of the plasma membrane in the context of a GFP fusion protein (Figure 5, A and B, and Table 3). In contrast, the Mep2^{D186A} mutant protein was unstable, and instead of plasma membrane localization, it seemed to be localized to endogenous membranes, consistent with a recent report (Marini *et al.*, 2006). Cells expressing the Mep2^{H194A} and Mep2^{H348A} proteins did not undergo pseudohyphal growth in response to low ammonium, even though both proteins were stably expressed and correctly localized (Figure 5C) (Table 3). Un-

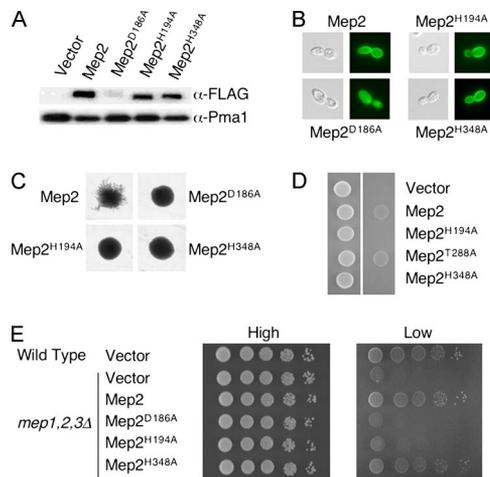


Figure 5. Mep2 transport function is linked to pseudohyphal differentiation. (A) The levels of Mep2-N4Q-FLAG and derived mutants within the membrane fraction of cells grown in low- (50 μ M) ammonium sulfate medium were quantified by Western analysis. Levels of the plasma membrane H⁺-ATPase Pma1 served as a loading control. (B) Localization of Mep2 was visualized using diploid *mep2Δ/mep2Δ* cells that contained plasmids expressing wild-type and mutant GFP-tagged Mep2 that had been grown in low-ammonium sulfate (50 μ M) medium for 2 h. (C) Pseudohyphal growth was assayed using diploid *mep2Δ/mep2Δ* cells that contained plasmids expressing wild-type and mutant Mep2 that were grown on low ammonium sulfate (50 μ M) medium for 6 d. (D) Haploid *mep2Δ* mutant cells containing relevant plasmids were spotted onto low-ammonium sulfate (50 μ M) medium lacking uracil and grown for 6 d. Panels show cells before (left) and after (right) cells have been washed off the surface of the medium to reveal the extent of invasive growth. (E) Wild-type and mutant cells containing the relevant plasmids were grown overnight in complete medium (CMD-ura), washed, and serially diluted onto medium containing high or low (50 μ M) levels of ammonium sulfate.

der ammonium limiting conditions, the Mep2^{H194A} protein did not support growth of a strain lacking the three Mep permeases of *S. cerevisiae*, whereas cells expressing Mep2^{H348A} grew to the same extent as wild-type cells. Thus, neither the transport-defective H194A mutant nor the transport-proficient H348A mutant support pseudohyphal differentiation, indicating that transport is not sufficient for signaling, and that signaling requires functions in addition to transport (Figure 5E and Table 3).

In addition, the ability of these Mep2 mutants to support haploid invasive growth under ammonium limiting conditions was assessed. Consistent with the analysis of diploid pseudohyphal growth, haploid cells expressing the Mep2^{H194A}

Table 3. Summary of phenotypes of diploid (pseudohyphal growth, growth on low ammonium) and haploid (invasive growth) *mep1,2,3Δ* cells expressing wild-type or mutant Mep2 permeases (see Fig. 5)

	Mep2 localized to membrane	Pseudohyphal growth	Growth on low ammonium	Invasive growth
Mep2	+	+	+	+
Mep2 ^{D186A}	-	-	-	NT
Mep2 ^{H194A}	+	-	-	-
Mep2 ^{H348A}	+	-	+	-

and Mep2^{H348A} proteins failed to undergo invasive growth in response to low ammonium. Conversely, expression of a Mep2^{T288A} mutant protein that exhibits defects in pseudohyphal growth supported haploid invasive growth under the same conditions (Figure 5D). The T288 residue of Mep2 is a potential PKA phosphorylation site that may link the PKA signaling cascade to Mep2 signaling activities under certain conditions (Smith *et al.*, 2003).

Mep2 Levels Correlate with Extent of Pseudohyphal Growth

Analysis in *C. albicans* has established that the ability of Mep2 to induce pseudohyphal growth is dependent on its expression levels (Biswas and Morschhäuser, 2005). In accord with these studies, we have found that in *S. cerevisiae*, Mep2 expression levels are important for its regulatory function. *MEP2* expression from a high copy plasmid resulted in more robust pseudohyphal growth compared with expression from a low copy number centromeric plasmid (Figure 6A). Similarly, growth of cells on galactose as opposed to glucose resulted in a significant reduction in the level of pseudohyphal growth, and this was correlated with reduced Mep2-N4Q-FLAG expression (Figure 6, B and C).

These findings, together with studies on *C. albicans* Mep2, suggested that uncoupling *MEP2* expression from nitrogen catabolite repression might induce pseudohyphal growth under noninducing conditions. To test this hypothesis, a diploid wild-type strain was transformed with a plasmid expressing *MEP2* from a galactose-inducible promoter. Limited, but distinct, pseudohyphae were observed in cells grown in galactose nitrogen-replete conditions, whereas no pseudohyphae were observed in control cells (Figure 6D). This establishes that ammonium limitation per se is not required for the induction of the ammonium-responsive dimorphic switch in *S. cerevisiae*.

The influence of Mep2 expression levels suggested an explanation for an intriguing observation with the Mep2^{T288A} mutant protein. Mep2^{T288A} lacks a putative PKA phosphorylation site and does not induce pseudohyphal growth in *mep2* mutant cells (Smith *et al.*, 2003) (Figure 6E). However, when the Mep2^{T288A} protein is expressed in cells lacking all three

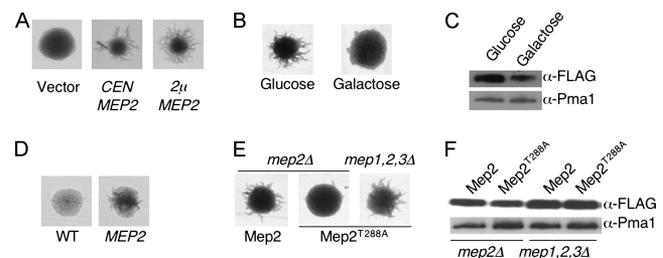


Figure 6. Mep2 function correlates with its expression levels. Pseudohyphal growth was assayed after growth for 6 d by using diploid *mep2Δ/mep2Δ* cells that contained either low or high copy plasmids expressing *MEP2* (A); wild-type cells grown with either glucose or galactose as the carbon source (B), wild-type cells grown with or without galactose-regulated *MEP2* were grown on synthetic medium with galactose as the carbon source and high levels of ammonium (D); and diploid *mep2Δ/mep2Δ* cells or diploid *mep1,2,3Δ/mep1,2,3Δ* cells expressing the Mep2^{T288A} variant (E). The levels of Mep2-FLAG within wild-type cells grown on different carbon sources and low (50 μ M)-ammonium sulfate (C) or Mep2^{T288A}-FLAG within diploid *mep2Δ/mep2Δ* cells and diploid *mep1,2,3Δ/mep1,2,3Δ* cells grown in low (50 μ M)-ammonium sulfate medium (F) were quantified by Western analysis. Levels of the plasma membrane H⁺-ATPase Pma1 served as a loading control.

ammonium permeases pseudohyphal growth is induced at the wild-type level (Figure 6E). One potential explanation for this phenotype is that cells lacking all three ammonium permeases are more starved for nitrogen and therefore induce the expression of Mep2-T288A to a higher level. This increased expression could compensate for the reduced activity of the Mep2^{T288A} protein. When levels of Mep2-FLAG and Mep2^{T288A}-FLAG were analyzed in *mep2* versus *mep1*, -2, and -3 mutant cells, both Mep2 proteins were more highly expressed in the *mep1,2,3Δ* strain (Figure 6F), supporting the interpretation that increased levels of the signaling compromised T288A mutant enable signaling function.

MEP2 Overexpression Induces Transcription of Genes Implicated in Filamentous Growth and Known Targets of Ste12

One hypothesis that is consistent with the role of Mep2 as a sensor of ammonium availability is that it interacts with a signal transduction pathway that activates downstream effectors that control gene expression. To test this hypothesis, the transcriptional profile of S288C haploid cells expressing the galactose-inducible *MEP2* allele was assessed during growth in ammonium-rich medium. Initially we determined whether the polymorphisms in the Σ 1278b genome would affect hybridizations on our yeast open reading frame (ORF) microarrays by comparing global gene expression between S288C and Σ 1278b diploid wild-type strains. Only 24 and 43 genes exhibited a greater than twofold increase and decrease in expression, respectively, between the two genetic backgrounds. Among the differentially regulated genes, the only functional enrichment observed was for glycolysis genes (*CDC19*, *TDH1-3*) that were down-regulated greater than twofold in Σ 1278b (Gene Ontology: 0006096; $p = 2.7 \times 10^{-6}$). These results indicate that the polymorphisms in the Σ 1278b strains have minimal effects on hybridization of our yeast ORF microarrays.

The high expression of *MEP2* under ammonium-rich conditions compared with cells with normal endogenous *MEP2* expression levels resulted in induction of 219 genes that exhibited a twofold or greater increase in expression or more (Supplemental Table 1). These include genes involved in the cell cycle (e.g., *CLB1*, *STB1*, *FAR3*, *SIC1*, and *WHI5*), cell wall maintenance (e.g., *MOB2*, *SKG1*, and *EXG1*) and signal transduction (e.g., *CDC25*, *SDC25*, *STE12*, *RGAI*, and *RTS1*). The most striking feature of the *MEP2*-induced profile in haploid cells was the induction of 35 transposable element genes, consisting mainly of members of the Ty1 family of retrotransposons (Supplemental Table 1). These mobile genetic elements replicate through an RNA intermediate and consist of two direct long terminal repeats that flank the TYA and TYB open reading frames. The promoter of Ty1 elements contains binding sites for multiple transcription factors. Important for this study, the Ty1 promoter contains a filamentation response element within its promoter that responds to nitrogen limitation via the combinatorial activity of Ste12 and Tec1 (Conte *et al.*, 1998; Morillon *et al.*, 2002). Interestingly, in addition to the Ty1 genes, other genes where Ste12 is known to bind to their promoters were also induced in response to constitutive *MEP2* expression. These genes include *AGA1*, *SCW11*, *HXX1*, *GAP1*, *PRY3*, and *PRM6* (Zeitlinger *et al.*, 2003; Borneman *et al.*, 2006, 2007). This profile, when considered in conjunction with those data that support a signaling role for Mep2, suggests that the Ste12 transcription factor is a downstream effector of Mep2 function.

The identification of a Mep2-dependent transcriptional profile in haploid cells that included genes involved in

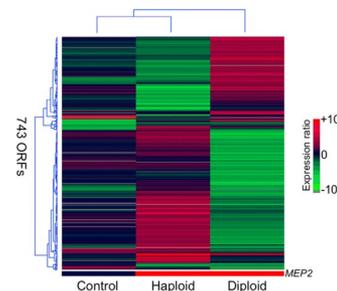


Figure 7. Transcriptional profiles of *MEP2* overexpression in a S288C haploid (middle) and Σ 1278b diploid (right) wild-type strain. Genes that are differentially regulated greater than twofold in at least one of the microarray experiments are shown in the cluster-gram. The control experiment (left) compares S288C and Σ 1278b diploid wild-type strains to determine whether hybridizations differ between the two genetic backgrounds on the yeast ORF microarrays. Only 77 genes were differentially regulated greater than twofold between the two genetic backgrounds. The bottom bar indicates *MEP2* expression in each microarray experiment.

pseudohyphal growth prompted similar experiments in pseudohyphal-competent Σ 1278b diploid yeast cells. The transcriptional profile of wild-type diploid cells was compared with diploid cells expressing *MEP2*. Remarkably, the profile of genes exhibiting twofold or greater differential expression was the reciprocal of that identified in the haploid experiment. Therefore, in most cases, genes that were induced by the constitutive expression of *MEP2* in haploid cells were repressed in diploid cells. Conversely, genes induced in diploid cells containing the *GAL-MEP2* plasmid were repressed in haploid cells (Figure 7).

The genes induced in diploid cells by *MEP2* expression are involved in similar cellular processes as those identified in haploid cells, although the same individual genes are rarely induced in both experiments. Therefore, genes involved in cell wall maintenance, the cell cycle, and mitochondrial function were induced in response to *MEP2* overexpression in diploid cells (Supplemental Table 2). In contrast to the haploid profile, however, the transposable element genes were not collectively induced. This observation may be due in part to lower transcription rates of Ty1 elements reported in diploid cells compared with haploid cells (Morillon *et al.*, 2002). In addition, a large number of genes involved in glucose, methionine, and sulfur metabolism were uniquely induced in diploid cells. Several genes linked to dimorphic growth were induced, including *BEM4* (bud emergence), *CBK1* (protein kinase), and with just under a twofold induction, *GIC2* (bud emergence), *FLO11* (cell surface glycoprotein), and *DIA1* (invasive growth). Interestingly, *FLO11* (*MUC1*), which is essential for pseudohyphal growth, is induced to a higher level in diploid cells (1.8-fold increase) than in haploid cells (1.3-fold increase) in response to Mep2 overexpression. Similar to the Mep2 induced profile in haploid cells, many genes that are targets of Ste12 were induced in diploid cells. These genes include *GIC2*, *CDC19*, *SCH9*, *FLO11*, *CWP2*, and *AGA1* (Zeitlinger *et al.*, 2003; Borneman *et al.*, 2006, 2007).

The Mep2 transcriptional profile also included genes that have a role in cell signaling in addition to the MAPK–Ste12 pathway. *TSC11* encodes a member of the TORC2 complex. Consistent with the involvement of Tor signaling, the gene encoding the Tsc11 subunit of the TORC2, which is proposed to regulate actin cytoskeleton polarization and cell integrity, is induced by Mep2 expression in diploid cells (Barbet *et al.*, 1996; Schmidt *et al.*, 1996; Schmidt *et al.*, 1997;

Cutler *et al.*, 2001; Wedaman *et al.*, 2003). *SCH9* encodes a protein kinase recently shown to be a major target of TORC1 for regulating ribosome biogenesis, translation initiation, and entry into G0 phase, was similarly induced (Urban *et al.*, 2007). The product of *RTS3* is predicted to form part of the protein phosphatase type 2A complex (PP2A) and physically interacts with catalytic and regulatory subunits of protein phosphatase 2A and the PP2A-related phosphatase Sit4–Sap185 complex (Ho *et al.*, 2002; Samanta and Liang, 2003; Gavin *et al.*, 2006). Interestingly, *RTS3* is one of the few genes to be induced in both haploid and diploid cells overexpressing Mep2. In addition, the functional homologue of *RTS3*, *RTS1*, and the regulator of PP2A activity, *RRD2*, were induced by Mep2 expression in haploid and diploid cells, respectively. Rrd2 interacts with the Tap42-PP2Ac complex that is required for a Tor-dependent filamentous signaling pathway (Cutler *et al.*, 2001; Zheng and Jiang, 2005). This suggests that Mep2 expression under nitrogen-sufficient conditions results in the differential regulation of PP2A activity, which might explain the number of identified genes that are involved in both the cell cycle and cell wall integrity and cAMP pathways (Cutler *et al.*, 2001).

Constitutive Mep2 Expression Restores Pseudohyphal Growth in Mutants Lacking cAMP–PKA Signaling but Not the MAP Kinase-regulated Transcription Factor Ste12

The established role of the MAP kinase pathway in pseudohyphal growth suggested that Mep2-dependent induction of Ty1 elements resulted from activity of the Ste12/Tec1 transcription factors. The absence of induction of the equivalent elements in diploid cells could be the result of loss of Ste12/Tec1 activity in diploid cells or to a cell-type-specific regulatory mechanism. The pleiotropic transcriptional regulator Mcm1 is a MADS box protein involved in cell cycle progression, arginine metabolism, cell wall maintenance, cell type determination, pheromone response, and mating (Sengupta and Cochran, 1990; Lydall *et al.*, 1991; Messenguy and Dubois, 1993). The transcriptional activation of α -specific genes results from dimerization of Ste12 and Mcm1 at target promoters (Sengupta and Cochran, 1990; Mueller and Nordheim, 1991). Conversely, Mata2–Mcm1 complexes repress the expression of α -specific genes in diploid cells (Keleher *et al.*, 1989). In addition, Mcm1 regulates Ty1 expression and binds to regulatory sequences independent of Ste12, unlike the pheromone-induced genes that are regulated by a Mcm1–Ste12 heterodimer (Errede, 1993). The cell-type-specific response to Mep2 expression observed in the microarray studies and the identification of genes regulated by Ste12 and the cell cycle suggested that Ste12 and/or Mcm1 could be downstream effectors of Mep2.

To examine this hypothesis, the ability of the *GAL-MEP2* allele to induce pseudohyphal growth in mutants lacking components of the PKA pathway or Ste12 was analyzed. *MEP2* overexpression restored pseudohyphal growth in mutants lacking Tpk2, Gpa2, Gpr1, or Ras2 (Figure 8A). However, *MEP2* overexpression did not restore pseudohyphal growth in a strain lacking Ste12 (Figure 8A). As a control, complementation of the *ste12* Δ mutant with a plasmid expressing *STE12* or a hyperactive *RAS2* allele restored wild-type pseudohyphal growth in the *ste12* Δ strain (Figure 8B). Analogous experiments were conducted to determine whether activation of either the PKA or the MAP kinase pathway restores pseudohyphal growth in a *mep2* Δ mutant. Expression of alleles that constitutively activate the PKA pathway, the MAP kinase pathway, as well as overexpression of Ste12, all restored pseudohyphal growth of the *mep2* Δ mutant (Figure 8C). Together, these experiments are consistent

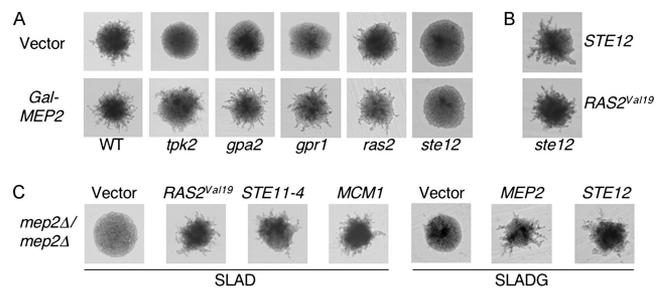


Figure 8. Epistasis analysis provides evidence that Mep2 and Ste12 are functionally related. After growth on galactose low-ammonium sulfate (50 μM) plates for 6 d at 30°C, pseudohyphal growth was assayed in diploid mutants lacking Tpk2, Gpa2, Gpr1, Ras2 and Ste12 that expressed *MEP2* from a galactose-inducible promoter (A) and diploid *ste12* Δ /*ste12* Δ cells that contained either a galactose-inducible *STE12* gene or the *RAS2*^{Val19} allele (B). (C) Cells lacking Mep2 and containing plasmids expressing *MCM1*, the *RAS2*^{Val19} allele, the *STE11-4* allele, *GAL-MEP2*, or *GAL-STE12*.

with Mep2 and Ste12 acting in either the same pathway during pseudohyphal growth or in parallel interdependent pathways. Overexpression of the Ste12 interacting transcription factor Mcm1 also restored pseudohyphal growth in the *mep2* Δ strain, providing further functional evidence linking Mep2 and the MAP kinase pathway (Figure 8C).

DISCUSSION

Nitrogen availability regulates the initiation of diploid filamentous growth, haploid invasive growth, and mating in different model fungi. These developmental processes occur when ammonium is limiting, and they are dependent on members of the AmtB/Mep/Rh family of ammonium permeases. Here, we present evidence that supports the hypothesis that Mep2 functions as an ammonium sensor; importantly, we document that the transport activity of Mep2 is linked to both differentiation and the control of gene expression.

The localization and oligomerization state of Mep2 are consistent with other members of the AmtB/Mep2/Rh family of transporters. We have demonstrated that Mep2 monomers interact to form a trimeric complex. Our data do not exclude the possibility that Mep2 could form heterotrimeric complexes with the nonpseudohyphal-inducing Mep1 and Mep3 ammonium permeases. When initially expressed, Mep2 is localized to the growing bud or the bud neck, a process that is dependent on the exocyst complex. Thereafter, Mep2 is localized throughout the plasma membrane.

A central question relating to Mep2 function is the extent to which ammonium binding, transport, or both are necessary to induce pseudohyphal growth. In previous studies, it was reported that Mep2-dependent pseudohyphal growth occurs only with limiting ammonium as a nitrogen source (Lorenz and Heitman, 1998a). This suggested that ammonium binding/transport via Mep2 is important for its regulatory function. Contrary to this, studies with *C. albicans* found that Mep2-dependent pseudohyphal growth occurs when this yeast is grown on a variety of nitrogen sources (Biswas and Morschhäuser, 2005). Similarly we find that *S. cerevisiae* also undergoes Mep2-dependent pseudohyphal growth in response to growth on glutamate and proline. In our previous *S. cerevisiae* studies, growth and filamentation assays were conducted on noble agar or agarose (Lorenz and Heitman, 1998a). Under these conditions, pseudohyphal growth is more limited, and even more so with glutamine or

proline as the nitrogen source in which there was no clear obligate role for Mep2 in pseudohyphal growth. When growth assays are conducted on standard SLAD medium containing agar (including contaminating minor nitrogen sources), more robust pseudohyphal growth occurs and under these conditions Mep2 plays a clearly demonstrable role in promoting pseudohyphal differentiation, even with glutamine and proline as nitrogen source. During the course of this work, Marini and colleagues published similar findings implicating Mep2 in physiological roles during growth on nitrogen sources other than ammonium (Boeckstaens *et al.*, 2007). They found that cells leak ammonium when grown on a variety of nitrogen sources, and they advanced the hypothesis that one role for Mep2 is to reimport ammonium that exits the cell during growth on alternative nitrogen sources. In the context of this model, pseudohyphal growth on nitrogen sources such as glutamine and proline still requires the ability of Mep2 to transport and sense ammonia, in this case involving ammonia that exits the cell from internal sources.

Mutational analysis of Mep2 supports the hypothesis that ammonium transport through Mep2 is required for the induction of pseudohyphal growth. A Mep2^{D186E} variant that is correctly expressed and localized to the plasma membrane fails to transport ammonium and does not induce pseudohyphal growth (Marini *et al.*, 2006). Our present study establishes that residues H194 and H348, which are conserved residues lining the pore of Mep2, are important in the initiation of pseudohyphal growth. The conservation of these residues throughout the AmtB/Mep/Rh family, and their position within the hydrophobic pore, suggest that their function is to bind ammonium as it translocates through the protein (Khademi *et al.*, 2004). The importance of these residues for ammonium transport has been underscored by mutational analysis of the equivalent residues in AmtB from *E. coli* (Javelle *et al.*, 2006). In *S. cerevisiae*, the Mep2^{H194A} and Mep2^{H348A} variants are stably expressed and localized to the cell membrane. The Mep2^{H194A} protein has a significantly reduced ability to transport ammonium and does not induce pseudohyphal growth. The Mep2^{H348A} protein transports ammonium but does not signal pseudohyphal growth. Somewhat unexpectedly, the ability of Mep2^{H348A} to support growth on low ammonium contrasts to the lack of transport activity resulting from the equivalent mutation in AmtB from *E. coli*. Together, these data suggest ammonium translocation through Mep2 is required to initiate pseudohyphal growth and that mutation of residues D186 and H194 prevents this signaling process. The phenotype of the Mep2^{H348A} protein suggests that the binding of ammonium ions to this residue could cause a conformational change in Mep2 that initiates pseudohyphal growth. Consistent with this, a Mep2 mutant that is hyperactive for both ammonium transport and pseudohyphal growth contains a mutation in the adjacent residue, G349 (Boeckstaens *et al.*, 2007).

The requirement for Mep2 transport activity may explain the inability of mutants lacking the Npr1 kinase to undergo pseudohyphal growth (Lorenz and Heitman, 1998a). Our data establish that Mep2 is clearly localized to the plasma membrane and highly expressed in mutants lacking the Npr1 kinase. However, in the absence of Npr1, Mep2 does not transport ammonium (Boeckstaens *et al.*, 2007). The Mep2^{G349C} variant has increased transport activity and restores pseudohyphal growth in an *npr1Δ* strain (Boeckstaens *et al.*, 2007). This is consistent with Npr1 being required to activate Mep2-dependent ammonium transport, but not expression or localization, which then enables a Mep2-dependent induction of pseudohyphal growth. Interestingly,

Mep2 is also correctly localized in a mutant lacking the Ure2 transcriptional repressor. Further studies will be required to determine whether the inability of a *ure2Δ* mutant to initiate dimorphic growth is a result of a loss of Mep2 transport function or some other regulatory process in which constitutive induction of the nitrogen catabolite repression response blocks pseudohyphal growth.

We have established a correlation between the level of Mep2 expression and the degree of pseudohyphal growth in *S. cerevisiae*. Increased expression of Mep2 results in greater pseudohyphal growth and a modest increase in Mep2 expression can compensate for the pseudohyphal growth induction defect in cells expressing the Mep2^{T288A} protein. In *C. albicans*, high expression levels of Mep2 are required for its ability to induce hyphal growth (Biswas and Morschhäuser, 2005). It is interesting to note that in each yeast that undergoes ammonium-dependent development, the relevant inducing permease is the most highly expressed of the family of ammonium permeases (Marini *et al.*, 1997; Smith *et al.*, 2003; Biswas and Morschhäuser, 2005; Rutherford *et al.*, 2008). Mep2 is required for the induction of haploid invasive growth in response to low-ammonium growth, similar to other haploid yeasts. This takes place under conditions that are distinct from the invasive growth that occurs in response to carbon source availability and the presence of fusel alcohols (Lorenz *et al.*, 2000b), which probably accounts for the novel finding of the involvement of Mep2 in this process. Similar to the diploid Mep2 pseudohyphal pathway, the Tpk2 catalytic subunit of PKA, the Npr1 kinase, and the Ste12 and Tec1 transcription factors are required for haploid invasive growth. The Tpk1/Tpk3 catalytic subunits of PKA repress ammonium-responsive invasive growth and Mep2 mutants altered in the conserved H194 and H348 residues do not undergo invasive growth. Cells that have undergone invasive growth in response to ammonium limitation can adopt an elongated morphology similar to that observed during diploid pseudohyphal growth. Together, these data support the view that the Mep2-dependent signaling processes that occur during haploid invasive growth are similar to those occurring during pseudohyphal growth. Our data contradict a previous study that reported that Mep2 is not required for haploid invasive growth when cells are grown on medium lacking or containing minimal (100 μ M) ammonium (Van Nuland *et al.*, 2006). The complementation of the *mep2Δ* strain with *MEP2* expressed from a plasmid confirms that under these conditions, Mep2 is required for ammonium induced haploid invasive growth. Ammonium permease haploid invasive growth may be widely conserved within yeast, because this conservation has now been observed in haploid *S. pombe*, *C. neoformans*, and *S. cerevisiae*.

The expression of Mep2 under conditions that do not normally induce pseudohyphal growth generates a transcriptional profile that includes genes that are involved in cellular processes that are likely to be differentially regulated during pseudohyphal growth. Of particular interest is a group of genes controlled by Ste12 involved in the cell wall (*AGA1*, *SED1*, and *CWP2*), cell cycle (*CLB1* and *SCH9*), MAP kinase pathway (*GIC2*), PKA pathway (*SCH9*), and pseudohyphal growth (*STE12*, *FLO11*, and *HMS1*). Also of interest is the induction of genes involved in the activity of Cdc42, a GTPase that establishes cell polarity and is also a component of the MAP kinase pathway (*CDC25*, *SDC25*, *RGA1*, and *GIC2*). Therefore, Mep2 expression results in the induction of a subset of Ste12-regulated genes and genes involved in the regulation of the MAP kinase pathway, indicative of Mep2-dependent differential activation of the pathway.

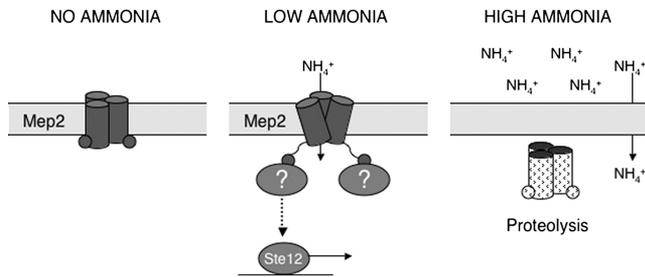


Figure 9. Model of Mep2 signaling during pseudohyphal growth. Mep2 forms a trimer within the membrane and acts as a sensor of ammonium availability. When no ammonium is present in the environment the signaling function of Mep2 is repressed. When limiting ammonium becomes available, its transport causes a conformational change in Mep2, which enables the permease to engage signaling partners that initiate pseudohyphal growth. High levels of ammonium result in the loss of Mep2 signaling because Mep2 is removed from the membrane and *MEP2* transcription is repressed.

Consistent with a link between Ste12 and Mep2, activation of the MAP kinase pathway, through either overexpression of Ste12 or the hyperactive *STE11-4* allele restored pseudohyphal growth in a diploid strain lacking Mep2, in contrast to a previous report (Lorenz and Heitman, 1998a). In addition, overexpression of Mep2 from the *GAL* promoter restored pseudohyphal growth in mutants lacking elements of the RAS–cAMP pathway but not in a mutant that lacks Ste12. This evidence strongly supports a link between Ste12 activity and the putative transceptor role of Mep2 during pseudohyphal growth. This, together with previous data that pseudohyphal growth in a *S. cerevisiae* strain lacking Mep2 can be restored by the exogenous addition of cAMP to cells (Lorenz and Heitman, 1998a), is similar to the epistatic relationship of *C. albicans* Mep2 and the PKA and MAP kinase pathways during filamentous growth. This mechanistic conservation in different fungi is consistent with the finding that the expression of certain Mep2 fungal homologues restores pseudohyphal growth in an *S. cerevisiae* strain lacking the endogenous Mep2, suggesting that significant aspects of the signaling role of these homologues is conserved in some fungal species (Montanini *et al.*, 2002; Javelle *et al.*, 2003; Smith *et al.*, 2003).

Although the constitutive expression of Mep2 induces pseudohyphal growth under normally repressing conditions, overexpression of Ste12 under the same conditions does not (Borneman *et al.*, 2007). This may be due to the increased turnover of Ste12 in nitrogen-sufficient conditions, a process regulated by the Srb10 cyclin-dependent kinase, which itself becomes depleted during nitrogen limitation (Nelson *et al.*, 2003). This therefore argues against a simple model whereby Mep2 only activates the MAP kinase pathway. Mep2 could influence the turnover of Ste12 and/or regulate pathways that influence Ste12 activity. It is possible that mechanisms that regulate the differential output of the MAP kinase in haploid and diploid cells are responsible for the transcriptional profiles that we have identified. Interestingly, we have shown here and in previous work that Mcm1 and Tec1, two transcription factors that interact with Ste12 to modulate its activity, suppress the loss of pseudohyphal growth in cells lacking Mep2 (Lorenz and Heitman, 1998a). Additional candidates for signaling pathways that Mep2 might interact with were also identified in microarray experiments including PP2A and the Tor kinases.

Together, our data suggest a novel model of Mep2 function (Figure 9). The requirement for ammonium transport by

Mep2 suggests that the lack of ammonium in the environment is not the initiating signal for pseudohyphal growth as has been proposed. This is supported by the Mep2 dependent induction of pseudohyphal growth under ammonium-sufficient growth because presumably, under these conditions, Mep2 is transporting ammonium into the cell. This strongly suggests that the importance of Mep2 does not relate to its influence on the internal levels of nitrogen, because it will induce pseudohyphal growth irrespective of ammonium levels when expressed, further supporting the designation of Mep2 as a transceptor. We propose that when ammonium is absent from the external environment, Mep2 is expressed and localized on the plasma membrane (Figure 9). Under these conditions, Mep2 does not transport ammonium and the lowering of internal nitrogen levels prevents growth. When low levels of ammonium become available, the transport of ammonium through Mep2 triggers conformational changes that most likely involve the C-terminal domains, which signal to the cell that there is sufficient ammonium for growth. Support for the conformational fluidity of Mep2 comes from the finding that the C-terminal domain of each monomer of the *Arabidopsis thaliana* Mep2 homologue Amt1;1 interacts with the adjacent monomer to confer allosteric regulation (Loqué *et al.*, 2007). The physical act of transport through Mep2 enables the permease to engage signaling components that result in Ste12 activation and the possible regulation of the Tor and PP2A pathways. Together, these result in the induction of pseudohyphal growth. If increased levels of ammonium become available, the transcription of the *MEP2* gene is repressed via catabolite repression (Marini *et al.*, 1997), and Mep2 is internalized, localized to the vacuole, and degraded (Zurita-Martinez *et al.*, 2007). Under these conditions, ammonium is able to diffuse through the plasma membrane, and the loss of the pseudohyphal-inducing signal from Mep2 results in the repression of pseudohyphal growth.

Ammonium permeases have been implicated in regulating haploid invasive growth, diploid pseudohyphal growth, and mating in different model fungal systems. Members of the Amt/Mep/Rh family are also required for certain aspects of the development of *Dictyostelium discoideum* (Kirsten *et al.*, 2005; Singleton *et al.*, 2006). Together, these systems suggest that ammonium permease-mediated control of development may be widespread throughout unicellular, and possibly also multicellular, eukaryotic organisms. The molecular mechanisms that link Mep2 to pseudohyphal differentiation may therefore be conserved and relevant for studies in other model developmental systems. The present study establishes for the first time, Mep2-dependent regulation of gene expression that likely involves, at least in part, the transcription factor Ste12. The challenge now is to identify the putative regulatory proteins that physically interact with Mep2 to govern transcriptional cascades that evoke novel cellular fates in response to external nutrient sources.

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