Genetics: Published Articles Ahead of Print, published on April 19, 2006 as 10.1534/genetics.106.056895

# A defect in protein farnesylation suppresses a loss of *Schizosaccharomyces pombe* *tsc2*$^{+}$, a homolog of the human gene predisposing tuberous sclerosis complex (TSC).

Yukiko Nakase,[1,2] Keiko Fukuda,[1,2] Yuji Chikashige,[4] Chihiro Tsutsumi,[4] Daisuke Morita,[3] Shinpei Kawamoto,[3] Mari Ohnuki,[3] Yasushi Hiraoka[4] and Tomohiro Matsumoto[1,2]

1:Radiation Biology Center, Kyoto University, Yoshida-Konoe cho, Sakyo ku, Kyoto, Japan, 606-8501

2: Department of Biostudies, Kyoto University, Kitashirakawa-Oiwake cho, Sakyo ku, Kyoto, Japan, 606-8502

3: Department of Agriculture, Kyoto University, Kitashirakawa-Oiwake cho, Sakyo ku, Kyoto, Japan, 606-8502

4: Cell Biology Group, Kansai Advanced Research Center

National Institute of Information and Communications Technology,

Kobe, Hyogo, Japan, 651-2492

**Running head**: Suppression of *Δtsc2* in *S. pombe*

**Key words**:

Tuberous Sclerosis Complex (TSC)

Tsc1/2

Farnesyltransferase

Rhb1 GTPase

**Corresponding author**:

Name: Tomohiro Matsumoto

Mailing address: Yoshida-Konoe cho, Sakyo Ku, Kyoto, Japan 606-8501

Phone number: 81-75-753-7552

Fax: 81-75-753-7564

e-mail: tmatsumo@house.rbc.kyoto-u.ac.jp

**Abstract**: Mutations in the human *Tsc1* and *Tsc2* genes predispose to Tuberous Sclerosis Complex (TSC), a disorder characterized by the widespread of benign tumors. Tsc1 and Tsc2 proteins form a complex and serve as a GAP (GTPase activating protein) 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 gene expression profile in a genome-wide 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. Based on these results, we discuss TSC-pathology and possible improvement in chemotherapy for TSC.

**Introduction**: Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterized by the widespread of benign tumors called hamartomas in different organs including the brain, eyes, heart, kidney, skin, and lungs (GOMEZ 1995; KWIATKOWSKI and SHORT 1994). Seizures and learning and behavioral problems, which are likely due to development of tumors in brain, are also common in patients with TSC (KWIATKOWSKI and SHORT 1994; GOMEZ 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 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.* 2004). Therefore, TSC1 and TSC2 are generally considered as a complex (TSC1/2) with 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; GREEN *et al.* 1994b; 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 *gigas* phenotype in fly mutant highlights the capability of TSC1/2 in controlling cell size (ITO and RUBIN 1999). Studies have shown that TSC1/2 control cell growth/proliferation by regulating the activity of a small GTPase, RHEB (ZHANG *et al.* 2003). When the environment surrounding the cell is not in favor for growth/proliferation, TSC1 and TSC2, which has a GAP (GTPase activating protein) domain in its 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 (LONG *et al.* 2005; LI *et al.* 2004; MANNING and CANTLEY 2003; PAN *et al.* 2004). 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.

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*$^+$ (*Δtsc1*) and *tsc2*$^+$ (*Δtsc2*) are defective in uptake of nutrients such as amino acids and adenine. Consistently 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 *Δtsc1* and *Δtsc2*. Secondly, *Δtsc1* and *Δtsc2* are unable to induce *sxa2*$^+$ gene, which is usually expressed upon stimulation by a mating type pheromone, P-factor, in starved *h*$^-$ cells (IMAI and YAMAMOTO 1994). Based on these phenotypes, we postulate that *tsc1*$^+$ and *tsc2*$^+$ are required for sensing/responding to starvation. We speculate that *Schizosaccharomyces 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 *Δtsc2*, we identified a gene, *cpp1*$^{+}$, encoding a subunit of the enzyme required for protein farnesylation.

## Materials & Methods

### Yeast strains, Media, and Transformation

The *Schizosaccharomyces pombe* strains used in this study are listed in Table 1. The yeast cells were grown in YEA and EMM media with appropriate nutrient supplements as described previously (MORENO *et al*. 1991). All yeast transformations were carried out by lithium acetate methods (GIETZ *et al*. 1992; OKAZAKI *et al*. 1990).

### Screen for an extragenic suppressor of *Δtsc2*

Reversion rate of AE512 strain used for the screening was 1.25 x $10^{-6}$. The spontaneous revertants were grown at 26 °C 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 °C (for 3 days) or 36 °C (for 2 days) respectively. Among the revertants isolated through the primary screen, 11 strains exhibited a temperature sensitivity for growth at 36 °C. 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 *Schizosaccharomyces 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(*BamH*I)TTAGAATTTTGATGATTCTTG-3']. The resulting fragment was digested with *BamH*I and *Sal*I, and then cloned into pREP41

(Maundrell, 1993). Plasmid 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(*BamH*I)TTAGGCGATAACACAACCCTTTCC-3']. The resulting fragment was digested with *BamH*I and *Sal*I, and then cloned into pREP41 and pREP81, respectively. pREP41-rhb1$^{CVIL}$ was constructed similarly with the exception of the primer used for PCR that were the reverse primer R- rhb1$^{CVIL}$ [5'-CCCCCCGGATCC(*BamH*I)TTACAAGATAACACAACCC-3'].

**Generation of Anti-Rhb1 Antibody**

A His-tagged protein of Rhb1 produced in *E. coli* was used to raise polyclonal antibodies. His-Rhb1 was obtained as follows; A 558bp DNA fragment carrying the entire *rhb1* coding region was amplified by PCR with two oligonucleotides, 5'-GGGGGGATCC(*BamH*I)GCTCCTATTAAATCTCGTAGAATTG-3' and 5' -CCCCGTCGAC(*Sal*I)TTAGGCGATAACACAACCCTTTCC-3'. The amplified DNA was digested with *BamH*I 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 MagneHis™ Protein Purification System (Promega) 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 *Δtsc2 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 the lysis buffer (150 mM NaCl and 10 mM Tris-HCl [pH7.0]) 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 1x Protease Inhibitor Cooktail (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 [$\alpha$-$^{32}$P]dCTP using standard methods.

**Spot test**

Cells were cultured in liquid YES or EMM medium at a concentration of 1 x $10^7$ cells/ml and each cultures were diluted 10, 100 and 1,000-fold. Five μl of each suspension was spotted on appropriate media.

**Subcellular Fractionation**

Spheroplasts were prepared as follows. $10^{10}$ cells were incubated at 37 °C for 1 hr in spheroplasts buffer (50 mM citrate-phosphate [pH5.6] and 1.2 M sorbitol) containing 5 mg/ml Lysing enzyme (Sigma). Spheroplasts were resuspended in a lysis buffer (20 mM Hepes-KOH [pH7.5], 20 mM KOAc and 0.1 M sorbitol) containing 0.4 mM phenylmethylsulfonyl fluoride and 1 x Protease Inhibitor Cooktail (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 1h 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. C. and Y. H., manuscript in preparation). Cells of *Δtsc1* and *Δtsc2* 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 (three hour-incubation in EMM medium depleted of nitrogen sources), referred to as Mex (mutant/experimental), and the remainder kept under the reference condition for three hours (Mrf; mutant/reference). 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 more than 4,900 genes together with Cy3-labelled 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 Wex (wild-type/experimental) and Wrf (wild-type/reference). After measurement of fluorescent intensity for Cy5 and Cy3, the measured fluorescent intensity, I, was corrected as follows to give a corrected intensity, C:

$$C = I - M, \quad (\text{for } I \geqq M+2s)$$

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

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 were greater than 2s, the values were

considered to be effective data. Expression ratio r' of the each effective detection spot obtained thus was scaled as follows: r'=r-m, r=$\log_2$R, R=($C_{cy5}$/$C_{cy3}$), m is an average of r of all effective detection spots.

We identified transcripts that were not induced in each of the two mutants, *Δtsc1* and *Δtsc2*, with the following criteria; r' of the wild type strain was greater than 2 whereas that of each mutant was less than 2. Transcripts that were more induced in each of the two mutants were identified with the following criteria; r' of the mutant strain was greater than 2 whereas that of the wild type strain was less than 2. The original data of microarray experiments have been submitted to GEO (http://www.ncbi.nlm.nih.gov/geo/index.cgi) and are accessible with an accession number of GSE4449.

**Results:**

**Gene expression in *Δtsc1* and *Δtsc2***

We previously showed that *sxa2*$^{+}$ gene is not induced in *Δtsc1* (MATSUMOTO *et al.* 2002). Another study (VAN SLEGTENHORST *et al.* 2004) also indicated that expression of a number of genes in exponentially growing *Δtsc1* and *Δtsc2* cells is abnormal. Having shown that *Schizosaccharomyces 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, Northern blot analysis indicated that the level of the induction of *fnx1*$^{+}$ was indeed much lower in *Δtsc1* and *Δtsc2*. In order to obtain genome-wide information, we analyzed expression profile of *Δtsc1* and *Δtsc2* by DNA micro-arrays. RNAs were prepared from *Δtsc1* and *Δtsc2* cells grown in nitrogen-rich YE medium as well as the cells grown for 3 hours 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 as

probes. Our Quantitative analysis indicated that 131 genes are induced upon nitrogen depletion in the wild type strain, of which approximately 31 genes are not induced in *Δtsc1* and *Δtsc2* (Table 2). We also found that 32 genes are induced in *Δtsc1* and *Δtsc2*, but not in the wild type strain (Table 3). By performing Northern blots, we confirmed some of the results obtained by the DNA microarrays analysis. As shown in Figure 1, *mei2*$^{+}$, which was normally induced upon nitrogen starvation, was not induced in *Δtsc1* and *Δtsc2*. Furthermore, *inv1*$^{+}$, which was not expressed at a detectable level in the wild type strain, was induced in *Δtsc1* and *Δtsc2* cells.

**Screen for an extragenic suppressor of *Δtsc2***

In order to dissect a genetic pathway involving *tsc1*$^{+}$/*2*$^{+}$, we attempted to isolate extragenic suppressors of *Δtsc2*. 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 *Δtsc2* cells, a loss of function of a gene encoding a positive regulator for Rhb1 would suppress the phenotypes caused by *Δtsc2*.

As we previously showed (MATSUMOTO *et al*. 2002) *Δtsc2 leu1-32* strains are defective in uptake of amino acids and cannot grow on EMM medium containing leucine at 40 μg/ml. In the primary screen for the extragenic suppressors of *Δtsc2*, we isolated spontaneous revertants, which could grow at 26 °C on the EMM medium with leucine at 40 μg/ml. Sixty five revertants, obtained through the primary screen were tested for their temperature sensitivity at 36 °C in the secondary screen. Because *rhb1*$^{+}$ 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 °C. 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 *Δtsc2*. 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 *Δtsc2* 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 human. 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 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 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 *Δtsc2*. The two phenotypes associated with *Δtsc2*, (*i.e*. defects in uptake of leucine and gene induction upon nitrogen starvation) were suppressed by this allele. Firstly, *fnx1*$^{+}$ and *mei2*$^{+}$ could be induced upon nitrogen starvation in *Δtsc2 cpp1-1* (Figure 1). Secondly, *Δtsc2 cpp1-1* double mutants could grow on the EMM medium 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, *Δtsc2* 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 *Δtsc2* (Figure 2A). When the *cpp1*$^{+}$ gene was introduced into a *Δtsc2 cpp1-1* strain, the strain became resistant to canavanine (Figure 2C). Introduction of each of the five multicopy suppressors into a *Δtsc2 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 *Δtsc1*. We found that *Δtsc1 cpp1-1* double mutants could grow on the EMM medium containing leucine at 40 μg/ml (Figure 2A), indicating that *cpp1-1* can suppress the defect in uptake of *Δtsc1*.

The Cpp1-dependent FTase likely farnesylates a number of proteins (SEBTI 2005). Although suppression of *Δtsc2* 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-terminal 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 *Δtsc2 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 *Δtsc2* by *cpp1-1*. Expression of the mutant Rhb1 did not rescue the temperature sensitivity of the *Δtsc2 cpp1-1* double mutant, indicating that a failure in farnesylation of another protein caused the temperature sensitivity.

**Rhb1 in *cpp1-1* mutant**

Having demonstrated that Rhb1 was involved in the suppression of *Δtsc2*, we prompted to investigate Rhb1 protein biochemically. An antibody to Rhb1 was raised in rabbit and 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. In order 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 °C. Six hours after the shift to the restrictive temperature 36 °C, the faster-migrating form of Rhb1 decreased. In cell extracts 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-terminal 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 °C for six hours (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 °C. We speculate that although the majority of Rhb1 would be a GTP-bound form in *Δtsc2* cells, a failure in farnesylation results in a partial loss of the Rhb1 function, which contributes to the suppression of *Δtsc2*.

**Discussion:**

In this study we first analyzed the gene expression profile in *Δtsc1* and *Δtsc2*. Secondly, 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 *Δtsc1* and *Δtsc2*:** The expression profiles of the *Δtsc1* and *Δtsc2* strains examined by the micro-arrays were very similar each other. We did not find any genes abnormally induced only 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 *Δtsc1* and *Δtsc2* 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 well accounts for the phenotypes of *Δtsc1* and *Δtsc2* (*i.e.* inefficient meiosis and low uptake).

We also found that 32 genes were induced at higher levels in *Δtsc1* and *Δtsc2* three hours 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 hours

after nitrogen starvation. If their induction is delayed in *Δtsc1* and *Δtsc2* and reaches a peak at later, the induction level in *Δtsc1* and *Δtsc2* may be higher than that in the wild type strain 3 hours 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 *Δtsc1* and *Δtsc2*. Among the 32 genes that are induced higher three hours after nitrogen starvation in *Δtsc1* and *Δtsc2*, we examined *inv1*$^+$ in detail and demonstrated that it is induced poorly up to 6 hours after nitrogen starvation in the wild type strain. *inv1*$^+$ is thereby induced specifically in *Δtsc1* and *Δtsc2*. 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 *Δtsc1* and *Δtsc2* even in the presence of glucose. A factor required for repression of *inv1*$^+$ may not be expressed in *Δtsc1* and *Δtsc2* 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 *Δtsc1* and *Δtsc2*. 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. Consistently 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 *Δtsc1* and *Δtsc2* by *cpp1-1* is a result of a decrease in the level of active Rhb1.

**Role of Tsc1/2 complex:** Considering that known biochemical function of Tsc1/2 complex has so far been to serves as a GAP for the Rhb1 GTPase, it is likely that the defect in gene induction in *Δtsc1* and *Δtsc2* 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. F. and T. M. unpublished result). 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 are 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 Tsc1/2 complex regulates Tor1/2 via Rhb1 GTPase,

While the *cpp1-1* mutant clearly restored the ability of *Δtsc2* to induce *mei2*$^+$ and *fnx1*$^+$ upon nitrogen starvation, it failed to repress abnormal induction of *inv1*$^+$ in *Δtsc1* and *Δtsc2*. Assuming that the *cpp1-1* mutation can suppress defects in Rhb1-dependent events, it is possible that the abnormal induction of *inv1*$^+$ in *Δtsc1* and *Δtsc2* may not a result of constitutive activation of Rhb1. In addition to GAP for Rhb1 GTPase, Tsc1/2 complex may play another role.

**TSC-pathology and treatment**: Tuberous Sclerosis Complex (TSC) is a disorder characterized by the widespread 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 Tsc1/2 complex regulates protein synthesis via Rheb and mTOR (LI *et al*. 2004; MANNING and CANTLEY 2003; PAN *et al*. 2004), it is currently considered that formation of hamartomas is due to deregulation of protein synthesis.

Our analysis of expression profile revealed that the *Δtsc1* and *Δtsc2* 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 *Δtsc1* and *Δtsc2* strains three hours after nitrogen starvation, retrotransposons (LTR Tf2) and a G1 cyclin (*pas1*$^{+}$) are expressed at a level higher than in the wild type strain. It has been previously reported that *pas1*$^{+}$ is expressed higher in exponentially growing *Δtsc1* and *Δtsc2* 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 hamartomas 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 anti-TSC drug as well. Combination of Rapamycin and FTI may enhance the specificity of the chemotherapy for TSC. FTIs were originally proposed as anti-cancer 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 (GRAAF *et al*. 2004; OMER and KOHL 1997).

**Acknowledgement**

The authors are grateful to Drs. Shimoda and Nakamura (Osaka City University, Japan) for providing the antibody to Spo14; Dr. K. Gull (University of Manchester, U. K.) for providing the anti-α-tubulin antibody, TAT-1. This work was supported by U. S. Army Medical Research and Material Command "Tuberous Sclerosis Complex Research Program" (to T. M.) and the Japan Science and Technology Agency (to Y. H.).

**Legends for Figures and Tables**

**Figure 1.** Gene expression upon nitrogen stravation. Wild type cells (SP6), *Δtsc1* cells (AE413), *Δtsc2* cells (AE512) and *Δtsc2 cpp1-1* cells (YKK25) transformed with pAL-KS vector were precultured overnight in EMM+N medium and then transferred to EMM-N medium. Total RNAs were analyzed by Northern blot hybridization with *fnx1*$^+$ (A), *mei2*$^+$ (B) and *inv1*$^+$ (C) as probes.

**Figure 2.** *cpp1-1* is an extragenic suppressor of *Δtsc2*. (A) Genetic interaction among *cpp1-1*, *Δtsc1* and *Δtsc2*. Each strain was spotted on the indicated media and grown for 2 days (YEA 36 °C) or 3days (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 4 species are shown in white against black and the amino acids conserved among 3 species are shaded. Sp, *Schizosaccharomyces pombe*; Sc, *Saccharomyces cerevisiae*; Hs, *Homo sapiens*; Dm, *Drosophila*

*melanogaster*. (C) The multicopy suppressors of *Δtsc2 cpp1-1* cells. *Δtsc2 cpp1-1* cells carrying each of the five multicopy suppressors were grown in liquid EMM (1 x $10^7$ cells/ml) and spotted on EMM or EMM with canavanine (60 μg/ml). They were incubated for 2 days (EMM, 36 °C) or 3 days (EMM or EMM containing canavanine, 26 °C).

**Figure 3.** Expression of SpRheb (rhb1$^{CVIL}$). *Δtsc2 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 medium at a concentration of 1 x $10^7$ cells/ml and 5 μl of the suspension was spotted on solid EMM or solid EMM medium containing canavanine (60 μg/ml). They were incubated for 2 days (EMM, 36 °C) or 3 days (EMM or EMM containing canavanine, 26 °C).

**Figure 4.** Antibody to Rhb1. (A) Wild type cells (SP6) carrying pREP81-rhb1 was grown to mid-log phase in EMM medium containing thiamine (50 μg/ml) and then transferred to thiamine free EMM medium for 17 hours at 30 °C. 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).

**Figure 5.** Modification of Rhb1 in *cpp1-1*. (A) Wild type cells (SP740) and *cpp1-1* cells (YKK55) were grown to mid-log phase in YEL medium at 26 °C and then shifted to 36 °C. 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 *Δtsc2 cpp1-1* cells (YKK25) were grown at 36 °C for 6 hours. 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 anti-Spo14 antibody, respectively.

**Table 1.** Strains used in this study.

**Table 2.** Genes less induced in *Δtsc1* and *Δtsc2*. *ORFs* which were induced more than four-fold in the wild type but not in *Δtsc1* nor *Δtsc2* 3 hours after nitrogen starvation were listed with their ORF ID, gene name (if available) and possible function.

**Table 3.** Genes more induced in *Δtsc1* and *Δtsc2*. *ORFs* which were induced more than four-fold in *Δtsc1* and *Δtsc2* but not in the wild type 3 hours after nitrogen starvation were listed with ORF ID, gene name (if available) and possible function.

**Table 4.** Multicopy suppressors of *cpp1-1*. Genes responsible for multicopy suppression of *cpp1-1* are listed with their accession number and possible function.

**Literature Cited**

ABRAHAM, R. T., and G. J. WIEDERRECHT, 1996 Immunopharmacology of rapamycin. Annu Rev Immunol **14:** 483-510.

ARELLANO, M., P. M. COLL, W. YANG, A. DURAN, F. TAMANOI *et al.*, 1998 Characterization of the geranylgeranyl transferase type I from Schizosaccharomyces pombe. Mol Microbiol **29:** 1357-1367.

BECK, T., and M. N. HALL, 1999 The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature **402:** 689-692.

CAFFERKEY, R., M. M. MCLAUGHLIN, P. R. YOUNG, R. K. JOHNSON and G. P. LIVI, 1994 Yeast TOR (DRR) proteins: amino-acid sequence alignment and identification of structural motifs. Gene **141:** 133-136.

CARBONARA, C., L. LONGA, E. GROSSO, C. BORRONE, M. G. GARRE *et al.*, 1994 9q34 loss of heterozygosity in a tuberous sclerosis astrocytoma suggests a growth suppressor-like activity also for the TSC1 gene. Hum Mol Genet **3:** 1829-1832.

CASEY, P. J., P. A. SOLSKI, C. J. DER and J. E. BUSS, 1989 p21ras is modified by a

farnesyl isoprenoid. Proc Natl Acad Sci U S A **86:** 8323-8327.

DIMITROV, K., and S. SAZER, 1998 The role of fnx1, a fission yeast multidrug resistance protein, in the transition of cells to a quiescent G0 state. Mol Cell Biol **18:** 5239-5246.

DUVEL, K., A. SANTHANAM, S. GARRETT, L. SCHNEPER and J. R. BROACH, 2003 Multiple roles of Tap42 in mediating rapamycin-induced transcriptional changes in yeast. Mol Cell **11:** 1467-1478.

EUROPEAN CHROMOSOME 16 TUBEROUS SCLEROSIS CONSORTIUM, 1993 Identification and characterization of the tuberous sclerosis gene on chromosome 16. Cell **75:** 1305-1315.

GIETZ, D., A. ST JEAN, R. A. WOODS and R. H. SCHIESTL, 1992 Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res **20:** 1425.

GOMEZ, M. R., 1995 History of the tuberous sclerosis complex. Brain Dev **17 Suppl:** 55-57.

GOMEZ, M. R., J. R. SAMPSON and V. H. WHITTEMORE, 1999 *Tuberous Sclerosis Complex*, Ed. 3. Oxford University Press, London.

GRAAF, M. R., D. J. RICHEL, C. J. VAN NOORDEN and H. J. GUCHELAAR, 2004 Effects of statins and farnesyltransferase inhibitors on the development and progression of cancer. Cancer Treat Rev **30:** 609-641.

GREEN, A. J., P. H. JOHNSON and J. R. YATES, 1994a The tuberous sclerosis gene on chromosome 9q34 acts as a growth suppressor. Hum Mol Genet **3:** 1833-1834.

GREEN, A. J., M. SMITH and J. R. YATES, 1994b Loss of heterozygosity on chromosome 16p13.3 in hamartomas from tuberous sclerosis patients. Nat Genet **6:** 193-196.

HENGSTSCHLAGER, M., D. M. RODMAN, A. MILOLOZA, E. HENGSTSCHLAGER-OTTNAD, M. ROSNER *et al.*, 2001 Tuberous sclerosis gene products in proliferation control. Mutat Res **488:** 233-239.

HENSKE, E. P., B. W. SCHEITHAUER, M. P. SHORT, R. WOLLMANN, J. NAHMIAS *et al.*, 1996 Allelic loss is frequent in tuberous sclerosis kidney lesions but rare in brain lesions. Am J Hum Genet **59:** 400-406.

IMAI, Y., and M. YAMAMOTO, 1994 The fission yeast mating pheromone P-factor: its molecular structure, gene structure, and ability to induce gene expression and G1 arrest in the mating partner. Genes Dev **8:** 328-338.

ITO, N., and G. M. RUBIN, 1999 gigas, a Drosophila homolog of tuberous sclerosis gene product-2, regulates the cell cycle. Cell **96:** 529-539.

JENSEN, R., G. F. SPRAGUE, JR. and I. HERSKOWITZ, 1983 Regulation of yeast mating-type interconversion: feedback control of HO gene expression by the mating-type locus. Proc Natl Acad Sci U S A **80:** 3035-3039.

KANOH, J., A. SUGIMOTO and M. YAMAMOTO, 1995 Schizosaccharomyces pombe zfs1+ encoding a zinc-finger protein functions in the mating pheromone recognition pathway. Mol Biol Cell **6:** 1185-1195.

KWIATKOWSKI, D. J., and M. P. SHORT, 1994 Tuberous sclerosis. Arch Dermatol **130:** 348-354.

KWIATKOWSKI, D. J., H. ZHANG, J. L. BANDURA, K. M. HEIBERGER, M. GLOGAUER *et al.*, 2002 A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells. Hum Mol Genet **11:** 525-534.

LI, Y., K. INOKI and K. L. GUAN, 2004 Biochemical and functional characterizations of small GTPase Rheb and TSC2 GAP activity. Mol Cell Biol **24:** 7965-7975.

LI, Y., M. N. CORRADETTI, K. INOKI and K. L. GUAN, 2004 TSC2: filling the GAP in the mTOR signaling pathway. Trends Biochem Sci **29:** 32-38.

LONG, X., Y. LIN, S. ORTIZ-VEGA, K. YONEZAWA and J. AVRUCH, 2005 Rheb binds and regulates the mTOR kinase. Curr Biol **15:** 702-713.

MACH, K. E., K. A. FURGE and C. F. ALBRIGHT, 2000 Loss of Rhb1, a Rheb-related GTPase in fission yeast, causes growth arrest with a terminal phenotype similar to that caused by nitrogen starvation. Genetics **155:** 611-622.

MANNING, B. D., and L. C. CANTLEY, 2003 Rheb fills a GAP between TSC and TOR. Trends Biochem Sci **28:** 573-576.

MATSUMOTO, S., A. BANDYOPADHYAY, D. J. KWIATKOWSKI, U. MAITRA and T. MATSUMOTO, 2002 Role of the Tsc1-Tsc2 complex in signaling and transport across the cell membrane in the fission yeast Schizosaccharomyces pombe. Genetics **161:** 1053-1063.

MCNEW, J. A., M. SOGAARD, N. M. LAMPEN, S. MACHIDA, R. R. YE *et al.*, 1997 Ykt6p, a prenylated SNARE essential for endoplasmic reticulum-Golgi transport. J Biol Chem **272:** 17776-17783.

MORENO, S., A. KLAR and P. NURSE, 1991 Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods Enzymol **194:** 795-823.

NAKAMURA-KUBO, M., T. NAKAMURA, A. HIRATA and C. SHIMODA, 2003 The fission yeast spo14+ gene encoding a functional homologue of budding yeast Sec12 is required for the development of forespore membranes. Mol Biol Cell **14:** 1109-1124.

OKAZAKI, K., N. OKAZAKI, K. KUME, S. JINNO, K. TANAKA *et al.*, 1990 High-frequency transformation method and library transducing vectors for cloning mammalian cDNAs by trans-complementation of Schizosaccharomyces pombe. Nucleic Acids Res **18:** 6485-6489.

OMER, C. A., and N. E. KOHL, 1997 CA1A2X-competitive inhibitors of farnesyltransferase as anti-cancer agents. Trends Pharmacol Sci **18:** 437-444.

PAN, D., J. DONG, Y. ZHANG and X. GAO, 2004 Tuberous sclerosis complex: from Drosophila to human disease. Trends Cell Biol **14:** 78-85.

PARK, H. W., S. R. BODULURI, J. F. MOOMAW, P. J. CASEY and L. S. BEESE, 1997 Crystal structure of protein farnesyltransferase at 2.25 angstrom resolution.

Science **275:** 1800-1804.

ROHDE, J. R., and M. E. CARDENAS, 2003 The tor pathway regulates gene expression by linking nutrient sensing to histone acetylation. Mol Cell Biol **23:** 629-635.

SEBTI, S. M., 2005 Protein farnesylation: implications for normal physiology, malignant transformation, and cancer therapy. Cancer Cell **7:** 297-300.

STRICKLAND, C. L., W. T. WINDSOR, R. SYTO, L. WANG, R. BOND *et al.*, 1998 Crystal structure of farnesyl protein transferase complexed with a CaaX peptide and farnesyl diphosphate analogue. Biochemistry **37:** 16601-16611.

SUGIMOTO, A., Y. IINO, T. MAEDA, Y. WATANABE and M. YAMAMOTO, 1991 Schizosaccharomyces pombe ste11+ encodes a transcription factor with an HMG motif that is a critical regulator of sexual development. Genes Dev **5:** 1990-1999.

TANAKA, K., T. YONEKAWA, Y. KAWASAKI, M. KAI, K. FURUYA *et al.*, 2000 Fission yeast Eso1p is required for establishing sister chromatid cohesion during S phase. Mol Cell Biol **20:** 3459-3469.

TANAKA, N., N. OHUCHI, Y. MUKAI, Y. OSAKA, Y. OHTANI *et al.*, 1998 Isolation and

characterization of an invertase and its repressor genes from Schizosaccharomyces pombe. Biochem Biophys Res Commun **245:** 246-253.

THOMAS, P. A., K. S. BHAT and K. M. KOTIAN, 1980 Antibacterial properties of dilute formocresol and eugenol and propylene glycol. Oral Surg Oral Med Oral Pathol **49:** 166-170.

URANO, J., M. J. COMISO, L. GUO, P. J. ASPURIA, R. DENISKIN *et al.*, 2005 Identification of novel single amino acid changes that result in hyperactivation of the unique GTPase, Rheb, in fission yeast. Mol Microbiol **58:** 1074-1086.

URANO, J., A. P. TABANCAY, W. YANG and F. TAMANOI, 2000 The Saccharomyces cerevisiae Rheb G-protein is involved in regulating canavanine resistance and arginine uptake. J Biol Chem **275:** 11198-11206.

VAN SLEGTENHORST, M., A. MUSTAFA and E. P. HENSKE, 2005 Pas1, a G1 cyclin, regulates amino acid uptake and rescues a delay in G1 arrest in Tsc1 and Tsc2 mutants in Schizosaccharomyces pombe. Hum Mol Genet **14:** 2851-2858.

VAN SLEGTENHORST, M., E. CARR, R. STOYANOVA, W. D. KRUGER and E. P. HENSKE, 2004 Tsc1+ and tsc2+ regulate arginine uptake and metabolism in

Schizosaccharomyces pombe. J Biol Chem **279:** 12706-12713.

VAN SLEGTENHORST, M., R. DE HOOGT, C. HERMANS, M. NELLIST, B. JANSSEN *et al.*, 1997 Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. Science **277:** 805-808.

YANG, W., A. P. TABANCAY, JR., J. URANO and F. TAMANOI, 2001 Failure to farnesylate Rheb protein contributes to the enrichment of G0/G1 phase cells in the Schizosaccharomyces pombe farnesyltransferase mutant. Mol Microbiol **41:** 1339-1347.

YANG, W., J. URANO and F. TAMANOI, 2000 Protein farnesylation is critical for maintaining normal cell morphology and canavanine resistance in Schizosaccharomyces pombe. J Biol Chem **275:** 429-438.

YEUNG, R. S., 2003 Multiple roles of the tuberous sclerosis complex genes. Genes Chromosomes Cancer **38:** 368-375.

ZHANG, Y., X. GAO, L. J. SAUCEDO, B. RU, B. A. EDGAR *et al.*, 2003 Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nat Cell Biol **5:** 578-581.

Table 1 Strains used in this study

| Strain | Genotype | Source |
|---|---|---|
| SP6 | *h⁻ leu1-32* | Lab. stock |
| AE512 | *h⁻ tsc2::ura4⁺ ura4-D18 leu1-32* | Lab. stock |
| AE413 | *h⁻ tsc1::ura4⁺ ura4-D18 leu1-32* | Lab. 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⁻* | Lab. stock |
| YKK55 | *h⁻ ura4-D18 leu1-32 cpp1-1* | This work |
| SP740 | *h⁻ ura4-D18 leu1-32* | Lab. stock |

Table 2 Genes not induced in *Δtsc1* and *Δtsc2*

| 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 |

Table 3 Genes induced higher in *Δtsc1* and *Δtsc2* 3 hrs 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 |

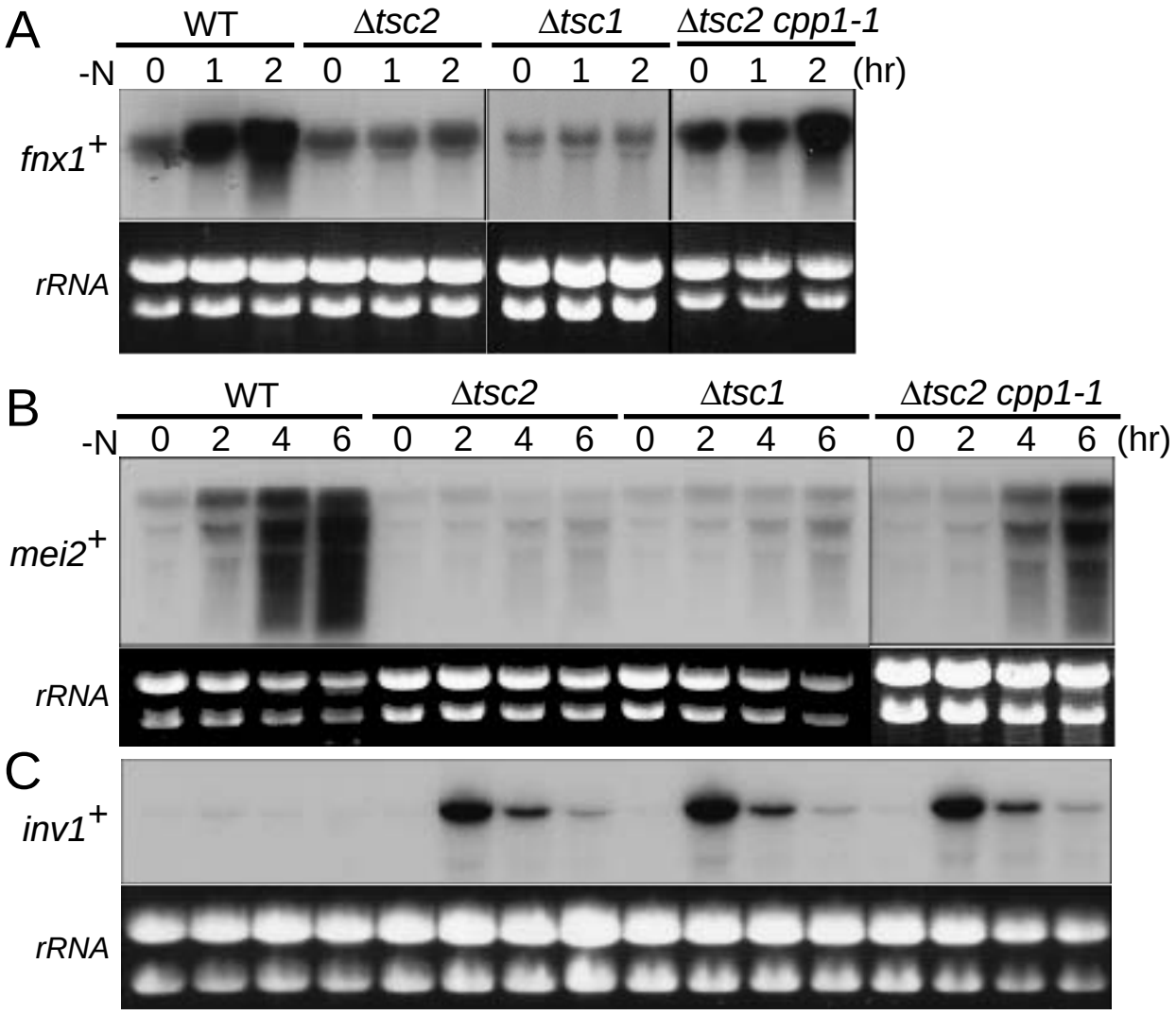
| | | | |
|---|---|---|---|
| SPAC3F10.16c | | | GTPase |
| SPBC1271.08c | | | hypothetical protein |
| SPBC1271.07c | | | Acetyltransferase |
| SPAC57A10.01 | SPAC19E9.03 | *pas1* | Pcl-like cyclin |
| SPBC2G2.04c | | *mmf1* *pmf1* | conserved protein |
| SPBP4H10.12 | | | conserved protein |
| SPAC821.10c | | *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 |

Table 4 Multicopy suppressors of *Δtsc2 cpp1-1* cells.

| Gene name (systematic name) | Accession number | Possible function |
|---|---|---|
| *cwp1* (SPAPB1A10.04c) | CAC21477.1 | alpha 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 |

Figure 1


A
WT
Δtsc2
Δtsc1
Δtsc2 cpp1-1
-N 0 1 2 0 1 2 0 1 2 0 1 2 (hr)
fnx1+
rRNA
B
WT
Δtsc2
Δtsc1
Δtsc2 cpp1-1
-N 0 2 4 6 0 2 4 6 0 2 4 6 0 2 4 6 (hr)
mei2+
rRNA
C
inv1+
rRNA

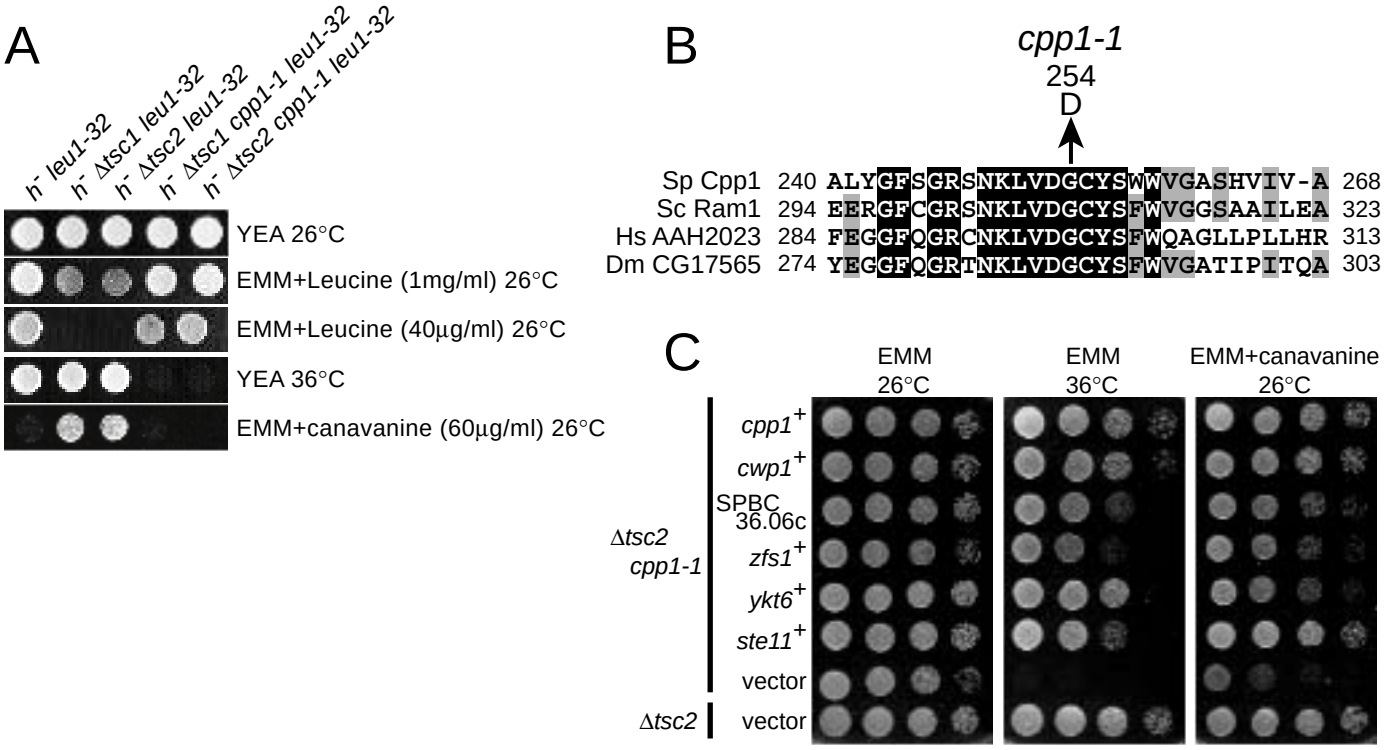

A
h- leu1-32
h- Δtsc1 leu1-32
h- Δtsc2 leu1-32
h- Δtsc1 cpp1-1 leu1-32
h- Δtsc2 cpp1-1 leu1-32
YEA 26°C
EMM+Leucine (1mg/ml) 26°C
EMM+Leucine (40µg/ml) 26°C
YEA 36°C
EMM+canavanine (60µg/ml) 26°C
B
cpp1-1
254
D
Sp Cpp1 240 ALYGFSGRSNKLVDGCYSWWVGASHVIV-A 268
Sc Ram1 294 EERGFCGRSNKLVDGCYSFWVGGSAAILEA 323
Hs AAH2023 284 FEGGFQGRCNKLVDGCYSFWQAGLLPLLHR 313
Dm CG17565 274 YEGGFQGRTNKLVDGCYSFWVGATIPITQA 303
C
EMM 26°C
EMM 36°C
EMM+canavanine 26°C
Δtsc2 cpp1-1
cpp1+
cwp1+
SPBC 36.06c
zfs1+
ykt6+
ste11+
vector
Δtsc2
vector

Figure 3

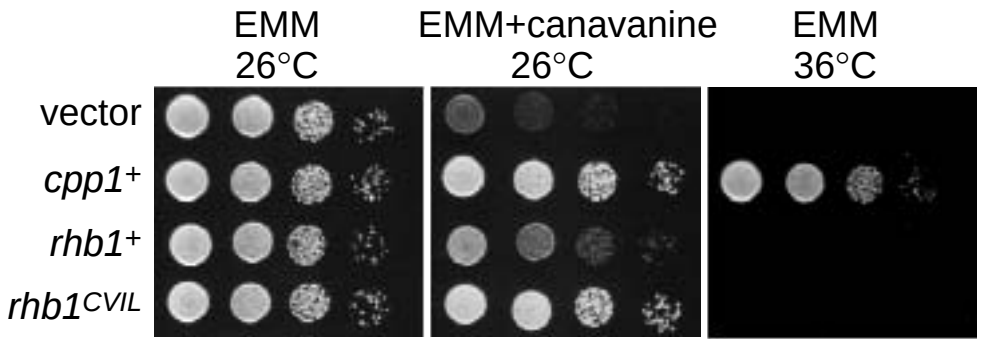

EMM
26°C
EMM+canavanine
26°C
EMM
36°C
vector
cpp1+
rhb1+
rhb1CVIL

Figure 4

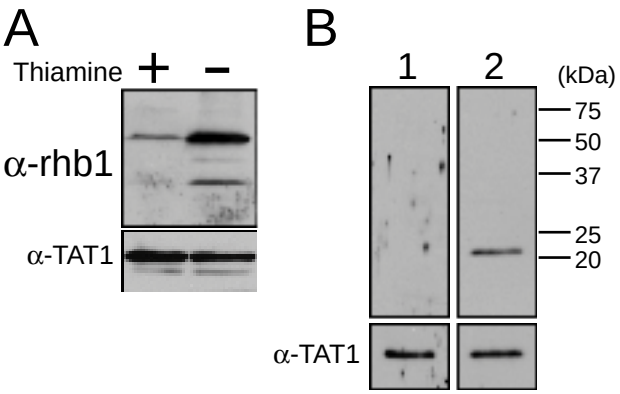
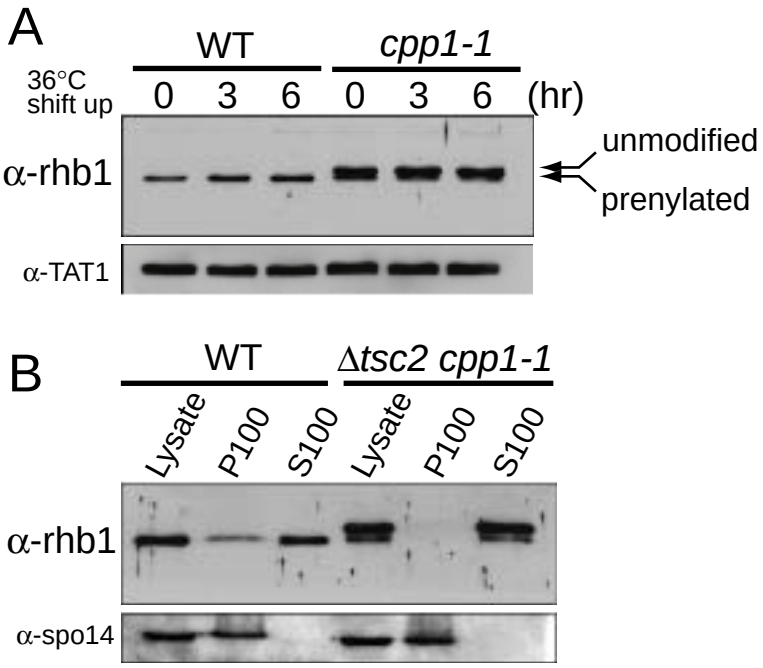


Figure 5


A
WT
cpp1-1
36°C
shift up
0 3 6 0 3 6 (hr)
α-rhb1
unmodified
prenylated
α-TAT1
B
WT
Δtsc2 cpp1-1
Lysate
P100
S100
Lysate
P100
S100
α-rhb1
α-spo14