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INTRODUCTION:

This Idea Development Award application is focused on the *TSC1* and *TSC2* gene homologs in the fission yeast *Schizosaccharomyces pombe* (*S. pombe*). Tuberous sclerosis complex (TSC) is an autosomal dominantly inherited disease whose manifestations can include seizures, mental retardation, autism, and tumors of the brain, heart, kidney, and skin. The *TSC2* gene encodes tuberin, a 200 kD protein with homology to a GTPase activating protein (GAP) for Rap1. The *TSC1* gene encodes hamartin, a 130 kD protein. Both TSC genes are tumor suppressor genes: germline *TSC1* and *TSC2* mutations are predicted to inactivate protein function, and loss of heterozygosity occurs in TSC tumors.

Tuberin and/or hamartin appear to be involved in multiple cellular pathways: vesicular trafficking, signaling via the small GTPase Rap1, cell cycle regulation, steroid hormone function, cell adhesion via the small GTPase Rho, and signaling via ribosomal protein S6 and mTOR (mammalian target of rapamycin), which regulate protein synthesis and cell growth. The *in vivo* importance of these different activities and their contributions to human TSC are not yet entirely understood.

BODY:

Our central hypothesis is that the high degree of sequence conservation between the human and yeast TSC genes reflects conservation of key cellular pathways.

Statement of Work:

- Aim 1: To determine whether the phenotypes observed in the *tsc1* and *tsc2* knockout *S. pombe* strains are the result of defects in the cell cycle.
- Aim 2: To determine the functional consequences of missense mutations within the highly conserved GAP domain of tuberin.
- Aim 3: To identify proteins in yeast that function in the same cellular pathways as the TSC protein homologs using a genetic screen.

We report for the first time that $\Delta tsc1$ and $\Delta tsc2$ have a defect in arginine uptake, which is regulated through *rhb1*⁺ in *S. pombe*, providing evidence that TSC-Rheb signaling is conserved in *S. pombe*. A mutation in the GAP domain of *tsc2*⁺ at a site corresponding to a patient-derived missense mutation could not rescue the uptake defects, strengthening the relationship of the *S. pombe* model to human TSC. The transcriptional expression profile and intracellular amino acid levels associated with $\Delta tsc1$ and $\Delta tsc2$ overlapped extensively, suggesting similar roles for *tsc1*⁺ and *tsc2*⁺ in *S. pombe*. These findings support *S. pombe* as a model for TSC and indicate that the *S. pombe* Tsc1 and Tsc2 proteins play central roles in amino acid biosynthesis and sensing.

F15 $\Delta tsc1$ and F15 $\Delta tsc2$ have growth defect

As a first step towards understanding the physiological functions of *tsc1*⁺ and *tsc2*⁺, we disrupted *tsc1*⁺ and *tsc2*⁺ in the *S. pombe* genome by one-step gene replacement. To initiate phenotypic analysis, the F15 $\Delta tsc1$ strain was mated with the CHP428 strain (*h*⁺, *leu1-32*, *ura4-D18*, *ade6-210*, *his7-366*) and spores were analyzed on yeast extract (YE) medium, supplemented with 50 μ g/ml leucine, uracil, adenine and histidine (YES). Dissection of asci from heterozygous diploid cells showed that two out of four colonies were smaller in size. These smaller colonies were found by PCR to be $\Delta tsc1$. Similar results were obtained for the F15 $\Delta tsc2$

strain. The slower growth phenotype on YES media was quantified in exponential liquid growing cultures. The generation time (time required for cell population to double) of F15 $\Delta tsc1$ and F15 $\Delta tsc2$ was approximately 5 hours compared to 3.5 hours for the F15 strain. To test if growth was further affected by temperature stress, F15 $\Delta tsc1$ and F15 $\Delta tsc2$ were plated on YES plates and incubated at 25°C and 37°C. No temperature-induced growth defect was observed in the F15 $\Delta tsc1$ and F15 $\Delta tsc2$ strains.

F15 $\Delta tsc1$ and F15 $\Delta tsc2$ are conditionally lethal

We found a more severe growth defect in the F15 $\Delta tsc1$ and F15 $\Delta tsc2$ strains when they were grown on essential minimal medium (EMM) plates. F15 $\Delta tsc1$ and F15 $\Delta tsc2$ yeast could not grow on EMM supplemented with normal amounts (50 μ g/ml) uracil, histidine, adenine and leucine, but increasing supplements to 1000 μ g/ml partially restored growth. These results are in agreement with the previously reported defect in uptake of leucine, adenine and histidine. To verify that the growth defect was due to deletion of the *tsc1*⁺ and *tsc2*⁺ genes, Tsc1 and Tsc2, expressed from a plasmid with *ura4*⁺, were transformed into the F15 $\Delta tsc1$ and F15 $\Delta tsc2$ strains and were plated on EMM without uracil. Expression of Tsc1 restored growth in F15 $\Delta tsc1$ yeast, but failed to rescue F15 $\Delta tsc2$, while Tsc2 expressed restored growth in F15 $\Delta tsc2$, but not in F15 $\Delta tsc1$.

972 $\Delta tsc1$ and 972 $\Delta tsc2$ have a defect in arginine uptake

Previously, Rhb1 was shown to regulate arginine uptake in *S. pombe*, prompting us to determine whether Tsc1 and Tsc2 also regulate arginine uptake. Since F15 $\Delta tsc1$ and F15 $\Delta tsc2$ have a growth defect, we crossed $\Delta tsc1$ and $\Delta tsc2$ into the 972 background. This strain does not require amino acid supplements and 972 $\Delta tsc1$ and 972 $\Delta tsc2$ did not show a growth defect on EMM. We found that 972 $\Delta tsc1$ and 972 $\Delta tsc2$ are resistant to 60 μ g/ml canavanine, a toxic analog of arginine. This dose of canavanine was toxic to the wild-type 972 strain. To determine whether the canavanine resistance was due to decreased uptake, the uptake of ³H- arginine was measured. After 10 minutes, arginine uptake was approximately 3.5-fold less in the 972 $\Delta tsc1$ and 972 $\Delta tsc2$ strains compared to wild-type 972, indicating that the canavanine resistance is due to decreased uptake.

Dominant negative Rhb1 can rescue the arginine uptake in *ura4* $\Delta tsc2$

A recent screen in *S. pombe* identified a dominant negative Rhb1 mutation, Rhb1-D60K, that is unable to bind GTP or GDP. We generated this mutation in the pSLF373-*ura4*⁺ expression vector and crossed $\Delta tsc2$ into the *ura4*-D18 strain to allow selection for cells expressing from the pSLF373-*ura4*⁺ plasmid. We found that the decreased arginine uptake in $\Delta tsc2$ was restored by expression of Rhb1-D60K, but not by wild-type Rhb1, suggesting that arginine uptake is regulated through Tsc1, Tsc2 and Rhb1 in *S. pombe*.

A missense mutation in the GAP domain of *tsc2*⁺ does not rescue the conditional lethality or arginine uptake in $\Delta tsc2$

The Tsc2 GAP domain in *S. pombe* is 39% identical to the GAP domain in human tuberlin, and the conserved residues include the sites of 3 patient-derived TSC2 missense mutations. To determine whether these residues are crucial for the function of Tsc2 in *S. pombe*, we constructed one of them, Tsc2-N1292K, which corresponds with N1643K in human, in the HA-tagged pSLF373-*ura4*⁺ expression vector. Western blot analysis showed protein expression

for both Tsc2 and Tsc2-N1292K. Next, the Tsc2 and Tsc2-N1292K expression constructs were transformed into F15 Δ tsc2 and cells were plated on EMM plates without uracil, but with 50 μ g/ml leucine, adenine and histidine. The wild-type Tsc2 expression construct restored growth, but no growth was detected when the Tsc2-N1292K mutation was expressed. We next asked whether re-introducing Tsc2-N1292K could revert the canavanine resistance in the *ura4* Δ tsc2 strain. Wild-type Tsc2 restored the canavanine sensitivity, while Tsc2-N1292K did not. The decreased arginine uptake was similarly rescued by wild-type Tsc2 but not by Tsc2-N1292K. These results suggest that the function of Tsc2 in regulating arginine uptake requires the GAP domain, and support the use of *S. pombe* as a model system for human TSC.

Δ tsc1 and Δ tsc2 show a significant overlap in expression profile

To elucidate the mechanism through which *tsc1*⁺ and *tsc2*⁺ regulate amino acid uptake, we compared the expression profile of 972 Δ tsc1 and 972 Δ tsc2. Total RNA was isolated from 972, 972 Δ tsc1 and 972 Δ tsc2 yeast, labeled, and hybridized to cDNA arrays (Eurogentec, Belgium). The expression profile of 972 Δ tsc1 was compared to 972 on two separate arrays, including a dye-flip experiment. The 972-972 Δ tsc2 comparison was completed using the same design. Since all four arrays showed a linear relation between cy3 and cy5, a linear regression normalization was applied to the data. In addition, the dye-flip experiment for Δ tsc1 was highly correlated. The expression data was also validated by the absence of *tsc1*⁺ expression in the Δ tsc1 and absence of *tsc2*⁺ in the Δ tsc2 arrays, serving as internal controls.

There was a high degree of overlap in expression profile between 972 Δ tsc1 and 972 Δ tsc2. In total 14 genes were downregulated at least 1.5 fold and 26 genes were upregulated at least 1.5 fold both in 972 Δ tsc1 and 972 Δ tsc2. Many of the upregulated genes have predicted roles in iron transport and amino acid metabolism, including the arginase gene, *car1*⁺.

Many of the downregulated genes were putative transporters, including three amino acid permeases, two oligopeptide transporters, two polyamine transporters, and one with homology to vitamin/cofactor transporters. Interestingly, the three downregulated amino acid permeases had homology to the Gap1p (general amino acid permease) in *S. cerevisiae*. The expression change for these three permeases was confirmed by Northern blotting. The fold-change on the Northern blot was determined by densitometry and was in each case slightly greater than the fold-change on the array, further validating the array result. These data support that Tsc1 and Tsc2 function in the same pathway in *S. pombe*, and suggest that they have a central role in the regulation of the biosynthesis and uptake of amino acids, oligopeptides and polyamines.

Intracellular amino acid concentrations are decreased in 972 Δ tsc1 and 972 Δ tsc2

The downregulation of permease expression and decreased uptake of amino acids in the 972 Δ tsc1 and 972 Δ tsc2 strains could represent an appropriate response to high intracellular amino acid concentrations. However, we found that the intracellular levels of multiple amino acids were low in 972 Δ tsc1 and 972 Δ tsc2 compared to 972 wild-type yeast. Ornithine, which is a product of both glutamate and arginine metabolism, showed the largest relative decrease, from approximately 15 nM in 972 wild-type to nearly undetectable levels in 972 Δ tsc1 and 972 Δ tsc2, while lysine was not changed. A decrease of at least 40% was detected for alanine, asparagine, histidine, glutamine, ornithine, citrulline and arginine. Interestingly, the latter four are linked to arginine biosynthesis. The low intracellular amino acid levels, combined with the low amino acid permease expression levels and the decreased arginine uptake, strongly suggest that yeast lacking *tsc1*⁺ or *tsc2*⁺ have an intrinsic defect in amino acid sensing.

Pas1 is a suppressor of $\Delta tsc1$ and $\Delta tsc2$

A genetic screen for suppressors of the $\Delta tsc1$ phenotype was performed, and Pas1, a putative G1 cyclin, was identified. Overexpression of Pas1 could rescue the conditional lethal growth phenotype of $\Delta tsc1$ and $\Delta tsc2$. As described before, $\Delta tsc1$ and $\Delta tsc2$ were shown to be required for arginine and leucine uptake in *S. pombe*, prompting us to determine whether Pas1 has a defect in amino acid uptake similar to $\Delta tsc1$ and $\Delta tsc2$. We found that $\Delta pas1$ as well as $\Delta pas1 \Delta tsc1$ and $\Delta pas1 \Delta tsc2$ are resistant to 60 $\mu\text{g/ml}$ canavanine, a toxic analog of arginine. Furthermore, radiolabeled arginine and leucine uptake was significantly reduced in the $\Delta tsc2$ and $\Delta pas1$ strain relative to wild type. We also found that the decreased arginine uptake in $\Delta tsc1$ was partially restored by expression of $pas1^+$ almost to a similar degree seen with $tsc1^+$, suggesting that arginine uptake is regulated through $tsc1^+$ and $pas1^+$ in *S. pombe*. In addition $pas1^+$ expression is upregulated in $\Delta tsc1$ and $\Delta tsc2$. These data suggest that Pas1 may be a target of the Tsc1/Tsc2 complex.

KEY RESEARCH ACCOMPLISHMENTS:

- F15 $\Delta tsc1$ and F15 $\Delta tsc2$ have growth defect
- F15 $\Delta tsc1$ and F15 $\Delta tsc2$ are conditionally lethal
- 972 $\Delta tsc1$ and 972 $\Delta tsc2$ have a defect in arginine uptake
- Dominant negative Rhb1 can rescue the arginine uptake in *ura4* $\Delta tsc2$
- A missense mutation in the GAP domain of $tsc2^+$ does not rescue the conditional lethality or arginine uptake in $\Delta tsc2$
- $\Delta tsc1$ and $\Delta tsc2$ show a significant overlap in expression profile
- Intracellular amino acid concentrations are decreased in 972 $\Delta tsc1$ and 972 $\Delta tsc2$
- Pas1, a putative G1 cyclin, was identified in a suppressor screen and $\Delta pas1$ has similar defects in amino acid uptake as $\Delta tsc1$ and $\Delta tsc2$
- Pas1 may be a target of the Tsc1/Tsc2 complex that links nutrient availability to cell cycle progression in *S. pombe*

REPORTABLE OUTCOMES:

van Slegtenhorst M, Carr E, Stoyanova R, Kruger W, Henske EP. $Tsc1^+$ and $tsc2^+$ regulate arginine uptake and metabolism in *Schizosaccharomyces pombe*. *Journal of Biological Chemistry* (2004) 279; 12706-12713 (see Appendix).

CONCLUSIONS:

In conclusion, our data show for the first time that Tsc1 and Tsc2 regulate arginine uptake and arginine biosynthesis in *S. pombe*. Rescue of the arginine uptake defect by a dominant negative form of Rhb1 suggests that Rhb1 is downstream of Tsc2 in *S. pombe*, as well as in other species. The complexity of the amino acid phenotype is suggestive of an intrinsic defect in amino acid sensing, involving amino acids and enzymes closely linked to ornithine and arginine. If similar pathways are affected in mammalian cells lacking *TSC1* or *TSC2*, defects in polyamines and/ or nitric oxide levels could be pathogenically linked to the clinical manifestations of TSC, including refractory seizures.

REFERENCES: None

BIBLIOGRAPHY OF PUBLICATIONS:

van Slegtenhorst M, Carr E, Stoyanova R, Kruger W, Henske EP. *Tsc1*⁺ and *tsc2*⁺ regulate arginine uptake and metabolism in *Schizosaccharomyces pombe*. *Journal of Biological Chemistry* (2004) 279; 12706-12713 (see Appendix).

LIST OF PERSONNEL:

Elizabeth Henske, M.D., Principal Investigator
Marjon A. van Slegtenhorst, Ph.D., Postdoctoral Associate

APPENDIX:

van Slegtenhorst M, Carr E, Stoyanova R, Kruger W, Henske EP. *Tsc1*⁺ and *tsc2*⁺ regulate arginine uptake and metabolism in *Schizosaccharomyces pombe*. *Journal of Biological Chemistry* (2004) 279; 12706-12713.

Tsc1⁺ and *tsc2*⁺ Regulate Arginine Uptake and Metabolism in *Schizosaccharomyces pombe**

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Mutations in either *TSC1* or *TSC2* cause tuberous sclerosis complex, an autosomal dominant disorder characterized by seizures, mental retardation, and benign tumors of the skin, brain, heart, and kidneys. Homologs for the *TSC1* and *TSC2* genes have been identified in mouse, rat, *Fugu*, *Drosophila*, and in the yeast *Schizosaccharomyces pombe*. Here we show that *S. pombe* lacking *tsc1*⁺ or *tsc2*⁺ have similar phenotypes including decreased arginine uptake, decreased expression of three amino acid permeases, and low intracellular levels of four members of the arginine biosynthesis pathway. Recently, the small GTPase Rheb was identified as a target of the GTPase-activating domain of tuberin in mammalian cells and in *Drosophila*. We show that the defect in arginine uptake in cells lacking *tsc2*⁺ is rescued by the expression of a dominant negative form of *rhb1*⁺, the Rheb homolog in *S. pombe*, but not by expressing wild-type *rhb1*⁺. Expression of the *tsc2*⁺ gene with a patient-derived mutation within the GAP domain did not rescue the arginine uptake defect in *tsc2*⁺ mutant yeast. Taken together, these findings support a model in which arginine uptake is regulated through *tsc1*⁺, *tsc2*⁺, and *rhb1*⁺ in *S. pombe* and also suggest a role for the Tsc1 and Tsc2 proteins in amino acid biosynthesis and sensing.

Tuberous sclerosis complex (TSC)¹ is a tumor suppressor syndrome that is characterized by the development of a variety of benign tumors (hamartomas) and severe neurological problems including seizures, mental retardation, and autism. TSC is caused by mutations in either *TSC1* (1) or *TSC2* (2). Hamartin, the *TSC1* gene product, and tuberin, the *TSC2* gene product, are known to interact (3, 4) and function in a complex. Tuberin has a highly conserved GTPase-activating protein (GAP) domain with activity for Rheb1 (Ras homolog enriched in brain) (5–11), a small GTPase that may be involved in nutrient signaling and cell cycle regulation (12).

Studies in *Drosophila* and mammalian systems have shown that the hamartin-tuberin complex negatively regulates p70S6 kinase (pS6K) within the PI3K signaling pathway (13, 14). The

regulation of pS6K is mediated by Rheb and by the target of rapamycin, which are components in pathways that control cell size by integrating mitogenic signals and nutrient availability with protein synthesis (11, 13, 15–18). Both hamartin and tuberin are regulated by phosphorylation. Hamartin is phosphorylated by cyclin-dependent kinase CDK1 (19), and tuberin is a substrate for Akt (protein kinase B) (14, 20, 21), p38-activated kinase MK2 (22), and the AMP-activated protein kinase (23).

Schizosaccharomyces pombe contains genes with significant similarity to *TSC1* and *TSC2*, which were named *tsc1*⁺ and *tsc2*⁺ (24). The GAP domain of tuberin is particularly highly conserved with 39% identity. In addition to *TSC1* and *TSC2* homologs, *S. pombe* also has a Rheb homolog, *rhb1*⁺. Loss of *rhb1*⁺ in *S. pombe* results in growth arrest, similar to that caused by nitrogen starvation (25), and loss of farnesylation of the Rhb1 protein has been postulated to regulate arginine uptake in *S. pombe* (26).

Recently, *S. pombe* strains lacking *tsc1*⁺ ($\Delta tsc1$) or *tsc2*⁺ ($\Delta tsc2$) were shown to have abnormal localization of the amino acid permease *c359.03*⁺, suggesting that the *S. pombe* Tsc1-Tsc2 protein complex regulates protein trafficking (24). Here we report for the first time that $\Delta tsc1$ and $\Delta tsc2$ have a defect in arginine uptake, which is regulated through *rhb1*⁺ in *S. pombe*, providing evidence that TSC-Rheb signaling is conserved in *S. pombe*. A mutation in the GAP domain of *tsc2*⁺ at a site corresponding to a patient-derived missense mutation could not rescue the uptake defects, strengthening the relationship of the *S. pombe* model to human TSC. The transcriptional expression profile and intracellular amino acid levels associated with $\Delta tsc1$ and $\Delta tsc2$ overlapped extensively, suggesting similar roles for *tsc1*⁺ and *tsc2*⁺ in *S. pombe*. These findings support *S. pombe* as a model for TSC and indicate that the *S. pombe* Tsc1 and Tsc2 proteins play central roles in amino acid biosynthesis and sensing.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Growth Conditions—The yeast strains used in this study are listed in Table I. CHP428 and CHP429 were constructed by Charlie Hoffman (Boston College) and were a gift from Janet Leatherwood (University of New York, Stony Brook, NY). Wild-type strain 972 (27) and *ura4*-D18 (28) were gifts from J. Bähler (Sanger Institute). *S. pombe* cells were grown in essential minimal medium (EMM, Qbiogene, Carlsbad, CA) or yeast extract complete medium with 50 μ g/ml uracil, histidine, adenine, and leucine (YES) at 30 °C unless otherwise stated. Transformations were performed with Frozen-EZ yeast transformation II kit (Zymo Research, Orange, CA).

Construction of *tsc1*⁺ and *tsc2*⁺-deficient Strains—*Tsc1*⁺ and *tsc2*⁺-deficient strains were constructed with the PCR one-step homologous recombination method (29). The kanamycin cassette was amplified from plasmid pFA6a-kanMX6 (a gift from J. Bähler) using primers with 75 extra bases corresponding to sequences immediately upstream of the start codon of the *tsc* genes and primers whose gene-specific portions correspond to sequences 75 bases downstream of the gene. For gene disruption of *tsc1*⁺, the entire open reading frame was deleted from the genome of the

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¹ The abbreviations used are: TSC, tuberous sclerosis complex; GAP, GTPase-activating protein; Rheb, *ras* homolog enriched in brain; EMM, essential minimal medium; YES, yeast extract medium with supplements; HA, hemagglutinin.

TABLE I
S. pombe strains used in this study

Strain	Genotype	Source
972	<i>h</i> ⁻	Ref. 27
MVS1	<i>h</i> ⁻ , $\Delta tsc1::kan^+$	This study
MVS2	<i>h</i> ⁻ , $\Delta tsc2::kan^+$	This study
ura4-D18	<i>h</i> ⁺ , <i>ura4-D18</i>	Ref. 28
MVS3	<i>h</i> ⁺ , <i>ura4-D18</i> , $\Delta tsc1::kan^+$	This study
MVS4	<i>h</i> ⁺ , <i>ura4-D18</i> , $\Delta tsc2::kan^+$	This study
CHP428	<i>h</i> ⁺ , <i>leu1-32</i> , <i>ura4-D18</i> , <i>ade6-210</i> , <i>his7-366</i>	C. Hoffman
CHP429	<i>h</i> ⁻ , <i>leu1-32</i> , <i>ura4-D18</i> , <i>ade6-216</i> , <i>his7-366</i>	C. Hoffman
MVS5	<i>h</i> ⁻ , <i>leu1-32</i> , <i>ura4-D18</i> , <i>ade6-216</i> , <i>his7-366</i> , $\Delta tsc1::kan^+$	This study
MVS6	<i>h</i> ⁻ , <i>leu1-32</i> , <i>ura4-D18</i> , <i>ade6-216</i> , <i>his7-366</i> , $\Delta tsc2::kan^+$	This study

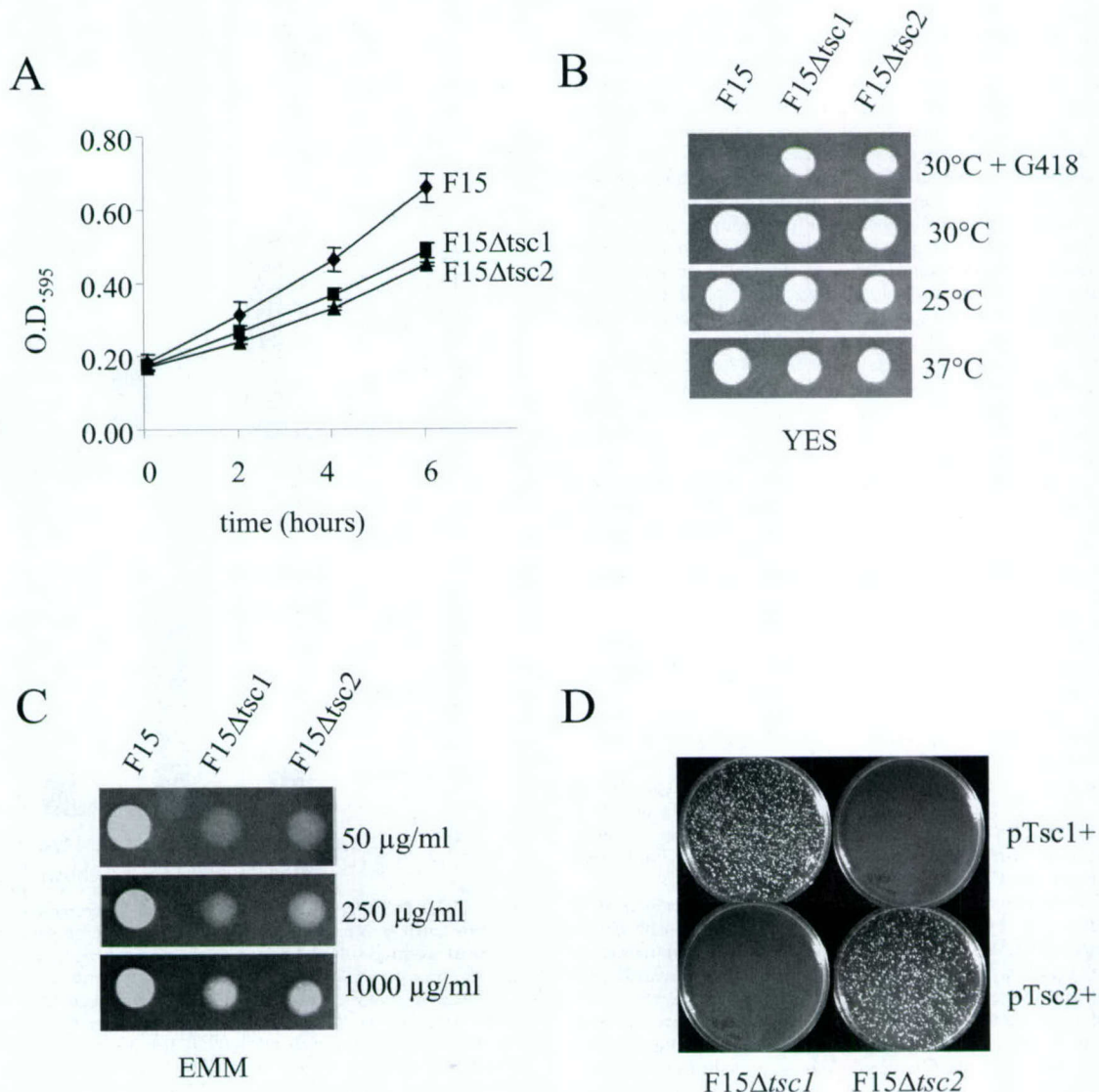


FIG. 1. F15Δtsc1 and F15Δtsc2 exhibit growth defects. A, F15, F15Δtsc1, and F15Δtsc2 strains were grown in rich liquid medium with supplements (YES) overnight to mid-log phase ($A_{595} = 0.4-0.6$) and diluted to $A_{595} = 0.2$. Cells were grown for an additional 6 h, and the generation time was determined between 3 and 6 h. The F15Δtsc1 and F15Δtsc2 needed ~5 h to complete one generation compared with 3.5 h for wild-type strains. B, 40,000 F15, F15Δtsc1 and F15Δtsc2 cells were spotted on YES + G418 (200 μg/ml). F15Δtsc1 and F15Δtsc2 were G418-resistant as expected. The cells were also spotted on YES plates and grown at 25, 30, and 37 °C for 3 days. No growth differences were seen among F15, F15Δtsc1 and F15Δtsc2. C, F15, F15Δtsc1, and F15Δtsc2 cells were spotted (40,000 cells) onto EMM plates with different amounts of adenine, leucine, histidine, and uracil. At regular amounts (50 μg/ml), the F15Δtsc1 and F15Δtsc2 could not grow but growth was partially restored by increasing the amount of supplements to 1 mg/ml. D, Tsc1 and Tsc2 expression constructs in pREP4X (*ura4*⁺) were transformed into F15Δtsc1 and F15Δtsc2 and plated onto EMM plates supplemented with 50 μg/ml leucine, adenine, and histidine. pREP4X-Tsc1 expression rescued the growth of F15Δtsc1, and pREP4X-Tsc2 expression rescued the growth of F15Δtsc2.

haploid strain CHP429 (*h*⁻, *leu1-32*, *ura4-D18*, *ade6-216*, *his7-366*) and replaced by the kanamycin cassette to create MVS5. We will refer to $\Delta tsc1$ with this genotype as F15Δtsc1. $\Delta tsc2$ was constructed using identical strategy and resulted in MVS6, which will be referred to as F15Δtsc2 in figures and text. Correct integration of the kanamycin cassette into the

yeast genome was confirmed by PCR over the integration site, by Southern blotting, and by sequencing. Subsequently, F15Δtsc1 and F15Δtsc2 were crossed into the *ura4-D18* strain to generate MVS3 (*ura4Δtsc1*) and MVS4 (*ura4Δtsc2*) and into wild-type 972 to generate MVS1 (972Δtsc1) and MVS2 (972Δtsc2) using random spore analysis on selective plates.

Construction of Plasmids—Tsc1 and Tsc2 expression constructs were generated by a PCR-cloning approach. The *tsc1*⁺ and *tsc2*⁺ genes were amplified from the cosmids c23F3 and c630C13 (a gift from J. Bähler) using primers with SalI restriction sites and cloned into the pREP4X expression vector (ATCC). The Rhb1 expression construct was generated by PCR of full-length *rhb1*⁺ from total cDNA using primers with SalI and XmaI and cloned into pREP4X. After sequence verification, *tsc1*⁺, *tsc2*⁺, and *rhb1*⁺ were inserted in-frame into the HA-tagged pSLF173/273/373 series with different *mt* (no message in thiamin) promoter strength (ATCC). The GAP (Tsc2-N1191K) and the Rhb dominant negative (Rhb-D60K) mutations were introduced into the pSLF373 constructs using site-directed mutagenesis (Stratagene, La Jolla, CA). All of the constructs were verified by sequencing.

Expression Profiling—Yeast were grown overnight in EMM to early log phase ($A_{595} = 0.2-0.3$), and total RNA was isolated by phenol extraction and purified using RNeasy (Qiagen, Valencia, CA). The total RNA of three independent biological samples was pooled (10 μ g of each sample), reverse-transcribed into cDNA, and labeled with Cy3 and Cy5 (Amersham Biosciences). Hybridizations were carried out overnight at 42 °C. The slides were scanned with a GMS 428 scanner (Affymetrix, Santa Clara, CA), and spot quantification was performed with the ImaGene software (BioDiscovery, Marina del Rey, CA). Each of the 4976 *S. pombe* genes was present in duplicate on each slide, and the experiments were repeated using opposite labels (dye-flip), resulting in a total of four measurements for each gene per sample. Genes were considered expressed when all four measurements exceeded a threshold of 3.5 times above the background. A linear regression normalization was applied to the data (30), and fold changes were calculated. Genes were grouped and annotated on the basis of predicted function in the Proteome Knowledge Library (Incyte, Beverly, MA).

Western Blot Analysis—Yeast were grown to mid-log phase ($A_{595} = 0.4-0.6$) and washed once in ice-cold buffer containing 50 mM Tris-HCl, pH 7.5, 25 mM NaCl, and 0.1 mM phenylmethylsulfonyl fluoride. Cells were lysed in the same buffer with 0.5 mm-glass beads in a BeadBeater (Biospec Products, Bartlesville, OK). 20 μ g of each sample was run on 4–20% SDS-PAGE gel (Bio-Rad) and transferred to nitrocellulose membranes using standard methods. The immobilized proteins were detected using enhanced chemiluminescence (Amersham Biosciences). Antibodies used were anti-HA (Roche Applied Science) and monoclonal TAT1 for *S. pombe* tubulin (gift from K. Gull, University of Manchester, United Kingdom).

Northern Blot Analysis—10 μ g of total RNA was run on a 1% formaldehyde gel at 60 V for 4 h and transferred to nylon membrane overnight in 20 $\times$ SSC. Probes for *p7G5.06*⁺, *c869.10*⁺, *isp5*⁺, and *gpd3*⁺ were PCR-amplified from cDNA, cleaned over 0.8% agarose gel, and labeled with [α -³²P]dCTP (PerkinElmer Life Sciences) using standard methods. Hybridizations were performed in rapid hybridization buffer (Amersham Biosciences).

Canavanine Sensitivity—Cells were grown overnight to mid-log phase ($A_{595} = 0.4-0.6$), and A_{595} was adjusted to 0.4 (10,000 cells/ μ l). 4 μ l of 1 $\times$, 10 $\times$, and 100 $\times$ dilutions was spotted onto EMM as a growth control or EMM containing canavanine (60 μ g/ml) (Sigma) and incubated for 3 days at 30 °C.

Arginine Uptake Assays—Arginine uptake assays were performed in triplicate as described by Urano *et al.* (31) with minor modifications. Cells were grown in EMM minimal medium with no supplements to mid-log phase. 1 μ Ci of L-[³H]arginine (40–70 Ci/mmol) (PerkinElmer Life Sciences) and 100 μ M of non-radioactive arginine (Sigma) were added to 25,000 cells in 600 μ l of EMM. 200- μ l aliquots were removed at 0 and 10 min, injected into 5 ml of deionized water, and immediately subjected to vacuum manifold filtration. Cells were collected on Whatman glass microfiber filters, washed twice, and dried. [³H]Arginine was measured by scintillation counting.

Measurements of Intracellular Amino Acid Pools—Protein extracts were prepared as described under Western blot analysis, quantified using the Bradford assay (Bio-Rad), and diluted to 1 μ g/ μ l. Proteins were precipitated by treatment of 100 μ l of sample with 100 μ l of 10% 5-sulfosalicylic acid at 4 °C for 1 h. The pH value of the supernatant was adjusted to 2.2 with 3 M LiOH. 100 μ l of sample was injected into the Biochrom 30 amino acid analyzer (Biochrom, Cambridge, United Kingdom) including a standard amino acid mixture of 10 nM (Sigma).

RESULTS

F15 Δ tsc1 and F15 Δ tsc2 Have Growth Defect—As a first step toward understanding the physiological functions of *tsc1*⁺ and *tsc2*⁺, we disrupted *tsc1*⁺ and *tsc2*⁺ in the *S. pombe* genome by one-step gene replacement. To initiate phenotypic analysis, the

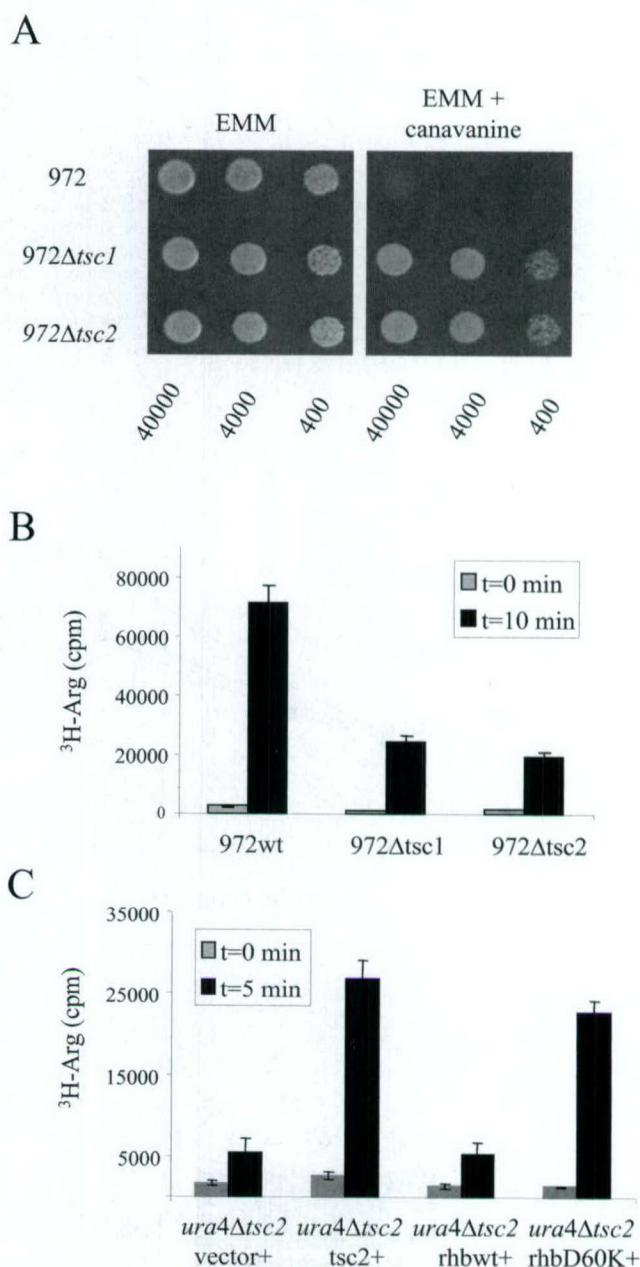


FIG. 2. The 972 Δ tsc1 and 972 Δ tsc2 have a decreased uptake of arginine, which can be restored in the 972 Δ tsc2 by expressing a dominant negative Rhb1 mutation. A, wild-type 972, 972 Δ tsc1, and 972 Δ tsc2 were grown in EMM without supplements overnight to mid-log phase, and cells were diluted to $A_{595} = 0.4$. Three different dilutions (40,000–4000–400 cells) were spotted on EMM plates with and without 60 μ g/ml canavanine. Plates were incubated at 30 °C for 3 days. Canavanine killed wild-type cells, but 972 Δ tsc1 and 972 Δ tsc2 were resistant to 60 μ g/ml canavanine. B, cells were grown until mid-log phase ($A_{595} = 0.5$) in EMM, and 25,000 cells were resuspended in 100 μ M L-arginine with 1 μ Ci of L-[³H]arginine (40–70 Ci/mmol) in 600 μ l. Aliquots of 200 μ l were injected into 5 ml of EMM in a vacuum manifold at 0–10 min, washed twice with 5 ml of deionized water, and assayed for L-[³H]arginine uptake. The arginine uptake was 3.5-fold less in the 972 Δ tsc1 and 972 Δ tsc2 strains compared with wild-type 972. Experiments were done in triplicate, and similar results were seen in two independent experiments. C, the arginine uptake was measured for the *ura4* Δ tsc2 strain expressing empty vector, Tsc2, Rhb1, and dominant negative Rhb-D60K from a plasmid with *ura4*⁺. Expression of either Tsc2 or Rhb1-D60K restored the arginine uptake.

F15 Δ tsc1 strain was mated with the CHP428 strain (*h*⁺, *leu1*–32, *ura4*–D18, *ade6*–210, *his7*–366) and spores were analyzed on yeast extract medium supplemented with 50 μ g/ml leucine, uracil, adenine, and histidine (YES). Dissection of asci from het-

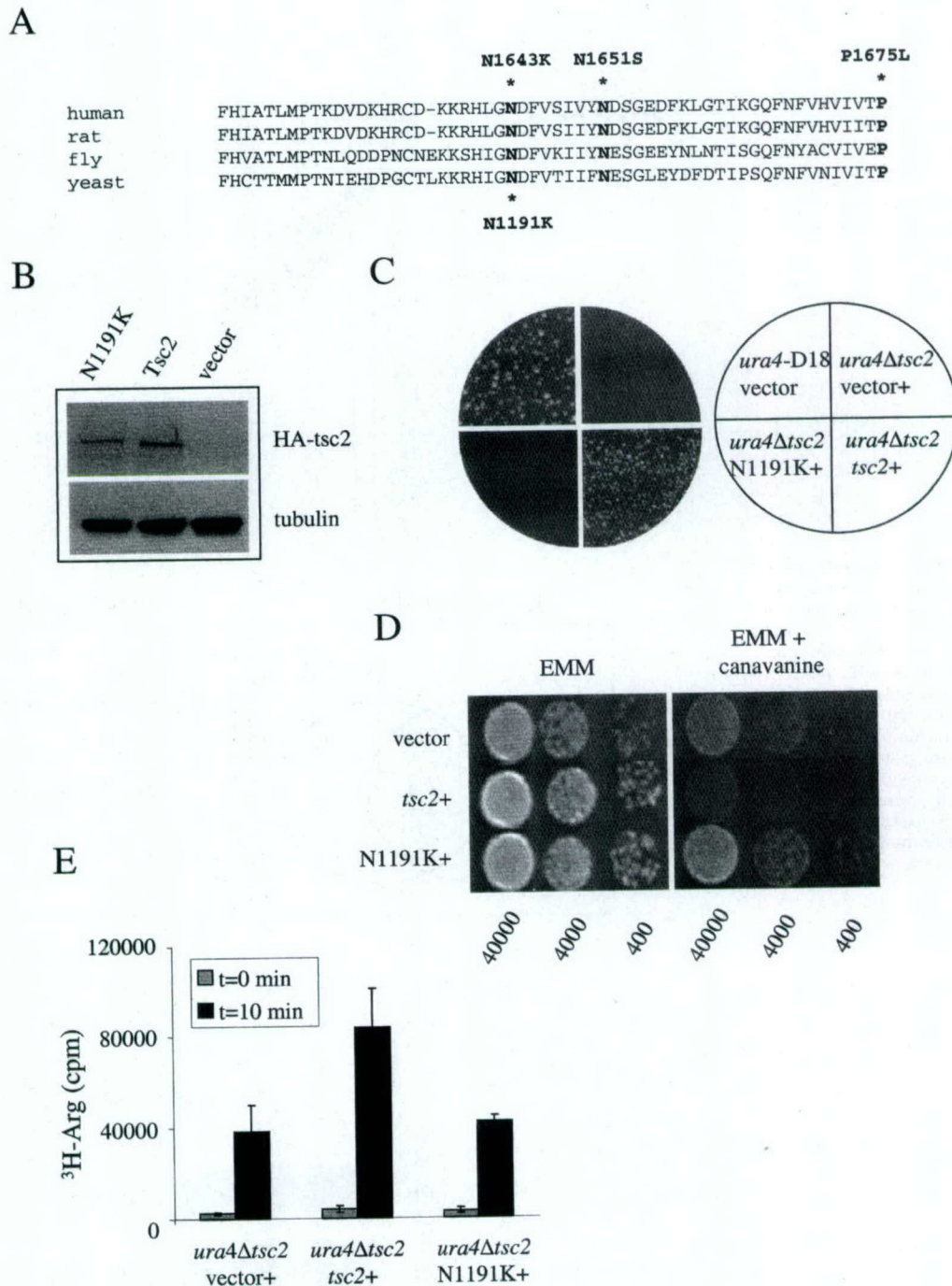


FIG. 3. **A** patient-derived mutant form of *tsc2* does not rescue the arginine uptake. **A**, alignment of the GAP domain amino acids 1609–1675. Three human disease-causing mutations (N1643K, N1651S, and P1675L), indicated by asterisks above the alignment, are conserved in *S. pombe*. **B**, expression of HA-Tsc2 and HA-Tsc2-N1191K was confirmed in *ura4Δtsc2* by Western immunoblot with an anti-HA antibody. Tubulin is shown as a loading control. **C**, constructs expressing wild-type Tsc2 and Tsc2-N1191K were transformed into the *F15Δtsc2* strain and plated onto EMM plates supplemented with 50 μ g/ml leucine, adenine, and histidine and without uracil. Tsc2 expression rescued growth, whereas the Tsc2-N1191K mutation did not. **D**, canavanine sensitivity was measured in *ura4Δtsc2* expressing empty vector, Tsc2, or Tsc2-N1191K from a plasmid with *ura4*⁺. Tsc2 expression restored the canavanine sensitivity in *Δtsc2*, whereas Tsc2-N1191K expression did not. **E**, arginine uptake was measured in *ura4Δtsc2* expressing empty vector, Tsc2, or Tsc2-N1191K. The uptake defect was rescued by expressing Tsc2 but not by Tsc2-N1191K.

erozygous diploid cells showed that two of four colonies were smaller in size (data not shown). These smaller colonies were found by PCR to be *Δtsc1*. Similar results were obtained for the *F15Δtsc2* strain. The slower growth phenotype on YES medium was quantified in exponential liquid-growing cultures. The generation time (time required for cell population to double) of *F15Δtsc1* and *F15Δtsc2* was ~5 h compared with 3.5 h for the

F15 strain (Fig. 1A). To test whether growth was further affected by temperature stress, *F15Δtsc1* and *F15Δtsc2* were plated on YES plates and incubated at 25 and 37 °C. No temperature-induced growth defect was observed in the *F15Δtsc1* and *F15Δtsc2* strains (Fig. 1B).

***F15Δtsc1* and *F15Δtsc2* Are Conditionally Lethal**—We found a more severe growth defect in the *F15Δtsc1* and *F15Δtsc2*

A

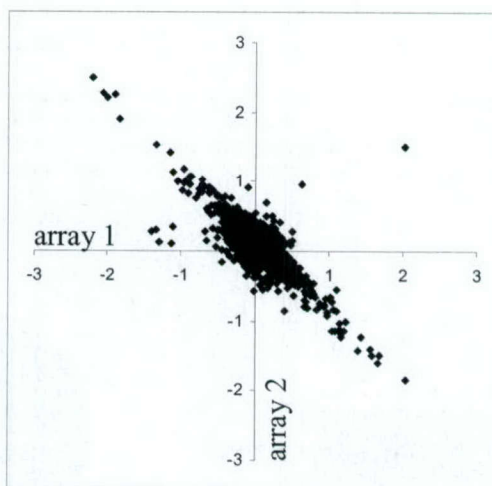
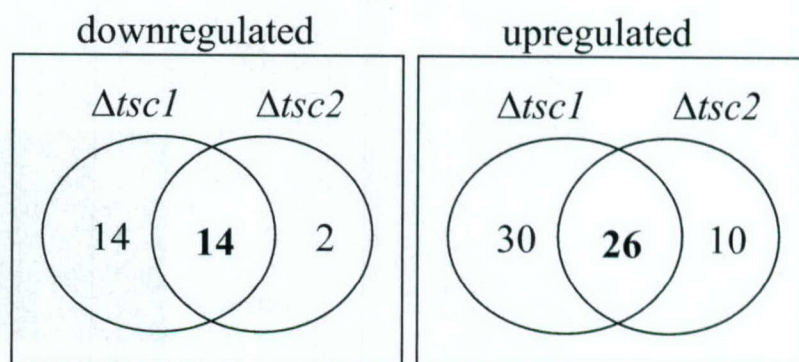
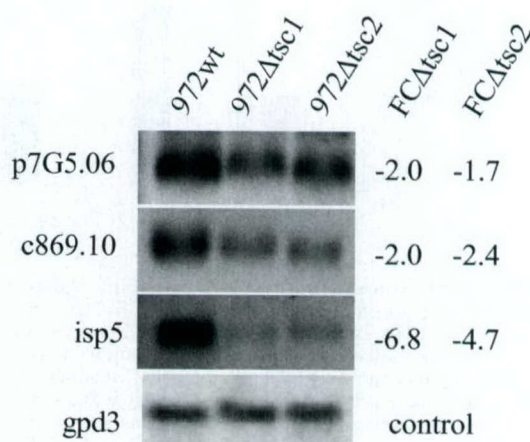


FIG. 4. *972Δtsc1* and *972Δtsc2* show a significant overlap in expression profile. A, correlation plot of the average gene expression ratios from the *Δtsc1* dye-flip experiment. B, expression profiles were compared among 972, *972Δtsc1*, and *972Δtsc2*. At fold change of >1.5, there were 14 down-regulated and 26 up-regulated genes in common. C, expression of three permease genes, *p7G5.06*⁺, *c869.10*⁺, and *isp5*⁺ was determined by Northern blots. Fold changes were determined by densitometry. All three genes were down-regulated in *972Δtsc1* and *972Δtsc2*, consistent with the array result.

B



C



strains when they were grown on EMM plates. *F15Δtsc1* and *F15Δtsc2* yeast could not grow on EMM supplemented with normal amounts (50 μg/ml) of uracil, histidine, adenine, and leucine, but increasing supplements to 1000 μg/ml partially restored growth (Fig. 1C). These results are in agreement with the previously reported defect in uptake of leucine, adenine, and histidine (24). To verify that the growth defect was attributed to deletion of the *tsc1*⁺ and *tsc2*⁺ genes, *Tsc1* and *Tsc2*, expressed from a plasmid with *ura4*⁺, were transformed into

the *F15Δtsc1* and *F15Δtsc2* strains and were plated on EMM without uracil. Expression of *Tsc1* restored growth in *F15Δtsc1* yeast but failed to rescue *F15Δtsc2*, whereas *Tsc2* expressed restored growth in *F15Δtsc2* but not in *F15Δtsc1* (Fig. 1D).

972Δtsc1 and *972Δtsc2* Have a Defect in Arginine Uptake—Previously, *Rhb1* was shown to regulate arginine uptake in *S. pombe* (26), prompting us to determine whether *Tsc1* and *Tsc2* also regulate arginine uptake. Because *F15Δtsc1* and *F15Δtsc2* have a growth defect, we crossed *Δtsc1* and *Δtsc2* into the 972

TABLE II
Genes upregulated at least 1.5-fold in 972Δ*tsc1* and 972Δ*tsc2*

Gene name	Fold change ^a		Predicted function ^b
	Δ <i>tsc1</i>	Δ <i>tsc2</i>	
<i>bfr1</i>	1.8	1.9	ABC transporter
<i>c359.05</i>	1.6	1.6	ABC transporter
<i>c569.05c</i>	1.9	1.7	Sugar transporter
<i>c1020.03</i>	1.7	1.6	Iron ion transporter
<i>fio1</i>	1.5	1.8	Iron permease
<i>fip1</i>	1.7	1.8	Iron permease
<i>c750.05c</i>	2.5	1.9	Vitamin/cofactor transporter
<i>c1002.16c</i>	2.4	1.8	Vitamin/cofactor transporter
<i>car1</i>	3.1	2.6	Arginase
<i>plr</i>	1.5	2.2	Pyridoxal reductase
<i>c29B12.04</i>	1.9	1.7	Pyridoxine biosynthesis
<i>c5H10.10</i>	2.8	2.0	NADPH dehydrogenase
<i>c750.01</i>	2.1	3.6	Oxidoreductase
<i>p8B7.18c</i>	2.3	2.2	Phosphomethylpyrimidine kinase
<i>c977.14c</i>	2.0	3.3	Alcohol dehydrogenase
<i>c9E9.09c</i>	2.0	1.9	Aldehyde dehydrogenase
<i>pB24D3.08c</i>	1.6	2.1	Alcohol dehydrogenase
<i>c1271.07c</i>	2.3	1.7	N-acetyltransferase
<i>c21E11.04</i>	3.1	1.7	N-acetyltransferase
<i>obr1</i>	2.1	2.9	DNA binding
<i>c530.07c</i>	2.4	2.0	Unknown
<i>c70.08c</i>	4.0	2.0	Unknown
<i>c1348.02</i>	2.0	1.6	Unknown
<i>c186.05c</i>	8.7	8.1	Unknown
<i>c977.01</i>	2.2	1.8	Unknown
<i>c1271.08c</i>	2.2	1.7	Unknown

^a Fold change is the average of four independent comparisons and was only included when all four fold changes were >1.5.

^b Predicted function derived from Proteome Knowledge Library.

background. This strain does not require amino acid supplements, and 972Δ*tsc1* and 972Δ*tsc2* did not show a growth defect on EMM. We found that 972Δ*tsc1* and 972Δ*tsc2* are resistant to 60 μg/ml canavanine, a toxic analog of arginine (Fig. 2A). This dose of canavanine was toxic to the wild-type 972 strain. To determine whether the canavanine resistance was the result of decreased uptake, the uptake of [³H]arginine was measured. After 10 min, arginine uptake was ~3.5-fold less in the 972Δ*tsc1* and 972Δ*tsc2* strains compared with wild-type 972 (Fig. 2B), indicating that the canavanine resistance is the result of decreased uptake.

Dominant Negative Rhb1 Can Rescue the Arginine Uptake in *ura4*Δ*tsc2*—A recent screen in *S. pombe* identified a dominant negative Rhb1 mutation, Rhb1-D60K, that is unable to bind GTP or GDP (32). We generated this mutation in the pSLF373-*ura4*⁺ expression vector and crossed Δ*tsc2* into the *ura4*-D18 strain to allow selection for cells expressing from the pSLF373-*ura4*⁺ plasmid. We found that the decreased arginine uptake in Δ*tsc2* was restored by expression of Rhb1-D60K but not by wild-type Rhb1 (Fig. 2C), suggesting that arginine uptake is regulated through Tsc1, Tsc2, and Rhb1 in *S. pombe*.

A Missense Mutation in the GAP Domain of *tsc2*⁺ Does Not Rescue the Conditional Lethality or Arginine Uptake in Δ*tsc2*—The Tsc2 GAP domain in *S. pombe* is 39% identical to the GAP domain in human tuberlin, and the conserved residues include the sites of three patient-derived TSC2 missense mutations (Fig. 3A). To determine whether these residues are crucial for the function of Tsc2 in *S. pombe*, we constructed one of them, Tsc2-N1191K, which corresponds with N1643K in human, in the HA-tagged pSLF373-*ura4*⁺ expression vector. Western blot analysis showed protein expression for both Tsc2 and Tsc2-N1191K (Fig. 3B). The Tsc2 and Tsc2-N1191K expression constructs were transformed next into F15Δ*tsc2*, and cells were plated on EMM plates without uracil but with 50 μg/ml leucine, adenine, and histidine. The wild-type Tsc2 expression construct restored growth, but no growth was detected when the Tsc2-N1191K mutation was expressed (Fig. 3C). We next asked

TABLE III
Genes downregulated at least 1.5-fold in 972Δ*tsc1* and 972Δ*tsc2*

Gene name	Fold change ^a		Predicted function ^b
	972Δ <i>tsc1</i>	972Δ <i>tsc2</i>	
<i>isp5</i>	3.6	2.6	Amino acid permease
<i>c869.10c</i>	1.6	1.5	Amino acid permease
<i>p7G5.06</i>	1.7	1.5	Amino acid permease
<i>isp4</i>	4.4	3.7	Oligopeptide transporter
<i>ptr2</i>	2.4	2.3	Oligopeptide transporter
<i>c409.08</i>	1.6	1.5	Polyamine transporter
<i>c794.04c</i>	3.3	2.3	Polyamine transporter
<i>c11D3.18c</i>	3.4	2.5	Vitamin/cofactor transporter
<i>c1039.10</i>	2.3	1.8	Translation initiation inhibitor
<i>c11D3.14c</i>	3.6	2.3	5-Oxoprolinase
<i>c11D3.15</i>	3.7	2.3	5-Oxoprolinase
<i>c2H10.01</i>	2.1	1.6	Transcription factor
<i>c1223.09</i>	1.7	1.6	Urate oxidase
<i>c5H10.01</i>	6.7	3.1	Unknown

^a Fold change is the average of four independent comparisons and was only included when all four fold changes were >1.5.

^b Predicted function derived from Proteome Knowledge Library.

whether re-introducing Tsc2-N1191K could revert the canavanine resistance in the *ura4*Δ*tsc2* strain. Wild-type Tsc2 restored the canavanine sensitivity, whereas Tsc2-N1191K did not (Fig. 3D). The decreased arginine uptake was similarly rescued by wild-type Tsc2 but not by Tsc2-N1191K (Fig. 3E). These results suggest that the function of Tsc2 in regulating arginine uptake requires the GAP domain and support the use of *S. pombe* as a model system for human TSC.

Δ*tsc1* and Δ*tsc2* Show a Significant Overlap in Expression Profile—To elucidate the mechanism through which *tsc1*⁺ and *tsc2*⁺ regulate amino acid uptake, we compared the expression profile of 972Δ*tsc1* and 972Δ*tsc2*. Total RNA was isolated from 972, 972Δ*tsc1*, and 972Δ*tsc2* yeast, labeled, and hybridized to cDNA arrays (Eurogentec, Liege, Belgium). The expression profile of 972Δ*tsc1* was compared with 972 on two separate arrays including a dye-flip experiment. The 972–972Δ*tsc2* comparison was completed using the same design. Because all four arrays showed a linear relation between Cy3 and Cy5, a linear regression normalization was applied to the data (30). In addition, as shown in Fig. 4A, the dye-flip experiment for Δ*tsc1* was highly correlated. The expression data were also validated by the absence of *tsc1*⁺ expression in the Δ*tsc1* and absence of *tsc2*⁺ in the Δ*tsc2* arrays, serving as internal controls.

There was a high degree of overlap in expression profile between 972Δ*tsc1* and 972Δ*tsc2*. In total, 14 genes were down-regulated at least 1.5-fold and 26 genes were up-regulated at least 1.5-fold both in 972Δ*tsc1* and 972Δ*tsc2* (Fig. 4B). Table II lists the genes that were up-regulated at least 1.5-fold in both Δ*tsc1* and Δ*tsc2*. Many of the up-regulated genes have predicted roles in iron transport and amino acid metabolism including the arginase gene, *car1*⁺.

Table III lists the genes that were down-regulated at least 1.5-fold in both Δ*tsc1* and Δ*tsc2*. Many of the down-regulated genes were putative transporters including three amino acid permeases, two oligopeptide transporters, two polyamine transporters, and one with homology to vitamin/cofactor transporters. Interestingly, the three down-regulated amino acid permeases had homology to the Gap1p (general amino acid permease) in *Saccharomyces cerevisiae*. The expression change for these three permeases was confirmed by Northern blotting (Fig. 4C). The fold change on the Northern blot was determined by densitometry and was in each case slightly greater than the fold change on the array, further validating the array result. These data support that Tsc1 and Tsc2 function in the same pathway in *S. pombe* and suggest that they have a central role in the regulation of the biosynthesis and uptake of amino acids, oligopeptides, and polyamines.

A

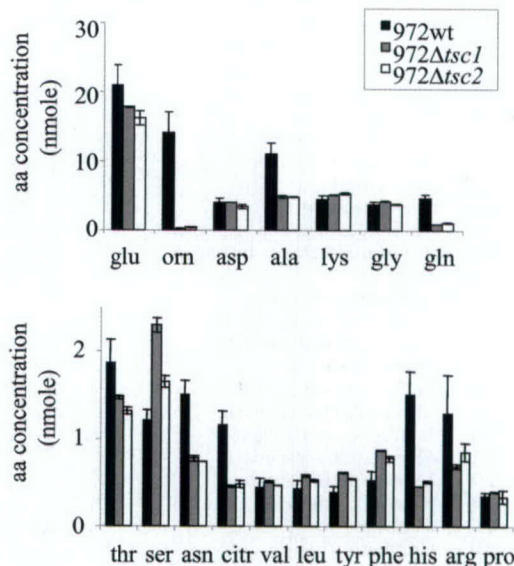
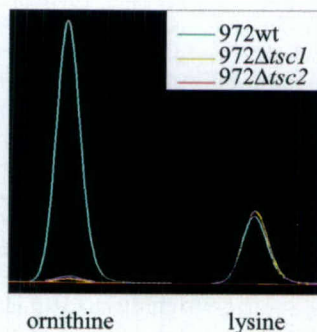
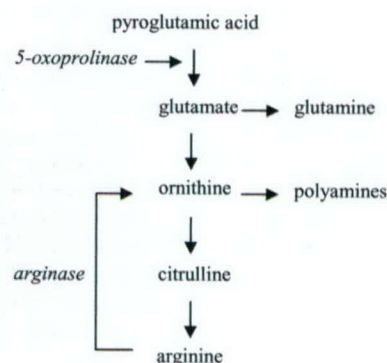


FIG. 5. Intracellular amino acid levels are low in the 972Δ*tsc1* and 972Δ*tsc2*. A, intracellular amino acid levels in 972Δ*tsc1* and 972Δ*tsc2* were compared with 972 wild-type yeast. A decrease of at least 40% was detected for alanine, asparagine, histidine, glutamine, ornithine, citrulline, and arginine in 972Δ*tsc1* and 972Δ*tsc2*. Two biological replicates were run for each sample, and similar results were seen in two independent experiments. B, ornithine and lysine amino acid profile in wild-type 972, 972Δ*tsc1*, and 972Δ*tsc2*. Ornithine levels were greatly decreased in 972Δ*tsc1* and 972Δ*tsc2*, whereas lysine levels were similar for wild-type 972, 972Δ*tsc1*, and 972Δ*tsc2*. C, arginine metabolism in *S. pombe*. Enzymes are in *italic*. Arginase converts arginine into ornithine, a precursor of polyamines.

B



C



Intracellular Amino Acid Concentrations Are Decreased in 972Δ*tsc1* and 972Δ*tsc2*—The down-regulation of permease expression and decreased uptake of amino acids in the 972Δ*tsc1* and 972Δ*tsc2* strains could represent an appropriate response to high intracellular amino acid concentrations. However, we found that the intracellular levels of multiple amino acids were low in 972Δ*tsc1* and 972Δ*tsc2* compared with 972 wild-type yeast (Fig. 5A). Ornithine, which is a product of both glutamate and arginine metabolism, showed the largest relative decrease from ~15 nM in wild-type 972 to nearly undetectable levels in 972Δ*tsc1* and 972Δ*tsc2*, whereas lysine was not changed (Fig. 5B). A decrease of at least 40% was detected for alanine, asparagine, histidine, glutamine, ornithine, citrulline, and arginine. Interestingly, the latter four are linked to arginine biosynthesis (Fig. 5C). The low intracellular amino acid levels, combined with the low amino acid permease expression levels and the decreased arginine uptake, strongly suggest that yeast lacking *tsc1*⁺ or *tsc2*⁺ have an intrinsic defect in amino acid sensing.

DISCUSSION

We report here that *S. pombe* lacking *tsc1*⁺ or *tsc2*⁺ have defects in amino acid transport, involving not only the permease localization reported previously (24) but also decreased

expression of amino acid permeases, decreased uptake of arginine, and low intracellular amino acid levels.

The decreased uptake of arginine in the Δ*tsc2* cells could be restored by expressing wild-type *tsc2*⁺ but not by expressing the *tsc2*⁺ gene carrying a mutation in the highly conserved GAP domain. This mutation is homologous to the patient-derived N1643K. Interestingly, the small GTPase Rheb was recently identified as the key target of the GAP domain of the TSC2 gene product, tuberlin, in mammals and *Drosophila* (5–7, 9). From previous studies, it was known that Rbh1 regulates arginine uptake in *S. pombe* (25) as well as in *S. cerevisiae* (31). We found that the arginine uptake defect in the Δ*tsc2* yeast was rescued by expression of a dominant negative form of *S. pombe* Rbh1, D60K. The rescue by Rbh1-D60K suggests that Rbh1 is downstream of Tsc2 in *S. pombe* as well as in other species and further strengthens the relevance of the *S. pombe* model to human TSC.

Previously, the mislocalization of an amino acid permease *c359.03*⁺ (GenBankTM accession number CAB91572) in Δ*tsc1* and Δ*tsc2* was postulated to be the result of aberrant protein trafficking (24). However, we found that the expression of three other amino acid permeases with high homology to the general amino acid permease (Gap1p) in *S. cerevisiae* were down-regulated both in Δ*tsc1* and Δ*tsc2* cells. The permease *c359.03*⁺

was not down-regulated in $\Delta tsc1$ or $\Delta tsc2$, suggesting that permeases are regulated at both the transcriptional and post-translational levels in $\Delta tsc1$ and $\Delta tsc2$. In *S. cerevisiae*, decreased expression of *GAP1* and sorting of Gap1p from the plasma membrane to the vacuole are the appropriate response to high levels of intracellular amino acids (33). In contrast, the decreased permease expression in $\Delta tsc1$ or $\Delta tsc2$ yeast was associated with low intracellular amino acids including alanine, asparagine, histidine, glutamine, ornithine, citrulline, and arginine. The inability to respond appropriately to low amino acid levels suggests that Tsc1 and Tsc2 play a role in amino acid sensing and would argue that the expression levels of permeases as well as their localization are crucial in coordinating sensing and growth in *S. pombe*.

Altered intracellular amino acid levels have not been detected in mammalian cells lacking tuberlin or hamartin, although to our knowledge only limited studies have looked into this phenomenon. The only study published so far measured the levels of valine, leucine, phenylalanine, and lysine in *Drosophila* S2 cells treated with TSC2 small interference RNA (17). A difference in intracellular levels was not detected, but those four amino acids were not changed in *S. pombe* lacking *tsc1*⁺ and *tsc2*⁺.

The expression profile of $\Delta tsc1$ and $\Delta tsc2$ cells showed extensive overlap, consistent with similar phenotypes of *TSC1* and *TSC2* mutations in humans, rodents, *Drosophila*, and *S. pombe*. In addition to the down-regulated amino acid permeases, two enzymes linked to arginine biosynthetic pathways were differentially expressed: arginase and 5-oxoprolinase. Two genes with homology to mammalian 5-oxoprolinase were down-regulated in both $\Delta tsc1$ and $\Delta tsc2$. 5-Oxoprolinase hydrolyzed pyroglutamic acid to glutamate and was down-regulated in some human tumors (34). Arginase was up-regulated in both $\Delta tsc1$ and $\Delta tsc2$ despite the low intracellular arginine levels. Arginase plays an important role in the production of ornithine (35), so the increase in arginase mRNA could be a response to the drop in ornithine levels from 15 nM in wild type to nearly undetectable levels in the $\Delta tsc1$ and $\Delta tsc2$ strains. Ornithine is the precursor of polyamines including spermidine. Spermidine is essential for growth and cell cycle progression in *S. pombe* (36). Polyamines are also critical to the growth and differentiation of mammalian cells and are elevated in many human cancers (37, 38). Finally, arginase is important in mammalian cells because it competes with nitric-oxide synthetase for arginine, which is the substrate for both arginase and nitric-oxide synthetase. In mammalian cells, nitric oxide is a key second messenger regulating many processes including neuronal signaling (39). It will clearly be important to determine whether expression of permeases, arginase, or 5-oxoprolinase is regulated by mammalian *TSC1* and *TSC2*.

Mice with conditional inactivation of Tsc1 in brain astrocytes develop seizures (40). Seizures are a major clinical problem in TSC, affecting 80% of patients, and are often refractory to treatment. Interestingly, the *Tsc1*^{-/-} astrocytes have decreased uptake of the excitatory neurotransmitter glutamate and decreased expression of two glutamate transporters (40). It is postulated that reduced astrocyte clearance of glutamate from the synaptic cleft slows the decay of excitatory stimuli, lowering the seizure threshold. If the decreased glutamate uptake is mechanistically related to the decreased amino acid uptake in *S. pombe*, the yeast model could provide a novel system for the study of epilepsy.

In conclusion, our data show for the first time that Tsc1 and Tsc2 regulate arginine uptake and arginine biosynthesis in *S. pombe*. Rescue of the arginine uptake defect by a dominant negative form of Rhb1 suggests that Rhb1 is downstream of Tsc2 in *S. pombe* as well as in other species. The complexity of the amino acid phenotype is suggestive of an intrinsic defect in amino

acid sensing, involving amino acids and enzymes closely linked to ornithine and arginine. If similar pathways are affected in mammalian cells lacking *TSC1* or *TSC2*, defects in polyamines and/or nitric oxide levels could be pathogenically linked to the clinical manifestations of TSC, including refractory seizures.

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