A Role for the Rap GTPase YlRsr1 in Cellular Morphogenesis and the Involvement of YlRsr1 and the Ras GTPase YlRas2 in Bud Site Selection in the Dimorphic Yeast Yarrowia lipolytica

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Yarrowia lipolytica is a dimorphic yeast species that can grow in the ovoid yeast form or in the elongated pseudohyphal or hyphal form depending on the growth conditions. Here, we show that the Rap GTPase Rsr1 of Y. lipolytica (YlRsr1) plays an important role in cellular morphogenesis in this microorganism. Cells deleted for YlRSR1 exhibited impaired paired growth during yeast-form growth. Pseudohyphal and hyphal development were also abnormal. YlRsr1 is also important for cell growth, since the deletion of YlRSR1 in cells lacking the Ras GTPase YlRas2 caused lethality. Y. lipolytica cells bud in a bipolar pattern in which the cells produce the new buds at the two poles. YlRsr1 plays a prominent role in this bud site selection process. YlRsr1’s function in bud site selection absolutely requires the cycling of YlRsr1 between the GTP- and GDP-bound states but its function in cellular morphogenesis does not, suggesting that the two processes are differentially regulated. Interestingly, the Ras GTPase YlRas2 is also involved in the control of bud site selection, as Ylras2Δ cells were severely impaired in bipolar bud site selection. The GTP/GDP cycling and the plasma membrane localization of YlRas2 are important for YlRas2’s function in bud site selection. However, they are not essential for this process, suggesting that the mechanism by which YlRas2 acts is different from that of YlRsr1. Our results suggest that YlRsr1 is regulated by the GTPase-activating protein (GAP) YlBud2 and partially by YlCdc25, the potential guanine nucleotide exchange factor (GEF) for YlRas2.

The correct positioning of cell surface growth or a cell division plane along a defined polarity axis in response to internal or external cues is important for cellular morphogenesis and cell differentiation (1). In budding yeasts, the position of a new budding site, which also defines the future cell division plane, is often not randomly chosen. There are specific patterns for the ordered selection of a new budding site depending on cell type (2). For example, in diploid Saccharomyces cerevisiae strains, the newly formed daughter cells choose the distal pole (the old bud tip) as the new budding site, whereas the mother cells can choose either the distal pole or the proximal pole (the old bud neck) as the new site. This pattern of bud site selection is called the bipolar pattern, since the bud scars are located at the two poles of a cell (2). This type of spatial control on bud formation is thought to evolve for better cell survival in the wild, since wild S. cerevisiae strains are diploids and normally respond to a shortage of nitrogen source in the environment by switching to pseudohyphal growth. This switch is accompanied by a series of cellular changes, including the change of the budding pattern from bipolar to unipolar and delayed separation of daughter cells from the mother cells, leading to the formation of long filaments (3), which may help the cells spread and forage for nutrients.

S. cerevisiae Rsr1 is essential for proper bud site selection in diploid cells as well as in haploid cells, the latter of which bud in an axial pattern. Deletion of RSR1 (also called BUD1) randomized bud site selection in both cell types (4, 5). Rsr1 is a Rap family member of small GTP-binding proteins. Rap proteins are closely related to Ras, the archetype of small GTPases, and share an identical core effector domain, which is implicated in the interaction with downstream effectors (6), with Ras. Like Ras, Rsr1 functions as a molecular switch by cycling between the GTP- and GDP-bound states (7). The rapid GTP/GDP cycling of Rsr1 relies on Bud5 and Bud2. Bud5 is the guanine nucleotide exchange factor (GEF) for Rsr1 that promotes the binding of Rsr1 to GTP (8-10), whereas Bud2 is the GTPase-activating protein (GAP) for Rsr1 that stimulates the hydrolysis of Rsr1-bound GTP to GDP (11). Rsr1 in the GTP-bound form is known to bind Cdc42 (12, 13), a Rho GTPase, and Cdc24, the GEF for Cdc42 (14). Rsr1 in the GDP-bound form binds Bem1 (14), a scaffold protein that binds Cdc42 and Cdc24 and is important for Cdc42 activation and downstream signaling. Rsr1 is thought to function in bipolar bud site selection by linking Cdc42 to the spatial landmark in the beginning of the cell cycle, which in turn activates Cdc42, leading to bud formation at the chosen site (15). The landmark proteins are delivered by polarized secretion to the bud tip during and shortly after bud emergence (to mark the distal pole) and to the bud neck during cytokinesis (to mark the proximal pole).

The Rsr1 homologue in the pathogenic yeast Candida albicans, CaRsr1, also plays a key role in the control of an axial-like budding pattern (16, 17). However, unlike S. cerevisiae rsr1Δ cells, which did not exhibit defects in cell growth or cellular morphogenesis (4), C. albicans cells deleted for CaRSR1 were invisible at 42°C. The cells were large and round and formed small cell clusters during yeast-form (YF) growth at 30°C. Hyphal development was also...
defective (16, 17). In the filamentous yeast *Ashbya gossypii*, cells lacking AgRsl1 displayed an abnormal zig-zag shape and irregular thickening of hyphal segments (18). Rsl1 homologues in amoeba, fruit fly, and mammals also regulate cellular morphogenesis (19–22). For example, Rap1 controls the positioning and formation of E-cadherin-based cell-cell adhesion in mammalian epithelial cells (20, 21). This process bears some resemblance to the selection of a budding site and subsequent formation of a bud in yeast cells.

*Yarrowia lipolytica* is a dimorphic yeast species that grows in the yeast form in liquid yeast-peptone-dextrose (YPD) medium and forms pseudohyphae or hyphae in synthetic yeast nitrogen base and dextrose (YNBD) medium (23–25). This species buds in a bipolar pattern in both haploid and diploid cells (26). Here, we report that the Rsl1 homologue YlRsl1 plays an important role in cellular morphogenesis and in bud site selection. In addition, YlRsl1’s function in cellular morphogenesis does not absolutely require the cycling of YlRsl1 between the GTP- and GDP-bound states. We further show that the Ras GTPase YlRas2 is required for cellular morphogenesis and in bud site selection. These discoveries provided new insights into the functions of Rsl1 in cellular morphogenesis as well as the regulation of bud site selection in yeast.

**MATERIALS AND METHODS**

**Strains, media, and growth conditions.** *Y. lipolytica* strains used in this study are listed in Table S1 in the supplemental material. PO1a (MATa leu-270 ura-3-302) and Fil354 (PO1a except YlYdc25::mYn1-URA3) (27) were kindly provided by Claude Gaillardin and Mathias Richard (UMR INRA, Thiverval Grignon, France). *Escherichia coli* strains were routinely grown at 37°C in YPD medium (2% peptone, 1% yeast extract, and 2% glucose) or synthetic YNBD medium (0.67% yeast nitrogen base without amino acid, 2.94% trisodium citrate dehydrate, 1% glucose, pH 7.0) was used for *Yarrowia lipolytica*. *Yarrowia lipolytica* was grown at 30°C in YPD medium (2% peptone, 1% yeast extract, and 2% glucose) or synthetic YNBD medium (0.67% yeast nitrogen base without amino acid, 2.94% trisodium citrate dehydrate, 1% glucose, pH 7.0) was used for *Yarrowia lipolytica* growth. YNB medium (0.67% yeast nitrogen base without amino acid, 1% glycerol) was used to keep the cells in the ovoid yeast form when they carry plasmids. Synthetic media were supplemented with 80 μg/ml of leucine, 20 μg/ml of uracil, or both when required. For solid medium, 15 g/liter of agar was added to 2%.

**Plasmid and yeast strain construction.** Plasmids and oligonucleotides used in this study are listed in Tables S2 and S3, respectively, in the supplemental material. YlRsr1, YlBUD2, and YlYDC25 were deleted in *Y. lipolytica* strain PO1a by homologous recombination using a method identical to that for the deletion of YlTEC1 (see Fig. S1A) (25). Yeast transformants were examined by PCR to identify the clones that bear the correct replacement of each gene, yielding YLX260 (YlYdr1Δ::loxR/P, YLX229 (YlBud2Δ::loxR/P), and YLX258 (YlYdc25Δ::loxR/P) (see Fig. S1). Strain YLX81 (YlYdr2A) was constructed previously by the deletion of YlYdr2 using a two-step pop-in/pop-out method (28). The YlYRA3 marker strain Fil354 (PO1a except YlYdc25::mYn1-URA3) (27) was excised by Cre::loxP site-specific recombination, yielding YLX58 (YlYdc25Δ::loxP).

To generate pWU24-YlYRAS1 for the complementation of the YlYdr1Δ strain, the 1.90-kb YlYRAS1 gene containing a 966-bp promoter and 174-bp 3′-untranslated region (UTR) sequence was amplified from genomic DNA by PCR and ligated into BamH1- and Kpn1-digested pWU24 (integrative; YlURA3) (25). pWU24-YlYRAS2 and pWU24-YlBUD2 were constructed similarly. The 1.95-kb HindIII-Clal fragment of the YlYRAS2 gene carrying a 658-bp promoter and 193-bp 3′-UTR was inserted into pWU24, yielding pWU24-YlYRAS2. To introduce pWU24 and pWU24-based plasmids into yeast cells for the complementation test, plasmids were linearized by NotI at a unique site in the 3′ UTR of YlURA3 and integrated at the ara1-302 locus on the chromosome.

To generate pWU24-YlYRAS1G12V, pWU24-YlYRAS1K16N, and pWU24-YlYRAS1Q63L, the 2.69-kb BamH1-SalI fragment of the YlYRAS1 gene carrying a 966-bp promoter and 924-bp 3′-UTR sequence was amplified by PCR and inserted into pWU24. These YlYRAS1 and YlYRAS2 fragments were also amplified by PCR as BamH1-SalI fragments and inserted into pINA445 (CEN, YILEU2). YlYRAS1 amplified by PCR as a BamH1-SalI fragment was inserted into pINA443 (CEN, YlURA3), yielding pINA443-YlYRAS1. The YlG6L, C245S, and C246S mutations were introduced into YlYRAS2 by site-directed DNA mutagenesis, yielding pWU24-YlYRAS2G64L, pWU24-YlYRAS2C245S, and pWU24-YlYRAS2C246S.

To generate the YlYdr2Δ YlYdr1Δ strain covered by plasmid-borne YlYRAS2 or YlYRAS1, YlYRAS1 was deleted from YLX81 (YlYrs2A) carrying plasmid pINA445-YlYRAS2. The resulting strain, YLX383 (YlYdr2Δ::loxR::YlYRA3-loxP, pINA445-YlYRAS2), was confirmed to carry YlYRAS1 by PCR. Plasmid pUB4-CRE (CEN, hph, CRE) (29) then was transformed into YLX383 to excise the YlYRA3 marker, yielding strain YLX407 (PO1a except YlYdr2A YlYdr1Δ::loxR::P, pINA445-YlYRAS2). Lastly, plasmid pINA443-YlYRAS1 was transformed into YLX407 to replace pINA445-YlYRAS2, yielding YLX473 (PO1a except YlYdr2A YlYdr1Δ::loxR::P, pINA443-YlYRAS1). For the examination of the budding pattern of the YlYdr2Δ YlYdr1Δ strain, pWU24-YlYRAS2Q63L was linearized by NotI and transformed into strain YLX407. Plasmid pINA445-YlYRAS2 in this strain was lost, yielding YLX480 (YlYdr2A YlYdr1Δ, pWU24-YlYRAS1Q63L).

To generate pYL4 for the overexpression of YlYRAS1 and YlYRAS1 mutants, the 406-bp SacI-XbaI fragment of YlYRAS1 was amplified by PCR and inserted into pBlueScript KS (+). The Kpn1 fragment of the YlYRA3 gene then was inserted into this intermediate plasmid, yielding pYL4 (integrative, YlYRA3, pYYTREP). The open reading frames (ORFs) of YlYRAS1 and YlYRAS1 mutants plus the 3′-UTR sequence were amplified by PCR from appropriate templates and inserted into pYL4, yielding pYL4-YlYRAS1, pYL4-YlYRAS1K16N, and pYL4-YlYRAS1Q63L. The resulting plasmids were linearized and integrated on the chromosome in *Y. lipolytica*.

**Y. lipolytica transformation.** The lithium acetate method of *Saccharomyces cerevisiae* transformation (31) was used for *Yarrowia lipolytica* transformation, except cells were heat shocked at 37°C for 15 min before plating on selective medium.

**Microscopy.** An Olympus BX51 microscope (Tokyo, Japan) and a Retiga 2000R charge-coupled-device (CCD) camera (QImaging Corporation, Canada) were used to visualize cell morphology and GFP-tagged proteins by differential interference contrast (DIC) and fluorescence microscopy. The images were acquired using QCapture Suite (QImaging Corporation, Canada). The budding pattern of a yeast strain was examined by staining the bud scars with calcofluor white (Sigma-Aldrich). Only cells that carry three or more bud scars were scored for budding pattern. The budding pattern of a cell could be unipolar (Uni; bud scars were at the same pole), bipolar (Bi; bud scars were at the two poles), or random (R; bud scars were randomly distributed). For DNA staining, yeast cells were fixed by formaldehyde and stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) at 1 μg/ml. The length of yeast cells was measured on DIC or calcofluor white staining images using...
and examined Ylrs1Δ cells during YF and filamentous growth. Successful deletion of YLRS1 was confirmed by PCR analysis (see Fig. S1B in the supplemental material) and YLRS1 complementation (see below). Ylrs1Δ cells did not exhibit a detectable growth defect at 30°C but did show a morphological defect during YF and filamentous growth. When grown in liquid YPD medium (YF growth), most Ylrs1Δ cells (60%; n = 306) were bigger than wild-type cells, and 8% of cells became large and round (Fig. 2A), indicating a cell polarity defect. This phenotype is very similar to that of C. albicans cells lacking CaRsr1 (16, 17), suggesting that, like C. albicans CaRsr1, Ylrs1Δ is important for polarized growth in YF cells. Interestingly, we observed that 50% (n = 204) of Ylrs1Δ cells contained two or more nuclei, which was not found in wild-type cells (Fig. 2B) or reported for C. albicans cells lacking CaRsr1. This phenotype could result from a defect in polarized growth, a process required for the active transport of the nucleus into the daughter cell. Reintroduction of YLRS1 into Ylrs1Δ cells restored cell morphology and the number of nuclei back to normal (Fig. 2A and B, right). A fraction of C. albicans cells lacking CaRsr1 displayed multiple buds or short cell chains in YPD medium (16). This phenotype can also be seen in Ylrs1Δ cells. Twenty percent (n = 264) of Ylrs1Δ cells formed multiple buds or short cell chains that superficially resemble pseudohyphae (Fig. 2A). These results suggest that CaRsr1 and Ylrs1Δ are also required for efficient cell separation.

C. albicans CaRsr1 is important for hyphal development. Carsr1Δ/Carsr1Δ cells formed less hyphae under hypha-inducing conditions, and the hyphal cells were larger, wider, and more like pseudohyphae cells (16, 17). Thus, we examined Ylrs1Δ cells for their ability to form pseudohyphae and hyphae. Y. lipolytica cells adopted an elongated morphology and formed some pseudohyphae and a few hyphae when grown in YNBD medium (25). We found that Ylrs1Δ cells formed markedly more pseudohyphae (42%; n = 304) than wild-type cells did (6%; n = 292) in YNBD medium (Fig. 2C, middle). Under this growth condition, Ylrs1Δ cells became closer to wild-type cells in cell morphology. Nuclear division and segregation appeared to be normal, as the percentage of multinucleated cells was less than 1% (see Fig. S2 in the supplemental material). However, 16% (n = 202) of cells still were wider than wild-type cells. In addition, the mother cells in both single cells and pseudohyphae were less elongated than wild-type cells (Fig. 2C and D), indicative of insufficient apical growth. The differences in cell length were statistically significant (P < 0.001). These results suggest that Ylrs1Δ is important for normal pseudohyphal development.

When grown in hypha-inducing YNDC7 medium, Ylrs1Δ cells formed abundant filaments that looked more like pseudohyphae than hyphae because they contained constrictions (Fig. 2E). Chitin staining confirmed that Ylrs1Δ cells formed fewer hyphae (3%; n = 310) than wild-type cells did (30%; n = 284), but Ylrs1Δ cells formed more pseudohyphae (40%) than wild-type cells did (2%). This phenotype is very similar to that of C. albicans cells lacking CaRsr1 (17), suggesting that, like C. albicans CaRsr1, Ylrs1Δ is important for hyphal development. Ylrs1Δ seems not to be important for hyphal elongation, because the average length of mother cells in hyphae formed by Ylrs1Δ cells was 22.0 ± 7.7 μm (n = 30), which was close to that of wild-type cells (22.2 ± 7.1 μm; n = 100). Nuclear division and segregation were normal (see Fig. S2 in the supplemental material).

**RESULTS**

**YLrs1Δ is important for cellular morphogenesis during YF and filamentous growth.** The protein encoded by the ORF YAL10F23177 from the Y. lipolytica genome database (http://www.genolevures.org) shares the highest degree of amino acid sequence identity (62%) with S. cerevisiae Rsr1, a yeast Rap GTPase. YAL10F23177 is 254 amino acids (aa) in length and contains GTP-binding motifs (G1-G4 box) commonly found in small GTP-binding proteins, as well as a CaaX motif (a, aliphatic acid; X, any amino acid) at the C terminus (Fig. 1). It also shares a high degree of sequence identity with C. albicans Rsr1 (66%), human Rap1A, human H-Ras, and Ylras2. Identical and similar residues are highlighted in black and gray shades, respectively. The GTP/GDP-binding motifs (G1-G4 box), core effector domain, highly variable region (HVR), and CaaX motif are indicated by lines. The Gln (Q) residue at position 61 in Ras and position 63 in Rap sequences are marked by an asterisk.

**FIG 1** Sequence alignment of YLRs1 with Rap and Ras homologues. Aligned sequences include YLRs1, S. cerevisiae Rsr1, CaRsr1, human Rap1A, human H-Ras, and Ylras2. Identical and similar residues are highlighted in black and gray shades, respectively. The GTP/GDP-binding motifs (G1-G4 box), core effector domain, highly variable region (HVR), and CaaX motif are indicated by lines. The Gln (Q) residue at position 61 in Ras and position 63 in Rap GTPases are marked by an asterisk.

ImagePro Plus software (Glen Mills, PA). For counting the percentage of cells with abnormal morphology, budding pattern, or length of cells, at least 200 cells were counted unless otherwise noted.
Together, our results suggest that YLrsr1 is important for cellular morphogenesis during YF and filamentous growth.

The GTP/GDP cycling of YLrsr1 is not crucial for YLrsr1’s function in cellular morphogenesis during YF and filamentous growth. In *C. albicans*, the rapid cycling of CaRsr1 between the GTP- and GDP-bound states is critical for CaRsr1 function in regulating polarized growth during YF and hyphal growth (17). To determine whether the GTP/GDP cycling of YLrsr1 is important for YLrsr1’s function in cellular morphogenesis, we constructed YLrsr1<sup>Q63L</sup> and YLrsr1<sup>K16N</sup> mutants, analogous to the constitutively active GTP-bound human *S. cerevisiae* rsr1<sup>G12V</sup> and the constitutively inactive GDP-bound *S. cerevisiae* rsr1<sup>K38E</sup> mutants, respectively (7, 32), and expressed them in YLrsr1Δ cells in single copy numbers under the control of its own promoter. We observed that YLrsr1<sup>Q63L</sup>, but not YLrsr1<sup>K16N</sup>, largely rescued the morphological defect of YLrsr1Δ cells during YF and pseudohyphal growth. When grown in YPD medium, 90% (n = 234) of YLrsr1<sup>Q63L</sup> cells exhibited a normal cell size and shape. There were only 8% of cells that were bigger than wild-type cells and 2% of cells that were large and round. Cells with multiple buds or short cell chains were absent (Fig. 3A, left; also see Table S4 in the supplemental material). When grown in YNBD medium, YLrsr1<sup>Q63L</sup> cells no longer formed extensive pseudohyphae (Fig. 3A, right). Only 8% (n = 212) pseudohyphae was detected, which is close to the 6% (n = 292) pseudohyphae formed by wild-type cells. Moreover, the average length of mother cells in single cells was close to that of wild-type cells (see Table S4). In contrast, YLrsr1<sup>K16N</sup> cells exhibited the same defective morphology as YLrsr1Δ cells did under these growth conditions (see Table S4). We also generated an YLrsr1<sup>G12V</sup> mutant analogous to the constitutively active GTP-bound *S. cerevisiae* rsr1<sup>G12V</sup> mutant (7). YLrsr1<sup>G12V</sup> cells exhibited morphology similar to that of YLrsr1<sup>Q63L</sup> cells during YF and pseudohyphal growth (Fig. 3A; also see Table S4). These results suggest that YLrsr1 locked in the GTP-bound form is largely functional. Thus, unlike *C. albicans* CaRsr1, GTP/GDP cycling is not crucial for YLrsr1’s function in cellular morphogenesis.

In an alternative approach, we wanted to examine the morphology of cells deleted for the gene encoding the GAP for YLrsr1. In *S. cerevisiae*, Bud2 functions as the GAP for Rsr1 by accelerating the rate of GTP hydrolysis by Rsr1 (11). To look for the Bud2 homologue, we searched the *Y. lipolytica* genome database and identified YALI0D23903 as a protein that shares the highest amino acid sequence identity with *S. cerevisiae* Bud2 (24% identity) and with *C. albicans* CaBud2 (20% identity). YALI0D23903 is 1,139 aa in length and contains a conserved RasGAP domain (aa 568 to 912) (Fig. 3B). The RasGAP domain of YALI0D23903 shares 29% and 24% identity, the highest among *Y. lipolytica* proteins, with that of Bud2 and CaBud2, respectively. Therefore, we named it YlBud2. Bud2 and CaBud2 contain a C2 domain (24% identity). Although the analysis with the SMART program (http://smart.embl-heidelberg.de/) failed to identify a C2 domain in YlBud2, we found that the region of aa 463 to 564 of YlBud2 contained a C2 domain (Fig. 3B), which locates immediately upstream of the RasGAP domain, shares 21% and 18% of amino acid sequence identity with the C2 domains of Bud2 and CaBud2, respectively (Fig. 3C). This region of YlBud2 could be a C2 domain.

We deleted YlBUD2 by homologous recombination (see Fig. S1C).
in the supplemental material) and examined YlBud2Δ cells for phenotypes. In theory, cells lacking YlBud2, the GAP for YlRsr1, would accumulate GTP-bound YlRsr1. As a result, YlBud2Δ cells would morphologically resemble Ylrsr1Q63L cells. Indeed, we found that, like Ylrsr1Q63L cells, YlBud2Δ cells were relatively normal in cell morphology during YF growth, except that a small fraction (7%; n = 301) of cells were slightly bigger than wild-type cells (Fig. 3D, top; also see Table S4 in the supplemental material), which is clearly different from the defective morphology of Ylrsr1Δ cells. YlBud2Δ cells also underwent cell elongation and formed an amount of pseudohyphae similar to that of wild-type cells in YNBD medium (Fig. 3D, bottom; also see Table S4). Therefore, this result supports the view that the GTP/GDP cycling of YlRsr1 is not crucial for YlRsr1’s function in cellular morphogenesis.

YlRsr1 shares an essential function with YlRas2 in cell growth. Ras GTPases in yeast play important roles in cell growth and hyphal development. There are three Ras proteins in Y. lipolytica. However, only cells lacking YlRas2 were severely impaired in filamentous growth (27, 28). Because YlRsr1 and YlRas2 are closely related in amino acid sequence, we wanted to determine whether YlRsr1 and YlRas2 share overlapping functions. To this end, we tried to delete YlRSR1 in Ylras2Δ cells. We tried several times, but no correct double mutants could be obtained. Later, we obtained correct deletion of YlRSR1 in Ylras2Δ cells that carry the YlRAS2 gene on a LEU2-marked centromere-based plasmid. The LEU2-marked plasmid was later switched to a URA3-marked plasmid carrying YlRSR1. Interestingly, the URA3-marked cover plasmid in this strain could not be lost, as no colonies grew on YNBD medium containing 5-fluoroorotic acid (5-FOA), a drug that selectively kills cells carrying URA3 gene (represented by Fig. 4, Vec). This result suggests that cells lacking YlRsr1 and YlRas2 are inviable, and that YlRsr1 shares an essential function with YlRas2 in cell growth.

Surprisingly, the URA3-marked cover plasmid in Ylras2Δ Ylrsr1Δ cells could be lost when the Ylrsr1Q63L or Ylrsr1G12V, but not the Ylrsr1K66N, allele on a LEU2-marked plasmid was introduced into the double mutant strain (Fig. 4), indicating that the constitutively active Ylrsr1Q63L and Ylrsr1G12V mutants could function as the sole source of YlRsr1 in supporting cell growth when YlRas2 is absent. Therefore, the function of YlRsr1 in regu-
lating cell growth does not appear to require rapid GTP/GDP cycling of YlRsr1.

**YlRsr1 plays a prominent, but nonessential, role in bud site selection.** During YF growth in liquid YPD medium, cells of the haploid wild-type strain PO1a exhibited a bipolar pattern of bud site selection in more than 90% (n = 206) of cells (Fig. 5A and B). As Rsr1 plays a key role in bud site selection in *S. cerevisiae*, we wanted to determine if YlRsr1 also controls bud site selection in *Y. lipolytica*. To this end, we examined the budding pattern of Ylrsr1Δ cells grown in liquid YPD medium. Like *S. cerevisiae*, *Y. lipolytica* cells lacking YlRsr1 exhibited a defect in bipolar bud site selection, as the percentage of bipolar budding cells was reduced to 40% (n = 312) (Fig. 5A and B). Introduction of YlRsr1 into the Ylrsr1Δ strain complemented the defect (Fig. 5B). However, the bud site selection defect of Ylrsr1Δ cells (40% bipolar budding cells) was not as penetrant as that of *S. cerevisiae* rsr1Δ cells (less than 5% bipolar budding cells). Additionally, the percentage of bipolar budding Ylrsr1Δ cells could increase to 78% (n = 233) under the growth condition that favors pseudohyphal growth (YNBD medium) (Fig. 5C). Thus, the defect in bud site selection is only partly in Ylrsr1Δ cells, whereas it is more complete in *S. cerevisiae* rsr1Δ cells. Other regulators may exist in *Y. lipolytica* that function redundantly with YlRsr1 in bud site selection.

**YlRas2 is required for bud site selection.** The observation mentioned above implies that *Y. lipolytica* has an YlRsr1-independent pathway regulating bud site selection. YlRsr1 is a Rap GTPase. Given that Rap and Ras GTPases share an identical core effector domain, along with our observation that Ylrsr1Δ is synthetically lethal with Ylras2Δ, we suspected that YlRas2 provided us an opportunity to examine the budding pattern of Ylras2Δ Ylrsr1Δ cells. We found that less than 10% (n = 280) of Ylras2Δ Ylrsr1Δ cells budded in a bipolar pattern (Fig. 5B), lower than the ~20% bipolar budding population of cells carrying Ylras2Δ alone. This result suggests that YlRsr1 and YlRas2 share a function in the control of bud site selection. To our knowledge, this is the first demonstration of the involvement of a Ras GTPase in yeast bud site selection.

The GTP/GDP cycling of YlRsr1 is critical for YlRsr1’s function in bud site selection. The function of *S. cerevisiae* Rsr1 in bud site selection requires the rapid cycling of Rsr1 between the GTP- and GDP-bound states (7). To determine whether the GTP/GDP cycling of YlRsr1 is also critical for YlRsr1’s role in bud site selection, we examined the budding pattern of Ylrsr1Δ cells expressing the Ylrsr1Q63L or Ylrsr1K16N allele in single copy numbers under the control of its own promoter in YPD medium. We found that neither the Ylrsr1Q63L nor Ylrsr1K16N allele rescued the bipolar budding defect of Ylrsr1Δ cells (Table 1). In addition, overexpression of Ylrsr1Q63L or Ylrsr1K16N in wild-type cells under the control of the strong constitutive YEF1 promoter caused a...
A significant increase in random budding cells, whereas overexpression of YIR31 did not (Table 1). These results suggest that YIR31 functions similarly to S. cerevisiae Rsr1. They both absolutely require GTP/GDP cycling for their function in bud site selection.

We also examined the budding pattern of YLbud2Δ cells. Like YLR1Δ cells, YLbud2Δ cells exhibited a moderate, but not a complete, defect in bud site selection, as 33% (n = 275) of YLbud2Δ cells budded bipolar (Table 1). Reintroduction of YLbud2Δ into YLbud2Δ cells restored bipolar budding. Together with the findings for the YLR1Q63L mutant, our results suggest that, like S. cerevisiae Rsr1, the cycling of YIR2 between the GTP- and GDP-bound states is necessary for YIR2’s function in bud site selection.

The GTP/GDP cycling and plasma membrane localization of YIR2 are important for YIR2’s function in bud site selection. To determine whether YIR2 acts in bud site selection in a way similar to that of S. cerevisiae Rsr1, we first investigated whether the GTP/GDP cycling of YIR2 is critical for its function in bud site selection. To this end, we constructed a constitutively active YIR2Q66L mutant, analogous to the oncogenic H-RasQ61L mutant, and examined the budding pattern of YIR2Δ cells expressing YIR2Q66L under the control of its endogenous promoter. We found that YIR2Δ cells expressing YIR2Q66L displayed normal bipolar budding in 45% (n = 280) of cells, a much lower level than that of cells expressing wild-type YIR2 (95%; n = 201) (Table 1), suggesting that the GTP/GDP cycling of YIR2 is important for its function in bud site selection. However, unlike S. cerevisiae Rsr1 and YIR2, the constitutively active YIR2Q66L mutant did not exhibit a complete loss of function, as the percentage of bipolar budding cells was still higher than that of cells expressing the empty vector (21%, n = 243), suggesting that the constitutively active YIR2Q66L mutant has partial function. Remarkably, while the expression of YIR2Q66L in wild-type cells under the control of its endogenous promoter caused a marked reduction of bipolar budding cells, the expression of YIR2Q66L under the control of its own promoter did not impair bipolar budding (Table 1). This feature of YIR2 is clearly different from that of Y. lipolytica YIR2 and S. cerevisiae Rsr1.

We next investigated whether the plasma membrane localization of YIR2 is required for its function in bud site selection. S. cerevisiae Rsr1 normally localizes to the plasma membrane. The plasma membrane localization of Rsr1 is essential for its role in bud site selection, as Rsr1K260S, K260S and Rsr1G266S mutants, which mainly localize to the endomembranes and to the cytoplasm, respectively, but no longer to the plasma membrane, completely lost their function in bud site selection (34). Like Rsr1, S. cerevisiae Ras2 also localizes to the plasma membrane. This localization is known to be mediated by the palmitoylation of cysteine 318 and the farnesylation of cysteine 319 in the CaaX motif at the C terminus (35). The Ras2S318I mutant lacks palmitoylation and localizes primarily to the endomembranes, whereas the Ras2K318N mutant lacks both palmitoylation and farnesylation and localizes to the cytoplasm (35, 36). None of them localizes to the plasma membrane. Based on these findings, we constructed YRas2S318I and YRas2K318N mutants, analogous to the S. cerevisiae ras2S318I and ras2K318N mutants, respectively, in the CaaX motif at YRas2’s C terminus by site-directed mutagenesis. As expected, YRas2S318I localized to the endomembranes, whereas YRas2K318N localized to the cytoplasm in the cells as detected by GFP fusion proteins (28). When the budding patterns of YRas2Δ cells carrying these mutant alleles were examined, we found that the expression of the YRas2S318I mutant completely failed to rescue the bipolar budding defect of YRas2Δ cells during YF growth. The YRas2S318I mutant also did not completely complement the defect of YRas2Δ cells (Table 1), suggesting that the association of YRas2 with the plasma membrane is important for YRas2’s function in bud site selection. Interestingly, 57% (n = 262) of cells expressing YRas2S318I exhibited bipolar budding, whereas 21% of YRas2Δ cells expressed the empty vector (Table 1). This finding suggests that YRas2 can partially function on the endomembranes. This feature of YRas2 is not found in S. cerevisiae Rsr1.

### Table 1 Patterns of bud site selection in Y. lipolytica mutant cells

| Parent strain and derivative | Presence (%) of each budding cell type |
|-----------------------------|--------------------------------------|
|                             | Uni | Bi | R   |
| YLX260                      |     |    |     |
| YLR1Δ/Vec                   | 0   | 39 | 61  |
| YLR1Δ/YIR31                 | 1   | 96 | 3   |
| YLR1Δ/YIR31Q63L             | 2   | 36 | 62  |
| YLR1Δ/YIR31K16N             | 1   | 40 | 59  |
| PO1a                        |     |    |     |
| WT/Vec                      | 2   | 93 | 5   |
| WT/YIR31                    | 0   | 97 | 3   |
| WT/YIR31Q63L†               | 5   | 48 | 47  |
| WT/YIR31K16N†               | 28  | 42 | 30  |
| PO1a and YLY229†            |     |    |     |
| WT/Vec                      | 2   | 93 | 5   |
| YLbud2Δ/Vec                 | 3   | 33 | 64  |
| YLbud2Δ/IBUD2               | 3   | 92 | 5   |
| YLX81†                      |     |    |     |
| YLR2Δ/Vec                   | 10  | 21 | 69  |
| YLR2Δ/YIR31                 | 2   | 95 | 3   |
| YLR2Δ/YIR31Q63L             | 5   | 45 | 50  |
| YLR2Δ/YIR31K16N             | 12  | 57 | 31  |
| YLR2Δ/YIR31Q63L             | 13  | 20 | 67  |
| PO1a                        |     |    |     |
| WT/YIR32                  | 2   | 95 | 3   |
| WT/YIR32Q63L               | 2   | 91 | 7   |
| WT/YIR31                   | 0   | 97 | 3   |
| WT/YIR31Q63L               | 2   | 48 | 50  |

*Cells of each strain were grown in liquid YPD medium for 12 h at 30°C and stained for bud scars with calcofluor white. The percentage of uninuclear (Uni), bipolar (Bi), and random (R) budding cells was scored in at least 200 cells.

a Strain YLX260 (YLR1Δ) integrated with Not1-linearized plasmid pWU24 (YLR1Δ/ Vec), pWU24-YIR31 (YLR1Δ/YIR31), pWU24-YIR31Q63L (YLR1Δ/YIR31Q63L), or pWU24-YIR31K16N (YLR1Δ/YIR31K16N).

b Strain PO1a integrated with Sall-linearized plasmid pY4 (WT/Vec), pY4-YIR31 (WT/YIR31), pY4-YIR31Q63L (WT/YIR31Q63L), or pY4-YIR31K16N (WT/YIR31K16N).

c Strain PO1a integrated with Not1-linearized pWU24 (WT/Vec) and strain YLY229 (YLbud2Δ) integrated with linearized pWU24 (YLbud2Δ/Vec) or pWU24-IBUD2 (YLbud2Δ/IBUD2).

d Strain YLX81 (YLR1Δ2) integrated with Not1-linearized pWU24 (YLR1Δ2/Vec), pWU24-YIR32 (YLR1Δ2/YIR32), pWU24-YIR32Q63L (YLR1Δ2/YIR32Q63L), or pWU24-YIR32K16N (YLR1Δ2/YIR32K16N).

e Strain PO1a integrated with Not1-linearized pWU24-YIR32 (WT/YIR32), pWU24-YIR32Q63L (WT/YIR32Q63L), pWU24-YIR31 (WT/YIR31), or pWU24-YIR31Q63L (WT/YIR31Q63L).
Together, our results suggest that, like \textit{S. cerevisiae} Rsr1, the GTP/GDP cycling and the plasma membrane localization of YlRas2 are important for YlRas2’s function in bud site selection. However, some differences exist in the way by which the two small GTPases act.

\textbf{YlCdc25 may function as the GEF for YlRas2 and YlRsr1.} In \textit{S. cerevisiae}, Bud5 is thought to function as the GEF for Rsr1. Deletion of \textit{BUD5} randomized bud site selection (8). To look for a Bud5 homologue in \textit{Y. lipolytica}, we used the Bud5 sequence to perform a BLAST search of the \textit{Y. lipolytica} genome. Two proteins, YALI0E12705 (1,250 aa) and YALI0D22286 (1,189 aa), stood out as the proteins that share the two highest amino acid sequence identities with Bud5, 29% and 25%, respectively. Like Bud5, both proteins contain a REM and RasGEF domain at their C termini (Fig. 6A).

Because YALI0E12705 shares 29% sequence identity with Bud5 in the RasGEF domain, the highest level for \textit{Y. lipolytica} proteins, YALI0E12705 appears to be the \textit{Y. lipolytica} protein that is the most homologous to Bud5. For comparison, the known Bud5 homologue in \textit{C. albicans}, CaBud5 (Fig. 6A) (37), also shares a low 32% sequence identity with Bud5 in the RasGEF domain. YALI0E12705 was named YlCdc25 in an early study (27), and the \textit{Y. lipolytica} genome database has since kept this name for it. We found that, like \textit{Ylras2} cells and the cells lacking all three Ras proteins, cells of the \textit{Ylcdc25} mutant were rounder than wild-type cells (see Fig. S3 in the supplemental material). \textit{Ylcdc25} cells were also severely defective in bipolar budding, as only 10% (\(n = 327\)) of cells budded in the bipolar pattern. Eighty-seven percent of cells budded randomly (Fig. 6B). The extent of the bipolar budding defect is stronger than that of \textit{Ylrsr1} cells but close to that of \textit{Ylras2} and \textit{Ylras2} \textit{Ylrsr1} cells. Together with our observation that \textit{Ylcdc25} cells were severely defective in hyphal development to an extent similar to that of the cells lacking all three YlRas proteins (28), these results suggest that YlCdc25 functions as the major GEF for YlRas2. YlCdc25 may also function as the GEF for YlRsr1, because we observed that about 10% (\(n = 200\)) of \textit{Ylcdc25} cells showed marked enlargement in size compared to \textit{Ylras2} cells and the cells lacking all three Ras proteins (see Fig. S3). However, because the number of nuclei in \textit{Ylcdc25} cells was normal, which is different from \textit{Ylrsr1} cells, YlCdc25 may partially function on YlRsr1. The major GEF for YlRsr1 is not yet known.

Compared to YlCdc25, YALI0D22286 more closely resembles \textit{S. cerevisiae} Cdc25 because it shares 35% sequence identity with Cdc25, which is higher than the 26% identity between YlCdc25 and \textit{S. cerevisiae} Cdc25.
and Cdc25. When comparing only the RasGEF domain, the sequence identity of YALI0D22286 with Cdc25 is 45%, much higher than the 30% identity between YICdc25 and Cdc25 in that domain. Thus, we designated YALI0D22286 YlSdc25 and investigated whether YlSdc25 was the major GEF for YlRsr1. We deleted Y1SDC25 (see Fig. S1D in the supplemental material) and examined the budding pattern of YlSdc25Δ cells. We found that the deletion of Y1SDC25 did not impair bud site selection, as 90% (n = 306) of YlSdc25Δ cells budded bipolarly (Fig. 6B). In addition, no dramatic differences in cell morphology or hyphal growth could be observed between YlSdc25Δ cells and wild-type cells (data not shown). Because there is a possibility that the function of YlSdc25 could be masked by YICdc25, we also deleted Y1SDC25 in a YlCdc25 mutant strain. Cells of the YlCdc25 YlSdc25Δ strain were viable and did not display a growth defect (data not shown). The percentage of bipolar budding cells for the double mutant was 10% (n = 284), close to that of YlCdc25 cells. Based on these data, we cannot reach a conclusion on whether or not YlSdc25 functions as the GEF for YlRsr1.

In S. cerevisiae, apart from a general plasma membrane localization, Rsr1 was enriched on the bud cortex in small-budded cells and at the bud neck in large-budded cells during YF growth (34). Bud5 (the GEF for Rsr1) also localized to the bud cortex in small-budded cells and to the bud neck in medium- to large-budded cells (38, 39). In contrast, Ras2 was not enriched on the bud cortex (35, 36), and Cdc25 (the GEF for Ras1 and Ras2) localized mainly to the nucleus and also to the cytoplasm (40). We then examined the localization of YlRsr1, YlRas2, YlCdc25, and YlSdc25 by using the GFP-YlRsr1, GFP-YlRas2, GFP-YICdc25, and GFP-Y1SDC25 fusion constructs expressed from the YITEF1 promoter. Like S. cerevisiae Rsr1, GFP-YlRsr1 was enriched on the bud cortex in small-budded cells and at the bud neck in large-budded cells (Fig. 6C). A large fraction of unbudded cells also had one patch of GFP-YlRsr1, which is thought to be inherited from the previous cell cycle and serves as the spatial landmark for directing the positioning of the new budding site in the next cell cycle. In contrast to YlRsr1, GFP-YlRas2 localized to the plasma membrane uniformly and did not enrich as a patch in unbudded cells (Fig. 6C). Interestingly, GFP-YICdc25 localized to the bud tip as a patch in small-budded cells and also to the bud neck at a later cell cycle stage. A large fraction of unbudded cells also carried one or two patches of GFP-YICdc25 at the two poles. In contrast, GFP-YlSdc25 did not exhibit bud cortex localization in small-budded cells. Although GFP-YlSdc25 localizes to the bud neck in some medium- and large-budded cells (Fig. 6C, arrow), the bud neck localization was fainter and the number of cells displaying the bud neck localization was fewer. More importantly, GFP-YlSdc25 did not appear as a patch on the cell surface in unbudded cells. The similar localization patterns of YlRsr1 and YlCdc25 support the view that YlCdc25 functions as a GEF for YlRsr1. The localization of YlCdc25 as one or two patches in unbudded cells also suggests that YlRas2 could be locally activated in these patches. This may explain why YlRas2, which seems to be uniformly distributed on the plasma membrane, is implicated in bud site selection.

Cells grown in hypha-inducing VNDC7 medium showed GFP-YlRsr1 and GFP-YlRas2 localization on the entire plasma membrane and at the septum in hyphal cells. There was no obvious enrichment of these proteins at the hyphal tip (Fig. 6D). Interestingly, GFP-YlCdc25 showed a localization to the hyphal tip as well as to the septum (Fig. 6D). This pattern of localization is identical to that of C. albicans CaRas1 (37). This finding supports the view that YlCdc25 functions as the GEF for YlRsr1. It may activate YlRsr1 at the hyphal tip during hyphal growth. We did not observe a clear localization of GFP-YlSdc25 on the cell surface. The GFP-YlRsr1Δ, GFP-YlRAS2, and GFP-YlCdc25 fusion construct were functional, as they complemented the bipolar bud site selection and filamentous growth defects of Ylrsr1Δ, Ylras2Δ, and YlCdc25 cells, respectively (data not shown). We also examined the localization of C-terminally GFP-fused YlCdc25-GFP and YlSdc25-GFP under the control of their endogenous promoter. The localization pattern was essentially the same, except that the fluorescence signal was weaker (data not shown).

Taken together, our results support the view that YlCdc25 functions as the GEF for YlRas2. YlCdc25 may also partially function as the GEF for YlRsr1.

DISCUSSION

Roles of YlRsr1 and the GTP/GDP cycling of YlRsr1 in cellular morphogenesis and cell growth. Rap GTPases in yeast are known to regulate cellular morphogenesis and growth. In this study, we show that YlRsr1 in Y. lipolytica plays important roles in the proper selection of a budding site as well as in polarized growth during yeast-form growth and filamentous growth. How does YlRsr1 function in cellular morphogenesis? In S. cerevisiae, YlRsr1 in the GTP-bound form binds Cdc24 and Cdc42, whereas YlRsr1 in the GDP-bound form binds Bem1 (12–14). It is thought that YlRsr1’s function in bud site selection is mediated by the YlRsr1→Cdc24→Cdc42 signaling pathway (15). In mammalian epithelial cells, Rap1 is known to function upstream of Cdc42 in the formation of E-cadherin-based cell-cell adhesion (20). It seems likely that YlRsr1 also controls cellular morphogenesis via the conserved Rsr1→Cdc24→Cdc42 signaling pathway.

Interestingly, YlRsr1’s function in bud site selection absolutely requires the cycling of YlRsr1 between the GTP- and GDP-bound states, while YlRsr1’s functions in cellular morphogenesis and supporting growth do not. Why is there a difference? We speculate that the difference reflects YlRsr1’s functions at different cell cycle stages. Bud site selection is an early cell cycle event occurring prior to bud emergence. In S. cerevisiae, it is known that YlRsr1, Bud5 (the GEF for Rsr1), and Bud2 (the GAP for Rsr1) all localize to the presumptive budding site (39, 41). Thus, Rsr1, and possibly YlRsr1 as well, may cycle rapidly between the GTP- and GDP-bound states, and this process is critical for the recruitment of downstream effectors, such as Cdc24, to the budding site. After bud emergence, the GEF and GAP for Rsr1 become spatially separated. Bud2 stays at the bud neck, whereas Bud5 becomes enriched at the bud tip (39, 41). Rsr1 itself is also enriched on the bud cortex (34). In this study, we observed that YlCdc25, the potential GEF for YlRsr1, also localizes to the bud tip in small-budded cells, similar to S. cerevisiae Bud5. Thus, the pool of YlRsr1 at the bud tip may be kept active by YlCdc25 but may not be able to cycle rapidly between the GTP- and GDP-bound states because YlBud2 likely is absent. We hypothesize that this pool of active YlRsr1 at the bud tip in small-budded cells plays a role in the local activation of Cdc24, which in turn activates Cdc42, contributing to polarized growth. A similar scenario may occur in hyphal cells, because we observed that YlCdc25 localizes to the hyphal tip.

It has been shown that C. albicans CaRas1 is also involved in cellular morphogenesis, and CARS1Δ/CARS1Δ cells exhibited defective cell morphology resembling Ylrsr1Δ cells (16, 17). How-
ever, CaRsr1 behaves differently from YlRsr1 with respect to the requirement of cycling between the GTP- and GDP-bound states. CaRsr1 absolutely requires the GTP/GDP cycling for its function in cellular morphogenesis, as cells lacking CaRsr1 or CaBud2 exhibited identical defective cell morphology during YF and hyphal growth (17). It is not clear why YlRsr1 and CaRsr1 have different requirements for GTP/GDP cycling in the regulation of the same cellular process.

Based on the amino acid sequence homology to S. cerevisiae Bud2 and the phenotypes of Ylbud2A cells, the GAP that helps YlRsr1 hydrolyze GTP, which is important for the rapid cycling of YlRsr1 between the GTP- and GDP-bound states, likely is YlBud2 (YALI0D23903). The GEF for YlRsr1, which helps YlRsr1 load GTP, however, is less clear. Our findings suggest that YlCdc25 functions partially as the GEF for YlRsr1 based on the cell enlargement phenotype of YlCdc25 mutant cells and YlCdc25’s subcellular localization pattern being similar to that of S. cerevisiae Bud5. However, the major target of YlCdc25’s GEF activity appears to be YlRas2, since YlCdc25 cells phenotypically resemble Ylras2 mutant cells in cell morphology, dimorphic transition, and bud site selection. Future investigation is needed to test whether YlCdc25 could function as the GEF for both YlRas2 and YlRsr1.

Mechanism of YlRas2 function in bipolar bud site selection. In this study, we show that the Ras GTAPase YlRas2 is required for bipolar bud site selection in Y. lipolytica. However, YlRas2 appears to act differently from S. cerevisiae Ras1, a Rap GTAPase specialized in regulating bud site selection. First, the constitutively active YlRas2Q66L mutant is partially functional in bud site selection. Second, the endomembrane-restricted YlRas2C245S mutant could also partially function in this process. Third, unlike Ras1, YlRas2 is distributed uniformly on the plasma membrane. It is not enriched at the presumptive budding site or at the bud neck during a late cell cycle stage. How does YlRas2 function in bipolar bud site selection? We propose two models. In the first model, YlRas2 directly regulates bud site selection, similar to Ras1. This possibility is supported by the localization of YlCdc25, the potential GEF for YlRas2 (27). YlCdc25 localizes to the bud tip and the bud neck in a cell cycle-dependent manner similar to that of S. cerevisiae Bud5 (the GEF for Ras1) but not S. cerevisiae Cdc25 (the GEF for Ras1 and Ras2). We observed that a large fraction of un budded cells carry one or two patches of YlCdc25 at the two poles of a cell where the bipolar bud site selection machinery normally locates. This finding suggests that a pool of YlRas2 is activated and undergoes cycling between the GTP- and GDP-bound states at one of the two poles. If YlRas2 can interact with Cdc42 or Cdc24, it might cause a local activation of and GDP-bound states at one of the two poles. If YlRas2 can interact with Cdc42 or Cdc24, it might cause a local activation of

The second model also could explain our observation that the bipolar budding defect of Ylrsr1A cells becomes less pronounced under hypha-inducing growth conditions. Previous studies of S. cerevisiae have pointed out an effect of prolonged apical growth on the fidelity of bipolar bud site selection (47, 48). We speculate that the increased fidelity in bud site selection results from enhanced polarized growth caused by YlRas2-mediated pseudohyphal development. Prolonged apical growth during filamentous growth could cause the continuous deposition of spatial landmark proteins to the bud tip when the buds become bigger and stabilize the pool of these proteins at that site, leading to increased fidelity in bipolar bud site selection.

The two models are not mutually exclusive. One important question is whether YlRas2 could bind YlCdc24 or YlCdc42. This is not clear at present. It also remains to be tested whether YlCdc25 has GEF activity for YlRas2. The role of Ras GTAPase in bipolar bud site selection may not be limited to Y. lipolytica. In C. albicans, the deletion of CaRsr1 also did not completely eliminate bipolar budding, as ~30% of CarSR1Δ/Carsr1Δ cells still bud in a bipolar pattern (16). Further investigation will be needed to elucidate whether Ras GTAPase plays a general role in bud site selection in yeast.

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