The conserved Pkh–Ypk kinase cascade is required for endocytosis in yeast

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Internalization of activated signaling receptors by endocytosis is one way cells downregulate extracellular signals. Like many signaling receptors, the yeast α-factor pheromone receptor is downregulated by hyperphosphorylation, ubiquitination, and subsequent internalization and degradation in the lysosome-like vacuole. In a screen to detect proteins involved in ubiquitin-dependent receptor internalization, we identified the sphingoid base–regulated serine–threonine kinase Ypk1. Ypk1 is a homologue of the mammalian serum– and glucocorticoid-induced kinase, SGK, which can substitute for Ypk1 function in yeast. The kinase activity of Ypk1 is required for receptor endocytosis because mutations in two residues important for its catalytic activity cause a severe defect in α-factor internalization. Ypk1 is required for both receptor-mediated and fluid-phase endocytosis, and is not necessary for receptor phosphorylation or ubiquitination. Ypk1 itself is phosphorylated by Pkh kinases, homologues of mammalian PDK1. The threonine in Ypk1 that is phosphorylated by Pkh1 is required for efficient endocytosis, and pkh mutant cells are defective in α-factor internalization and fluid-phase endocytosis. These observations demonstrate that Ypk1 acts downstream of the Pkh kinases to control endocytosis by phosphorylating components of the endocytic machinery.

Introduction

The internalization of plasma membrane proteins into the endocytic pathway is a key regulatory event in signal transduction, nutrient uptake, and ion homeostasis. The activity and interaction of the network of proteins and lipids that comprise the endocytic machinery is carefully controlled in response to intra- and extracellular signals. This control is exerted by many types of regulatory proteins, including protein and lipid kinases, phosphatases, GTPases, and proteins that regulate actin dynamics. Lipid messengers, such as phosphoinositides, sphingolipids, and sterols, also play key roles in endocytosis (for review see D’Hondt et al., 2000). Sphingolipids and their precursors, sphingoid bases and ceramides, have recently emerged as important but poorly understood activators of endocytosis. Exogenous addition of Ceramide and sphingoid bases may be crucial for endocytosis because they activate regulatory phosphorylation cascades. Inactivation of protein phosphatase 2A, or overexpression of either the Pkc1 or Yck2 kinase, suppresses the endocytosis defects of an lcb1 mutant, suggesting that sphingoid base–stimulated kinase activity is important for receptor endocytosis (Friant et al., 2000). Endocytic proteins that are kinase substrates include clathrin (Wilde et al., 1999), amphiphysin (Bauerfeind et al., 1997), dynamin (Robinson et al., 1993), synaptojanin (McPherson et al., 1994), Eps15 (Fazioli et al., 1993), and epsin (Chen et al., 1999). The regulated phosphorylation of these proteins is likely to be critical for the assembly and disassembly of the network required for internalization (Slepnev et al., 1998).

Many of the proteins comprising the internalization machinery are conserved from yeast to mammals, and yeast has been exploited to identify novel proteins that participate in receptor internalization (for review see D’Hondt et al., 2000). Receptor-mediated endocytosis has been studied in Saccharomyces cerevisiae using Ste2, a G protein–coupled signaling receptor that is rapidly internalized in response to binding its ligand, α-factor (Jenness and Spatrick, 1986). The isolation of mutants defective in Ste2 internalization has
revealed a novel role for the sphingoid base–regulated Pkh and Ypk kinases in the internalization step of endocytosis.

Results and discussion

Ypk1 is required for endocytosis

Ubiquitination of the Ste2 cytoplasmic tail is required before internalization (Hicke and Riezman, 1996). We performed a screen of ethyl methanesulfonate–mutagenized cells to identify novel proteins involved in ubiquitin-dependent receptor internalization. One mutant, udi5–1 (ubiquitin-dependent internalization), was defective in α-factor internalization at both 24°C and 37°C (Fig. 1A), showed reduced growth on YPUAD + 2 mM EGTA. We screened a genomic DNA library for plasmids that rescued this growth defect and identified a plasmid carrying the YPK1 gene. A centromere-based plasmid carrying YPK1 restored the ability of udi5–1 both to grow on YPUAD + 2 mM EGTA (unpublished data) and to internalize α-factor (Fig. 1A). A ypk1Δ strain had an internalization defect similar to the udi5–1 strain (Fig. 1B), suggesting that the mutation in the udi5–1 strain was in YPK1. We rescued the ypk1 gene from udi5–1 cells, and found that it had a single point mutation in the coding region for the Ypk1 catalytic domain that changed glycine 490 to arginine. Expression of Ypk1G490R in ypk1Δ cells did not rescue internalization, whereas expression of Ypk1 did (unpublished data). These results demonstrate that UD15 is allelic to YPK1, and hereafter we refer to udi5–1 as ypk1G490R.

Ypk1 is a serine–threonine kinase involved in sphingolipid signaling (Sun et al., 2000). Ypk1 has a S. cerevisiae homologue, Ypk2 (68% identical), and a mammalian homologue, SGK (50% identical) (Casamayor et al., 1999) (Fig. 1C). The amino acid mutated in ypk1G490R cells, G490, is conserved in Ypk1 homologues. Unlike ypk1Δ cells, ypk2Δ cells exhibited no α-factor internalization defect (Fig. 1B). Ypk1 is important for endocytosis and cannot be replaced by Ypk2, despite their high degree of conservation, whereas Ypk2 plays either no role in endocytosis or a role that can be fully assumed by Ypk1. Ypk1 and Ypk2 perform at least one redundant, essential function because ypk1Δ ypk2Δ cells are dead, even though each single-null mutant is viable (Chen et al.,...
Pkh and Ypk kinases are required for endocytosis (deHart et al., 1993; Casamayor et al., 1999). ypk1<sup>G490R</sup> ypk2<sup>Δ</sup> cells are also dead, suggesting that the Ypk1<sup>G490R</sup> protein is completely inactive.

To investigate the role of Ypk1 in fluid-phase endocytosis, we assayed the ability of ypk1 cells to deliver Lucifer yellow (LY)* to the vacuole (Fig. 2, A and B). Both ypk1<sup>G490R</sup> and ypk1<sup>Δ</sup> cells were significantly impaired in LY transport compared with their congenic wild-type strains. Ypk1 was also required for internalization of receptors carrying the linear peptide internalization signal NPFXD (Tan et al., 1996), instead of a ubiquitin signal (unpublished data). Ypk1 is not generally required for membrane trafficking, because carboxypeptidase Y was transported through the biosynthetic pathway to the vacuole with normal kinetics in ypk1<sup>G490R</sup> cells incubated at the restrictive temperature (unpublished data). These observations indicate that Ypk1 is necessary for fluid-phase endocytosis and for the internalization of plasma membrane proteins carrying different types of internalization signals.

*Abbreviations used in this paper: DIC, differential interference contrast; HA, hemagglutinin; LY, Lucifer yellow.

The kinase activity of Ypk1 is required for endocytosis downstream of receptor phosphorylation and ubiquitination

To test whether the kinase activity of Ypk1 is required for its function in internalization, we made two mutant forms of epitope-tagged Ypk1 that abolish its kinase activity, Ypk1<sup>K376R</sup> and Ypk1<sup>D488A</sup> (Casamayor et al., 1999; Sun et al., 2000). We assayed cells expressing similar levels of the different Ypk proteins (Fig. 3 A) for their ability to internalize α-factor. Both ypk1<sup>K376R</sup> and ypk1<sup>D488A</sup> mutants showed internalization defects similar to that of ypk1<sup>Δ</sup> (Fig. 3 B), demonstrating that the kinase activity of Ypk1 is required for its role in endocytosis.

We used two approaches to test whether Ypk1 is involved in Ste2 phosphorylation, which is a prerequisite to receptor ubiquitination and internalization (Hicke et al., 1998). First, we assayed α-factor internalization by a Ste2-Ub chimeric protein that does not require posttranslational ubiquitination for endocytosis (Terrell et al., 1998; Dunn and Hicke, 2001). In ypk1<sup>G490R</sup> cells, internalization of Ste2-Ub was as defective as the internalization of a similar receptor carrying posttranslational ubiquitination sites but no fused ubiquitin (Ste2–378Stop) (Fig. 3 C), consistent with the
idea that Ypk1 functions after receptor ubiquitination. Second, we demonstrated that Ste2 was phosphorylated and ubiquitinated normally in ypkl\textsuperscript{G490R} and ypkl\textsuperscript{H9004} cells (Fig. 3D; unpublished data). The modified forms accumulated in ypkl\textsuperscript{G490R} cells when compared with wild-type cells, as observed in other endocytic mutants (Hicke and Riezman, 1996). These results indicate that the kinase activity of Ypk1 is required for receptor internalization, and that Ypk1 is not involved in ligand-stimulated receptor modifications.

\textbf{Pkh-dependent phosphorylation of Ypk1 is required for efficient internalization}

Ypk1 contains two conserved phosphorylation sites, T504 and T662 (Fig. 1C). T504 is phosphorylated by Pkh1, a yeast homologue of the phosphoinositide-dependent kinase, PDK1 (Casamayor et al., 1999; Inagaki et al., 1999). To determine if either T504 or T662 is involved in the endocytic function of Ypk1, we mutated these residues to alanine alone or in combination. We integrated the constructs into ypkl\textsuperscript{H9004} cells, identified transformants with equivalent expression levels of epitope-tagged Ypk1 variants (Fig. 4A), and performed \textalpha{}-factor internalization assays on these cells. Cells expressing Ypk1\textsuperscript{T662A} showed a slight defect in internalization, whereas the cells expressing Ypk1\textsuperscript{D488A} were more strongly impaired (Fig. 4B). These results indicate that the conserved phosphorylation sites of Ypk1 are required for efficient internalization. T504 appears to play a critical role in internalization, suggesting that phosphorylation by Pkh1 is important for endocytosis. The modest but significant internalization defect observed with cells expressing Ypk1\textsuperscript{T662A} suggests Ypk1 endocytic activity may also be regulated by phosphorylation at T662.

Due to the importance of T504, we investigated the role of Pkh1 in endocytosis. Pkh1 shares significant homology with two other yeast kinases, Pkh2 and Pkh3. Functions of these kinases are at least partially redundant because null mutations in individual \textit{PKH} genes are not lethal, whereas a \textit{pkh1} \textit{pkh2} mutant is dead or slow growing (Casamayor et al., 1999; Inagaki et al., 1999; see below). We tested the ability of \textit{pkh1} \textit{pkh2} and \textit{pkh3} cells to internalize \textalpha{}-factor and deliver LY to the vacuole. None of the single \textit{pkh} mutants showed a defect in either assay as compared with isogenic wild-type cells (unpublished data). We then created double mutants to examine if Pkh kinases function redundantly in internalization. It has been reported that cells carrying a deletion of both \textit{PKH1} and \textit{PKH2} are inviable (Casamayor et al., 1999; Inagaki et al., 1999). In our genetic background, \textit{pkh1} \textit{pkh2} cells were viable, but grew slowly at 24°C (Fig. 5A). We found that the
A conserved, sphingoid base-regulated kinase cascade in endocytosis

Sphingoid bases are required for the internalization step of endocytosis in yeast. A lcb1–100 strain defective in sphingolipid biosynthesis is defective in receptor and fluid-phase endocytosis, and the addition of sphingoid bases suppresses this defect (Munn and Riezman, 1994; Zanolari et al., 2000). In addition, our screen identified a lcb2 mutant strain with an α-factor internalization defect similar to that of lcb1–100 (unpublished data). Pkh and Ypk kinases are two members of a kinase cascade that function downstream of sphingoid bases. Ypk1 was isolated as a suppressor of growth inhibition by ISP-1, a toxic compound that acts by depleting intracellular sphingoid base levels. Furthermore, phosphorylation of Ypk1, which is required for its kinase activity, is dependent on the level of sphingoid bases in cells (Sun et al., 2000). ypk1 mutants are synthetically lethal with both lcb1 and lcb2 mutants (unpublished data), an observation that supports a connection between sphingoid base synthesis and Ypk activity. Unlike the Yck and Pkc kinases, overexpression of Ypk1 does not suppress the internalization defect of lcb1 or lcb2 (Friant et al., 2000; unpublished data). This may be because overexpression does not increase the level of active, phosphorylated Ypk1, or because sphingoid bases regulate other endocytic proteins whose activity is not increased in a Ypk1-overexpressing cell.

Pkh kinases are likely to be the link between sphingoid bases and Ypk1 activation. Pkh-dependent phosphorylation is required for Ypk activity and the overexpression of Pkh1, like Ypk1, suppresses growth inhibition by ISP-1 (Sun et al., 2000). PDK1, the functional mammalian homologue of Pkh1 (Casamayor et al., 1999), is activated by sphingoid bases (King et al., 2000). Like Pkh1, Ypk1 has a mammalian homologue and the Pkh–Ypk kinase cascade is conserved in mammalian cells. PDK1 phosphorylates SGK at T256, a position equivalent to T504 in Ypk1 (Fig. 1 A) (Park et al., 2000). Although a role for PDK or SGK in endocytosis has not been reported, another Ypk homologue, PKB/Akt, is required to activate a small GTPase, Rab5, that is involved in endocytic trafficking (Barbieri et al., 1998).

Sphingoid base–dependent phosphorylation may regulate Ypk1 by controlling its localization. Treatment of yeast cells with sphingoid bases appears to cause increased association of overexpressed GFP-Ypk1 with the plasma membrane (Sun et al., 2000); however, we did not see this effect using subcellular fractionation of Ypk1 expressed at normal levels. Cellular substrates of the Ypk kinases have not been reported. Friant et al. (2000) suggested that a target of sphingoid base regulation may be an actin-binding protein because the overexpression of sphingoid bases suppresses both the endocytic and the actin cytoskeleton defects in a lcb1 mutant. One of the many proteins that link actin to endocytosis in yeast may be regulated by Ypk1-dependent phosphorylation.

Regulation of the internalization step of endocytosis requires multiple kinases that receive input from different sources for efficient assembly of endocytic vesicles. We have shown that the Pkh–Ypk1 kinase cascade is an important regulatory component of the endocytic machinery.

Materials and methods

Reagents
All strains were grown in minimal (SD) or rich (YPUD) medium (Sherman, 1991) as indicated in the figure legends. Table I lists the strains used.
in this study. BY4741 strains containing deletions in YPK1, YPK2, PKH1, PKH2, and PKH3 were obtained from EUROSCARF. pkh1Δ pkh2Δ and related strains were a gift from Kunihiro Matsumoto (Nagoya University, Nagoya, Japan). bar1Δ derivatives were generated by single-step gene transplacement or by crossing to a bar1Δ strain. Ste2 antiserum and [35S]-α-factor were purified as previously described (Dulic et al., 1991). The gapped plasmid repair and [35S]-α-factor were determined by gapped plasmid repair (Dulic et al., 1991). Under these conditions, LHY1451 cells internalized 68 ± 8% of the surface-bound α-factor. Mutagenized cells that consistently internalized <40% of bound α-factor at 20 min were crossed to wild-type cells at least three times.

Cloning of YPK1
YPK1 was cloned from a centromeric genomic library made in the YCpKan101 vector, provided by Jon Binkley and David Botstein (Stanford University, Stanford, CA) by complementation of the growth defect of ypk1Δ cells on YPUAD + 2 mM EGTA at 37°C. The YPK1 gene was subcloned from this plasmid by ligating the 2.8-kb BstBI fragment into the ClaI site of YCp50, resulting in LHP1086. The mutation in ypk1Δ was determined by gapped plasmid repair (Roberts, 1991). The gapped YPK1 plasmid was purified and transformed into ypk1Δ cells. Repaired plasmids were purified from yeast cells and transformed into E. coli. Bacterial plasmid DNA was purified and sequenced. Two plasmids recovered from ypk1Δ cells were each found to have a single point mutation in codon 490.

Figure 5. Phk kinases are required for receptor-mediated and fluid-phase endocytosis. (A) Growth of pkh1Δ pkh2Δ cells and pkh1Δ pkh2Δ leu2::PKH1 cells from the same tetrad (LHY2716 and LHY2714, respectively) or pkh1Δ pkh2Δ (LHY3030) and an isogenic pkh2Δ strain (LHY3032) on YPUAD at 24°C or 30°C or on YPUAD + 1.2 M sorbitol at 30°C after 4 d. The differences in strain background between the two pkh mutants are likely to account for the difference in suppression of the growth defect on sorbitol medium. (B) The same pkh1Δ pkh2Δ (●) and pkh1Δ pkh2Δ leu2::PKH1 (○) strains as in (A) were grown overnight in YPUAD + 1.2 M sorbitol. Internalization of [35S]-α-factor was measured by the continuous presence protocol at 30°C in YPUAD + 1.2 M sorbitol. (C) Ly localization was assayed for wild-type cells (LHY2762), pkh1Δ cells (LHY2759), pkh2Δ cells (LHY2760), pkh1Δ pkh2Δ cells (LHY2716), and end4Δ cells (LHY37). Cells were grown to early logarithmic phase in YPUAD + 1.2 M sorbitol at 24°C, shifted to 30°C for 15 min, and then incubated with Ly at 30°C for 60 min. Images were taken using DIC optics (top) and fluorescence optics (bottom). The pkh1Δ pkh2Δ cells that are brightly stained throughout the whole cell are likely lysed cells. (D) pkh1Δ cells (LHY3031, ●), pkh2Δ cells (LHY3032, ○), and pkh1Δ pkh2Δ (LHY3030, □) were grown overnight in YPUAD. Internalization of [35S]-α-factor was measured by the continuous presence protocol at 30°C in YPUAD.

Screen for udi mutants
LHY1451, which expressed a variant α-factor receptor with only ubiquitin-independent internalization signals (Ste2–378Stop), was treated with 3% ethyl methanesulfonate for 90 min. Mutagenized cells were tested for their ability to internalize α-factor using an assay modified from the method of Dulic et al. (1991). Cells were grown in YPUAD to mid logarithmic phase, then incubated at 37°C for 15 min before the addition of [35S]-α-factor. 20 min after α-factor addition, aliquots of cells were placed in either pH1 or pH6 buffer and cell associated radioactivity in each sample was determined as described (Dulic et al., 1991). Under these conditions, LHY1451 cells internalized 68 ± 8% of the surface-bound α-factor. Mutagenized cells that consistently internalized <40% of bound α-factor at 20 min were crossed to wild-type cells at least three times.

Cloning of YPK1
YPK1 was cloned from a centromeric genomic library made in the YCpKan101 vector, provided by Jon Binkley and David Botstein (Stanford University, Stanford, CA) by complementation of the growth defect of ypk1Δ cells on YPUAD + 2 mM EGTA at 37°C. The YPK1 gene was subcloned from this plasmid by ligating the 2.8-kb BstBI fragment into the ClaI site of YCp50, resulting in LHP1086. The mutation in ypk1Δ was determined by gapped plasmid repair (Roberts, 1991). The gapped YPK1 plasmid was purified and transformed into ypk1Δ cells. Repaired plasmids were purified from yeast cells and transformed into E. coli. Bacterial plasmid DNA was purified and sequenced. Two plasmids recovered from ypk1Δ cells were each found to have a single point mutation in codon 490.
Internalization assays
All α-factor internalization assays were performed essentially as described (Dulic et al., 1991) using the continuous presence protocol. Conditions for growth are indicated in the figure legends. Cells were harvested in early logarithmic phase and shifted to the assay temperature for 15 min before the addition of 125 I-factor. Lysates were resolved by SDS-PAGE on 10% gels and transferred to nitrocellulose. Membranes were first probed with α-HA antibodies, then with HRP-conjugated goat α-mouse antibodies (Sigma-Aldrich). The blots were developed using SuperSignal ECL reagents (Pierce Chemical Co.). Lysates for Ste2 immunoblots were prepared as described by Hick and Riezman (1996), except cycloheximide treatment was not performed.

Cell lysates and immunoblots
Lysates for HA-Ypk1 immunoblots were prepared after growth in YPUAD at 24°C (Horvath and Riezman, 1994). Lysates were resolved by SDS-PAGE on 10% gels and transferred to nitrocellulose. Membranes were first probed with α-HA antibodies, then with HRP-conjugated goat α-mouse antibodies (Sigma-Aldrich). The blots were developed using SuperSignal ECL reagents (Pierce Chemical Co.). Lysates for Ste2 immunoblots were prepared as described by Hick and Riezman (1996), except cycloheximide treatment was not performed.

We gratefully acknowledge the gifts of the YCpKan101-based genomic library from Jon Binkley and David Botstein and the pkh+ and related pkh strains from Kunihiko Matsumoto. We thank Robert Lamb for generously providing HA antibodies and for the use of his confocal microscope. We thank Melissa Starkey for her assistance in this project. The manuscript was improved by the critical comments of Greg deHart and Marija Tesic.

This research was supported by the Burroughs Wellcome Fund, the Searle Scholar program, and the National Institutes of Health (R01 DK 53257).

Submitted: 31 July 2001
Revised: 27 November 2001
Accepted: 27 November 2001

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Table I. Strains used in this study

| Strain          | Genotypea                                      |
|-----------------|------------------------------------------------|
| LHY1            | his4 leu2 lys2 ura3 bar1-1                   |
| LHY10           | ste2α::LEU2 his3 leu2 trp1 ura3 bar1-1       |
| LHY37           | end4::LEU2 his4 leu2 lys2 ura3 bar1-1        |
| LHY291          | his3 leu2 lys2 trp1 ura3 bar1-1              |
| LHY558          | ste2α::LEU2 his3 leu2 trp1::STEG488-UBG488::URA3 bar1-1 |
| LHY825          | ste2α::LEU2 his3 leu2 trp1::STEG488-UBG488::URA3 bar1-1 |
| LHY1451         | ste2α::TRP1 GAL2 ade2 his3 leu2 lys2 trp1 ura3 bar1-1 | pGalSTE2-378Stop[URA3] |
| LHY2536         | ypk1Δ::KAN his3 leu2Δ met15A ura3 bar1-1    |
| LHY2537         | his3 leu2 trp1 ura3                           |
| LHY2543         | ypk1G490R GAL2 his3 leu2 lys2 trp1 ura3 bar1-1 |
| LHY2563         | ypk1Δ::KAN his3::YPK1::HIS3 leu2Δ met15A ura3 bar1-1 |
| LHY2564         | ypk1Δ::KAN his3::YPK1G490::HIS3 leu2Δ met15A ura3 bar1-1 |
| LHY2565         | ypk1Δ::KAN his3::YPK1T504A::HIS3 leu2Δ met15A ura3 bar1-1 |
| LHY2567         | ypk1Δ::KAN his3::YPK1T504A,T662A::HIS3 leu2Δ met15A ura3 bar1-1 |
| LHY2568         | ypk1Δ::KAN his3::YPK1T504A,TK376R::HIS3 leu2Δ met15A ura3 bar1-1 |
| LHY2569         | ypk1Δ::KAN his3::YPK1T504A,TK376R::HIS3 leu2Δ met15A ura3 bar1-1 |
| LHY2632         | his3Δ leu2Δ met15A ura3Δ bar1Δ::URA3        |
| LHY2633         | ypk2Δ::KAN his3Δ leu2Δ met15A ura3Δ bar1Δ::URA3 |
| LHY2684         | ypk1G490R ste2α::LEU2 GAL2 his3 leu2 lys2 trp1 ura3::STEG488-UBG488::URA3 bar1-1 |
| LHY2690         | ypk1G490R ste2α::LEU2 GAL2 his3 leu2 lys2 trp1 ura3::STEG488-UBG488::URA3 bar1-1 |
| LHY2712         | ypk1G490R GAL2 his3 leu2 lys2 trp1 ura3 bar1 pYPK1[URA3] |
| LHY2714         | pkh1Δ::KAN pkh2Δ::KAN his3Δ leu2::PKH1::LEU2 met15A ura3Δ bar1-1 |
| LHY2716         | pkh1Δ::KAN pkh2Δ::KAN his3Δ leu2 met15A ura3Δ bar1-1 |
| LHY2759         | pkh1Δ::KAN his3 leu2 trp1 ura3 bar1-1        |
| LHY2760         | pkh2Δ::KAN his3 leu2 trp1 ura3 bar1-1        |
| LHY2761         | MATa GAL2 his3 leu2 lys2 trp1 ura3 bar1-1    |
| LHY2762         | MATa leu2 his3 met15 ura3                   |
| LHY3030         | pkh1G488::HIS3 pkh2Δ::LEU2 ade1 his2 leu2 trp1 ura3 bar1Δ::URA3 |
| LHY3031         | pkh1Δ::LEU2 ade1 his2 leu2 trp1 ura3 bar1Δ::URA3 |
| LHY3032         | pkh2Δ::LEU2 ade1 his2 leu2 trp1 ura3 bar1Δ::URA3 |

aAll strains are MATa unless otherwise noted.
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