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The Tsc/Rheb signaling pathway controls basic amino acid uptake via the Cat1 permease in fission yeast

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Abstract

The Tsc/Rheb signaling pathway plays critical roles in the control of growth and cell cycle. Studies in fission yeast have also implicated its importance in the regulation of amino acid uptake. Disruption of *tsc2*⁺, one of the *tsc*⁺ genes, has been shown to result in decreased arginine uptake and resistance to canavanine. A similar effect is also seen with other basic amino acids. We have identified a permease responsible for the uptake of basic amino acids by genetic complementation and disruption. SPAC869.11 (termed Cat1 for cationic amino acid transporter) contains 12 predicted transmembrane domains and its overexpression in wild type fission yeast leads to the increased uptake of basic amino acids and sensitivity to canavanine. Disruption of *cat1*⁺ in the $\Delta tsc2$ background interfered with the suppression of the canavanine-resistant phenotype of $\Delta tsc2$ mutants by a dominant negative Rheb. In $\Delta tsc2$ mutant strains, the amount of Cat1 was not altered, but instead was mislocalized. This mislocalization was suppressed by the expression of dominant negative Rheb. In addition, we found that the loss of the E3 ubiquitin ligase, Pub1, also restores proper localization. These results provide a crucial link between Tsc/Rheb signaling and the regulation of the basic amino acid permease in fission yeast.

Keywords

Tsc; Rheb; Permease; Basic amino acids; Fission yeast; Intracellular localization

Introduction

Rheb G-protein belongs to a unique family of the Ras superfamily GTP-binding proteins (Aspuria and Tamanoi 2003; Aspuria et al. 2007). Rheb is conserved from yeast to human and possesses unique structural features including the presence of arginine at residue 15 that corresponds to glycine-12 in Ras. Rheb is downregulated by the complex of Tsc1 and Tsc2 proteins that acts as a GTPase activating protein (GAP) for Rheb. Mutations in the *TSC1* or *TSC2* gene result in tuberous sclerosis complex (TSC), a genetic disorder that is associated with the appearance of benign tumors at various sites in the body including the kidneys, lungs and brain (Gomez et al. 1999; Crino et al. 2006).

Studies in yeast have led to the idea that Tsc/Rheb signaling regulates uptake of basic amino acids such as arginine and lysine. Our initial observation was made using budding yeast (Urano et al. 2000). In this organism, disruption of the *RHB1* gene encoding the budding yeast Rheb

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led to the increased uptake of arginine and lysine and increased sensitivity to canavanine and thialysine, toxic analogues of arginine and lysine, respectively. Recent studies have shown that fission yeast is an ideal system to further investigate Tsc/Rheb/TOR signaling. Fission yeast has both *tsc1*⁺ and *tsc2*⁺ genes and, like mammalian cells, these gene products form a complex that act to downregulate Rheb (Matsumoto et al. 2002). Loss of Tsc function in fission yeast by disrupting *tsc1*⁺ or *tsc2*⁺ leads to decreased uptake of arginine and a dramatic increase in resistance to canavanine (van Slegtenhorst et al. 2004). The pool of arginine and lysine is decreased in the mutants. The canavanine-resistant phenotype of Δtsc mutants is reversed by the expression of dominant negative Rheb protein. Inhibition of Rheb function by the inhibition of protein farnesyltransferase leads to hypersensitivity to canavanine and increased uptake of arginine (Yang et al. 2001). On the other hand, hyperactivation of Rheb causes resistance to canavanine and thialysine (Urano et al. 2006). These results suggest that the Tsc/Rheb signaling pathway negatively regulates the uptake of arginine and, presumably, lysine.

To further understand the regulation of basic amino acid uptake by the Tsc/Rheb signaling pathway, we sought to identify the permease responsible for the uptake of basic amino acids in fission yeast. To accomplish this, we developed a two-step assay that utilized both budding yeast and fission yeast. We first took advantage of the availability of a budding yeast mutant defective in Can1, a permease for arginine (Grenson et al. 1966; Ahmad and Bussey 1986). Since this mutant is resistant to canavanine, we sought to identify a fission yeast gene whose expression in the $\Delta can1$ mutant restores canavanine sensitivity. The candidate permease genes were then disrupted in fission yeast. Finally overexpression in the wild type fission yeast was used to confirm the involvement of the putative permease in the uptake of basic amino acids. This led to the identification of SPAC869.11 as a fission yeast permease responsible for the uptake of basic amino acids (cationic amino acid transporter, Cat1). Characterization of Cat1 showed that its amount is unchanged in the $\Delta tsc2$ mutant strain; rather its intracellular localization is affected.

Materials and methods

Yeast strains, media and reagents

Budding yeast cells were grown in YPD or SD with the appropriate supplements (Sherman 1991). Fission yeast cells were grown in yeast extract complete medium with 225 $\mu\text{g}/\mu\text{l}$ adenine, leucine, histidine, uracil (YES) or Edinburgh minimal media (EMM) + 225 $\mu\text{g}/\mu\text{l}$ adenine at 30°C (Moreno et al. 1991). Transformations were performed using the lithium acetate method (Kanter-Smoler et al. 1994). Yeast strains used are listed in Table 1. The *tsc2*⁺, *spac869.11c*⁺, *spac359.03c*⁺, *sbpb2b2.01c*⁺ and *pub1*⁺ genes were disrupted by the PCR-based method using the kanamycin resistance cassette kanMX, URA cassette, and the hygromycin B resistance cassette hphMX (Bahler et al. 1998; Sato et al. 2005). The 3xHA-hphMX and GFP-hphMX cassettes were also integrated into the *spac869.11*⁺ C-terminus via the same PCR-based method. Canavanine and thialysine were purchased from Sigma and Research Organics, respectively. [³H]arginine (40 Ci/mmol), [³H]lysine (60 Ci/mmol), [³H]histidine (40 Ci/mmol), [³H]proline (20 Ci/mmol), and [³H]leucine (72.5 Ci/mmol) were obtained from American Radiolabeled Chemicals.

Constructs

p416a-ADH-fission yeast permeases were created by a PCR-cloning approach. The putative *Schizosaccharomyces pombe* permeases were amplified from a genomic DNA prep from the wild-type strain FY972 using primers with *Xho*I and *Xba*I restriction sites and cloned into the p416a-ADH expression vector. pREP41-*c869.11*⁺-Myc was also created by a PCR-cloning approach by using primers with *Sal*I and *Bam*HI restriction sites and cloned into the thiamine repressible pREP41 expression vector.

Amino acid uptake assays

Amino acid uptake assays were performed essentially as previously described with some minor modifications (Urano et al. 2000). Cells were grown to mid log phase (OD_{600} 0.4–0.8) in EMM with appropriate supplements, collected, and washed with dH_2O . Cells were resuspended in 1.2 ml of media without amino acids to an OD_{600} 2.0. 4.9 μ l of [3H]amino acid and 50 μ M cold amino acid were then added. Two hundred-microliter aliquots were taken at the indicated time points, injected into 5 ml of dH_2O , and immediately filtered and washed twice in a vacuum manifold. Filtration was performed on glass fiber filter circles (Fisher Scientific). Filters were dried under a heat lamp and counted in a Beckman LS-6500 scintillation counter using Econosafe scintillation solution (Resource Product International).

Northern blot analysis

Ten micrograms of total RNA was run on a 4% formaldehyde gel at 100 V for 1 h and transferred to a nylon membrane for 3 h in $10\times$ SSC. Probes for *spac869.11c⁺* and *tub1⁺* were PCR-amplified from cDNA, cleaned via a Quick Spin column (Roche Diagnostics) and labeled with [$\alpha^{32}P$] dATP (American Radiolabeled Chemicals) using standard methods. Hybridizations were performed in Quickhyb buffer (Stratagene).

Western blot analysis

Whole cell extracts were made essentially as described previously with minor modifications (Umebayashi and Nakano 2003). Fifty milliliters of cells were grown to an OD_{600} 1.0–2.0, collected, and washed with dH_2O . The cells were resuspended in 250 μ l of lysis buffer (20 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1.6% SDS, 6 M urea) containing a protease inhibitor mixture [1 mM PMSF and $1\times$ protease inhibitor cocktail (Roche diagnostics)]. Cells were lysed with glass beads and large cell debris and unbroken cells were removed by centrifugation for 10 min at 3,000 rpm. Protein concentration was determined using the Bradford method (Bio-Rad protein assay). Fifty micrograms of the sample was subjected to SDS-PAGE and immunoblotting with anti-HA antibody (HA11 from BabCO) or anti-PCNA antibody (Ab1 from Oncogene).

Fluorescence microscopy

For Cat1-GFP localization, cells were grown to midlog phase in EMM medium. The cells were then spotted directly onto poly-L-lysine slides and visualized using a Zeiss Microscope. Images were captured using AxioVision software.

Results

The $\Delta tsc2$ mutant cells have a defect in basic amino acid uptake, which can be suppressed by the expression of dominant negative RhebD60K.

The Tsc/Rheb signaling pathway has been shown to regulate arginine uptake (Yang et al. 2001; van Slegtenhorst et al. 2004; Urano et al. 2006). To determine whether this is applied to all basic amino acids, we examined the uptake of lysine, arginine and histidine in $\Delta tsc2$ mutant cells. In these mutant cells, Rheb is activated because Tsc2 of the Tsc1/Tsc2 complex that acts as a GAP is missing. As can be seen, there is a three- to fivefold decrease in the uptake of basic amino acids in $\Delta tsc2$ mutant cells compared with the control cells that have *tsc2⁺* expressed in the mutant (Fig. 1a). This point was further confirmed by the use of canavanine and thialysine, as $\Delta tsc2$ mutant cells were resistant to these toxic amino acid analogues (Fig. 8).

The decreased uptake of basic amino acids in $\Delta tsc2$ mutant cells was reversed by the expression of a dominant negative Rheb. This is shown in Fig. 1c, where we examine the uptake of lysine, arginine and histidine in $\Delta tsc2$ mutant cells expressing a dominant negative Rheb mutant,

RhebD60K (rhb1D60K⁺ construct) (Tabancay et al. 2003). As can be seen, expression of dominant negative Rheb causes an increase in the uptake of these basic amino acids. In addition, expression of dominant negative Rheb restores sensitivity of $\Delta tsc2$ mutant cells to canavanine (Fig. 8).

Identification of SPAC869.11 as a cationic amino acid permease in fission yeast

In order to understand how Rheb signaling controls the uptake of basic amino acids, we sought to identify the cationic amino acid permease in fission yeast. We first examined putative permeases with sequence similarity to *Saccharomyces cerevisiae* Can1p according to the BLAST search of the Sanger Center *Schizosaccharomyces pombe* database. Twelve amino acid permeases were identified by this analysis and the result of this analysis is shown in Fig. 2. As can be seen, the overall percent identity among these putative permeases is rather low. Based upon homology, it is possible to come up with a dendrogram for these putative permeases. However, it is difficult to choose a particular permease that is highly similar to Can1p from this analysis.

We decided to experimentally identify the fission yeast permease responsible for the uptake of basic amino acids using a two-step assay described in Fig. 3. In this approach, we first take advantage of the expected functional similarity between the fission yeast permease and the budding yeast Can1p. Disruption of the budding yeast *CAN1* gene leads to a defect in arginine uptake, highlighted by a resistance to the toxic arginine analogue, canavanine. Therefore, we initially screened putative *Schizosaccharomyces pombe* amino acid permease ORFs for their ability to convert resistance of the $\Delta can1$ mutant to a canavanine-sensitive phenotype. In the second step, these candidates were disrupted in *Schizosaccharomyces pombe* and it was examined whether the disruption caused canavanine resistance. To overexpress these fission yeast ORFs in *Saccharomyces cerevisiae* $\Delta can1$ strain and assay for restoration of canavanine sensitivity, the ORFs were placed under the control of the ADH promoter. As summarized in Fig. 4, expression of *spb2b2b2.01*⁺, *spbc359.03*⁺ or *spac869.11*⁺ restored canavanine sensitivity to the $\Delta can1$ mutant. To further characterize the three *Schizosaccharomyces pombe* basic amino acid permease candidates, we disrupted each of them by the kanMX or URA4 cassette in the fission yeast strain SP812. These strains were then assayed for their resistance to canavanine. Disruption of *spac869.11*⁺ was able to confer resistance to canavanine, whereas the disruption of *spb2b2b2.01*⁺ and *spbc359.03*⁺ did not (Fig. 5a). Similar results were obtained with thialysine, when *spac869.11*⁺ was disrupted (unpublished observation). This was also furthered by the analysis of double-disrupted strains, wherein canavanine resistance was observed when *spac869.11*⁺ was disrupted.

Because canavanine resistance is likely due to a defect in arginine uptake, the wild-type strain as well as the $\Delta tsc2$, $\Delta spbc359.03$, and $\Delta spac869.11$ strains were assayed for their ability to take up [³H]arginine. Cells with *spac869.11*⁺ disrupted, including the double mutant, exhibited a defect in arginine uptake, similar to the $\Delta tsc2$ mutants compared to wild type (Fig. 5b). On the other hand, loss of *spbc359.03*⁺ had no effect. Disruption of *spac869.11*⁺ also had a major effect on lysine uptake (Fig. 5c). As shown in Fig. 5c, $\Delta spac869.11$ cells failed to take up [³H]lysine and the level of decreased uptake was similar to that seen with the *tsc2*⁺ disruption strain. These uptake defects appear to be specific for arginine and lysine, as the uptake of non-basic amino acids such as tyrosine, proline, and leucine was not decreased and instead slightly increased (data not shown). From these results, we conclude that the *spac869.11*⁺ gene encodes the major permease specifically involved in arginine and lysine uptake in fission yeast.

Features of SPAC869.11

spac869.11⁺ encodes a protein of 580 amino acids, which contains the PROSITE amino acid permeases signature. While the amino acid sequences of SPAC869.11 and *Saccharomyces*

cerevisiae Can1 are only 14.8% identical, we found that they are structurally similar. Using a prediction server of transmembrane topology, ConPredII (Arai et al. 2004), we deduced the predicted transmembrane architectures of SPAC869.11 and *Saccharomyces cerevisiae* Can1. As can be seen in Fig. 6, the structures of these permeases are predicted to be very similar. SPAC869.11 has 12 predicted transmembrane domains with an intracellular 87 amino acid N-terminal tail and 47 amino acid C-terminal tail. This overall structure is strikingly similar to that of the *Saccharomyces cerevisiae* Can1. Interestingly, both transporters have a conserved glutamate residue in the periplasmic loop 7, which has been speculated to be involved in cationic substrate recognition (Regenberg and Kielland-Brandt 2001).

Overexpression of *spac869.11*⁺ leads to an increase in the uptake of basic amino acids

We overexpressed *spac869.11*⁺ in the wild-type strain SP812 and measured the rate of uptake of arginine, lysine and histidine. Overexpressing *spac869.11*⁺ using the pREP41 or pREP81 promoter was sufficient to cause a marked increase in the amount of arginine, lysine and histidine uptake (Fig. 7a). The increase in arginine uptake occurred in a dose-dependent manner, as we observed higher uptake when using a stronger promoter (data not shown). In contrast, the overexpression of *spac869.11*⁺ did not alter the uptake of proline, thus implying specificity for basic amino acids. The overexpression of *spac869.11*⁺ also led to the increase in sensitivity to canavanine (Fig. 7b). As can be seen, the strain overexpressing *spac869.11*⁺ grew significantly less on a plate containing canavanine, compared to the control strain carrying the vector. This, along with the disruption data, suggested that *spac869.11*⁺ is a major *Schizosaccharomyces pombe* arginine transporter, which we have designated as *cat1*⁺ (cationic amino acid transporter).

Cat1 is required for RhebD60K suppression of $\Delta tsc2$ mutants' resistance to canavanine

With the identification of a permease responsible for the uptake of basic amino acids, we returned our attention to the situation in $\Delta tsc2$ mutant cells. Based upon the results in Fig. 1d, we asked whether *cat1*⁺ is required for the ability of dominant negative Rheb to restore canavanine sensitivity in $\Delta tsc2$ mutant cells. As shown in Fig. 8, the canavanine resistance of $\Delta tsc2$ mutants is reversed by the expression of *rhb1D60K*⁺ to a level comparable to that seen with the $\Delta tsc2$ mutant cells expressing *tsc2*⁺ on a plasmid. In contrast, when the *cat1*⁺ gene is disrupted, *rhb1D60K*⁺ expression did not lead to the restoration of canavanine sensitivity in $\Delta tsc2$ mutant cells. Therefore, these results are consistent with the idea that Cat1 functions downstream of Rheb to regulate the uptake of arginine.

Cat1 expression is unchanged but its localization is altered in $\Delta tsc2$ mutant cells

The above results suggest that there is a decrease in the function of Cat1 in $\Delta tsc2$ mutant cells. To further characterize this point, we first examined expression of the *cat1*⁺ gene by performing Northern analysis of wild type and $\Delta tsc2$ mutant cell lysates using a full-length *cat1*⁺ probe. *tub1*⁺ was used as a loading control. A single band of 1.7 kb was detected and the intensity of the bands between wild type and the $\Delta tsc2$ mutant was similar (Fig. 9a). Thus, *cat1*⁺ expression is unaffected by the disruption of the *tsc2*⁺ gene. We then performed Western analysis to examine Cat1 protein level. A 3xHA epitope tag was chromosomally integrated in the C-terminus of *cat1*⁺. Anti-HA antibody was used to examine the level of Cat1 protein. On a gel, a band was observed at roughly 60 kD, the expected size of Cat1. As shown in Fig. 9b, total amount of the permease protein is not altered in $\Delta tsc2$ mutant cells.

Since protein levels are not affected, we examined if Cat1 was altered in its intracellular localization in $\Delta tsc2$ mutant cells. This was examined using strains with a GFP tag chromosomally integrated into the C-terminus of *cat1*⁺. Cat1-GFP is functional as assessed by the strain's sensitivity to canavanine (data not shown). In wild-type cells, Cat1-GFP was predominantly found on the periphery of cell tips with minor internal staining (Fig. 9c). This

is similar to the plasma membrane ABC transporter, Pmd1 (Iwaki et al. 2006). In $\Delta tsc2$ mutant cells, however, there was a more dispersed and mostly cytoplasmic fluorescence of the Cat1 protein. This punctate staining is similar to that of the golgi protein Ynd1 (Matsuyama et al. 2006). Therefore, Cat1 appears to be mislocalized in the $\Delta tsc2$ mutant. Since RhebD60K could revert the $\Delta tsc2$ mutant's defect in arginine uptake (Fig. 8), we assessed whether Cat1 localization was being restored. Indeed, the expression of RhebD60K could restore proper Cat1-GFP localization to the cell tips in the $\Delta tsc2$ mutant (Fig. 9c).

We also found that loss of the E3 ubiquitin ligase Pub1 restores proper localization of Cat1 in the $\Delta tsc2$ mutant. Cat1-GFP localization was examined in the $\Delta pub1 \Delta tsc2$ double mutant and was found properly localized to the periphery of cell tips as well as mild intracellular punctate staining (Fig. 9c). Based on these results, it appears that Pub1 is required for the control of Tsc2 in Cat1-GFP localization.

Discussion

In this paper, we report the identification of a fission yeast permease for cationic amino acids. This permease, Cat1, is capable of replacing the function of the arginine permease, Can1p, in *Saccharomyces cerevisiae*. Disruption of *cat1*⁺ in fission yeast causes dramatic decrease in the uptake of arginine or lysine and resistance to canavanine and thialysine. Overexpression of *cat1*⁺ in fission yeast results in the significant increase in the uptake of arginine, lysine and histidine. These results provide convincing evidence for the assignment of *spac869.11*⁺ as a major permease responsible for the uptake of basic amino acids in fission yeast.

The predicted overall structure of Cat1 is very similar to that of budding yeast Can1p. Both N-terminal and C-terminal sequences are predicted to be located inside the cell and a crucial residue speculated to be involved in the recognition of cationic amino acids is present in the same periplasmic loop (Regenberg and Kielland-Brandt 2001). Thus, the basic mechanism of the function of these permeases may be similar between the fission yeast and budding yeast proteins. On the other hand, there are intriguing differences between the two permeases. In budding yeast, separate permeases are used for the uptake of arginine, lysine and histidine (Can1p, Lyp1p and Hip1p for arginine, lysine and histidine, respectively). In contrast, our study suggests that a single permease can take up all three basic amino acids in fission yeast. In support of this idea, the uptake of lysine into fission yeast cells is competed by L-arginine, L-histidine and D-lysine (Sychrova et al. 1989).

Cat1 appears to be the major route for the uptake of arginine in the presence of ammonium. Interestingly, preliminary data suggest that when ammonium is depleted, $\Delta tsc2$ cells as well as $\Delta cat1$ are no longer canavanine resistant (unpublished observation). These results may suggest that another permease that can take up arginine can function in the absence of ammonium. It has been speculated that there are two systems involved in the uptake of arginine (Fantes and Creanor 1984). System I is the major route for the uptake of arginine, while system II is inhibited in the presence of ammonium in the medium. In support of this idea, the double-disrupted $\Delta cat1 \Delta spbc359.03$ strain is still canavanine-resistant under ammonium depletion (unpublished observation). This may suggest that Cat1 and SPBC359.03 are components of system I and system II, respectively. This idea is consistent with our observation that SPBC359.03 could confer canavanine sensitivity to the *Saccharomyces cerevisiae* $\Delta can1$ mutant when overexpressed. In addition, this gene product is most similar to Cat1.

Our results show that the Cat1 permease is enriched toward the growing ends of fission yeast, possibly reflecting its increased utilization at the growing ends. Similar localization of another fission yeast permease, SPBC359.03, has also been reported (Matsumoto et al. 2002). This intracellular distribution of both permeases is altered significantly in $\Delta tsc2$ cells; Cat1 and

SPBC359.03 staining appear dispersed as intracellular dots. Thus, the Tsc/Rheb signaling pathway regulates the localization of these permeases. On the other hand, we did not detect any significant changes in the expression of *cat1*⁺ mRNA and the total amount of Cat1 permease. It has been previously reported that the transcription of *isp5*⁺, *spac869.10*⁺, and *spap7g5*⁺ is decreased in $\Delta tsc2$, $\Delta tor1$, and in rapamycin-treated cells (van Slegtenhorst et al. 2004; Weisman et al. 2005, 2007). In addition, we have observed the induction of expression of a variety of putative amino acid permeases upon inhibition of Tor2 (Matsuo et al. 2007). Therefore, it appears that there are transcriptional as well as post-translational mechanisms used to regulate the uptake of amino acids in fission yeast.

What could account for the mislocalization of the permease? While further work is needed, it is tempting to speculate that control of the ubiquitination machinery is involved in this regulation. In budding yeast, ubiquitination of amino acid permeases by the ubiquitin ligase RSP5 is known to serve as a post-translational modification/signal for intracellular sorting (Hein et al. 1995; Soetens et al. 2001; Blondel et al. 2004). Interestingly, it has been shown that the *Schizosaccharomyces pombe* Rsp5 homologue, Pub1, is required for the downregulation of leucine uptake (Karagiannis et al. 1999). Therefore, we looked to see if Pub1 was involved in the Tsc2 regulation of arginine uptake. Disruption of *pub1*⁺ in a wild-type background resulted in hypersensitivity to canavanine (unpublished observation). In addition, ($\Delta pub1 \Delta tsc2$ double mutants were also sensitive to canavanine (unpublished observation). This suggested that perhaps Cat1 mislocalization in the $\Delta tsc2$ mutants was being suppressed by the loss of *pub1*⁺. We have shown that the E3 ubiquitin ligase, Pub1, is important for Cat1-GFP mislocalization in $\Delta tsc2$ cells. Further work is needed to investigate whether ubiquitination plays a role in the regulation of Cat1 localization by Tsc/Rheb signaling. Also, it is unclear as to where in Cat1 trafficking, anterograde or retrograde transport to and from the plasma membrane, is targeted by Tsc/Rheb signaling. Further studies on how Tsc/Rheb signaling affects transcription and localization of various permeases, as well as its interplay with its downstream effectors, may provide a deeper understanding of how this signaling pathway controls amino acid transport.

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Fig. 1.

$\Delta tsc2$ Cells defect in basic amino acid uptake and resistance to canavanine can be suppressed by the expression of dominant negative RhbD60K. **a** $\Delta tsc2$ cells transformed with pREP1- $tsc2^+$ or pREP1 were assayed for the uptake of arginine, lysine and histidine. Assays were done in triplicate. **b** $\Delta tsc2$ cells transformed with pREP1- $tsc2^+$ or pREP1 were spotted onto EMM + Ade plates either with canavanine or thialysine. **c** $\Delta tsc2$ cells transformed with pREP41- $rhb1D60K^+$ or pREP41 were assayed for the uptake of arginine, lysine and histidine. Assays were done in triplicate

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Fig. 3.

A scheme for the two-step assay to identify the *Schizosaccharomyces pombe* cationic amino acid transporter (CAT)

Fig. 4. ScCan1 complementation assay. Budding yeast $\Delta can1$ mutant cells were transformed with putative fission yeast amino acid permeases that are placed under control of the ADH promoter. **a** Three positive transformants (*spbp2b2.01⁺*, *spbc359.03⁺* and *spac869.11⁺*), a negative transformant (*spac869.10⁺*), and a vector control were streaked onto SD-Ura with and without canavanine. Strains that complement ScCan1 restore sensitivity to canavanine. **b** Spotting of three positives (*spbp2b2.01⁺*, *spbc359.03⁺* and *spac869.11⁺*) on a plate containing canavanine. Two clones each were tested for *spbp2b2.01⁺* and *spbc359.03⁺*

Fig. 5.

Loss of SPAC869.11 results in canavanine resistance and a defect in basic amino acid uptake.

a *Schizosaccharomyces pombe* strains with *spac869.11*⁺, *spac359.03*⁺, and *spbpb2b2.01*⁺ disrupted with the kanMX or URA cassette were serially diluted and spotted onto EMM + Ade plates with and without 60 mg/l canavanine. $\Delta spac869.11$ as well as $\Delta spac869.11$ double-mutant cells were resistant to canavanine. **b** and **c** Arginine and lysine uptake assays were performed on $\Delta spac869.11$ and $\Delta spac359.03$ cells. $\Delta spac869.11 \Delta spbc359.03$ double-mutant cells were also assayed for their ability to take up arginine. Cells lacking *spac869.11*⁺ have a defect in arginine and lysine uptake. Wild type and $\Delta tsc2$ cells were used as positive and negative controls, respectively. Assays were done in triplicate

Fig. 6. Transmembrane architecture analysis of SPAC869.11 and ScCan1. The amino acid sequences of both permeases were analyzed by ConPredII (Arai et al. 2004), a transmembrane prediction software program. In and out designate intracellular and extracellular matrices, respectively. The conserved glutamate residue is marked by an *asterisk*

Fig. 7. Overexpression of *cat1*⁺ in wild type cells increases basic amino acid uptake and canavanine sensitivity. **a** *spac869.11*⁺ was overexpressed under the control of the pREP41 promoter in wild type cells. Cells were assayed for their ability to take up [³H] arginine, lysine, histidine, and proline. Cells overexpressing *spac869.11*⁺ had a significantly higher uptake of basic amino acids. Assays were done in triplicate. **b** The same cells were spotted onto EMM + Ade plates with and without 10 mg/l canavanine to test for sensitivity

Fig. 8.

Cat1 is required for RhebD60K to suppress the resistance of $\Delta tsc2$ to canavanine. $\Delta tsc2$ and $\Delta tsc2 \Delta cat1$ cells transformed with pREP1- $tsc2^+$, pREP1, or pREP41- $rhb1D60K^+$ were spotted onto EMM + Ade plates with and without 60 mg/l canavanine. Plates were photographed after 3 days of incubation

Fig. 9.

Loss of *tsc2*⁺ does not affect *cat1*⁺ expression, but results in the mislocalization of Cat1. **a** Northern analysis of *cat1*⁺ in wild type and *Δtsc2* mutant cells. *tub1*⁺ is used as a loading control. **b** Western analysis of Cat1-3xHA in wild type and *Δtsc2* mutant cells. PCNA is used as a loading control. **c** Fluorescence microscopy of Cat1-GFP in wild type, *Δtsc2* mutant cells transformed with or without pREP1-*RhebD60K*⁺, and *Δtsc2Δpub1* cells

Table 1

Yeast strains used in this study

Strain name	Genotype	Reference/source
TD1	<i>MATa trp1 ura3 his4 gal2</i>	Urano et al. (2000)
JU46-1	<i>MATa trp1 ura3 his4 gal2 can1::kanMX</i>	Urano et al. (2000)
SP812	<i>h⁻ ade6-210 leu-1-32 ura4-d18</i>	Matsumoto et al. (2002)
AE502	<i>h⁻ ade6-210 leu-1-32 ura4-d18 tsc2::ura4</i>	Matsumoto et al. (2002)
FY972	<i>h⁻</i>	ATCC
PJ001	<i>h⁻ tsc2::kanMX</i>	This study
PJ101	<i>h⁻ ade6-210 leu-1-32 ura4-d19 spbc869.11::kanMX</i>	This study
PJ102	<i>h⁻ ade6-210 leu-1-32 ura4-d20 spbc359.03::ura4</i>	This study
PJ103	<i>h⁻ ade6-210 leu-1-32 ura4-d21 spbpb2b2.01::kanMX</i>	This study
PJ104	<i>h⁻ ade6-210 leu-1-32 ura4-d22 spbc869.11::kanMX spbc359.03::ura4</i>	This study
PJ105	<i>h⁻ ade6-210 leu-1-32 ura4-d23 spbc869.11::kanMX spbpb2b2.01::ura4</i>	This study
PJ106	<i>h⁻ ade6-210 leu-1-32 ura4-d24 spbpb2b2.01::kanMX spbc359.03::ura4</i>	This study
PJ201	<i>h⁻ ade6-210 leu-1-32 ura4-d18 spbc869.11-3xHA-hphMX</i>	This study
PJ202	<i>h⁻ ade6-210 leu-1-32 ura4-d18 tsc2::ura4 spbc869.11-3xHA-hphMX</i>	This study
PJ385	<i>h⁻ Cat1-GFP-hphMX</i>	This study
PJ380	<i>h⁻ tsc2::kanMX Cat1-GFP-hphMX</i>	This study
PJ404	<i>h⁻ tsc2::kanMX Cat1-GFP-hphMX pREP1-HA-rhebD60K⁺</i>	This study
PJ405	<i>h⁻ tsc2::kanMX pub1::ura4 Cat1-GFP-hphMX</i>	This study