Proteomic Analysis of in Vivo 14-3-3 Interactions in the Yeast *Saccharomyces cerevisiae*†

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ABSTRACT: The yeast *Saccharomyces cerevisiae* produces two 14-3-3 proteins, Bmh1 and Bmh2, whose exact functions have remained unclear. Here, we performed a comprehensive proteomic analysis using multistep immunoaffinity purification and mass spectrometry and identified 271 yeast proteins that specifically bind to Bmh1 and -2 in a phosphorylation-dependent manner. The identified proteins have diverse biochemical functions and cellular roles, including cell signaling, metabolism, and cell cycle regulation. Importantly, there are a number of protein subsets that are involved in the regulation of yeast physiology through a variety of cell signaling pathways, including stress-induced transcription, cell division, and chitin synthesis at the cell wall. In fact, we found that a yeast mutant deficient in Bmh1 and -2 had defects in signal-dependent response of the MAPK (Hog1 and Mpk1) cascade and exhibited an abnormal accumulation of chitin at the bud neck. We propose that Bmh1 and -2 are common regulators of many cell signaling modules and pathways mediated by protein phosphorylation and regulate a variety of biological events by coordinately controlling the identified multiplex phosphoprotein components.

The 14-3-3 proteins comprise a family of acidic, dimeric proteins with subunit molecular masses of ∼30 kDa distributed widely among eukaryotic cells. This protein family recognizes the phosphopeptide consensus sequence motifs RXX*pS/T*XP and RXXX*pS/T*XP (where *pS/T* is phospho-Ser/Thr and X is any amino acid) (*1*, *2*) and binds a variety of enzymes and signaling molecules to modulate their activity, conformation, stability, intracellular localization, and function (for reviews, see refs *3*−*6*). In mammalian cells, there are currently more than 150 proteins that have been identified as 14-3-3-binding partners, including enzymes involved in monoamine synthesis, regulators of cell proliferation and development, and proteins in pro-apoptotic pathways. Recent proteomic studies suggest that mammalian cells contain several hundred additional proteins whose functions might be regulated by 14-3-3 (*7*−*12*). Although these findings suggest that 14-3-3 proteins may be an integral part of various signal transduction pathways mediated by protein phosphorylation, the extent and overall mechanisms of the 14-3-3 interactions remain to be elucidated. Furthermore, recent evidence, particularly from plant cells, has suggested that the 14-3-3 interactions may vary by cell type (*13*). This cell type variation may be attributed to the preference of 14-3-3 isoforms for distinct cell-specific signaling targets and/or phosphorylation states. Thus, systematic analysis of these interactions is necessary to elucidate the diverse roles of the 14−3-3-mediated regulation in complex signaling networks in eukaryotic cells.

The budding yeast *Saccharomyces cerevisiae* was the first eukaryote whose genome was completely sequenced. Subsequently, it became one of the key organisms for genome-wide studies of eukaryotic cells with respect to mRNA abundance and stability, biochemical activity, transcriptional regulation, gene disruption phenotype, protein abundance, and protein−protein interactions. The *S. cerevisiae* genome contains only two genes, *BMH1* and *BMH2*, encoding 14-3-3 proteins, thus providing a simple model for functional studies of 14-3-3 interactions as compared with mammalian and plant cells that express 9−13 14-3-3 isoforms. Previous studies have revealed that Bmh1 and Bmh2 are involved in the control of the signaling pathway from the GTPase Ras via protein kinase A, receptor-mediated endocytosis of clathrin-coated vesicles, and the Ras-MAPK (Kss1) cascade that functions during pseudohyphal development (for a review, see ref *14*). It has also recently been demonstrated that Bmh1/2 positively regulates the TOR[1]-mediated cell

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[1] Abbreviations: TOR, target of rapamycin; MEF, myc-TEV-FLAG; GST, glutathione *S*-transferase; WT, wild type; MS, mass spectrometry.

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signaling (*14*). These findings suggest that, like the higher eukaryotic 14-3-3 proteins, Bmh1/2 may play fundamental roles in diverse kinase-mediated processes. However, few phosphoprotein targets as well as the molecular mechanisms of action have yet to be described in these and many other processes.

We recently described the MS-based proteomics technology combined with a novel tandem affinity purification tag, called MEF [myc-TEV-FLAG (*15*)]. The MEF procedure was originally developed for the comprehensive proteomic characterization of mammalian 14-3-3 interacting partners, but it also is amenable to studies of other interactions, including viral proteins. Using this approach, we show that Bmh1/2 participates in diverse cellular processes through association with at least 271 yeast proteins. Combining the proteomic results with the published large-scale genetic and biochemical information on *S. cerevisiae*, we find that the detected associations are strongly enriched in a variety of cell signaling and regulatory pathways involved in the control of yeast physiology.

MATERIALS AND METHODS

Plasmids and Strains. To produce the MEF−Bmh1/2 fusion constructs, the MEF tag cDNA cassette of pcDNA3-MEF (*15*) was first digested with *Hin*dIII and *Xho*I and inserted into these same cloning sites of pYTF2 [based on pYES2 (Invitrogen), a kind gift from Y. Amaya] to create pYTF2-MEF. The original Gal1 promoter in pYES2 was replaced with the TEF2 promoter to yield constitutive expression of the desired proteins under standard culture conditions (e.g., without addition of galactose). The *BMH1* and *BMH2* ORFs were amplified by PCR using oligonucleotides 5′-GGAATTCAAGTCAACCAGTCGTGAAGA-3′ and 3′-GCTCTAGATTACTTTGGTGCTTCACC-5′ (for *BMH1*) and 5′-GGAATTCAAGTCCCAAACTCGTGAAGA-3′ and 3′-GCTCTATATTTGGTTGGTTCACC-5′ (for *BMH2*) using the yeast S288C genomic DNA as a template. The PCR fragments were then inserted into pYTF2-MEF via *Eco*RI and *Xho*I sites (termed pYTF2-MEF-Bmh1 and pYTF2-MEF-Bmh2, respectively). To simultaneously transform yeast cells with these plasmids, the selection marker gene (*His3*) of the pYTF2-MEF-Bmh2 plasmid was replaced with the *Leu2* gene by amplifying the *LEU2* ORF using oligonucleotides 5′-GAAAGGTGAGAGCGCCGGAAC-3′ and 3′-CTTATCACGTTGAGCCATTAG-5′ with the S288C genomic DNA as a template and then inserted via the *Nhe*I and *Nco*I sites of pYTF2-MEF-BMH2 (termed pYTF2-MEF-Bmh2-LEU2). The *bmh* null mutant RRY1216 strain [*bmh1*::HIS3 *bmh2*::HIS3 *ura3-52 his3 leu2* (*16*)] was then transformed with the pYTF-MEF-Bmh1 and pYTF-MEF-Bmh2-LEU2 vectors using a lithium acetate method. The transformants were selected for histidine and leucine prototrophy.

Cell Culture and MEF Purification. The RRY1216 cells simultaneously expressing both MEF-Bmh1 and MEF-Bmh2 were grown in SD medium to midlog phase. The cells were harvested, suspended in a lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 100 mM NaF, 10 mM EGTA, 1 mM Na_3VO_4, 1% (w/v) Triton X-100, 5 μM $ZnCl_2$, 2 mM phenylmethanesulfonyl fluoride, 10 μg/mL aprotinin, and 1 μg/mL leupeptin], and lysed with glass beads by vortexing. The cell debris was pelleted, and the supernatant was subjected to MEF purification, which was carried out essentially as described previously (*15*). In brief, the expressed MEF−Bmh1/2 and associated proteins were recovered from the supernatant by immunoprecipitation with anti-myc-conjugated Sepharose beads. After the mixture had been washed, TEV protease (Invitrogen) was added to release bound materials from the beads. The eluates were incubated with FLAG−agarose beads for the second immunoprecipitation. After the mixture had been washed, proteins bound to the immobilized Bmh1/2 on FLAG beads were dissociated with 1 mM synthetic phosphopeptide [LSQRQRST*p*STPNVHA, based on residues 250−265 of cRaf-1 (*1*)]. We used the Raf-1 phosphopeptide instead of a FLAG peptide because the protein profiles of the dissociated proteins by either peptide were essentially identical and because the bait FLAG−Bmh1/2 proteins were mostly absent from the Raf-1 eluate. Approximately 5 μg of protein (0.01% of starting materials) was routinely recovered by this procedure from a 2 L culture.

Protease Digestion and Tandem Mass Spectrometry. Purified proteins (10−30 μg) were precipitated as described previously (*15*) and redissolved in 10 μL of 8 M urea in 100 mM Tris-HCl (pH 8.0), and 1 μL of endo-Lys-C (0.5 μg, Wako) was added to the solution and the mixture incubated for 4 h at 37 °C. The urea concentration was diluted with 64 μL of 10 mM Tris-HCl (pH 8.0), and then 5 μL of trypsin (0.125 μg, Promega) was added and the mixture incubated for an additional 20 h at 37 °C. The resulting peptide mixtures were analyzed directly by an automated microscale two-dimensional (2D) LC−MS/MS system equipped with a cation-exchange Bioassist S column [0.75 μm (inside diameter) × 40 mm, Tosho] for the first-dimension LC and a reversed-phase Mightysil-C18 column [150 μm (inside diameter) × 50 mm, Kanto Kagaku] for the second dimension LC, a high-resolution Q-TOF hybrid mass spectrometer (Q-Tof Ultima, Micromass), and an automated data analysis system (*17*). All MS/MS spectra were processed by the MASCOT search algorithm (Matrix Science, Ltd.) for peptide assignment with the publicly available yeast ORF database (Saccharomyces Genome Database, Stanford University, Stanford, CA). The parameters for the protein identification were as follows. We first screened the candidate peptides with probability-based Mowse scores that exceeded their threshold ($p < 0.05$) and then applied more strict criteria for final assignment, as follows (*17*). (i) When the match scores exceeded their threshold by 10, identifications were accepted without further consideration. (ii) When scores were lower than 10 above the threshold, or if identifications were based on a single matched MS/MS spectrum, we manually inspected the raw data for confirmation prior to acceptance (*17*).

Far-Western Blotting. Far-Western blotting was performed as described previously (*18*) with some modifications. In brief, proteins were separated by 10% SDS−PAGE and transferred to PVDF membranes. After the membranes were blocked with 5% skim milk in PBS for 60 min at room temperature, they were incubated overnight at 4 °C with GST−Bmh1 and GST−Bmh2 proteins (5 μg/mL each) in PBS. The bound GST proteins were visualized by Western blotting with a monoclonal antibody against GST (Santa Cruz Biotechnology).

Assay of MAPKs. Midlog cultures of cells were stimulated with or without the inducers listed in the legend of Figure 3. Western blotting was performed with anti-phospho-p38 (Cell Signaling Technology), anti-phospho-p44/42 (New England Biolabs), anti-Hog1 (Santa Cruz Biotechnology), and anti-Mpk1 (Santa Cruz Biotechnology).

Measurement of Chitin and 1,3-β-Glucan. Chitin was assessed enzymatically as described previously (*19*). 1,3-β-Glucan was quantitated using alanine blue (*20*).

Fluorescence Microscopy. Calcofluor White staining was carried out as described previously (*21*). Images were collected using a fluorescence microscope (Zeiss Axioskop), a CCD camera (Nippon Roper), and MetaMorph software (Universal Imaging Corp.).

RESULTS

Affinity Purification of in Vivo Bmh1/2-Binding Partners. The recently described multistep immunoaffinity purification procedure, the MEF method (*15*), in which proteins in eukaryotic cells are expressed as fusions with the MEF affinity tag, was used to screen for Bmh-binding partners. The MEF tag cassette was fused to the amino terminus of both Bmh1 and Bmh2, and the MEF-fused Bmh1 and -2 were simultaneously expressed in the *BMH1*- and *BMH2*-deficient *S. cerevisiae* mutant, RRY1216 (Supporting Information Figure S1A,B). RRY1216 has a Σ1278b genetic background that is widely used for Bmh functional studies (*16*, *22*). We examined whether the MEF-fused Bmh1/2 expressed in *S. cerevisiae* shares similar properties with the wild-type (WT) Bmh1/2. First, we used the "double-transfection assay" described by Jones et al. (*23*) to test for dimer formation in the cells. Each of the expressed fusions bound the coexpressed nontagged Bmh1 and Bmh2 proteins in this assay (Figure S1C), suggesting that, like the WT protein (*16*), the recombinant fusions can form homo- and heterodimers in cells. In the second control, we examined whether the expressed MEF−Bmh1/2 protein restores the reported growth defect phenotypes of mutant RRY1216 under various stress conditions (*16*). RRY1216 expressing either or both of these fusions grew normally under all conditions that were tested (Figure S1D).

Furthermore, these Bmh transformants exhibited resistance to rapamycin treatment and had normal morphology like the transformants expressing WT Bmh1/2 did (data not shown). Thus, we concluded that this procedure using a MEF tag would yield functional Bmh1/2-associated complexes in the yeast cell.

The expressed MEF−Bmh1/2 proteins were then purified by the MEF method. Ultimately, proteins bound to MEF−Bmh1/2 proteins were dissociated from the ligand binding groove of Bmh1/2 with a synthetic phosphopeptide [LSQRQRST*p*-STPNVHA (*1*)] that mimics a 14-3-3-binding site of Raf-1 kinase (see also Materials and Methods). Figure 1A shows SDS−PAGE analysis of the dissociated proteins. Approximately 50 protein bands with molecular masses ranging between 20 and 200 kDa were reproducibly detected by this procedure (lane 4), whereas no background proteins were detected using a control WT cell lysate that did not express MEF−Bmh1/2 protein (termed RRY3, lane 3). To verify that the MEF purification procedure indeed enriched Bmh-binding partners, we performed a far-Western analysis. The

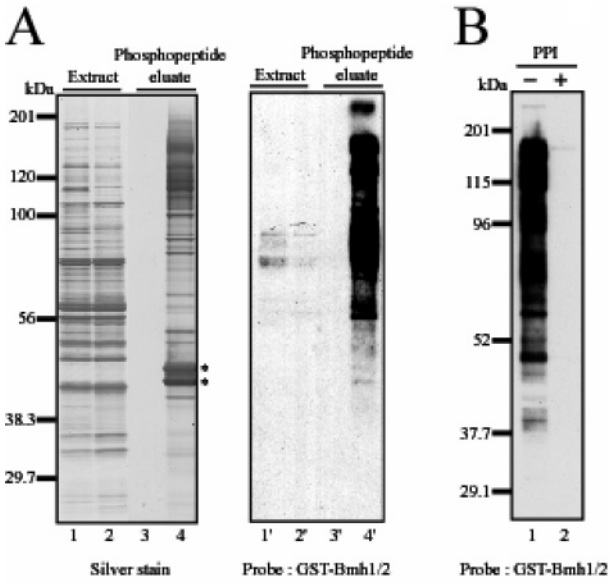

FIGURE 1: Characterization of proteins copurified with MEF-fused Bmh1/2. (A) In the left panel are Whole-cell extract of WT RRY3 cells (lane 1, 20 μg) or the Bmh-deficient RRY1216 mutant expressing MEF-bound Bmh1/2 (lane 2, 20 μg). The proteins recovered by the MEF method from RRY3 (lane 3) or RRY1216 expressing MEF−Bmh1/2 protein (lane 4, ∼5 μg) were analyzed by SDS−PAGE and silver staining. The positions of the fused Bmh1/2 are indicated with an asterisk. In the right panel, the proteins shown in the left panel were transferred to a PVDF membrane, and the membrane was overlaid with GST−Bmh1 and GST−Bmh2 proteins (each 5 μg/mL). (B) The proteins shown in lane 4 of panel A were transferred to the PVDF membrane and analyzed as in the right portion of panel A except that the proteins were preincubated with (lane 2) or without (lane 1) protein phosphatase-1 (10 units, Promega) for 60 min prior to SDS−PAGE.

same protein fractions shown in Figure 1A (left) were transferred to a PVDF membrane that was probed with GST-fused Bmh1/2. As expected, GST-fused Bmh1/2 bound the proteins in the fully purified fraction more strongly than those in the starting cell extract (lane 4′, compared with lanes 1′ and 2′). We also used protein phosphatase-1 (PP1) to test whether the observed association required phosphorylation of target proteins. As shown in Figure 1B, PP1 treatment completely abolished the binding (lane 2). Thus, the majority of proteins recovered by the MEF method were prospective phosphoproteins that specifically associated with Bmh1/2 in a phosphorylation-dependent manner.

Proteomic Identification and Characterization of Bmh-Binding Proteins. The proteins thus recovered were digested with proteases, and the resulting polypeptides were directly analyzed by two-dimensional liquid chromatography−tandem mass spectrometry (2D LC−MS/MS) to identify Bmh-binding proteins (see Materials and Methods). We repeated this analysis seven times with three independent preparations and obtained a total of 12 337 MS/MS spectra that were assigned to 5902 peptides, leading to the identification of 271 unique proteins at the saturated level (Figure 2A). In these experiments, each protein was identified using between 1 and 47 corresponding peptides with an average of 3.1 corresponding peptides per protein per single assay. In contrast, no yeast proteins were detected by LC−MS/MS with three independently prepared mock samples from the WT RRY3 cell extracts, which confirms purification specificity (data not shown). The complete data sets from

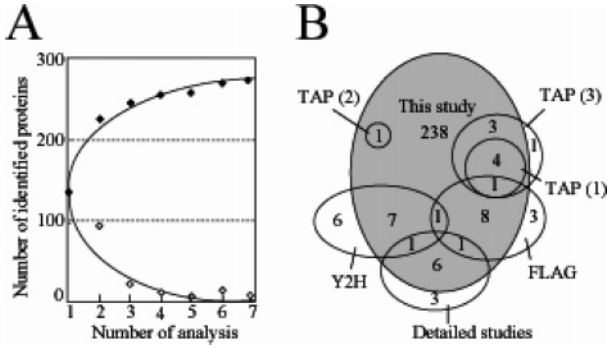

FIGURE 2: Identification of Bmh1/2-interacting partners. (A) Comprehensive analysis of Bmh1/2-associated proteins by LC−MS/MS. The graph shows the number of newly identified proteins in each analysis (○) and the total number identified (●). (B) Comparison of the Bmh interactors identified from this study with those from other studies. See the text for details. TAP, tandem affinity purification. TAP (1), (2), and (3) were from refs *28*−*30*, respectively.

all of these experiments are available as Table 1 and Supporting Information Table S1.

Previously published data regarding Bmh interactions, including data from detailed case-by-case analyses and high-throughput two-hybrid screen and immunoprecipitation assays, were available for a total of 46 proteins (*14*, *24*−*31*). We found that our approach covered the majority of the previously reported interactors: 8 of 11 proteins in case-by-case analysis [Acm1, Cdh1, Fin1, Mks1, Msn2, Msn4, Reg1, and Yak1 (*14*, *25*)], 9 of 15 in a two-hybrid screen (*26*, *27*), all (*28*, *29*) or 8 of 9 (*30*) in tandem affinity purifications (TAPs), and 11 of 14 in a FLAG-tagged immunoprecipitation approach (*31*), allowing 60−100% of interactors listed in the respective data sets (Figure 2B). Accordingly, 238 other proteins were simultaneously identified in this study as new interacting partners of Bmh1/2 (Figure 2B), reflecting the importance of using the MEF method for global screening to identify novel interactions.

Overview of the Bmh1/2-Associated Proteins. The identified Bmh-associated proteins were used as queries to search the Munich Information Center for Protein Sequences (MIPS) database and the genome-wide protein expression data sets (*32*). The proteins were sorted according to their annotated molecular weight, isoelectric point, copy number, subcellular localization, phenotype, and biological function (Supporting Information Figure S2; see also Table S1). This analysis revealed that Bmh1/2 can bind diverse proteins that fall into almost all functional classes and subcellular localizations. Notably, there was no observed correlation between the detection and copy number (abundance) of the identified proteins (Figure S2C), suggesting that the interaction with Bmh1/2 is contingent on posttranslational modification, most likely phosphorylation. We found 31 protein kinases and 19 transcription factors in our list, which accounts for nearly 30 and 10% of total protein kinases and transcription factors encoded in the *S. cerevisiae* genome, respectively (Supporting Information Table S2). These results are consistent with the fact that these two groups represent the major phosphoprotein classes of yeast cells (*33*).

Interactions Are Strongly Enriched in a Variety of Signaling or Other Regulatory Pathways. Previous genetic studies have suggested that Bmh1/2 could be global regulators in TOR-mediated cell survival (*14*, *22*, *34*). In support of this hypothesis, our analysis identified 11 TOR pathway proteins, including eight previously uncharacterized proteins [Bul1, Bul2, Gat1, Glu3, Npr1, Pam1, Rsp5, and Rtg2 (Figure 3A)]. To identify the involvement of Bmh1/2 in other cell signaling pathways, we combined our proteomic results with the published results in the YPD (Yeast Proteome Database). We assigned each binding protein to the reported signaling or regulatory pathways and then evaluated the extracted information by literature miming. We found that the interactions are also strongly enriched in at least 20 other signaling or regulatory pathways that have been identified mainly using genetic approaches (Supporting Information Figure S3). The extracted pathways include one known Bmh1/2-regulated pathway, the MAPK (Kss1) pathway (*16*), as well as 19 unreported pathways involved in control of two independent MAPK (Hog1 and Mpk1) signaling cascades, endocytosis, exocytosis, chitin synthesis, phosphoinositide metabolism, ion transport, cell cycle entry, and cell division. Most of the identified interactors and other molecules are functionally linked with each other in control of the activity of the respective pathways: inactivation of any of these proteins leads to either improved or worsened functional outcomes (Figure S3, shown as arrows and bars). Because the 271 proteins identified in this study comprise only 4.4% of the *S. cerevisiae* proteome (271 of 6632 proteins), we assume that this enrichment is specific and thus may be biologically relevant.

Bmh1 and -2 Are Required for the Signal-Dependent Activation of the Hog1 and Mpk1 Pathways. We analyzed whether Bmh1 and -2 are involved in the control of the Hog1 and Mpk1 pathways in vivo (Figure 3B; see also Supporting Information Table S3). WT and RRY1216 mutant cells were grown to log phase in YPD medium and then stimulated with NaCl or rapamycin [known activators of these MAPKs (*35*, *36*)], and samples were removed at various time points and analyzed by Western blotting with anti-phospho-p38 and anti-p44/42. As shown in Figure 4 (lanes 1−3 and 1′−3′), Hog1 and Mpk1 phosphorylation was induced in the WT RRY3 strain with kinetics almost identical to those previously reported (*35*, *36*). However, the Bmh-deficient RRY1216 mutant did not exhibit these MAPK phosphorylation kinetics (lanes 4−6 and 4′−6′), although higher overall levels of Mpk1 phosphorylation, but not Hog1 phosphorylation, were detected before and during stimulation (Figure 4B, lanes 4′−6′). The differences found between the phosphorylation levels in the WT and Bmh-deficient mutant cells were not the result of altered MAPK protein levels, as the expression levels of these MAPKs were almost equal between the two strains (Figure 4, bottom panels). These results suggest that Bmh1 and -2 are necessary for the stimulus-induced activation of not only the Kss1 MAPK pathway (16) but also the Hog1 and Mpk1 pathways in vivo. This could explain the reported phenotypes that the Bmh-deficient mutant cannot grow under several stress conditions (*16*, *22*) because the precise control of these MAPK activities is prerequisite for yeast survival via stress responses (*37*).

Bmh1/2 Is Also Involved in the Control of Cellular Chitin Levels. To further explore the functional implication of Bmh1/2 in other pathways in vivo, we examined the influence of Bmh deletion on chitin synthesis. Two regulatory pathways have previously been implicated in controlling the biosynthesis of this polysaccharide, a minor but essential

Table 1: Summary of Bmh1/2-Associated Proteins Identified by LC−MS/MS

gene	ID	description[a]
ABD1	YBR236C	mRNA cap methyltransferase
ACC1	YNR016C	acetyl-CoA carboxylase
AGE1	YDR524C	similarity to ADP-ribosylation factor (ARF) GTPase activating protein (GAP) with effector functions
AGE2	YIL044C	weak similarity to zinc finger protein Gcs1p
AKL1	YBR059C	Ark family kinase-like protein
ALD6	YPL061W	aldehyde dehydrogenase, cytosolic
ALK1	YGL021W	DNA damage-responsive protein
APM4	YOL062C	AP-2 complex subunit, mu2 subunit, 55 kDa
ARK1	YNL020C	actin regulating kinase
ARP3	YJR065C	actin-related protein
ASK10	YGR097W	involved in Skn7p-dependent transcription
AT(P)G13	YPR185W	protein required for the autophagic process
ATG11	YPR049C	peripheral membrane protein required for stable binding of precursor API to its target membrane
AVT4	YNL101W	involved in amino acid efflux from the vacuole
BBP1	YPL255W	cell division control protein
BCK1	YJL095W	Ser/Thr protein kinase of the MEKK family
BCK2	YER167W	suppressor of mutations in protein kinase C pathway components
BFA1	YJR053W	spindle assembly checkpoint protein
BIM1	YER016W	binding to microtubules
BNI4	YNL233W	bud neck involved
BNI5	YNL166C	similarity to *Schizosaccharomyces pombe* SPBC1711.05 serine-rich repeat protein of unknown function
BNR1	YIL159W	regulator of budding
BOI1	YBL085W	BEM1 protein-binding protein
BOI2	YER114C	budding protein
BOP3	YNL042W	hypothetical protein
BUB2	YMR055C	cell cycle arrest protein
BUL1	YMR275C	ubiquitination pathway protein
BUL2	YML111W	strong similarity to ubiquitination protein Bul1p
CDC25	YLR310C	GDP/GTP exchange factor for Ras1p and Ras2p
CDH1	YGL003C	substrate-specific activator of APC-dependent proteolysis
CIK1	YMR198W	spindle pole body-associated protein
CIN8	YEL061C	kinesin-related protein
CKA1	YIL035C	casein kinase II, catalytic α chain
CKA2	YOR061W	casein kinase II α′ chain
CKI1	YLR133W	choline kinase
CSR2	YPR030W	nuclear protein with a potential regulatory role in utilization of galactose and nonfermentable carbon sources
CYK3	YDL117W	similarity to hypothetical *S. pombe* protein, protein possibly involved in cytokinesis
CYR1	YJL005W	adenylate cyclase
DED1	YOR204W	ATP-dependent RNA helicase
DIG1	YPL049C	MAP kinase-associated protein, downregulator of invasive growth and mating
DIG2	YDR480W	MAP kinase-associated protein, downregulator of invasive growth and mating
DOT6	YER088C	involved in derepression of telomeric silencing
ECM21	YBL101C	involved in cell wall biogenesis and architecture
EFR3	YMR212C	weak similarity to myosins, conserved, ubiquitous membrane protein required for cell viability
ENT4	YLL038C	weak similarity to YJR125C and YDL161W
ERB1	YMR049C	protein required for maturation of the 25S and 5.8S ribosomal RNAs
ERG3	YLR056W	C-5 sterol desaturase
FIN1	YDR130C	weak similarity to sea urchin myosin heavy chain
FIR1	YER032W	interacts with the poly(A) polymerase in the two-hybrid system
FLO8	YER109C	protein required for diploid filamentous growth, pseudogene in S288C
FRQ1	YDR373W	regulator of phosphatidylinositol-4-OH kinase protein
FRT1	YOR324C	component of calcineurin-mediated stress response
FUN21	YAL031C	cytoplasmic protein of unknown function, potential Cdc28p substrate
GAL4	YPL248C	transcription factor
GAT1	YFL021W	transcription factor for nitrogen regulation
GCR2	YNL199C	glycolytic gene transcriptional activator
GIC2	YDR309C	Cdc42 GTPase-binding protein
GIN4	YDR507C	Ser/Thr protein kinase
GIP2	YER054C	Glc7p-interacting protein
GIP3	YPL137C	Glc7p-interacting protein whose overexpression relocalizes Glc7p from the nucleus
GIS4	YML006C	hypothetical protein
GLC7	YER133W	Ser/Thr phosphoprotein phosphatase 1, catalytic chain
GLN3	YER040W	transcription factor for positive nitrogen regulation
GTS1	YGL181W	transcription factor of the Gcs1p/Glo3p/Sps18p family
HAA1	YPR008W	similarity to transcription factor
HAL5	YJL165C	Ser/Thr protein kinase
HLR1	YDR528W	similarity to Lre1p
HOF1	YMR032W	involved in cytokinesis
HOT1	YMR172W	transcription factor required for the transient induction of glycerol biosynthetic genes GPD1 and GPP2 in response to high osmolarity
HRK1	YOR267C	similarity to Ser/Thr protein kinases

Table 1. (Continued)

gene	ID	description[a]
HRR25	YPL204W	casein kinase I, Ser/Thr/Tyr protein kinase
HSL1	YKL101W	Ser/Thr protein kinase, coupling septin ring assembly to cell cycle progression
HYM1	YKL189W	component of the RAM signaling network that is involved in regulation of Ace2p activity and cellular morphogenesis
ILV6	YCL009C	acetolactate synthase, regulatory subunit
IML3	YBR107C	outer kinetochore protein
IMP2′	YIL154C	sugar utilization regulatory protein
INP53	YOR109W	phosphatidylinositol phosphate phosphatase
IPP1	YBR011C	inorganic pyrophosphatase, cytoplasmic
JIP4	YDR475C	Jumonji interacting protein
JSN1	YJR091C	suppresses the high-temperature lethality of tub2-150
KAR2	YJL034W	nuclear fusion protein
KAR3	YPR141C	kinesin-related protein
KCS1	YDR017C	potential transcription factor of the BZIP type
KIN1	YDR122W	Ser/Thr protein kinase
KIN2	YLR096W	Ser/Thr protein kinase
KIN4	YOR233W	Ser/Thr protein kinase
KIN82	YCR091W	Ser/Thr protein kinase
KIP2	YPL155C	kinesin-related protein
KKQ8	YKL168C	Ser/Thr protein kinase
KSP1	YHR082C	Ser/Thr protein kinase
LAT1	YNL071W	dihydrolipoamide *S*-acetyltransferase
LEU2	YCL018W	*β*-isopropylmalate dehydrogenase
LHS1	YKL073W	chaperone of the ER lumen
LRE1	YCL051W	involved in laminarinase resistance
LSB3	YFR024C-A	possible role in the regulation of actin cytoskeletal organization
LSC1	YOR142W	succinate-CoA ligase α subunit
LSP1	YPL004C	strong similarity to YGR086C
LST4	YKL176C	protein required for regulated transport of nitrogen-regulated permeases from the Golgi to the plasma membrane
MAF1	YDR005C	protein required for sorting of Mod5p
MCM10	YIL150C	protein required for S-phase initiation or completion
MDS3	YGL197W	negative regulator of early meiotic expression
MET18	YIL128W	involved in NER repair and RNA polymerase II transcription
MHP1	YJL042W	microtubule-associated protein
MIG1	YGL035C	transcriptional repressor
MKS1	YNL076W	pleiotropic regulatory factor
MLF3	YNL074C	similarity to YIL135C
MPT5	YGL178W	multicopy suppressor of POP2
MSB1	YOR188W	morphogenesis-related protein
MSB3	YNL293W	similarity to Mic1p and human transforming protein tre-2, and strong similarity to YOL112W
MSC3	YLR219W	hypothetical protein
MSG5	YNL053W	dual-specificity protein phosphatase
MSN2	YMR037C	stress responsive regulatory protein
MSN4	YKL062W	transcriptional activator
MSO1	YNR049C	secretion protein, multicopy suppressor of SEC1
MSS4	YDR208W	phosphatidylinositol-4-phosphate 5-kinase
NAM7	YMR080C	nonsense-mediated mRNA decay protein
NAP1	YKR048C	nucleosome assembly protein I
NPR1	YNL183C	Ser/Thr protein kinase
NTH1	YDR001C	neutral trehalase (α,α-trehalase)
NTH2	YBR001C	α,α-trehalase
NUG1	YER006W	nuclear GTPase (involved in ribosome biogenesis)
NUP60	YAR002W	nuclear pore protein
OSH2	YDL019C	similarity to Swh1p
PAM1	YDR251W	coiled-coil protein multicopy suppressor of loss of PP2A
PBS2	YJL128C	tyrosine protein kinase of the MAP kinase kinase family
PFK26	YIL107C	6-phosphofructose 2-kinase, isozyme 1
PHO87	YCR037C	low-affinity phosphate transporter
PIK1	YNL267W	phosphatidylinositol 4-kinase
PIL1	YGR086C	strong similarity to hypothetical protein YPL004C
PKH2	YOL100W	similarity to Ser/Thr protein kinases
PMD1	YER132C	negative regulator of early meiotic expression
PPS1	YBR276C	protein tyrosine phosphatase
PPZ2	YDR436W	protein Ser/Thr phosphatase of the PP-1 family
PRK1	YIL095W	Ser/Thr protein kinase involved in regulation of actin cytoskeleton organization
PRR1	YKL116C	protein kinase with a possible role in MAP kinase signaling in the pheromone response pathway
PSK1	YAL017W	PAS kinase involved in the control of sugar metabolism and translation
PSK2	YOL045W	PAS domain-containing Ser/Thr kinase
PSP1	YDR505C	high-copy number suppressor of ts mutations in DNA polymerase α
PTK1	YKL198C	polyamine transport enhancing protein
PTK2	YJR059W	involved in polyamine uptake
PTP3	YER075C	protein tyrosine phosphatase
PUF2	YPR042C	similarity to Jsn1p
PUF3	YLL013C	transcript-specific regulator of mRNA degradation

Table 1. (Continued)

gene	ID	description[a]
PXL1	YKR090W	similarity to chicken Lim protein kinase and Islet proteins
RAI1	YGL246C	nuclear protein that binds to and stabilizes the exoribonuclease Rat1p, required for pre-rRNA processing
RAM1	YDL090C	protein farnesyltransferase, β subunit
RBL2	YOR265W	β-tubulin binding protein
RDS1	YCR106W	regulator of drug sensitivity
REG1	YDR028C	regulatory subunit for protein phosphatase Glc7p
RGA2	YDR379W	similarity to Dbm1p and to the rat GAP-associated protein p190
RIM4	YHL024W	no sporulation
ROD1	YOR018W	*O*-dinitrobenzene, calcium and zinc resistance protein
ROM2	YLR371W	GDP−GTP exchange factor for Rho1p
ROX1	YPR065W	heme-dependent transcriptional repressor of hypoxic genes
RPS3	YNL178W	ribosomal protein S3.e
RPS31	YLR167W	ubiquitin/40S small subunit ribosomal protein
RRM3	YHR031C	DNA helicase involved in rDNA replication and Ty1 transposition
RSP5	YER125W	hect domain E3 ubiquitin-protein ligase
RTG2	YGL252C	retrograde regulation protein
RTS3	YGR161C	hypothetical protein
RTT102	YGR275W	regulator of Ty1 transposition
SAC7	YDR389W	suppressor of actin mutation
SAM50	YNL026W	sorting and assembly machinery (SAM) complex subunit
SAP1	YER047C	member of the AAA protein family
SBE22	YHR103W	Golgi protein involved in yeast cell wall formation
SEC1	YDR164C	protein transport protein
SEC16	YPL085W	multidomain vesicle coat protein
SEF1	YBL066C	putative transcription factor
SFL1	YOR140W	transcription factor
SHE1	YBL031W	hypothetical protein
SIR4	YDR227W	silencing regulatory and DNA repair protein
SIS1	YNL007C	heat shock protein
SKT5(CHS4)	YBL061C	protoplast regeneration and killer toxin resistance protein
SLA1	YBL007C	cytoskeleton assembly control protein
SLX4	YLR135W	subunit of Slx1p/Ybr228p-Slx4p complex, required for cell growth in the absence of SGS1 or TOP3
SNF1	YDR477W	carbon catabolite derepressing Ser/Thr protein kinase
SNT1	YCR033W	component of meiotic-specific repressor of the sporulation
SOK1	YDR006C	high-copy number suppressor of a cyclic AMP-dependent protein kinase mutant
SPC110	YDR356W	spindle pole body component
SPC29	YPL124W	spindle pole body protein
SPO71	YDR104C	protein involved in spore wall formation
SPT6	YGR116W	transcription elongation protein
SRL3	YKR091W	similarity to YOR083W
SRV2	YNL138W	adenylate cyclase-associated protein, 70 kDa
SSA1	YAL005C	heat shock protein of the HSP70 family, cytosolic
SSA2	YLL024C	heat shock protein of the HSP70 family, cytosolic
SSA3	YBL075C	heat shock protein of the HSP70 family, cytosolic
SSA4	YER103W	heat shock protein of the HSP70 family, cytosolic
SSB1	YDL229W	heat shock protein of the HSP70 family
SSB2	YNL209W	heat shock protein of the HSP70 family, cytosolic
SSD1	YDR293C	involved in the tolerance to high concentrations of Ca^{2+}
STB2	YMR053C	SIN3 binding protein
STB6	YKL072W	SIN3 binding protein
STC1	YKR089C	strong similarity to YOR081C
STU1	YBL034C	mitotic spindle protein
SVL3	YPL032C	strong similarity to Pam1p
SYT1	YPR095C	guanine nucleotide exchange factor, containing a conserved Sec7p domain
TCO89	YPL180W	TOR complex 1, 89 kDa subunit
TDH2	YJR009C	glyceraldehyde-3-phosphate dehydrogenase 2
TDH3	YGR192C	glyceraldehyde-3-phosphate dehydrogenase 3
TEF2	YBR118W	translation elongation factor eEF1 α-A chain, cytosolic
TSA1	YML028W	thiol-specific antioxidant
UBP12	YJL197W	ubiquitin C-terminal hydrolase
UME6	YDR207C	negative transcriptional regulator
URA2	YJL130C	multifunctional pyrimidine biosynthesis protein
UTP6	YDR449C	U3 snoRNP protein
VAC17	YCL063W	vacuole-specific receptor of Myo2p
VHS2	YIL135C	multicopy suppressor of cell cycle arrest at the G1−S transition
WHI3	YNL197C	involved in regulation of cell size
WHI4	YDL224C	strong similarity to WHI3 protein
XBP1	YIL101C	stress-induced transcriptional repressor
YAK1	YJL141C	Ser/Thr protein kinase
YBL054W	YBL054W	weak similarity to transforming protein (B-myb)
YBL060W	YBL060W	weak similarity to probable guanine nucleotide exchange factor, *S. pombe*
YBL111C	YBL111C	protein of unknown function
YBR007C	YBR007C	hypothetical protein

Table 1. (Continued)

gene	ID	description[a]
YBR225W	YBR225W	hypothetical protein
YCL005W	YCL005W	protein of unknown function localized to lipid particles
YDR186C	YDR186C	hypothetical protein
YDR266C	YDR266C	protein of unknown function
YDR326C	YDR326C	strong similarity to YHR080C, similarity to YFL042C and YLR072W
YDR338C	YDR338C	protein of unknown function
YDR348C	YDR348C	similarity to hypothetical protein YHR097C
YER071C	YER071C	hypothetical protein
YFL049W	YFL049W	weak similarity to Npl6p
YFR017C	YFR017C	protein of unknown function localized to cytoplasm
YFR022W	YFR022W	revertant of glycogen synthase kinase mutation
YGR015C	YGR015C	similarity to hypothetical protein YGR031W
YGR058W	YGR058W	similarity to mouse calcium binding protein
YGR068C	YGR068C	weak similarity to Rod1p
YGR237C	YGR237C	weak similarity to YOR019w
YHL008C	YHL008C	similarity to *Methanobacterium formicicum* formate dehydrogenase
YHR080C	YHR080C	similarity to hypothetical protein YDR326C, YFL042C, and YLR072W
YHR097C	YHR097C	strong similarity to hypothetical protein YDR348C
YHR209W	YHR209W	similarity to hypothetical protein YER175C
YHR218W	YHR218W	strong similarity to subtelomeric encoded proteins
YIL092W	YIL092W	hypothetical protein
YIR003W	YIR003W	weak similarity to mammalian neurofilament triplet H proteins
YJL084C	YJL084C	protein of unknown function localized to cytoplasm
YKL105C	YKL105C	similarity to YMR086W
YKL171W	YKL171W	weak similarity to Ser/Thr protein kinase
YLR001C	YLR001C	protein of unknown function localized to vacuolar membrane
YLR149C	YLR149C	weak similarity to hypothetical protein SPCC4G3.03 from *S. pombe*
YLR152C	YLR152C	similarity to YOR3165W and YNL095C
YLR177W	YLR177W	similarity to suppressor protein Psp5p
YLR247C	YLR247C	similarity to *S. pombe* Rad 8p and Rdh54p
YLR253W	YLR253W	protein of unknown function localized to mitochondria
YLR404W	YLR404W	hypothetical protein
YML059C	YML059C	similarity to *Caenorhabditis elegans* ZK370.4 protein
YMR086W	YMR086W	similarity to YKL105C
YMR124W	YMR124W	weak similarity to YLR031W
YNL152W	YNL152W	similarity to hypothetical protein from *S. pombe*
YNR014W	YNR014W	weak similarity to hypothetical protein YMR206W
YNR047W	YNR047W	similarity to Ser/Thr protein kinases
YNR071C	YNR071C	strong similarity to aldose 1-epimerase and UDP-glucose 4-epimerase
YOR220W	YOR220W	hypothetical protein
YOR227W	YOR227W	protein of unknown function localized to cytoplasm
YOR283W	YOR283W	protein of unknown function localized to cytoplasm and nucleus
YOX1	YML027W	homeodomain protein
YPL141C	YPL141C	strong similarity to protein kinase Kin4p
YPL150W	YPL150W	similarity to Ser/Thr protein kinases
YPL247C	YPL247C	similarity to human HAN11 protein and petunia an11 protein
YPL267W	YPL267W	weak similarity to *C. elegans* transcription factor unc-86
YPR013C	YPR013C	putative transcription factor
YPR015C	YPR015C	similarity to transcription factors
YPR115W	YPR115W	similarity to probable transcription factor Ask10p, and to YNL047C and YIL105C
YSC84	YHR016C	strong similarity to hypothetical protein YFR024c-a
YSH1	YLR277C	component of pre-mRNA polyadenylation factor PF I
YTA6	YPL074W	similarity to Vps4p and YER047C
ZAP1	YJL056C	metalloregulatory protein involved in zinc-responsive transcriptional regulation
ZDS1	YMR273C	protein involved in negative regulation of cell polarity

[a] Information was obtained from the MIPS database.

component of the yeast cell wall (Figure 3C; see also Table S3). We first used Calcofluor White, a widely used indicator for detecting cell wall defects in yeast mutants (*38*). As shown in Figure 5A, the RRY1216 mutant, but not the WT control, was sensitive to this drug, suggesting that the chitin level in the mutant might not be normal. To directly assess this observation, the chitin content of the mutant was then measured (Figure 5B). A significant increase in the chitin level was detected in the *bmh* null mutant (230.2 ± 9.3 μg of *N*-acetyl-D-glucosamine/mg of protein, $n = 3$) compared to that in the WT (76.5 ± 4.2 μg of *N*-acetyl-D-glucosamine/mg of protein, $n = 3$). Fluorescence microscopy also confirmed this result and further indicated that the increased level of chitin accumulated mainly at the bud neck region of the mutant (Figure 5C).

The 1,3-β-glucan is another essential component of the yeast cell wall. We found, however, that the levels of 1,3-β-glucan remained unchanged between the two yeast strains (Figure 5B, right two columns). Thus, the observed increase in the chitin levels in the *bmh* mutant reflects a specific defect in chitin biosynthesis rather than a more general effect on polysaccharide biosynthesis. In these experiments, expression of either Bmh1 or Bmh2 equally rescued the observed phenotypes (Figure 5A,B and data not shown). The one exception was that Bmh1 was more effective than Bmh2 in rescuing the Calcofluor-induced growth reduction of RRY1216

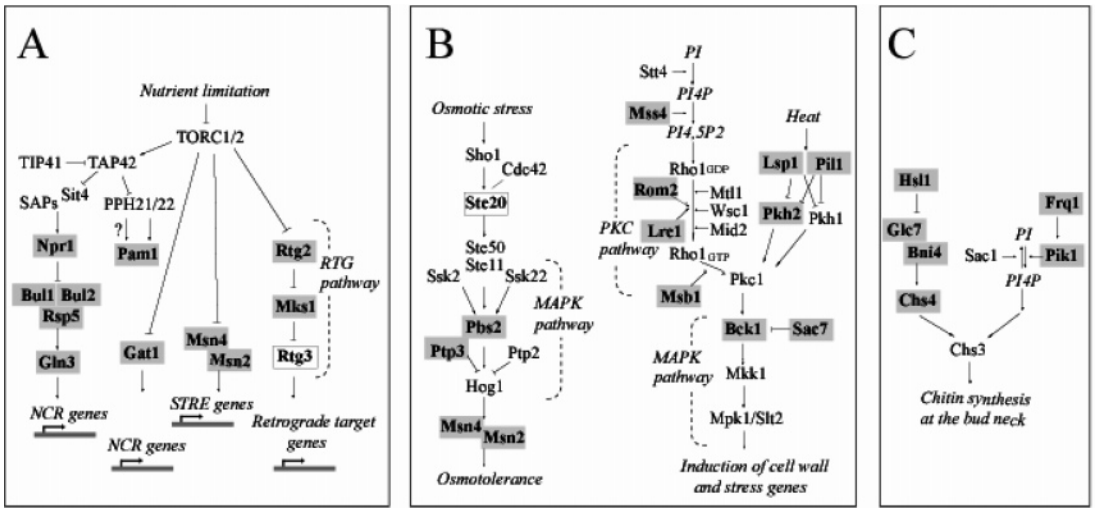

FIGURE 3: Summary of Bmh-binding proteins in (A) TOR pathways, (B) MAPK (Hog1 and Mpk1) pathways, and (C) the regulatory pathways controlling cellular chitin levels. Proteins in gray boxes indicate the Bmh-associated proteins identified in this study, and proteins in white boxes indicate those identified in other studies (*16*, *39*) but not in this study. Arrows denote activation and bars repression.

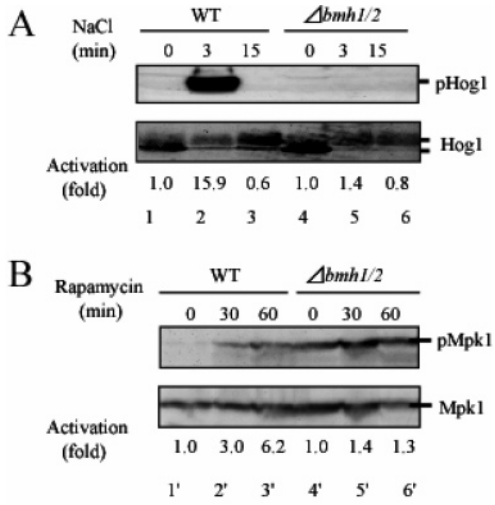

FIGURE 4: Bmh1/2 mediates MAPK (Hog1 and Mpk1) activation in response to NaCl and rapamycin. WT and Δbmh1/bmh2 cells were stimulated with (A) 1.4 M NaCl or (B) 200 ng/mL rapamycin at the indicated times. Cell extracts (50 μg each) were separated by SDS−PAGE and analyzed by immunoblotting with phospho-p38- and phospho-p44/42-specific antibodies to detect phosphorylated Hog1 and Mpk1, respectively (top) and with anti-Hog1 and anti-Mpk1 to determine the protein level of Hog1 and Mpk1, respectively (bottom). The intensity of each immunostained band shown in the top panel was also quantified by densitometry and normalized to 1.0 at time zero for each strain. Similar results were obtained for at least three independent analyses.

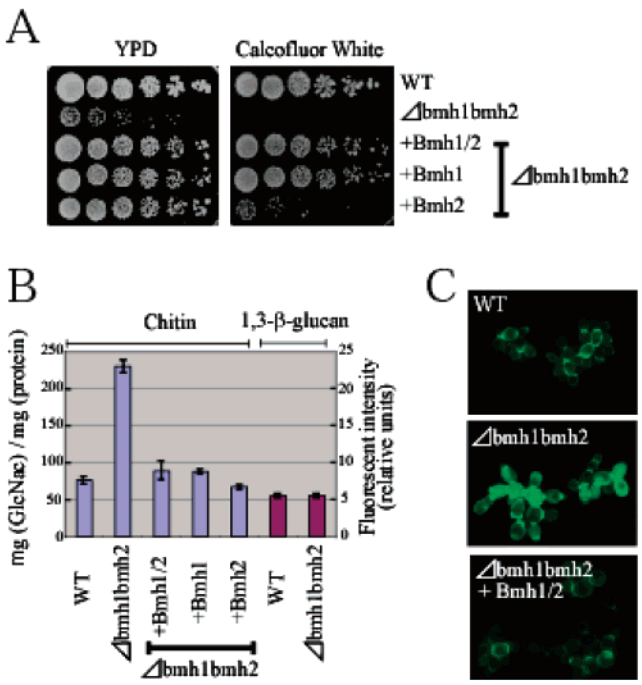

FIGURE 5: Bmh1 and -2 are involved in chitin biosynthesis. (A) WT, Δbmh1/bmh2, or Δbmh1/bmh2 cells carrying plasmids expressing the indicated proteins were plated onto YPD or YPD with 125 μg/mL Calcofluor White and grown at 30 °C. (B) The indicated cells were grown to an A_{600} of 1.0, and then chitin and 1,3-β-glucan contents were measured as described in Materials and Methods. Results shown in the bar diagrams represent means ± the standard deviation of three independent experiments. GlcNac, *N*-acetylglucosamine. (C) The indicated cells were grown to early log phase in YPD medium and stained with 1 mg/mL Calcofluor White.

through an unknown mechanism (Figure 5A, bottom two columns). Taken together, these data suggest that Bmh1 and -2 are also required for proper control of cellular chitin levels.

DISCUSSION

In this study, we performed a comprehensive proteomic analysis using multistep immunoaffinity purification and mass spectrometry to identify 271 yeast proteins that specifically associate with Bmh1/2 in a phosphorylation-dependent manner. For 33 of these 271 proteins, the interactions were already known or were predicted from the literature (Figure 2B). The Bmh interaction of the 238 remaining proteins is presented here for the first time (Figure 2B). The newly identified proteins are involved in many different aspects of signaling, metabolism, protein fate, cell fate, transcription, transport, cell cycle, DNA processing, etc. In addition, the identified interactors localize to various subcellular locations, including the cytoplasm, nucleus, cytoskeleton, mitochondria, endoplasmic reticulum, bud, and cell membrane. These data suggest that, like mammalian 14-3-3 proteins, Bmh1 and -2 are involved in a very broad range of cellular processes in concert with protein phosphorylation.

This proteomic procedure allowed us to analyze a wide range of proteins regardless of their physicochemical and expression characteristics. For example, our analysis showed a wide range of M_r values, from 12.4 (Rbl2) to 250.4 kDa (Acc1), pI valuess from 4.2 (Nap1) to 10.4 (She1), and absolute abundances from 49 (Yjl084) to 378 212 copies (Tsa1) per cell (Table S1). A search of a yeast database and the expression data set (*32*) for any of these parameters revealed that the analytical range of this analysis is also equivalent to 93.5% for M_r, 96.7% for pI, or 99.1% for copy number of the yeast proteome (listed in data sets), suggesting that our results represent the majority of Bmh interactions for exponentially growing yeasts. It should be noted, however, that several important Bmh1/2 complexes were not identified during our analysis, which may be attributed to several reasons. (i) The solubilizing buffer contained mild detergent (1% Triton X-100). (ii) The minor components were below our detection limit (e.g., <49 copies per cell). (iii) The complexes were very unstable and easily dissociated during our extraction−purification procedures. Indeed, our analysis failed to identify 3 of the 11 known Bmh ligands, Rtg3, Ste20, and Rim15, probably due to the reported low solubility of the Bmh−Rtg3 complex under mild conditions (*39*) and the low stability of Bmh−Ste20 and Bmh−Rim15 complexes required for overexpression of both types of components for detection of the association (*16*, *24*). We found that the observed Bmh interactions vary significantly after withdrawal of glucose or amino acids (60 min) from culture medium (as analyzed by SDS−PAGE, unpublished data), suggesting that alternative conditions might also be needed for isolation of additional complexes.

Our results provide strong candidates of Bmh interactors whose interaction could mediate the biological effects of Bmh1/2 in the previously reported cellular processes. For example, we identified 11 components (including 8 novel components) of the TOR signaling pathways (Figure 3A), supporting the previous proposal that Bmh1 and -2 could be global regulators of TOR-mediated cell survival (*14*, *22*, *34*). A pivotal role for Bmh1/2 in carbon source utilization has been suggested from recent transcriptomic studies (*40*,*41*) and is also supported by our analysis, which identified all core components of the Snf1 signaling pathway that is central to catabolite repression [Glc7, Reg1, Ssb1, Ssb2, Snf1, and Mig1 (Figure S3)] (*42*). The blanket identification of all members of the Ark1/Prk1 protein kinase family (Ark1, Prk1, and Akl1), which is implicated in the formation and/or maintenance of endocytic vesicles in fungal cells (*43*), is of great interest in light of a previous study suggesting a regulatory role for Bmh1/2 in receptor-mediated endocytosis via clathrin-coated vesicles (*44*).

We noted that enrichment in interactions was observed in a variety of cell signaling and other regulatory pathways (Figures 3 and S3). Furthermore, the physiological significance of such interactions was supported in part by the effect of *BMH* deletion, which led to an impaired activation of MAPK pathways and an abnormal accumulation of chitin at the bud neck (Figures 4 and 5). These results, together with the recent proteomic data (*7*−*12*), point strongly toward a role of a coordinator for 14-3-3 proteins, which could modulate the activity of the pathway components in a highly controlled fashion, possibly in concert with protein phosphorylation. A similar mechanism of control by 14-3-3 has recently been postulated for metabolic enzymes from plants and humans (*7*, *45*). For example, Alexander and Morris (*45*) found that four of five enzymes sequentially involved in sucrose biosynthesis from triose phosphates commonly interact with and possibly are regulated by plant 14-3-3 proteins. Such multistep control in sequential metabolic enzymes might ensure the coordinated production of metabolites or fine-tune biologically important events according to cellular requirements. Thus, our results complement other published data and further raise the possibility that this type of control might represent a general mechanism for 14-3-3-mediated regulation in eukaryotic cells.

A growing body of evidence suggests that many cellular processes are carried out by multiprotein complexes (*26*−*31*). For example, Gavin et al. (*30*) recently identified 491 unique complexes that differentially associate with additional attachment proteins or protein modules, thereby enabling potential functional diversification in *S. cerevisiae*. Comparing our proteomic results with their data sets, we found that many of the reported complexes (67 complexes, 14% of total complexes) also contain the identified Bmh-associated proteins (Supporting Information Table S4). Interestingly, however, we found that Bmh1 and -2 were not components in most of the complexes identified in the analysis of Gavin et al. (*30*).

Furthermore, nearly two-thirds of the interactors identified in our study were not found in these published complexes, suggesting that some of the Bmh complexes reported here belong to distinct pools when compared to those reported by Gavin et al. (*30*). Thus, our analysis suggests the existence of additional multiprotein complexes whose formation or interaction is dynamically regulated by protein phosphorylation and Bmh1/2 binding.

In addition to their published functions, our results suggest that Bmh1 and -2 are *bona fide* regulators of a wide variety of signaling modules and pathways mediated by protein phosphorylation. Because a number of the identified interactors are highly conserved from fungi to humans, our comprehensive identification of the Bmh interactors in *S. cerevisiae* will not only serve as a valuable resource for yeast 14-3-3 research but also provide insight into the regulatory roles of the 14-3-3 family in many eukaryotes.

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SUPPORTING INFORMATION AVAILABLE

Protein identification and analysis data. This material is available free of charge via the Internet at http://pubs.acs.org.

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