

# The Birt-Hogg-Dube and Tuberous Sclerosis Complex Homologs Have Opposing Roles in Amino Acid Homeostasis in *Schizosaccharomyces pombe*\*<sup>§</sup>

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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  $\Delta tsc1$  and  $\Delta tsc2$ , were up-regulated in  $\Delta bhd$ , and levels of specific intracellular amino acids known to be low in  $\Delta tsc1$  and  $\Delta tsc2$  were elevated in  $\Delta bhd$ . 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*<sup>+</sup> dramatically increased permease expression levels in  $\Delta bhd$  but not in wild-type yeast. Loss of Bhd sensitized yeast to rapamycin-induced increases in permease expression levels, and rapamycin induced lethality in  $\Delta bhd$  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)<sup>4</sup> 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*<sup>+</sup> 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*<sup>+</sup> (Rhb1<sup>G63D/S165N</sup>) (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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<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Tables S1 and S2.

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<sup>4</sup> 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.

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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*<sup>+</sup>, here called *bhd*<sup>+</sup>, and used homologous recombination to generate a novel deletion strain,  $\Delta bhd$ . Unexpectedly, we found that multiple amino acid permeases and transporters that are down-regulated in  $\Delta tsc1$  and  $\Delta tsc2$  are up-regulated in  $\Delta bhd$ , and that  $\Delta bhd$  *S. pombe* have elevated intracellular levels of specific amino acids that are low in  $\Delta tsc1$  and  $\Delta tsc2$ . Yeast lacking Bhd demonstrated hypersensitivity to rapamycin, a specific inhibitor of mTOR. These data strongly support 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 *ura4-D18* (27) were gifts from J. Bähler (Sanger Institute). The methionine auxotrophic strain 1945h<sup>+</sup> was obtained from the National Collection of Yeast Cultures, UK. *S. pombe* cells were grown in essential minimal medium (EMM; Qbiogene, 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*<sup>+</sup>, *bhd*<sup>+</sup>*tor1*<sup>+</sup>, and *bhd*<sup>+</sup>*tsc1*<sup>+</sup>-deficient Strains**—The entire open reading frame of *bhd*<sup>+</sup> 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.  $\Delta bhd$  was crossed into the *ura4-D18* strain to generate DK2 (*ura4* $\Delta bhd$ ) using random spore analysis on selective plates. *Tsc1*<sup>+</sup>-deficient strain MVS3 was used to generate  $\Delta tsc1\Delta bhd$  by isolating spores and using PCR to identify double mutants, which were verified by Northern blot. The  $\Delta tor1\Delta bhd$ -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*<sup>+</sup> gene was amplified from genomic DNA and cloned into the pREP4X expression vector. After sequence verification, *bhd*<sup>+</sup> 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  $\mu$ g 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 ( $A_{595} = 0.2-0.3$ ) 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  $\mu$ g of each sample), reverse transcribed into cDNA, and labeled with Cy3 and Cy5 (Amersham Biosciences). Hybridizations were carried out overnight at 42 °C. The slides were scanned with a GMS 428 Scanner (Affymetrix, Santa Clara, CA) and spot quantification was performed with the ImaGene software (BioDiscovery, Marina del Rey, CA). Each *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  $\mu$ g 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  $\mu$ g of total RNA was run on a 1% formaldehyde gel and transferred to nylon membrane overnight in 20 $\times$  SSC. Probes for *bhd*<sup>+</sup>, *c869.10*<sup>+</sup>, *isp4*<sup>+</sup>, *isp5*<sup>+</sup>, and *gpd3*<sup>+</sup> were PCR amplified from cDNA, labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (PerkinElmer Life Sciences), and hybridized using standard methods.

**Canavanine and DL-Ethionine Sensitivity**—Cells were grown overnight to midlog phase ( $A_{595} = 0.4-0.6$ ) and  $A_{595}$  was adjusted to 0.4 (10,000 cells/ $\mu$ l). 4  $\mu$ l of 1, 10, and 100 times dilutions was spotted onto EMM as a growth control, or EMM containing canavanine (60  $\mu$ g/ml) or DL-ethionine (30  $\mu$ g/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<sup>TM</sup> (Ambion, Austin, TX). RNA was quantified using the Agilent 2100 BioAnalyzer in combination with a

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**TABLE 1**  
***S. pombe* strains used in this study**

Strain	Genotype	Source
972	h <sup>-</sup>	Leupold
DK1	h <sup>-</sup> , <i>bhd::kan</i> <sup>+</sup>	This study
MVS1	h <sup>-</sup> , <i>tsc1::kan</i> <sup>+</sup>	van Slegtenhorst
K2	h <sup>-</sup> , <i>bhd::kan</i> <sup>+</sup> , <i>tsc1::kan</i> <sup>+</sup>	This study
DK3	h <sup>+</sup> , <i>bhd::kan</i> <sup>+</sup> , <i>ura4-D18</i>	This study
MVS9	h <sup>-</sup> , <i>Rhb1</i> <sup>G63D,S165N</sup>	van Slegtenhorst
MVS10	h <sup>+</sup> , <i>bhd::kan</i> <sup>+</sup> , <i>Rhb1</i> <sup>G63D,S165N</sup>	This study
1945h <sup>+</sup>	h <sup>+</sup> , <i>met3-1</i>	NCYC
DK4	h <sup>+</sup> , <i>bhd::kan</i> <sup>+</sup> , <i>met3-1</i>	This study
DK5	h <sup>+</sup> , <i>tsc1::kan</i> <sup>+</sup> , <i>met-1</i>	This study
TA99	h <sup>-</sup> , <i>tor1::ura</i> <sup>+</sup> , <i>ura4-D18</i> , <i>leu1-32</i> , <i>ade6-M216</i>	Weisman
DK6	h <sup>+</sup> , <i>tor1::ura</i> <sup>+</sup> , <i>ura4-D18</i>	This study
DK7	h <sup>-</sup> , <i>tor1::ura</i> <sup>+</sup> , <i>bhd::kan</i> <sup>+</sup>	This study

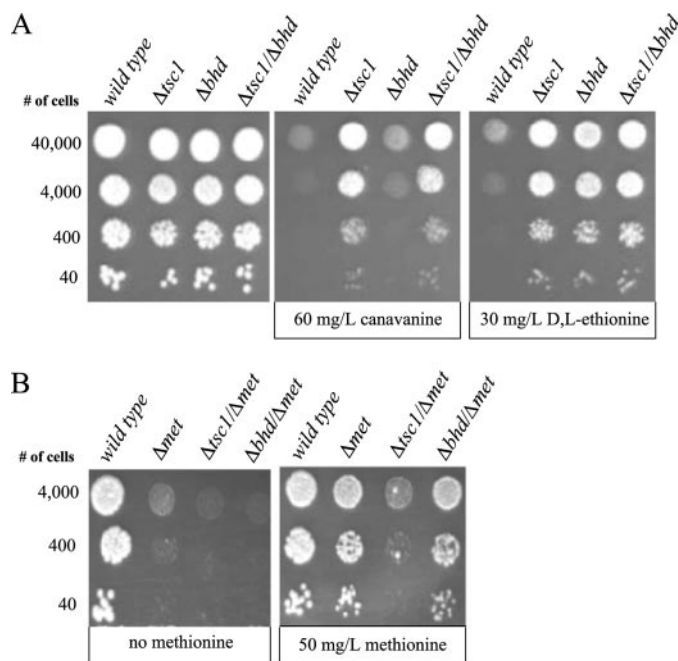
RNA 6000 Nano LabChip. RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Ambion) and a mixture of anchored oligo(dT) and random decamers. For each sample, 2 RT reactions were performed with inputs of 50 and 10 ng. A -RT control reaction with 50 ng of input was also 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 by treatment 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*<sup>+</sup>, referred to as *bhd*<sup>+</sup>. *Bhd*<sup>+</sup> 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*<sup>+</sup> was deleted by homologous recombination and replaced with a kanamycin cassette, to generate a haploid *bhd*<sup>+</sup> deletion strain,  $\Delta bhd$  (Table 1). The  $\Delta bhd$  strain had no evident defects in growth, proliferation, or mating.

**$\Delta bhd$  Is Resistant to DL-Ethionine, but the Mechanism of Resistance Is Different Than in  $\Delta tsc1$** —We previously found that  $\Delta tsc1$  and  $\Delta tsc2$  have decreased uptake of arginine, and are therefore resistant to L-canavanine, a toxic analog of arginine (28).  $\Delta tsc1$  and  $\Delta tsc2$  are also resistant to the toxic methionine analog, DL-ethionine (20). To determine whether  $\Delta bhd$  has a similar phenotype, the  $\Delta bhd$  strain was incubated with 60



**FIGURE 1.  $\Delta bhd$  *S. Pombe* are resistant to the toxic methionine analog, DL-ethionine.** A, 972 wild-type,  $\Delta tsc1$ ,  $\Delta bhd$ , and  $\Delta tsc1\Delta bhd$  yeast were grown in EMM overnight to midlog phase. Cells were then diluted to  $A_{595} = 0.4$ , and 10-fold different EMM 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  $\Delta tsc1$  and  $\Delta bhd$  were grown and spotted as described above on EMM (left panel) and on EMM with 50 mg/liter methionine (right panel).

mg/liter L-canavanine or 30 mg/liter DL-ethionine.  $\Delta bhd$  yeast were not resistant to L-canavanine (unlike  $\Delta tsc1$ ), but they were resistant to DL-ethionine, similar to  $\Delta tsc1$  (Fig. 1A). A  $\Delta tsc1\Delta bhd$  double mutant strain was resistant to canavanine and DL-ethionine. This partial phenotypic overlap between  $\Delta tsc1$  and  $\Delta bhd$  suggested that the Tsc1, Tsc2, and Bhd proteins may function in a common pathway.

Next, to determine whether the DL-ethionine resistance observed in  $\Delta bhd$  and  $\Delta tsc1$  was due to decreased uptake of methionine, we crossed the  $\Delta tsc1$  and  $\Delta bhd$  strains into a methionine auxotrophic strain, 1945h<sup>+</sup>, referred to as  $\Delta met$ , 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  $\Delta tsc1/\Delta met$  strain was unable to grow with this low level of supplemented methionine, consistent with a methionine uptake defect, but the  $\Delta bhd/\Delta met$  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  $\Delta tsc1$  and  $\Delta bhd$ .

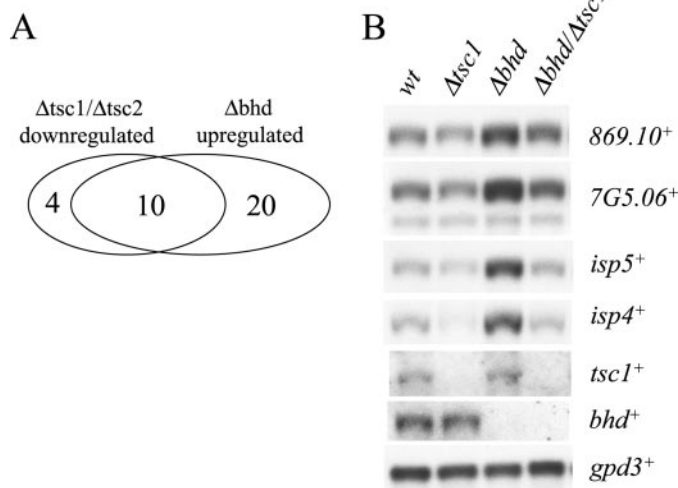
**$\Delta tsc1$  and  $\Delta bhd$  Have Opposite Expression Profiles for Transporters and Permeases**—Because these results did not support a role of Bhd in the Tsc/Rhb pathway, as we had originally hypothesized, we next compared the transcriptional expression profile of 2 separate colonies of  $\Delta bhd$  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”).

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**TABLE 2**

Genes up-regulated in  $\Delta bhd$  and down-regulated in  $\Delta tsc1 + \Delta tsc2$

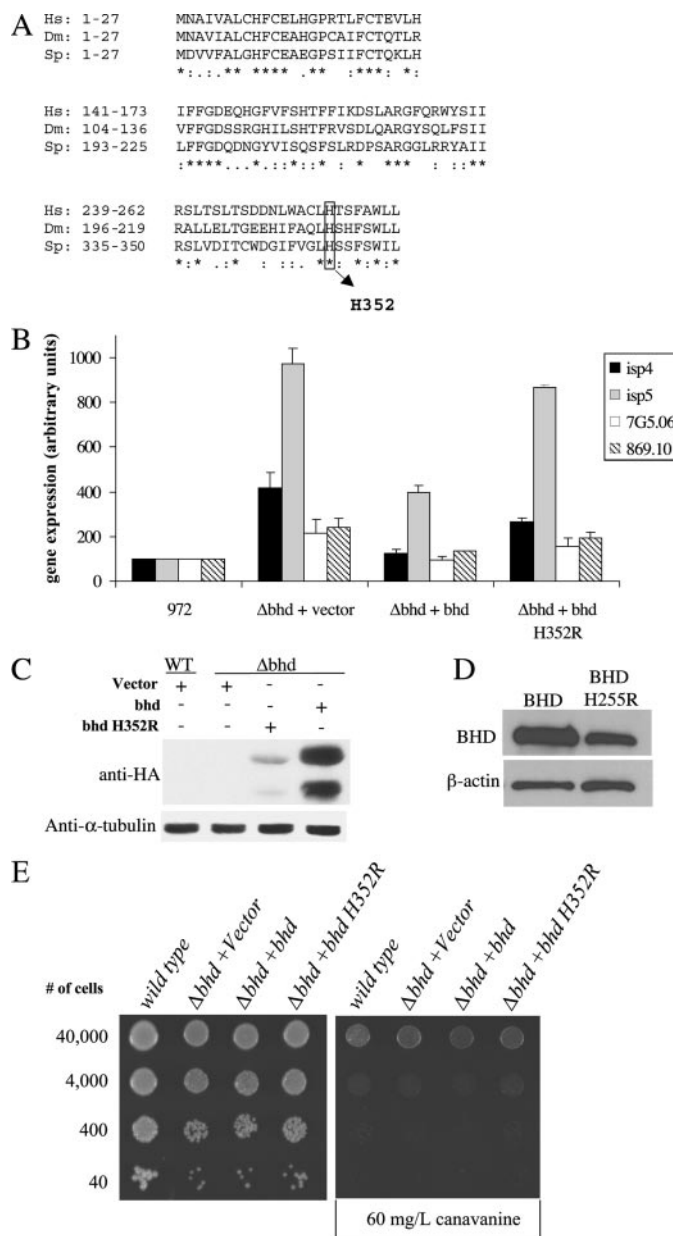
Gene name	Predicted function
SPAC896.10c <sup>+</sup>	Amino acid permease
SPAP7G5.06 <sup>+</sup>	Amino acid permease
isp5 <sup>+</sup>	Amino acid permease
isp4 <sup>+</sup>	Oligopeptide transporter
ptr2 <sup>+</sup>	Oligopeptide transporter
SPAC11D3.18c <sup>+</sup>	Membrane transporter
SPAC11D3.14 <sup>+</sup>	5-Oxoprolinase, proline metabolism
SPAC1223.09 <sup>+</sup>	Urate oxidase
SPAC5H10.01 <sup>+</sup>	Mitochondrial precursor
SPAC1039.10 <sup>+</sup>	Mitochondrial precursor



**FIGURE 2.  $\Delta bhd$  *S. pombe* have increased expression of a group of genes, including amino acid permeases, which are down-regulated in  $\Delta tsc1$  and  $\Delta tsc2$ .** A, expression profiles were compared among  $\Delta bhd$ ,  $\Delta tsc1$ , and  $\Delta tsc2$ . At a designated fold change of  $>1.5$ , there were 10 up-regulated genes that were down-regulated in  $\Delta tsc1$  and  $\Delta tsc2$ . B, expression of four permease genes,  $7G5.06^+$ ,  $869.10^+$ ,  $isp5^+$ , and  $isp4^+$ , was confirmed by Northern blot. All four genes were up-regulated in  $\Delta bhd$ , consistent with the array results.

Using a threshold of 1.5-fold change, 10 genes were down-regulated and 30 genes were up-regulated in  $\Delta bhd$  in at least 2 of the 4 arrays (supplemental materials Tables S1 and S2). Remarkably, 10 of the up-regulated genes in  $\Delta bhd$  were previously found to be down-regulated in  $\Delta tsc1$  and  $\Delta tsc2$  (Table 2 and Fig. 2A). Of particular interest,  $SPAC869.10^+$  (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  $\Delta tsc1$  and  $\Delta tsc2$  strains (28). Northern blotting confirmed the expression change for four of the genes (Fig. 2B). The  $\Delta bhd\Delta tsc1$  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*.

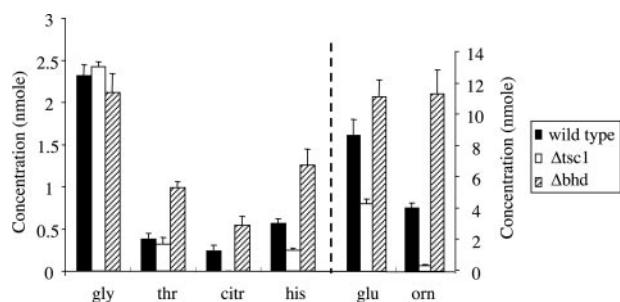
**The Permease Expression Defect in  $\Delta bhd$  Is Rescued by Wild Type  $bhd^+$** —Three regions of homology to the human and *Drosophila melanogaster* BHD proteins are shown in Fig. 3A. The histidine residue that is mutant in the German shepherd hereditary renal cancer syndrome (H255R) is conserved 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*<sup>+</sup> expression vector. The Bhd and Bhd-H352R expression



**FIGURE 3. The permease expression defect in  $\Delta bhd$  is rescued by wild-type BHD but not by the German shepherd homologous mutant.** A, three regions of conservation from the human (*Hs*), *D. melanogaster* (*Dm*), and *S. pombe* (*Sp*) BHD homologs are illustrated. The position of the histidine residue (His-255 in the human) at which a missense change causes renal carcinoma in German shepherd dogs is indicated in a box. Identical amino acids are denoted by an asterisk, conserved amino acids by a colon, and semi-conserved amino acids by a period. B, permease expression levels using real-time PCR for  $isp4^+$ ,  $isp5^+$ ,  $7G5.06^+$ , and  $869.10^+$  in wild-type (WT) and  $\Delta bhd$  over-expressing vector, Bhd, or Bhd-H352R mutant. Ct values were converted to quantities using a standard curve established with the wild-type 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. C, level of expression of Bhd or Bhd-H352R mutant in  $\Delta bhd$  by Western blotting. D, level of expression of BHD or the BHD-H255R mutant after transfection in HEK 293 cells by Western blotting. E, cells for  $\Delta bhd$  overexpressing vector, Bhd, or Bhd-H352R mutant were spotted on EMM plates (left panel) and on EMM with 60 mg/liter canavanine (right panel) as described before.

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.

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**FIGURE 4.  $\Delta bhd$  *S. pombe* have elevated levels of specific amino acids that are decreased in  $\Delta tsc1$  and  $\Delta tsc2$ .** Intracellular amino acid levels in  $\Delta bhd$  and  $\Delta tsc1$  were compared with 972 wild-type yeast. A 2-fold increase was detected for threonine (*thr*), citrulline (*citr*), histidine (*his*), glutamate (*glu*), and ornithine (*orn*) in  $\Delta bhd$  compared with wild type. In contrast, glycine (*gly*) was not significantly different in  $\Delta bhd$  compared with wild type. Triplicates were run for each sample and the mean  $\pm$  S.D. were plotted. Similar results were seen in two independent experiments. The dashed line separates the scales for lower (left) and higher (right) concentration ranges.

3B), confirming that the permease expression defect in the  $\Delta bhd$  yeast is Bhd dependent. The permease expression levels were not completely normalized, perhaps reflecting the level of expression of BHD, or indicating that the hemagglutinin tag partially interferes with the protein function. In contrast, the Bhd-H352R construct did not decrease permease expression. However, Western blot analysis showed that the dog mutant expressed at a lower level (Fig. 3C). The lack of correction by Bhd-H352R is of interest given the high degree of evolutionary conservation of this residue (Fig. 3A), but the lower expression level makes it impossible to distinguish an effect of this residue on the function *versus* the stability of the protein. To determine whether BHD H255R is also expressed at lower levels in mammalian cells we transfected HEK 293 cells with either wild-type BHD or BHD H255R. We found that BHD H255R was consistently expressed at a lower level than wild-type BHD (Fig. 3D). The  $\Delta bhd$  strain overexpressing Bhd-H352R did not show canavanine resistance (Fig. 3E), consistent with the fact that  $\Delta bhd$  is not resistant to canavanine.

To determine whether introduction of human BHD could revert the permease expression levels in the  $\Delta bhd$  yeast strain we cloned the human BHD into yeast pSLF173 expression vector. Expression was confirmed by Western blot. Human BHD did not decrease permease expression levels, indicating that the human gene does not complement for the yeast gene (data not shown).

**Intracellular Concentrations of Specific Amino Acids Are Increased in  $\Delta bhd$** —Previously we found that *S. pombe* lacking *tsc1*<sup>+</sup> or *tsc2*<sup>+</sup> have low intracellular levels of specific amino acids (28), particularly those in the arginine metabolic pathway (ornithine, citrulline, arginine, and glutamate). To further test the hypothesis that Tsc1-Tsc2 and Bhd have opposing functions, we measured the intracellular amino acid concentrations in  $\Delta bhd$ . Levels of specific amino acids were elevated in  $\Delta bhd$ , including glutamate, ornithine, and citrulline (Fig. 4), which are decreased in *S. pombe* lacking Tsc1 or Tsc2 (28). Notably, ornithine, which showed the highest fold increase in  $\Delta bhd$ , was previously shown to have the highest fold decrease in  $\Delta tsc1$  and  $\Delta tsc2$ .

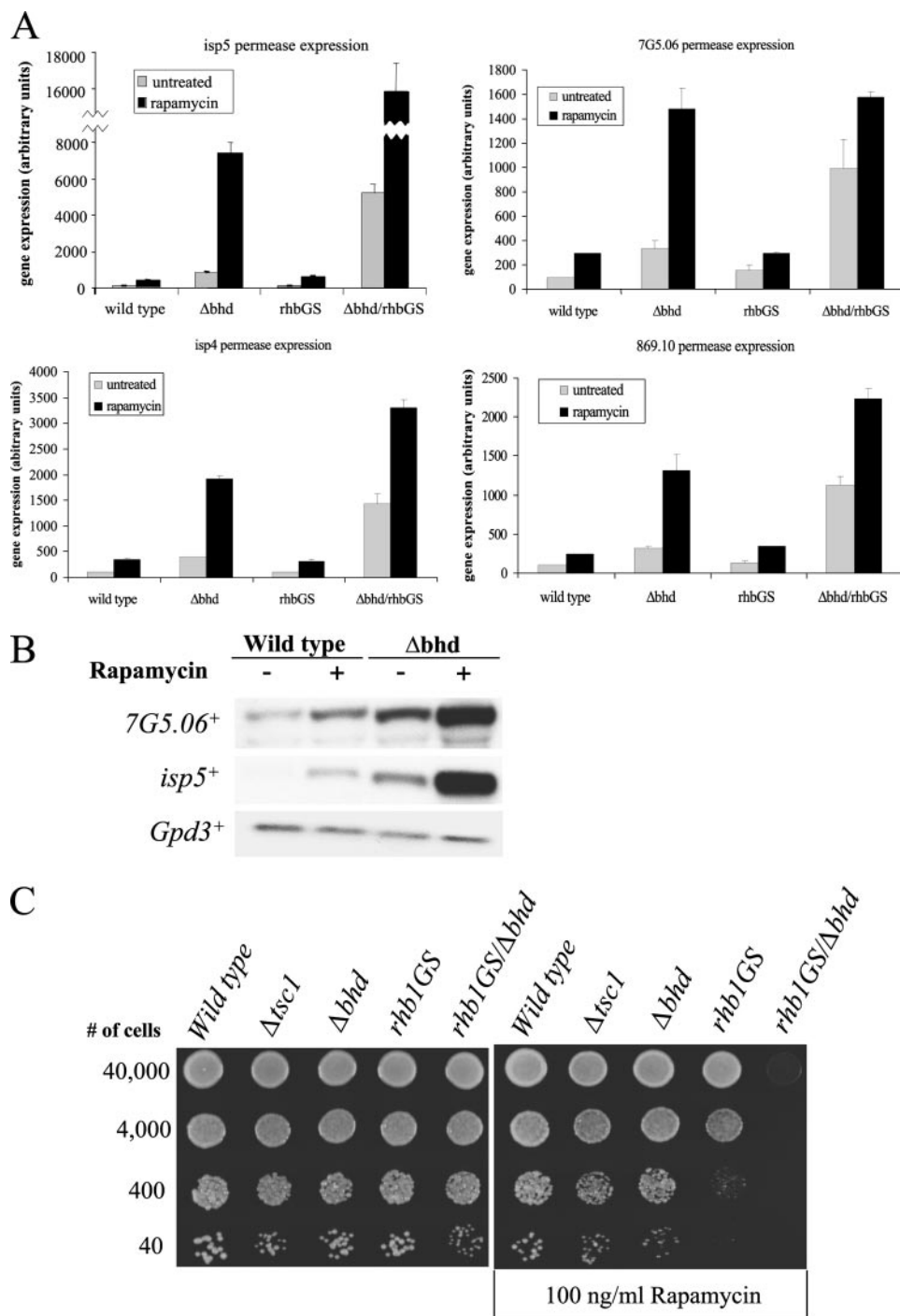
**Permease Expression in Bhd Mutants Is Increased by Expression of a Hypomorphic Rhb1 Allele and Rapamycin**—To determine whether permease expression in  $\Delta bhd$  is regulated by Rhb1, we analyzed permease expression in  $\Delta bhd$ /Rhb1GS using real time RT-PCR. Levels of the permeases *isp4*<sup>+</sup>, *isp5*<sup>+</sup>, *7G5.06*<sup>+</sup>, and *869.10*<sup>+</sup> were increased by 3–8-fold in the  $\Delta bhd$  strain compared with wild-type, with the greatest increase in *isp5*<sup>+</sup> (Fig. 5A). The Rhb1GS strain had levels similar to wild-type for all 4 permeases. However, the  $\Delta bhd$ /Rhb1GS strain showed permease expression levels that were substantially higher than  $\Delta bhd$ , ranging from 10- to 51-fold (relative to wild-type), with the highest fold change for *isp5*<sup>+</sup>. 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*<sup>+</sup>, *7G5.06*<sup>+</sup>, and *869.10*<sup>+</sup> permeases (25, 29). To determine whether the previously demonstrated rapamycin inhibition of permease expression is Bhd dependent, we treated wild-type and  $\Delta bhd$  *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*<sup>+</sup>, *7G5.06*<sup>+</sup>, *isp4*<sup>+</sup>, and *isp5*<sup>+</sup>) 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*<sup>+</sup> and *isp5*<sup>+</sup> (Fig. 5B). Interestingly, in each case, rapamycin induced a higher fold increase in permease expression in the  $\Delta bhd$  yeast, ranging from 4.3- to 8.9-fold in  $\Delta bhd$ , compared with 2.3–4-fold increases in wild-type yeast, with the greatest fold increases in both cases in *isp5*<sup>+</sup> expression. Treatment of the  $\Delta bhd$ /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  $\Delta tsc1$ ,  $\Delta bhd$ , Rhb1GS, and  $\Delta bhd$ /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  $\Delta tsc1$  and  $\Delta bhd$  strains, and only a minor effect on the growth of the Rhb1GS strains. However, rapamycin completely inhibited the growth of the  $\Delta bhd$ /Rhb1GS strain (Fig. 5C).

**Permease Expression in  $\Delta bhd$ / $\Delta tor1$  Strain Is Intermediate When Compared with  $\Delta bhd$  or  $\Delta tor1$** —To determine whether BHD regulates permease expression through a Tor1 dependent pathway, we analyzed permease expression in  $\Delta bhd$ / $\Delta tor1$  using real time RT-PCR. Levels of permeases *isp4*<sup>+</sup>, *isp5*<sup>+</sup>, and *869.10*<sup>+</sup> were intermediate when compared with the single mutants  $\Delta bhd$  and  $\Delta tor1$  (Fig. 6A). This result is consistent with a model in which Bhd and Tor1 function independently to regulate permease expression.

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**FIGURE 5. Rapamycin increases permease expression in  $\Delta bhd$  and inhibits growth in  $Rhb1GS\Delta bhd$ .** *A*, expression levels using real time PCR for *isp4*<sup>+</sup>, *isp5*<sup>+</sup>, *7G5.06*<sup>+</sup>, and *869.10*<sup>+</sup> in  $\Delta bhd$ ,  $Rhb1GS$ , and  $Rhb1GS\Delta bhd$  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  $\Delta bhd$  and in  $Rhb1GS\Delta bhd$ . For each sample, the 2 values of relative quantity (from 2 PCR) were averaged. *B*, increase in expression of permeases was confirmed for *isp5*<sup>+</sup> and *7G5.06*<sup>+</sup> on a Northern blot. *Gpd3*<sup>+</sup> is loading control. *C*, wild-type yeast and  $\Delta bhd$ ,  $Rhb1GS$ , and  $Rhb1GS\Delta bhd$  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  $\Delta bhd/Rhb1GS$  strain.

**Intracellular Amino Acid Levels in  $\Delta bhd/\Delta tor1$  Strain Are Intermediate When Compared with  $\Delta bhd$  or  $\Delta tor1$** —Next we analyzed the intracellular amino acid levels in the  $\Delta bhd/\Delta tor1$

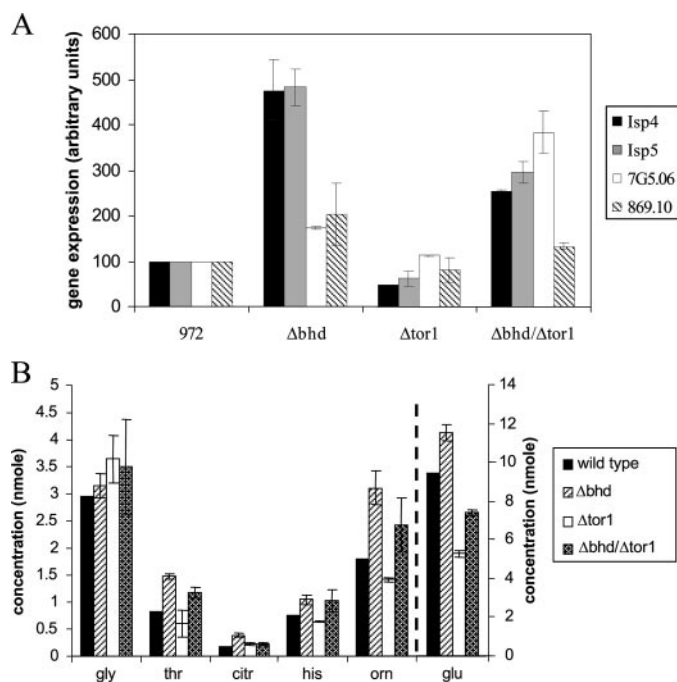
strain. Levels of threonine, glutamine, and ornithine were intermediate in the  $\Delta bhd/\Delta tor1$  strain, relative to the single mutants (Fig. 6B), again supporting the hypothesis that Bhd and Tor1 signal independently to common downstream targets (Fig. 6B).

### DISCUSSION

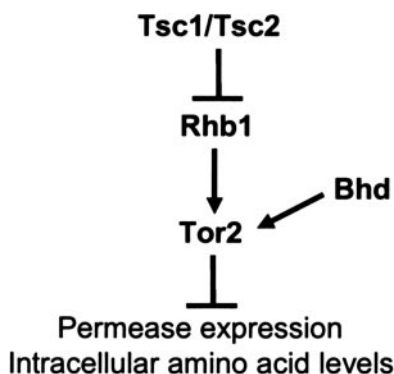
We report here that *S. pombe* lacking *bhd*<sup>+</sup>, the homolog of the human Birt-Hogg-Dube disease gene, have opposite phenotypes to yeast lacking *Tsc1* or *Tsc2*. These opposite phenotypes include increased expression of a group of amino acid permeases (*869.10*<sup>+</sup>, *7G5.06*<sup>+</sup>, and *isp5*<sup>+</sup>) and oligopeptide transporters (*ptr2*<sup>+</sup> and *isp4*<sup>+</sup>) 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*<sup>+</sup>, *isp5*<sup>+</sup>, *C869.10*<sup>+</sup>, and *7G5.06*<sup>+</sup>) that are up-regulated in  $\Delta bhd$  (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  $\Delta bhd$  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  $Rhb1GS$ ,  $\Delta bhd$ , and  $\Delta bhd/Rhb1GS$  strains, with a particularly striking effect in  $\Delta bhd$ . Furthermore, rapamycin induced complete growth inhibition in  $\Delta bhd/Rhb1GS$ , but had little effect on growth in other strains. The targets of rapamycin

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**FIGURE 6. Permease expression and intracellular amino acid levels in the  $\Delta bhd/\Delta tor1$  strain are intermediate when compared with  $\Delta bhd$  or  $\Delta tor1$ .** A, permease expression levels using real-time PCR for *isp4*<sup>+</sup>, *isp5*<sup>+</sup>, *7G5.06*<sup>+</sup>, and *869.10*<sup>+</sup> in wild-type,  $\Delta bhd$ ,  $\Delta tor1$ , and  $\Delta bhd/\Delta tor1$ . Ct values were converted to quantities using a standard curve established with the wild-type 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. B, intracellular amino acid levels in wild-type,  $\Delta bhd$ ,  $\Delta tor1$ , and  $\Delta bhd/\Delta tor1$  were compared. Intermediate levels for threonine (*thr*), ornithine (*orn*), and glutamate (*glu*) were detected in  $\Delta bhd/\Delta tor1$  when compared with  $\Delta bhd$  and  $\Delta tor1$ . Triplicates were run for each sample and the mean  $\pm$  S.D. plotted. Similar results were seen in two independent experiments. The dashed line separates the scales for lower (left) and higher (right) concentration ranges.



**FIGURE 7. Our data are consistent with a model in which Bhd and Tsc1/Tsc2/Rhb1 regulate permease gene expression and intracellular amino acid levels through Tor2.**

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  $\Delta bhd/\Delta tor1$  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-

sion 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  $\Delta bhd$  and  $\Delta tsc1$  phenotypes. First, the mechanism of DL-ethionine resistance in  $\Delta bhd$  is not known:  $\Delta bhd$  do not have decreased methionine uptake, in contrast to  $\Delta tsc1$ . 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  $\Delta tsc1$  and  $\Delta tsc1\Delta bhd$  are resistant to the toxic arginine analog, canavanine, and  $\Delta bhd$  did not exhibit enhanced sensitivity to canavanine. This suggests that arginine uptake is not part of the opposing phenotypes of  $\Delta bhd$  and  $\Delta tsc1$ , and therefore may be regulated through a separate Rhb1-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

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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 (lymphangiomyomatosis), whereas in BHD the cysts occur without a proliferative component.

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