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A Defect in Protein Farnesylation Suppresses a Loss of *Schizosaccharomyces pombe tsc2*$^+$, a Homolog of the Human Gene Predisposing to Tuberous Sclerosis Complex

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ABSTRACT

Mutations in the human *Tsc1* and *Tsc2* genes predispose to tuberous sclerosis complex (TSC), a disorder characterized by the wide spread of benign tumors. Tsc1 and Tsc2 proteins form a complex and serve as a GTPase-activating protein (GAP) for Rheb, a GTPase regulating a downstream kinase, mTOR. The genome of *Schizosaccharomyces pombe* contains *tsc1*$^+$ and *tsc2*$^+$, homologs of human *Tsc1* and *Tsc2*, respectively. In this study we analyzed the gene expression profile on a genomewide scale and found that deletion of either *tsc1*$^+$ or *tsc2*$^+$ affects gene induction upon nitrogen starvation. Three hours after nitrogen depletion genes encoding permeases and genes required for meiosis are less induced. Under the same condition, retrotransposons, G1-cyclin (*pas1*$^+$), and *inv1*$^+$ are more induced. We also demonstrate that a mutation (*cpp1-1*) in a gene encoding a β-subunit of a farnesyltransferase can suppress most of the phenotypes associated with deletion of *tsc1*$^+$ or *tsc2*$^+$. When a mutant of *rhb1*$^+$ (homolog of human *Rheb*), which bypasses the requirement of protein farnesylation, was expressed, the *cpp1-1* mutation could no longer suppress, indicating that deficient farnesylation of Rhb1 contributes to the suppression. On the basis of these results, we discuss TSC pathology and possible improvement in chemotherapy for TSC.

TUBEROUS sclerosis complex (TSC) is an autosomal dominant disorder characterized by the wide spread of benign tumors called hamartomas in different organs including the brain, eyes, heart, kidney, skin, and lungs (Kwiatkowski and Short 1994; Gomez 1995). Seizures and learning and behavioral problems, which are likely due to development of tumors in the brain, are also common in patients with TSC (Kwiatkowski and Short 1994; Gomez *et al.* 1999). Two human genes, *TSC1* and *TSC2*, are responsible for TSC (European Chromosome 16 Tuberous Sclerosis Consortium 1993; van Slegtenhorst *et al.* 1997), each of which encodes hamartin and tuberin, respectively. Inactivation of TSC1 and TSC2 causes phenotypes similar each other, suggesting that they might affect the same pathway. TSC1 and TSC2 form a heterodimer and the TSC1–TSC2 interaction appears to be important for the stability of the two proteins (Li *et al.* 2004b). Therefore, TSC1 and TSC2 are generally considered as a complex (TSC1/2) with a single biological function, and understanding functions of the TSC1/2 complex is clinically important.

Genetic studies in mammalian systems (Carbonara *et al.* 1994; Green *et al.* 1994a,b; Henske *et al.* 1996; Kwiatkowski *et al.* 2002) and Drosophila (Ito and Rubin 1999) have shown that TSC1/2 functions to inhibit cell growth as well as cellular proliferation (Hengstschlager *et al.* 2001). Appearance of giant cells within hamartomas from TSC patients and the *gigas* phenotype in the fly mutant highlight the capability of TSC1/2 in controlling cell size (Ito and Rubin 1999). Studies have shown that TSC1/2 controls cell growth/proliferation by regulating the activity of a small GTPase, RHEB (Zhang *et al.* 2003). When the environment surrounding the cell is not favorable for growth/proliferation, TSC1 and TSC2, which have a GTPase-activating protein (GAP) domain in their C-terminal region, convert RHEB into an inactive form. A kinase, mTOR is a target of RHEB and promotes protein synthesis when stimulated by RHEB GTPase (Manning and Cantley 2003; Li *et al.* 2004a,b; Pan *et al.* 2004; Long *et al.* 2005). It is postulated that formation of hamartomas in TSC is a result of abnormal regulation of RHEB GTPase. A loss of TSC1/2 would allow constitutive activation of the GTPase as well as its target, mTOR.

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TABLE 1

Strains used in this study

Strain	Genotype	Source
SP6	*h⁻ leu1-32*	Laboratory stock
AE512	*h⁻ tsc2::ura4⁺ ura4-D18 leu1-32*	Laboratory stock
AE413	*h⁻ tsc1::ura4⁺ ura4-D18 leu1-32*	Laboratory stock
YKK25	*h⁻ tsc2::ura4⁺ ura4-D18 leu1-32 cpp1-1*	This work
YKK59	*h⁻ tsc1::ura4⁺ ura4-D18 leu1-32 cpp1-1*	This work
972h⁻	*h⁻*	Laboratory stock
YKK55	*h⁻ ura4-D18 leu1-32 cpp1-1*	This work
SP740	*h⁻ ura4-D18 leu1-32*	Laboratory stock

Homologs of the mTOR kinase and RHEB can be found in lower eukaryotes. The genome of *Schizosaccharomyces pombe* contains two genes homologous to mTOR (*tor1*$^+$ and *tor2*$^+$) and a gene homologous to RHEB, *rhb1*$^+$ (Mach *et al.* 2000). It also contains genes *tsc1*$^+$ and *tsc2*$^+$, each of which corresponds to mammalian *TSC1* and *TSC2*, respectively (Matsumoto *et al.* 2002). Although the genome of *Saccharomyces cerevisiae* also encodes proteins homologous to mTOR (Cafferkey *et al.* 1994) and RHEB GTPase (Urano *et al.* 2000), it does not contain any obvious homologs to TSC1/2, suggesting that RHEB GTPase may be regulated by another mechanism.

In our previous study we showed that fission yeast strains lacking either *tsc1*$^+$ or *tsc2*$^+$ are viable in rich media, but exhibit several defects. First, deletion strains for *tsc1*$^+$ (Δ*tsc1*) and *tsc2*$^+$ (Δ*tsc2*) are defective in uptake of nutrients such as amino acids and adenine. Consistent with this defect, an amino acid permease, which is normally positioned on the plasma membrane, aggregates in the cytoplasm or is confined in vacuole-like structures in Δ*tsc1* and Δ*tsc2*. Second, Δ*tsc1* and Δ*tsc2* are unable to induce the *sxa2*$^+$ gene, which is usually expressed upon stimulation by a mating-type pheromone, *P* factor, in starved *h*$^-$ cells (Imai and Yamamoto 1994). On the basis of these phenotypes, we postulate that *tsc1*$^+$ and *tsc2*$^+$ are required for sensing/responding to starvation. We speculate that *S. pombe* Tsc1/2 regulates Tor1/2 via Rhb1 and plays a role in sensing/responding to starvation. In this study we continued to take advantage of this simple and tractable system and attempted to dissect genetic pathways to interact with Tsc1/2. Through a genetic screen of extragenic suppressors of Δ*tsc2*, we identified a gene, *cpp1*$^+$, encoding a subunit of the enzyme required for protein farnesylation.

MATERIALS AND METHODS

Yeast strains, media, and transformation: The *S. pombe* strains used in this study are listed in Table 1. The yeast cells were grown in YEA and EMM with appropriate nutrient supplements as described previously (Moreno *et al.* 1991). All yeast transformations were carried out by lithium acetate methods (Okazaki *et al.* 1990; Gietz *et al.* 1992).

Screen for an extragenic suppressor of Δ*tsc2*: The reversion rate of the AE512 strain used for the screening was 1.25×10^{-6}. The spontaneous revertants were grown at 26° for 4 days on EMM medium with leucine at 40 μg/ml. Sixty-five revertants obtained through the primary screen were tested for their temperature sensitivity in the secondary screen. The revertants were replicated on two YEA plates and incubated at 26° (for 3 days) or 36° (for 2 days), respectively. Among the revertants isolated through the primary screen, 11 strains exhibited a temperature sensitivity for growth at 36°. Finally, the 11 revertants were further tested for their ability to induce *fnx1*$^+$ and *mei2*$^+$ upon nitrogen starvation by Northern analysis. Two revertants satisfied the final criterion and were further examined genetically.

Cloning of *cpp1*$^+$: The *cpp1-1* mutant (YKK25) was transformed with an *S. pombe* genomic library containing partially digested *Sau*3AI DNA fragment constructed in a multicopy plasmid, pAL-KS (Tanaka *et al.* 2000). Plasmids were recovered from Ts$^+$ Leu$^+$ transformants and their nucleotide sequences were determined. BLAST search was performed for the obtained sequences, and the region covered by the inserted genomic sequence was determined.

Plasmid construction: Plasmid pREP41-cpp1 was constructed as follows. The *cpp1*$^+$ gene was amplified by PCR using the forward primer F-cpp1 [5′-CCCCCCGTCGAC(*Sal*I)GATGGATGAATTATCAGAAAC-3′] and the reverse primer R-cpp1 [5′-CCCCCCGGATCC(*Bam*HI)TTAGAATTTTGATGATTCTTG-3′]. The resulting fragment was digested with *Bam*HI and *Sal*I and then cloned into pREP41 (Maundrell 1993). Plasmids pREP41-rhb1 and pREP81-rhb1 were constructed as follows. The *rhb1*$^+$ gene was amplified by PCR using the forward primer F-rhb1 [5′- CCCCCCGTCGAC(*Sal*I)CATGGCTCCTATTAAATCTC -3′] and the reverse primer R-rhb1 [5′-CCCCCCGGATCC(*Bam*HI)TTAGGCGATAACACAACCCTTTCC-3′]. The resulting fragment was digested with *Bam*HI and *Sal*I and then cloned into pREP41 and pREP81, respectively. pREP41-rhb1^{CV1L} was constructed similarly with the exception of the primer used for PCR that was the reverse primer R-rhb1^{CV1L} [5′-CCCCCCGGATCC(*Bam*HI)TTACAAGATAACACAACCC-3′].

Generation of anti-Rhb1 antibody: A His-tagged protein of Rhb1 produced in *Escherichia coli* was used to raise polyclonal antibodies. His-Rhb1 was obtained as follows: A 558-bp DNA fragment carrying the entire *rhb1* coding region was amplified by PCR with two oligonucleotides, 5′-GGGGGGATCC(*Bam*HI)GCTCCTATTAAATCTCGTAGAATTG-3′ and 5′ - CCCCGTCGAC(*Sal*I)TTAGGCGATAACACAACCCTTTCC-3′. The amplified DNA was digested with *Bam*HI and *Sal*I and then inserted into the same sites of the His-tag expression vector pET-30-a to make pET(rhb1). The pET(rhb1) was transformed into *E. coli* Tuner. The fusion protein was purified from the MagneHis Protein Purification System (Promega, Madison, WI) and used to immunize rabbits.

Nucleotide sequence analysis of the *cpp1-1* mutant allele: The entire *cpp1* ORF was amplified by PCR using genomic DNA prepared from Δ*tsc2 cpp1-1* cells (YKK25) as a template and then cloned into pREP41. The nucleotide sequences of three clones derived from each independent PCR amplification were determined entirely. Comparison of the nucleotide sequences of *cpp1-1* with *cpp1*$^+$ revealed a single-nucleotide change, from G to A, which resulted in the replacement of glycine 254 with asparatate.

Western blotting: Total cell lysates were prepared as follows: Cells were lysed with glass beads in lysis buffer [150 mM NaCl and 10 mM Tris–HCl (pH 7.0)] containing 0.5% Triton X-100 and 0.5% deoxycholate. The following protease inhibitors were added to the cell extracts: 0.4 mM phenylmethylsulfonyl fluoride and 1× protease inhibitor cocktail (Nacalai Tesque). Equal amounts of total proteins were then loaded onto a 15% polyacrylamide gel and transferred to nitrocellulose membranes. Antibodies used were anti-Rhb1 and monoclonal TAT-1 for *S. pombe* tubulin (gift from K. Gull, University of Manchester, United Kingdom).

Northern blotting: Total RNAs were prepared from *S. pombe* culture as described (Jensen *et al.* 1983) and fractionated on a 0.8% gel containing 3.7% formaldehyde gel as previously reported (Thomas *et al.* 1980). Probes for *fnx1*$^+$, *mei2*$^+$, and *inv1*$^+$ were PCR amplified from a *S. pombe* genomic DNA library and labeled with [α-^{32}P]dCTP using standard methods.

Spot test: Cells were cultured in liquid YES or EMM medium at a concentration of 1×10^7 cells/ml and each culture was diluted 10, 100, and 1000-fold. Five microliters of each suspension were spotted on appropriate media.

Subcellular fractionation: Spheroplasts were prepared as follows. A total of 10^{10} cells were incubated at 37° for 1 hr in spheroplasts buffer [50 mM citrate–phosphate (pH 5.6) and 1.2 M sorbitol] containing 5 mg/ml lysing enzyme (Sigma, St. Louis). Spheroplasts were resuspended in a lysis buffer [20 mM Hepes-potassium hydroxide (pH 7.5), 20 mM potassium acetate, and 0.1 M sorbitol] containing 0.4 mM phenylmethylsulfonyl fluoride and 1× protease inhibitor cocktail (Nacalai Tesque) and downed ~20 times with a glass tissue homogenizer. The crude lysate was centrifuged at 300 × *g* to remove unlysed spheroplasts. The 300 × *g* supernatant was centrifuged at 100,000 × *g* for 1 hr to separate pellet (P100) and supernatant (S100) fractions.

Microarray analysis: Details of DNA-microarray construction, RNA isolation from fission yeast cells, sample labeling, microarray hybridization, and data processing will be described elsewhere (Y. Chicashige and Y. Hiraoka, unpublished data). Cells of Δ*tsc1* and Δ*tsc2* mutants were grown under the reference condition (YES liquid medium) to early-log phase and divided into two parts, one shifted to the experimental condition (3-hr incubation in EMM depleted of nitrogen sources), referred to as mutant/experimental (Mex), and the remainder kept under the reference condition for 3 hr (mutant/reference, Mrf). Poly(A)$^+$ RNA was extracted from Mex cells and cDNA probes were prepared, labeled with Cy5, and hybridized to a fission yeast cDNA microarray covering >4900 genes together with Cy3-labeled cDNA probes prepared from Mrf cells. To exclude false positives generated by the experimental condition, we performed the same analysis with a wild-type strain, designating the samples as wild type/experimental (Wex) and wild type/reference (Wrf). After measurement of fluorescent intensity for Cy5 and Cy3, the measured fluorescent intensity, *I*, was corrected as follows to give a corrected intensity, *C*,

$$C = I - M \qquad (\text{for } I \geq M + 2s)$$

$$C = I \times 2s/(M + 2s) \quad (\text{for } I \leq M + 2s),$$

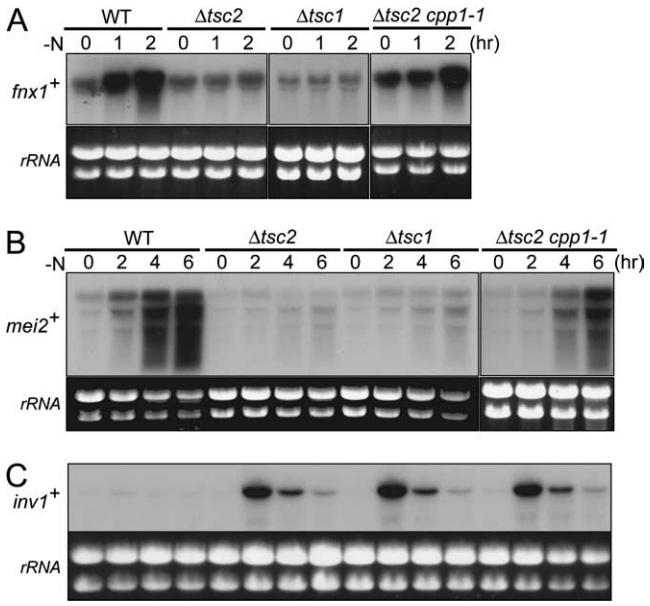

Figure 1.—Gene expression upon nitrogen starvation. Wild-type cells (SP6), Δ*tsc1* cells (AE413), Δ*tsc2* cells (AE512), and Δ*tsc2 cpp1-1* cells (YKK25) transformed with pAL-KS vector were precultured overnight in EMM plus N and then transferred to EMM minus N. Total RNAs were analyzed by Northern blot hybridization with *fnx1*$^+$ (A), *mei2*$^+$ (B), and *inv1*$^+$ (C) as probes.

where *M* and *s* are an average and a standard deviation of *I* of negative control spots for each wave length, respectively. When $I = M + 2s$, that is, $C = 2s$, it was set to be a detection limit. When *C* for either Cy3 or Cy5 or both was $>2s$, the values were considered to be effective data. Expression ratio r' of each effective detection spot obtained thus was scaled as follows: $r' = r - m$, $r = \log_2 R$, $R = (C_{\text{cy5}}/C_{\text{cy3}})$, and *m* is an average of *r* of all effective detection spots.

We identified transcripts that were not induced in each of the two mutants, Δ*tsc1* and Δ*tsc2*, with the following criteria: r' of the wild-type strain was >2 whereas that of each mutant was <2. Transcripts that were more induced in each of the two mutants were identified with the following criteria: r' of the mutant strain was >2 whereas that of the wild-type strain was <2. The original data of microarray experiments have been submitted to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/index.cgi) and are accessible with accession no. GSE4449.

RESULTS

Gene expression in Δ*tsc1* and Δ*tsc2*: We previously showed that the *sxa2*$^+$ gene is not induced in Δ*tsc1* (Matsumoto *et al.* 2002). Another study (van Slegtenhorst *et al.* 2004) also indicated that expression of a number of genes in exponentially growing Δ*tsc1* and Δ*tsc2* cells is abnormal. Having shown that *S. pombe* Tsc1/2 is required for sensing/responding to starvation, we thought that analysis of gene induction/repression upon starvation might provide more insightful information. It was previously demonstrated that expression of *fnx1*$^+$ is induced upon nitrogen starvation (Dimitrov and Sazer 1998). As shown in Figure 1,

TABLE 2

Genes not induced in Δ*tsc1* and Δ*tsc2*

ORF ID	Gene name	Possible function
SPCP31B10.09 SPCC962.01		Unknown
SPAC1039.04		Membrane transporter
SPBC1773.17c SPBP26C9.01c		Glycerate and formate dehydrogenase
SPAC1039.01		Amino acid permease
SPBC887.17		Uracil permease
SPAC1399.02		Membrane transporter
SPBP35G2.11c		Zinc finger protein
SPBC947.15c		Mitochondrial NADH dehydrogenase
SPAP7G5.06		Amino acid permease
SPAC1039.03		Esterase/lipase
SPBC24C6.06	*gpa1*	Guanine nucleotide-binding protein
SPAC13G7.04c	*mac1*	Membrane-anchored protein
SPAC27F1.05c		Aminotransferase
SPBC1604.03c		Hypothetical protein
SPCC1183.11 SPCC31H12.01		MS ion channel
SPAC31G5.09c	*spk1*	MAP kinase
SPAC11D3.03c		Conserved protein
SPAC13F5.07c		Hypothetical protein
SPAC27D7.03c	*mei2*	RNA-binding protein
SPAC11H11.04	*mam2*	Pheromone P-factor receptor
SPAC186.04c		Pseudogene
SPBC1683.02		Adenosine deaminase
SPBC660.07	*ntp1*	*O*-glycosyl hydrolase
SPBC1711.11		Sorting nexin
SPBC36B7.05c		Phosphatidylinositol(3)-phosphate-binding protein
SPBC25B2.02c SPBC2G5.09c	*mam1*	ABC transporter
SPBPB2B2.01		Amino acid permease
SPCC1682.11c		Hypothetical protein
SPCC550.07		Acetamidase
SPCC550.10	*meu8*	Betaine aldehyde dehydrogenase
SPCC622.11		Hypothetical protein

ORFs that were induced more than fourfold in the wild type but not in Δ*tsc1* or Δ*tsc2* 3 hr after nitrogen starvation are listed with their ORF ID, gene name (if available), and possible function.

Northern blot analysis indicated that the level of the induction of *fnx1*$^+$ was indeed much lower in Δ*tsc1* and Δ*tsc2*. To obtain genomewide information, we analyzed the expression profile of Δ*tsc1* and Δ*tsc2* by DNA microarrays. RNAs were prepared from Δ*tsc1* and Δ*tsc2* cells grown in nitrogen-rich YE medium as well as the cells grown for 3 hr in EMM medium lacking nitrogen. These RNAs were labeled with Cy3 or Cy5 and used as probes for hybridization to DNA microarrays. For the control experiment, we also prepared RNAs from the wild-type strain and used them as probes. Our quantitative analysis indicated that 131 genes are induced upon nitrogen depletion in the wild-type strain, of which ~31 genes are not induced in Δ*tsc1* and Δ*tsc2* (Table 2). We also found that 32 genes are induced in Δ*tsc1* and Δ*tsc2*, but not in the wild-type strain (Table 3). By performing Northern blots, we confirmed some of the results obtained by the DNA microarray analysis. As shown in Figure 1, *mei2*$^+$, which was normally induced upon nitrogen starvation, was not induced in Δ*tsc1* and Δ*tsc2*. Furthermore, *inv1*$^+$, which was not expressed at a detectable level in the wild-type strain, was induced in Δ*tsc1* and Δ*tsc2* cells.

Screen for an extragenic suppressor of Δ*tsc2*: To dissect a genetic pathway involving *tsc1*$^+$/*2*$^+$, we attempted to isolate extragenic suppressors of Δ*tsc2*. Assuming that Tsc2 serves as a GAP for Rhb1 GTPase and negatively regulates the Rhb1 function, we were particularly interested in a mechanism to positively regulate the GTPase. If Rhb1 is constitutively active in the Δ*tsc2* cells, a loss of function of a gene encoding a positive regulator for Rhb1 would suppress the phenotypes caused by Δ*tsc2*.

As we previously showed (Matsumoto *et al.* 2002), Δ*tsc2 leu1-32* strains are defective in uptake of amino acids and cannot grow on EMM containing leucine at 40 μg/ml. In the primary screen for the extragenic suppressors of Δ*tsc2*, we isolated spontaneous revertants, which could grow at 26° on the EMM with leucine at 40 μg/ml. Sixty-five revertants obtained through the primary screen were tested for their temperature sensitivity at 36° in the secondary screen. Because *rhb1*$^+$

TABLE 3

Genes induced more in Δ*tsc1* and Δ*tsc2* 3 hr after nitrogen starvation

ORF ID	Gene name	Possible function
SPAC21E11.04	*ppr1*	L-azetidine-2-carboxylic acid acetyltransferase
SPCC1020.14	Tf2-12 tf2-5	tf2-type transposon
SPCC794.05c		Pseudogene
SPAC9.04	Tf2-1 tf2-7	tf2-type transposon
SPAC26A3.13c	Tf2-4 tf2-2	tf2-type transposon
SPCC1494.11c	Tf2-13-pseudo	LTR retrotransposon tf2-type retrotransposon polyprotein with 1 frameshift
SPAC167.08 SPAC1F2.03	Tf2-2 tf2-3 tf2-4	tf2-type transposon
SPAC2E1P3.03c SPAC2E1P3.03	tf2-10 Tf2-3	tf2-type transposon
SPBC9B6.02c	tf2-8 Tf2-9	Retrotransposable element
SPAPB18E9.03c		Hypothetical protein
SPBC1E8.04c	Tf2-10-pseudo	Frameshifted LTR retrotransposon polyprotein
SPBC660.09		Hypothetical protein
SPAC3F10.16c		GTPase
SPBC1271.08c		Hypothetical protein
SPBC1271.07c		Acetyltransferase
SPAC57A10.01 SPAC19E9.03	*pas1*	Pcl-like cyclin
SPBC2G2.04c	*mmf1 pmf1*	Conserved protein
SPBP4H10.12		Conserved protein
SPAC821.10c	*sod1*	Cu,Zn-superoxide dismutase
SPBC211.07c	*ubc8*	Ubiquitin-conjugating enzyme
SPAC29B12.13		Hypothetical protein
SPAC2F3.08	*sut1*	α-Glucoside transporter
SPCC1450.13c		Riboflavin synthase
SPAC3C7.02c		Hypothetical protein
SPCC70.10		Hypothetical protein
SPAC25B8.09		Methyltransferase
SPCC70.08c		SAM-dependent methyltransferase
SPAC16E8.03	*gna1 spgna1*	Glucosamine-phosphate *N*-acetyltransferase
SPBC1773.05c	*tms1*	Dehydrogenase
SPBC16A3.17c		Transporter
SPBC839.06	*cta3*	Ca^{2+}-ATPase
SPCC191.11	*inv1*	Invertase

ORFs that were induced more than fourfold in Δ*tsc1* and Δ*tsc2* but not in the wild type 3 hr after nitrogen starvation are listed with ORF ID, gene name (if available), and possible function.

is an essential gene for growth (Mach *et al.* 2000), we postulated that if a revertant carried a mutation on a positive regulator for Rhb1, it might be lethal under a more severe condition. Among the 65 revertants isolated through the primary screen, 11 strains exhibited a temperature sensitivity for growth at 36°. Finally, the revertants, which appeared to be temperature sensitive in the secondary screen, were further tested for their ability to induce *fnx1*$^+$ and *mei2*$^+$ upon nitrogen starvation. Two revertants satisfied the final criterion and were further examined genetically.

Gene cloning of extragenic suppressor: Genetic analysis indicated that the two revertants carried a single mutation at the same locus responsible for the suppression of Δ*tsc2*. Furthermore, the suppression activity was found to link to the temperature sensitivity. We attempted to clone the corresponding gene by complementation of the temperature sensitivity and identified six genes. Integration mapping and sequence analysis of the genome of the suppressors indicated that *cpp1*$^+$ is the gene responsible for the suppression. *cpp1*$^+$ encodes a β-subunit of a farnesyltransferase (FTase), which farnesylates proteins. The two suppressors of Δ*tsc2* carry a mutation at the identical amino acid position Glycine-254 (Figure 2B). We refer to this allele as *cpp1-1* hereafter. Genes encoding the β-subunit of FTase have been identified from yeast to humans. According to a study of the human FTase (Park *et al.* 1997), the mutation site of *cpp1-1* corresponds to the catalytic center that traps the zinc ion.

The other five genes appeared to be a multicopy suppressor of *cpp1-1*. Overexpression of the *cwp1*$^+$ (Arellano *et al.* 1998) gene encoding an α-subunit of the FTase suppressed the temperature sensitivity of *cpp1-1* comparatively to that by *cpp1*$^+$. The remaining four genes, SPBC36.06c encoding a farnesyl pyrophosphate synthetase, *zfs1*$^+$ (Kanoh *et al.* 1995), *ykt6*$^+$ (McNew *et al.* 1997), and *ste11*$^+$ (Sugimoto *et al.* 1991), suppressed the

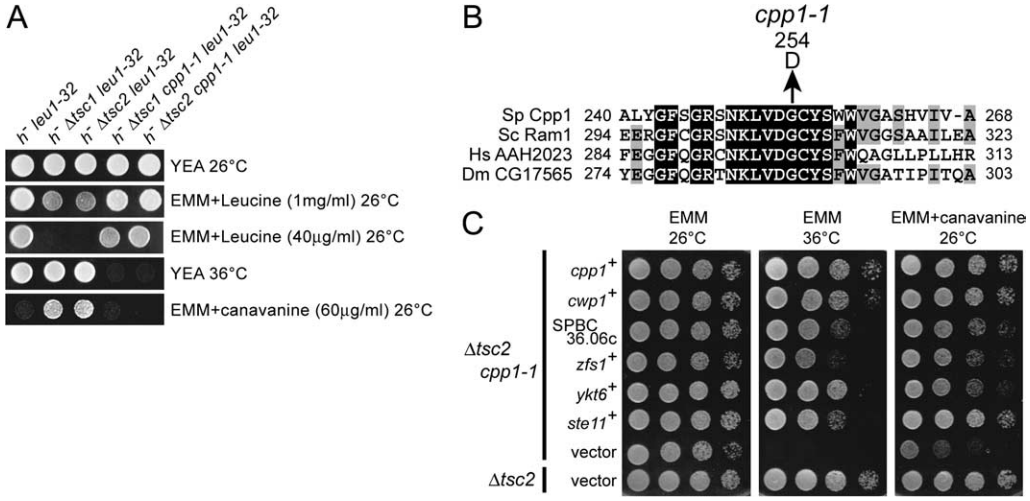

FIGURE 2.—*cpp1-1* is an extragenic suppressor of Δ*tsc2*. (A) Genetic interaction among *cpp1-1*, Δ*tsc1*, and Δ*tsc2*. Each strain was spotted on the indicated media and grown for 2 days (YEA, 36°) or 3 days (other conditions). (B) Comparison of the amino acid sequences of Cpp1 and other members of β-subunit of FTases. The *cpp1-1* mutant allele carries a single-nucleotide change (from G to A) that results in the replacement of Gly-254 with Asp. Identical amino acids among four species are shown in white against black and the amino acids conserved among three species are shaded. Sp, *Schizosaccharomyces pombe*; Sc, *Saccharomyces cerevisiae*; Hs, *Homo sapiens*; Dm, *Drosophila melanogaster*. (C) The multicopy suppressors of Δ*tsc2 cpp1-1* cells. Δ*tsc2 cpp1-1* cells carrying each of the five multicopy suppressors were grown in liquid EMM (1×10^7 cells/ml) and spotted on EMM or EMM with canavanine (60 μg/ml). They were incubated for 2 days (EMM, 36°) or 3 days (EMM or EMM containing canavanine, 26°).

temperature sensitivity of *cpp1-1* to a lesser extent (Figure 2C and Table 4).

Genetic interaction of *cpp1-1*: The *cpp1-1* allele was originally isolated through a screen for an extragenic suppressor of Δ*tsc2*. The two phenotypes associated with Δ*tsc2* (*i.e.*, defects in uptake of leucine and gene induction upon nitrogen starvation) were suppressed by this allele. First, *fnx1*$^+$ and *mei2*$^+$ could be induced upon nitrogen starvation in Δ*tsc2 cpp1-1* (Figure 1). Second, Δ*tsc2 cpp1-1* double mutants could grow on the EMM containing leucine at 40 μg/ml (Figure 2A). Interestingly, the *cpp1-1* mutation could not suppress the abnormal induction of *inv1*$^+$ (Figure 1).

Owing to the defect in uptake, Δ*tsc2* cells are resistant to canavanine, a toxic analog of arginine (Figure 2A). The *cpp1-1* mutation abolished the resistance to canavanine in the background of Δ*tsc2* (Figure 2A). When the *cpp1*$^+$ gene was introduced into a Δ*tsc2 cpp1-1* strain, the strain became resistant to canavanine (Figure 2C). Introduction of each of the five multicopy suppressors into a Δ*tsc2 cpp1-1* strain also conferred a resistance to canavanine (Figure 2C).

As it has been postulated that Tsc1 and Tsc2 function together in the same pathway, we were tempted to test if *cpp1-1* could also suppress Δ*tsc1*. We found that Δ*tsc1 cpp1-1* double mutants could grow on the EMM containing leucine at 40 μg/ml (Figure 2A), indicating that *cpp1-1* can suppress the defect in uptake of Δ*tsc1*.

The Cpp1-dependent FTase likely farnesylates a number of proteins (SEBTI 2005). Although suppression of Δ*tsc2* by *cpp1-1* suggested that a failure in farnesylation of a protein contributed to the suppression, it was not clear which protein was involved in this process. A likely protein was Rhb1 GTPase, which is believed to be a target of Tsc1/2. Its amino acid sequence ends with the consensus sequence of the FTase substrates, Cys–Val–Ile–Ala (STRICKLAND *et al.* 1998). The previous study (STRICKLAND *et al.* 1998) showed that the requirement of the Cpp1-dependent FTase could be bypassed by alteration of the amino acid sequence of the C terminus

TABLE 4

Multicopy suppressors of Δ*tsc2 cpp1-1* cells

Gene name (systematic name)	Accession no.	Possible function
cwp1 (SPAPB1A10.04c)	CAC21477.1	α-Subunit of geranylgeranyltransferase I (GGTase I) and farnesyltransferase (FTase)
No name (SPBC36.06c)	CAA19054.1	Farnesyl pyrophosphate (FPP) synthetase
zfs1 (SPBC1718.07c)	BAA08654.1	Zinc-finger protein involved in mating and meiosis
ykt6 (SPBC13G1.11)	CAA18664.1	Protein with high similarity to *S. cerevisiae* Ykt6p, which is a synaptobrevin (v-SNARE) homolog that is essential for endoplasmic reticulum–Golgi transport
ste11 (SPBC32C12.02)	CAA18162.1	Transcription factor that regulates genes required for mating

Genes responsible for multicopy suppression of *cpp1-1* are listed with their accession number and possible function.

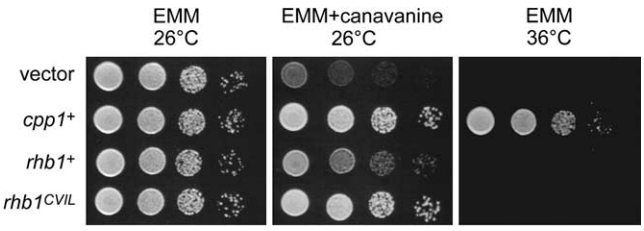

FIGURE 3.—Expression of SpRheb (rhb1^{CVIL}). Δ*tsc2 cpp1-1* cells were transformed with vector, plasmids expressing *rhb1*$^{+}$ wild type, *rhb1*^{CVIL} mutant, or *cpp1*$^{+}$ wild type. Transformants were suspended in liquid EMM at a concentration of 1×10^7 cells/ml and 5 μl of the suspension was spotted on solid EMM or solid EMM containing canavanine (60 μg/ml). They were incubated for 2 days (EMM, 36°) or 3 days (EMM or EMM containing canavanine, 26°).

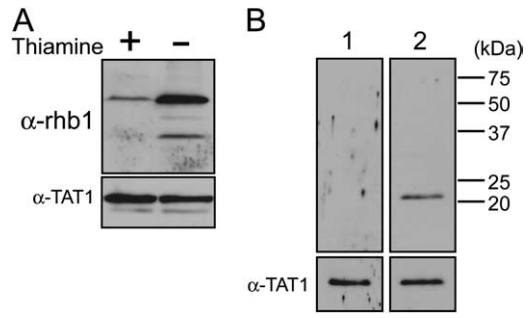

FIGURE 4.—Antibody to Rhb1. (A) Wild-type cells (SP6) carrying pREP81-rhb1 were grown to midlog phase in EMM containing thiamine (50 μg/ml) and then transferred to thiamine-free EMM for 17 hr at 30°. Protein extracts were subjected to immunoblot analysis with antibody to Rhb1 as well as with anti-α-tubulin antibody (TAT-1) as a loading control. (B) The antibody was first incubated with an excess amount of recombinant Rhb1 proteins immobilized on beads and the unbound fraction was used for Western blot (lane 1). The antibody was incubated with beads alone and the unbound fraction was used for Western blot (lane 2).

to Cys–Val–Ile–Leu, which is recognized by geranylgeranyl transferase, another enzyme to isoprenylate proteins. It was shown previously that proteins, which are normally farnesylated, can remain functional if modified by a geranylgeranyl group (YANG *et al.* 2001). We expressed a mutant of Rhb1-CVIL in a Δ*tsc2 cpp1-1* double mutant, which was sensitive to canavanine, and found that expression of Rhb1-CVIL could confer a resistance to canavanine (Figure 3). The result would suggest that Rhb1 is, at least in part, a protein involved in the suppression of Δ*tsc2* by *cpp1-1*. Expression of the mutant Rhb1 did not rescue the temperature sensitivity of the Δ*tsc2 cpp1-1* double mutant, indicating that a failure in farnesylation of another protein caused the temperature sensitivity.

Rhb1 in the *cpp1-1* mutant: Having demonstrated that Rhb1 was involved in the suppression of Δ*tsc2*, we were prompted to investigate Rhb1 protein biochemically. An antibody to Rhb1 was raised in rabbit and we tested its specificity. As shown in Figure 4, a band corresponding to a 20.5-kDa protein on SDS–PAGE was recognized by the antibody. Because the intensity of this band increased upon overexpression of Rhb1 (Figure 4A), we concluded that the 20.5-kDa protein was Rhb1. To further examine the specificity of the antibody, the antibody was first incubated with an excess amount of recombinant Rhb1 proteins immobilized on beads and the unbound fraction was used for Western blot. The incubation with the Rhb1 beads clearly abolished the 20.5-kDa band, whereas after incubation with beads alone, the antibody could still recognize the 20.5-kDa band (Figure 4B). These results indicated that the antibody specifically recognized the Rhb1 protein in fission yeast cell extracts.

It was likely that the *cpp1-1* mutation causes a defect in the FTase activity and that Rhb1 in the *cpp1-1* background may not be properly modified by a farnesyl group. To test this, we prepared cell extracts from the *cpp1-1* mutant and examined the mobility of Rhb1 on SDS–PAGE. It was shown previously that Rhb1 GTPase in fission yeast (YANG *et al.* 2000) migrates faster on SDS–PAGE if properly modified. As shown in Figure 5A, Rhb1 was detected as a doublet in cell extracts prepared from the *cpp1-1* mutant grown at 26°. Six hours after the shift to the restrictive temperature of 36°, the faster-migrating form of Rhb1 decreased. In cell extracts

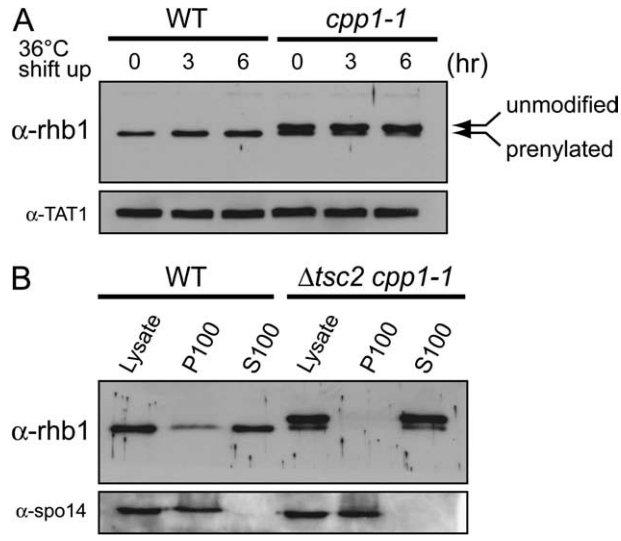

FIGURE 5.—Modification of Rhb1 in *cpp1-1*. (A) Wild-type cells (SP740) and *cpp1-1* cells (YKK55) were grown to midlog phase in YEL medium at 26° and then shifted to 36°. Cell extracts were analyzed with SDS–PAGE and immunoblotted with the anti-Rhb1 and with TAT-1 antibody. (B) Subcellular fractionation of Rhb1. Wild-type cells (SP6) and Δ*tsc2 cpp1-1* cells (YKK25) were grown at 36° for 6 hr. Cells were converted to spheroplasts, homogenized, and subjected to differential centrifugation to fractionate into P100 (membrane fraction) and S100 (supernatant). Each fraction was resolved by SDS–PAGE and subjected to immunoblot analysis using either the anti-Rhb1 or the anti-Spo14 antibody, respectively.

prepared from the wild-type strain, only the faster-migrating form was detected. These results indicated that the *cpp1-1* mutant was indeed defective in protein farnesylation and that the Rhb1 protein was not modified by a farnesyl group.

Farnesylation of the C termini of GTPases is thought to be important for membrane association (Casey *et al.* 1989). We fractionated cell extracts into a membrane fraction and a cytosolic fraction, and examined which fraction contained Rhb1 GTPase. Spo14, a membrane-bound protein (Nakamura-Kubo *et al.* 2003), was used as a probe for the membrane fraction. While a majority of Rhb1 was found in the cytosolic fraction, a small amount of Rhb1 was found in the membrane fraction prepared from the wild-type strain. On the other hand, Rhb1 was not detectable in the membrane fraction prepared from the *cpp1-1* mutant grown at 36° for 6 hr (Figure 5B). These results suggested that only a fraction of Rhb1 is farnesylated and thereby functional in the *cpp1-1* mutant even at the permissive temperature, 26°. We speculate that although the majority of Rhb1 would be a GTP-bound form in Δ*tsc2* cells, a failure in farnesylation results in a partial loss of the Rhb1 function, which contributes to the suppression of Δ*tsc2*.

DISCUSSION

In this study we first analyzed the gene expression profile in Δ*tsc1* and Δ*tsc2*. Second, we demonstrated that a mutation in a gene encoding the β-subunit of FTase can suppress most of the phenotypes associated with a loss of function of Tsc1/Tsc2.

Expression profile of Δ*tsc1* and Δ*tsc2*: The expression profiles of the Δ*tsc1* and Δ*tsc2* strains examined by the microarrays were very similar each other. We did not find any genes abnormally induced in either one of the two strains. The two genes, *tsc1*$^+$ and *tsc2*$^+$, thereby function together in the same pathway to regulate gene expression upon nitrogen starvation. The genes that cannot be induced in Δ*tsc1* and Δ*tsc2* are broadly classified in the following groups: genes required for meiosis, genes encoding permeases/transporter for nutrients, and genes encoding enzymes for biosynthesis. The defect in induction of these genes accounts well for the phenotypes of Δ*tsc1* and Δ*tsc2* (*i.e.*, inefficient meiosis and low uptake).

We also found that 32 genes were induced at higher levels in Δ*tsc1* and Δ*tsc2* 3 hr after nitrogen starvation. It should be noted that some of these genes may possibly be induced in the wild-type strains at earlier time points and already repressed 3 hr after nitrogen starvation. If their induction is delayed in Δ*tsc1* and Δ*tsc2* and reaches a peak later, the induction level in Δ*tsc1* and Δ*tsc2* may be higher than that in the wild-type strain 3 hr after nitrogen starvation. It is, therefore, necessary to examine the induction level of each gene in more detail to identify a gene, which is induced specifically in Δ*tsc1* and Δ*tsc2*. Among the 32 genes that are induced >3 hr after nitrogen starvation in Δ*tsc1* and Δ*tsc2*, we examined *inv1*$^+$ in detail and demonstrated that it is induced poorly up to 6 hr after nitrogen starvation in the wild-type strain. *inv1*$^+$ is thereby induced specifically in Δ*tsc1* and Δ*tsc2*. It is normally derepressed upon glucose starvation in fission yeast (Tanaka *et al.* 1998). At present, it is not clear why *inv1*$^+$ is induced in Δ*tsc1* and Δ*tsc2* even in the presence of glucose. A factor required for repression of *inv1*$^+$ may not be expressed in Δ*tsc1* and Δ*tsc2* when nitrogen is depleted.

Activation of Rhb1 by FTase: Our genetic study demonstrated that a mutation (*cpp1-1*) in the β-subunit of FTase can suppress a loss of function of Tsc1/Tsc2. When a mutant of Rhb1 that bypasses the requirement of farnesylation was expressed, the *cpp1-1* mutation no longer suppressed Δ*tsc1* and Δ*tsc2*. The result indicated that a failure in farnesylation of Rhb1 contributes to the suppression. Only a small fraction of Rhb1 GTPase was found as a modified form in the *cpp1-1* mutant. It has been generally accepted that protein farnesylation at the C terminus of GTPases facilitates membrane association. Consistent with this notion, while a portion of Rhb1 in the wild-type strain was found in the P100 membrane fraction, no Rhb1, in the *cpp1-1* mutant, was found in this fraction. We speculate that activation of Rhb1 requires both GTP binding and farnesylation. The suppression of Δ*tsc1* and Δ*tsc2* by *cpp1-1* is a result of a decrease in the level of active Rhb1.

Role of the Tsc1/2 complex: Considering that the known biochemical function of the Tsc1/2 complex has so far been to serve as a GAP for the Rhb1 GTPase, it is likely that the defect in gene induction in Δ*tsc1* and Δ*tsc2* is due to constitutive activation of Rhb1. An active form of Rhb1, in turn, would continuously repress the gene induction even when nitrogen is removed. Supporting this notion, expression of a hyperactive mutant of Rhb1 (Urano *et al.* 2005) resulted in a failure in induction of *mei2*$^+$ and *fnx1*$^+$ (K. Fukuda and T. Matsumoto, unpublished results). It has also been reported that the two genes, *mei2*$^+$ and *fnx1*$^+$, are induced upon repression of a hypomorphic allele of *rhb1*$^+$ in the presence of nitrogen, demonstrating that expression of these genes is solely regulated by Rhb1 (Mach *et al.* 2000). In budding yeast, a number of studies demonstrated that Tor, a downstream target of Rhb1, is involved in transcriptional regulation (Beck and Hall 1999; Duvel *et al.* 2003; Rohde and Cardenas 2003). We speculate that fission yeast Tor1 and Tor2 as targets of Rhb1 play a role in a signal cascade to regulate transcription/translation in response to the availability of nutrients. In this cascade the Tsc1/2 complex regulates Tor1/2 via Rhb1 GTPase.

While the *cpp1-1* mutant clearly restored the ability of Δ*tsc2* to induce *mei2*$^+$ and *fnx1*$^+$ upon nitrogen starvation, it failed to repress abnormal induction of *inv1*$^+$ in Δ*tsc1* and Δ*tsc2*. Assuming that the *cpp1-1* mutation can

suppress defects in Rhb1-dependent events, it is possible that the abnormal induction of *inv1*$^+$ in Δ*tsc1* and Δ*tsc2* may not be a result of constitutive activation of Rhb1. In addition to GAP for Rhb1 GTPase, the Tsc1/2 complex may play another role.

TSC pathology and treatment: TSC is a disorder characterized by the wide spread of benign tumors, called hamartomas. The tumor cells exhibit abnormalities in cell size, number, morphology, and location, thereby implying a role of the Tsc1/2 complex in regulating cell growth, proliferation, differentiation, and migration (Yeung 2003). Because the Tsc1/2 complex regulates protein synthesis via Rheb and mTOR (Manning and Cantley 2003; Li *et al.* 2004a,b; Pan *et al.* 2004), it is currently considered that formation of hamartomas is due to deregulation of protein synthesis.

Our analysis of the expression profile revealed that the Δ*tsc1* and Δ*tsc2* strains exhibit an abnormality in induction of a number of genes upon nitrogen starvation. They cannot efficiently induce genes required for meiosis, a process of differentiation in fission yeast. We also found that in the Δ*tsc1* and Δ*tsc2* strains 3 hr after nitrogen starvation, retrotransposons (LTR Tf2) and a G1 cyclin (*pas1*$^+$) are expressed at a level higher than that in the wild-type strain. It has been previously reported that *pas1*$^+$ is expressed at a higher level in exponentially growing Δ*tsc1* and Δ*tsc2* strains as well (van Slegtenhorst *et al.* 2005). Although the consequence of abnormal induction of these genes remains to be examined, deregulation of these genes could result in alteration of the genome structure as well as a program of cell proliferation. Expression analysis of the hamartoma cells may allow identification of genes whose abnormal expression accounts for the complex pathology of TSC.

As Rapamycin targets mTOR, it is a good candidate for an anti-TSC drug. On the other hand, it also has immunosuppressive effects (Abraham and Wiederrecht 1996), suggesting that it is not an ideal drug for a long term-administration. Our model study in fission yeast demonstrated that a defect in FTase well suppresses the phenotypes associated with deletion of *tsc1/2*$^+$. We thereby postulate that an inhibitor of FTase (FTI) should be considered as an anti-TSC drug as well. A combination of Rapamycin and FTI may enhance the specificity of the chemotherapy for TSC. FTIs were originally proposed as anticancer agents because Ras-oncoproteins must be farnesylated for its transforming activity. A number of compounds, some of which competitively inhibit FTase with their structure mimicking the C-terminal C-A-A-X motif of the GTPase, have been developed and tested clinically (Omer and Kohl 1997; Graaf *et al.* 2004).

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