DOI: 10.1534/genetics.109.107797

Deleting the 14-3-3 Protein Bmh1 Extends Life Span in *Saccharomyces cerevisiae* by Increasing Stress Response

Chen Wang, Craig Skinner, Erin Easlon and Su-Ju Lin[1]

Department of Microbiology, College of Biological Sciences, University of California, Davis, California 95616

Manuscript received July 27, 2009
Accepted for publication September 24, 2009

ABSTRACT

Enhanced stress response has been suggested to promote longevity in many species. Calorie restriction (CR) and conserved nutrient-sensing target of rapamycin (TOR) and protein kinase A (PKA) pathways have also been suggested to extend life span by increasing stress response, which protects cells from age-dependent accumulation of oxidative damages. Here we show that deleting the yeast 14-3-3 protein, Bmh1, extends chronological life span (CLS) by activating the stress response. 14-3-3 proteins are highly conserved chaperone-like proteins that play important roles in many cellular processes. *bmh1*Δ-induced heat resistance and CLS extension require the general stress-response transcription factors Msn2, Msn4, and Rim15. The *bmh1*Δ mutant also displays a decreased reactive oxygen species level and increased heat-shock-element-driven transcription activity. We also show that *BMH1* genetically interacts with CR and conserved nutrient-sensing TOR- and PKA-signaling pathways to regulate life span. Interestingly, the level of phosphorylated Ser238 on Bmh1 increases during chronological aging, which is delayed by CR or by reduced TOR activities. In addition, we demonstrate that PKA can directly phosphorylate Ser238 on Bmh1. The status of Bmh1 phosphorylation is therefore likely to play important roles in life-span regulation. Together, our studies suggest that phosphorylated Bmh1 may cause inhibitory effects on downstream longevity factors, including stress-response proteins. Deleting Bmh1 may eliminate the inhibitory effects of Bmh1 on these longevity factors and therefore extends life span.

RECENT studies in genetically tractable model systems including yeasts, worms, flies, and mice demonstrated that longevity could be modulated by single-gene mutations (Kenyon 2001; Tissenbaum and Guarente 2002; Dilova *et al.* 2007). In addition to genetic interventions, calorie restriction (CR) has also been shown to extend life span in many species (Weindruch and Walford 1998; Roth *et al.* 2001) and to ameliorate age-related diseases such as cancer and diabetes (Weindruch and Walford 1998). Identification and study of novel longevity genes may therefore provide insights into the molecular mechanisms underlying CR, longevity regulation, and age-associated diseases.

The budding yeast *Saccharomyces cerevisiae* is an efficient model for studying longevity regulation. Yeast life span has been studied in two distinct ways: replicative life span (RLS) and chronological life span (CLS). RLS measures the number of cell divisions that an individual yeast cell undergoes before senescence. CLS measures the length of time that cells remain viable at a nondividing state. Yeast cells enter a nondividing stationary phase when nutrients are exhausted. This quiescent state has been suggested to resemble the G_0 state in higher eukaryotes (Werner-Washburne *et al.* 1993). Moderate CR can be imposed in yeast by reducing the glucose concentration from 2% to 0.5% in rich media (Lin *et al.* 2000; Easlon *et al.* 2007; Smith *et al.* 2007; Wei *et al.* 2008), which extends both CLS and RLS. CR is suggested to function through reducing the activities of conserved nutrient-sensing pathways to extend life span. Decreasing the activity of the Ras-cyclic AMP-activated protein kinase A (Ras-cAMP/PKA) pathway, which regulates cell growth and stress response, extends life span (Lin *et al.* 2000; Fabrizio *et al.* 2001). Deleting the nutrient-responsive kinases Sch9 (a homolog of mammalian S6K kinases) and Tor1 also promotes longevity (Fabrizio *et al.* 2001; Kaeberlein *et al.* 2005). The conserved Sir2 family proteins have been shown to play important roles in moderate CR-induced RLS extension (Lin *et al.* 2000; Lamming *et al.* 2005; Easlon *et al.* 2007). Interestingly, Sir2 appeared to be dispensable for CLS (Fabrizio *et al.* 2005; Smith *et al.* 2007) and for RLS (Jiang *et al.* 2002; Kaeberlein *et al.* 2004; Easlon *et al.* 2007) in certain CR models, which further underscored the complexity of CR and longevity regulation. Identification of novel longevity factors is therefore essential to help elucidate the underlying mechanisms.

An enhanced stress response has been shown to play important roles in extending longevity. An increased

Supporting information is available online at http://www.genetics.org/cgi/content/full/genetics.109.107797/DC1.

[1]*Corresponding author:* Department of Microbiology, College of Biological Sciences, University of California, 323 Briggs Hall, 1 Shields Ave., Davis, CA 95616. E-mail: slin@ucdavis.edu

stress response may protect cells from age-dependent accumulation of damages caused by reactive oxygen species (ROS) generated as metabolic by-products. In yeast, downregulation of the nutrient-sensing target of rapamycin (TOR)- and PKA-signaling activities confers resistance to various stresses such as heat shock and oxidative stress (Fabrizio *et al.* 2001; Kaeberlein *et al.* 2005). Stress-response transcription factors including Msn2, Msn4, Rim15, and Gis1 have been reported to mediate life-span extension and stress resistance in these nutrient-sensing mutants (Wei *et al.* 2008). In worms, the stress-response transcription factors DAF-16 and HSF-1 have also been shown to be required for increased life span and thermo-tolerance in the *daf-2* mutants (Lin *et al.* 1997; Hsu *et al.* 2003). In mammals, fibroblasts derived from long-lived Snell dwarf mice were more resistant to different forms of stresses than cells from normal mice (Salmon *et al.* 2005). Furthermore, mice carrying deletions in the $P66^{shc}$ redox-sensing protein were resistant to stress-induced cell death and exhibited longer life span (Migliaccio *et al.* 1999). These studies demonstrate a correlation between increased life span and enhanced stress resistance.

In this study, we employed an accelerated cell-death-based genetic screen to identify novel longevity genes. Our screen identified the yeast 14-3-3 proteins, which are evolutionarily conserved dimeric acidic proteins involved in cell signaling, cell cycle control, apoptosis, and transcription (Aitken 2006; van Heusden and Steensma 2006). Yeast 14-3-3 proteins are encoded by two highly homologous genes, *BMH1* and *BMH2*, and deleting both genes is lethal in most strains (van Heusden and Steensma 2006). 14-3-3 proteins function as molecular chaperones and protein tethers, which have >200 interacting partners (Kakiuchi *et al.* 2007). Most binding partners of 14-3-3 proteins contain either of the consensus sequence motifs RSX*pS/T*XP and RXXX*pS/T*XP (*pS/T*: phospho-Ser or phospho-Thr; X: any amino acid). Interactions of 14-3-3 proteins and their binding partners are regulated through protein phosphorylation (Aitken 2006; van Heusden and Steensma 2006). Genomewide gene/protein expression studies of yeast 14-3-3 protein (Bmh) mutants revealed that Bmh proteins affect the expression of many genes/proteins associated with carbon and nitrogen metabolism (Ichimura *et al.* 2004; Bruckmann *et al.* 2007). 14-3-3 proteins have also been shown to interact with the TOR and PKA pathways; however, it remains unclear how TOR and PKA regulate 14-3-3 proteins. In this study, we characterized the roles of yeast 14-3-3 protein Bmh1 in stress response and in CR-, TOR-, and PKA-mediated longevity pathways.

MATERIALS AND METHODS

Yeast strains and media: The yeast strain BY4742 *MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0* and the isogenic gene deletion collections were acquired from Open Biosystems (Brachmann *et al.* 1998). W303AR *MAT***a** *ura3-1, leu2-3, 112 his3-11,15 trp1-1 ade2-1 RDN1::ADE2 can1-100* were described previously (Kaeberlein *et al.* 1999; Lin *et al.* 2000). Rich media YPD and synthetic SD media were made as described (Burke *et al.* 2000). Media used for CLS analysis were minimal synthetic SD supplemented with 4× auxotrophic amino acids (leucine, histine, lysine, and uracil) and glucose to a final concentration of 2% or 0.5%. All gene deletions were generated and verified as described (Guldener *et al.* 1996). Strains used in this study are listed in Table 1. Strains overexpressing Bmh1 or Bmh2 were made by integrating the p*ADH1-BMH1* or *pADH1-BMH2* plasmid into the genome.

Genetic screen conditions: About 20,000 colonies, which represented about five copies of the yeast genome, were screened. Colonies carrying the 2μ genomic library were replica plated onto two sets of YPD plates. One set (assay plates) was upshifted to nonpermissive temperature (38°) and the other set (master plates) was incubated at 25°. After 4 days at 38°, the assay plates were shifted to permissive temperature (25°) for an additional 2 days. Cell patches that grew on the assay plates at 38° were excluded because they were likely to carry *cdc25-10*-specific suppressors that simply rescued the growth defects of the *cdc25-10* mutant at 38°. Cell patches on the assay plates that did not grow at 38° but grew after shifting to 25° were likely to carry genes extending survival. Four cell patches were identified under these conditions. Plasmid DNA conferring the strongest survival was recovered from the corresponding master plate and retransformed into the *cdc25-10* mutant to confirm the phenotype. Sequencing analysis of this DNA fragment indicated that it contained both the *NDJ1* and the *BMH2* genes. Each gene was cloned into an integrative vector driven by the *ADH1* promoter pPP81. Only *BMH2* overexpression extended survival.

Plasmid constructions: Bmh overexpression constructs p*ADH1-BMH1* and p*ADH1-BMH2* were made as follows: specific oligonucleotides were designed (with a *Not*I site added to the 5′-end and a *Nhe*I site to the 3′-end) to amplify the *BMH1* (or *BMH2*) coding region via PCR using *Pfu* polymerase. Amplified DNA was digested with *Not*I and *Nhe*I and then ligated to pPP81 (an integrative vector carrying a *LEU2* auxotrophic marker and an *ADH1* promoter).

Chronological life span: Four colonies from each strain were analyzed in each experiment as described (Fabrizio and Longo 2003) with some modifications. Cells were grown in 10 ml SD (at a starting OD_{600} of 0.1) in 50-ml tubes on a roller drum set at the maximum speed to ensure proper aeration. Cells were continuously grown in SD or shifted to sterile water after entering stationary phase (typically after 48 hr). Cells shifted to water showed more significant CLS extension compared to cells maintained in SD (Fabrizio *et al.* 2004) (C. Wang and S.-J. Lin, unpublished results). However, a regrowth phenomenon was often observed: after ~90–99% of cells died, the number of viable cells increased. This regrowth phenomenon has also been reported in several other strains (Fabrizio *et al.* 2004) and was likely due to nutrients released by dead cells and to increased adaptive mutations during prolonged culture (Fabrizio *et al.* 2004). We found that the regrowth problem could be alleviated by transferring 10-fold fewer cells to water at a final density of OD_{600} ~1 (10^7 cells/ml; ~10^8 cells were examined for each CLS assay in water). Cell viability was monitored every other day by plating a fraction of culture onto fresh YPD to determine the colony-forming units (CFU). The rate of cell survival was calculated by normalizing each CFU to the CFU obtained 48 hr after starting CLS in SD (when cells just entered the stationary phase).

Heat shock and oxidative stress resistance: Cells were first grown in SD containing 2% glucose (normal) or 0.5% glucose

TABLE 1

Yeast strains used in this study

Strain	Genotype	Reference
W303AR	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 RDN::ADE2 can1-100*	Lin *et al.* (2000)
LSC1041	W303AR *bmh1*Δ*::Kan*^{r}	This study
LSC14	W303AR *cdc25-10*	Lin *et al.* (2000)
BY4742	*MAT***a** *his*3Δ1 *leu*2Δ0 *lys*Δ0 *ura*3Δ0	Easlon *et al.* (2007)
LSC722	BY4742 *bmh1*Δ*::Kan*^{r}	This study
LSC716	BY4742 *bmh2*Δ*::Kan*^{r}	This study
LSC622	BY4742 p*ADH1-BMH1-LEU2*	This study
LSC623	BY4742 p*ADH1-BMH2-LEU2*	This study
LSC89	BY4742 p*ADH1-XXX-LEU2* (pPP81, vector control)	Easlon *et al.* (2007)
LSC586	BY4742 *tor1*Δ*::Kan*^{r}	This study
LSC157	BY4742 *cdc25-10*	Easlon *et al.* (2007)
LSC837	BY4742 *cdc25-10 bmh1*Δ*::Kan*^{r}	This study
LSC801	BY4742 *tor1*Δ *bmh1*Δ*::Kan*^{r}	This study
LSC1021	BY4742 *bmh1*Δ*::Kan*^{r} p*ADH1*-Bmh1-S189A-*LEU2*	This study
LSC1020	BY4742 *bmh1*Δ*::Kan*^{r} p*ADH1*-Bmh1-S238A-*LEU2*	This study
LSC327	BY4742 *msn2*Δ*msn4*Δ*::Kan*^{r}	This study
LSC971	BY4742 *msn2*Δ*msn4*Δ*bmh1*Δ*::Kan*^{r}	This study
LSC516	BY4742 *rtg3*Δ*::Kan*^{r}	This study
LSC997	BY4742 *bmh1*Δ*rtg3*Δ*::Kan*^{r}	This study
LSC1246	BY4742 *gnc4*Δ*::Kan*^{r}	This study
LSC1245	BY4742 *bmh1*Δ*gcn4*Δ*::Kan*^{r}	This study
LSC1244	BY4742 *tor1*Δ*gcn4*Δ*::Kan*^{r}	This study
LSC1218	BY4742 *rim15*Δ*::Kan*^{r}	This study
LSC1219	BY4742 *bmh1*Δ*rim15*Δ*::Kan*^{r}	This study

(CR) for 2 days with a starting OD_{600} of 0.1 prior to analysis. For heat-shock studies, cells were spotted onto YPD plates (2% glucose) in fivefold serial dilutions (started at OD_{600} of 1) and then were incubated at 55° or 25° for 45 min or 60 min. After heat shock, plates were transferred to 30° and continued to incubate for 2–3 days. For the hydrogen peroxide toxicity test, cells were spotted onto YPD plates (with 0 or 3 mm H_2O_2) in fivefold serial dilutions and were allowed to grow for 2 days. For the paraquat toxicity test, cell growth was monitored in SD containing indicated concentrations of paraquat with a starting OD_{600} of 0.05 after incubation at 30° for 16 hr.

ROS detection: Cells grown in minimal SD to stationary phase were washed twice with PBS buffer (pH 7.4) and then resuspended in PBS with 10 μm H_2DCFDA following incubation at 4° in the dark for 45 min (Zuin *et al.* 2008). Next, cells were washed once with PBS and then added to 96-well fluorescence assay plates (~5×10^6 cells/well). Fluorescence signals were detected using a plate reader with excitation at 485 nm, and emission was monitored at 535 nm.

Superoxide dismutase gel assay: Cells grown in minimal SD were harvested at indicated time points. Total protein extract was obtained by agitations using glass beads and the FastPrep beads beater. About 15 μg protein was loaded in each lane of a 12% native polyacrylamide gel. After electrophoresis, gel was stained in a buffer with 0.025% Nitro Blue Tetrazolium, 0.01% riboflavin, 0.01% *N,N,N′,N′*-tetramethylethylenediamine for 45 min in the dark at room temperature (Raychaudhuri *et al.* 2003). Stained gel was then exposed to intensive light. The superoxide dismutase (SOD)-active bands appeared white in a dark-blue background on the gel. Results shown in Figure 2G are inverted images of the original gels.

Epitope tagging: Bmh1 was tagged by the HA epitope tag in the genome using the pFA6a-3HA-KanMX6 plasmid as described (Longtine *et al.* 1998). Yeast strains expressing Myc-tagged Bmh1 proteins were made by introducing the p*BMH1*-13MycXX plasmid (Easlon *et al.* 2008).

Site-directed mutagenesis: The Bmh1-S189A and Bmh1-S238A mutants were generated using the QuickChange kit (Stratagene) by PCR. The p*ADH1*-Bmh1 plasmid was used as a template in a 50-μl reaction with 4 μl 2.5 mm dNTP, 5 μl 10× *Pfu* buffer, 1 μl p*ADH1*-Bmh1 plasmid DNA, 2 μl *Pfu* Turbo polymerase, and 1 μl of each pair of oligos: S189A-f (5′-GAAATTCAAAACGCTCCAGAC-3′) + S189A-r (5′-GTCT GGAGCGTTTTGAATTTC-3′) or S238A-f (5′-CAGACATG GCCGAGTCCGGTC-3′ + S238A-r (5′-GACCGGACTCGGC CATGTCTG-3′). The PCR products were digested with *Dpn*I and then introduced into *XL1-Blue* competent cells by electroporation. Plasmids with desired point mutations were verified by sequencing.

Antibody production: Antibodies to total Bmh1 proteins (anti-Bmh1-total) and phosphorylated Bmh1 proteins (anti-Bmh1-pS238) were generated in rabbits using the keyhole-limpet-hemocyanin-conjugated phosphopeptides SVFYYEIQN(p)SPDKAC (flanking Ser189) or TLWTSDM(p)SESGQAEDQ (flanking Ser238) from Antagene. Antibodies were purified from the resulting antiserum by column chromatography on phosphopeptide-conjugated affinity resin.

Protein extraction and Western blot analysis: Total protein extract was obtained as described (Easlon *et al.* 2008). About 15 μg of total proteins were loaded in each lane. After electrophoresis, proteins were transferred to nitrocellulose membranes (Whatman), which were then washed and blotted with anti-Bmh1-total, anti-Bmh1-pS238, anti-actin (Abcam), or anti-Myc antiserums (Covance). Proteins were visualized using anti-mouse or anti-rabbit antiserum conjugate to the horseradish peroxidase (Amersham) and the ECL reagents (Pierce). Chemiluminescent images were analyzed using the Alpha Innotech imaging system.

Calf intestinal alkaline phosphatase treatment: A total of 250 ml cells ($\sim 5 \times 10^9$) grown in SD to mid-log phase were harvested and resuspened in 500 μl breaking buffer: 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, and protease inhibitors (Roche). Cell suspensions were lysed by agitations using glass beads and the FastPrep beads beater. Monocolonal anti-Myc antibody (7 μl; Covance) was used to immunoprecipitate Myc epitope-tagged Bmh1 in 400 μl cell extract. For calf intestinal alkaline phosphatase (CIP) treatments (Ai *et al.* 2002), the immunocomplex was spun down, washed, and then resuspended in 200 μl CIP buffer (100 mM NaCl, 50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9). A total of 100 μl of this suspension was incubated with CIP (20 units, Roche) for 20 min at 37°. About 30 μl of the resultant protein suspension was used in Western blot analysis.

Cloning and purification of recombinant Tpk1: The coding region of *TPK1* was cloned into the 6xHis-tag-containing pET28b expression vector using engineered *Bam*HI and *Xho*I sites. This plasmid was then electroporated into *BL21*(*DE3*) cells using kanamycin selection. These cells were grown to OD_{600} ~1 in a total volume of 100 ml and induced for 2 hr with 0.4 mM IPTG. Following induction, cells were collected, and recombinant Tpk1-6xHis was purified using the His Bind purification kit (Novagen). Purified Tpk1 was concentrated by the 5000 NMWL filter unit (Millipore).

Kinase assay: Cells (15 ml) were harvested at OD_{600} ~10 and resuspended in 500 μl breaking buffer: 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, and 1 mM PMSF. Cell suspension was lysed by agitations using glass beads and the FastPrep beads beater. Monocolonal anti-HA antibody (5 μl; Covance) was used to immunoprecipitate HA epitope-tagged Bmh1 in 400 μl cell extract. After CIP treatment, beads were washed with buffer (50 mM Tris–HCL, pH 7.5, 100 mM NaCl, and 0.2% Triton X-100) to remove the CIP in the reaction and then resuspended in kinase buffer (20 mM Tris–HCl, pH 7.5, 20 mM β-glycerophosphate, 100 μM orthovanadate, 10 mM $MgCl_2$, 1 mM DTT, 50 μM ATP, and 1 mM NaF). Thirty microliters of recombinant Tpk1 (0.1 μg/μl) was added to initiate the kinase reaction at 30° for 30 min.

Heat-shock-element reporter assay: To monitor Hsf1-mediated transcription activity, we transformed yeast cells with the HSE4Ptt-*CYC1-LacZ* reporter plasmid (Bandhakavi *et al.* 2008). Cells carrying the plasmid were first grown in SD without URA at 30° with a starting OD_{600} of 0.1. After 2 days, cells were spotted onto YPD plates containing 4 mg X-gal. After incubation at 37° for 4 days, plates were transferred to room temperature for 2 days.

RESULTS

Deleting Bmh1 extends chronological life span: To further understand the mechanisms of longevity regulation, we screened for factors that could extend the survival of cells at a nondividing state (CLS). Since yeast cells could survive up to several months in stationary phase, we utilized a *cdc25-10* temperature-sensitive mutant to accelerate the screening process: an accelerated cell death system. *CDC25* encodes a GTP–GDP exchange factor that activates Ras in the cAMP/PKA pathway in response to glucose. When arrested at 38°, the *cdc25-10* mutant exhibited phenotypes similar to that of stationary phase cells (Gray *et al.* 2004) and survived only ~3 days. Therefore, factors that extended the survival of the *cdc25-10* mutant at 38° might also extend survival of wild type cells in stationary phase (CLS). We first introduced a 2μ-based yeast overexpression library into the *cdc25-10* mutant to obtain genes that could extend survival (see materials and methods). As shown in Figure 1A, a genomic DNA fragment containing the *BMH2* gene (clone 1) was identified, and its overexpression extended the survival of the *cdc25-10* mutant cells at 38°.

We then examined if both yeast 14-3-3 proteins indeed played important roles in longevity regulation. Wild-type (BY4742) cells overexpressing Bmh1 and Bmh2 (Bmh1-oe and Bmh2-oe) were subject to standard CLS analysis in minimal synthetic SD. As shown in Figure 1B, both Bmh1 and Bmh2 overexpressions significantly extended CLS. We then examined whether deleting *BMH1* or *BMH2* would also affect CLS. Surprisingly, the *bmh1*Δ mutant showed extended survival compared to wild-type cells (Figure 1C). This phenotype was not due to adaptive mutagenesis since the frequency of canavanine-resistant mutations did not increase significantly in the *bmh* mutants (Figure 1D). Deleting *BMH1* also extended the maximum CLS in a different strain, W303 (Figure 1E), indicating that the observed *bmh1*Δ life-span phenotype was not specific to the BY4742 strain. To further understand the role of Bmh in CLS, we examined the effects of Bmh on CLS under more stringent and stressful growth conditions by shifting cells to water after they had entered stationary phase. Shifting cells to water has been suggested to mimic the adverse growth conditions that yeast cells frequently encounter in a natural environment (Fabrizio and Longo 2003; Gray *et al.* 2004). Interestingly, under this condition, *bmh1*Δ still extended CLS (Figure 1F) whereas Bmh1-oe and Bmh2-oe failed to extend CLS (Figure 1G). Since *bmh1*Δ extends CLS regardless of growth conditions (in both SD and H_2O), it is more likely to function in conserved longevity pathways. Moreover, although *bmh1*Δ did not dramatically extend CLS, distribution of the mean and maximum life span from multiple experiments demonstrated that *bmh1*Δ indeed extended CLS consistently and significantly (Figure 1H).

***bmh1*Δ extends chronological life span by increasing stress response:** Many studies have associated increased CLS to activation of the stress response (Fabrizio and Longo 2003; Powers *et al.* 2006). As shown in Figure 2A (top), the *bmh1*Δ mutant exhibited increased resistance to heat stress at a level similar to that observed in CR cells and in the long-lived *tor1*Δ and *cdc25-10* mutants (Figure 2B). Interestingly, Bmh1-oe did not show increased resistance (Figure 2A, bottom), which was consistent with the finding that Bmh1-oe failed to extend CLS under more stressful growth conditions (Figure 1G). These results suggested that *bmh1*Δ might extend CLS by activating the stress response.

The heat-shock factor HSF-1 has been shown to be required for starvation-induced life-span extension as well as for responding to heat shock and oxidative stress

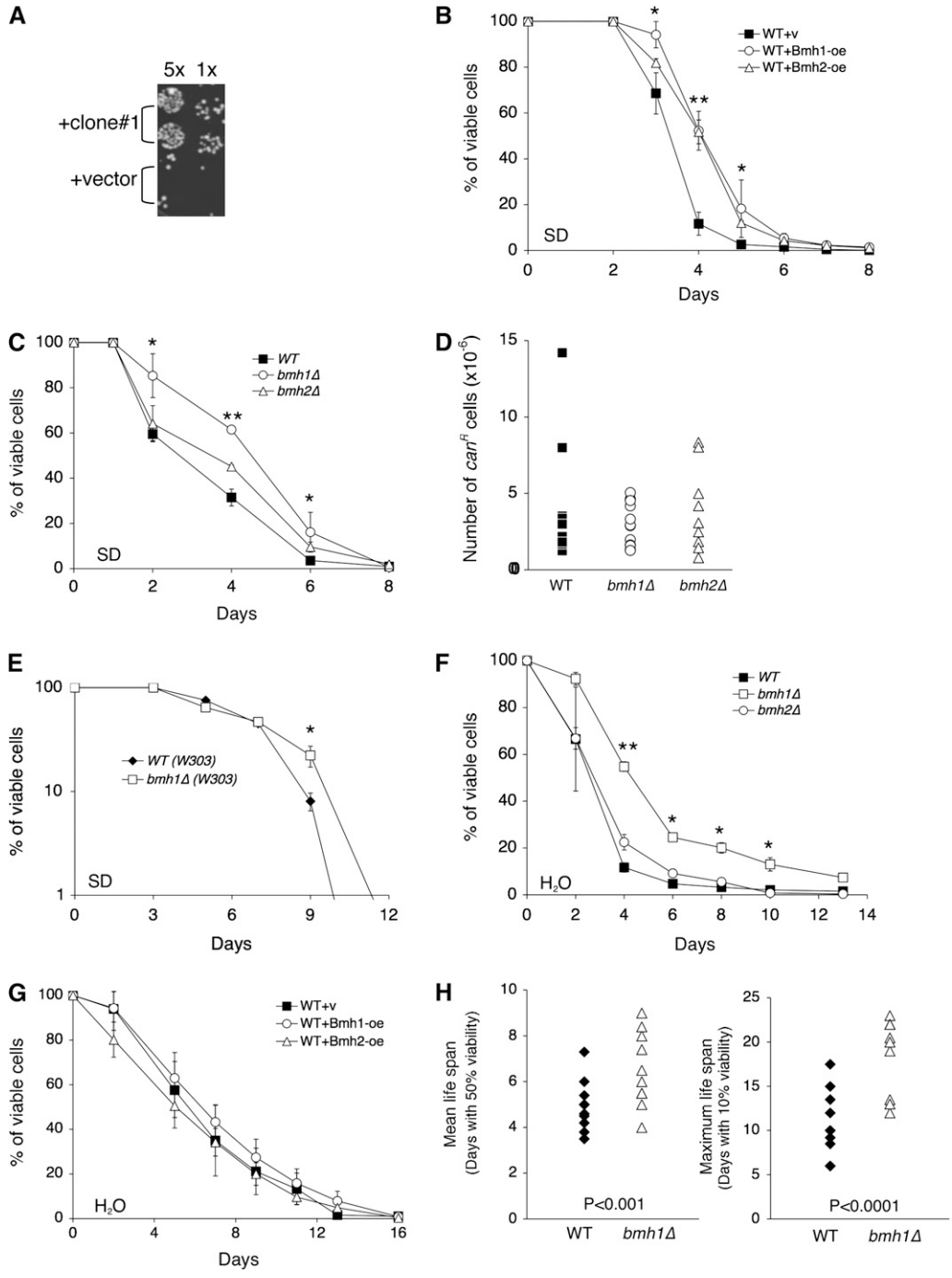

FIGURE 1.—Characterization of yeast 14-3-3 proteins as novel longevity factors that regulate CLS. (A) Overexpression of clone 1 (containing *BMH2*) extends survival in an accelerated cell death assay. Temperature-sensitive *cdc25-10* mutants carrying a control 2μ vector or clone 1 are first incubated at 38° for 4 days and then are incubated at 30° for 2 days. About 5×10^4 cells are spotted in the first column ("5×"). (B) Bmh1 and Bmh2 overexpressions extend the CLS of cells grown and kept in SD. (C) *bmh1*Δ extends the CLS of cells grown and kept in SD. (D) The *bmh1*Δ and *bmh2*Δ mutants do not show increased mutation rates in stationary phase. The mutation rates of wild-type and *bmh1*Δ and *bmh2*Δ mutant cells are determined by measuring the mutation frequency of the *CAN1* gene. Stationary-phase cells (day 4 in SD) are collected and then plated onto both YPD and SD −ARG (without arginine, and containing 60 mg/liter L-canavanine sulfate). Mutation frequency is calculated by normalizing the number of colonies that appeared on SD −ARG to that of the corresponding YPD. For each strain, 12 independent colonies were examined. (E) *bmh1*Δ extends maximum the CLS in a different strain, W303. (F) *bmh1*Δ extends the CLS of cells shifted to water. Cells are first grown in SD to stationary phase and then are shifted to sterile water. (G) Bmh1 and Bmh2 overexpressions do not extend the CLS of cells shifted to water. (H) *bmh1*Δ extends both mean and maximum CLS. Results show statistics of 11 independent experiments, each of which was conducted in triplicate or quadruplicate. Each symbol represents the average viability of four samples, each containing 10^7 cells. For CLS analysis, one representative set of three independent experiments, each conducted in quadruplicate, is shown. "Days" denotes the number of days that cells were in SD or H_2O. Error bars denote standard deviations. *P*-values were calculated using the Student's *t*-test (*$P < 0.05$; **$P < 0.01$). WT, wild-type control; v, vector control; oe, overexpression.

in worms (HSU *et al.* 2003). Since the *bmh1*Δ mutant showed increased heat resistance, we examined whether Hsf1-mediated transcription activity was increased in the *bmh1*Δ mutant using a reporter assay (BANDHAKAVI *et al.* 2008). Hsf1 recognizes **h**eat **s**hock **e**lements (HSEs) in promoters of target genes such as molecular chaperons and heat-shock proteins. As shown in Figure 2C, the *bmh1*Δ mutant displayed higher HSE-driven transcription activity at the *LacZ* (β-galactosidase) reporter gene. Yeast Bmh proteins have also been suggested to sequester the stress-sensing transcription factors Rim15 and Msn2/4 (VAN HEUSDEN and STEENSMA 2006) and to

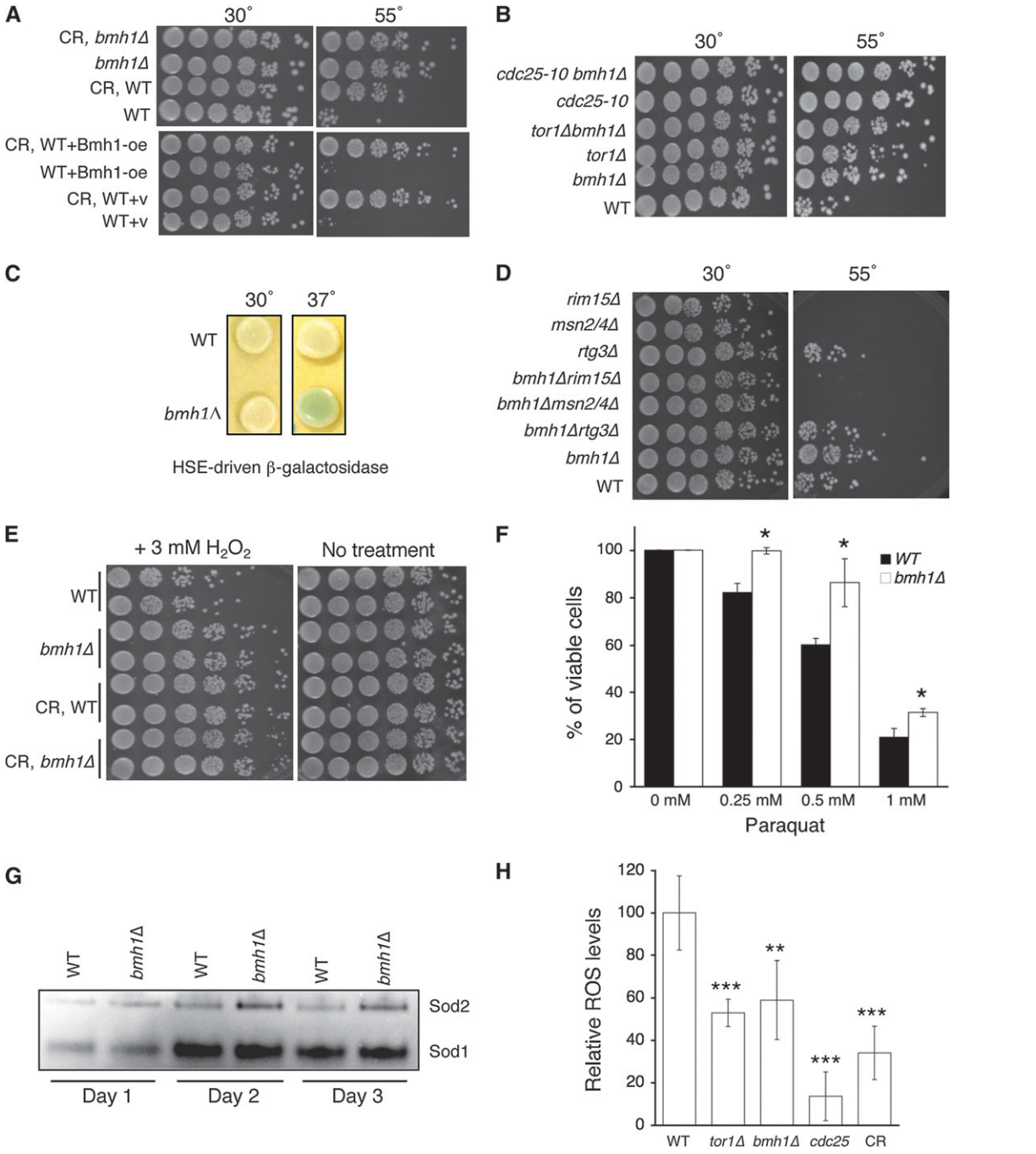

FIGURE 2.—The *bmh1*Δ mutant shows increased stress response and decreased ROS levels. (A) *bmh1*Δ increases heat resistance, which is not enhanced by CR (top). Bmh1-oe does not increase heat resistance (bottom). Results show fivefold serial dilutions of cells grown on YPD with or without heat shock. (B) *bmh1*Δ does not further increase heat resistance in the *tor1*Δ or *cdc25-10* mutants. (C) The *bmh1*Δ mutant shows increased HSE-driven transcription activity. Results show wild-type and *bmh1*Δ mutant cells (carrying an HSE-driven β-galactosidase reporter plasmid) grown on YPD containing 4 mg X-gal. The light green color shown in the *bmh1*Δ mutant grown at 37° indicates higher HSE-driven transcription activity. (D) *bmh1*Δ-induced heat resistance requires Msn2/4 and Rim15. (E) *bmh1*Δ confers resistance to H_2O_2-induced toxicity. (F) *bmh1*Δ confers resistance to paraquat-induced toxicity. (G) The *bmh1*Δ mutant has higher Sod2 activity in stationary phase using a SOD gel activity assay. (H) *bmh1*Δ decreases intracellular ROS levels. *P*-values are calculated using the Student's *t*-test (**$P < 0.01$; ***$P < 0.005$). WT, wild-type control; CR, cells pregrown in SD with 0.5% glucose prior to analysis.

inhibit the activity of the retrograde response that senses mitochondrial genome integrity (VAN HEUSDEN and STEENSMA 2006). We therefore examined whether *bmh1*Δ required these stress-response transcription factors to confer heat resistance. As shown in Figure 2D, deleting Msn2/4 and Rim15 abolished *bmh1*Δ-induced resistance to heat stress. Furthermore, deleting Msn2/4 also abolished *bmh1*Δ-induced CLS extension (supporting infor-

mation, Figure S1), suggesting that these stress-response factors played important roles in *bmh1*Δ-induced heat resistance and CLS extension.

Intracellular homeostasis of ROS has also been shown to affect the expectancy of life span (Reverter-Branchat *et al.* 2004). Increased intracellular ROS levels cause damages to macromolecules such as DNA, proteins, and lipids. To further understand the roles of Bmh1 in ROS homeostasis and life-span regulation, we first examined whether the *bmh1*Δ mutant was more resistant to oxidative stress by challenging cells with hydrogen peroxide (H_2O_2) and paraquat (generating superoxide anions). As shown in Figure 2E, the *bmh1*Δ mutant showed an ~10-fold increased resistance to H_2O_2 compared to wild type. In addition, *bmh1*Δ also conferred resistance to paraquat-induced toxicity (Figure 2F). These results demonstrate that deleting Bmh1 protects cells from oxidative stress. We next compared the activities of SODs in cell extracts of the *bmh1*Δ mutant and wild-type cells using a SOD gel assay (Raychaudhuri *et al.* 2003). Both *bmh1*Δ and wild-type cells showed higher Sod1 activities (cytosolic Cu/Zn-SOD) after entering the stationary phase (days 2 and 3) (Figure 2G). Interestingly, wild-type cells failed to show increased Sod2 activities (mitochondrial Mn-SOD) whereas the *bmh1*Δ mutant showed slightly higher Sod2 activities, suggesting that Sod2 might play an important role in *bmh1*Δ-induced stress response. Finally, we determined the level of ROS in the *bmh1*Δ mutant using a ROS-specific fluorescence dye (Zuin *et al.* 2008). Similar to CR-treated cells and to the *tor1*Δ and *cdc25-10* mutants, the *bmh1*Δ mutant showed a lower ROS level compared to wild type (Figure 2H). These data suggest that *bmh1*Δ activates the stress response, leading to increased stress resistance, decreased ROS level, and CLS extension.

***BMH1* genetically interacts with CR, PKA, and TOR longevity pathways to regulate life span:** To further understand the roles of Bmh1 in longevity regulation, we examined the effects of *bmh1*Δ on CR, *tor1*Δ- and *cdc25-10*-induced CLS extension to determine whether *bmh1*Δ functioned in these pathways. Figure 3A showed that *bmh1*Δ further extended CR-induced CLS, suggesting that *bmh1*Δ and CR functioned in parallel or in partially overlapping pathways to extend CLS. Figure 3, B and C, showed that *bmh1*Δ did not significantly further extend CLS of the *tor1*Δ and *cdc25-10* mutants, suggesting that *bmh1*Δ might function in the TOR and PKA pathways to extend CLS.

Since *bmh1*Δ further extended CR-induced CLS (Figure 3A), it was possible that *bmh1*Δ activated additional stress-response factors to extend the CLS of cells grown in CR. Deleting this *bmh1*Δ-specific stress-response factor should affect only *bmh1*Δ-induced but not CR-induced heat resistance. Since deleting Msn2/4, Rim15, and Rtg3 also abolished CR-induced heat resistance (Figure 3D), these factors were not only specific to the *bmh1*Δ pathway. Because our genetic studies suggested that *bmh1*Δ and *tor1*Δ might function in the same pathway to extend CLS (Figure 3C), we examined whether Gcn4, a downstream target of TOR, might play a role in *bmh1*Δ-induced heat resistance and CLS extension. Decreased TOR-signaling activities have been shown to enhance Gcn4 (a nutrient- and stress-sensing transcription factor) expression, which activates genes involved in nitrogen utilization (Valenzuela *et al.* 2001). Gcn4 was also required for the response and resistance to hydrogen peroxide (Mascarenhas *et al.* 2008). Figure 3E showed that *gcn4*Δ specifically abolished *bmh1*Δ- and *tor1*Δ-induced but not CR-induced heat resistance. Furthermore, Gcn4 was also required for *bmh1*Δ-induced CLS extension (Figure 3F). These results suggest that Gcn4 is an important factor in the *bmh1*Δ pathway, which works in concert with other factors such as Msn2/4 and Rim15 to regulate the stress response and CLS (Figure 3G).

The level of phosphorylated Bmh1-Ser238 is increased during chronological aging: We next examined the roles of Bmh1 phosphorylation in chronological aging. Studies of mammalian 14-3-3 proteins have shown that phosphorylation of the Ser185 or Ser/Thr233 residues affect the interactions between 14-3-3 proteins and their binding partners (Aitken 2006). The equivalent phosphorylation sites on yeast Bmh1 are Ser189 and Ser238. We attempted to raise peptide antibodies that specifically targeted phosphorylated Ser189 and Ser238 on Bmh1 and total Bmh1 proteins. However, only anti-Bmh-total (specific to both Bmh1 and Bmh2 proteins; Figure 4A), anti-Bmh1-total (specific to total Bmh1 proteins; Figure 4B), and anti-Bmh1-pS238 (specific to phosphorylated Ser238 on Bmh1) antibodies showed specificity. The anti-Bmh1-pS238 antibody specifically recognized phosphorylated Ser238 on Bmh1 since it was unable to recognize Bmh1 after phosphatase (CIP) treatment (Figure 4D) or when Ser238 was mutated to alanine (Figure 4E).

We next monitored the phosphorylation status of Bmh1-Ser238 during chronological aging using the anti-Bmh1-pS238 antibody. As shown in Figure 5, A–C, the level of phosphorylated Bmh1 proteins increased approximately three- to fourfold in wild-type cells upon entering stationary phase (48 hr). It was possible that phosphorylated Bmh1-Ser238 caused inhibitory effects on CLS because deleting Bmh1 extended CLS. In addition, both *tor1*Δ (Figure 5A) and CR (Figure 5B) largely prevented the increase in Bmh1 phosphorylation whereas the *cdc25-10* mutation did not significantly affect Bmh1 phosphorylation (Figure 5C). These results suggested that phosphorylated Bmh1-Ser238 might impede the beneficial effects induced by CR and *tor1*Δ and that CR and *tor1*Δ extend CLS in part by preventing Bmh1-Ser238 phosphorylation.

Protein kinase A can directly phosphorylate Bmh1 at Ser238: To gain further insight into the regulation of

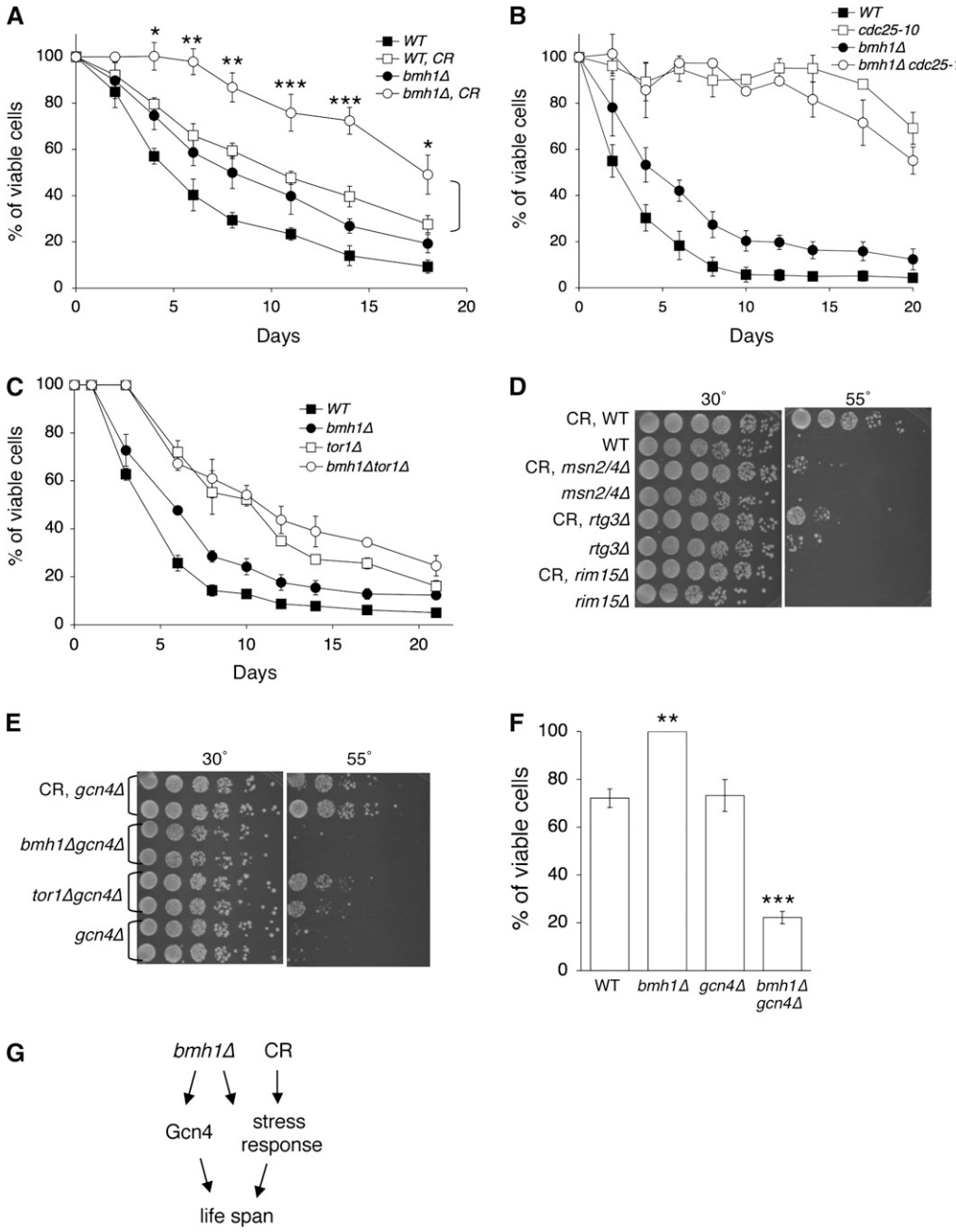

FIGURE 3.—Analyses of genetic interactions between *bmh1*Δ, CR, and long-lived nutrient-sensing mutants. (A) *bmh1*Δ further extends CR-induced CLS. (B) *bmh1*Δ does not significantly affect *cdc25-10*-induced CLS. (C) *bmh1*Δ does not significantly affect *tor1*Δ-induced CLS. For CLS, cells are first grown in SD containing 2% (normal) or 0.5% glucose (CR) to stationary phase (48 hr) and then are shifted to sterile water. "Days" denotes the number of days that cells are in H_2O. One representative set of three independent experiments, each conducted in quadruplicate, is shown. (D) CR-induced heat resistance requires the stress-response factors Msn2/4, Rtg3, and Rim15. (E) Deleting *GCN4* totally abolishes *bmh1*Δ-induced heat resistance. (F) *bmh1*Δ-induced increase in survival requires Gcn4. Results show cell viability determined in day 6 cultures (4 days after shifting to H_2O). (G) A proposed model for *bmh1*Δ- and CR-induced CLS extension. *bmh1*Δ and CR function in overlapping pathways to regulate CLS since they share common downstream stress-response factors such as Msn2/4 and Rim15. Gcn4 appears to be more specific to the *bmh1*Δ pathway. For simplicity, other factors/pathways are not shown. Error bars denote standard deviations. *P*-values were calculated using the Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.005$). WT, wild-type control; CR, cells pregrown in SD with 0.5% glucose prior to analysis.

Bmh1, we screened 132 putative protein kinase mutants (defined in the Saccharomyces Genome Database) for kinases involved in the phosphorylation of Bmh1-Ser238 (Table S1) by using the yeast nonessential gene deletion collections. We found that the levels of phosphorylated Bmh1 were significantly decreased (70% decrease) in the *bub1*Δ mutant (Figure 5D). *BUB1* encodes the protein kinase that plays a crucial role in the anaphase checkpoint control. Previous studies have linked 14-3-3 proteins to DNA checkpoint controls (USUI and PETRINI 2007). Our findings supported a role for 14-3-3-mediated cell cycle checkpoint controls in life-span regulation. Interestingly, *tpk1*Δ also reduced the levels of phosphorylated Bmh1-Ser238 (30% decrease). Yeast protein kinase A is encoded by three different genes: *TPK1*, *TPK2*, and *TPK3*. Although these Tpk proteins are functionally redundant for viability, they have also been reported to play different roles in many processes. Since the decrease in Bmh1-Ser238 phosphorylation was only 30% in the *tpk1*Δ mutant, it was possible that all Tpk proteins contributed to Bmh1 phosphorylation. We next directly examined whether Tpk1 could phosphorylate Bmh1-Ser238. As shown in Figure 5E, recombinant Tpk1 was able to phosphorylate

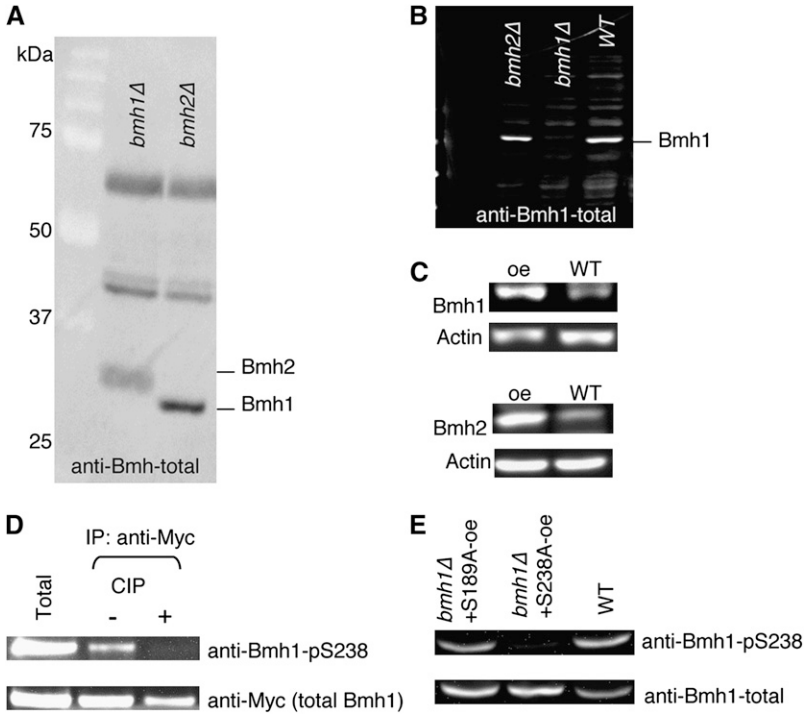

FIGURE 4.—Western blot analyses of anti-Bmh1 antibodies and Bmh1 and Bmh2 protein expression. (A) The anti-Bmh-total antibody recognizes both Bmh1 and Bmh2. Total protein extracts of the *bmh1*Δ and *bmh2*Δ mutants are analyzed. About 10 μl cell extract was loaded in each lane (A, B, C, and E). (B) The anti-Bmh1-total antibody specifically recognizes Bmh1 proteins. (C) Levels of protein expression in cells overexpressing Bmh1 or Bmh2 are analyzed using the anti-Bmh-total antibody. (D) The anti-Bmh1-pS238 specifically recognizes phosphorylated Ser238 on Bmh1. Results show CIP treatment of immunoprecipitated Myc-tagged Bmh1. CIP, calf intestinal alkaline phosphatase; IP, immunoprecipitation. (E) The anti-Bmh1-pS238 antibody is specific to phosphorylated Ser238 of Bmh1. Total protein extracts of wild-type and *bmh1*Δ cells expressing mutated Bmh1-S238A or Bmh1-S189A are analyzed. WT, wild-type control; oe, overexpression.

immunoprecipitated Bmh1 on the Ser238 residue *in vitro*. It remains highly possible that Tpk1 also phosphorylates other Ser/Thr residues on Bmh1. Phosphorylation of other Ser/Thr residues on Bmh1 by Tpk1 and/or other kinases may also play important roles in life-span regulation.

DISCUSSION

The yeast 14-3-3 proteins, Bmh1 and Bmh2, have been implicated in many cellular processes. Our studies demonstrate that yeast 14-3-3 proteins also play important roles in longevity regulation and stress response. In this study, 14-3-3 proteins were identified as longevity factors because their overexpressions extended cell survival in an accelerated cell death assay (Figure 1A) and extended CLS (Figure 1B). Interestingly, we discovered that Bmh1 deletion also extended CLS (Figure 1C). Since Bmh1-oe extended CLS only under certain growth conditions whereas *bmh1*Δ extended CLS regardless of growth conditions, *bmh1*Δ was more likely to function in a conserved pathway to extend CLS. Therefore, we focused on determining the mechanisms underlying *bmh1*Δ-induced CLS extension. We showed that the *bmh1*Δ mutant was more resistant to challenges of heat shock (Figure 2A) and oxidative stress-inducing reagents (Figure 2, E and F). Deleting Bmh1 also increased HSE-mediated transcription (Figure 2C). In addition, cells lacking Bmh1 had a lower intracellular ROS level (Figure 2H). Together, our results demonstrated that *bmh1*Δ-induced CLS extension was likely due to activation of stress-response mechanisms, which protects cells from ROS-induced damages during chronological aging.

In line with our findings, RNA-interference-mediated knockdown of specific 14-3-3 proteins in worms was also shown to increase the expression of antioxidant enzymes such as Sod3 (extracellular superoxide dismutase) (Li *et al.* 2007). It remained unclear why Bmh1-oe extended CLS only in cells grown and kept in SD but failed to extend CLS in cells shifted to H_2O (Figure 1, B and G). It is possible that Bmh1-oe extends CLS only in cells that are more metabolically active. It has been shown that cells remain at a high metabolic state (post-diauxic phase) if they are maintained in SD media (Fabrizio and Longo 2003). Conversely, shifting cells to water induces a low metabolic state (stationary phase), which mimics the adverse natural environment that yeast cells frequently encounter (Fabrizio and Longo 2003; Gray *et al.* 2004). Overexpressions of 14-3-3 proteins have also been reported to extend life span in worms (Berdichevsky *et al.* 2006; Wang *et al.* 2006); however, the detailed mechanisms remain unclear. Since 14-3-3 proteins have many interacting partners and affect many cellular pathways, they are likely to regulate life span via multiple mechanisms. Understanding how Bmh1-oe extends life span in yeast may provide further insight into the roles that 14-3-3 proteins play in longevity regulation and cellular metabolism.

Phosphorylated Bmh1-Ser238 might cause inhibitory effects on CLS. The amount of phosphorylated Bmh1-Ser238 increased in stationary phase, which was significantly delayed by CR (Figure 5A) and *tor1*Δ (Figure 5B). Although *cdc25-10* did not decrease the level of phos-

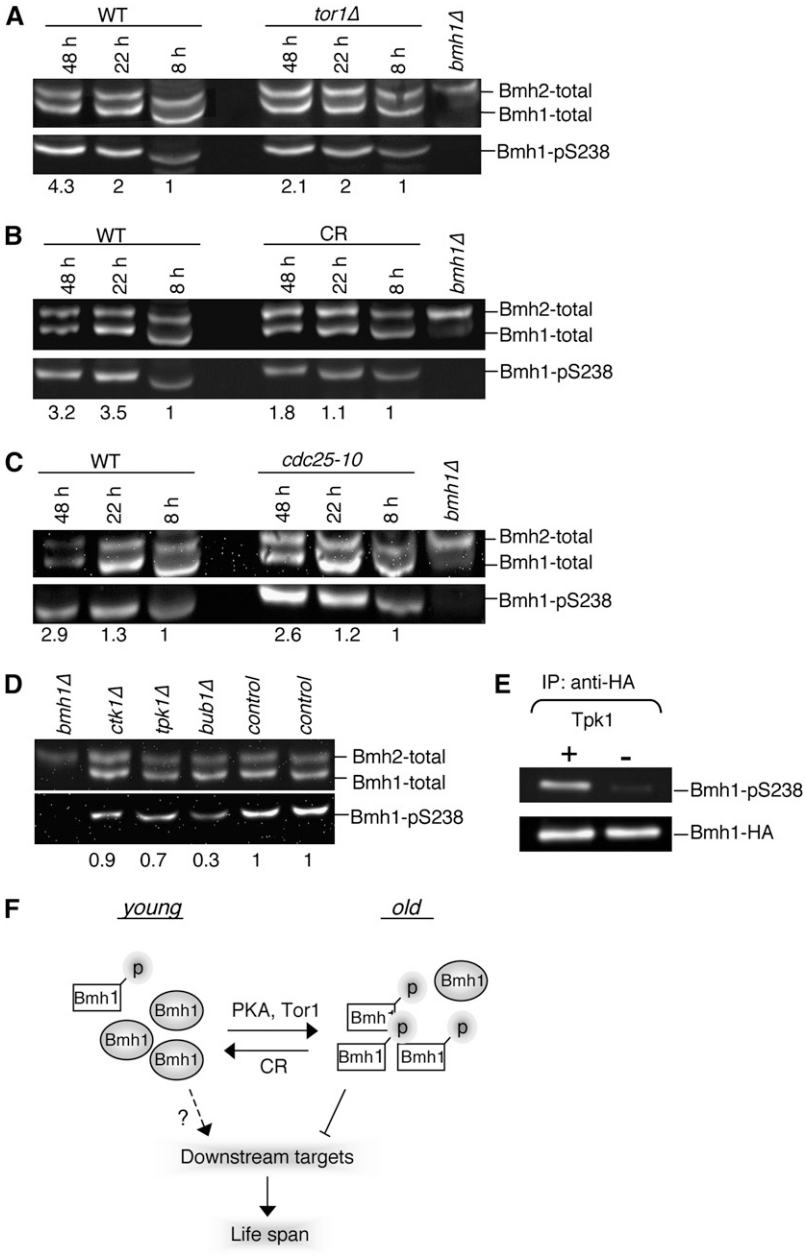

FIGURE 5.—Analyses of Bmh1-Ser238 phosphorylation levels using site-specific anti-Bmh1-pS238 antibody. (A) Phosphorylation of Ser238 on Bmh1 is increased in stationary phase, which is delayed by *tor1*Δ. Numbers below the bottom panels in A–E indicate the relative amount of Bmh1-pS238 normalized to the levels of Bmh1-pS238 at 8 hr. (B) CR reduces Bmh1-Ser238 phosphorylation in stationary phase. (C) *cdc-25-10* does not decrease Bmh1-Ser238 phosphorylation in stationary phase. (D) Screening kinase mutants that show reduced Bmh1-Ser238 phosphorylation. (A–D) Results show duplicated blots, each loaded with the same amount of proteins from the same sample in each lane. (A–C) The amount of Bmh1-pS238 is first normalized to an internal loading control and then normalized to the levels of Bmh1-pS238 at 8 hr. WT, wild-type control; CR, 0.5% glucose. (E) Recombinant Tpk1 can phosphorylate Bmh1 at Ser238 *in vitro*. (F) A proposed model for the role of Bmh1 phosphorylation in life-span regulation. Bmh1 phosphorylation (at Ser238) is increased in old cells, which may inhibit the downstream longevity factors, including stress-response proteins. CR, low PKA, and low TOR activities can decrease the level of phosphorylated Bmh1 (at Ser238), which may help release its inhibitory effects on downstream longevity factors. It is possible that phosphorylation of Bmh1 at other residues also plays an important role in life-span regulation and that nonphosphorylated Bmh1 may also induce certain beneficial effects on life span.

phorylated Bmh1-Ser238 (Figure 5C), our kinase screen (Figure 5D) and *in vitro* PKA kinase assay (Figure 5E) showed that PKA could directly phosphorylate Bmh1-Ser238. These results suggest that decreasing the level of phosphorylated Bmh1-Ser238 might promote longevity and that CR and low TOR and PKA activities might extend CLS in part by this mechanism (Figure 5F). In addition, Bmh proteins have been shown to interact with the retrograde response proteins (VAN HEUSDEN and STEENSMA 2006), the stress-sensing transcription factors Msn2/4 and Rim15 (VAN HEUSDEN and STEENSMA 2006), and the components of the autophagy pathway (KAKIUCHI *et al.* 2007). Our data showed that *bmh1*Δ required these components for heat resistance (Figure 2D) and/or CLS extension (Figure S1), suggesting that the ability to respond to various types of metabolic and oxidative stresses was essential for *bmh1*Δ-mediated CLS extension. It is possible that during chronological aging, an increased amount of phosphorylated Bmh1-Ser238 might enhance the ability of Bmh1 to sequester and/or interfere with the interactions of these stress-response factors with their interacting partners. According to this model, deleting Bmh1 was sufficient to extend CLS. Therefore, *bmh1*Δ-induced stress resistance and CLS extension was likely due to elimination of the inhibitory effects of Bmh1 on downstream longevity factors, including stress-response proteins.

Our data also suggest that *bmh1*Δ might function in the same pathway as the *tor1*Δ and low PKA activity mutations to extend CLS. We showed that *bmh1*Δ did not further extend the long life span induced by *tor1*Δ and *cdc25-10* (Figure 3, B and C). We also showed that

both TOR and PKA could affect Bmh1-238 phosphorylation (Figure 5, A and E). Although *bmh1*Δ further extended CR-induced CLS (Figure 3A), *bmh1*Δ and CR appeared to share common downstream stress-response factors such as Msn2/4 and Rim15 (Figure 2D and Figure 3D). In addition, CR also decreased the level of Bmh1-Ser238 phosphorylation during chronological aging (Figure 5B). Together, these data suggest that Bmh1 is a novel downstream target of the TOR, PKA, and CR pathways and functions in accordance with other longevity factors to regulate CLS. Consistent with our studies, mammalian 14-3-3 proteins have also been reported to interact with the PKA and TOR pathways. For example, mammalian PKA has been shown to phosphorylate the binding motif of 14-3-3-interacting proteins, therefore altering the conformation of these proteins and their functions (Aitken 2006). A novel mTOR-binding partner, PRAS40, was also suggested to associate with 14-3-3 proteins, causing insulin to stimulate mTOR activities (Vander Haar *et al.* 2007).

14-3-3 proteins have been suggested to play a role in several aging-associated diseases, including cancers and neurodegenerative diseases (Wilker and Yaffe 2004; Darling *et al.* 2005). However, it remains unclear how and which types of 14-3-3 protein abnormalities contribute to the cause of these diseases. 14-3-3 protein levels are abundant in the neurofibrillary tangle in patients with Alzheimer's disease (Umahara *et al.* 2004). Elevated 14-3-3 expressions in lung and breast cancers have also been described. It would be interesting to determine whether phosphorylation of 14-3-3 proteins plays an important role in these diseases as well as in life-span regulation in mammals.

We thank T. Powers for providing the pFA6a-13Myc-kanMX6 plasmid and suggestions and members of the Lin laboratory for discussions and suggestions. The HSE4Ptt-*CYC1-LacZ* reporter plasmid was kindly provided by T. Griffin. We also thank members of the Parales laboratory for assistance with oxygen consumption assays and members of the Hunter laboratory for assistance with image analysis. This study was supported by the National Institute on Aging, the Ellison Medical Foundation, and an American Cancer Society Institutional Research Grant.

LITERATURE CITED

Ai, W., P. G. Bertram, C. K. Tsang, T. F. Chan and X. F. Zheng, 2002 Regulation of subtelomeric silencing during stress response. Mol. Cell **10:** 1295–1305.

Aitken, A., 2006 14–3-3 proteins: a historic overview. Semin. Cancer Biol. **16:** 162–172.

Bandhakavi, S., H. Xie, B. O'Callaghan, H. Sakurai, D. H. Kim *et al.*, 2008 Hsf1 activation inhibits rapamycin resistance and TOR signaling in yeast revealed by combined proteomic and genetic analysis. PLoS One **3:** e1598.

Berdichevsky, A., M. Viswanathan, H. R. Horvitz and L. Guarente, 2006 C. elegans SIR-2.1 interacts with 14–3-3 proteins to activate DAF-16 and extend life span. Cell **125:** 1165–1177.

Brachmann, C. B., A. Davies, G. J. Cost, E. Caputo, J. Li *et al.*, 1998 Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast **14:** 115–132.

Bruckmann, A., P. J. Hensbergen, C. I. Balog, A. M. Deelder, H. Y. de Steensma *et al.*, 2007 Post-transcriptional control of the Saccharomyces cerevisiae proteome by 14–3-3 proteins. J. Proteome Res. **6:** 1689–1699.

Burke, D., D. Dawson and T. Sterns, 2000 *Methods in Yeast Genetics*, pp. 171–174. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Darling, D. L., J. Yingling and A. Wynshaw-Boris, 2005 Role of 14–3-3 proteins in eukaryotic signaling and development. Curr. Top. Dev. Biol. **68:** 281–315.

Dilova, I., E. Easlon and S. J. Lin, 2007 Calorie restriction and the nutrient sensing signaling pathways. Cell. Mol. Life Sci. **64:** 752–767.

Easlon, E., F. Tsang, I. Dilova, C. Wang, S. P. Lu *et al.*, 2007 The dihydrolipoamide acetyltransferase is a novel metabolic longevity factor and is required for calorie restriction-mediated life span extension. J. Biol. Chem. **282:** 6161–6171.

Easlon, E., F. Tsang, C. Skinner, C. Wang and S. J. Lin, 2008 The malate-aspartate NADH shuttle components are novel metabolic longevity regulators required for calorie restriction-mediated life span extension in yeast. Genes Dev. **22:** 931–944.

Fabrizio, P., and V. D. Longo, 2003 The chronological life span of Saccharomyces cerevisiae. Aging Cell **2:** 73–81.

Fabrizio, P., F. Pozza, S. D. Pletcher, C. M. Gendron and V. D. Longo, 2001 Regulation of longevity and stress resistance by Sch9 in yeast. Science **292:** 288–290.

Fabrizio, P., L. Battistella, R. Vardavas, C. Gattazzo, L. L. Liou *et al.*, 2004 Superoxide is a mediator of an altruistic aging program in Saccharomyces cerevisiae. J. Cell Biol. **166:** 1055–1067.

Fabrizio, P., C. Gattazzo, L. Battistella, M. Wei, C. Cheng *et al.*, 2005 Sir2 blocks extreme life-span extension. Cell **123:** 655–667.

Gray, J. V., G. A. Petsko, G. C. Johnston, D. Ringe, R. A. Singer *et al.*, 2004 "Sleeping beauty": quiescence in Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev. **68:** 187–206.

Guldener, U., S. Heck, T. Fielder, J. Beinhauer and J. H. Hegemann, 1996 A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res. **24:** 2519–2524.

Hsu, A. L., C. T. Murphy and C. Kenyon, 2003 Regulation of aging and age-related disease by DAF-16 and heat-shock factor. Science **300:** 1142–1145.

Ichimura, T., H. Kubota, T. Goma, N. Mizushima, Y. Ohsumi *et al.*, 2004 Transcriptomic and proteomic analysis of a 14–3-3 gene-deficient yeast. Biochemistry **43:** 6149–6158.

Jiang, J. C., J. Wawryn, H. M. Shantha Kumara and S. M. Jazwinski, 2002 Distinct roles of processes modulated by histone deacetylases Rpd3p, Hda1p, and Sir2p in life extension by caloric restriction in yeast. Exp. Gerontol. **37:** 1023–1030.

Kaeberlein, M., M. McVey and L. Guarente, 1999 The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. Genes Dev. **13:** 2570–2580.

Kaeberlein, M., K. T. Kirkland, S. Fields and B. K. Kennedy, 2004 Sir2-independent life span extension by calorie restriction in yeast. PLoS Biol. **2:** 1381–1387.

Kaeberlein, M., R. W. Powers, III, K. K. Steffen, E. A. Westman, D. Hu *et al.*, 2005 Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. Science **310:** 1193–1196.

Kakiuchi, K., Y. Yamauchi, M. Taoka, M. Iwago, T. Fujita *et al.*, 2007 Proteomic analysis of in vivo 14–3-3 interactions in the yeast Saccharomyces cerevisiae. Biochemistry **46:** 7781–7792.

Kenyon, C., 2001 A conserved regulatory system for aging. Cell **105:** 165–168.

Lamming, D. W., M. Latorre-Esteves, O. Medvedik, S. N. Wong, F. A. Tsang *et al.*, 2005 HST2 mediates SIR2-independent life-span extension by calorie restriction. Science **309:** 1861–1864.

Li, J., M. Tewari, M. Vidal and S. S. Lee, 2007 The 14–3-3 protein FTT-2 regulates DAF-16 in Caenorhabditis elegans. Dev. Biol. **301:** 82–91.

Lin, K., J. B. Dorman, A. Rodan and C. Kenyon, 1997 daf-16: an HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. Science **278:** 1319–1322.

Lin, S. J., P. A. Defossez and L. Guarente, 2000 Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae. Science **289:** 2126–2128.

Longtine, M. S., A. McKenzie, III, D. J. Demarini, N. G. Shah, A. Wach *et al.*, 1998 Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast **14:** 953–961.

Mascarenhas, C., L. C. Edwards-Ingram, L. Zeef, D. Shenton, M. P. Ashe *et al.*, 2008 Gcn4 is required for the response to peroxide stress in the yeast Saccharomyces cerevisiae. Mol. Biol. Cell **19:** 2995–3007.

Migliaccio, E., M. Giorgio, S. Mele, G. Pelicci, P. Reboldi *et al.*, 1999 The p66shc adaptor protein controls oxidative stress response and life span in mammals. Nature **402:** 309–313.

Powers, R. W., III, M. Kaeberlein, S. D. Caldwell, B. K. Kennedy and S. Fields, 2006 Extension of chronological life span in yeast by decreased TOR pathway signaling. Genes Dev. **20:** 174–184.

Raychaudhuri, S., M. M. Reddy, N. R. Rajkumar and R. Rajasekharan, 2003 Cytosolic iron superoxide dismutase is a part of the triacylglycerol biosynthetic complex in oleaginous yeast. Biochem. J. **372:** 587–594.

Reverter-Branchat, G., E. Cabiscol, J. Tamarit, J. Ros, E. Piulats *et al.*, 2004 Oxidative damage to specific proteins in replicative and chronological-aged Saccharomyces cerevisiae: common targets and prevention by calorie restriction oxidative stress promotes specific protein damage in Saccharomyces cerevisiae. J. Biol. Chem. **279:** 31983–31989.

Roth, G. S., D. K. Ingram and M. A. Lane, 2001 Caloric restriction in primates and relevance to humans. Ann. NY Acad. Sci. **928:** 305–315.

Salmon, A. B., S. Murakami, A. Bartke, J. Kopchick, K. Yasumura *et al.*, 2005 Fibroblast cell lines from young adult mice of long-lived mutant strains are resistant to multiple forms of stress. Am. J. Physiol. Endocrinol. Metab. **289:** E23–E29.

Smith, D. L., Jr., J. M. McClure, M. Matecic and J. S. Smith, 2007 Calorie restriction extends the chronological lifespan of Saccharomyces cerevisiae independently of the Sirtuins. Aging Cell **6:** 649–662.

Tissenbaum, H. A., and L. Guarente, 2002 Model organisms as a guide to mammalian aging. Dev. Cell **2:** 9–19.

Umahara, T., T. Uchihara, K. Tsuchiya, A. Nakamura, T. Iwamoto *et al.*, 2004 14–3-3 proteins and zeta isoform containing neurofibrillary tangles in patients with Alzheimer's disease. Acta Neuropathol. **108:** 279–286.

Usui, T., and J. H. Petrini, 2007 The Saccharomyces cerevisiae 14–3-3 proteins Bmh1 and Bmh2 directly influence the DNA damage-dependent functions of Rad53. Proc. Natl. Acad. Sci. USA **104:** 2797–2802.

Valenzuela, L., C. Aranda and A. Gonzalez, 2001 TOR modulates GCN4-dependent expression of genes turned on by nitrogen limitation. J. Bacteriol. **183:** 2331–2334.

Vander Haar, E., S. I. Lee, S. Bandhakavi, T. J. Griffin and D. H. Kim, 2007 Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nat. Cell Biol. **9:** 316–323.

van Heusden, G. P., and H. Y. Steensma, 2006 Yeast 14–3-3 proteins. Yeast **23:** 159–171.

Wang, Y., S. W. Oh, B. Deplancke, J. Luo, A. J. Walhout *et al.*, 2006 C. elegans 14–3-3 proteins regulate life span and interact with SIR-2.1 and DAF-16/FOXO. Mech. Ageing Dev. **127:** 741–747.

Wei, M., P. Fabrizio, J. Hu, H. Ge, C. Cheng *et al.*, 2008 Life span extension by calorie restriction depends on Rim15 and transcription factors downstream of Ras/PKA, Tor, and Sch9. PLoS Genet. **4:** e13.

Weindruch, W., and R. L. Walford, 1998 *The Retardation of Aging and Diseases by Dietary Restriction*. Charles C Thomas, Springfield, IL.

Werner-Washburne, M., E. Braun, G. C. Johnston and R. A. Singer, 1993 Stationary phase in the yeast Saccharomyces cerevisiae. Microbiol. Rev. **57:** 383–401.

Wilker, E., and M. B. Yaffe, 2004 14–3-3 proteins: a focus on cancer and human disease. J. Mol. Cell. Cardiol. **37:** 633–642.

Zuin, A., N. Gabrielli, I. A. Calvo, S. Garcia-Santamarina, K. L. Hoe *et al.*, 2008 Mitochondrial dysfunction increases oxidative stress and decreases chronological life span in fission yeast. PLoS ONE **3:** e2842.

Communicating editor: M. D. Rose

GENETICS

Supporting Information
http://www.genetics.org/cgi/content/full/genetics.109.107797/DC1

Deleting the 14-3-3 Protein Bmh1 Extends Life Span in *Saccharomyces cerevisiae* by Increasing Stress Response

Chen Wang, Craig Skinner, Erin Easlon and Su-Ju Lin

DOI: 10.1534/genetics.109.107797

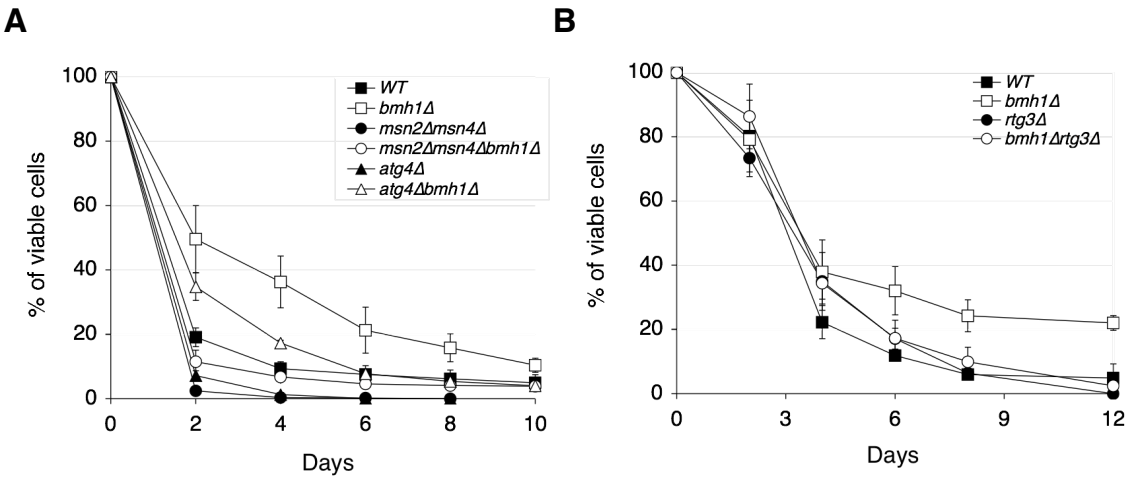

FIGURE S1.—Potential downstream factors required for *bmh1*Δ-induced chronological life span extension. (A) Msn2 and Msn4-mediated stress response and the autophagy pathway play important roles in *bmh1*Δ-induced life span extension. (B) A functional retrograde response is required for life span extension in the *bmh1*Δ mutant. WT: wild type control. One set of representative data is shown.

TABLE S1

List of genes encoding characterized and putative yeast kinases

No.	Standard Name	Position in KO collection	Annotation	Systematic Name
1	*CKA1*	129-B-5	Alpha' catalytic subunit of casein kinase 2, a Ser/Thr protein kinase with roles in cell growth and proliferation	*YIL035C*
2	*CKA2*	106-D-2	Alpha' catalytic subunit of casein kinase 2, a Ser/Thr protein kinase with roles in cell growth and proliferation	*YOR061W|YOR29-12*
3	*YCK2*	127-E-2	Palmitoylated, plasma membrane-bound casein kinase I isoform	*YNL154C*
4	*ATG1*	148-D-7	Protein serine/threonine kinase, required for autophagy and for the cytoplasm-to-vacuole targeting (Cvt) pathway	*YGL180W|APG1|AUT3|CVT10*
5	*YCK1*	114-F-11	Palmitoylated, plasma membrane-bound casein kinase I isoform	*YHR135C|CKI2*
6	*CMK1*	129-H-4	Calmodulin-dependent protein kinase	*YFR014C*
7	*YGL059W*	126-G-1	Putative protein kinase of unknown cellular role	*YGL059W*
8	*ARK1*	140-G-6	Serine/threonine protein kinase involved in regulation of the cortical actin cytoskeleton	*YNL020C*
9	*SNF1*	149-D-6	AMP-activated serine/threonine protein kinase found in a complex containing Snf4p and members of the Sip1p/Sip2p/Gal83p family	*YDR477W|CAT1|GLC2|CCR1|HAF3|PAS14*
10	*HRR25*	inviable	Protein kinase involved in regulating diverse events including vesicular trafficking, DNA repair, and chromosome segregation; binds the CTD of RNA pol II; homolog of mammalian casein kinase 1delta (CK1delta)	*YPL204W*
11	*BCK1*	133-H-6	Mitogen-activated protein (MAP) kinase kinase kinase acting in the protein kinase C signaling pathway, which controls cell integrity	*YJL095W|SAP3|LAS3|SSP31|SLK1*
12	*GIN4*	147-F-1	Protein kinase involved in bud growth and assembly of the septin ring, proposed to have kinase-dependent and kinase-independent activities	*YDR507C|ERC47*
13	*HAL5*	143-D-10	Putative protein kinase	*YJL165C*
14	*HRK1*	144-H-9	Protein kinase implicated in activation of the plasma membrane H(+)-ATPase Pma1p in response to glucose metabolism	*YOR267C*
15	*HSL1*	117-B-8	Nim1p-related protein kinase that regulates the	*YKL101W|NIK1*

			morphogenesis and septin checkpoints	
16	*NPR1*	127-C-12	Protein kinase that stabilizes several plasma membrane amino acid transporters by antagonizing their ubiquitin-mediated degradation	*YNL183C*
17	*PKH2*	131-B-9	Serine/threonine protein kinase involved in sphingolipid-mediated signaling pathway that controls endocytosis	*YOL100W*
18	*PPZ2*	111-G-4	Serine/Threonine protein phosphatase Z	*YDR436W*
19	*PRR1*	117-C-5	Serine/threonine protein kinase that inhibits pheromone induced signalling downstream of MAPK, possibly at the level of the Ste12p transcription factor	*YKL116C*
20	*PSK1*	101-D-2	One of two (see also PSK2) PAS domain containing S/T protein kinases	*YAL017W|FUN31*
21	*PSK2*	107-G-2	One of two (see also PSK1) PAS domain containing S/T protein kinases	*YOL045W*
22	*YPL150W*	109-B-1	Putative protein of unknown function	*YPL150W*
23	*YNR047W*	141-D-9	Putative protein of unknown function	*YNR047W*
24	*YKL171W*	117-F-9	Putative protein of unknown function	*YKL171W*
25	*PXL1*	135-A-5	LIM domain-containing protein that localizes to sites of polarized growth, required for selection and/or maintenance of polarized growth sites, may modulate signaling by the GTPases Cdc42p and Rho1p; has similarity to metazoan paxillin	*YKR090W*
26	*AKL1*	140-D-12	Ser-Thr protein kinase, member (with Ark1p and Prk1p) of the Ark kinase family	*YBR059C*
27	*DIG1*	123-F-3	Regulatory protein of unknown function, constitutively-expressed, involved in the regulation of mating-specific genes and the invasive growth pathway, required for MAP-kinase imposed repression, inhibits pheromone-responsive transcription	*YPL049C*
28	*DIG2*	147-D-4	Regulatory protein of unknown function, pheromone-inducible, involved in the regulation of mating-specific genes and the invasive growth pathway, required for MAP-kinase imposed repression, inhibits pheromone-responsive transcription	*YDR480W*
29	*PRK1*	129-D-8	Protein serine/threonine kinase	*YIL095W|PAK1*
30	*KIN1*	110-G-12	Serine/threonine protein kinase involved in regulation of exocytosis	*YDR122W*
31	*KIN2*	102-F-11	Serine/threonine protein kinase involved in regulation of exocytosis	*YLR096W*

32	*KIN4*	119-D-3	Serine/threonine protein kinase that inhibits the mitotic exit network (MEN) when the spindle position checkpoint is activated	*YOR233W|KIN31|KIN3*
33	*KIN82*	124-G-6	Putative serine/threonine protein kinase, most similar to cyclic nucleotide-dependent protein kinase subfamily and the protein kinase C subfamily	*YCR091W*
34	*KKQ8*	117-F-8	Putative serine/threonine protein kinase with unknown cellular role	*YKL168C*
35	*KSP1*	114-D-1	Nonessential putative serine/threonine protein kinase of unknown cellular role	*YHR082C*
36	*SKM1*	131-C-10	Member of the PAK family of serine/threonine protein kinases with similarity to Ste20p and Cla4p	*YOL113W*
37	*CBK1*	inviable	Serine/threonine protein kinase that regulates cell morphogenesis pathways	*YNL161W*
38	*SMK1*	133-D-2	Middle sporulation-specific mitogen-activated protein kinase (MAPK) required for production of the outer spore wall layers	*YPR054W*
39	*RIM11*	144-E-9	Protein kinase required for signal transduction during entry into meiosis	*YMR139W|GSK3|MDS1*
40	*DUN1*	138-F-2	Cell-cycle checkpoint serine-threonine kinase required for DNA damage-induced transcription of certain target genes, phosphorylation of Rad55p and Sml1p, and transient G2/M arrest after DNA damage	*YDL101C*
41	*RIM15*	150-A-8 and 150-C-2	Glucose-repressible protein kinase involved in signal transduction during cell proliferation in response to nutrients, specifically the establishment of stationary phase	*YFL033C|TAK1*
42	*PBS2*	145-C-9	MAP kinase kinase that plays a pivotal role in the osmosensing signal-transduction pathway, activated under severe osmotic stress	*YJL128C|SSK4|SFS4|HOG4*
43	*TFG1*	inviable	TFIIF (Transcription Factor II) largest subunit	*YGR186W|RAP74|SSU71*
44	*PKH1*	147-E-1	Serine/threonine protein kinase involved in sphingolipid-mediated signaling pathway that controls endocytosis	*YDR490C*
45	*PKH3*	147-C-8	Protein kinase with similarity to mammalian phosphoinositide-dependent kinase 1 (PDK1) and yeast Pkh1p and Pkh2p, two redundant upstream activators of Pkc1p	*YDR466W*
46	*MCK1*	105-D-4	Protein serine/threonine/tyrosine (dual-specificity) kinase involved in control of chromosome segregation and in regulating entry into meiosis	*YNL307C|YPK1*

47	*CLA4*	105-D-11	Cdc42p activated signal transducing kinase of the PAK (p21-activated kinase) family, involved in septin ring assembly and cytokinesis	*YNL298W|ERC10*
48	*IME2*	133-H-1	Serine/threonine protein kinase involved in activation of meiosis, associates with Ime1p and mediates its stability, activates Ndt80p	*YJL106W|SME1*
49	*YMR291W*	105-A-2	Putative kinase of unknown function	*YMR291W*
50	*SAT4*	115-F-2	Ser/Thr protein kinase involved in salt tolerance	*YCR008W|HAL4*
51	*YDL025C*	146-E-2	Putative protein kinase, potentially phosphorylated by Cdc28p	*YDL025C*
52	*SWE1*	119-G-12	Protein kinase that regulates the G2/M transition by inhibition of Cdc28p kinase activity	*YJL187C|WEE1*
53	*PRR2*	136-F-12	Serine/threonine protein kinase that inhibits pheromone induced signalling downstream of MAPK, possibly at the level of the Ste12p transcription factor	*YDL214C*
54	*SKY1*	104-E-1	SR protein kinase (SRPK) involved in regulating proteins involved in mRNA metabolism and cation homeostasis	*YMR216C*
55	*ISR1*	124-A-9	Predicted protein kinase, overexpression causes sensitivity to staurosporine, which is a potent inhibitor of protein kinase C	*YPR106W*
56	*STE11*	149-G-7	Signal transducing MEK kinase involved in pheromone response and pseudohyphal/invasive growth pathways where it phosphorylates Ste7p, and the high osmolarity response pathway, via phosphorylation of Pbs2p	*YLR362W*
57	*DBF2*	143-C-1	Ser/Thr kinase involved in transcription and stress response	*YGR092W*
58	*CAK1*	inviable	Cyclin-dependent kinase-activating kinase required for passage through the cell cycle, phosphorylates and activates Cdc28p	*YFL029C|CIV1*
59	*TOS3*	148-D-6	Protein kinase, related to and functionally redundant with Elm1p and Sak1p for the phosphorylation and activation of Snf1p	*YGL179C*
60	*MPS1*	inviable	Dual-specificity kinase required for spindle pole body (SPB) duplication and spindle checkpoint function	*YDL028C|RPK1*
61	*MKK1*	119-D-2	Mitogen-activated kinase kinase involved in protein kinase C signaling pathway that controls cell integrity	*YOR231W|SSP32*
62	*IKS1*	134-B-3	Putative serine/threonine kinase	*YJL057C*
63	*MKK2*	109-B-7	Mitogen-activated kinase kinase involved in protein kinase C signaling pathway that controls cell integrity	*YPL140C|LPI6|SSP33*

64	*IPL1*	inviable	Aurora kinase involved in regulating kinetochore-microtubule attachments	*YPL209C|PAC15*
65	*YKL161C*	117-F-2	Protein kinase implicated in the Slt2p mitogen-activated (MAP) kinase signaling pathway	*YKL161C|MLP1*
66	*SGV1*	inviable	Cyclin (Bur2p)-dependent protein kinase that functions in transcriptional regulation	*YPR161C|BUR11*
67	*KIN28*	inviable	Serine/threonine protein kinase, subunit of the transcription factor TFIIH	*YDL108W*
68	*ELM1*	116-G-3	Serine/threonine protein kinase that regulates cellular morphogenesis, septin behavior, and cytokinesis	*YKL048C|LDB9*
69	*BUD32*	171-D-4	Protein kinase proposed to be involved in bud-site selection, telomere uncapping and elongation, and transcription	*YGR262C|LDB14*
70	*FUS3*	125-F-7	Mitogen-activated serine/threonine protein kinase involved in mating	*YBL016W|DAC2*
71	*RIO1*	inviable	Essential serine kinase involved in cell cycle progression and processing of the 20S pre-rRNA into mature 18S rRNA	*YOR119C|RRP10*
72	*CDC5*	inviable	Polo-like kinase with similarity to Xenopus Plx1 and S. pombe Plo1p	*YMR001C|PKX2|MSD2*
73	*RIO2*	inviable	Essential serine kinase involved in the processing of the 20S pre-rRNA into mature 18S rRNA	*YNL207W*
74	*STE20*	121-E-10	Signal transducing kinase of the PAK (p21-activated kinase) family, involved in pheromone response and pseudohyphal/invasive growth pathways, activated by Cdc42p	*YHL007C*
75	*SPS1*	147-G-3	Putative protein serine/threonine kinase expressed at the end of meiosis and localized to the prospore membrane, required for correct localization of enzymes involved in spore wall synthesis	*YDR523C*
76	*SSN3*	123-F-8	Cyclin-dependent protein kinase, component of RNA polymerase II holoenzyme	*YPL042C|SRB10|UME5|RYE5|CDK8|GIG2|NUT7*
77	*CDC7*	inviable	DDK (Dbf4-dependent kinase) catalytic subunit required for firing origins and replication fork progression in mitosis through phosphorylation of Mcm2-7p complexes and Cdc45p	*YDL017W|SAS1|LSD6*
78	*YCK3*	148-F-4	Palmitoylated, vacuolar membrane-localized casein kinase I isoform	*YER123W|CKI3*
79	*KIN3*	101-E-6	Nonessential protein kinase with unknown cellular role	*YAR018C|FUN52|NPK1*
80	*MEC1*	inviable	Genome integrity checkpoint protein and PI kinase superfamily member	*YBR136W|ESR1|SAD3*

81	*CMK2*	132-C-5	Calmodulin-dependent protein kinase	*YOL016C*
82	*YPK1*	117-D-1	Serine/threonine protein kinase required for receptor-mediated endocytosis	*YKL126W|SLI2*
83	*YPK2*	145-D-12	Protein kinase with similarityto serine/threonine protein kinase Ypk1p	*YMR104C|YKR2*
84	*KSS1*	143-B-7	Mitogen-activated protein kinase (MAPK) involved in signal transduction pathways that control filamentous growth and pheromone response	*YGR040W*
85	*SCH9*	175-F-9	Protein kinase involved in transcriptional activation of osmostress-responsive genes	*YHR205W|KOM1*
86	*KIC1*	inviable	Protein kinase of the PAK/Ste20 kinase family, required for cell integrity possibly through regulating 1,6-beta-glucan levels in the wall	*YHR102W|NRK1*
87	*VPS15*	171-C-10	Myristoylated serine/threonine protein kinase involved in vacuolar protein sorting	*YBR097W|GRD8|VAC4|VPL19*
88	*CDC15*	inviable	Protein kinase of the Mitotic Exit Network that is localized to the spindle pole bodies at late anaphase	*YAR019C|LYT1*
89	*HRR25*	inviable	Protein kinase involved in regulating diverse events including vesicular trafficking, DNA repair, and chromosome segregation	*YPL204W*
90	*YGK3*	131-D-9	Protein kinase related to mammalian glycogen synthase kinases of the GSK-3 family	*YOL128C*
91	*DBF20*	124-A-11	Ser/Thr kinase involved in late nuclear division, one of the mitotic exit network (MEN) proteins	*YPR111W*
92	*YBR028C*	140-B-9	Putative protein kinase, possible substrate of cAMP-dependent protein kinase (PKA)	*YBR028C*
93	*SSK22*	144-B-6	MAP kinase kinase kinase of the HOG1 mitogen-activated signaling pathway	*YCR073C*
94	*SKS1*	123-G-8	Putative serine/threonine protein kinase	*YPL026C|SHA3*
95	*TEL1*	126-C-5	Protein kinase primarily involved in telomere length regulation	*YBL088C*
96	*MEK1*	107-B-4	Meiosis-specific serine/threonine protein kinase, functions in meiotic checkpoint, promotes recombination between homologous chromosomes by suppressing double strand break repair between sister chromatids	*YOR351C|MRE4*
97	*SLT2*	114-A-8	Serine/threonine MAP kinase involved in regulating the maintenance of cell wall integrity and progression through the cell cycle	*YHR030C|SLK2|BYC2|MPK1*
98	*CHK1*	137-D-4	Serine/threonine kinase and DNA damage checkpoint effector, mediates cell cycle arrest via phosphorylation	*YBR274W*

			of Pds1p	
99	*HOG1*	102-G-10	Mitogen-activated protein kinase involved in osmoregulation via three independent osmosensors	*YLR113W|SSK3*
100	*KNS1*	101-G-7	Nonessential putative protein kinase of unknown cellular role	*YLL019C|L124*
101	*SSK2*	141-C-8	MAP kinase kinase kinase of the HOG1 mitogen-activated signaling pathway	*YNR031C*
102	*ALK1*	126-D-11	Protein kinase	*YGL021W*
103	*ALK2*	125-F-1	Protein kinase	*YBL009W*
104	*MRK1*	146-H-9	Glycogen synthase kinase 3 (GSK-3) homolog	*YDL079C*
105	*GCN2*	128-E-5	Protein kinase, phosphorylates the alpha-subunit of translation initiation factor eIF2 (Sui2p) in response to starvation	*YDR283C|AAS1*
106	*YPL141C*	109-B-6	Putative protein kinase	*YPL141C*
107	*SAK1*	148-F-7	Upstream serine/threonine kinase for the SNF1 complex	*YER129W|PAK1*
108	*RCK1*	148-C-1	Protein kinase involved in the response to oxidative stress	*YGL158W*
109	*PKC1*	inviable	Protein serine/threonine kinase essential for cell wall remodeling during growth	*YBL105C|HPO2|STT1|CLY15*
110	*RCK2*	121-H-5	Protein kinase involved in the response to oxidative and osmotic stress	*YLR248W|CMK3|CLK1*
111	*BUB1*	134-F-4	Protein kinase that forms a complex with Mad1p and Bub3p that is crucial in the checkpoint mechanism required to prevent cell cycle progression into anaphase in the presence of spindle damage, associates with centromere DNA via Skp1p	*YGR188C*
112	*YPL236C*	108-D-7	Putative protein kinase that exhibits Akr1p-dependent palmitoylation	*YPL236C*
113	*FMP48*	117-H-12	Putative protein of unknown function	*YGR052W*
114	*PHO85*	123-G-5	Cyclin-dependent kinase, with ten cyclin partners	*YPL031C|LDB15*
115	*PTK1*	127-E-6	Putative serine/threonine protein kinase that regulates spermine uptake	*YKL198C|STK1|KKT8|YKL199C|YKT9|POT1*
116	*CDC28*	inviable	Catalytic subunit of the main cell cycle cyclin-dependent kinase (CDK)	*YBR160W|SRM5|HSL5|CDK1*
117	*PTK2*	138-E-6	Putative serine/threonine protein kinase involved in regulation of ion transport across plasma membrane	*YJR059W|STK2*
118	*VHS1*	128-B-8	Cytoplasmic serine/threonine protein kinase	*YDR247W*
119	*TPK1*	120-A-3	cAMP-dependent protein kinase catalytic subunit	*YJL164C|PKA1|SRA3*
120	*RAD53*	inviable	Protein kinase, required for cell-cycle arrest in response to DNA damage	*YPL153C|LSD1|MEC2|SPK1*

121	*TPK2*	108-F-4	cAMP-dependent protein kinase catalytic subunit	*YPL203W|YKR1|PKA2|PKA3*
122	*TPK3*	117-F-6	cAMP-dependent protein kinase catalytic subunit	*YKL166C*
123	*STE7*	139-A-8	Signal transducing MAP kinase kinase involved in pheromone response, where it phosphorylates Fus3p, and in the pseudohyphal/invasive growth pathway, through phosphorylation of Kss1p	*YDL159W*
124	*YAK1*	143-D-4	Serine-threonine protein kinase that is part of a glucose-sensing system involved in growth control in response to glucose availability	*YJL141C*
125	*CTK1*	143-E-10	Catalytic (alpha) subunit of C-terminal domain kinase I (CTDK-I), which phosphorylates the C-terminal repeated domain of the RNA polymerase II large subunit (Rpo21p) to affect both transcription and pre-mRNA 3' end processing	*YKL139W*
126	*KCC4*	115-C-3	Protein kinase of the bud neck involved in the septin checkpoint, associates with septin proteins, negatively regulates Swe1p by phosphorylation, shows structural homology to bud neck kinases Gin4p and Hsl1p	*YCL024W*
127	*TOR1*	149-C-4	PIK-related protein kinase and rapamycin target	*YJR066W|DRR1*
128	*TOR2*	inviable	PIK-related protein kinase and rapamycin target	*YKL203C|DRR2*
129	*IRE1*	114-C-10	Serine-threonine kinase and endoribonuclease	*YHR079C|ERN1*