The Birt-Hogg-Dube and Tuberous Sclerosis Complex Homologs Have Opposing Roles in Amino Acid Homeostasis in Schizosaccharomyces pombe*^[3]

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Birt-Hogg-Dube (BHD) is a tumor suppressor gene disorder characterized by skin hamartomas, cystic lung disease, and renal cell carcinoma. The fact that hamartomas, lung cysts, and renal cell carcinoma can also occur in tuberous sclerosis complex (TSC) suggests that the BHD and TSC proteins may function within a common pathway. To evaluate this hypothesis, we deleted the BHD homolog in Schizosaccharomyces pombe. Expression profiling revealed that six permease and transporter genes, known to be down-regulated in Δtsc1 and Δtsc2, were up-regulated in Δbhd, and levels of specific intracellular amino acids known to be low in Δtsc1 and Δtsc2 were elevated in Δbhd. This “opposite” profile was unexpected, given the overlapping clinical phenotypes. The TSC1/2 proteins inhibit Rheb in mammals, and Tsc1/Tsc2 inhibit Rhb1 in S. pombe. Expression of a hypomorphic allele of rhb1+ dramatically increased permease expression levels in Δbhd but not in wild-type yeast. Loss of Bhd sensitized yeast to rapamycin-induced increases in permease expression levels, and rapamycin induced lethality in Δbhd yeast expressing the hypomorphic Rhb1 allele. In S. pombe, it is known that Rhb1 binds Tor2, and Tor2 inhibition leads to up-regulation of permeases including those that are regulated by Bhd. Our data, therefore, suggest that Bhd activates Tor2. If the mammalian BHD protein, folliculin, similarly activates mammalian target of rapamycin, it will be of great interest to determine how mammalian target of rapamycin inhibition in BHD patients and mammalian target of rapamycin activation in TSC patients lead to overlapping clinical phenotypes.

Birt-Hogg-Dube (BHD) syndrome is an autosomal dominant disorder characterized by hamartomas of skin follicles, lung cysts, spontaneous pneumothorax, and renal cell carcinoma (1–3). The BHD gene was cloned in 2002 and encodes folliculin, which has no significant homology to other human proteins (4). BHD mRNA is expressed in many tissues, including skin, kidney, lung, brain, heart, placenta, testes, spleen, and pancreas. All reported human germline BHD mutations are predicted to result in premature protein truncation (4–8). Inactivating mutations of the remaining allele have been identified in renal carcinomas from BHD patients, indicating that BHD is a tumor suppressor gene (8). Germline nonsense mutations in BHD can also cause isolated hereditary spontaneous pneumothorax with lung cysts (6), without the renal or skin manifestations of BHD. Consistent with the role of BHD as a tumor suppressor gene, somatic BHD mutations have also been detected in endometrial carcinomas (9). Disease-causing BHD mutations have also been noted in animals. A 1-base pair insertion mutation in the BHD rat homolog resulting in premature truncation causes renal carcinoma in the Nihon rat (10). In German shepherd dogs, a missense mutation, H255R, causes hereditary multifocal renal cancer, uterine leiomyoma, and skin lesions (11).

Skin hamartomas, lung cysts, pneumothorax, and renal tumors, the clinical hallmarks of BHD, also occur in tuberous sclerosis complex (TSC). TSC is a tumor suppressor gene syndrome caused by mutations in either the TSC1 or TSC2 gene. The TSC1 and TSC2 proteins heterodimerize and inhibit the mammalian target of rapamycin (mTOR) via the small GTPase Rheb, which is the target of the highly conserved GTPase activating domain of TSC2 (12–18). Similar to the mammalian pathway, Schizosaccharomyces pombe Tsc1/Tsc2 function as a complex to regulate Rhb1, the S. pombe Rheb homolog. S. pombe mutants in which Tsc1 or Tsc2 is deleted have a distinctive phenotype, with low levels of amino acid permease expression, low intracellular amino acid levels (particularly ornithine and citrulline), and resistance to canavanine, a toxic analog of arginine. Deletion of rhb1+ in S. pombe results in increased sensitivity to canavanine (19), and a mutagenesis screen for rescue of the amino acid uptake phenotype of yeast lacking Tsc1 or Tsc2 yielded a mutant allele of rhb1+ (Rhb1G30D/S165N) (20), consistent with a conserved relationship between Tsc1, Tsc2, and Rheb in S. pombe. Similar to mammalian cells Rhb1 is known to interact with Tor2, one of the two Tor proteins in yeast homologous to mTOR (21–23). Tor2 is an essential gene. Down-regulation of Tor2 activity leads to up-regulation of nitrogen responsive genes including membrane transporters.

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4 The abbreviations used are: BHD, Birt-Hogg-Dube; TSC, tuberous sclerosis complex; mTOR, mammalian target of rapamycin; EMM, essential minimal medium; RT, reverse transcriptase; HEK, human embryonic kidney.
and amino acid permeases (24). In contrast, deletion of Tor1 protein leads to down-regulation of amino acid permeases (25) and the combination with Tsc1/Tsc2 deletion leads to even lower levels of permease expression (23) indicating that these proteins function in parallel pathways.

Folliculin, the BHD protein, has no significant homology to any other human proteins, and very little is known about how BHD mutations lead to renal tumorigenesis or the lung disease. The clinical similarities between TSC and BHD patients led us to hypothesize that BHD and the TSC proteins function within a common cellular pathway. To address this, we identified the S. pombe BHD ortholog, SPBC24C6.08c, here called bhd, and used homologous recombination to generate a novel deletion strain, Δbhd. Unexpectedly, we found that multiple amino acid permeases and transporters that are down-regulated in Δtsc1 and Δtsc2 are up-regulated in Δbhd, and that Δbhd S. pombe have elevated intracellular levels of specific amino acids that are low in Δtsc1 and Δtsc2. Yeast lacking Bhd demonstrated hypersensitivity to rapamycin, a specific inhibitor of mTOR. These data strongly oppose an opposing role of Bhd to that of Tsc1/Tsc2 in regulating amino acid homeostasis in S. pombe. If this relationship is conserved in mammals, it will implicate a surprising mechanism in which both inappropriate mTOR inhibition in BHD and inappropriate mTOR activation in TSC lead to renal tumorigenesis and cystic lung disease.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Media, and Growth Conditions—**CHP428 and CHP429 were constructed by Charlie Hoffman (Boston College, MA) and were a gift from Janet Leatherwood (Stony Brook University of New York, New York). Wild-type strain 972 (26) and urad-D18 (27) were gifts from J. Bähler (Sanger Institute). The methionine auxotrophic strain 1945h was obtained from the National Collection of Yeast Cultures, UK. S. pombe cells were grown in essential minimal medium (EMM; Obiogene, Carlsbad, CA) at 30 °C unless otherwise stated. Transformations were performed with Frozen-EZ Yeast Transformation II kit (Zymol Research, Orange, CA). Where indicated, cells were treated with rapamycin (100 ng/ml) for 3 h prior to harvest.

**Construction of bhd**, bhd**tor1**, and bhd**tsc1**-deficient Strains—**The entire open reading frame of bhd was deleted from the genome of the haploid strain 972h, using double fusion PCR homologous recombination and replaced by the kanamycin cassette to create DK1. Correct integration was confirmed by PCR. Δbhd was crossed into the urad-D18 strain to generate DK2 (urad4Δbhd) using random spore analysis on selective plates. Tsc1−-deficient strain MVS3 was used to generate Δtsc1Δbhd by isolating spores and using PCR to identify double mutants, which were verified by Northern blot. The Δtor1Δbhd-deficient strain was generated by crossing TA99 (gift from R. Weisman) and DK2 using random spore analysis on selective plates. During mating, Tor1 was being expressed in TA99 from a plasmid to compensate for sterility of this mutant strain.

**Construction of Plasmids—**The bhd+ gene was amplified from genomic DNA and cloned into the pREP4X expression vector. After sequence verification, bhd+ was inserted in-frame into the hemagglutinin-tagged pSLF173/273/373 series with different nmt (no message in thiamine) promoter strength (ATCC, Manassas, VA). The BHD H352R mutation was introduced into the pSLF373 constructs using site-directed mutagenesis (Stratagene, La Jolla, CA) to generate Bhd-H352 and was verified by sequencing. Human BHD was amplified from cDNA (the generous gift of Laura Schmidt) using BHD-specific oligos and inserted in-frame into the hemagglutinin-tagged yeast pSLF173.

**BHD Plasmid Construction and Transfection—**Myc-BHD plasmid DNA was created by amplifying human cDNA (the generous gift of Laura Schmidt) using BHD-specific oligos. PCR product was ligated into myc-tagged pCMV-Tag3 vector (Stratagene). The Myc-BHD-H255R plasmid DNA was created using site-directed mutagenesis of the myc-BHD plasmid and was verified by sequencing.

Two µg of myc-BHD or myc-BHD-H255R DNA was transfected into HEK 293 cells using FuGENE 6 reagent (Roche Applied Sciences). After 48 h cells were lysed and analyzed by immunoblot using anti-folliculin or anti-actin antibodies.

**Expression Profiling—**Yeast were grown overnight in EMM to early log phase (A0.95 = 0.2–0.3) and total RNA was isolated by phenol extraction and purified using RNeasy (Qiagen, Valencia, CA). Total RNA from two independent biological samples was pooled (10 µg of each sample), reverse transcribed into cDNA, and labeled with Cy3 and Cy5 (Amersham Biosciences). Hybridizations were carried out overnight at 42 °C. The slides were scanned with a GMS 428 Scanner (Affymetrix, Santa Clara, CA) and spot quantification was performed with the ImaGene software (BioDiscovery, Marina del Rey, CA). Each S. pombe gene was present in duplicate on each slide, and the experiments were repeated using opposite labels (dye-flip), resulting in a total of eight measurements for each gene per sample. Genes were considered expressed when all eight measurements exceeded a threshold of 3.5 times above the background. A linear regression normalization was applied to the data and fold changes were calculated. Genes were grouped and annotated on the basis of predicted function in the Sanger Institute S. pombe Gene Data base.

**Western and Northern Blot Analyses—**For Western blots, 20 µg of each sample was run on 4–20% SDS-PAGE gel (Bio-Rad) and transferred to nitrocellulose using standard methods. The immobilized proteins were detected using enhanced chemiluminescence (Amersham Biosciences). For Northern blots, 10 µg of total RNA was run on a 1% formaldehyde gel and transferred to nylon membrane overnight in 20× SSC. Probes for bhd+, c869 10+, isp4+, isp5+, and gpd3+ were PCR amplified from cDNA, labeled with [α-32P]dCTP (PerkinElmer Life Sciences), and hybridized using standard methods.

**Canavanine and DL-Ethionine Sensitivity—**Cells were grown overnight to midlog phase (A0.95 = 0.4–0.6) and A0.95 was adjusted to 0.4 (10,000 cells/ml). 4 µl of 1, 10, and 100 times dilutions was spotted onto EMM as a growth control, or EMM containing canavanine (60 µg/ml) or DL-ethionine (30 µg/ml) (both from Sigma) and incubated for 3 days at 30 °C.

**Real Time Reverse Transcriptase-PCR (RT-PCR)—**Contaminating DNA from RNA preparations was removed using TURBO DNA-free™ (Ambion, Austin, TX). RNA was quantified using the Agilent 2100 BioAnalyzer in combination with a
RNA 6000 Nano LabChip. RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Ambion) and a mixture of anchored oligo(dT) and random decamers. For each sample, 2 RT reactions were performed with inputs of 50 and 10 ng. A – RT control reaction with 50 ng of input was also performed for each sample. 5’-Nuclease assays using TaqMan chemistry were run on a 7900 HT sequence detection system (Applied Biosystems, Foster City, CA). TaqMan sets were designed using Primer Express™ version 2.0 software from Applied Biosystems. The 5’ and 3’ ends of the probes were labeled with the reporter dye 6-FAM (6-carboxyfluorescein) (Glen Research, Sterling, VA) and the quencher dye BHQ1 (Black Hole Quencher) (Biosearch Technologies, Novato, CA), respectively. Cycling conditions were 95 °C, 15 min followed by 40 (two steps) cycles (95 °C, 15 s; 60 °C, 60 s). Ct (cycle threshold) values were converted to quantities (in arbitrary units) using a standard curve (5 points, 5-fold dilutions) established with a calibrator sample. For each sample, the 2 values of relative quantity (from 2 PCR) were averaged.

**Measurements of Intracellular Amino Acid Pools**—100 μg of protein extract (1 μg/μl) was precipitated with 100 μl of 10% 5-sulfosalicylic acid at 4 °C for 1 h. The pH value of the supernatant was adjusted to 2.2 with 3 M LiOH. 100 μl of sample was injected into the Biochrom 30 amino acid analyzer (Biochrom, Cambridge, United Kingdom) including a standard amino acid mixture of 10 nM (Sigma).

**RESULTS**

Deletion of the *S. pombe* Homolog of BHD—Comparison of the human BHD with the fission yeast genome identified a single homologous gene, SPBC24C6.08c+, referred to as bhd+ . Bhd+ encodes a 367-amino acid protein with 21% identity and an additional 36% similarity to the human BHD gene. The entire open reading frame of bhd+ was deleted by homologous recombination and replaced with a kanamycin cassette, to generate a haploid bhd+ deletion strain, Δbhd (Table 1). The Δbhd strain had no evident defects in growth, proliferation, or mating.

Δbhd Is Resistant to DL-Ethionine, but the Mechanism of Resistance Is Different Than in Δtscl—We previously found that Δtscl and Δtscl2 have decreased uptake of arginine, and are therefore resistant to l-canavanine, a toxic analog of arginine (28). Δtscl and Δtscl2 are also resistant to the toxic methionine analog, dl-ethionine (20). To determine whether Δbhd has a similar phenotype, the Δbhd strain was incubated with 60 mg/liter l-canavanine or 30 mg/liter dl-ethionine. Δbhd yeast were not resistant to l-canavanine (unlike Δtscl1), but they were resistant to dl-ethionine, similar to Δtscl1 (Fig. 1A). A Δtscl1Δbhd double mutant strain was resistant to canavanine and dl-ethionine. This partial phenotypic overlap between Δtscl1 and Δbhd suggested that the Tsc1, Tsc2, and Bhd proteins may function in a common pathway.

Next, to determine whether the dl-ethionine resistance observed in Δbhd and Δtscl1 was due to decreased uptake of methionine, we crossed the Δtscl1 and Δbhd strains into a methionine auxotrophic strain, 1945h+, referred to as Δmet, which is unable to synthesize methionine and is therefore dependent on methionine uptake for growth, and tested growth on essential minimal medium plates with and without 50 mg/liter methionine. The Δtscl1Δmet strain was unable to grow with this low level of supplemented methionine, consistent with a methionine uptake defect, but the ΔbhdΔmet grew similarly to wild-type (Fig. 1B). Therefore, despite the similar phenotype of resistance to the toxic analog of methionine, the mechanism of dl-ethionine resistance appeared to be different between Δtscl1 and Δbhd.

Δtscl1 and Δbhd Have Opposite Expression Profiles for Transporters and Permeases—Because these results did not support a role of Bhd in the Tsc/Rhb pathway, as we had originally hypothesized, we next compared the transcriptional expression profile of 2 separate colonies of Δbhd with wild-type yeast, using a dye-flip design, for a total of four arrays. A linear regression normalization was applied to the data and fold changes were calculated (see “Experimental Procedures”).

**TABLE 1**

*S. pombe* strains used in this study

| Strain        | Genotype  | Source                |
|---------------|-----------|-----------------------|
| 972           | h         | Leupold               |
| DK1           | h−, bhd::kan+ | This study            |
| MV51          | h−, tsc1::kan+ | This study            |
| K2            | h−, bhd::kan+, tsc1::kan+ | This study            |
| DK3           | h−, bhd::kan+, ura4::D18 | This study            |
| MV59          | h−, Rhb1::GAD7,Stn7::Nmt1 | This study            |
| MV510         | h−, bhd::kan+, Rhb1::GAD7,Stn7::Nmt1 | This study            |
| 1945h−        | h−, met3-1 | NCYC                  |
| DK4           | h+, bhd::kan+, met5-1 | This study            |
| DK5           | h−, tsc1::kan+, met-1 | This study            |
| TA99          | h−, tor1::ura, ura4::D18, leu1-32, ade6-M216 | Weisman              |
| DK6           | h+, tor1::ura, ura4::D18 | This study            |
| DK7           | h+, tor1::ura, bhd::kan+ | This study            |

**FIGURE 1. Δbhd S. Pombe are resistant to the toxic methionine analog, dl-ethionine.** A, 972 wild-type, Δtscl1, Δbhd, and Δtscl1Δbhd yeast were grown in EMM overnight to midlog phase. Cells were then diluted to A900 = 0.4, and 10-fold different dilutions (40,000 to 40 cells) were spotted on EMM plates (left panel), on EMM with 60 mg/liter canavanine (middle panel), and on EMM with 30 mg/liter dl-ethionine (right panel). Plates were incubated at 30 °C for 3 days and then photographed. B, wild type yeast and methionine mutants of Δtscl1 and Δbhd were grown and spotted as described above on EMM (left panel) and on EMM with 50 mg/liter methionine (right panel).
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TABLE 2
Genes up-regulated in Δbhd and down-regulated in Δtsc1 + Δtsc2

| Gene name              | Predicted function                  |
|------------------------|-------------------------------------|
| SPAC1096.10c           | Amino acid permease                 |
| SPAP7G5.06            | Amino acid permease                 |
| isp5                  | Amino acid permease                 |
| isp4                  | Oligopeptide transporter            |
| ptr2                  | Oligopeptide transporter            |
| SPAC11D3.18c          | Membrane transporter                |
| SPAC11D3.14c          | 5-Oxoprolinase, proline metabolism |
| SPAC1223.09c          | Urate oxidase                       |
| SPAC5H10.01          | Mitochondrial precursor             |
| SPAC1039.10          | Mitochondrial precursor             |

Using a threshold of 1.5-fold change, 10 genes were down-regulated and 30 genes were up-regulated in Δbhd in at least 2 of the 4 arrays (supplemental materials Tables S1 and S2). Remarkably, 10 of the up-regulated genes in Δbhd were previously found to be down-regulated in Δtsc1 and Δtsc2 (Table 2 and Fig. 2A). Of particular interest, SPAC869.10c (which will be referred to as 869.10), SPAP7G5.06 (referred to as 7G5.06), SPAC11D3.18c, ptr2, isp4, and isp5 are permeases or transporters that are down-regulated in Δtsc1 and Δtsc2 strains (28). Northern blotting confirmed the expression change for four of the genes (Fig. 2B). The ΔbhdΔtsc1 double mutant had intermediate mRNA expression levels for these permeases, comparable with wild type. These data suggested, unexpectedly, that Tsc1/Tsc2 and Bhd have opposing functions in S. pombe.

The Permease Expression Defect in Δbhd Is Rescued by Wild Type bhd—Three regions of homology to the human and Drosofila melanogaster BHD proteins are shown in Fig. 3A. The histidine residue that is mutant in the German shepherd hereditary renal cancer syndrome (H255R) is conserved in S. pombe and flies (Fig. 3A). To determine whether this mutant also disrupts the function of bhd in S. pombe, we generated the mutant, Bhd-H352R, in the hemagglutinin-tagged pSLF373-ura4+ expression vector. The Bhd and Bhd-H352R expression constructs were transformed into uraΔbhd and cells were plated on EMM plates without uracil. Relative expression of the permeases was studied by real time PCR. Wild-type Bhd expression decreased the expression of four permeases (Fig. 2).
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Permease Expression in Bhd Mutants Is Increased by Expression of a Hypomorphic Rhb1 Allele and Rapamycin—To determine whether permease expression in ∆bhd is regulated by Rhb1, we analyzed permease expression in ∆bhd/Rhb1GS using real time RT-PCR. Levels of the permeases isp4\(^{+}\), isp5\(^{+}\), 7G5.06\(^{+}\), and 869.10\(^{+}\) were increased by 3–8-fold in the ∆bhd strain compared with wild-type, with the greatest increase in isp5\(^{+}\) (Fig. 5A). The Rhb1GS strain had levels similar to wild-type for all 4 permeases. However, the ∆bhd/Rhb1GS strain showed permease expression levels that were substantially higher than ∆bhd, ranging from 10- to 51-fold (relative to wild-type), with the highest fold change for isp5\(^{+}\). These data suggest that loss of Bhd acts synergistically with decreased Rhb1 activity to regulate permease expression.

Rapamycin is a highly specific inhibitor of mTOR complex 1 in mammalian cells. In S. pombe, the effects of rapamycin are not well understood, but it is believed to inhibit both Tor1- and Tor2-related functions and to inhibit the expression of isp5\(^{+}\), 7G5.06\(^{+}\), and 869.10\(^{+}\) permeases (25, 29). To determine whether the previously demonstrated rapamycin inhibition of permease expression is Bhd dependent, we treated wild-type and ∆bhd S. pombe with the same dose of rapamycin used by Weisman et al. (100 ng/ml), and measured the expression of the four permeases (869.10\(^{+}\), 7G5.06\(^{+}\), isp4\(^{+}\), and isp5\(^{+}\)) using real-time RT-PCR. In contrast to the work of Weisman et al. (25), we found that in wild-type cells, rapamycin increased the expression of the permeases. These rapamycin-induced increases were confirmed by Northern blot for 7G5.06\(^{+}\) and isp5\(^{+}\) (Fig. 5B). Interestingly, in each case, rapamycin induced a higher fold increase in permease expression in the ∆bhd yeast, ranging from 4.3- to 8.9-fold in ∆bhd, compared with 2.3–4-fold increases in wild-type yeast, with the greatest fold increases in both cases in isp5\(^{+}\) expression. Treatment of the ∆bhd/Rhb1GS yeast with rapamycin resulted in additional 1.6–3-fold increases in the already high levels of expression. These data indicated that rapamycin can increase permease expression, and that loss of Bhd sensitizes S. pombe to the effects of rapamycin.

Rapamycin Induces Lethality in Bhd/Rhb1GS Double Mutants—To determine whether rapamycin influences the growth of cells lacking bhd, we treated ∆tsc1, ∆bhd, Rhb1GS, and ∆bhd/Rhb1GS yeast with rapamycin. All strains grew normally in the absence of rapamycin (Fig. 5C, left panel). Rapamycin had no effect on the growth of the ∆tsc1 and ∆bhd strains, and only a minor effect on the growth of the Rhb1GS strains. However, rapamycin completely inhibited the growth of the ∆bhd/Rhb1GS strain (Fig. 5C).

Permease Expression in ∆bhd/∆tor1 Strain Is Intermediate When Compared with ∆bhd or ∆tor1—To determine whether BHD regulates permease expression through a Tor1 dependent pathway, we analyzed permease expression in ∆bhd/∆tor1 using real time RT-PCR. Levels of permeases isp4\(^{+}\), isp5\(^{+}\), and 869.10\(^{+}\) were intermediate when compared with the single mutants ∆bhd and ∆tor1 (Fig. 6A). This result is consistent with a model in which Bhd and Tor1 function independently to regulate permease expression.
Intracellular Amino Acid Levels in Δbhd/Δtor1 Strain Are Intermediate When Compared with Δbhd or Δtor1—Next we analyzed the intracellular amino acid levels in the Δbhd/Δtor1 strain. Levels of threonine, glutamine, and ornithine were intermediate in the Δbhd/Δtor1 strain, relative to the single mutants (Fig. 6B), again supporting the hypothesis that Bhd and Tor1 signal independently to common downstream targets (Fig. 6B).

DISCUSSION

We report here that S. pombe lacking bhd, the homolog of the human Birt-Hogg-Dube disease gene, have opposite phenotypes to yeast lacking Tsc1 or Tsc2. These opposite phenotypes include increased expression of a group of amino acid permeases (isp4+/+, isp5+/+, 7G5.06+/+, and 869.10+/+) and oligopeptide transporters (ptr2+ and isp4+) known to have diminished expression in yeast lacking Tsc1 or Tsc2, and increased intracellular levels of specific amino acids including ornithine and citrulline. These data suggest that S. pombe Bhd and Tsc1/Tsc2 regulate common downstream targets. Several factors suggest that Tor2 is one of the key downstream targets regulated by Bhd and Tsc1/2. First, the TSC genes function as inhibitors of Rhb1, and Rhb1 was previously shown to bind and activate Tor2 in S. pombe (21–23). Second, it was very recently found that Tor2 negatively regulates the expression of the same permeases (isp4+/+, isp5+/+, 7G5.06+/+, and 869.10+/+) that are up-regulated in Δbhd (24). Third, our data presented here indicate that Bhd and Tor1 function in separate pathways (Fig. 6). Therefore, taken together with our data that the expression of a hypomorphic Rhb1 allele (Rhb1GS) in the Δbhd strain further elevates permease gene expression, indicating that Bhd and Rhb1 are in parallel pathways, we propose a working model (Fig. 7) in which Rhb1 and Bhd independently activate Tor2.

Rapamycin increased permease expression in Rhb1GS, Δbhd, and Δbhd/Rhb1GS strains, with a particularly striking effect in Δbhd. Furthermore, rapamycin induced complete growth inhibition in Δbhd/Rhb1GS, but had little effect on growth in other strains. The targets of rapamycin

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**FIGURE 5.** Rapamycin increases permease expression in Δbhd and inhibits growth in Rhb1GS, Δbhd. A, expression levels using real time PCR for isp4+, isp5+, 7G5.06+, and 869.10+ in Δbhd, Rhb1GS, and Rhb1GSΔbhd yeast were compared with wild-type yeast either untreated (gray bars), or after treatment with rapamycin (black bars). Ct values were converted to quantities using a standard curve established with the wild-type untreated sample. An arbitrary unit of 100 was given to the amount of each transcript present in that sample. No signal was detected in the RT controls. Permease expression levels increased after rapamycin treatment in all strains, but the greatest increase was present in Δbhd and in Rhb1GSΔbhd. For each sample, the 3 values of relative quantity (from 2 PCR) were averaged. B, increase in expression of permeases was confirmed for isp5+ and 7G5.06+ on a Northern blot. Gpd3+ is loading control. C, wild-type yeast and Δbhd, Rhb1GS, and Rhb1GSΔbhd mutants were grown and spotted as described in the legend to Fig. 2 on EMM (left panel) and on EMM with 100 ng/ml rapamycin (right panel). Rapamycin completely inhibited the growth of the Δbhd/Rhb1GS strain.

Intracellular Amino Acid Levels in Δbhd/Δtor1 Strain Are Intermediate When Compared with Δbhd or Δtor1—Next we analyzed the intracellular amino acid levels in the Δbhd/Δtor1 strain. Levels of threonine, glutamine, and ornithine were intermediate in the Δbhd/Δtor1 strain, relative to the single mutants (Fig. 6B), again supporting the hypothesis that Bhd and Tor1 signal independently to common downstream targets (Fig. 6B).
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in wild-type S. pombe. This contrasts with a recent report that rapamycin down-regulates permease expression (25). The reason for this discrepancy is not known, because the same yeast strain and dose of rapamycin were used. One possible factor is the duration of rapamycin treatment, because this was not specified in the prior report.

Based on the partially overlapping clinical phenotypes of BHD and TSC patients, we were surprised to find opposite phenotypes in S. pombe. It is possible that the relationship between the BHD, TSC, and TOR proteins varies between species. However, recently Baba et al. (30) showed that expression of BHD in a BHD-null cell line resulted in decreased phosphorylation of ribosomal protein p70 S6K in serum deprivation (similar to TSC1/TSC2 expression), but increased phosphorylation of p70 S6K in amino acid deprivation, suggesting that BHD can both inhibit and activate mTOR. Our data may indicate that the fundamental role of mammalian BHD is to oppose TSC1/TSC2, through common downstream effectors. To our knowledge this would be the first link between inappropriate mTOR inhibition and human disease. One possible mechanism through which mTOR inhibition might lead to tumorigenesis involves the balance between the two distinct complexes of mTOR in mammalian cells, mTORC1 (mTOR and raptor) and mTORC2 (mTOR, rictor, and SIN1) (31). Inhibition of mTORC1 with rapamycin alters the stoichiometry between mTORC1 and mTORC2 in a cell type-specific manner, with loss of mTOR-raptor binding at early time points and loss of mTOR-rictor binding at later time points (32). Further studies will be required to determine whether BHD similarly disrupts the balance between mTORC1 and mTORC2 in mammalian cells, and whether this promotes renal tumorigenesis or lung cyst formation.

Our model does not explain at least two aspects of the Δbhd and Δtsc1 phenotypes. First, the mechanism of DL-ethionine resistance in Δbhd is not known: Δbhd do not have decreased methionine uptake, in contrast to Δtsc1. As a toxic methionine analog, DL-ethionine inhibits the initiation step of protein synthesis. Little is known about the pathways impacted by DL-ethionine in S. pombe and whether they include Tor1 and Tor2. In mammalian cells DL-ethionine was studied in the 1930s through the 1970s as a treatment for diabetes, and found to be associated with major alterations in the translational apparatus, including altered ribosomal RNA synthesis and ribosomal protein S6 phosphorylation, and also induction of several enzymes including hepatic adenylate cyclase (33). The second unexplained phenotype is that the Δtsc1 and Δtsc1Δbhd are resistant to the toxic arginine analog, canavanine, and Δbhd did not exhibit enhanced sensitivity to canavanine. This suggests that arginine uptake is not part of the opposing phenotypes of Δbhd and Δtsc1, and therefore may be regulated through a separate Rbh1-dependent arm of the pathway.

In conclusion, we found that in S. pombe, the Bhd and Tsc1/Tsc2 proteins have opposing functions in the regulation of amino acid permease expression and intracellular levels of specific amino acids in the arginine pathway, suggesting that Bhd and Tsc1/Tsc2 regulate common downstream targets. If this relationship between BHD and TSC1/TSC2 is recapitulated in mammalian cells and mTOR is inhibited in cells lacking BHD, there may be important clinical implications for BHD patients.

in S. pombe likely include both Tor1 and Tor2 (23, 25, 29). The Tor mutants exhibit significant defects: deletion of Tor2 results in lethality, and deletion of Tor1 results in growth defects and loss of mating (29). We speculate that the effect of rapamycin in the Δbhd/Rbh1GS strain is the consequence of complete Tor2 inhibition. Further studies, which are clearly required to specifically test this hypothesis, will need to circumvent the significant growth defects associated with deletion of either Tor1 or Tor2.

Of note, we found using both real time RT-PCR and Northern blot that rapamycin treatment increased permease expres-
because the mTOR inhibitor CCI-779, which has significant efficacy in the treatment of sporadic renal cell carcinoma, would not be predicted to benefit BHD patients with renal cell carcinoma. Finally, the relationship of the lung cysts to mTOR regulation is of particular interest, because in TSC, lung cysts occur in association with abnormal cell proliferation (lymphangiogenesis), whereas in BHD the cysts occur without a proliferative component.

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REFERENCES

1. Birt, A. R., Hogg, G. R., and Dube, W. J. (1977) Arch. Dermatol. 113, 1674–1677
2. Toro, J. R., Glenn, G., Duray, P., Darling, T., Weirich, G., Zbar, B., Linehan, M., and Turner, M. L. (1999) Arch. Dermatol. 135, 1195–1202
3. Zbar, B., Alvord, W. G., Glenn, G., Turner, M., Pavlovich, C. P., Schmidt, L., Walther, M., Choyke, P., Weirich, G., Hewitt, S. M., Duray, P., Gabri, F., Greenberg, C., Merino, M. J., Toro, J., and Linehan, W. M. (2002) Cancer Epidemiol. Biomarkers Prev. 11, 393–400
4. Nickerson, M. L., Warren, M. B., Toro, J. R., Matrosova, V., Glenn, G., Turner, M. L., Duray, P., Merino, M., Choyke, P., Pavlovich, C. P., Sharma, N., Walther, M., Munroe, D., Hill, R., Maher, E., Greenberg, C., Lerman, M. I., Linehan, W. M., Zbar, B., and Schmidt, L. S. (2002) Cancer Cell 2, 157–164
5. Khoo, S. K., Giraud, S., Kahnoski, K., Chen, J., Motorna, O., Nickolov, R., Binet, O., Lambert, D., Friedel, J., Levy, R., Ferlicot, S., Wolkenstein, P., Hammel, P., Bergerheim, U., Hedblad, M. A., Bradley, M., Teh, B. T., Nordenskjold, M., and Richard, S. (2002) J. Med. Genet. 39, 906–912
6. Painter, J. N., Tapanainen, H., Somer, M., Tukiainen, P., and Aittomaki, K. (2005) J. Hum. Genet. 50, 522–527
7. Schmidt, L. S., Nickerson, M. L., Warren, M. B., Glenn, G. M., Toro, J. R., Merino, M. J., Turner, M. L., Choyke, P. L., Sharma, N., Peterson, J., Morison, P., Maher, E. R., Walther, M. M., Zbar, B., and Linehan, W. M. (2005) Am. J. Hum. Genet. 76, 1023–1033
8. Vocke, C. D., Yang, Y., Pavlovich, C. P., Schmidt, L. S., Nickerson, M. L., Torres-Cabala, C. A., Merino, M. I., Walther, M. M., Zbar, B., and Linehan, W. M. (2005) J. Natl. Cancer Inst. 97, 931–935
9. Fujii, H., Iwamatsu, A., Miyai, K., Sashara, K., Ohtsutsi, N., and Hino, O. (2006) J. Pathol. 209, 328–335
10. Okimoto, K., Sakurai, J., Kobayashi, T., Mitanii, H., Hirayama, Y., Nickerson, M. L., Warren, M. B., Zbar, B., Schmidt, L. S., and Hino, O. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 2023–2027
11. Lingaas, F., Comstock, K. E., Kirkness, E. F., Sorensen, A., Aarskaug, T., Hitte, C., Nickerson, M. L., Moe, L., Schmidt, L. S., Thomas, R., Breen, M., Galibert, F., Zbar, B., and Ostrander, E. A. (2003) Hum. Mol. Genet. 12, 3043–3053
12. Inoki, K., Li, Y., Xu, T., and Guan, K. L. (2003) Genes Dev. 17, 1829–1834
13. Castro, A. F., Rebhun, J. F., Clark, G. J., and Quilliam, L. A. (2003) J. Biol. Chem. 278, 32493–32496
14. Garami, A., Zwartkruis, F. J., Nobukuni, T., Joaquim, M., Roccio, M., Stocker, H., Koaza, S. C., Hafen, E., Bos, J. L., and Thomas, G. (2003) Mol. Cell 11, 1457–1466
15. Tee, A. R., Manning, B. D., Roux, P. P., Cantley, L. C., and Blenis, J. (2003) Curr. Biol. 13, 1259–1268
16. Zhang, Y., Gao, X., Saucedo, L. J., Ru, B., Edgar, B. A., and Pan, D. (2003) Nat. Cell Biol. 5, 578–581
17. Saucedo, L. J., Gao, X., Chiarelli, D. A., Li, L., Pan, D., and Edgar, B. A. (2003) Nat. Cell Biol. 5, 566–571
18. Stocker, H., Radimerski, T., Schindelholz, B., Wittwer, F., Belawat, P., Daram, P., Breuer, S., Thomas, G., and Hafen, E. (2003) Nat. Cell Biol. 5, 559–565
19. Yang, W., Urano, J., and Tamaoni, F. (2000) J. Biol. Chem. 275, 429–438
20. van Slegtenhorst, M., Mustafa, A., and Henske, E. P. (2005) Hum. Mol. Genet. 14, 2851–2858
21. Uritani, M., Hidaka, H., Hotta, Y., Ueno, M., Ushimaru, T., and Toda, T. (2006) Genes Cells 11, 1367–1379
22. Urano, J., Sato, T., Matsuo, T., Otsubo, Y., Yamamoto, M., and Tamaoni, F. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 3514–3519
23. Weisman, R., Roitburg, I., Schonbrun, M., Harari, R., and Kupiec, M. (2007) Genetics 175, 1153–1162
24. Matsu, T., Otsubo, Y., Urano, J., Tamaoni, F., and Yamamoto, M. (2007) Mol. Cell Biol. 27, 3154–3164
25. Weisman, R., Roitburg, I., Nahari, T., and Kupiec, M. (2005) Genetics 169, 559–560
26. Leupold, U. (1970) Methods Cell Physiol. 4, 169–177
27. Grimm, C., and Kohli, J. (1988) Mol. Gen. Genet. 215, 87–93
28. van Slegtenhorst, M., Carr, E., Stoyanova, R., Kruger, W. D., and Henske, E. P. (2004) J. Biol. Chem. 279, 12706–12713
29. Weisman, R. (2004) Curr. Top. Microbiol. Immunol. 279, 85–95
30. Baba, M., Hong, S. B., Sharma, N., Warren, M. B., Nickerson, M. L., Iwamatsu, A., Esposito, D., Gillette, W. K., Hopkins, R. F., 3rd, Hartley, J. L., Furuhata, M., Oishi, S., Zhen, W., Burke, T. R., Jr., Linehan, W. M., Schmidt, L. S., and Zbar, B. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 15552–15557
31. Sabatini, D. M. (2006) Nat. Rev. Cancer 6, 729–734
32. Sarbassov, D. D., Ali, S. M., and Sabatini, D. M. (2005) Curr. Opin. Cell. Biol. 17, 596–603
33. Friedman, M. A., Berry, D. E., and Elzay, R. P. (1977) Cancer Lett. 3, 71–76