Roles of the three Ras proteins in the regulation of dimorphic transition in the yeast Yarrowia lipolytica

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Abstract

Ras proteins in the budding yeast Saccharomyces cerevisiae are essential for growth and dimorphic transition. The dimorphic yeast Yarrowia lipolytica is distantly related to S. cerevisiae. Its genome encodes three Ras proteins. Here, we show that the three Ras proteins in Y. lipolytica are critical for dimorphic transition but are dispensable for growth. Among the three Ras proteins, YlRas2 plays a major role in the regulation of dimorphic transition, whereas YlRas1 plays a minor role in this process. The additional Ras protein, YlRas3, which resembles mammalian K-Ras4B at the C-terminus, does not seem to have a significant role in dimorphic transition. Thus, the three Ras proteins do not act equally in the regulation of dimorphic transition. We also show that the expression of YlRAS2 was increased dramatically at the transcriptional level during yeast-to-hypha transition, consistent with a major role of YlRas2 in the regulation of dimorphic transition. YlRas2’s function in dimorphic transition depends on the active GTP-bound form of YlRas2 and its localization to the plasma membrane. YlRas2 could also partially function on the endomembranes. In addition, we identified the transcription factor Mhy1 as a potential signal transducer downstream of YlRas2 in the control of dimorphic transition. This finding suggests that novel signaling pathway controlled by Ras proteins regulating dimorphic transition may exist in Y. lipolytica.

Introduction

Many yeast species and some filamentous fungi could grow either in ovoid, single-celled yeast form or in long, multicellular filaments called hyphae or pseudohyphae (Lengeler et al., 2000; Berman & Sudbery, 2002; Palecek et al., 2002). The ability to switch between these forms is called dimorphism. Dimorphic transition is normally induced by environmental factors including temperature, the accessibility of nitrogen source, or pH and is thought to be important for cell survival in response to environmental changes (Berman & Sudbery, 2002; Palecek et al., 2002; Ruiz-Herrera & Sentandreu, 2002). In the human pathogen Candida albicans, dimorphic transition is an important virulence factor for pathogenesis.

Ras proteins are small GTP-binding proteins widespread from yeast to humans. They control signal transduction pathways important for cell proliferation, cell differentiation, and cell survival (Malumbres & Barbacid, 2003). In yeast and filamentous fungi, Ras proteins have a critical role in cellular morphogenesis (Weeks & Spiegelman, 2003). For example, Ras proteins are important for the maintenance of a normal cell shape in the yeast species Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Cryptococcus neoformans (Fukui et al., 1986; Nadin-Davis et al., 1986; Ho & Bretscher, 2001; Waugh et al., 2002). Ras proteins are also implicated in dimorphic transition in yeast species S. cerevisiae, C. albicans, and in the yeast-like fungus Ustilaginoidea maydis (Mösch et al., 1996; Leberer et al., 2001; Lee & Kronstad, 2002; Zhu et al., 2009). Moreover, Ras proteins also regulate filamentous growth in filamentous fungi such as Trichoderma reesei (Zhang et al., 2012).

Ras proteins localize primarily to the plasma membrane in yeast cells (Bhattacharya et al., 1995). The plasma membrane targeting of Ras proteins depends on the post-translational modification of the cysteine residue in the CaaX motif (a, aliphatic acid; X, any amino acid).
at their C-termini with a farnesyl moiety (Hancock et al., 1989; Fujiiyama & Tamanoi, 1990). The removal of the CaaX motif or substitution of serine for the cysteine residue renders Ras proteins completely cytosolic. The palmitoylation of a second cysteine residue preceding the CaaX motif is also important for the plasma membrane localization of Ras (Dong et al., 2003; Onken et al., 2006; Nichols et al., 2009; Piispanen et al., 2011). The palmitoylation-deficient Ras mutants localize primarily to the endomembranes but not to the plasma membrane.

Ras proteins function as molecular switches (Takai et al., 2001). Ras in the GTP-bound form binds downstream effectors, transducing signals downstream. The hydrolysis of Ras-bound GTP to GDP by either its intrinsic GTPase activity or by the stimulation of its GTPase activity by GTPase-activating proteins (GAP) could terminate Ras function. Conversely, the activation of guanine nucleotide exchange factors (GEF) of Ras by upstream molecules could activate Ras (Takai et al., 2001; Malumbres & Barbacid, 2003). In S. cerevisiae, the GEFs for two Ras proteins, Ras1 and Ras2, are Cdc25 and Sdc25. The two GAPs for Ras are Ira1 and Ira2 (Weeks & Spiegelman, 2003).

Yarrowia lipolytica is a dimorphic yeast species distantly related to S. cerevisiae and C. albicans (Dujon et al., 2004; Jimenez-Bremont et al., 2012). Unlike other yeast species which usually has one or two Ras proteins, Y. lipolytica has three Ras proteins. In this study, we show that the three Ras proteins, particularly YIRAS1 and YIRAS2, are implicated in the control of dimorphic transition. The function of YIRas2 in this process does not absolutely require the cycling of YIRas2 between the GTP- and GDP-bound states and the plasma membrane localization of YIRas2. In addition, we identified the transcription factor Mhy1 as a potential downstream signal transducer of YIRas2 in the regulation of dimorphic transition. Our study builds foundations for future investigations on the regulatory mechanisms of Ras proteins in Y. lipolytica.

Materials and methods

Strains and growth conditions

Yarrowia lipolytica strains PO1a (MatA leu2-270 ura3-302) and FI354 (PO1a except YlKdc25::mTnY1-URA3; Richard et al., 2001) were kindly provided by Dr Claude Gaillardin and Mathias Richard. E122 (MatA leu2-270 ura3-302 lys8-11) and mhy1K09 (E122 except mhy1A::URA3; Hurtado & Rachubinski, 1999) were kindly provided by Dr Richard Rachubinski. Escherichia coli strains DH12S and DH5α were used for plasmid amplification. Standard culture media and genetic techniques were used. Yarrowia lipolytica strains were routinely grown at 30 °C in YPD medium (2% peptone, 1% yeast extract, and 2% glucose) or YNBD medium (0.67% yeast nitrogen base without amino acids, 2% glucose). When required, YNBD medium was supplemented with 80 mg L⁻¹ leucine, 20 mg L⁻¹ uracil, or both. For solid medium, agar was added to 2%. YNDC7 medium (0.67% yeast nitrogen base without amino acids, 2.94% trisodium citrate dehydrate, 1% glucose, pH 7.0) was used for hyphal growth. YNB+4% serum agar medium (0.67% yeast nitrogen base without amino acids, 4% bovine serum, supplemented with leucine and uracil) was used for the detection of radial filament formation.

Plasmid and yeast strain construction

Plasmid pINA445-YIRAS2 was constructed by inserting the 1.95-kb YIRAS2 gene containing 658-bp promoter, 1098-bp ORF, and 193-bp 3′ UTR amplified by PCR into HindIII- and ClaI-digested pINA445 (CEN, YILEU2). Site-directed DNA mutagenesis of YIRAS2 was performed in pINA445-YIRAS2, yielding pINA445-YIRAS2G17V and pINA445-YIRAS2C266L. Correct nucleotide substitutions were confirmed by DNA sequencing.

To overexpress YIRAS1, YIRAS2, and YIRAS3 under the control of the strong YITEF1 promoter, DNA fragment containing the ORF and 3′ UTR of YIRAS1 was amplified from PO1a genomic DNA by PCR, digested with BamHI, and ligated into pYL13 (CEN, YILEU2, PYITEF1; Zhao et al., 2013), yielding pYL13-YIRAS1. DNA fragments containing the ORF and 3′ UTR of YIRAS2 and YIRAS3 were amplified by PCR, digested with HindIII and ClaI, and ligated into pYL13, yielding pYL13-YIRAS2 and pYL13-YIRAS3. Similarly, DNA fragment containing the ORF and 3′ UTR of MHY1 was inserted into pYL13, yielding pYL13-MHY1.

To examine the subcellular localization of YIRas2, pYL15 (CEN, YILEU2, PYITEF1-EGFP) was constructed by inserting a 0.72-kb SpeI-BamHI EGFP fragment from pEGFP-C1 (Clontech) into XbaI- and BamHI-digested pYL13 (Zhao et al., 2013). Then, the 1.29-kb DNA fragment containing YIRAS2 ORF and 193-bp 3′ UTR was amplified from genomic DNA, digested by HindIII and ClaI, and ligated into pYL15, yielding pYL15-YIRAS2. EGFP was fused in frame to the N-terminus of YIRas2.

To evaluate the contributions of the two C-terminal cysteine residues in YIRas2 function, the 1.75-kb YIRAS2 gene containing 658-bp promoter and 1098-bp ORF without 3′ UTR was inserted into pYL11 (pINA445-TYIRAS2; Zhao et al., 2013), yielding pYL11-YIRAS2. Site-directed DNA mutagenesis of YIRAS2 was performed in pYL11-YIRAS2, yielding pYL11-YIRAS2C245S and pYL11-YIRAS2C246S. Correct nucleotide substitutions were confirmed by DNA sequencing. Site-directed DNA mutagenesis of YIRAS2 was
also performed in pYL15-YlRAS2, yielding pYL15-YlRAS2::Yl lacZ and pYL15-YlRAS2::Yl lacZ.

To measure the transcriptional activity of YlRAS1, YlRAS2, and YlRAS3 promoters during dimorphic transition, YlRAS promoters plus the first 6- to 9-bp ORF sequence were fused to lacZ by inserting the c. 2000-bp BamHI-SalI fragment of Yl lacZ promoter and BamHI-HindIII fragment of YlRAS and BamHI-HindIII promoters amplified by PCR into pINA445-lacZ (Zhao et al., 2013), yielding pINA445-YlRAS1-lacZ, pINA445-YlRAS2-lacZ, and pINA445-YlRAS3-lacZ.

YlRAS1 and YlRAS3 were deleted in the strain PO1a by homologous recombination with loxR-YURA3-loxP marker (Zhao et al., 2013). Uracil+ transformants were analyzed by PCR. At least two independent correct clones were obtained for each gene deletion. The YlURA3 marker was later excised by Cre-mediated site-specific DNA recombination between loxR and loxP sites (Zhao et al., 2013), yielding YLX288 (Ylras1::loxR/P) and YLX209 (Ylras3::loxR/P). YlRAS2 were deleted in the ORF region in the strain PO1a by the two-step 'pop-in/pop-out' method (Morin et al., 2007), yielding YLX81 (Ylras2Δ). YlRAS1 and YlRAS3 were deleted individually in strain YLX81 (Ylras2Δ), yielding YLX469 (Ylras1Δ::loxR/P Ylras2Δ) and YLX470 (Ylras2Δ Ylras3Δ::loxR/P), respectively. YlRAS3 was deleted in strains YLX288 (Ylras1Δ::loxR/P) and YLX469 (Ylras1Δ::loxR/P Ylras2Δ), yielding YLX471 (Ylras1Δ::loxR/P Ylras3Δ::loxR/P) and YLX472 (Ylras1Δ::loxR/P Ylras2Δ Ylras3Δ::loxR/P), respectively. The YlURA3 marker in strain Fil354 (PO1a except Yl ras1::loxR/Yl ras2/ Yl ras3::loxR) was excised by Cre/loxP site-specific recombination, yielding YLX58 (Ylcd25::loxP).

Yarrowia lipolytica transformation

The lithium acetate method of S. cerevisiae transformation (Gietz et al., 1995) was used for Y. lipolytica transformation, except cells were heat-shocked at 37 °C for 15 min before plating on selective medium.

Invasive growth assay

Yarrowia lipolytica cells were streaked onto YNBD agar medium supplemented with leucine and uracil if necessary and grown at 30 °C. Cells were gently rubbed off the agar surface under running water. Agar that contains invaded cells was cut into slices to visualize cell morphology by light microscopy.

β-Galactosidase assay

β-Galactosidase activity in crude cell extracts was assayed following a previously described method (Zhao et al., 2013). The specific β-galactosidase activity of the cell extracts was normalized by the amount of total protein in each extract and was calculated according to the following formula: 

\[
U = \frac{(\text{OD}_{420} \times 1.4) \times \text{protein concentration (mg mL}^{-1}) \times \text{extract volume (mL)}}{\text{time (min)}}
\]

The specific activity was expressed as nmoles (min mg protein)\(^{-1}\). Assays were performed in triplicate.

Microscopy

An Olympus BX51 microscope (Tokyo, Japan) and a Retiga 2000R CCD camera (QImaging Corporation, Canada) were used to visualize cell morphology and GFP by differential interference contrast (DIC) and fluorescent microscopy. The images were acquired using QCapture Suite (QImaging Corporation).

Results

The Y. lipolytica genome encodes three Ras proteins

By searching the Y. lipolytica genome database (http://www.genolevures.org/blast.html#), we identified five proteins that share the highest degree of amino acid sequence identity with S. cerevisiae Ras1 and Ras2. They are YALI0E08756, YALI0B16984, YALI0F23177, YALI0E30943, and YALI0E29887 in the order of decreased identity. Examination of key motifs in these five proteins, such as Ras-specific QEEY motif right after the G2 box (Fig. 1), revealed that only YALI0E08756 (223 a.a.), YALI0B16984 (258 a.a.), and YALI0E29887 (249 a.a.) belong to the Ras family of small GTP-binding proteins. The other two are Rap and Rheb homologues. Among the three Ras proteins, YALI0E08756 shares the highest amino acid sequence identity with S. cerevisiae Ras1 (67%) and Ras2 (64%). Next to it is YALI0B16984, which shares 57% and 48% of sequence identity with S. cerevisiae Ras1 and Ras2, respectively. YALI0E29887 was already named YlRas2 (Richard et al., 2001). It actually shares the lowest sequence identity with Ras1 (41%) and Ras2 (43%). We thus designated YALI0E08756 as YlRas1 and YALI0B16984 as YlRas3.

The coding genes of YlRas1 and YlRas2 both contain one intron near the beginning of the ORF, whereas the coding gene of YlRas3 does not contain intron. All the three Ras proteins contain the conserved G1-G4 boxes required for GTP binding. They share identical core effector domain with S. cerevisiae and human Ras proteins (Fig. 1). YlRas2 contains two small inserts in the region between the G2 and G3 boxes, which are absent in S. cerevisiae and human Ras. Like S. cerevisiae Ras1 and Ras2, the three Y. lipolytica Ras proteins contain a longer C-terminal domain.
hypervariable region (HVR) compared to the 25 amino acid region in H-Ras and the 24 a.a. region in K-Ras4B (Fig. 1). However, the HVR domains of the three YlRas proteins are shorter than those of Ras1 and Ras2, the latter of which are known to negatively regulate Ras function (Marshall et al., 1987).

The three YlRas proteins contain the conserved CaaX motif at their C-termini commonly found in all Ras proteins (Fig. 1). Like *S. cerevisiae* Ras1 and Ras2, YlRas1 and YlRas2 also contain a cysteine residue immediately upstream of the CaaX motif. The extra cysteine residue in *S. cerevisiae* Ras proteins is known to be palmitoylated and plays a crucial role for targeting Ras to the plasma membrane (Bhattacharya et al., 1995; Dong et al., 2003). H-Ras also relies on the palmitoylation of the two cysteine residues preceding the CaaX motif for plasma membrane targeting (Hancock et al., 1990). Interestingly, YlRas3 does not contain extra cysteine residues preceding the CaaX motif, but instead, it contains a stretch of basic lysines (Fig. 1). This feature resembles human K-Ras4B but not H-Ras. The polybasic stretch in K-Ras4B is known to be required for targeting K-Ras4B to the plasma membrane (Hancock et al., 1990).

**Fig. 1.** Sequence alignment of YlRas1, YlRas2, and YlRas3 with *S. cerevisiae* Ras1 and Ras2 and human H-Ras and K-Ras4B. Identical and similar residues are highlighted in dark and gray shades, respectively. The conserved G1-G4 boxes involved in GTP binding and GTP hydrolysis, the core effector domain involved in downstream effector binding, the hypervariable region (HVR), and the CaaX motif are marked on top by a line. The lysine residues in the polybasic region next to the CaaX motif are marked by bold letters.

**Roles of the three Ras proteins in cellular morphogenesis during yeast-form growth, filamentous growth, and invasive growth**

To explore the cellular functions of the three *Y. lipolytica* Ras proteins, we deleted the entire open reading frame of YlRas1, YlRas2, and YlRas3 genes individually in the wild-type strain PO1a. Successful deletion of each gene was confirmed by PCR analysis (data not shown) and gene complementation (see below). Except that cells deleted for YlRas2 grew slightly slower than wild-type cells at 30 °C, the deletion of YlRas2 cells did not cause a detectable growth defect (data not shown). When
grown in liquid YNDC7 medium, a hypha-inducing condition, Ylras2Δ cells were still round and failed to form pseudohyphae or hyphae as wild-type cells did (Fig. 2b, right panel). Ylras2Δ cells were also rounder than oval-shaped wild-type cells in liquid YPD medium (Fig. 2b, left panel). These results indicate that Ylras2Δ cells have a severe defect in filamentous growth and a moderate defect in cell morphology during yeast-form growth. Ylras1Δ cells also exhibited a defect in filamentous growth because the extent of pseudohyphal or hyphal development in YNDC7 medium was markedly reduced compared to the wild-type control (Fig. 2b, right panel). Unlike Ylras1Δ and Ylras2Δ cells, Ylras3Δ cells did not show detectable defect in filamentous growth under these growth conditions. The defect of Ylras2Δ cells in filamentous growth was due to deletion of YLRAS2 as reintroduction of YLRAS2 restored normal filamentous growth to Ylras2Δ cells (Supporting information, Fig. S1). The same is true for Ylras1Δ cells (Fig. S1). These results suggest that YLRas2 plays a major role in cellular morphogenesis during yeast-form growth and filamentous growth among the three Ras proteins. YLRas1 plays a minor role in filamentous growth, whereas YLRas3 seems to be dispensable for morphogenesis.

It is known that the introduction of LEU2 gene into cells of Y. lipolytica strain with leu2 genotype enhances filamentous growth (Fickers et al., 2003). Interestingly, we found that, under a stronger hypha-inducing condition (YNDC7 medium plus the introduction of LEU2 gene), a large fraction of Ylras2Δ cells adopted an elongated cell morphology (Fig. S1c, Ylras2Δ/Vec), indicating that Ylras2Δ cells did not completely lose their ability to undergo dimorphic transition. This view is supported by another observation. When grown on YNB+4% serum agar medium at 30 °C, wild-type cells as well as Ylras1Δ and Ylras3Δ cells robustly formed long radial filaments protruding from the periphery of colonies in 2 days. In contrast, Ylras2Δ cells did not develop long, easily detectable filaments during the same period (Fig. 2c, left panel). The few filaments they formed were very short, consistent with a defect in filamentous growth. Ylras2Δ cells did form longer, easily detectable filaments in 5 days (Fig. 2c, right panel). However, the filaments were still fewer and shorter than those of wild-type cells. These results demonstrate that the deletion of YLRAS2 does not completely abolish filamentous growth. It could still occur under strong hypha-inducing condition.

Cells of wild-type strain PO1a could invade agar on YNBD agar medium. While Ylras1Δ and Ylras3Δ cells acted normally in invasive growth, Ylras2Δ cells showed reduced invasive growth (Fig. 2d). In addition, the depth of agar penetration by Ylras2Δ cells was significantly shallower.
than that of wild-type cells (Fig. 2e). Moreover, unlike wild-type cells whose invading filaments were composed of oval-shaped cells at the leading front and elongated cells at the back, the invading filaments formed by Ylras2Δ cells were made of entirely round cells. These results indicate that YlRas2 also plays a prominent role in invasive growth.

We also generated double and triple YlrasΔ mutant strains and examined these strains for their ability to undergo filamentous growth. The three Ras proteins are not essential for cell growth as the double and triple YlrasΔ mutants grew well at 30 °C, except that the mutants bearing Ylras2Δ grew slightly slower than wild-type cells (data not shown). Cells of the double mutants Ylras1Δ Ylras2Δ and Ylras2Δ Ylras3Δ as well as the triple mutant Ylras1Δ Ylras2Δ Ylras3Δ bearing the deletion of YIRAS2 exhibited a severe defect in cell morphology during yeast-form growth and hyphal growth similar to Ylras2Δ cells. In comparison, Ylras1Δ Ylras3Δ cells only exhibited a moderate reduction in hyphal growth (Fig. 3a). Ylras1Δ Ylras3Δ cells also exhibited a moderate defect in the development of radial filaments on YNB+4% serum agar medium (Fig. 3b). These results support the view that YlRas2 plays a major role, whereas YlRas1 plays a minor role in filamentous growth. Ylras2Δ cells developed filaments of moderate length and density in 5 days (Fig. 3b). In contrast, Ylras1Δ Ylras2Δ cells failed to develop filaments in 5 days (Fig. 3b), which is much worse than the defect of Ylras2Δ cells, suggesting that YlRas1 shares a similar function with YlRas2 in filamentous growth. Ylras2Δ Ylras3Δ cells also exhibited a defect in filament formation worse than that of Ylras2Δ cells as Ylras2Δ Ylras3Δ cells developed fewer and shorter filaments than those of Ylras2Δ cells in 5 days (Fig. 3b). This is the only evidence in this study that suggests that YlRas3 may play a role in filamentous growth. Ylras1Δ Ylras2Δ Ylras3Δ triple mutant cells exhibited a defect in filament formation to an extent similar to that of Ylras1Δ Ylras2Δ cells (Fig. 3b).

We also examined the cell morphology and filamentous growth in Ylcdc25 mutant cells (Richard et al., 2001). Yl-Cdc25 (YALI0E12705) is a putative guanine nucleotide exchange factor (GEF) for YlRas proteins. We found that Ylcdc25 cells grew normally but they exhibited severe defects in cell morphology during yeast-form and hyphal growth (Fig. 3a). Ylcdc25 cells also failed to develop radial filaments in 5 days on serum agar medium (Fig. 3b). Because the phenotype of Ylcdc25 cells resembles that of Ylras1Δ Ylras2Δ Ylras3Δ cells, our data support the view that YlCdc25 may function as the GEF for the three YlRas proteins.

Together, our results demonstrate that among the three Ras proteins in Y. lipolytica, YlRas2 plays a major role in cellular morphogenesis during yeast-form growth and filamentous growth as well as in invasive growth. YlRas1 plays a minor role in filamentous growth. YlRas3, however, is largely dispensable for these processes. The activation of the three Ras proteins may involve YlCdc25.

**YIRAS overexpression and the expression of constitutively active Ylras2 mutants enhance filamentous growth**

As a complementary approach to the gene deletion study, we wanted to examine the effect of YIRAS overexpression
on filamentous growth in wild-type cells and Ylras2Δ cells. To this end, we overexpressed YIRAS1, YIRAS2, and YIRAS3 under the control of the strong constitutive YITEF1 promoter. We found that the overexpression of YIRAS2, but not YIRAS1 and YIRAS3, mildly enhanced filamentous growth in wild-type cells (Fig. 4a, top panel). YIRAS1 overexpression also partially suppressed the filamentous growth defect of Ylras2Δ cells. However, YIRAS3 overexpression had no effect (Fig. 4a, bottom panel). Later examination of the overall intensity of GFP fluorescence in cells expressing the GFP-YIRAS2 and GFP-YIRAS3 fusion constructs under the control of YITEF1 promoter suggests that the expression level of YIRAS3 may be markedly lower than that of YIRAS2 (data not shown). These findings suggest that Ylras1 may share overlapping functions with Ylras2. Both of them promote filamentous growth.

Constitutively active S. cerevisiae ras2G19V and C. albicans Caras1G13V mutants could enhance filamentous growth (Mösch et al., 1996; Feng et al., 1999). We then examined the effect of analogous mutation on Ylras2. We found that the expression of Ylras2G17V in wild-type cells under the control of its endogenous promoter on centromere-based plasmid enhanced filamentous growth, albeit with lower efficiency, whereas the expression of Ylras2Q66L, another constitutively active mutant, markedly enhanced filamentous growth (Fig. 4b, top panel). In the Ylcdc25 mutant strain that is defective in the activation of Ras proteins, the expression of both Ylras2G17V and Ylras2Q66L mutants significantly enhanced filamentous growth (Fig. 4b, bottom panel). These results demonstrate that, like other yeast Ras proteins, Ylras2's function in filamentous growth also requires the active GTP-bound form of Ylras2. The rapid cycling of Ylras2 between the GTP- and GDP-bound states seems not essential for the function of Ylras2 in the control of filamentous growth.

**YIRAS2 transcription is increased during dimorphic transition**

To explore whether the transcription of YIRAS genes might increase during yeast-to-hypha transition, we fused the c. 2000-bp promoter plus the first 6- to 9-bp ORF sequence of each YIRAS gene to lacZ and monitored the

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**Fig. 4. YIRAS overexpression and the expression of constitutively active Ylras2 mutants.** (a) YIRAS overexpression. Cells of strains PO1a and YLX81 (Ylras2A) carrying pYL13 (Vec), pYL13-YIRAS1 (YIRAS1↑), pYL13-YIRAS2 (YIRAS2↑), or pYL13-YIRAS3 (YIRAS3↑) were grown at 30 °C in liquid YNBD medium supplemented with uracil. DIC images were taken after 18 h. (b) The expression of constitutively active Ylras2 mutants. Cells of strains PO1a (WT) and YLXS8 (Ylcdc25) carrying pINA445 (Vec), pINA445-YIRAS2, pINA445-YIRAS2G17V, or pINA445-YIRAS2Q66L were grown at 30 °C in liquid YNBD and YNDC7 medium supplemented with uracil, respectively. DIC images were taken after 20 h. Scale bars, 5 μm.
expression of YlRAS-lacZ reporter constructs in cells grown under yeast- and hypha-inducing conditions. Cells of strain PO1a carrying the empty vector grown in liquid YNDC7 medium formed extensive long hyphae, whereas cells grown in YNBD medium formed a mixture of elongated yeast cells and pseudohyphae (Fig. 5a). We found that the expression levels of YIRAS1-lacZ, YIRAS2-lacZ, and YIRAS3-lacZ in cells grown in YNDC7 medium were all higher, but to variable extent, than those of cells grown in YNBD medium (Fig. 5b). The increase in expression of YIRAS1-lacZ and YIRAS3-lacZ were less than twofold. In comparison, the increase in YIRAS2-lacZ expression was about fourfold, the highest among these reporter constructs. This result suggests that the expression of YIRAS2 increases during yeast-to-hypha transition.

**Endomembrane-restricted YIRas2 could partially function in dimorphic transition**

Ras proteins were once thought to function exclusively on the plasma membrane. A growing body of evidence demonstrates that Ras proteins can also signal from the endomembranes including endoplasmic reticulum and Golgi for certain cellular functions (Hancock, 2003; Mor & Philips, 2006). For example, oncogenic H-RasG12V or H-RasG12V mutant that is restricted to the endomembranes by the substitution of serines for two cysteines preceding the CaaX motif can still transform cells, albeit with reduced efficiency (Hancock et al., 1989; Cheng et al., 2011). In the fission yeast S. pombe, the plasma membrane localization of Ras1 is essential for Ras1’s function in mating and sporulation but not for Ras1’s function in the maintenance of an elongated cell shape. Artificially restricting S. pombe Ras1 to the endomembranes could support Ras1’s role in cellular morphogenesis (Onken et al., 2006). In light of these observations, we wanted to examine whether YIRas2’s function in the regulation of dimorphic transition is also compartmentalized.

YIRas2 contains a cysteine residue (Cys 245) immediately upstream of the CaaX motif at its C-terminus (Fig. 6a). It is known that the cysteine residues of Ras preceding the CaaX motif are palmitoylated, whereas the cysteine residue within the CaaX motif is farnesylated in yeast as well as in metazoans (Hancock et al., 1989; Bhattacharya et al., 1995; Dong et al., 2003; Onken et al., 2006). Substitution of serine for the cysteine residue within the CaaX motif abolishes the farnesylation of Ras and renders Ras cytosolic, unable to target to the membranes. Substitution of serine for the cysteine residues preceding the CaaX motif, however, prevents Ras from getting palmitoylated, causing the accumulation of Ras on the endomembranes but not on the plasma membrane (Hancock et al., 1989, 1990; Bhattacharya et al., 1995; Dong et al., 2003; Onken et al., 2006). To examine whether YIRas2 could function on the endomembranes, we mutated cysteine 245 of YIRas2 to serine to prevent the palmitoylation of YIRas2 by site-directed DNA mutagenesis. We found that the YIRas2C245S mutant could support dimorphic transition because cells expressing YIRas2C245S as the sole source of YIRas2 under the control of its own promoter formed rough, bowl-shaped colonies on YNBD agar medium after 5 days at 30 °C, indicative of filamentous growth. A representative picture taken after 7 days was shown in Fig. 6b (top panel). The YIRas2C245S cells were elongated in morphology and could form pseudohyphae when grown in liquid YNBD medium (Fig. 6b, bottom panel). In comparison, cells carrying the empty vector were round in shape in liquid YNBD medium and formed smooth colonies on YNBD agar medium. However, the YIRas2C245S mutant appears to be less efficient than wild-type YIRas2 in supporting dimorphic transition because the colonies formed by cells expressing wild-type YIRas2 started to adopt a bowl shape after 3 days at 30 °C, while the colonies formed by cells expressing YIRas2C245S were still smooth and round at that time (data not shown). As expected, YIRas2C245S mainly
localized to the endomembranes but not to the plasma membrane as indicated by GFP-YlRas2C245S fusion protein, while wild-type YlRas2 localized mainly to the plasma membrane and also to the endomembranes (Fig. 6c). These results indicate that the plasma membrane localization of YlRas2 is important for its function in the control of dimorphic transition. However, endomembrane-restricted YlRas2 could also carry out its function in dimorphic transition, albeit with reduced efficiency.

We also mutated cysteine 246 of YlRas2, which is normally farnesylated and required for Ras’s membrane attachment, to serine. The analogous mutation in S. cerevisiae Ras2 and S. pombe Ras1 renders Ras cytosolic and completely nonfunctional (Bhattacharya et al., 1995; Onken et al., 2006). Like its mutated counterparts in S. cerevisiae and S. pombe, we found that GFP-YlRas2C246S localized primarily to the cytoplasm (Fig. 6c). Surprisingly, while the YlRas2C246S mutant was severely defective for its function in the control of dimorphic transition, it did not completely lose its function as the colonies formed by cells expressing YlRas2C246S as the sole source of YlRas2 developed ridges on the surface and had irregular edges after 5 days at 30 °C on YNBD agar medium, indicative of weak filamentous growth. A representative picture taken after 7 days is shown in Fig. 6b (top panel). In addition, YlRas2C246S cells were more elongated than the negative control when grown in liquid YNBD medium (Fig. 6b, bottom panel). This result suggests that the farnesylation-deficient YlRas2C246S mutant may still have some residual activity.

Together, our results reveal that cysteine 245 and cysteine 246 both contribute to YlRas2’s function in dimorphic transition. The plasma membrane localization of YlRas2 is important but does not seem to be absolutely required for the interaction of YlRas2 with its GEF and downstream effectors in the regulation of dimorphic transition. YlRas2 could partially signal from the endomembranes in dimorphic transition.
The transcription factor Mhy1 may function downstream of YlRas2 in the control of dimorphic transition

YlRas2 plays a major role in the control of dimorphic transition as Ylras2Δ cells exhibit a severe defect in filamentous growth. In an attempt to identify signaling transducers involved in YlRas2 signaling in dimorphic transition, we identified MHY1 as a gene whose overexpression could restore filamentous growth to Ylras2Δ cells. Ylras2Δ cells carrying an empty vector formed smooth colonies on YNBD agar medium. In contrast, Ylras2Δ cells overexpressing MHY1 formed fluffy colonies (Fig. 7a), indicative of filamentous growth. Consistently, Ylras2Δ cells overexpressing MHY1 formed abundant hyphae in liquid YNBD medium, while the vector control cells were still in the yeast form (Fig. 7b). MHY1 encodes a C2H2-type zinc finger transcription factor that promotes hyphal development. Similar to Ylras2Δ cells, mhy1Δ cells do not form either pseudohyphae or hyphae (Hurtado & Rachubinski, 1999). Interestingly, we found that YlRAS2 overexpression failed to induce filamentous growth in mhy1Δ cells (Fig. 7c), suggesting that Mhy1 is required for YlRas2 function. These results suggest that Mhy1 could be a novel potential signal transducer downstream of YlRas2 in the control of dimorphic transition.

Discussion

The three Y. lipolytica Ras proteins and their functions

Most yeast species in the subphylum Saccharomycotina where Y. lipolytica belongs contain only one RAS gene. Saccharomyces cerevisiae is an exception for having two RAS genes, which are thought to evolve from whole-genome duplication (Wolfe & Shields, 1997). The encoded Ras proteins in these yeast species share a conserved CCaaX motif at their C-termini and rely on the lipid modification of the two cysteine residues in this motif for plasma membrane targeting (Bhattacharya et al., 1995; Dong et al., 2003). They are thus similar to mammalian H-Ras and N-Ras, which also carry extra cysteine residues preceding the CaaX motif (Hancock et al., 1990). In this study, we show that the Y. lipolytica genome encodes three Ras proteins. Two of them, YlRas1 and YlRas2, have the CCaaX motif at their C-termini. This feature is similar to Ras proteins from other yeast species. The third one, YlRas3, however, carries no cysteines next to or preceding the CaaX motif. Instead, there is a polybasic region containing multiple lysines next to it (Fig. 1). This feature is very similar to human K-Ras4B, whose polybasic region contributes to the plasma membrane targeting of K-Ras4B via the electrostatic interaction with the phospholipids (Hancock et al., 1990). Remarkably, the majority of the higher fungal species including the basidiomycetous yeast C. neoformans, the plant pathogenic fungus U. maydis, and the filamentous fungus Neurospora crassa contain two Ras proteins (Waugh et al., 2002; Muller et al., 2003; Nichols et al., 2009). One resembles H-Ras and N-Ras, whereas the other one resembles K-Ras4B at their C-termini. Our gene deletion and gene overexpression studies indicate that the K-Ras4B-like Ras protein in Y. lipolytica, YlRas3, does not have a detectable function in dimorphic transition except under certain condition. It will be interesting to examine whether it may play a role in other cellular processes.

The expression of constitutively active S. cerevisiae Ras2G19V mutant in single copy could enhance filamentous growth in diploid wild-type cells (Mösch et al., 1996). We also find that the expression of YlRas2G19V and YlRas2Q66L mutant as the sole source of YlRas2 under the control of its own promoter enhanced hyphal development in wild-type cells. However, the effect of YlRas2G19V mutant appears to be less stronger than S. cerevisiae Ras2G19V.

![Fig. 7. Mhy1 may function downstream of YlRas2 in dimorphic transition.](image-url)
mutant. In *S. cerevisiae*, cells of laboratory strains in the Σ1278b genetic background could invade agar on rich media, whereas cells of strains from other backgrounds could not. The expression of constitutively active Ras2G12V mutant in strains of Σ1278b background also has only a minimal effect on invasiveness, whereas it induced strong invasive growth in SP1, a non-Σ1278b background laboratory strain that normally does not undergo invasive growth (Stanhill *et al.*, 1999). It is thought that the ability to undergo invasive growth for Σ1278b strains is due to an overactive Ras2 signaling pathway (Stanhill *et al.*, 1999).

The *Y. lipolytica* strain PO1a used in this study is a derivative of wild-type strain. Because strain PO1a shows stronger capability in dimorphic transition than other inbred lines (Richard *et al.*, 2001), we reasoned that Ras signaling in PO1a may also be somehow overactive compared with other inbred lines. This could explain the weak effect of YlrRas2G12V on filamentous growth in strain PO1a.

**How does YlrRas2 function in filamentous growth**

The mechanism by which Ras proteins control filamentous growth is best studied in *S. cerevisiae* and *C. albicans*. *Saccharomyces cerevisiae* Ras2 and *C. albicans* CaRas1 are known to promote filamentous growth via two signaling pathways, the Ste20-mitogen-activated protein kinase (MAPK) pathway and the cAMP-protein kinase A (PKA) pathway (Mösch *et al.*, 1996; Robertson & Fink, 1998; Leberer *et al.*, 2001). In *Y. lipolytica*, homologues of signaling components for both pathways are present. The MAPK pathway in *Y. lipolytica* appears to play a prominent, positive role in the control of filamentous growth based on the observation that cells lacking YlSte11, a MAPKK kinase, failed to form hyphae or pseudohyphae in YNBD medium (Cervantes-Chavez & Ruiz-Herrera, 2006). The cAMP-PKA pathway, however, does not promote but rather repress filamentous growth (Ruiz-Herrera & Sentandreu, 2002; Cervantes-Chavez *et al.*, 2009). Therefore, the MAPK and the cAMP-PKA pathways act antagonistically in *Y. lipolytica*. This feature is clearly different from those in *S. cerevisiae* and *C. albicans*.

*Saccharomyces cerevisiae* Ras2 is thought to activate the Ste11-Ste7-Kss1 MAP kinase cascade indirectly by activating Cdc42 first, which in turn activates Ste20, leading to the activation of Ste11 (Mösch *et al.*, 1996), the yeast MAPKKK homologue of mammalian Raf. This pattern of MAPK activation is different from what is known in the fission yeast *S. pombe* and in mammalian cells, in which Ras in the GTP-bound form directly binds Raf (called Byr2 in *S. pombe*), causing the activation of the Raf-MEK-ERK MAP kinase cascade (Moodie *et al.*, 1993; Masuda *et al.*, 1995). So far, it is not clear whether Ras proteins may control the MAPK signaling pathway in *Y. lipolytica*, and if they do, whether Ras proteins may directly bind Ste11 or indirectly activate it via Cdc42. In *S. cerevisiae* and *C. albicans*, Ras proteins also control the cAMP-PKA pathway by directly activating Cyr1, the adenylylate cyclase (Uno *et al.*, 1985; Fang & Wang, 2006).

At present, it is not known whether Ras proteins may control the CAMP-PKA pathway in *Y. lipolytica*. In this study, we identified that the transcription factor Mhy1 may function downstream of YlrRas2. This finding suggests the existence of a novel Ras signaling pathway in the regulation of dimorphic switch. At present, it is not known how Mhy1 is regulated by YlrRas2. Further investigations are needed to address this question.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Complementation of Ylras1Δ and Ylras2Δ mutants with corresponding genes.