Tsc1\(^+\) and tsc2\(^+\) regulate arginine uptake and metabolism in

*Schizosaccharomyces pombe*

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Running Title: Regulation of arginine by tsc1\(^+\) and tsc2\(^+\) in *S. pombe*
SUMMARY

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 tuberin’s GTPase activating domain in mammalian cells and in Drosophila. We show that the defect in arginine uptake in cells lacking tsc2+ is rescued by 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.
INTRODUCTION

Tuberous sclerosis complex (TSC) 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 to 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 (TOR), 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 (AMPK) (23).

Schizosaccharomyces pombe (S. 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\(^+\) (Δtsc1) or tsc2\(^+\) (Δtsc2) 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 Δtsc1 and Δtsc1 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 Δtsc1 and Δtsc2 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.

\(^1\)The abbreviations used are: TSC, tuberous sclerosis complex; GAP, GTPase activating domain; TOR, target of rapamycin; Rheb, ras homolog enriched in brain; *S. pombe*, *Schizosaccharomyces pombe*; EMM, essential minimal medium; YES, yeast extract medium with supplements
EXPERIMENTAL PROCEDURES

Yeast Strains, Media and Growth Conditions. The yeast strains used in this study are listed in Table 1. CHP428 and CHP429 were constructed by Charlie Hoffman (Boston College) and were a gift from Janet Leatherwood (Stony Brook). 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 µg/ml of 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 (gift from J. Bähler, Sanger Institute) 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 haploid strain CHP429 (*hi, leu1-32, ura4-D18, ade6-216, his7-366*) and replaced by the kanamycin cassette to create MVS5. We will refer to Δ*tsc1* with this genotype as F15Δ*tsc1*. Δ*tsc2* was constructed using identical strategy and resulted in MVS6, which will be referred to as F15Δ*tsc2* in figures and text. Correct integration of the kanamycin cassette into the yeast genome was confirmed by PCR over the integration site, Southern blotting and sequencing. Subsequently, F15Δ*tsc1* and F15Δ*tsc2* were crossed into the *ura4*-D18 strain to generate MVS3 (*ura4Δ*tsc1*) and MVS4 (*ura4Δ*tsc2*) and into 972 wild-type to generate MVS1 (972Δ*tsc1*) and MVS2 (972Δ*tsc2*) 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 (gift from J. Bähler, Sanger Institute) 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 \( nmt \) (no message in thiamin) promoter strength (ATCC). The GAP (Tsc2-N1292K) and the Rhb dominant negative (Rhb-D60K) mutations were introduced into the pSLF373 constructs using site-directed mutagenesis (Stratagene, La Jolla, CA). All constructs were verified by sequencing.

Expression Profiling. Yeast were grown overnight in EMM to early log phase (OD\(_{595}\)=0.2-0.3) and total RNA was isolated by phenol extraction and purified using RNeasy (Qiagen, Valencia, CA). Total RNA of three independent biological samples was pooled (10 \( \mu \)g of each sample), reverse transcribed into cDNA and labeled with Cy3 and Cy5 (Amersham Biosciences, Piscataway, NJ). Hybridizations were carried out overnight at 42\( ^\circ \)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 3.5x 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 midlog phase (OD<sub>595</sub>=0.4-0.6) and washed once in ice-cold buffer containing 50mM Tris-HCl pH 7.5, 25mM NaCl, and 0.1mM phenylmethylsulfonyl fluoride. Cells were lysed in the same buffer with 0.5 mm glass beads in a BeadBeater (Biospec Products, Bartlesville, OK). 20 µg of each sample was run on 4-20% SDS-Page gel (Bio-Rad, Hercules, CA) and transferred to Nitrocellulose using standard methods. The immobilized proteins were detected using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). Antibodies used were anti-HA (Roche Applied Science, Indianapolis, IN) and monoclonal TAT1 for *S. pombe* tubulin (gift from K. Gull, University of Manchester).

**Northern Blot Analysis.** Ten micrograms of total RNA was run on a 1% formaldehyde gel at 60V for 4 hours and transferred to Nylon membrane overnight in 20X SSC. Probes for p7G5.06<sup>+</sup>, c869.10<sup>+</sup>, isp5<sup>+</sup> and gpd3<sup>+</sup> were PCR amplified from cDNA, cleaned over 0.8% agarose gel and labeled with α<sup>32</sup>P-dCTP (Perkin Elmer, Wellesley, MA) using standard methods. Hybridizations were performed in rapid hybridization buffer (Amersham Biosciences, Piscataway, NJ).

**Canavanine Sensitivity.** Cells were grown overnight to midlog phase (OD<sub>595</sub>=0.4-0.6) and OD<sub>595</sub> was adjusted to 0.4 (10,000 cells/µl). 4 µl of 1X, 10X and 100X dilutions was spotted onto EMM as a growth control, or EMM containing canavanine (60 µg/ml) (Sigma, St. Louis, MO) and incubated for three 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 midlog phase. One µCi of L-[<sup>3</sup>H]-arginine (40-70 Ci/mmol) (Perkin Elmer, Wellesley, MA) and 100µM of non-radioactive arginine (Sigma, St. Louis, MO) were added to 25,000 cells in 600 µl of EMM. 200 µl aliquots were removed at 0 and 10 minutes, 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. $^3$H-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, Hercules, CA), and diluted to 1 µg/µl. Proteins were precipitated by treatment of 100µl of sample with 100µl of 10% 5-sulphosalicylic acid at 4°C for 1 hour. The pH of the supernatant was adjusted to 2.2 with 3M LiOH. 100 µl of sample was injected into the Biochrom 30 amino acid analyzer (Biochrom, Cambridge, UK) including a 10 nM standard amino acid mixture (Sigma, St. Louis, MO).
RESULTS

F15∆tsc1 and F15∆tsc2 have growth defect.

As a first step towards understanding the physiological functions of \( tsc1^+ \) and \( tsc2^+ \), we disrupted \( tsc1^+ \) and \( tsc2^+ \) in the \( S. \ pomb\ e \) genome by one-step gene replacement. To initiate phenotypic analysis, the F15∆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\( \mu \)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 (data not shown). These smaller colonies were found by PCR to be \( \Delta tsc1 \). Similar results were obtained for the F15∆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∆tsc1 and F15∆tsc2 was approximately 5 hours compared to 3.5 hours for the F15 strain (Figure 1A). To test if growth was further affected by temperature stress, F15∆tsc1 and F15∆tsc2 were plated on YES plates and incubated at 25\( ^\circ \)C and 37\( ^\circ \)C. No temperature-induced growth defect was observed in the F15∆tsc1 and F15∆tsc2 strains (Figure 1B).

F15∆tsc1 and F15∆tsc2 are conditionally lethal.

We found a more severe growth defect in the F15∆tsc1 and F15∆tsc2 strains when they were grown on essential minimal medium (EMM) plates. F15∆tsc1 and F15∆tsc2 yeast could not grow on EMM supplemented with normal amounts (50 \( \mu \)g/ml) uracil, histidine, adenine and leucine, but increasing supplements to 1000 \( \mu \)g/ml partially restored growth (Figure 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 due to deletion of the \( tsc1^+ \) and \( tsc2^+ \) genes, Tsc1 and Tsc2, expressed from a plasmid with \( ura4^+ \), were transformed into the F15∆tsc1 and
F15Δtsc2 strains and were plated on EMM without uracil. Expression of Tsc1 restored growth in F15Δtsc1 yeast, but failed to rescue F15Δtsc2, while Tsc2 expressed restored growth in F15Δtsc2, but not in F15Δtsc1 (Figure 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. Since F15Δtsc1 and F15Δtsc2 have a growth defect, we crossed Δtsc1 and Δtsc2 into the 972 background. This strain does not require amino acid supplements and 972Δtsc1 and 972Δtsc2 did not show a growth defect on EMM. We found that 972Δtsc1 and 972Δtsc2 are resistant to 60 µg/ml canavanine, a toxic analog of arginine (Figure 2A). 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 972Δtsc1 and 972Δtsc1 strains compared to wild-type 972 (Figure 2B), indicating that the canavanine resistance is due to decreased uptake.

**Dominant negative Rhb1 can rescue the arginine uptake in ura4Δtsc2.**

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 Δtsc2 into the ura4-D18 strain to allow selection for cells expressing from the pSLF373-ura4+ plasmid. We found that the decreased arginine uptake in Δtsc2 was restored by expression of Rhb1-D60K, but not by wild-type Rhb1 (Figure 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 \( \Delta tsc2 \).

The Tsc2 GAP domain in \( S. pombe \) is 39% identical to the GAP domain in human tuberin, and the conserved residues include the sites of 3 patient-derived \( TSC2 \) missense mutations (Figure 3A). 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 (Figure 3B). Next, the Tsc2 and Tsc2-N1292K expression constructs were transformed into F15\( \Delta tsc2 \) and cells were plated on EMM plates without uracil, but with 50 \( \mu \)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 (Figure 3C). We next asked whether re-introducing Tsc2-N1292K could revert the canavanine resistance in the \( ura4\Delta tsc2 \) strain. Wild-type Tsc2 restored the canavanine sensitivity, while Tsc2-N1292K did not (Figure 3D). The decreased arginine uptake was similarly rescued by wild-type Tsc2 but not by Tsc2-N1292K (Figure 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.

\( \Delta tsc1 \) and \( \Delta tsc2 \) 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\( \Delta tsc1 \) and 972\( \Delta tsc2 \). Total RNA was isolated from 972, 972\( \Delta tsc1 \) and 972\( \Delta tsc2 \) yeast, labeled, and hybridized to cDNA arrays (Eurogentec, Belgium). The expression profile of 972\( \Delta tsc1 \) was compared to 972 on two separate arrays, including a dye-flip experiment. The 972-972\( \Delta tsc2 \) 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 (30). In addition, as shown in Figure 4A, the dye-flip experiment for ∆tsc1 was highly correlated. The expression data was also validated by the absence of tsc1+ expression in the ∆tsc1 and absence of tsc2+ in the ∆tsc2 arrays, serving as internal controls.

There was a high degree of overlap in expression profile between 972∆tsc1 and 972∆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∆tsc1 and 972∆tsc2 (Figure 4B). Table 2 lists the genes that were upregulated at least 1.5 fold in both ∆tsc1 and ∆tsc2. Many of the upregulated genes have predicted roles in iron transport and amino acid metabolism, including the arginase gene, car1+

Table 3 lists the genes that were downregulated at least 1.5 fold in both ∆tsc1 and ∆tsc2. 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 (Figure 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.

**Intracellular amino acid concentrations are decreased in 972∆tsc1 and 972∆tsc2.**

The downregulation of permease expression and decreased uptake of amino acids in the 972∆tsc1 and 972∆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 972Δtsc1 and 972Δtsc2 compared to 972 wild-type yeast (Figure 5A).

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 972Δtsc1 and 972Δtsc, while lysine was not changed (Figure 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 (Figure 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 ∆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, tuberin, in mammals and *Drosophila* (5-7,9). From previous studies it was known that Rhb1 regulates arginine uptake in *S. pombe* (25) as well as *S. cerevisiae* (31). We found that the arginine uptake defect in the ∆tsc2 yeast was rescued by expression of a dominant negative form of *S. pombe* Rhb1, D60K. The rescue by Rhb1-D60K suggests that Rhb1 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*⁺ (accession No. CAB91572) in ∆tsc1 and ∆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 downregulated both in ∆tsc1 and ∆tsc2 cells. The permease *c359.03*⁺ was not downregulated in ∆tsc1 or ∆tsc2, suggesting that permeases are regulated at both the transcriptional and post-translational levels in ∆tsc1 and ∆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 ∆tsc1 or ∆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 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 tuberin 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 siRNA (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 Δtsc1 and Δtsc2 cells showed extensive overlap, consistent with similar phenotypes of TSC1 and TSC2 mutations in humans, rodents, Drosophila, and S. pombe. In addition to the downregulated 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 downregulated in both Δtsc1 and Δtsc2. 5-oxoprolinase hydrolyzes pyroglutamic acid to glutamate, and is downregulated in some human tumors (34). Arginase was upregulated in both Δtsc1 and Δtsc2, 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 Δtsc1 and Δtsc2 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 (NOS) for arginine, which is the substrate for both arginase and NOS. 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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FIGURE LEGENDS

Figure 1. F15\textit{Δtsc1} and F15\textit{Δtsc2} exhibit growth defects.

(A) F15, F15\textit{Δtsc1} and F15\textit{Δtsc2} strains were grown in rich liquid medium with supplements (YES) overnight to midlog phase (OD\textsubscript{595} = 0.4-0.6) and diluted to OD\textsubscript{595} = 0.2. Cells were grown for an additional 6 hours and the generation time was determined between 3 and 6 hours. The F15\textit{Δtsc1} and F15\textit{Δtsc2} needed approximately 5 hours to complete one generation compared to 3.5 hours for wild-type strains (B) 40,000 F15, F15\textit{Δtsc1} and F15\textit{Δtsc2} cells were spotted on YES + G418 (200 µg/ml). F15\textit{Δtsc1} and F15\textit{Δtsc2} were G418 resistant as expected. The cells were also spotted on YES plates and grown at 25°C, 30°C and 37°C for 3 days. No growth differences were seen between F15, F15\textit{Δtsc1} and F15\textit{Δtsc2}. (C) F15, F15\textit{Δtsc1} and F15\textit{Δtsc2} cells were spotted (40,000 cells) onto essential minimal medium (EMM) plates with different amounts of adenine, leucine, histidine and uracil. At regular amounts (50 µg/ml) the F15\textit{Δtsc1} and F15\textit{Δtsc2} 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 \textit{(ura4')} were transformed into F15\textit{Δtsc1} and F15\textit{Δtsc2} and plated onto EMM plates supplemented with 50 µg/ml leucine, adenine and histidine. pREP4X-Tsc1 expression rescued the growth of F15\textit{Δtsc1}, and pREP4X-Tsc2 expression rescued the growth of F15\textit{Δtsc2}. 
Figure 2. The 972Δtsc1 and 972Δtsc2 have a decreased uptake of arginine, which can be restored in the 972Δtsc2 by expressing a dominant negative Rhb1 mutation.

(A) 972 wild-type, 972Δtsc1 and 972Δtsc2 were grown in EMM without supplements overnight to midlog phase and cells were diluted to OD₅₉₅=0.4. Three different dilutions (40,000-4000-400 cells) were spotted on EMM plates with and without 60 µg/ml canavanine. Plates were incubated at 30°C for 3 days. Canavanine killed wild-type cells, but 972Δtsc1 and 972Δtsc2 were resistant to 60 µg/ml canavanine. (B) Cells were grown till midlog phase (OD₅₉₅=0.5) in EMM and 25,000 cells were resuspended in 100 µM of L-arginine with 1 µCi of L-H³ arginine (40-70 Ci/mmol) in 600 µl. Aliquots of 200 µl were injected into 5 ml of EMM in a vacuum manifold at 0 and 10 minutes, washed twice with 5 ml of deionized water and assayed for L-H³ arginine uptake. The arginine uptake was 3.5-fold less in the 972Δtsc1 and 972Δtsc2 strains compared to 972 wild-type. Experiments were done in triplicate and similar results were seen in two independent experiments. (C) The arginine uptake was measured for the ura4Δtsc2 strain expressing either 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.
Figure 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-N1292K 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-N1292K were transformed into the F15Δtsc2 strain and plated onto EMM plates supplemented with 50 µg/ml of leucine, adenine, histidine, without uracil. Tsc2 expression rescued growth, while the Tsc2-N1292K mutation did not. (D) Canavanine sensitivity was measured in *ura4Δtsc2* expressing either empty vector, Tsc2, or Tsc2-N1292K, from a plasmid with *ura4*+. Tsc2 expression restored the canavanine sensitivity in Δtsc2, while Tsc2-N1292K expression did not. (E) Arginine uptake was measured in *ura4Δtsc2* expressing either empty vector, Tsc2 or Tsc2-N1292K. The uptake defect was rescued by expressing Tsc2, but not by Tsc2-N1292K.
Figure 4. 972Δtsc1 and 972Δtsc2 show a significant overlap in expression profile.

(A) Correlation plot of the average gene expression ratios from the Δtsc1 dyeflip experiment. (B) Expression profiles were compared between 972, 972Δtsc, and 972Δtsc2. At fold change > 1.5, there were 14 downregulated and 26 upregulated 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 downregulated in 972Δtsc1 and 972Δtsc2, consistent with the array result.

Figure 5. Intracellular amino acid levels are low in the 972Δtsc1 and 972Δtsc2.

(A) Intracellular amino acid levels in 972Δtsc1 and 972Δtsc2 were compared to 972 wild-type yeast. A decrease of at least 40% was detected for alanine, asparagine, histidine, glutamine, ornithine, citrulline and arginine in 972Δtsc1 and 972Δtsc2. 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 972 wild-type, 972Δtsc1 and 972Δtsc2. Ornithine levels were greatly decreased in 972Δtsc1 and 972Δtsc2, while lysine levels were similar for 972 wild-type, 972Δtsc1 and 972Δtsc2. (C) Arginine metabolism in S. pombe. Enzymes are in italic. Arginase converts arginine into ornithine, a precursor of polyamines.
| Strain   | Genotype                        | Source         |
|----------|---------------------------------|----------------|
| 972      | \( h^- \)                       | Leupold        |
| MVS1     | \( h^- \), \( \Delta tsc1::kan^+ \) | This study     |
| MVS2     | \( h^- \), \( \Delta tsc2::kan^+ \) | This study     |
| ura4-D18 | \( h^+ \), \( ura4-D18 \)       | Grimm and Kohli|
| MVS3     | \( h^+ \), \( ura4-D18, \Delta tsc1::kan^+ \) | This study     |
| MVS4     | \( h^+ \), \( ura4-D18, \Delta tsc2::kan^+ \) | This study     |
| CHP428   | \( h^+ \), \( leu1-32, ura4-D18, ade6-210, his7-366 \) | Hoffman       |
| CHP429   | \( h^+ \), \( leu1-32, ura4-D18, ade6-216, his7-366 \) | Hoffman       |
| MVS5     | \( h^- \), \( leu1-32, ura4-D18, ade6-216, his7-366, \Delta tsc1::kan^+ \) | This study     |
| MVS6     | \( h^- \), \( leu1-32, ura4-D18, ade6-216, his7-366, \Delta tsc2::kan^+ \) | This study     |
| Gene name | fold change<sup>1</sup> | fold change<sup>1</sup> | predicted function<sup>2</sup> |
|-----------|------------------|------------------|------------------|
|           | $\Delta tsc1$  | $\Delta tsc2$   |                  |
| bfr1      | 1.8             | 1.9             | ABC transporter  |
| c359.05   | 1.6             | 1.6             | ABC transporter  |
| c569.05c  | 1.9             | 1.7             | sugar transporter|
| c1020.03  | 1.7             | 1.6             | iron ion transporter|
| fio1      | 1.5             | 1.8             | iron permease    |
| fip1      | 1.7             | 1.8             | iron permease    |
| c750.05c  | 2.5             | 1.9             | vitamin/cofactor transporter|
| c1002.16c | 2.4             | 1.8             | vitamin/cofactor transporter|
| car1      | 3.1             | 2.6             | arginase         |
| plr       | 1.5             | 2.2             | pyridoxal reductase|
| c29B12.04 | 1.9             | 1.7             | pyridoxine biosynthesis|
| c5H10.10  | 2.8             | 2.0             | NADPH dehydrogenase|
| c750.01   | 2.1             | 3.6             | oxidoreductase   |
| p8B7.18c  | 2.3             | 2.2             | phosphomethylpyrimidine kinase|
| c977.14c  | 2.0             | 3.3             | alcohol dehydrogenase|
| c9E9.09c  | 2.0             | 1.9             | aldehyde dehydrogenase|
| pB24D3.08c| 1.6             | 2.1             | alcohol dehydrogenase|
| c1271.07c | 2.3             | 1.7             | N-acetyltransferase|
| c21E11.04 | 3.1             | 1.7             | N-acetyltransferase|
| obr1      | 2.1             | 2.9             | DNA binding      |
| c530.07c  | 2.4             | 2.0             | unknown          |
| c70.08c   | 4.0             | 2.0             | unknown          |
| c1348.02  | 2.0             | 1.6             | unknown          |
| c186.05c  | 8.7             | 8.1             | unknown          |
| c977.01   | 2.2             | 1.8             | unknown          |
| c1271.08c | 2.2             | 1.7             | unknown          |

<sup>1</sup>fold change is the average of four independent comparisons and was only included when all four fold changes were >1.5

<sup>2</sup>predicted function derived from Proteome Knowledge Library
| Gene name | fold change<sup>1</sup> | fold change<sup>1</sup> | predicted function<sup>2</sup> |
|-----------|-------------------------|-------------------------|-------------------------------|
| isp5      | 3.6                     | 2.6                     | amino acid permease           |
| c869.10c  | 1.6                     | 1.5                     | amino acid permease           |
| p7G5.06   | 1.7                     | 1.5                     | amino acid permease           |
| isp4      | 4.4                     | 3.7                     | oligo peptide transporter     |
| ptr2      | 2.4                     | 2.3                     | oligo peptide transporter     |
| c409.08   | 1.6                     | 1.5                     | polyamine transporter         |
| c794.04c  | 3.3                     | 2.3                     | polyamine transporter         |
| c11D3.18c | 3.4                     | 2.5                     | vitamin/cofactor transporter   |
| c1039.10  | 2.3                     | 1.8                     | translation initiation inhibitor |
| c11D3.14c | 3.6                     | 2.3                     | 5-oxoprolinase                |
| c11D3.15  | 3.7                     | 2.3                     | 5-oxoprolinase                |
| c2H10.01  | 2.1                     | 1.6                     | transcription factor          |
| c1223.09  | 1.7                     | 1.6                     | urate oxidase                 |
| c5H10.01  | 6.7                     | 3.1                     | unknown                       |

<sup>1</sup>fold change is the average of four independent comparisons and was only included when all four fold changes were >1.5

<sup>2</sup>predicted function derived from Proteome Knowledge Library
Figure 1
**Figure 2**

A) A comparison of growth on plates with EMM and EMM + canavanine for strains 972, 972Δtsc1, and 972Δtsc2.

B) Bar graph showing the levels of labeled arginine (3H-Arg (cpm)) at t=0 min and t=10 min for 972wt, 972Δtsc1, and 972Δtsc2.

C) Bar graph showing the levels of labeled arginine (3H-Arg (cpm)) at t=0 min and t=5 min for strains ura4Δtsc2, ura4Δtsc2 tsc2+, ura4Δtsc2 rhbwt+, and ura4Δtsc2 rhbD60K+.
**Figure 3**

**A**

|             | N1643K | N1651S | P1675L |
|-------------|--------|--------|--------|
| human       | *      | *      | *      |
| rat         | FHIATLMPTKDVHKHRC-KKRHLGGDFVSIVY\(NDSGEDFKLGTIKQFNVFHVHTP\) |
| fly         | FHVATLMPTNLQDDPNCNEKKSHICDFVVIY\(NESGEEYNLNTISGQFYACVIVEP\) |
| yeast       | FHCTTMMPNIEHDPGCTLKKRHLGGDFVTIIF\(NESGLEYDFDTSQFNFVINVETP\) |

**B**

- **N1292K**
  - **Tsc2**
  - **vector**

**C**

- ura4-D18
  - u4\(\Delta\)tsc2 vector
  - u4\(\Delta\)tsc2 u4\(\Delta\)tsc2 vector

**D**

- EMM
  - EMM + canavanine
  - vector
  - tsc2
  - N1292K

**E**

| 3H-Arg (cpm) | t=0 min | t=10 min |
|--------------|---------|----------|
| ura4\(\Delta\)tsc2 vector+ | 40000   | 40000    |
| ura4\(\Delta\)tsc2 tsc2+    | 40000   | 40000    |
| ura4\(\Delta\)tsc2 tsc2-N1292K+ | 40000  | 40000    |
Figure 4

A

B

downregulated upregulated

\( \Delta tsc1 \) \( \Delta tsc2 \) \( \Delta tsc1 \) \( \Delta tsc2 \)

14 14 2

30 26 10

C

| Gene    | 972wt | 972Δtsc1 | 972Δtsc2 | FCΔtsc1 | FCΔtsc2 |
|---------|-------|----------|----------|---------|---------|
| p7G5.06 |       |          |          | -2.0    | -1.7    |
| c869.10 |       |          |          | -2.0    | -2.4    |
| isp5    |       |          |          | -6.8    | -4.7    |
| gpd3    |       |          |          | control |         |
Figure 5

(A) Graph showing amino acid concentrations.

(B) Image depicting ornithine and lysine peaks.

(C) Metabolic pathway diagram:
- ornithine → pyroglutamic acid
  - 5-oxoprolinase → glutamate → glutamine
  - arginase → citrulline
  - Ornithine → polyamines

Figure 5
Tsc1+ and tsc2+ regulate arginine uptake and metabolism in Schizosaccharomyces pombe
Marjon van Slegtenhorst, Erieka Carr, Radka Stoyanova, Warren Kruger and Elizabeth Petri Henske

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